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EDITORIAL

Dear Readers;

We are greatly pleased and honored to present to you the third issue of our journal for the year 2024. In this issue, we feature a total of 15 articles, comprising 12 research papers, 2 case reports, and 1 review article.

Esteemed researchers, as of June, the Journal Impact Factors (JIF values) and Quartile rankings for journals indexed in ESCI and SCIE for the year 2023 have been published. In this classification by Clarivate, our journal continues to be ranked among the Q3 journals. On this occasion, I extend my gratitude to all our stakeholders, especially our esteemed readers and the Journal Editorial Board Members, for their contributions.

Dear readers, on May 31st, we lost Prof. Dr. Rıfkı Hazıroğlu, who served as the Chief Editor of our journal between 2003-2009. We extend our condolences and wish patience to his family and the professional community.

Dear readers, I offer my heartfelt respect to all of you, with the hope that the latest issue of our journal will contribute to the world of science.

Best Regards.

Dr. Levent ALTINTAŞ
Editor in Chief
Ankara Üniversitesi Veteriner Fakültesi Dergisi

Views of veterinary faculty students on the concept of Artificial Intelligence and its use in Veterinary Medicine practices: An example of Ankara University Faculty of Veterinary Medicine

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ABSTRACT

The study was carried out to determine the knowledge levels of the students of Ankara University Faculty of Veterinary Medicine, on the concept of artificial intelligence and its use in veterinary practices. For this purpose, an online questionnaire was applied to a total of 529 students in the study, covering all grades of the faculty. The questionnaire consists of two parts. In the first part, there are 10 questions including demographics, knowledge about the concept of artificial intelligence, etc. The second part consists of 26 5-point Likert-type questions to determine students' thoughts on artificial intelligence applications. Data were analyzed using statistical tests. Consequently, the students participating in the study are partially knowledgeable about artificial intelligence (52.9%). They know the importance of following the developments in artificial intelligence for the profession (45.2%). They think that artificial intelligence applications will improve their professional skills (53.5%). They have the opinion that a robot cannot replace a veterinary surgeon (36.7%) and artificial intelligence cannot cause unemployment in veterinary medicine in the future (35.3%). In addition, they believe that artificial intelligence can cause ethical problems (39.3%) and that applications made with this technology should be developed in an ethical sense (42.4%). As a result, while the students think that artificial intelligence will have positive effects in the field of veterinary medicine, they also think that artificial intelligence can bring negative ethical implications. It can be concluded that including elective courses on artificial intelligence applications in veterinary faculties and conducting further research on the subject would be beneficial.

Introduction

As a branch of computer science, artificial intelligence (AI) is an emerging science and technology used to mimic and expand human behavior and thinking (5). AI is a general term that means creating a model of intelligent behavior using computers with minimal human intervention (21). AI, which has become very popular today, especially with the increase in processor powers, is applied in many different areas such as the public and private sectors and academy (2). It is generally accepted that AI started with the invention of robots (21). The term AI was first mentioned by John McCarthy at Dartmouth College in a scientific meeting held in 1956, and it was defined as *"the science and engineering of making intelligent machines, especially intelligent computer programs"* (11, 16, 24). AI, which basically targets human intelligence, is a field of study that can imitate human intelligence through computers and aims to place products in different areas of life (13) It is an artificial operating system that is expected to exhibit high cognitive features such as recognizing, perceiving, analyzing, grouping, choosing, responding, learning, imitating, speaking, chatting, educating, connecting plural concepts, thinking, problem-solving, and communicating (5). Human

intelligence can forget the events and subjects that it has learned or experienced over time. Apart from some limitations, AI provides advantages in terms of using what it has learned very quickly and is permanent in the information it has learned (1, 13, 16, 28). Systems using AI have the potential to provide unprecedented benefits to humanity (32). Many researchers have started to work intensively on the topic since the introduction and use of AI in the literature. It is suggested that the technology of AI, which attracts the attention of almost everyone from all professions at any age, can especially be useful in health-related topics such as early diagnosis, accurate diagnosis, and clinical decision support to protect and maintain health (30, 39). AI is expected to significantly impact various fields of medicine in the future and, if designed and used appropriately, has the potential to strengthen many of the weaknesses encountered in current healthcare practices (29).

Becoming a very popular and developed subject in the field of veterinary medicine, AI is used in many areas such as animal health, welfare, production, public health, and herd health management (19). It is used as an important tool, especially in veterinary clinics, for performance management of dairy cows, and body condition score applications (37). In addition, it is known that AI will provide benefits such as prevention of zoonotic diseases, improvement of diagnosis, treatment, pharmacovigilance reporting, determination of new preventive medicine strategies, and reduction of unnecessary drug (antibiotic) use through diagnosis and treatment protocols (18). It is foreseen that the use of AI, 3D software, and gene technology will become prevalent in veterinary practices in the near future. Predictions have become widespread that the concept of AI for the medical field, which is a fairly new concept, will be on the agenda of the health sector in the future (2). Studies on integrating AI technologies into the education systems have started in many countries (27). Although AI applications are quite common globally, they have only just begun to be put into practice in Türkiye. Various AI projects are prepared in organizations providing services in education including the Ministry of National Education, the Council of Higher Education, and universities, and studies are carried out for the realization of these projects. These projects cover areas such as digital classrooms, lifelong learning approach, talent hunting for qualified human resources, and new education models (36).

Although there is no training within the scope of AI in the curricula of veterinary faculties, pioneering steps are coming from Türkiye's well-established faculties as the subject is on the rise in the world. Within the framework of the "Artificial Intelligence and Digitalization Training Program in Veterinary Medicine" organized at Ankara University Faculty of Veterinary Medicine (AUFVM) in 2021, experts invited from Türkiye and abroad provided information to veterinarians and students about AI (4). With this training program, which was organized for the first time in Türkiye and set an example for other veterinary faculties, raising awareness about AI applications is aimed.

AI has an increasing interest day by day and attracts the attention of various professional groups and many studies address the views of university youth on the concept of AI (13, 14, 17, 35, 39). However, no study has been found in Türkiye that deals with the knowledge level and opinions of students on AI in the field of veterinary medicine. This study was conducted to determine the knowledge level of AUFVM students on AI and their views on its use in veterinary medicine.

Materials and Methods

The data were collected with the "AI Knowledge Level Questionnaire" prepared by the researchers. In order to prepare the questions that serve the purpose, literature research was conducted, previous research on university students was examined, and a pilot study was conducted with 25 veterinary students. After the pilot study, the students' feedback was received and the questions were finalized. In the first part of the questionnaire, there are 10 questions including demographics, level of computer use, knowledge about the concept of AI, etc. The second part consists of 26 5-point Likert-type questions to determine students' thoughts on AI applications [Strongly Disagree (1), Disagree (2), Undecided (3), Agree (4), Strongly Agree (5)]. The study was carried out with 529 students studying in 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} grades enrolled in XUFVM 2021-2022 Spring Semester Turkish Program. Informed consent and survey forms have been made online using Google Forms through the existing Google account of the researchers. The created link was delivered to the students via each grade's own WhatsApp group. The participants were provided with the questionnaire form after reading the consent form and giving their informed consent. Those who did not consent to the study were prevented from accessing the data collection tool. The answers were recorded in Excel via Google Forms Online and the analyzes were carried out. Descriptive statistics on the data to be obtained are determined as mean and standard deviation for quantitative data, frequency, and percentage for qualitative data. In addition, whether there is a relationship between the rate of answers given to the questions and demographic characteristics was examined by Chi-Square analysis. Pearson Chi-Square or Fisher's Exact Test was used according to the distribution of the observed values and expected values to the cells in the cross table. All statistical analyzes were conducted with SPSS 14.01 package program. The statistical significance limit was accepted as $P<0.05$.

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Results

It was determined that the majority of participants are women (54.6%) and the highest participation is between the ages of 18-22 (65.6%). Among the participants, 27.2% (n=144) are fourth grade, 20.8% (n=110) are third grade, 18.3% (n=97) are fifth grade, 18.3% (n=97) are first grade and 15.3% (n=81) are second grade students. The average level of computer use is average at a 46.7% rate (n=247). Considering the time spent on the internet daily, the average is 4.88±2.42 hours and 3.5-6 hours is the most preferred range by 54.6% (n=288) (Table 1).

Among the students, 93% (n=492) of them stated that they heard about the concept of AI before and 52.9% (n=279) stated that they have partial knowledge. 45.9% (n=242) said that they do not have knowledge about the use of AI in veterinary medicine. 87.3% (n=460) first heard about the concept of AI from social media and the internet and 61.4% (n=325) used an application using AI (Table 2).

Table 1. Distribution of demographics of students.

Variables	**Number (n)**	**Percentage (%)**
Gender		
Female	289	54.6
Male	240	45.4
Total	529	100
Age (mean±std.dev)	**22.10±3.07**	
	Number (n)	**Percentage (%)**
18-22 (1)	347	65.6
23-27 (2)	163	30.8
28-32 (3)	12	2.3
33 or older (4)	7	1.3
Total	529	100
Grade	**Number (n)**	**Percentage (%)**
1	97	18.3
2	81	15.3
3	110	20.8
4	144	27.2
5	97	18.3
Total	529	100
Level of computer use?	**Number (n)**	**Percentage (%)**
Bad	22	4.2
Average	247	46.6
Good	197	37.2
Very good	63	11.9
Total	529	100
Average time spent online per day?	**Number (n)**	**Percentage (%)**
0-3 hours	141	26.6
3,5-6 hours	288	54.4
6,5-9 hours	62	11.7
9,5- 12 hours	35	6.6
12,5 and above hours	3	0.6
Total	529	100

Table 2. Descriptive information on the concept of artificial intelligence.

Variables	**Number (n)**	**Percentage (%)**
Have you heard the concept of AI before?		
Yes	492	93.4
Partially	33	6.3
No	2	0.4
Total	527	100
Do you have information on the concept of AI?	**Number (n)**	**Percentage (%)**
Yes	208	39.5
Partially	279	52.9
No	40	7.6
Total	527	100
Do you have information on the use of AI in veterinary medicine?	**Number (n)**	**Percentage (%)**
Yes	84	15.9
Partially	201	38.1
No	242	45.9
Total	527	100
Have you ever used an application (simple or advanced) that uses AI?	**Number (n)**	**Percentage (%)**
Yes	325	61.4
No	204	38.6
Where did you first hear about the concept of AI?	**Number (n)**	**Percentage (%)**
Social media, internet	460	87.3
University environment	25	4.7
Circle of family, friends	27	5.1
Other	15	2.8
Total	527	100

As seen in Table 3, "AI should be used in the field of veterinary medicine." (4.28±0.780) got the highest score and this is followed by "It is important for me to follow the developments in AI for my profession." (4.26±0.876), "AI applications need to be improved from an ethical point of view." (4.21±0.799) and "The use of AI in veterinary medicine would contribute to the early diagnosis, treatment, and prevention of animal diseases." (4.17±0.642). The answers "AI would leave veterinarians unemployed in the future." (2.51±1.123) and "A robot can replace a veterinary surgeon with AI technology." (2.52±1.153) received the lowest scores.

***Chi-Square Analyzes*:** A significant relationship was found between male and female students for the *"Have you heard the concept of AI before?"* question (P=0.044). A significant relationship was found between male and female students for the *"Do you have information on the concept of AI?"* question (P<0.001). A significant relationship was found between male and female students for the *"Do you have information on the use of AI in veterinary medicine?"* question (P=0.008). No significant relationship was found between male and female students for the *"Where did you first hear about the concept of AI?"* question (P=0.488). A significant relationship was found between male and female students for the *"Have you ever used an application (simple or advanced) that uses AI?"* question (P=0.005).

Table 3. Distribution of 5-point likert-type questions related to measuring thoughts on the concept of AI.

	1 Strongly disagree	**2 Disagree**	**3 Undecided**	**4 Agree**	**5 Strongly agree**	**mean ± std. deviation**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**	
It is important for me to follow the developments in AI for my profession.	15 (2.8)	7 (1.3)	40 (7.6)	228 (43.1)	239 (45.2)	4.26 ± 0.876
AI should be used in the field of veterinary medicine.	4 (0.8)	8 (1.5)	59 (11.2)	221 (41.8)	237 (44.8)	4.28 ± 0.780
AI applications need to be improved from an ethical point of view.	4 (0.8)	8 (1.5)	76 (14.4)	223 (42.4)	215 (40.9)	4.21 ± 0.799
AI is the science and engineering of making intelligent computer programs.	4 (0.8)	43 (8.1)	152 (28.7)	254 (48)	76 (14.4)	3.67 ± 0.847
The developments for the use of AI in veterinary medicine excite me.	3 (0.6)	18 (3.4)	91 (17.3)	263 (49.9)	152 (28.8)	4.03 ± 0.806
The term AI reminds me of robotic systems.	9 (1.7)	66 (12.5)	78 (14.8)	296 (56.1)	79 (15)	3.70 ± 0.929
The decision support feature of AI can help veterinarians.	5 (0.9)	24 (4.5)	72 (13.6)	297 (56.1)	131 (24.8)	3.99 ± 0.807
AI applications reduce the risk of making medical errors.	3 (0.3)	36 (6.8)	128 (24.2)	263 (49.7)	99 (18.7)	3.79 ± 0.843
I can provide better healthcare to patients and patient owners by using AI.	1 (0.2)	10 (1.9)	67 (12.7)	322 (60.9)	129 (24.4)	4.07 ± 0.677
I am excited to chat about AI applications for my profession.	5 (0.9)	42 (7.9)	117 (22.2)	249 (47.2)	115 (21.8)	3.81 ± 0.899

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AI applications enrich my professional skills.	6 (1.1)	22 (4.2)	85 (16.1)	283 (53.5)	133 (25.1)	3.97 ± 0.825
As AI becomes more widespread, the need for healthcare workers would gradually decrease.	36 (6.8)	137 (25.9)	133 (25.1)	163 (30.8)	60 (11.3	3.14 ± 1.130
Developments in AI applications scare me.	57 (10.8)	191 (36)	171 (32.5)	79 (15)	28 (5.3)	2.68 ± 1.028
A robot can replace a veterinary surgeon with AI technology.	105 (19.8)	194 (36.7)	108 (20.4)	93 (17.5)	29 (5.5)	2.52 ± 1.153
Training on AI should be provided in the curricula of veterinary medicine education.	7 (1.3)	30 (5.7)	91 (17.2)	279 (52.7)	122 (23.1)	3.91 ± 0.861
AI applications should be placed in veterinary faculties.	5 (0.9)	13 (2.5)	74 (14)	295 (55.9)	141 (26.7)	4.05 ± 0.768
The use of AI can replace practical training in veterinary medicine in cases where practical training is insufficient.	9 (1.7)	28 (5.3)	110 (20.8)	276 (52.3)	105 (19.9)	3.83 ± 0.864
The use of AI in veterinary medicine would be useful in preventing zoonotic diseases transmitted from animals to humans.	4 (0.8)	21 (4)	117 (22.1)	270 (51)	117 (22.1)	3.90 ± 0.812
The use of AI in veterinary medicine would contribute to the early diagnosis, treatment, and prevention of animal diseases.	2 (0.4)	6 (1.1)	41 (7.8)	330 (62.6)	148 (28.1)	4.17 ± 0.641
Herd health management would be improved with the use of AI in veterinary medicine.	3 (0.6)	6 (1.1)	69 (13)	300 (56.6)	151 (28.5)	4.12 ± 0.707
AI technologies would prevent the unnecessary use of antibiotics and drugs in animals.	6 (1.1)	34 (6.5)	154 (29.3)	231 (44)	100 (19)	3.73 ± 0.881
Artificial intelligence technologies would benefit the profitability and sustainability of livestock enterprises.	4 (0.88)	6 (1.1)	66 (12.5)	312 (59)	141 (26.7)	4.10 ± 0.705
AI also causes ethical problems.	15 (2.8)	57 (10.8)	174 (32.9)	208 (39.3)	75 (14.2)	3.51 ± 0.960
AI is useful as long as it does not harm humanity.	14 (2.7)	27 (5.1)	84 (15.8)	265 (50.3)	137 (26)	3.92 ± 0.926
AI would leave veterinarians unemployed in the future.	102 (19.4)	186 (35.3)	135 (25.6)	74 (14)	30 (5.7)	2.51 ± 1.123

Table 4. Effects of gender on AI knowledge levels (n = 529).

Have you heard the concept of AI before?					P
		Female	**Male**	**Total**	
Yes	N	264	228	492	
	%	53.70%	46.30%	100.00%	
Partially	N	23	10	33	
	%	69.70%	30.30%	100.00%	0.044*
No	N	0	2	2	
	%	0.00%	100.00%	100.00%	
Total	N	287	240	527	
	%	54.50%	45.50%	100.00%	

*P<0.05.

Do you have information on the concept of AI?					P
		Female	**Male**	**Total**	
Yes	N	87	121	208	
	%	41.80%	58.20%	100.00%	
Partially	N	175	104	279	
	%	62.70%	37.30%	100.00%	<0.001*
No	N	25	15	40	
	%	62.50%	37.50%	100.00%	
Total	N	287	240	527	
	%	54.50%	45.50%	100.00%	

*P<0.05.

Do you have information on the use of AI in veterinary medicine?					P
		Female	**Male**	**Total**	
Yes	N	40	44	84	
	%	47.60%	52.40%	100.00%	
Partially	N	127	74	201	
	%	63.20%	36.80%	100.00%	0.008*
No	N	121	121	242	
	%	50.00%	50.00%	100.00%	
Total	N	288	239	527	
	%	54.60%	45.40%	100.00%	

Where did you first hear about the concept of AI?					P
		Female	**Male**	**Total**	0.488
Social media, internet	N	253	207	460	
	%	55.00%	45.00%	100.00%	
Circle of family, friends	N	15	10	25	
	%	60.00%	40.00%	100.00%	
University environment	N	11	16	27	
	%	40.70%	59.30%	100.00%	
Other	N	9	7	16	
	%	56.30%	43.80%	100.00%	
	N	288	240	528	
	%	54.50%	45.50%	100.00%	

Have you ever used an application (simple or advanced) that uses AI?					P
		Female	**Male**	**Total**	
Yes	N	162	163	325	
	%	49.80%	50.20%	100.00%	
No	N	127	77	204	0.005*
	%	62.30%	37.70%	100.00%	
	N	289	240	529	
	%	54.60%	45.40%	100.00%	

Discussion and Conclusion

With the developments in technology, the internet stands out as a tool that provides easier access to information in today's information societies (12). As like in the study of Akyüz et al. (2), the students participating in this study stated that they heard the concept of AI for the first time over the internet and social media (87.3%, n=460). It is seen that most of the students can use computers at average level (46.6%, n=247) and spend an average of 3.5-6 hours a day on the internet (54.4%, n=288). In terms of knowledge levels, it was determined that 93.4% (n=492) heard the concept of AI before and have partial knowledge about it (52.9%, n= 279). AI reminds robotic systems to students (56.1%, n=296) and is known as the science and engineering of making intelligent computer programs (48%, n=254). In addition, 61.4% (n=325) of the students reported that they used an AI application, simple or advanced. From these statements, it can be said that the students have more or less general knowledge about AI. When AI in veterinary medicine was examined, it was seen that 45.9% of the students (n=242) answered *"no"* to the *"Do you have information on the use of AI in veterinary medicine?"* question. As it is known, the usage areas of AI in the field of veterinary medicine are expanding globally. Kour et al. (23) in their article on AI in veterinary medicine report the areas where AI can be used as detection of left atrial enlargement on canine thoracic radiology, predicting survivability and need for surgery in horses with colic, detection of subclinical mastitis in cows with the help of machine learning, discriminating between meningiomas and gliomas in canines MRI's and using a xenograft platform and machine learning in development of exosomal gene to detect residual disease in dogs with osteosarcoma. Data sets from patient images and test results are being used to train AI software to detect complex diseases with growing accuracy. Some practices use AI to evaluate test results and suggest alternative diagnostics and further steps. There are even AI-built-in stethoscopes available that can detect arrhythmias and other conditions relating to the heart. These are only a few of the many applications of AI in veterinary care (3). Despite these developments in the world, the fact that veterinarian candidates in Türkiye do not have sufficient knowledge about the use of AI may be due to the new recognition of technology, and the fact that it has not yet been included in the veterinary medicine education system. Even if the students do not have enough knowledge about the use of AI in veterinary medicine, it is seen that they understand the importance of this technology. As a matter of fact, when the knowledge levels about the use of AI in veterinary medicine were examined, answers like *"It is important for me to follow the developments in AI for my profession."* (45.2%, n=239), *"AI should be used in the field of veterinary medicine."* (44.8%, n=237), *"AI applications enrich my professional skills."* (53.5%, n=283), *"The developments for the use of AI in veterinary medicine excite me."* (49.9%, n=263), *"The use of AI in veterinary medicine would contribute to the early diagnosis, treatment, and prevention of animal diseases."* (62.6%, n=330) indicate that they are aware of the importance of this technology and the benefits it will bring.

One of the areas where AI is being developed is the field of zoonotic diseases, which has an important place in public health. Students think that the use of AI would be beneficial in preventing zoonotic diseases transmitted from animals to humans (22.1%, n=117). Relatedly, researchers such as Deng (15) and Silva Motta et al. (34) mention positive scientific outcomes of the use of AI zoonotic diseases.

Park et al. (29) reported that the most important criterion for the adoption of the use of AI technology in medical applications is that it allows a better healthcare service for patients. Choudhury and Asan (10) noted that the results of AI studies are mostly positive for patient safety, and that AI improves or outperforms traditional methods, and AI-enabled decision support systems, when implemented correctly, can aid in enhancing patient safety by improving error detection, patient stratification, and drug management. The students stated that by using AI, they could provide better health care to patients and the owners (60.9%, n=322), that AI systems would reduce the risk of making medical mistakes (49.7%, n=263), and that AI would help veterinarians with its decision support feature (56.1%, n=297). These statements show that they have accurate predictions on the subject.

Students agreeing (59%, n=312) with the statements *"Herd health management would be improved with the use of AI in veterinary medicine."* (56.6%, n=300) and *"AI technologies would benefit the profitability and sustainability of livestock enterprises."* (59%, n=312) demonstrates their knowledge of the subject. In fact, Gökçen and Gökçen (20), while explaining the place and importance of AI technologies in animal husbandry, stated that to make predictions in extensive livestock management, a large number of different datasets such as weather, air quality, sound signals, and visual animal behaviors should be collected throughout the year. However, it is not possible to store and process large volumes of data with an ordinary system, and it is necessary to have a larger computing and storage power. At this stage, AI technologies such as sensors, big data, cloud computing, machine learning, etc. get involved, and that way, protection of animal health, productivity, profitability, and sustainability could be guaranteed.

On the matter, The European Coordination Committee on Veterinary Training (ECCVT), by considering the outcomes of a 2018 workshop on digital

technologies in veterinary medicine, has decided to establish a joint group of experts on assessing opportunities, risks, and impacts related to the use of digital technologies and AI applications in veterinary medicine (19).

ECCVT (19) emphasized that relevant authorities urgently need to develop policies and regulations and be incorporated into education systems to ensure the efficient, safe, ethical, and legal use of AI in veterinary medicine. According to this study, the students think that *"Training on AI should be provided in the curricula of veterinary medicine education."* (52.7%, n=279), *"AI applications should be placed in veterinary faculties."* (55.9%, n=295) and *"The use of AI can replace practical training in veterinary medicine in cases where practical training is insufficient."* (52.3%, n=276). These statements help to think that veterinarian candidates know that AI technologies can be used in the fields of health and education and that these technologies are needed to increase the quality of education. Wartman and Combs (38) report that teaching medical students to practice successfully in a healthcare environment transformed by AI applications should become the main focus of curriculum reform today. Moreover, Chen et al (8) emphasize that AI has a great impact on education, especially on the management, teaching, and learning areas of the education sector or in the context of individual learning institutions. It is thought that in veterinary medicine, which is a part of the health field, the subjects related to AI applications should be included in the curricula and the importance of this subject will increase day by day.

What AI can do, whether it can outperform human intelligence, and the effects this technology may create in the future are still being investigated (33). Although there are different opinions about the effects of AI, the main concern is the contribution of AI to the unemployment level (26). Chiacchio et al. (9) and Bardot (6) state that as a result of increasing efficiency in AI, robots may replace humans in the future and cause unemployment. Rigby (31), on the other hand, predicts that no matter how much patient care is improved with AI, patients will still be treated by doctors and that the practice of medicine will always have a human element. The students participating in the study stated that with the spread of AI, the need for healthcare professionals will gradually decrease (30.8%, n=163), but that AI will not cause unemployment in the future (35.3%, n=186), a robot cannot replace a veterinary surgeon with this technology (36.7%, n=194) and that they are not afraid of developments in the field of AI (36%, n=191). It can be said that a longer period is needed to observe the effects of this technology on unemployment in veterinary medicine in the future.

In various studies (7, 22, 25) that deal with the issue of AI in an ethical sense, it is generally stated that technology may create ethical problems in the future and that AI designs should be compatible with the ethical principles and moral values of human beings. Students in this study think that AI applications should be developed in an ethical sense (42.4%, n=223), that AI can cause ethical problems (39.3%, n=208) and that AI is beneficial as long as it does not harm humanity (50.3%, n=265). The findings from the study can be evaluated as that the students are aware of the ethical problems that may arise related to the designs of AI technologies, and if considered carefully in that sense, AI can be positively perceived by the students.

As a result, in this study, which deals with the opinions of veterinary faculty students on AI for the first time in Türkiye, it can be said that the students have partial knowledge about the subject and its use in veterinary medicine. Considering the benefits of AI to humanity, it can be argued that inclusion of this subject in the undergraduate and graduate curricula of veterinary faculties would be beneficial in terms of following the developments in the world. Large-scale studies detailing the subject are needed for the recognition and development of AI applications.

Ethical Statement

This study received ethical approval at the Ankara University Ethics Committee meeting dated 13.04.2022 with the decision number 07/70. In addition, additional permissions were obtained from the Dean's Office of the AUFVM for the application of the study to the students.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

NY conceived and planned the study. NY and ÖK conducted literature review and writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

References

1. **Adalı, E** (2017): *İnsanlaşan Makineler ve Yapay Zekâ*. İTÜ Vakfı Dergisi, **75**, 14-17.
2. **Akyüz HÖ, Alkan S, Yücebaş SC** (2021): *Sağlık Hizmetleri Meslek Yüksek Okulu Öğrencilerinin Yapay Zeka Hakkında Bilgi Düzeylerinin İncelenmesi*. Medical Research Reports, **4**, 28-35.
3. **Anonymous** (2022). Artificial intelligence in veterinary medicine leads to efficiency and superior accuracy. Available at https://www.idexx.com. (Accessed July 27, 2022).
4. **Anonymous** (2022): Veteriner Hekimlikte Yapay Zeka ve Dijitalizasyon Eğitim Programı Başarıyla Gerçekleştirildi. Available at http://www.veterinary.ankara.edu.tr/2021/04/06/veteriner-hekimlikte-yapay-zeka-ve-dijitalizasyon-egitim-programi-basariyla-gerceklestirildi/. (Accessed July 29, 2022).
5. **Attila AŞ** (2022): Yapay Zekâ Teknolojisi ve Uygulamaları, Dikeyeksen Yayıncılık, İstanbul.
6. **Bardot F** (2022): *Artificial intelligence, robots and unemployment: evidence from OECD countries.* JIE&M, **1**, 117-138.
7. **Bostrom N, Yudkowsky E** (2011): The ethics of artificial intelligence. 1-20. Cambridge Handbook of Artificial Intelligence, Cambridge University Press.
8. **Chen L, Chen P, Lin Z** (2020): *Artificial Intelligence in Education: A Review*. *IEEE Access*, **8**, 75264-75278.
9. **Chiacchio F, Petropoulos G, Pichler D** (2018): *The impact of industrial robots on EU employment and wages: A local labour market approach*. Bruegel Working Paper, **2**, 1-35.
10. **Choudhury A, Asan O** (2020): *Role of Artificial Intelligence in Patient Safety Outcomes. Systematic Literature Review*. JMIR Med Inform, **8**, 1-30.
11. **Coşkun F, Gülleroğlu HD** (2021): *Yapay Zekanın Tarih İçindeki Gelişimi ve Eğitimde Kullanılması.* AÜEBFD, **54**, 947-966.
12. **Çalık D, Çınar ÖP** (2009): Geçmişten Günümüze Bilgi Yaklaşımları Bilgi Toplumu ve İnternet. XIV. Türkiye'de İnternet Konferansı Bildirileri. 77-88. İstanbul, Türkiye.
13. **Çam MB, Çelik N, Göktepe ET, et al** (2021): *Öğretmen Adaylarının Yapay Zekâ Teknolojileri ile İlgili Farkındalıklarının Belirlenmesi*. M Kemal Üni Sosyal Bilimler Enstitüsü Dergisi, **18**, 263-285.
14. **Doğaner A** (2021): *The approaches and Expectations of the Health Sciences Students towards Artificial Intelligence*. Karya J of Health Sci, **2**, 5-11.
15. **Deng B** (2015): *Artificial intelligence joins hunt for human–animal diseases*. Nature News, 1-2.
16. **Dick S** (2019): *Artificial intelligence.* HDSR, **1**, 1-8.
17. **Dos Santos DP, Giese D, Brodehl S, et al** (2019): *Medical Students' attitude towards Artificial İntelligence: A Multicentre Survey*. Eur Radiol, **29**, 1640-1646.
18. **ECCVT** (2018): Report of The ECCVT Expert Working Group on The Impact of Digital Technologies & Artificial Intelligence in Veterinary Education and Practice.
19. **ECCVT** (2020): Report of The ECCVT Expert Working Group on The Impact of Digital Technologies & Artificial Intelligence in Veterinary Education and Practice, Final Report.
20. **Gökçen H, Gökçen İ** (2021): *Yapay zeka teknolojilerinin hayvan yetiştiriciliğindeki yeri ve önemi*. Rum Vet Hek Derg, **93**, 56-64.
21. **Hamet P, Tremblay J** (2017): *Artificial intelligence in medicine.* Metabolism Clinical and Experimental, **69**, 36-40.
22. **Keskinbora KH** (2019): *Medical ethics considerations on artificial intelligence*. Journal of Clinical Neuroscience, **64**, 277- 282.
23. **Kour S, Agrawa R, Sharma N, et al** (2022): *Artificial Intelligence and its Application in Animal Disease Diagnosis.* J Anim Res, **12**, 1-10.
24. **McCarthy J** (2007): What is Artificial Intelligence? Available at https://www-formal.stanford.edu/jmc/whatisai.pdf (Accessed July 20, 2022).
25. **Morone G, Pirrera A, Meli P, et al** (2022): *Ethics and Automated Systems in the Health Domain: Design and Submission of a Survey on Rehabilitation and Assistance Robotics to Collect*. Healthcare, 1-13.
26. **Mutascu M** (2021): *Artificial intelligence and unemployment: New insights*. Economic Analysis and Policy, **69**, 653-667.
27. **Nabiyev V, Erümit AK** (2020): Eğitimde Yapay Zeka Kuramdan Uygulamaya. Pegem Akademi Yayınları, Ankara.
28. **Öztürk K, Şahin Ergin M** (2018): *Yapay Sinir Ağları ve Yapay Zekaya Genel Bir Bakış*. Takvim-i Vekayi, **6**, 25-36.
29. **Park SH, Do KH, Kim S, et al** (2019): *What should medical students know about artificial intelligence in medicine?* J Educ Eval Health Prof, 1-6.
30. **Reddy S, Fox J, Purohit MP** (2019): *Artificial intelligence-enabled healthcare delivery*. J R Soc Med, **112**, 22-28.
31. **Rigby M** (2019): *Ethical dimensions of using artificial intelligence in health care.* AMA Journal of Ethics, **21**, 121-124.
32. **Russell S, Dewey D, Tegmark M** (2015): *Research Priorities for Robust and Beneficial Artificial Intelligence.* AI Magazine, **36**, 105-114.
33. **Shabbir J, Anwer T** (2015): *Artificial Intelligence and its Role in Near Future.* Journal of Latex Class Files, **14**, 1-11.
34. **Silva MD, Badaro R, Santos A, et al** (2019): Use of Artificial Intelligence on the Control of Vector-Borne Diseases. Vectors and Vector-Borne Zoonotic Diseases. Published February 20th, 2019.
35. **Sit C, Srinivasan R, Amlani A, et al** (2020): *Attitudes and Perceptions of UK Medical Students towards Artificial İntelligence and Radiology: a Multicentre Survey.* Insights Into Imaging, **11**, 1-6.
36. **Tamer HY, Övgün B** (2020): *Yapay Zeka Bağlamında Dijital Dönüşüm Ofisi*. Ankara Üniversitesi SBF Dergisi, **75**, 775-803.
37. **Ural DA** (2016): *The use of new practices for assessment of body condition score*. Rev MVZ Córdoba, **21**, 5154-5162.
38. **Wartman SA, Combs CD** (2018): *Medical Education Must Move From the Information Age to the Age of Artificial Intelligence.* Academic Medicine, **93**, 1107-1109.
39. **Yılmaz Y, Uzelli Yılmaz D, Yıldırım D, et al** (2021): *Yapay Zeka ve Sağlıkta Yapay Zekanın Kullanımına Yönelik Sağlık Bilimleri Fakültesi Öğrencilerinin Görüşleri.* SDÜ Sağlık Bilimleri Dergisi, **12**, 297-308.

Histochemical and immunohistochemical investigations on pyloric tonsil in Turkeys (*Meleagris gallopavo*)

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ABSTRACT

The structural patterns of pyloric tonsil in turkey (*Meleagris gallopavo*) were investigated in this study. Turkeys, less resistant to disease than chickens, are a crucial source of protein with low cholesterol levels and a high protein/calorie ratio. Pyloric tonsil, involved in the chicken defense barrier, is a component of GALT. To evaluate the specific region, adult turkeys were taken from slaughterhouses. Crossman's modified triple staining demonstrated the general histological structure, PAS revealed the density of goblet cells synthesizing mucus, and Safranin O showed the capsule around the follicles. The width of lymphoid follicles in the pyloric tonsil (204.0±22.33) differed significantly from that of the middle part of the duodenum (111.7±4.741). It was noticed that the specific area harbors T cells, B cells, and follicular dendritic cells. Interfollicular regions were infiltrated with $CD3^{+}$T cells, but the $CD268^{+}$B cells occupied primarily germinal centers. However, it was noticed that T cells were present in germinal centers and some B cells in the interfollicular region. Anti-Vimentin antibody revealed follicular dendritic cells in the pyloric zone. It was noted that the epithelial layer of the pyloric tonsil is devoid of CK18-positive cells. Findings of the semi-thin section indicated that some columnar epithelial cells were stained differently in the transition zone (lymphoepithelium). To sum up, a pyloric region in turkeys is structurally dissimilar to pyloric tonsils detected in chicken, chukar partridge, and duck. More research is needed to reveal the effect of pyloric tonsil on the alimentary tract in poultry in terms of defense mechanisms.

Introduction

The immune system acts as a defensive tool for preventing or limiting infections. It has been suggested that the genes regulating the immune system have made notable evolutionary progress to block pathogens. In this regard, the immune surveillance system can recognize effectively and eliminate pathogens. However, pathogens continue to evolve new tactics to evade the immune system (25). In response to pathogens escaping defending mechanisms, the host increases its resistance over time. This well-established co-evolutionary dynamic has been postulated as the "host-pathogen arms race (6).

The poultry immune system has provided a crucial model for basic immunological studies. Chickens (*Gallus gallus domesticus*) have been included in most immunological investigations. With the seminal findings of avian immunology studies, basic immunological concepts are better understood. Undoubtedly, the remarkable contribution of the studies is to reveal in detail the development of humoral and cellular (cellular) responses, which are the two main branches of acquired immunity. The identification of developing T and B cell-dependent lymphocyte lines has been achieved smoothly. Furthermore, poultry species have contributed to the development of vaccine science (5).

Avian immunity depends on the function of lymphoid organs categorized as primary and secondary. Primary lymphoid organs such as bursa Fabricius and thymus are immune-privileged areas for antigen-independent differentiation and lymphocyte proliferation. Spleen, harderian gland, CALT (Conjunctiva-associated lymphoid tissue), NALT (Nasal region-associated lymphoid tissue), BALT (Bronch-associated lymphoid tissue), and GALT (Gut-associated lymphoid tissue) are

classified as secondary lymphoid organs and are the hub of adaptive immune responses (18). Studies have shown that GALT plays a key role in inducing local immune responses and protecting against mucosal pathogens (15). GALT contains pharyngeal tonsils, esophageal tonsils, Peyer's patches, caecal tonsils, and Meckel's diverticulum (18). In addition to these lymphoid structures, proventricular lymphoid tissue is involved in the poultry defense system (22).

The pyloric tonsil was first defined at the initial segment of the duodenum in a study performed on chickens. It has been reported that Lieberkühn crypts in this tonsillar area transformed into tonsillar invaginations, and the surface of the cryptic zone is covered with lymphoepithelium. Moreover, it was clarified that the localization of the lymphoid tissue of the pyloric region, unlike Peyer's patches on the antimesenteric side of the intestine, lies the entire wall of the intestine, thus forming a complete ring. In addition, it has been determined that the pyloric region contains at least 15-20 tonsillar units (16). Similarly, Gedam et al. (10) and Arugh and Hamedi (1) detected pyloric tonsillar units in ducks and chuckar partridges, respectively. It has been stated that tonsillar focal units were located in the lamina propria, especially adjacent to the muscularis mucosa layer. In addition, the capsular network surrounding each lymphoid structure fused with the connective tissue of the lamina muscularis layer (16). It has been declared that these tonsillar units were encountered in the submucosa and even in the lamina muscularis layer of ducks (10).

It is assumed that this lymphoid tissue has two pivotal functions. Firstly, the tonsillar region is an essential lymphoid structure and is heavily exposed to environmental antigens. Secondly, tonsillar areas may participate in the development of B-lymphocytes occurring mainly in Peyer's patches (18). Oláh et al. (17) stated that the tonsils function as a "gateway" for antigens. They stressed that an influx of antigens or allergens in the pyloric tonsil constantly stimulates the immune system and keeps it active.

This study, which was a determined road map based on previous studies revealing general features of the pyloric tonsil of avian species, aimed to define the structural characteristic of the pyloric tonsil in turkeys. It was planned to perform histochemical and immunohistochemical examinations to understand that this region is organized lymphoid tissue rather than lymphocyte infiltration.

Materials and Methods

Sampling: Both sexes of American bronze turkeys were utilized in this study [male: five adults (10-12 kg); female: five adults (8-9 kg)]. Ankara University Animal Experiments Local Ethics Committee approved this descriptive study (Ethics committee approval date: 15.03.2017, Decision number: 2017-6-50). The pyloric tonsilar region, located at the beginning of the duodenum, was taken from a total of 10 animals slaughtered in several slaughter houses around Ankara province. Both routine histological and electron microscopic fixation protocols were applied to tissue materials to reveal general structural configuration.

Preparation of samples for paraffin embedding and histochemical staining: The tissues were fixated with neutral buffered formalin (NBF) for 24 hours to detect normal histological structure. After the fixation process, they were exposed gradually to alcohol series (70%, 80%, 96%, and 100%) and immersed in methyl benzoate and benzol series. Following routine tissue processing steps, tissues were embedded in paraffin wax (2). Tissue blocks were sectioned by using the microtome (Leica RM2125 RTS). Afterward, 5-6 µm sections were kept in an oven (Core EN 500P) at 37 °C for 24 hours. Before staining paraffin sections, they were cleaned in xylene and rehydrated by immersing in serial dilutions of ethyl alcohol and water mixture. In the subsequent step, Crossman's modified triple staining method, PAS (Periodic acid-Schiff), and Safranin O staining methods were applied to different sections (2). Finally, they were photographed with a light microscope (Leica DM2500).

Immunohistochemical staining: To evaluate the pyloric tonsillar area immunohistochemically, tissue samples were firstly fixated with 10% NBF solution. Routine histological preparation protocol was conducted on samples, followed by tissue embedding step. After sectioning, serial paraffin sections (5µm thickness) mounted on poly-lysine-coated slides were used for immunohistochemical investigations. They were dried in the oven at 37 °C for 24 hours. Afterward, the sections were put in the oven at +58 °C for 30 to thaw parafin wax. After deparaffinization with xylene and alcohol series, they were washed in PBS for 5 min twice. Next, antigen retrieval was performed in 0.01 M citrate buffer (pH 6) at 100 °C for 20 min and then cooled at room temperature. 3% hydrogen peroxide solution were applied to sections for 20 minutes to prevent the activation of endogenous peroxides. Antibodies were diluated at a ratio of 1:50 for Anti-CD79A, Anti-Vimentin, and Anti-cytokeratin18 (CK18), 1:200 for Anti-CD268, and 10 µg/ml for Anti-CD3ϵ (Table 1). Prior to treated with primary antibody, serum blocking (Ultra V Block, TA-060-UB, Thermo Fisher Scientific) was performed in a humid chamber for 10 min. Following blocking nonspecific binding, antibody incubations were applied overnight at +4 °C and PBS were dropped on the negative controls. They were then incubated with biotinylated Goat Anti-Polyvalent secondary antibody (TP-060-BN, Thermo Fisher

Scientific) at room temperature for 30 min. Biotin-specific HRP (Horseradish Peroxidase) with Streptavidin was treated for 30 minutes. After this step, AEC (3-amino-9-ethyl carbazole) chromogen was applied to the sections in the dark for 1-5 minutes, and 3,3'-Diaminobenzidine (DAB) chromogen was performed on the section to which Anti-CD268 primer antibody was dropped only. Cell nuclei were stained with Gill's hematoxylin for 5 min. Subsequently, samples were coated with coverslips. Finally, images were captured under a light microscope (Leica DM2500).

Semi-thin sections: Additional tissue samples collected from a male and a female animal were prepared for the ultrastructural examination. For this purpose, tissues were immersed in glutaraldehyde-paraformaldehyde solution for 2-6 h. Washing step was carried out with 0.1 M Cacodylate buffer (pH 7.4) for 3 h. They were post-fixed in 1% osmic acid at 2 h. Removing osmic acid was achieved using cacodylate buffer for 20 min. The tissues were soaked in 30, 50, and 70% alcohol for 30 min, respectively. After immersing into uranyl dye, they were kept at +4°C for 2 h. Before embedding with Araldite, the tissues were rinsed with 96, 100, and propylene oxide for 1 h. Serial sections (1 µ thickness) were obtained by using a pyramitome, and then dyed with Toluidine blue. The sections were closed and photographed using a Leica DM2500 brand-fluorescent filter attachment microscope. Light micrograph images were acquired with the help of Leica DM2500 microscope.

Statistical Analysis: Statistical analyses were performed using GraphPad Prism (Version 5.00) software. Data (n=4) were given as mean ± standard error (SE). The diameter of lymphoid follicles was analyzed with the nonparametric Mann-Whitney U test to evaluate differences between the initial (pyloric region) and middle areas of the duodenum. Statistical significance was interpreted according to $P<0.05$ level.

Results

Triple staining: In this study, it was observed that a transitional region defined as the pyloric tonsil and localized at pyloroduodenal junction was filled with tonsillar units in turkeys. It was detected that lymphoid follicles and lymphoepithelium were primary components of the pyloric tonsil present at the initial segment of the duodenum. Lymphoid follicles in the tonsillar region of turkeys were scattered along the entire junctional wall rather than in the upper or lower part of the intestine, thus forming a complete ring. Lymphoid follicles (Width: 204.0±22.33) were located in the villi rather than in Lieberkühn crypts. It was noticed that lamina propria of villi contained 1-2 or more lymphoid follicles (3-5) of variable diameter. Additionally, the villi tended to expand in different directions, depending on the number and diameter of the follicles. It was noted that the number of follicles has a spatial relationship with the localization of lymphoid tissue. In this context, 1-2 follicles occupied the middle part of a villus, while 3-5 follicles were distributed from the bottom to the tip of a villus. This was a crucial indicator for the villus to demonstrate a capability of compensating for configurational alterations. However, some villi are devoid of lymphoid follicles (Figure 1. A-C). The epithelial transformation was another finding of this study. The epithelial cell layer lining the villi containing the lymphoid follicle exhibited a gradual alteration from columnar to squamous. However, this lymphoepithelial change did not occur along the entire length of the villi, the lymphoepithelium was detected commonly at contact sites where an epithelial layer of a villus fused with an epithelial layer of another villus. Finally, it was determined that goblet cells were in this area (Figure 1. D).

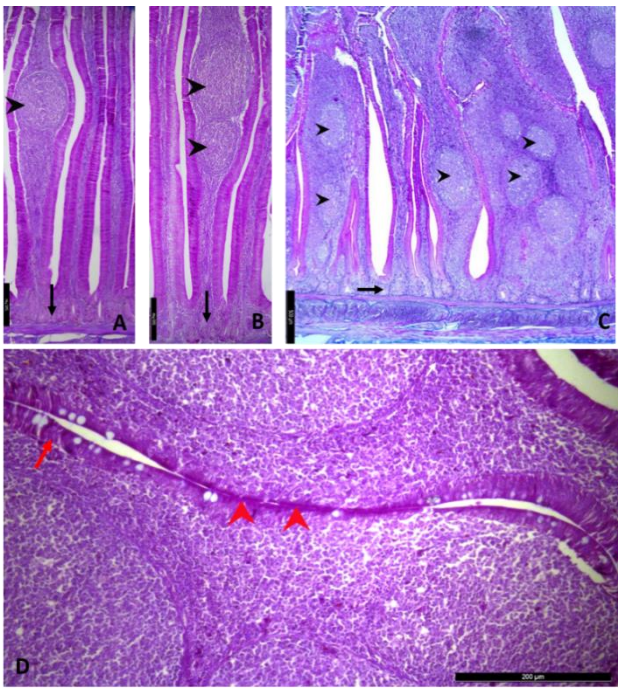

Figure 1. General view of the pyloric tonsil area.
A-B: Lymphoid follicles in villi, X100. C: Villi of different diameters in the pyloric tonsil area, X50. D: Lymphoepithelium, 200X. Staining: Crossmann's modified triple. Black arrow: Lieberkühn crypt. Black arrowhead: Lymphoid follicle. Red arrow: Simple columnar epithelium. Red arrowhead: Lymphoepithelium.

Safranin O and PAS staining: To visualize a capsular network around the germinal center and yield reliable results, the Safranin O staining procedure was applied on sections. The presence of a capsule around the germinal center was detected. It was revealed that the capsule reacting positively to Safranin O was stained pink (Figure

2. A). After the junction area, it was noticed that the lymphoid follicles (Width: 111.7±4.741) in the villi decreased both numerically and volumetrically in Figure 2-B. Width of lymphoid follicles in middle portion of duodenum showed statistical difference ($P<0.05$) with that of pyloric tonsillar follicles (Figure 3). The villi were devoid of fusion sites any longer. Another result of the PAS staining method was the detection of goblet cells in the villous epithelium covering lymphoid follicles. It was determined that the capsule, which reacted positively with safranin O, was also stained with PAS (Figure 2. C).

Immunohistochemical staining: Results obtained by immunohistochemical stainings are summarized in Table 1. Detection of T cells in the pyloric tonsil region was achieved using an Anti CD3ϵ antibody. From a general perspective, lymphoid follicles are localized along the pyloric region of the digestive tract to form a ring-shaped structure. This finding was compatible with the triple result. Black arrows represented the interfollicular area and black arrowheads demonstrated positive T cells in the germinal center in Figure 4. CD3+ T cells populated widely in the interfollicular area, but small quantities of T cells were also determined in germinal centers (Figure 4. A-B). Apart from this, T lymphocytes (red arrowhead) were detected in the intraepithelial area (Figure 4. A).

Two different clones were exerted to show B lymphocytes in the region. Anti-CD268 BAFF-R (MCA6011GA) was conjugated with CD-268, thereby giving a positive reaction. Conversely, the Anti CD79a-HM57 (NB100-64347) clone failed to bind to the cell surface antigen. Unlike T lymphocytes expressing CD3, B lymphocytes expressing CD268 antigen were primarily observed in the germinal center of the follicle (black arrow). They were also present in the interfollicular areas where T cells were abundant. In other words, $CD268^{+}$ B cells were noticed both in the follicle and the interfollicular area (Figure 5. A-C).

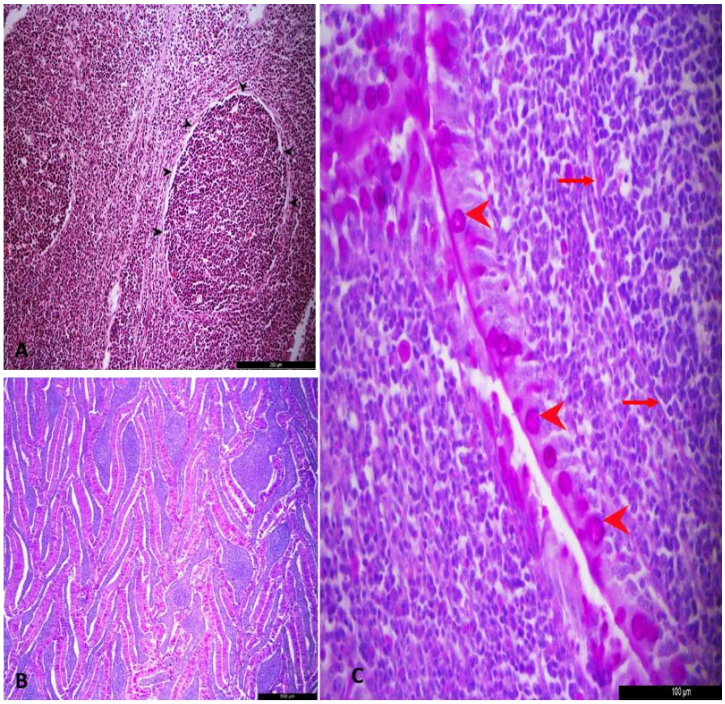

Figure 2. A: Image of a capsular structure around a follicle, X200. Staining: Safranin O. Black arrowhead: Capsule. B: The general structural patterns of the villi in the area slightly ahead of the pyloric tonsil region and the showing of considerably shrunken lymph follicles in some villi by PAS staining, X50. C: Presence of capsule and distribution of goblet cells in pyloric tonsil, X400. Staining: PAS. Red arrow: Capsule. Red arrowhead: Goblet cell.

Table 1. Antibody types, dilutions, and immunohistochemical staining results.

Antibody	Type	Clone	Dilution	Result
Anti-CD3ϵ	Polyclonal	CD3 A0452 Dako	10 µg/ml	Positive
Anti-CD79a	Monoclonal	Anti CD79A-HM57 (NB100-64347)	1:50	Negative
Anti-CD268	Monoclonal	Anti CD268 BAFF-R (MCA6011GA)	1:200	Positive
Anti-Vimentin (RV202)	Monoclonal	OMA 1-06001	1:50	Positive
Anti-CK18	Monoclonal	Anti Cytokeratin18 (NBP1-97715)	1:50	Negative

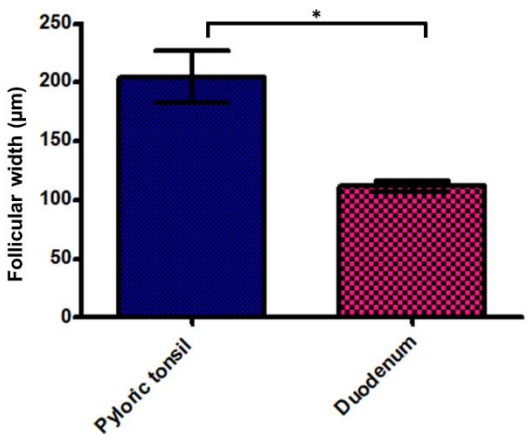

Figure 3. The width of lymphoid follicles measured in pyloric area and midportion of duodenum.
* P<0.05. Mann-Whitney U test was used.

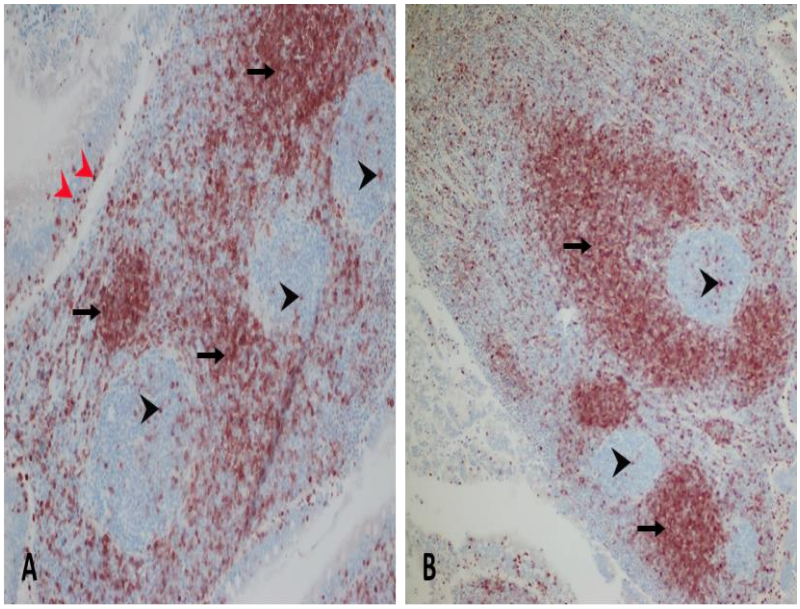

Figure 4. Demonstration of positive reaction in different regions and at different magnifications.
A: Detection of $CD3^+$ T cells in interfollicular region (black arrow) germinal center (black arrowhead) and epithelium (red arrowhead), 100X. B: Manifestation of $CD3^+$ T cells in interfollicular region (black arrow) and germinal center (black arrowhead), 200X.

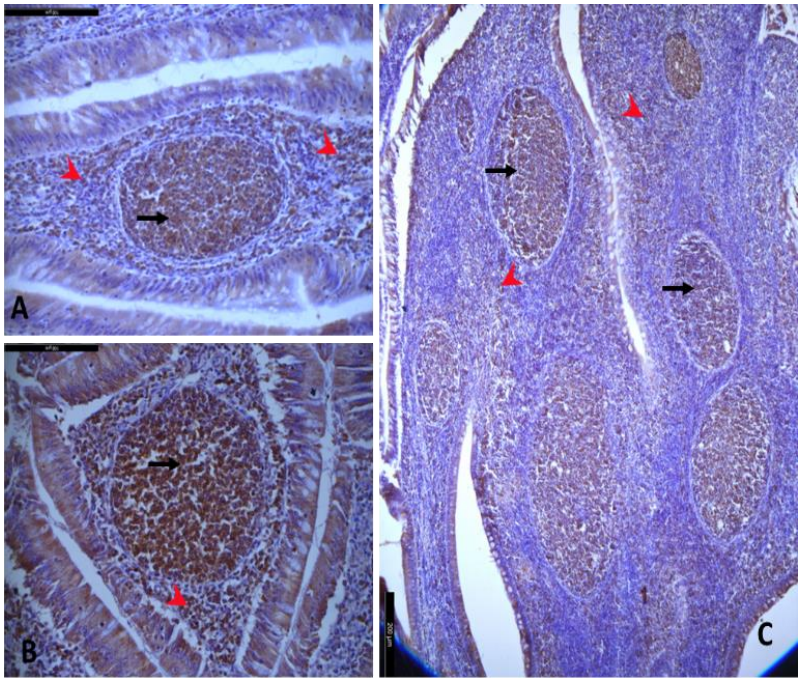

Figure 5. Detection of positive reactions in three different areas and at different magnifications.
A-B: Localization of CD268+ B cells in germinal center (black arrow) and interfollicular area (red arrowhead), X400. C: CD268+ B cells in germinal center (black arrow) and interfollicular area (red arrowhead), X100.

To identify follicular dendritic cells in the pyloric tonsil, serial sections were incubated with Anti-Vimentin antibody. As a result of immunohistochemical staining, it was noted that follicular dendritic cells (black arrows), an antigen-presenting cell in the follicles, were stained positively. Similarly, positive reactions were observed in some other cells (black arrowheads) in the outer part of the follicle (Figure 6). The reason why those cells showed positive reactions may be that vimentin is one of the basic elements of the cytoskeleton.

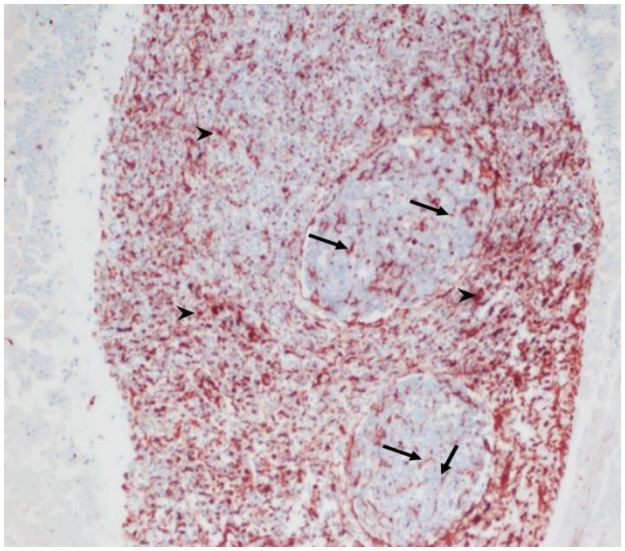

Figure 6. Localization of follicular dendritic cells in the pyloric tonsil, X200.
Black arrow: Follicular dendritic cells. Black arrowhead: Other cells expressing Vimentin.

The main purpose of using the anti-CK 18 antibodies was to reveal the localization of M cells in the lymphoepithelium. However, no cells expressing cytokeratin 18 were detected in the epithelial layer. This result indicated that M cells in the pyloric tonsil could not synthesize the intermediate protein.

Semi-thin sections: The presence of lymphoepithelial transformation in semi-thin sections stained with toluidine blue was detected at various magnifications (200X, 400X, and 1000X). Both the preparatory phase and the places where the transformation from the columnar epithelium to the lymphoepithelium took place were determined in the same area (200X). A group of blue-stained prismatic cells, which were the same size as the normal single-layered columnar epithelium and were thought to be preparing to transform into lymphoepithelium, were observed (Figure 7. B). The color of these epithelial cells changed from white to blue. It was assumed to be the case that cells first altered their functional character and then shortened their length. The localization of squamous epithelial cells, which we previously found out with triple staining (Figure 1), was revealed in more detail with 1µ sections. It was noticed that lymphocytes migrating inside lymphoepithelium were packed in the epithelium. The number of these lymphocytic cells (black arrowheads) reached roughly 5-6 (Figure 7. C). It has been concluded that the epithelial cells associated with lymphoid follicles may function the way packaging lymphocytes similar to M cells.

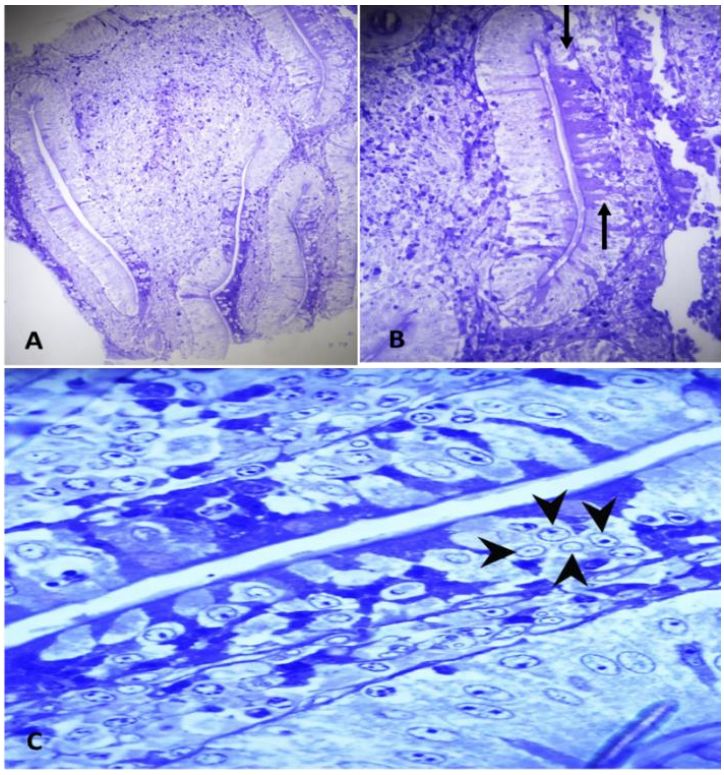

Figure 7. Demonstration of lymphoepithelium in serial semi-thin sections (Staining: Toluidine Blue).
A: Detection of general structural features, 200X. B: Presence of a group of blue-stained prismatic cells, 400X. Black arrow: Epithelial area occupied by blue-stained prismatic cells. C: Manifestation of lymphocytes packed by epithelial cells, 1000X. Black arrowhead: Intraepithelial lymphocytes.

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Discussion and Conclusion

The pyloric tonsil, absent from mammalian species, is located in the entrance region of the duodenum and was first determined in chickens. Researchers suggested that the pyloric tonsil takes part in fighting against pathogens (4, 16). After revealing this structure in chickens, it has been a matter of curiosity whether this tonsillar area is similar in other poultry. For this purpose, similar studies were carried out in ducks (10) and chukar partridges (1), and the presence of the pyloric tonsil in these poultry species was detected. No study has been found in the literature on the histological structure of this region in turkeys. In the present study, the histological structure of the part referred to as the pyloric tonsil region in turkeys was unveiled and discussed with literature data in this area. It has been stated that these tonsillar units consisting of 15-20 lymphoid follicles are localized in the intestine's upper and lower walls, while lymphoid follicles in the ileum and cecum are located in the anti-mesenteric region (16, 18). Our findings regarding the distribution of lymphoid tissue in turkeys were similar to the results of other studies.

It has been reported that the digestive and respiratory tracts contain tonsillar structures since they are exposed to the invasion of many foreign antigens (4). Tonsils are generally defined as lymphoid follicles located within the mucosal layer (7, 21). Perry (19) mentioned crypts ending with many blind ends in the tonsillar region, their lateral branches, and lymphoid tissues in these parts. However, Oláh et al. (17) described the tonsil as a complex structure containing a depression called a crypt surrounded by dense lymphoid tissue. Casteleyn et al. (4), conducted on esophageal, proventricular, pyloric, and caecal tonsils, stated that lymphoid tissues were all observed at the cryptic regions and close to muscular mucosae. Considering the results of this study, the localization of lymphoid tissue in the pyloric tonsil region was different. In this context, it was determined that the villi and crypts in the pyloric tonsil region of turkeys were similar to a normal intestinal structure. The lymphoid follicles were mostly located at different levels within the villi rather than the crypts. Furthermore, it was revealed that these lymphoid follicles give rise to a volumetric increase and shape alteration in villi. All the dissimilarities mentioned above have been one of the main differences between our findings and other studies.

On the one hand, Nagy and Oláh (16) stated that Lieberkühn crypts transformed into lymphoepithelial tonsillar crypts and that there were primary and secondary lymphoid follicles and interfollicular regions in the lamina propria. On the other hand, Gedam et al. (10) stated that there are lymphoid follicles in the submucosa and even in the muscularis mucosa. In this study, lymph follicles were not detected along the crypts. Moreover, no lymphoid follicles were observed in the muscularis mucosa or the submucosa. It has been revealed that there is a positive relationship between the number of lymph follicles and their localization. Accordingly, it was determined that lymphoid follicles (1-2) were commonly located in the middle part of a villus and had a different localization (from the base to the tip) into a villus, depending on the number of follicles. Apart from this, the width of lymphoid follicles in the pyloric tonsil (204.0±22.33) differed significantly from that of the middle part of the duodenum (111.7±4.741). The reason why there are larger follicles in the pyloric tonsil may be that it may be exposed to a high microbial load. Unlike other studies (1, 4, 10, 16) 5-6 lymph follicles were observed in a villus. In addition, it has been demonstrated that the villi can expand depending on the localization of the lymph follicles.

Nagy and Oláh (16) reported that most of the circular-shaped germinal centers of lymph follicles are located close to the muscularis mucosa of the intestine. They stressed that the capsule, which consists of type III collagen around these follicles, fuses with the connective tissue capsule of the muscularis mucosa layer. Nonetheless, no fusion has been noted based on the difference in the location of the lymphoid follicles in the present study. On the other hand, the capsule surrounding one germinal center did not merge with that of another germinal center in conjunction with the findings of other researchers (10, 16).

Mason et al. (14) stated that B cells, including mammalian progenitor B cells, express the CD79a antigen. Furthermore, the expression of CD79a antigen on the surface of B cells was detected at all stages of the differentiation process involving plasma cells. However, it was revealed that B-lymphocytes in the turkey pyloric tonsil did not synthesize this receptor in the study. To identify B cells in the region, CD268 antigen expression of peripheral B cells (20) was demonstrated immunohistochemically. B cells were densely located in germinal centers. The presence of positive-reacting B cells in the interfollicular areas, the T-lymphocyte region, revealed similarity with the BALT tissue study (8). These lymphocytes were also found in the intraepithelial area. Oláh et al. (18) stated that T cells accumulate T cell-dependent regions, interfollicular areas, in the caecal tonsils and Peyer's plaques. Similarly, Nagy and Oláh (16) showed that T cells densely located in interfollicular regions stained positively with anti-CD3 antibodies. Apart from this, they revealed that T cells were also present substantially in the germinal center. The presence and localization of CD3+ T cells in turkeys were similar to other studies.

It has been reported that heterogeneous intraepithelial lymphocytes settle between epithelial cells in the mucosal layer along the gastrointestinal tract (9).

Generally, T cells inhabit the epithelial layer, while B-lymphocytes mostly remain in the lamina propria (11). Vervelde and Jeurissen (24) noticed that the stratified squamous epithelium of the esophagus and the simple columnar epithelium of the intestines harbor lymphocytes. CD3+ intraepithelial lymphocytes are predominantly CD8a+ cells, most of which express TCRγδ (3). In the current study, localization of CD3+ T-lymphocytes in the epithelial layer was also observed in turkeys.

Follicular dendritic cells (FDCs), a rare type of stromal cell, resides in follicles of secondary lymphoid tissues. FDC, which defines the light zone of the GC, is crucial for GC formation and maintenance. They bind and store antigens in the form of immune complexes (ICs) for presentation to GC B cells (23). In the study revealing the pyloric tonsil region for the first time in chickens (16), the presence of vimentin intermediate filament in follicular dendritic cells was indicated. Follicular dendritic cells expressing vimentin were also detected within the germinal centers in the present study.

Nagy and Oláh (16), conducted on chickens, stated that a capsule encircled germinal centers of lymph follicles in the pyloric tonsil region. Likewise, it was observed that the capsule surrounded lymph follicles of the pyloric area in turkeys. Hondo et al. (12) stated that they observed CK18-positive M cells in the follicle-associated epithelium of the jejunal and ileal region in cattle. As a result of examining semi-thin sections, some specialized epithelial cells packaging of a few lymphocytes were noticed in this study. That is why they were considered M-cell-like cells. However, the sections incubated with CK18 stained negatively.

While lymphoepithelium was observed along the crypt in the tonsils (17), it was reported that lymphoepithelial transformation was detected only dome region in the ileum (13). In this study, lymphoepithelium was observed in the contact site of the villi. It was revealed that the simple columnar epithelium gradually became the lymphoepithelium in semithin sections.

In conclusion, the pyloric tonsil, stated in the literature to be related to the defense mechanism, localized between the stomach and duodenum, structurally different from the duodenum and pylorus, has been revealed by using histological and immunohistochemical methods in turkeys. Contrary to other studies, it was observed that the pyloric tonsil region of turkeys was in a typical intestinal structure. Furthermore, it was determined that the lymphoid follicles, which are the main components of this region, were not located at the crypt as claimed in the literature. As a result of the examination, a remarkable finding was revealed that follicles developed in different numbers and sizes within the villi. Further studies, however, are required elaborately to demonstrate the defense mechanism of the digestive tract in poultry.

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Conflict of Interest

The author declared that there is no conflict of interest.

Author Contributions

NY conceived and planned the experiments, carried out the experiments, interpreted the results, and took the lead in writing the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

The author confirm that he has adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Arugh PA, Hamedi S** (2019): *A histomorphometric study on age-related changes in selected lymphoid structures of Chukar partridge (Alectoris chukar).* Iran J Vet Res, **20,** 186-191.
2. **Bancroft JD, Gamble M** (2008): Theory and Practice of Histological Techniques. 6th edn. Churchill Livingstone, Elsevier, China.
3. **Bucy RP, Chen CL, Cihak J, et al** (1988): *Avian T cells expressing γδ receptors localize in the splenic sinusoids and the intestinal epithelium.* J Immunol, **141**, 2200-2205.
4. **Casteleyn C, Doom M, Lambrechts E, et al** (2010): *Locations of gut-associated lymphoid tissue in the 3-monthold chicken: a review.* Avian Pathol, **39**, 143–150.
5. **Davison F, Kaspers B, Schat KA, et al** (2011): Avian Immunology. 1st edn. MA: Elsevier/Academic Press, Boston.
6. **Decaestecker E, Gaba S, Raeymaekers JA, et al** (2007): *Host-parasite 'Red Queen' dynamics archived in pond sediment*. Nature, **450**, 870-873.
7. **Eurell JA, Frappier BL** (2006): Textbook of Veterinary Histology, 6th edn. Blackwell Publishing Ltd, Oxford.
8. **Fagerland JA, Arp LH** (1993): *Structure and development of bronchus-associated lymphoid tissue in conventionally reared broiler chickens.* Avian Dis, **37**, 10-18.

9. **Gartner LP, Hiatt JL** (1997): Digestive system II– Alimentary Canal. Color Textbook of Histology. 1st edn. PA: Saunders, Philadelphia.
10. **Gedam PM, Nandeshwar NC, Salankar AM, et al** (2018): *Histomorphological and histochemical studies on lymphoid tissue of pyloric region in khaki campbell breed of duck (Anas platyrhynchos).* J Entomol Zool Stud, **6**, 1222-1224.
11. **Guy-Grand D, Cerf-Bensussen N, Malissen B, et al** (1991): T*wo gut intraepithelial CD8+ lymphocyte populations with different T cell receptors: a role for the gut epithelium in T cell differentiation.* J Exp Med, **173**, 471-481.
12. **Hondo T, Kanaya T, Takakura I, et al** (2011): *Cytokeratin 18 is a specific marker of bovine intestinal M cell.* Am J Physiol Gastrointest Liver Physiol, **300**, G442-G453.
13. **Kido T, Ohwada S, Watanabe K, et al** (2003): *Differential characteristics of microfold cells on the dome epithelium of porcine ileum*. Anim Sci J, **74**, 375-382.
14. **Mason DY, Cordell J L, Brown MH, et al** (1995): *CD79a: a novel marker for B-cell neoplasms in routinely processed tissue samples.* Blood, **86**, 1453-1459.
15. **Mörbe UM, Jørgensen PB, Fenton TM, et al** (2021): *Human gut-associated lymphoid tissues (GALT); diversity, structure, and function.* Mucosal Immunol, **14**, 793-802.
16. **Nagy N, Oláh I** (2007): *Pyloric tonsil as a novel gut associated lymphoepithelial organ of the chicken.* J Anat, **211**, 407-411.
17. **Oláh I, Nagy N, Magyar A, et al** (2003): *Esophageal tonsil: a novel gut associated lymphoid organ.* Poultry Sci, **82**, 767–770.
18. **Oláh I, Nagy N, Vervelde L** (2014): Structure of the avian lymphoid system. 11-44. In: Avian Immunology. F Davison (Ed), B Kaspers (Ed), KA Schat (Ed), P Kaiser (Ed). Elsevier, Philadelphia.
19. **Perry ME** (1994): *The specialised structure of crypt epithelium in the human palatine tonsil and its functional significance.* J Anat, **185**, 111-127.
20. **Reddy SK, Hu T, Gudivada R, et al** (2008): *The BAFF-Interacting receptors of chickens.* Dev Comp Immunol, **32**, 1076-1087.
21. **Samuelson DA** (2007): Textbook of Veterinary Histology. Saunders– Elsevier, St. Louis, Missouri.
22. **Somayeh H JL** (2021): *Histological characterization of the gut-associated lymphoid tissue in three-month old guinea fowls (Numida meleagris).* Philipp J Vet Med, **58**, 96-100.
23. **van der Poel CE, Bajic G, Macaulay CW, et al** (2019): *Follicular dendritic cells modulate germinal center B cell diversity through FcγRIIB*. Cell Rep, **29**, 2745-2755.
24. **Vervelde L, Jeurissen SHM** (1993): *Postnatal development of intra-epithelial leukocytes in the chicken digestive tract: phenotypical characterisation in situ.* Cell Tissue Res, **274**, 295-301.
25. **Woolhouse ME, Webster JP, Domingo E, et al** (2002): *Biological and biomedical implications of the co-evolution of pathogens and their hosts.* Nat Genet, **32**, 569-577.

Investigation of the biocompatibility and in vivo wound healing effect of *Cotinus coggygria* extracts

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ABSTRACT

Cotinus coggygria is widely recognized its antiseptic, anti-inflammatory, antimicrobial, antihemorrhagic, and wound-healing properties. In this, aimed to evaluate the phenolic contents, cytotoxicity/proliferation, hemolytic, antimicrobial, genotoxic, apoptotic, necrotic activities, and *in vivo* wound healing effects of *C. coggygria*, a plant species known to have beneficial effects on wound healing. TOF-LC/MS analyzes revealed that the methanol extract of *C. coggygria* leaves contained flavonoids and phenolic compounds such as gallic acid (18.5 mg/kg), catechin (4.6 mg/kg), protocatesic acid (0.6 mg/kg), vanillic acid (8.4 mg/kg), ellagic acid (0.1 mg/kg), rosmarinic acid (0.1 mg/kg), quercetin (15 ppb) and *C. coggygria* stems contained such as gallic acid (24.6 mg/kg), catechin (155.1 mg/kg), chlorogenic acid (1.9 mg/kg), 4-hydroxybenzoic acid (383.3 mg/kg), rutin (2.5 mg/kg), ellagic acid (15.1 mg/kg), apigenin 7-glycoside (10.5 mg/kg), rosmarinic acid (0,4 mg/kg), quercetin (15.2 mg/kg), naringenin (279.1 mg/kg). Consequently, C. coggyria has a positive effect on wound healing with antibacterial properties, particularly against E. coli, and without cytotoxic, genotoxic, or hemolytic effects at test concentrations. In the *in vivo* burn model, wounds treated with leaf and stem extracts healed faster than the control group. Thus, *C. coggygria* is an effective plant for wound healing with antibacterial properties, particularly against E. coli, and without cytotoxic, genotoxic, and hemolytic effects.

Introduction

The skin is the human body's largest organ, providing protection to internal organs from microbial infection, mechanical damage, radiation, and extreme heat (34). The breakdown of the anatomical and physiological integrity of the skin due physical damage is called a “wound” (7). There are wound types determined according to different characteristics such as acute wound, chronic wound, burn wound and necrotic wound (3, 7, 38). Acute wounds are characterized by a healing process that can be completely healed by the body and not longer than 8 weeks while chronic wounds take a minimum of 12 weeks to heal. A burn is an injury to the skin caused by thermal, electrical, chemical, or radiation. Dry or wet sources account for 80% of all burns (19, 35). Burn wounds are classified based on the layer of skin affected as first, second, third and fourth degree burns. First-degree burns affect only the epidermis while second-degree burns are partial thickness and involve the epidermis and dermis. In third-degree burns, the reticular part of the dermis is affected and in fourth-degree burns the skin and tissues such as muscle and bone are damaged (31). Wound healing is a complex process that involves the systematic work of many cells, in which a series of cellular, physiological and biochemical events enabling the damaged tissue to regain its structure and function (18, 23, 33). The inflammation phase is the first phase of the wound healing process beginning after the injury and lasting 4-6 days during which a vascular and cellular response develops. The first event that occurs when bleeding is stopped is hemostasis.

During the proliferative phase, there is the development of granulation tissue to fill the wound cavity, along with the formation of epithelium to line the wound site. The formation of granulation tissue includes the proliferation of fibroblasts, the deposition of collagens and other extracellular matrixes, and the development of new blood vessels. When new tissue is formed in the wound area, the matrix formation and remodeling phase begins by restoring the structural integrity and functionality of the tissue (25, 32, 41). Depending on the condition of the wound, the selection of methods for wound healing is crucial to the healing process as it can reduce the risk of complications, sped up the wound healing process or minimize scar formation after complete healing. Skin grafts, wound dressings, cell therapy, hyperbaric oxygen therapy, ozone therapy and medicinal plants are used in wound healing treatment (30, 39). Using medicinal plants or herbal products in the treatment of various skin problems has become widespread, especially in recent years (17). Many herbs are of great importance in the wound healing process as they support natural repair mechanisms. It is used as a wound healing agent thanks to bioactive molecules such as flavonoids, tannins, terpenoids, saponins and phenolic compounds in medicinal plants. The purpose of using medicinal plants in wound healing is to contribute to the wound healing process by facilitating blood clotting, fighting infection, and speeding up wound healing (8, 15, 22). The genus *Cotinus* and *Cotinus coggygria* species are widely distributed in southern Europe, the Himalayas, Southwest China, and Southwest America. *C. coggygria Scop*. (Anacardiaceae), as is growing common in some parts of Turkey, in some parts of Balkans, in the Himalayas, is a plant that grows in the southwest of China and the United States. In traditional medicine, the plant is used for its anti-inflammatory, antimicrobial, antiseptic, antipyretic, antidiarrheal, antihemorrhagic and wound-healing properties. According to the researches, some phenolic compounds isolated from the *C. coggygria* plant have been determined to be effective in wound healing (1, 2, 10, 26, 27). The study aimed to investigate the *in vitro* biocompatibility and antimicrobial activity of extracts of *C. coggygria* (leaves and stem parts). In addition, the study aimed to demonstrate the proteins that play a critical role in wound healing by immunocytochemical techniques, to determine gylcosaminoglycans (GAGs) expressions and to investigate the *in vivo* wound healing properties.

Materials and Methods

Materials: *Cotinus coggygria* collected from Kümeevler locality in Akyurt district of Ankara in June 2013. Voucher number: M.Türk 6089 (ADO: Kırıkkale University, Faculty of Arts and Sciences Department of Biology Anatolian Herbarium). DMEM, FBS, Tyrpsin/EDTA solution and Penicillin streptomycin used in cytotoxicity and genotoxicity testing were obtained from Biological Industries (USA). For antibacterial analyses, the cultures of *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *C. albicans* (ATCC 10231) were taken from Kırıkkale University Scientific and Technological Research Laboratories (KÜBTUAM). The solid and liquid broth used in antibacterial tests and bacterial culture were obtained from Sigma-Aldrich (Germany). For cell proliferation assay e-plate was obtained from Elips (Roche). Hemolysis test standards-chemicals and DMMB assay chemicals were obtained from Sigma-Aldrich (Germany).

Cell Culture: L929 fibroblast cells obtained from the cell culture collection of the ŞAP Institute in Turkey were used. L929 cells were cultivated in DMEM (Biological Industries, USA) medium supplemented 10% Fetal bovine Serum (Biological Industries, USA) and 1% penicillin and streptomycin (Biological Industries, USA) in a humidified incubator at 37°C and 5% CO_2 atmosphere.

Preparation of Extracts: Plant extracts were prepared by two different methods. The first of the methods used was to prepare the water extract by boiling the leaves and stems of the *C. coggygria* at 6 g/L for 15 minutes. The second method is the preparation of methanol extract by approximately 10 g of the leaves and stems of the *C. coggygria* were taken and extracting in 100 ml of methanol with an automatic extraction system (BÜCHİ, B-811) for 2 hours. After extraction, solid extract was obtained by removing methanol with a rotary evaporator (BÜCHİ, R210) system (24).

TOF-LC / MS Analysis: Quantitative analysis was performed by TOF/LC-MS to determine the phenolic compounds and flavonoids contained in *C. coggygria*. Separation was performed on the ZORBAX SB column. After the analysis protocol was determined as mobile phase and gradient program, it was carried out in positive ion mode in 30 minutes (13).

In vitro WST-1 Assay for Cytotoxicity: L929 fibroblast cells were placed per well ($10x10^3$ cells) in 96 well plates. After the cells were incubated for 24 hours (37 °C in 5% CO^2), *C. coggygria* extracts were applied at concentrations (1.2 and 0.6 mg/ml). After the cells were incubated with the extracts for 24 hours, 15 µL of WST-1 (water-soluble tetrazolium salt) reagent was added to the wells. It was measured on an Elisa Microplate Reader (BioTek, USA) at 440 nm after 4 hours of incubation (9, 21).

In vitro Cell Proliferation Assay: The proliferation of L929 fibroblast cells applied to *C. coggygria* (leaves and

stem extract) was determined with Real Time Analyzer (RTCA) SP (Roche, Germany). Cell growth was followed by seeding L929 fibroblast cells ($5x10^3$ cells per well) with e-plate 96 (Roche). Leaves and stem extracts (1.2 - 0.6 - 0.3 mg/mL concentrations) were applied at 18th and 40th hours of incubation. The logarithmic increase of L929 fibroblast cells incubated in 5% CO_2 at 37 °C for 65 hours was measured (9).

In vitro Genotoxicity Assay: The micronucleus test was performed according to the ISO 10993-3 OECD guidelines 487. The micronucleus test, in which the genotoxic effect of *C. coggygria* water extracts was investigated. CHO cells-Chinese hamster ovary cells (20 x 10^3 cells per well) were seeded in 48-well plates with DMEM medium containing 10% fetal calf serum and 1% penicillin-streptomycin. L929 cells were treated at two concentrations of extracts (1.2 and 0.6 mg/ml) for 24 h. Mitomycin C, known to have genotoxic effects, was used as a positive control in the experiment. 3 µg/ml Cytochalazine-B (Santa Cruz) was added to the culture media prepared for the micronucleus test in order to obtain cells with binucleus at the 44th hour of incubation. At the 72nd h, the media in the wells were discarded and incubated with cold 0.075 M KCI (Ambresco) hypotonic solution at room temperature for 4 min in order to allow the cells to swell and disintegrate and chromosomes to separate from each other. At the end of the incubation, methanol: glacial acetic acid (Merck, Germany) in a ratio of 3:1 was fixed with a freshly prepared fixative in order to detect the opened metaphase plates. The fixation process was repeated 3 times. Propodium iodide (Life Technologies, USA) was dripped and incubated in the dark for 15 minutes to visualize binucleus cells with micronuclei formation at the final stage. Binucleated cells with micronuclei were examined under a fluorescent microscope (Leica, DMI6000B, Germany) (37).

In vitro Antimicrobial Activity: To determine the antimicrobial activity of water and methanol extracts of *C. coggygria*, agar applied to *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *C. albicans* (ATCC 10231) strains by disk diffusion method. The 0.5 Macfarland turbidity standard prepared from 24 h fresh cultures of bacteria. Homogenized bacterial suspensions were inoculated into petri dishes with Mueller Hinton Agar. Blank discs impregnated with 20 µl of water and methanol extracts (1.2 mg/ml and 0.6 mg/ml) of *C. coggygria* were placed on agar. Penicillin (Bioanalyse P10) for *S. aureus*, Nystatin (Bioanalyse NY100) for *C. albicans*, tetracycline (Bioanalyse TE10) for *E. coli*, antibiotic discs were used as controls. Petri dishes were incubated for 18-24 h at 37 °C. The zone diameters around the disc were then measured (42).

In vitro Hemolysis Assay: The hemolysis test was performed according to the TS EN ISO 10993-4 standard. Hemoglobin standard was prepared according to ASTM F756-00 standard. In order to determine the amount of hemoglobin in plasma in blood samples taken from rabbits, 3 ml of blood was transferred to falcon tubes. 500 µl of the plasma part of the blood samples centrifuged at 700 G-force was taken and transferred to the eppendorf tube. 500 µl of Drabkin's reagent was added to tubes and incubated for 15 minutes at room temperature. After incubation, blood samples transferred to well plates were measured at 540 nm wavelength in Elisa reader. The amount of hemoglobin in the plasma should be less than 2 mg/ml. 20 µl of blood was added to 5 ml of Drabkin's reagent and incubated for 15 minutes. The amount of hemoglobin in whole blood was determined by measuring at a wavelength of 540 nm. Blood samples were diluted to 10 mg/ml of hemoglobin. Blood samples diluted with PBS were incubated with *C. coggygria* water extract in falcon tubes for 3.5 hours at 37 °C. Control groups were also treated with blood for 3.5 hours. Water was used as positive control and PBS was used as negative control. At the end of the incubation, blood samples interacted with plant extracts in falcon tubes were centrifuged at 750 G-force for 15 minutes. Plant extracts were transferred to Eppendorf tubes by taking 500 µl from the supernatant part of the tubes that were interacted with the positive and negative control. 500 µl of Drabkin's reagent was added to it. It was incubated for 15 minutes at room temperature. Measurements were made at 540 nm by placing 100 µl on 96 well plate. Percent hemolysis rates were calculated with the determined absorbance values (5). According to ASTM F756-00 standard directed by ISO 10993-4 standard: <2% not hemolytic, 2-5% mild hemolytic and> 5% hemolytic.

In vivo Burn Wound Healing: In vivo burn studies were performed at Kırıkkale University Hüseyin Aytemiz Experimental Research and Application Center. A total of 9 male Sprague-Dawley rats (250 - 300 g weight), 3 each (n=3) for the leaves, stem and control groups, were used to evaluate the efficacy of the extracts in wound healing. 50 µl of 0.6 mg/ml *C. coggygria* leaves and stem water extracts were applied to the burned areas.

After intraperitoneal ketamine-xylazine anesthesia was applied to the rats, their back regions were shaved. A cylindrical disc with a diameter of 1 cm was kept in 100 ◦C water and contacted with the back of the rats for 20 s to create a burn wound. Wound healing rates were calculated by measuring the wound areas at regular intervals for 27 days. The percentage wound contraction was determined by the following formula (36).

$$\%\ wound\ closure = \frac{\text{wound area on day 0} - \text{wound area on day n}}{\text{wound area on day 0}} x100$$

n is the number of days (3^{rd}, 7^{th}, 14^{th}, 21^{st} and 27^{th}).

Statistical Analysis: The data was first analyzed if it met the parametric test assumptions. For this Shapiro Wilk and Levene tests were performed. The results revealed that the data did not normally distributed. Therefore, Kruskal Wallis test was performed to see if there are group differences. Mann-Whitney U test was performed when group difference was existed for pairwise comparison. The Bonferroni correction was applied, and new significance levels was calculated as P=0.017 (for three groups comparison).

Results

TOF-LC/MS Analysis: Phenolic compounds, chemical compounds and amino acids in the leaves and stem methanol extract of *C. coggygria* were determined by TOF-LC/MS analysis. According to TOF-LC/MS phenolic compounds and flavonoids found in methanol extract of *C. coggygria* leaves and stem parts; gallic acid, catechin, protocatechic acid, vanillic acid, ellagic acid, rosmarinic acid, quercetin, 4-hydroxybenzoic acid, chlorogenic acid, routine and apigenin 7-glycoside (Table 1).

In vitro Cytotoxicity Assay: WST-1 test was performed to determine the toxicity of *C. coggygria* leaves and stem extracts (methanol-water). The % viability values of L929 fibroblast cells are given in Table 2 and Figure 1. The extracts were applied at two different concentrations, 0.6 mg/ml and 1.2 mg/ml. Cell viability was calculated as 117±3.5% and 114±4.6%, respectively, in cells treated with 0.6 mg/ml water and methanol extracts of leaves. While it was determined that the viability of L929 fibroblast cells, in which 1.2 mg/ml *C. coggygria* leaves water and methanol extracts were applied, increased viability, the % viability was calculated as 125±2.8% and 118±4.3%, respectively. L929 fibroblast cells treated with 0.6 mg/ml water and methanol extract of stem the % viability was calculated as 118±5.1 and 117±5 respectively. In cells treated with 1.2 mg/ml water and methanol extracts of stem, the % viability rates were calculated as 128±3.6 and 123±4.7, respectively.

Table 1. Total phenolic and flavonoid contents various parts of *Cotinus coggygria.*

Plant part used	
Leaves methanol extract Main compounds (mg/kg)	**Stem metanol extract Main compounds (mg/kg)**
Gallic acid (18.5), Catechin (4.6), Protokatesic acid (0.6), Vanilic acid (8.4), Ellagic acid (0.1), Rosmarinic acid (0.1), Quercetin (15 ppb)	Gallic acid (24.6), Catechin (155.1), Chlorogenic acid (1.9), 4-hidroksibenzoic acid (385.3), Rutin (2.5), Ellagic acid (15.1), Apigenin-7-glycoside (10.5), Rosmarinic acid (0.4), Quercetin (15.2), Naringenin (279.1)

Table 2. % viability values of L929 fibroblast cells to which *C. coggygria* leaves-stem water and methanol extracts were applied.

Concentration mg/mL	Leaves water extract	Stem water extract	Leaves methanol extract	Stem methanol extract
Control	100±2.4	100±4.2	100±3.7	100±3.9
0.6 mg/mL	117±3.5	118±5.1	114±4.6	117±5.0
1.2 mg/mL	125±2.8	128±3.6	118±4.3	123±4.7

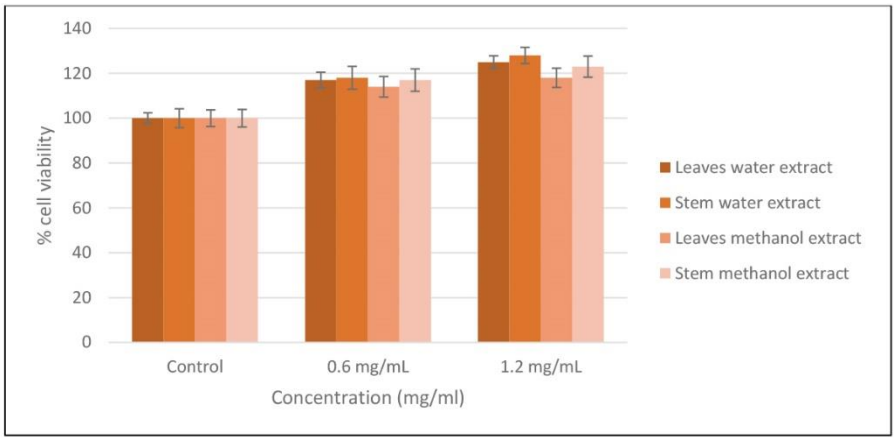

Figure 1. % Viability graph of L929 fibroblast cells.

In vitro Cell Proliferation Assay: The effect of *C. coggygria* (leaves and stem) aqueous extracts on cell proliferation was analyzed with the xCELLigence RTCA system. Proliferation graphs of L929 fibroblast cells to which water extracts were applied (*C. coggygria*-leaves) are shown in figure 2 and (*C. coggygria* stem) in figure 3. Water extracts were applied at 3 different concentrations (1.2 mg/ml- 0.6 mg/ml- 0.3 mg/ml). The first application was made at the 18th hour when the cells showed a logarithmic increase. After the first application of *C. coggygria* leaves-water extract, a rapid increase in proliferation was observed until the 25th hour, especially in cells where 1.2 mg/ml concentration was applied (Figure 2). It was observed that after the second application of the plant extracts at the 42nd hour, the concentration of 0.3 mg/ml increased the cell proliferation more rapidly compared to the control group. In the first application at the 18th hour, it was observed that *C. coggygria* stem-water extract increased the proliferation of L929 fibroblast cells at a concentration of 1.2 mg/ml. The increase in proliferation continued until the 25th hour (Figure 3). The increase in cell proliferation continued until the 45th hour with the second application of the plant extracts at the 42nd hour. It was seen that there was no previous study in the literature on the effect of *C. coggygria* plant extracts on L929 fibroblast cell proliferation. After applying *C. coggygria* leaves and stem extracts with the xCELLigence RTCA real-time cell analysis system, it was observed that it caused a faster increase in proliferation compared to the control group.

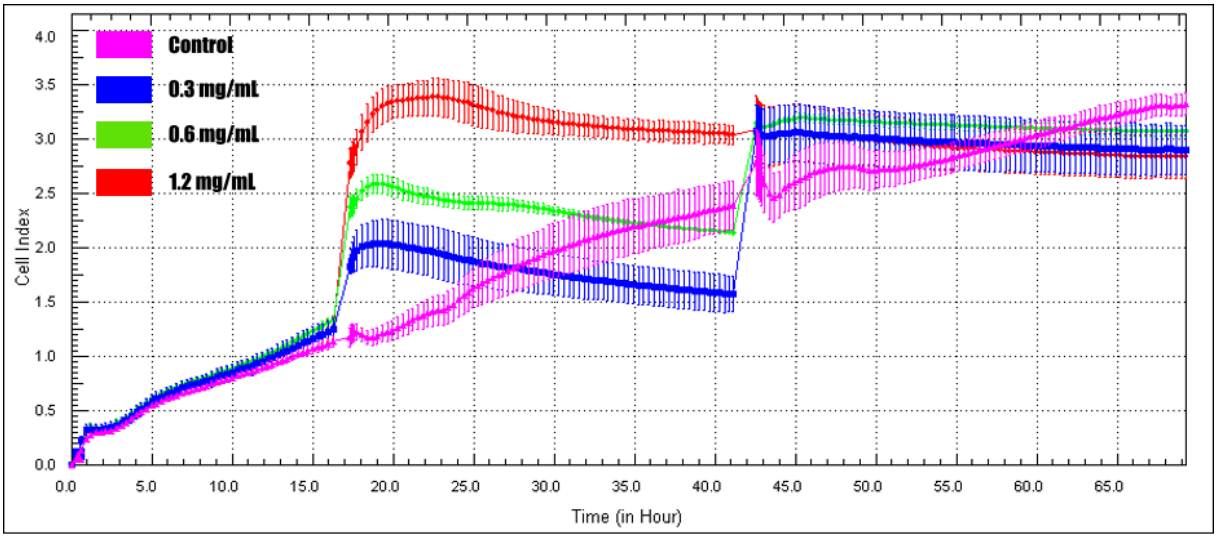

Figure 2. Proliferation graph of L929 fibroblast cells to which *C. coggygria* leaves water extracts were applied.

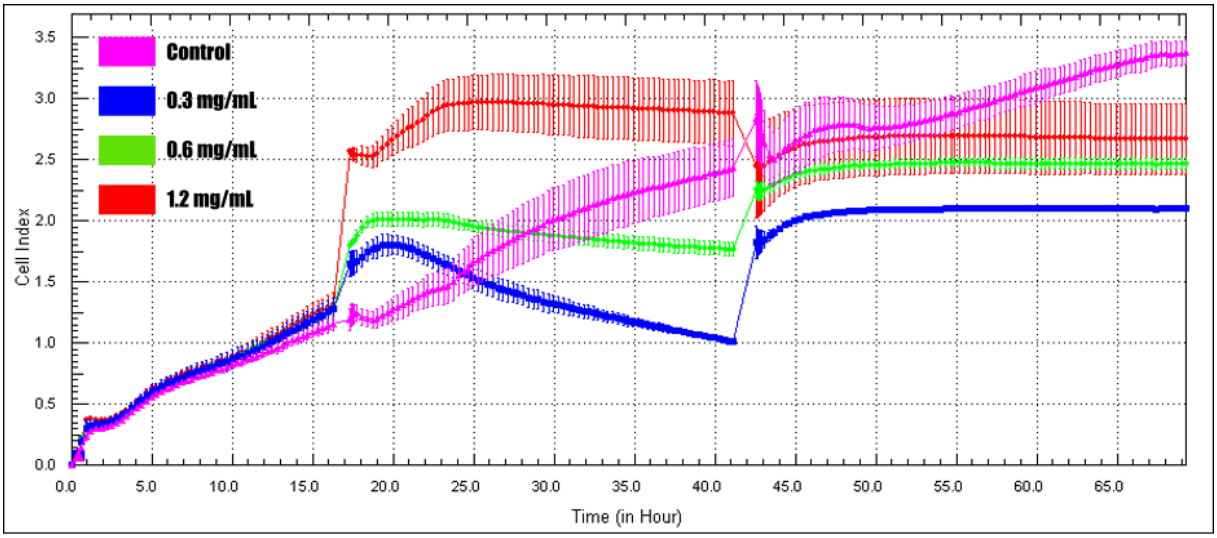

Figure 3. Proliferation graph of L929 fibroblast cells to which *C. coggygria* stem water extracts were applied.

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In vitro Genotoxicity Assay: The genotoxic effect of *C. coggygria* leaves - stem water extracts was determined by the OECD 487 micronucleus test. The genotoxicity rate was determined by calculating the number of binucleated cells, multinucleated cells, micronuclei in the binucleated cells and the total number of cells in the treated wells (Figure 4). The medium was used as a negative control. Mitomycin C was used as a positive control. When the negative control and water-extracted cells were evaluated microscopically, it was observed that there was no difference in terms of cells with micronuclei. When the positive control and water extract treated CHO cells were compared microscopically, there was a difference between the two groups in terms of cells with micronuclei. According to the micronucleus test results, it was determined that *C. coggygria* water extracts had no genotoxic effect on CHO cells.

In vitro Antimicrobial Activity: *C. coggygria* (leaves and stem parts) water and methanol extracts were impregnated on empty anti discs to be placed in petri dishes. Extract-impregnated discs and antibiotic discs were placed in petri dishes in which *S. aureus*, *E. coli* and *C. albicans* were cultivated, and the zone diameters of the formed zone were observed after 24 hours of incubation. Table 3. and the measured zone diameters and images are given in figure 5. Gentamicin was used for *E. coli* and *S. aureus*, ketoconazole was used for *C. albicans* as positive control. No inhibition zone was observed in microorganisms (*S. aureus* and *C. albicans*) to which water and methanol extracts were applied. The zone diameters formed in *E. coli* were calculated as 9 mm in 1.2 mg/ml methanol (leaves and stem) extract and 8 mm in 0.6 mg/ml water extract (leaves and stem).

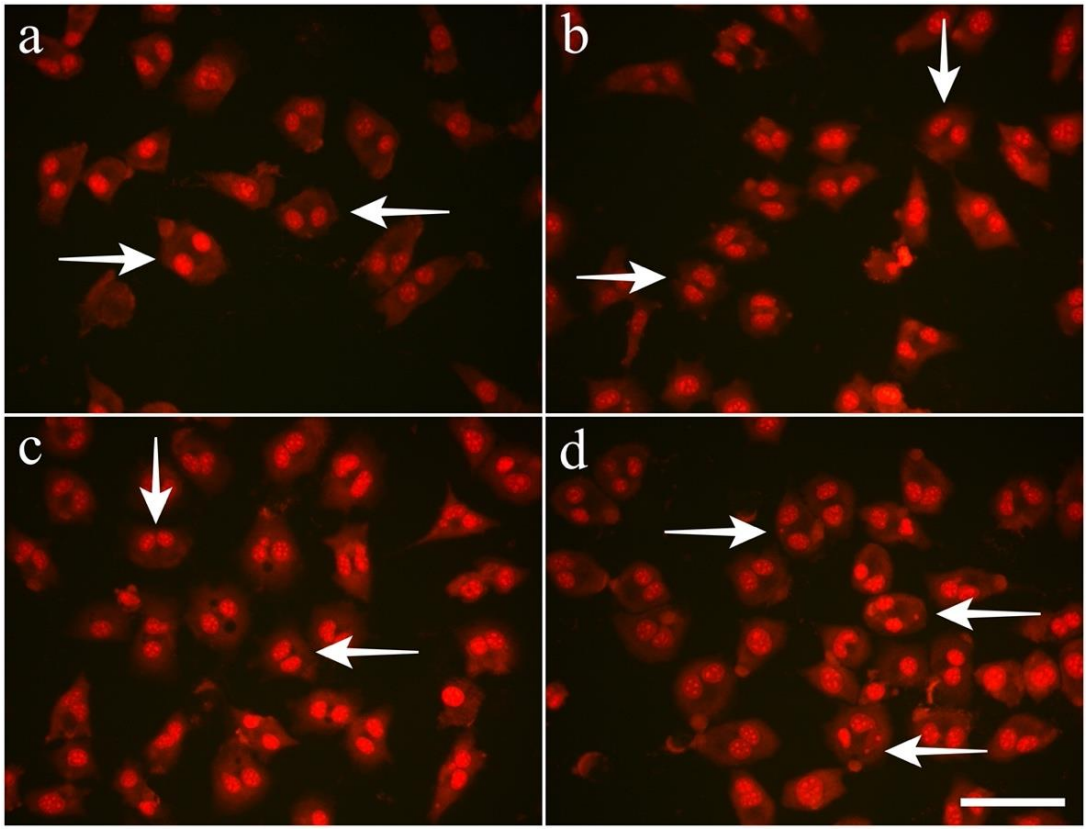

Figure 4. Binucleated and micronucleated CHO cells.
a) Binucleated CHO cells treated with 1.2 mg/mL *C. coggygria* (leaves) water extract. b) Binucleated CHO cells of 1.2 mg/mL *C. coggygria* (stem) water extract applied. c) CHO cells treated with medium alone as negative control. d) CHO cells treated with mitomycin C as positive control. Cells with micronuclei are indicated by the arrow. Scale bar = 100 µm.

Table 3. Inhibition zones of bacteria and fungi treated with *C. coggygria* extracts.

C.coggyria	***E. coli*** **(mm)**	**Ant(*E. coli*) Gentamicin (mm)**	***S. aerus*** **(mm)**	**Ant(*S. aerus*) Gentamicin (mm)**	***C.Albicans*** **(mm)**	**Ant(*C.Albicans*) Ketokonazol (mm)**
Leaves methanol extract	9	18	-	12	-	18
Leaves water extract	8	18	-	12	-	18
Stem methanol extract	9	18	-	12	-	18
Stem water extract	8	18	-	12	-	18

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In vitro Hemolysis Assay: Hemolysis is the release of hemoglobin from the cell by damaged erythrocyte membranes, showing a deterioration in RBC membrane integrity. Hemolysis test is used in blood compatibility evaluation according to TS EN ISO 10993-4 standard. The method we apply is the hemolysis test performed according to the ASTM F756, to which the TS EN ISO 10993-4 standard refers. According to ASTM F756-0 standard evaluation criteria, if the hemolytic index is <2%, is not hemolytic. The hemolytic index for the *C. coggygria* leaves water extract was 1.25%, and the hemolytic index for the *C. coggygria* stem water extract was calculated as 1.56%. In addition, the hemolytic effect of C. coggyria was investigated for the first time according to the literature search and it was determined that have an anti-hemolytic effect.

In vivo Burn Wound Healing: Wound healing activity of *C. coggygria* (leaves-stem) water extracts was determined by measuring burn wound areas in rats on certain days. Wound sites were followed for 21 days. Wound contraction percentage of plant extracts and control group is shown in Figure 6. Photographs of wounds during the treatment period are shown in Figure 7. At the end of 21 days, it was observed that the wounds healed faster in the *C. coggygria* (leaves-stem) applied group compared to the control group. When the burn wound was created, the wound diameter was measured as 1.8 cm. The recovery rate was 11.1±0.3% in animals treated with leaf extract on the 7th day, 38.9±0.2% in animals treated with stem extract, and 11.1±0.4% in the control group. On the 7th day, there was a statistically significant difference between the stem and control groups. At the end of the 21st day, the recovery rate was 55.6±0.1% in the leaf extract applied group, 50.0±0.1% in the stem extract applied group, and 27.8±0.5% in the control group. *C. coggygria* (leaves-stem) extracts are thought to accelerate wound healing.

Table 4. *In vivo* wound healing statistical analysis results of plant extracts applied at 0.6 mg/ml concentration on days 1, 3, 7, 14 and 21 (n=3).

	Mean±SE	Median	IQR	Mean Rank	P value
Group					0.55
Leaves	1.14±0.15	1.5	0.8	22.43	
Stem	1.08±0.15	1.1	0.8	20.73	
Control	1.32±0.11	1.5	0.7	25.83	
Day 1					0.96
Leaves	1.67±0.33	1.7		4.83	
Stem	1.67±0.33	1.7		4.83	
Control	1.67±0.08	1.7		5.33	
Day3					0.78
Leaves	1.60±0.06	1.6		5.67	
Stem	1.57±0.09	1.6		5.17	
Control	1.43±0.18	1.5		4.17	
Day7					0.04
Leaves	1.47±0.33	1.5		5.67	
Stem	1.10±0.06	1.1		2.00	
Control	1.57±0.07	1.5		7.33	
Day14					0.12
Leaves	0.77±0.14	0.8		3.00	
Stem	0.93±0.09	0.9		4.50	
Control	1.27±0.17	1.2		7.50	
Day21					0.37
Leaves	0.20±0.06	0.2		5.00	
Stem	0.13±0.03	0.1		3.50	
Control	0.67±0.28	0.9		6.50	

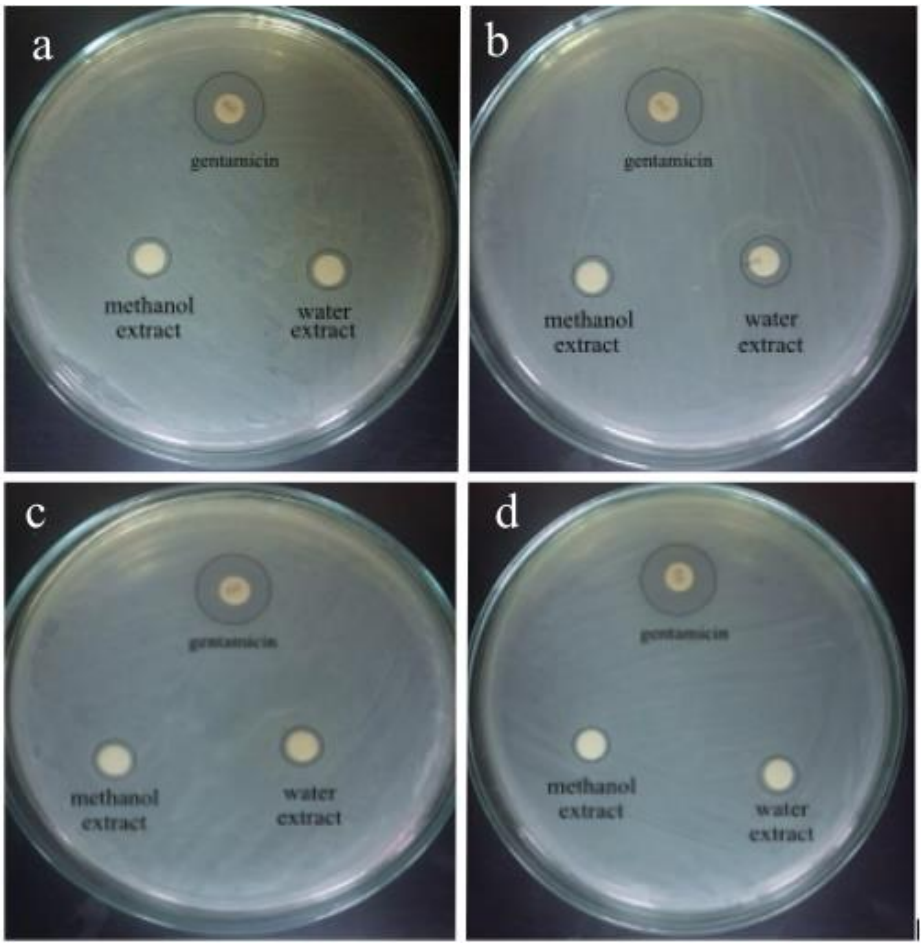

Figure 5. Inhibition zones formed by *C. coggygria* extracts in *E. coli*.
a) Inhibition zones formed by 1.2 mg/ml stem extract.
b) Inhibition zones formed by 0.6 mg/ml stem extract.
c) Inhibition zones formed by 1.2 mg/ml leaves extract.
d) Inhibition zones formed by 0.6 mg/ml leaves extract.

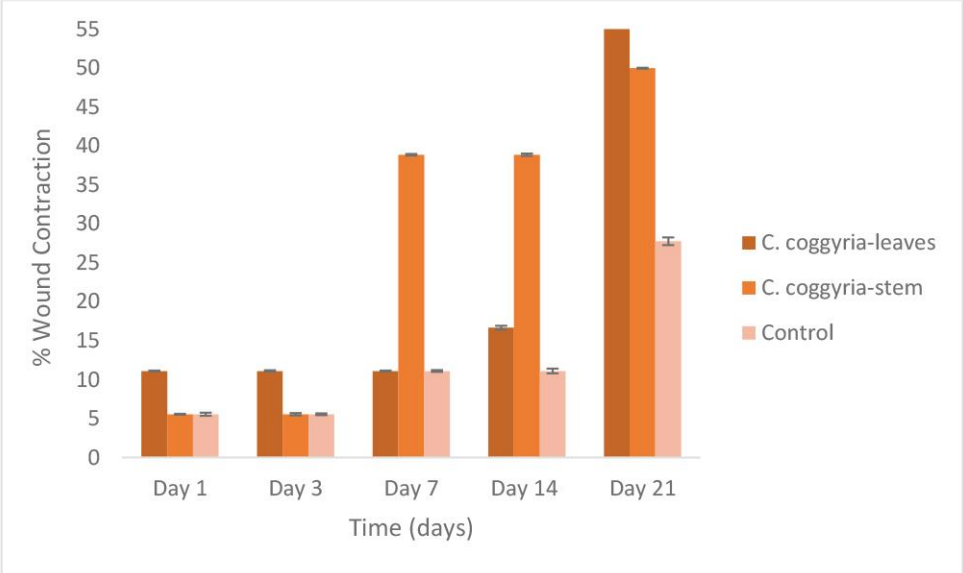

Figure 6. % Wound contraction of test, standard, and control groups at the 1st, 3rd, 7th, 14th and 21st days.

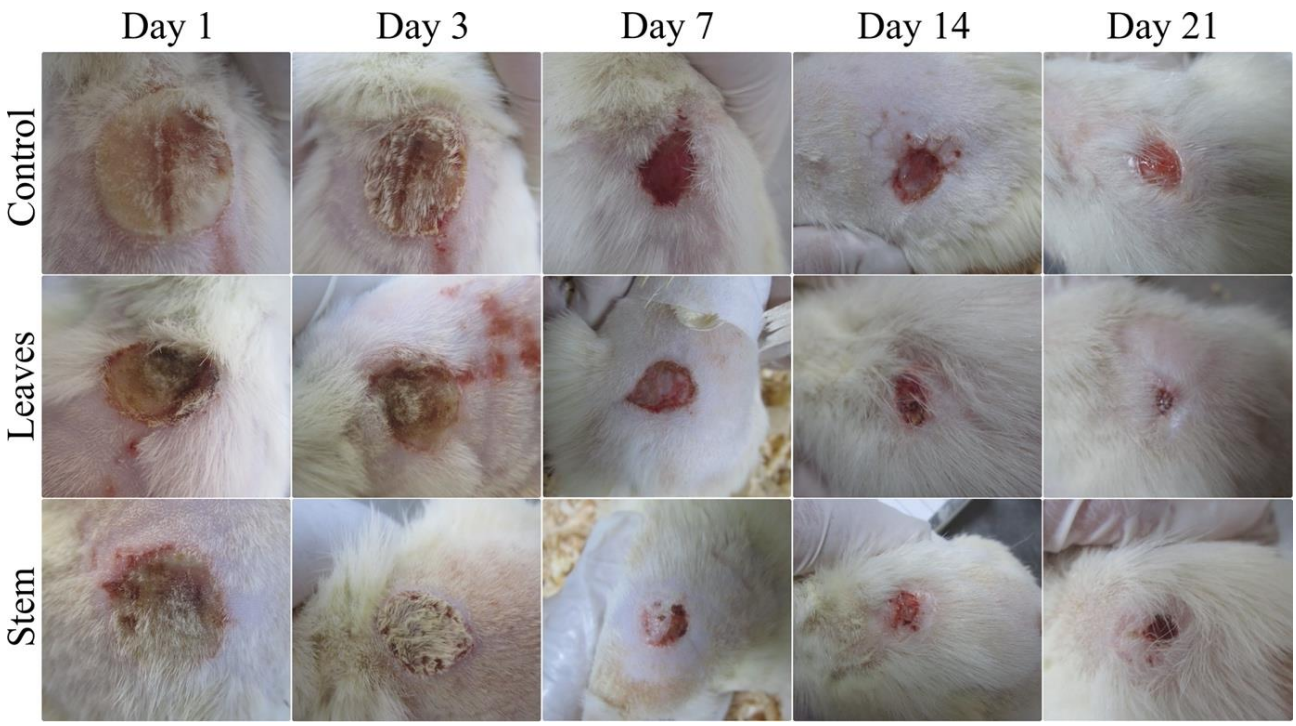

Figure 7. Photographs of 0.6 mg/ml *C. coggygria* extract applied to burn wounds on days 1, 3, 7, 14 and 21.

Discussion and Conclusion

The use of *C. coggygria* in medicine dates back to many years, especially among the public. It possesses antimicrobial and anti-inflammatory properties, as well as anti-hemorrhagic and wound healing properties. *C. coggygria* shows these therapeutic properties thanks to the phenolic compounds and flavonoids it contains (40). Matić et al. (26) reported that methanol extract of stem of *C. coggygria* contained 3.78 mg gallic acid in total phenolics, 8.29 mg rutin in total flavonoids. HPLC analysis also revealed that myricetin is the main component of *C. coggygria* extract along with varying amounts of hydroxyl derivatives of cinnamic acids (chlorogenic, caffeic, coumaric, ferulic and rosmaric acid) were identified in the extract. Previous research has shown that gallic acid, catechin and chlorogenic acid regulate wound healing. Gallic acid, which is found in almost all plants, is included in the literature with its anti-inflammatory, analgesic, anticancer and antidiabetic properties, as well as its powerful antioxidant effect. Yang et al. (43) reported that gallic acid reduces oxidative stress in human keratinocyte cells and speeds up wound healing. Reports have shown that chlorogenic acid is effective as an antidiabetic, antihypertensive, antitumor, and anti-inflammatory agent in the prevention of gastric lesions and liver injuries. In addition, chlorogenic acid regulates the secretion of collagens and matrix metalloproteinases, which are effective in scar tissue formation in the final stage of wound healing (29).

Flavonoids, which have antioxidant and antibacterial properties, also provide blood circulation in the body and prevent vascular occlusion. The antibacterial effect of catechins can suppress the proliferation of pathogenic bacteria in the wound area and prevents the possibility of infection in case of injury. In our study, flavonoids and phenolic compounds contained in *C. coggygria* were determined by TOF/LC-MS as gallic acid, catechin, protocatechin acid, vanillic acid, ellagic acid, rosmarinic acid, quercetin, 4-hydroxybenzoic acid, chlorogenic acid, rutin and apigenin 7-glycoside. The antioxidant, antibacterial, antidiabetic and anti-inflammatory properties of the flavonoid and phenolic compounds contained in *C. coggygria* show that they can be used in the healing of acute and chronic wounds. Ferrazzano et al. (16), the cytotoxicity of *C. coggygria* methanol extract in HGF-1 and Hacat cells was evaluated by XTT test. The study revealed that the water extract was less toxic than the ethanol extract. Iliev et al. (20) evaluated the antiproliferative effect of *C. coggygria* ethyl acetate extract in MCF-10A cells. They have reported an increase in cell proliferation at a concentration of 3 µg/ml. Artun et al. (4) the toxicity of *C. coggygria* methanol extract in Hela and vero cells was evaluated. While the IC50 value was calculated as 293 µg/ml in Hela cells, it was calculated as > 1000 µg/ml in vero cells. İn another study (43) it was reported that gallic acid, one of the phenolic compounds of *C.coggygria*, increased fibroblast proliferation. According to previous studies in the literature, *Cotinus* plant has different cytotoxic effects in

different cell lines, depending on the extract (such as water, ethanol, methanol) and concentrations. In present study, the cytotoxic effects of water and methanol extracts of leaves and stem parts of *C. coggygria* at two different concentrations (1.2 mg/ml - 0.6 mg/ml) were evaluated. It was determined that water and methanol extracts did not cause any toxicity (Table 2). Since the % viability was higher in L929 fibroblast cells treated with water extracts, only water extracts were applied *in vitro* cell proliferation, genotoxicity, hemolysis and *in vivo* wound healing assays. Varanka et al. (12) reported that *C. coggygria* methanol extract at 5% concentration was genotoxic and induced sex-linked recessive lethal mutations in the X chromosome of *Drosophila melanogaster* males. On the other hand, it has been reported that 2% methanol extract reduces genotoxicity. The mutagenicity of *C. coggygria* methanol extract was determined by in vivo SLRL and alkaline comet test. The SLRL test, 5% methanol extract has been reported to be genotoxic in spermatozoid. The comet test, the extracts were applied as 500, 1000, 2000 mg/kg (extract/body weight) and followed for 24 and 72 hours. It has been reported that 500 mg/kg extract was not significantly different from the control group in the group administered. Genotoxic damage was reported in the groups in which 1000 and 2000 mg/kg extract were applied (27). In our study, it was determined that water extracts of *C. coggygria* leaves and stem parts (1.2 mg/ml - 0.6 mg/ml) did not cause genotoxicity on CHO cells. When our results are compared with previous studies, the genotoxic and mutagenic effects of the extracts may vary depending on the applied method and concentration. Matic et al. (28) reported the antimicrobial activity of *C. coggygria* methanol extract was determined by macro broth dilution technique. Methanol extract was applied to different bacterial species and *C. albicans*. Inhibition zones of *C. coggygria* methanol extract in 150 ug and 300 ug concentrations in *E. coli* were determined as 29 mm and 17 mm, respectively. *C. coggygria* methanol extract has been reported to have the highest antimicrobial effect on *E. coli*. Dulger et al. (11), ethanol extract of *C. coggygria* was applied to some bacterial and yeast cultures. It has been reported that 10.4 to 22.8 mm inhibition zones are formed for bacteria, while inhibition zones ranging from 11.8 to 16.9 mm are formed for yeasts. In the study, it was shown that *S. aureus* is more sensitive to *C. coggygria* extract. In our study, water and methanol extracts (1.2 mg/ml and 0.6 mg/ml) of *C. coggygria* were prepared by impregnating 20 µl empty antibiotic discs and tested for antimicrobial activity and the extracts showed antibacterial activity against *E. coli* (Table 3). *Cotinus* shows anticancer, anticoagulant, hemolytic and antioxidant properties because of the phenolic compounds and flavonoids it contains (40). Some diseases and disorders, including cancers, are the result of excessive production of ROS and can aggravate others, such as hemolytic anemia. Antioxidant molecules derived from plants are effective in neutralizing ROS, thereby compromising their harmful effects on cells. Such studies in the literature provide evidence that plant extracts and derivatives have protective effects against ROS-mediated damage to red blood cells (6). *C. coggygria*'s lack of hemolytic effect shows it can be used in open wounds with bleeding. It was determined that the water extracts of *C. coggygria* plant (leaves and stem parts), whose anti-hemolytic effect was investigated for the first time in the literature, had no hemolytic effect. Wound healing is a complex process that includes hemostasis, inflammatory phase, proliferation phase, formation of granulation tissue, and epithelialization and remodeling phases. Ertaş et al. (14) *C. coggygria* has studied the effect of ethanol extract on burn wound healing. PGE2 levels, which play a role at the beginning of the inflammation phase and hydroxyproline levels, which extracellular protein in the granulation tissue, are measured by biochemical analyzes and speed up the wound healing of the ethanol extract. In this study, the effect of *C. coggygria* (leaves and stem) water extracts on wound healing was evaluated by measuring the burn site. At the end of the 21st day, it was determined that leaves and stem extracts accelerated wound healing compared to the control group.

In conclusion; the present study the phenolic compounds of the *C. coggygria*, were determined by TOF/LC-MS. It was determined by MTT test that *C. coggygria* had no cytotoxic effect on L929 fibroblast cells, and it increased L929 fibroblast cell proliferation with xCELLigence (Real Time Cell Analysis System). In addition, the hemolytic effect of *C. coggygria* was investigated for the first time in the literature and it was determined that it was anti-hemolytic. It was observed that there was no genotoxic effect by micronucleus test. It has been determined that it has an antimicrobial effect on *E. coli*. Finally, in the in vivo burn model, the rats were followed for 21 days, and it was observed that the extracts were effective in wound healing, and the wound area closed faster than the control group. As a result, it is thought that *C. coggygria* can lead to *in vivo* studies aimed at the use of various skin defects in the treatment.

Acknowledgments

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Ethical Statement

The study was approved by Kırıkkale University Animal Experiments Local Ethics Committee with the decision dated 16.12.2013, numbered 2013/15, meeting numbered 13/02.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

EB, MT, HE and SK conceived and planned the experiments. EB and HE carried out the experiments. EB and HE planned and carried out the simulations. EB and HE contributed to sample preparation. EB, MT, HE and SK contributed to the interpretation of the results. EB. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

References

1. **Aksoy H, Sancar M, Sen A, et al** (2016): *The effect of topical ethanol extract of Cotinus coggygria Scop. on cutaneous wound healing in rats*. Nat Prod Res, **30**, 452-455.
2. **Aksoy H, Sen A, Sancar M, et al** (2016): *Ethanol extract of Cotinus coggygria leaves accelerates wound healing process in diabetic rats*. Pharm Biol, **54**, 2732-2736.
3. **Alam G, Singh MP, Singh A** (2011): *Wound healing potential of some medicinal plants*. Int J Pharm Sci Rev Res, **9**, 136-145.
4. **Artun T, Karagoz A, Ozcan G, et al** (2016): *In vitro anticancer and cytotoxic activities of some plant extracts on HeLa and Vero cell lines*. J BUON, **21**, 720-725.
5. **Astm F** (2000): 756-00. Standard practice for assessment of hemolytic properties of materials. ASTM.
6. **Bhat SH, Ullah MF, Abu-Duhier FM** (2019): *Anti-hemolytic activity and antioxidant studies of Caralluma quadrangula: potential for nutraceutical development in cancers and blood disorders*. Int J Pharm Res Allied Sci, **8**, 121-129.
7. **Bozkaya O, Arat E, Gök ZG, et al** (2022): *Production and characterization of hybrid nanofiber wound dressing containing Centella asiatica coated silver nanoparticles by mutual electrospinning method*. Eur Polym J, **166**, 111023.
8. **Budovsky A, Yarmolinsky L, Ben-Shabat S** (2015): *Effect of medicinal plants on wound healing*. Wound Repair Regen, **23**, 171-183.
9. **Ciftci H, Türk M, Tamer U, et al** (2013): *Silver nanoparticles: cytotoxic, apoptotic, and necrotic effects on MCF-7 cells*. Turk J Biol, **37**, 573-581.
10. **Demirci B, Demirci F, Başer KHC** (2003): *Composition of the essential oil of Cotinus coggygria Scop. from Turkey*. Flavour Fragr J, **18**, 43-44.
11. **Dülger B, Hacıoglu N, Bilen S** (2009): *Antimicrobial activity of Cotinus coggyria from Turkey*. Asian J Chem, **21**, 4139-4140.
12. **Eck-Varanka B, Kováts N, Horváth E** (2019): *Assessing Genotoxic Potential of Cofinus Coggygria Scop: Using the Mussel Micronucleus Test*. J Bot Res, **2**, 57-59.
13. **Erenler R, Telci I, Ulutas M, et al** (2015): *Chemical Constituents, Quantitative Analysis and Antioxidant Activities of E chinacea purpurea (L.) M oench and E chinacea pallida (N utt.) N utt*. J Food Biochem, **39**, 622-630.
14. **Ertaş B, Okuyan B, Şen A, et al** (2022): *The effect of Cotinus coggygria L. ethanol extract in the treatment of burn wounds*. J Res Pharm, **26**, 554-564.
15. **Farahpour MR** (2019): *Medicinal plants in wound healing*. Wound Heal Curr Perspect, **2019**, 33-47.
16. **Ferrazzano GF, Roberto L, Catania MR, et al** (2013): *Screening and scoring of antimicrobial and biological activities of Italian vulnerary plants against major oral pathogenic bacteria*. Evid Based Complementary Altern Med, **2013.**
17. **Firdous SM, Sautya D** (2018): *Medicinal plants with wound healing potential*. Bangladesh J Pharmacol, **13**, 41-52.
18. **Gizaw M, Thompson J, Faglie A, et al** (2018): *Electrospun fibers as a dressing material for drug and biological agent delivery in wound healing applications*. Bioeng, **5**, 9.
19. **Hemmati A, Rezaie A, Tamri P, et al** (2017): *Evaluation of the healing effects of Onosma bolbutrichum root extract on second degree burn wound in rabbit*. J Appl Pharm Sci, **7**, 168-171.
20. **Iliev I, Ivanov I, Todorova K, et al** (2020): *Effects of a Cotinus coggygria ethyl acetate extract on two human normal cell lines*. Acta Morphol Anthropol, **27**, 25-29.
21. **Kantar C, Mavi V, Baltaş N, et al** (2016): *Novel zinc (II) phthalocyanines bearing azo-containing schiff base: Determination of pKa values, absorption, emission, enzyme inhibition and photochemical properties*. J Mol Struct, **1122**, 88-99.
22. **Kumarasamyraja D, Jeganathan N, Manavalan R** (2012): *A review on medicinal plants with potential wound healing activity*. Int J Pharm Pharm Sci, **2**, 105-111.
23. **Kumari P, Yadav P, Verma PR, et al** (2013): *A review on wound healing properties of Indian medicinal plants*. Ind J Fund Appl Life Sci, **3**, 220-232.
24. **Kunene P, Mahlambi P** (2020): *Optimization and application of ultrasonic extraction and Soxhlet extraction followed by solid phase extraction for the determination of triazine pesticides in soil and sediment*. J Environ Chem Eng, **8**, 103665.

25. **Li J, Chen J, Kirsner R** (2007): *Pathophysiology of acute wound healing*. Clin Dermatol, **25**, 9-18.
26. **Matić S, Stanić S, Bogojević D, et al** (2013): *Methanol extract from the stem of Cotinus coggygria Scop., and its major bioactive phytochemical constituent myricetin modulate pyrogallol-induced DNA damage and liver injury*. Mutat Res Genet Toxicol Environ Mutagen, **755**, 81-89.
27. **Matić S, Stanić S, Mihailović M, et al** (2016): *Cotinus coggygria Scop.: An overview of its chemical constituents, pharmacological and toxicological potential*. Saudi J Biol Sci, **23**, 452-461.
28. **Matić S, Stanić S, Solujić S, et al** (2011): *Biological properties of the Cotinus coggygria methanol extract*. Period Biol, **113**, 87-92.
29. **Moghadam SE, Ebrahimi SN, Salehi P, et al** (2017): *Wound healing potential of chlorogenic acid and Myricetin-3-O-β-Rhamnoside isolated from Parrotia persica*. Molecules, **22**, 1501.
30. **Mutlu S, Yilmaz E** (2019): *Yara Yönetiminde Yenilikçi Yaklaşımlar*. Gümüşhane Univ Sağlık Bilim Derg, **8**, 481-494.
31. **Noorbakhsh SI, Bonar EM, Polinski R, et al** (2021): *Educational Case: Burn Injury—Pathophysiology, Classification, and Treatment*. Acad Pathol, **8**, 23742895211057239.
32. **Reinke JM, Sorg H** (2012): *Wound repair and regeneration*. Eur Surg Res, **49**, 35-43.
33. **Rodero MP, Khosrotehrani K** (2010): *Skin wound healing modulation by macrophages*. Int J Clin Exp Pathol, **3**, 643-653.
34. **Rodrigues M, Kosaric N, Bonham CA, et al** (2019): *Wound healing: a cellular perspective*. Physiol Rev, **99**, 665-706.
35. **Rowan MP, Cancio LC, Elster EA, et al** (2015): *Burn wound healing and treatment: review and advancements*. Crit Care, **19**, 1-12.
36. **Şahin Y, Zehra G, Alçiğir ME, et al** (2023): *Effects of functional poly (ethylene terephthalate) nanofibers modified with sericin-capped silver nanoparticles on histopathological changes in parenchymal organs and oxidative stress in a rat burn wound model*. Ankara Univ Vet Fak Derg, **70,** 131-140.
37. **Şekeroğlu V, Atlı Şekeroğlu Z** (2011): *Micronucleus test for determining genotoxic damage*. Turk Bull Hyg Exp Biol, **68**, 241-252.
38. **Trucillo P, Di Maio E** (2021): *Classification and Production of Polymeric Foams among the Systems for Wound Treatment*. Polymers, **13**, 1608.
39. **Vural F, Savcı A** (2017): *Yara bakımında yeni uygulamalar*. Turkiye Klinikleri J Surg Nurs Special Topics, **3**, 224-232.
40. **Wang G, Wang J, Du L, et al** (2015): *Effect and mechanism of total flavonoids extracted from Cotinus coggygria against glioblastoma cancer in vitro and in vivo*. Biomed Res Int, **2015.**
41. **Wang P-H, Huang B-S, Horng H-C, et al** (2018): *Wound healing*. J Chin Med Assoc, **81**, 94-101.
42. **Yahaya I, Chemchem M, Aydıner B, et al** (2019): *Novel fluorescent coumarin-thiophene-derived Schiff bases: Synthesis, effects of substituents, photophysical properties, DFT calculations, and biological activities*. J Photochem Photobiol A, **368**, 296-306.
43. **Yang DJ, Moh SH, Son DH, et al** (2016): *Gallic acid promotes wound healing in normal and hyperglucidic conditions*. Molecules, **21**, 899.

Head and neck tumors detected in dogs and cats between 2011 and 2021: A retrospective study

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ABSTRACT

The aim of this study is to determine and compare the distribution, localization, breed, age and gender incidences of head and neck region tumors by years, by evaluating the results of dog-cat biopsies and/or operation material brought to Ankara University, Faculty of Veterinary Medicine, Department of Pathology between the years 2011 and 2021. In the study, 244 (15.9%) of 1533 tumors diagnosed in dogs and cats between 2011 and 2021 belonged to the head and neck region, of which 159 were in dogs and 85 in cats. Dogs with tumors were generally more than 6 years old (n=108) and cats were mostly 1 year old and older (n=75). In the head and neck region tumors observed in dogs, malignant and benign tumors of epithelial origin were most common (n=81; 50.9%), with sebaceous tumors (n=22) being the most common, and mostly observed around the eyes (n=26), while malignant and benign tumors of mesenchymal origin were most commonly found in the mouth region. Among the tumors of this region, epithelial tumors were mostly encountered in cats (n=39, 45.8%), and 51.2% of the epithelial tumors were squamous cell cancer (n=20), with epithelial tumors being the most common in the mouth (n=12) and nose (n=9). As a result, epithelial malignant and benign tumors were mostly seen in the eyes, and mesenchymal malignant and benign tumors were detected in the mouth. Among tumor types, papilloma in benign tumors and squamous cell carcinoma in malignant tumors were noted.

Introduction

Tumor formations are common in dogs and cats. Where skin, mammae, uro-genital system, spleen and soft tissue come to the fore in dogs according to their anatomical-topographic features; in cats, it has been reported frequently in the eyes, digestive system, nasal cavity, lymph nodes, bones and mammae (10). Although skin tumors can be seen in almost every part of the body, localization differs according to the cells from which they originate. As a matter of fact, squamous cell and basal cell cancers, which are frequently seen, are especially localized in the head and neck region (4, 11).

Studies on the head and neck region are limited. These tumors in dogs are mostly presented as survey studies and these tumors constitute 7% of all canine tumors. Half of these are malignant tumors and consist of squamous cell cancer, melanocytic tumors, soft tissue sarcomas and adenocarcinomas, respectively (1, 11). In cats, head and neck tumors were mostly observed in the mouth region and epulis were frequently mentioned (12).

Although the age range, breed and gender predisposition of head and neck region tumors shows equality, it has been reported that especially oro-pharyngeal region tumors are observed in young terrier or mixed breed dogs and old tabby cats, often in females (10, 12).

In the study, the results of canine-cat biopsy and/or operation material brought to Ankara University Faculty of Veterinary Medicine Department of Pathology between 2011 and 2021 were evaluated, and the results of tumors in the head and neck region (including skin, ear, eye, nose, brain, skull, oropharyngeal region and mouth) were evaluated over the years. It was aimed to determine and compare their distribution, localization, race, age and gender incidences according to each other. With this retrospective study, in which tumor data observed in the head and neck region over a ten-year period were

examined in detail, it was thought to contribute to both veterinary pathology and clinicians as a result of sharing up-to-date data.

Materials and Methods

The material for the study consisted of biopsy and/or operation materials from 1399 dogs and 587 cats, which were examined in the Pathology Department of Ankara University Faculty of Veterinary Medicine between 2011 and 2021. Tumors were detected in 1100 dogs and 433 cats from these materials. Among them, those belonging to the head and neck region were evaluated.

Sampling: 1 cm thick samples were taken from the materials and fixed in 10% formaldehyde solution. These were then routinely processed, embedded in paraffin blocks, and 5 µm thick sections were taken and stained with hematoxylin & eosin. Afterwards, the sections were examined under a light microscope.

Primarily, the distribution of these tumors by years, breed and age details in dogs and cats, localizations (skin, ear, eye, nose, brain, skull and mouth (including the oropharyngeal region)) are detailed in Tables 1, 2, 3 and 4. Although it was adhered to the original data in the records of the Department in the diagnosis of tumors, Meuten (8) was used for nomenclature and classification. When the diagnoses were evaluated in general, they were interpreted after being divided into six groups: as epithelial tumors, mesenchymal tumors, melanocytic tumors, odontogenic tumors, nervous system tumors and other tumors. In some cases involving the head and neck region, the location of the tumor was not specified. These are included in the "skin, localization not-specified" section.

Results

In this study, 244 (15.9%) of 1533 tumors diagnosed in dogs and cats between 2011 and 2021 belonged to the head and neck region. Of these, 159 were in dogs and 85 were in cats. Two different tumors were detected simultaneously in six dogs and two cats. It was determined that 14.4% of the detected tumors in dogs and 19.6% in cats were seen in the head and neck region. The distribution of head and neck tumors in dogs and cats by years is given in Figure 1. Accordingly, head-neck region tumors were most common in dogs in 2018 (n=19, 11.8%); in cats, they were most commonly observed in 2021 (n=16, 18.6%).

Of the dogs with tumors, 71 were female, 70 were male, whereas 38 of the cats were male and 41 were female. The genders of 14 dogs and 5 cats were not specified.

It was noted that commonly, dogs with these tumors were older than 6 years (n=108) and cats were 1 year and older (n=75). The distribution of animals according to breeds and age ranges is given in Tables 1 and 2.

Tumors in dogs were found in Terrier (n=21), German Shepherd (n=11), Boxer (n=8), Golden Retriever (n=25), Pekignese (n=2), Bulldog (n=2), Labrador (n=3), Rottweiller (n=1), Cocker Spaniel (n=11), American Cocker Spaniel (n=1), Kangal (n=4), Husky (n=4), Japanese Spaniel (n=1), King Charles Cavalier Spaniel (n=3), Jack Russell Terrier (n=1), Alaskan Malamute (n=1), Chow Chow (n=1), Pomeranian (n=1), and mixed (n=29) breeds. In cats, tumors were observed in Orange Tabby (n=8), Siameese (n=1), Tabby (n=14), Turkish Van (n=1), Turkish Angora (n=8), Himalayan (n=1), Bombay (n=1), Persian (n=1), and mixed (n=33) breeds. The breeds of 31 dogs and 19 cats were not specified. When evaluated, except for those of unknown breed, head-neck region tumors were mostly observed in crossbreeds in both dogs and cats. While the most common breeds in dogs after crossbreeds were Golden Retriever and Terrier, respectively; Tabby was the most common breed in cats.

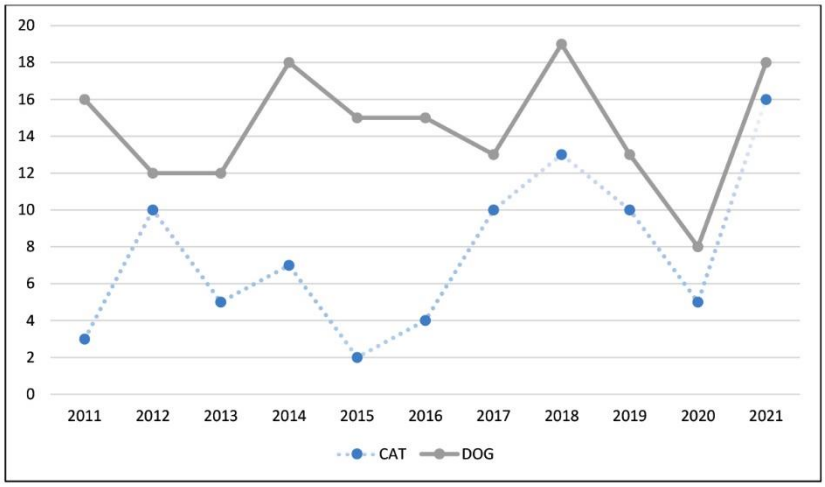

Figure 1. Distribution of the number of head and neck tumors in dogs and cats by years.

Table 1. Breed and age distributions of dogs with head and neck region tumors.

Breed	Age 0-1	Age 1-5	Age 6-10	Age 11 +	Age Unknown	Total
Terrier	1	2	5	12	1	21
German Shepherd	1	3	5	2	-	11
Boxer	-	-	4	3	1	8
Golden Retriever	1	3	12	8	1	25
Pekingese	-	-	1	1	-	2
Bulldog	-	2	-	-	-	2
Labrador Retriever	-	-	3	-	-	3
Rottweiler	-	-	1	-	-	1
Cocker Spaniel	-	-	5	6	-	11
American Cocker Spaniel	-	1	-	-	-	1
Sivas Kangal	-	-	3	-	-	3
Husky	-	1	1	2	-	4
Japon Spaniel	-	-	-	1	-	1
King Charles Cavalier Spaniel	-	-	1	-	2	3
Jack Russell Terrier	1	-	-	-	-	1
Malamute	-	-	1	-	-	1
Pomeranian	-	-	1	-	-	1
Chow chow	-	-	1	-	-	1
Mixed	1	10	8	7	2	28
Unknown	-	6	8	9	8	31
Total	5	28	60	51	15	159

Table 2. Breed and age distributions of cats with head and neck region tumors.

Breed	Age 0-1	Age 1-5	Age 6-10	Age 11 +	Age Unknown	Total
Orange Tabby	-	2	2	3	1	8
Siameese	-	-	1	-	-	1
Tabby	-	5	2	5	2	14
Turkish Van	-	-	1	-	-	1
Turkish Angora	-	1	5	1	1	8
Himalayan	-	1	-	-	-	1
Bombay	-	1	-	-	-	1
Persian	-	1	-	-	-	1
Mixed	3	11	12	8	1	35
Unknown	1	2	6	6	4	19
Total	4	24	29	23	9	89

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Table 3. Canine Head-Neck Tumors.

Tumor Types	Localization									
	Eye	Nose	Sinus	Mouth	Skin	Ear	Neck	Brain	Cranium	Total
Epithelial Tumors										
Benign Epithelial Tumors										
Papilloma	2	-	-	5	1	1	-	-	-	9
Adenoma	2	-	-	-	1	-	-	-	-	3
Cutaneous Sebaseous Epithelioma	1	-	-	-	-	-	-	-	-	1
Meibomian Adenoma	5	-	-	-	-	-	-	-	-	5
Sebaseous Adenoma	1	-	-	1	-	2	-	-	-	4
Sebaseous Ductal Adenoma	-	-	-	-	-	1	-	-	-	1
Ceruminous Adenoma	-	-	-	-	-	1	-	-	-	1
Trichoblastoma	1	-	-	-	1	1	-	-	-	3
Malign Epithelial Tumors										
Mix Ceruminous Carcinoma	-	-	-	-	-	1	-	-	-	1
Meibomian Carcinoma	2	-	-	-	-	-	-	-	-	2
Ceruminous Adenocarcinoma	7	-	-	-	1	-	1	-	-	9
Squamous Cell Carcinoma	3	-	2	5	3	3	-	-	-	16
Indifferential Squamous Cell Carcinoma	-	-	1	1	1	-	-	-	-	3
Basal Cell Carcinoma	1	-	-	-	2	5	1	-	-	9
Baso-squamous Cell Carcinoma	1	-	-	-	-	1	-	-	-	2
Adenocarcinoma	-	-	2	-	-	-	-	-	-	2
Sweat Gland Carcinoma	-	-	-	-	1	-	1	-	-	2
Solid Carcinoma	-	-	-	2	-	-	-	-	-	2
Ethmoidal Adenocarcinom	-	-	1	-	-	-	-	-	-	1
Cystic Papillary Adenocarcinoma	-	-	-	-	-	1	-	-	-	1
Indifferential Carcinoma	-	1	-	-	-	-	-	-	-	1
Nasal Carcinoma	-	1	1	-	-	-	-	-	-	2
Indifferential Nasal Carcinoma	-	-	1	-	-	-	-	-	-	1
Total	26	2	8	14	11	17	3	-	-	81
Mesenchymal Tumors										
Benign Mesenchymal Tumors										
Lipoma	-	-	-	-	-	-	2	-	-	2
Fibromyoma	-	-	-	1	-	-	-	-	-	1
Chondroma	-	-	-	-	-	-	1	-	-	1
Chondroosteoma	-	-	-	-	-	1	-	-	-	1
Malign Mesenchymal Tumors										
Fibrosarcoma	-	-	1	1	-	-	1	-	-	3
Chondrosarcoma	-	1	1	1	-	-	-	-	1	4
Osteosarcoma	1	1	2	-	-	-	-	-	1	5
Rhabdomyosarcoma	-	-	-	3	-	-	-	-	-	3
Fibromyxolipochondro-osteosarcoma	1	-	-	-	-	-	-	-	-	1
Hemangiosarcoma	-	-	-	1	-	-	1	-	-	2
Hemangioendothelioma malignum	1	-	-	-	-	-	-	-	-	1
Total	3	2	4	7	-	1	5	-	2	24

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Melanocytic Tumors										
Benign Melanocytic Tumors										
Benign Melanoma	5	1	-	1	-	-	-	-	-	7
Amelanotic Melanoma	1	-	-	2	-	-	-	-	-	3
Malign Melanocytic Tumors										
Malign Melanoma	-	1	-	6	-	1	-	-	-	8
Amelanotic Malign Melanoma	-	-	-	-	-	1	-	-	-	1
Total	6	2	-	9	-	2	-	-	-	19
Odontogenic Tumors										
Epulis Granulomatosis	-	-	-	1	-	-	-	-	-	1
Fibromatous Epulis	-	-	-	1	-	-	-	-	-	1
Ossified Epulis	-	-	-	1	-	-	-	-	-	1
Odontogenic Fibroma	-	-	-	2	-	-	-	-	-	2
Ameloblastoma	-	-	-	2	-	-	-	-	-	2
Ameloblastic fibroadenoma	-	-	-	2	-	-	-	-	-	2
Total	-	-	-	9	-	-	-	-	-	9
Nervous System Tumors										
Meningothelial Meningioma	-	-	-	-	-	-	-	1	-	1
Oligodendroglioma	-	-	-	-	-	-	-	2	-	2
Indifferential Astrocytoma	-	-	-	-	-	-	-	1	-	1
Psammomatous Meningioma	-	-	-	-	-	-	-	1	-	1
Total	-	-	-	-	-	-	-	5	-	5
Other Tumors										
Extragenital TVT	1	-	1	-	2	-	-	-	-	4
Mastocytoma	-	-	1	1	1	1	-	-		4
Histiocytoma	1	-	-	-	-	-	-	-		1
Cutaneous Histiocytoma	-	-	-	-	-	3	-	-		3
Atypical Histiocytoma	-	-	-	1	-	-	-	-		1
Malignant Fibrous Histiocytoma	-	-	-	2	-	-	-	-		2
Round Cell Tumor	1	-	-	-	-	-	1	-		2
Plasmacytoma	1	-	-	2	-	-	-	-		3
Lymphoma	-	-	-	-	-	-	1	-		1
Total	4	-	2	6	3	4	2	-	-	21
TOTAL TUMORS	39	6	14	45	14	24	10	5	2	159
	24.5%	3.7%	8.8%	28.3%	8.8%	15.0%	6.2%	3.1%	1.2%	

Epithelial tumors were most common in the head and neck region of dogs (n=81, 50.9%). The most common epithelial benign tumors were sebaceous tumors (n=11) and most of them were in the eye (n=7). The most common tumor type after sebaceous tumors was papillomas (n=9) most of which were found in the mouth region. Epithelial malignant tumors (n=54) were observed more frequently than benign ones (n=27). The most common epithelial malignant tumor was squamous cell carcinoma (n=19), 9.2% of which were in the mouth. Although there was no significant difference between tumor types of mesenchymal origin (n=24, 15.0%) in dogs, they were frequently encountered in the mouth region (n=7). Also in the head and neck region, other tumors including melanocytic tumors (n=19, 11.9%), odontogenic tumors (n=9, 5.6%), nervous system tumors (n=5, 3.1%), and usually round cell tumors (n=21, 13.2%) were observed. It was noted that the most common head and neck tumors observed in dogs were in the mouth (n=45, 28.3%), in the nose (n=6, 3.7%), in the sinus (n=14, 8.8%) and in the eye (n=39, 24.5%). The types and localizations of the tumors are detailed in Table 3.

Table 4. Feline Head-Neck Tumors.

Tumor Types	Localization									
	Eye	Nose	Sinus	Mouth	Skin	Ear	Neck	Brain	Cranium	Total
Epithelial Tumors										
Benign Epithelial Tumors										
Polyp	-	1	-	-	-	-	-	-	-	1
Squamous cell papilloma	-	-	-	1	-	-	-	-	-	1
Malign Epithelial Tumors										
Squamous Cell Carcinoma	1	4	-	8	-	2	1	-	-	16
Basal Cell Carcinoma	-	-	-	-	-	1	-	-	-	1
Baso-squamous Cell Carcinoma	2	2	-	1	1	-	-	-	-	6
Papillary Cystic Adenocarcinoma	-	1	-	-	-	1	-	-	-	2
Indifferential Squamous Cell Carcinoma	-	1	1	1	-	-	1	-	-	4
Carcinoma	-	-	-	-	-	1	-	-	-	1
Adenocarcinoma	-	-	-	1	-	-	-	-	-	1
Indifferential Carcinoma	1	-	1	-	-	-	-	-	-	2
Apocrine Carcinoma	-	-	-	-	-	1	-	-	-	1
Acinar Cell Carcinoma	-	-	-	-	-	-	1	-	-	1
Nasal Carcinoma	-	-	2	-	-	-	-	-	-	2
Total	4	9	4	12	1	6	3	-	-	39
Mesenchymal Tumors										
Benign Mesenchymal Tumors										
Fibroma	-	1	-	-	-	2	-	-	-	3
Fibromyxoma	-	-	-	-	-	1	-	-	-	1
Fibrochondroosteoma	-	1	-	-	-	-	-	-	-	1
Hemangioma capillare	-	-	-	1	-	-	-	-	-	1
Hemangioendothelioma	-	-	-	-	1	-	-	-	-	1
Malign Mesenchymal Tumors										
Fibrosarcoma	-	-	-	-	-	1	-	-	-	1
Fibromyosarcoma	-	-	-	-	-	1	2	-	-	3
Fibromyxosarcoma	-	-	-	-	-	-	1	-	-	1
Fibromyxomyosarcoma	-	-	-	-	-	-	1	-	-	1
Hemangiosarcoma	-	1	1	1	-	-	-	-	-	3
Osteosarcoma	-	-	-	1	-	-	-	-	-	1
Giant Cell Osteosarcoma	-	1	-	-	-	-	1	-	-	2
Osteoblastic Osteosarcoma	-	-	-	-	-	-	-	-	1	1
Giant Cell Sarcoma	-	-	-	-	-	-	1	-	-	1
Nasal Sarcoma	-	1	-	-	-	-	-	-	-	1
Total	-	5	1	3	1	5	6	-	1	22
Melanocyctic Tumors										
Melanoma	3	-	-	-	-	-	-	-	-	3
Malign Melanoma	1	-	-	-	-	-	-	-	-	1
Total	4	-	-	-	-	-	-	-	-	4
Odontogenic Tumors										
Giant Cell Epulis	-	-	-	1	-	-	-	-	-	1
Ameloblastoma	-	-	-	1	-	-	-	-	-	1
Total	-	-	-	2	-	-	-	-	-	2

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Nervous System Tumors										
Fibroblastic Meningioma	-	-	-	-	-	-	-	1	-	1
Peripheral Nerve Sheath Tumor	-	-	-	-	1	-	-	-	-	1
Transitional Meningioma	-	-	-	-	-	-	-	1	-	1
Psammomatous Meningioma	-	-	-	-	-	-	-	1	-	1
Meningioma	-	-	-	-	-	-	-	1	-	1
Total	-	-	-	-	1	-	-	4	-	5
Other Tumors										
Plasmacytoma	-	-	-	7	-	-	-	-	-	7
Round Cell Tumor	-	-	-	2	-	-	-	-	-	2
Round Cell Sarcoma	-	-	-	1	-	-	-	-	-	1
Histiocytoma	-	-	-	1	-	-	-	-	-	1
Lymphoma	-	-	-	1	-	-	-	-	-	1
Mastocytoma	-	-	-	-	-	-	1	-	-	1
Total	-	-	-	12	-	-	2	-	-	13
TOTAL TUMORS	8	14	5	29	3	11	10	4	1	85
	9.4%	16.4%	5.8%	34.1%	3.5%	12.9%	11.7%	4.7%	1.1%	

Epithelial tumors were most frequently observed in cats (n=39), with a relatively small number of benign tumors (n=2). Squamous cell carcinoma (n=20) constituted 54% of epithelial malignant tumors, and half of them were in the mouth region. When epithelial tumors were considered as general localization; they were most frequently observed in the mouth (n=12) and nose (n=9). No significant difference was observed between the types of tumors of mesenchymal origin (n=22, 25.8%). Other tumors in the head and neck region also include melanocytic tumors (n=4, 4.7%), odontogenic tumors (n=2, 2.3%), nervous system tumors (n=5, 5.8%), and round cell tumors (n=13, 15.2%). It was noted that the head and neck tumors observed in cats were mostly located in the mouth (n=29, 34.1%) and nose (n=14, 16.4%). The types and localizations of these tumors are given in Table 4.

Discussion and Conclusion

Diseases, tumors and types of cases observed in animals, their incidence, and distribution according to identity information emerge as a result of screening and research carried out within a certain period of time. Especially in the examination of tumoral cases in domestic animals, cities such as Ankara, where the rate of dog and cat feeding is high and conscious populations are interested in animals, gain importance (3, 6, 7, 9, 12). In this study, the types and distributions of head and neck tumors, which are frequently observed in dogs and cats, were examined and compared over an 11-year period. By this, actuality and and continuity of results of the study by Kutlu et al. in Ankara University Faculty of Veterinary Medicine Department of Pathology will also be provided.

Considering the age range in which head and neck tumors were seen, it was observed that the average decreased to earlier ages. It is known that the incidence of tumors increases with age in domestic animals. Kutlu et al. (7) in their study examining the tumors observed in the head region that the average age was 10 years in dogs and 11 years in cats between 2000 and 2010. Contrary to a similar study (7), which reported that there was no significant difference between breeds when racial predisposition was examined, the most common breeds in dogs after crossbreeds were Golden Retriever and Terrier, respectively; in cats, it was observed that Tabby is the most common. Despite these results, it is thought that breed predisposition should be considered according to tumor types. For example, melanomas, which are common in the head region from skin and mouth tumors, are more common in dark-colored breeds with more intense pigment. In our study, it was reported that sebaceous tumors, one of the most frequently observed tumors, were mostly observed in English Cocker Spaniel, Cocker Spaniel, Siberian Husky, Samoyed, and Alaskan Malamute (4).

In the study, Kutlu et al. (7) similarly did not report any gender differences in the occurrence of head tumors in dogs, but they found that they were more common in female cats than in male cats. In a study investigating head and neck tumors in dogs, it was reported that they were more common in females than males (1). When the genders of dogs and cats were evaluated, it was seen that the percentage of males and females was approximately 50%.

Tumors detected in this study were considered as epithelial, mesenchymal tumors, melanocytic tumors, odontogenic tumors, nervous system tumors and other

tumors, considering their diagnoses. Accordingly, it was noted that tumors of epithelial origin (n=81), especially malignant ones (n=54) were observed most frequently in dogs and cats. In the studies, the most common melanomas in the head region in dogs (1); and squamous cell cancer (1) are; odontogenic tumors, papillomas, (12) and melanomas (12) in the oropharyngeal region (2, 5). Although this study is partially similar to some others (1, 12) due to the prevalence of epithelial tumors, it was found that sebaceous gland tumors were most common among the epithelial tumors. These tumors, which are generally classified as sebaceous adenoma, sebaceous ductal adenoma, sebaceous epithelioma and sebaceous carcinoma, have been reported to occur frequently in the head region, especially in dogs (4). They were observed most frequently in the mouth and eyes, respectively, which was consistent with the study of Brønden et al. (1). Further examination of the eye as biopsy and/or operation material (n=27) and the diagnosis of sebaceous tumors in most of them (59.2%) had associated results in terms of tumor type and localization. It has been reported that the study is similar to the general information in terms of the frequent observation of tumors in the mouth region in dogs, with the most common being melanocytic tumors (30-40%), squamous cell cancer (15-25%) and fibrosarcoma (4). In this study, contrary to previously stated, oral melanocytic tumors (42.8%) and odontogenic tumors including epulis (42.8%) come to the fore.

In cats, studies in the head and neck region are quite limited, and the most common epithelial tumors observed in this study and the higher percentage of squamous cell cancer among epithelial tumors are similar to other studies (1, 7).

As a result, epithelial tumors were observed most frequently in dogs. There were papillomas in benign tumors and squamous cell carcinomas in malignant tumors. Considering their localization, epithelial benign and malignant tumors were mostly observed in the eyes, while mesenchymal benign and malignant tumors were encountered in the mouth. Although there was not much difference between the number of mesenchymal tumors, tumors were most frequnently encountered in the mouth. Epithelial tumors were also observed most frequently in cats, and squamous cell cancer was the most prominent tumor type. Epithelial tumors were most frequently encountered in the nose and mouth in cats. Although there is not much difference between the number of mesenchymal tumors, malignant and benign mesenchymal tumors were most common in the ear and neck regions, and fibromas, fibrosarcomas, and hemangiosarcomas were the most common.

Of all other tumors in cats, plasmacytoma and melanoma, as mentioned in the tables and texts, were the most striking. Among the other tumors seen in dogs, mostly benign and malignant melanomas were diagnosed.

In the study, while the results and general characteristics of two different species were examined in detail, comparisons between both species were avoided.

When the number of cases observed by year is evaluated, it is noteworthy that there is a decrease in the number of tumors in both dogs and cats in 2020. This was undoubtedly due to the decrease in the total number of materials experienced due to the Covid-19 pandemic. The number of benign and malignant cases by year is as indicated in Figure 2.

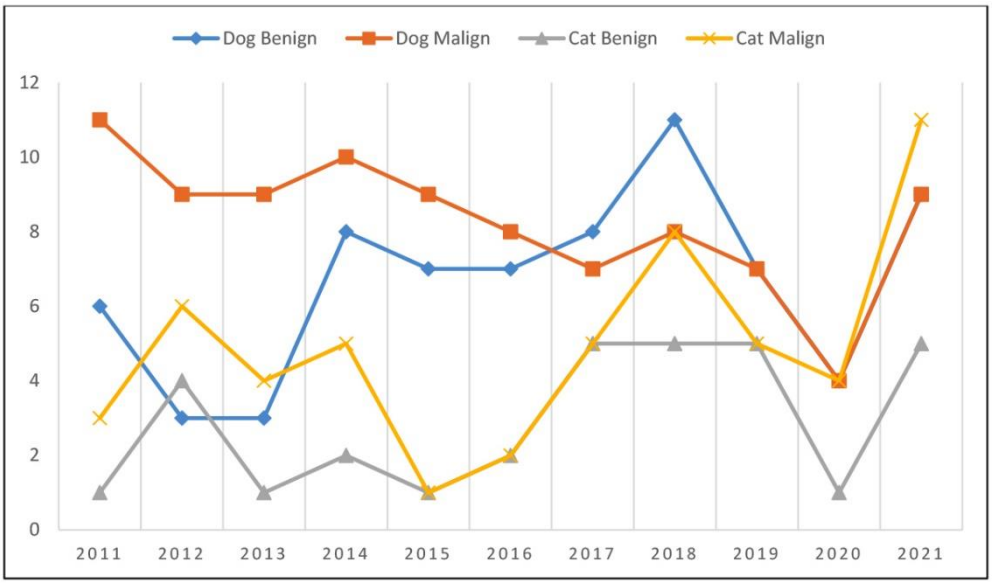

Figure 2. Distribution of benign and malignant tumors in the head and neck region of cats and dogs by years.

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Author Contributions

GYT conceived the idea and planned the manuscript for this review article. OBD made the archive researches for the samples dated in between the years 2011-2021 and prepared the tables. GYT took the lead in the literatüre review and writing the manuscript. SAV and OK have made significant scientific support and also contributed to the interpretation of the results. All authors provided significant contributions by giving feedback and help shape the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

Ethical approval was found unnecessary due to the fact that no experiments were done on animals.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Brønden LB, Eriksen T, Kristensen AT** (2009): *Oral malignant melanomas and other head and neck neoplasms in Danish dogs-data from the Danish Veterinary Cancer Registry.* Acta Vet Scand, **51**, 1-6.
2. **Dorn CR, Priester WA** (1976): *Epidemiologic analysis of oral and pharyngeal cancer in dogs, cats, horses, and cattle.* JAVMA, **169**, 1201-1206.
3. **Ertürk E, Tanzer F, Bulucu M** (1971): *Patolojik anatomi kürsüsünde 1964-70 yılları arasında incelenen köpek ve kedi tümörleri.* Ankara Univ Vet Fak Derg, **18**, 383-386.
4. **Goldschmidt MH, Goldschmidt, KH** (2016): Epithelial and melanocytic tumors of the skin. 88-141. In: Meuten (Ed), Tumors in Domestic Animals. WILEY Blackwell, Iowa.
5. **Gorlin RJ, Barron CN, Chaudhry AP, et al** (1959): *The oral and pharyngeal tumours of domestic animals: a study of 487 cases.* Am J Vet Res, **20**, 1032-1061.
6. **Gülçubuk A, Gürel A** (2003): *1995-2000 Yılları arasında İstanbul'da saptanan köpek tümörleri*. İstanbul Üniv Vet Fak Derg, **29**, 83-91.
7. **Kutlu T, Yücel G, Ekebaş G, et al** (2015): *2000-2010 yılları arasında incelenen kedi ve köpeklerdeki baş bölgesi tümörleri.* Vet Hekim Der Derg, **86**, 34-41.
8. **Meuten DJ** (2016): Tumors in domestic animals. 5th Ed. John Wiley & Sons, Iowa.
9. **Pamukçu AM, Ertürk E** (1962): *Ankara'da köpeklerde görülen tümör çeşitleri.* Ankara Univ Vet Fak Derg, **9**, 1-9.
10. **Pinello K, Pires I, Castro AF, et al** (2022): *Cross Species Analysis and Comparison of Tumors in Dogs and Cats, by Age, Sex, Topography and Main Morphologies.* Vet Sci, **9**, 167.
11. **Tannehill-Gregg SH, Levine AL** (2006): *Rosol, T.J. Feline head and neck squamous cell carcinoma: A natural model for the human disease and development of a mouse model*. Vet Comp Oncol, **4**, 84–97.
12. **Vural SA, Keleş H, Özsoy ŞY, et al** (2007): *Köpek ve kedilerde 1977-2005 yılları arasında saptanan orofaringeal bölge tümörleri: retrospektif çalışma.* Ankara Univ Vet Fak Derg, **54**, 197-203.

Evaluation of tumor-suppressive properties and apoptotic functions of Mad Honey and Vincristine applications in a rat model of breast cancer

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ABSTRACT

In this study, the suppressive effects of vincristine and Turkish mad honey alone and in co-applications were biochemically, hematologically, and histopathologically investigated in a mammary tumor model induced with 7,12-dimethylbenz[a]anthracene (DMBA) in rats. A total of 72 rats, 43-49 days old, were divided into 6 groups of 12 rats each. The control group (CG) consisted of healthy rats. The vehicle group (VG) received only vehicle substance and the cancer control group (CCG) was given only DMBA. DMBA and the honey group (HG) given group. DMBA and the vincristine (VinG) given group, and DMBA, the vincristine-honey group (VHG) received both Turkish mad honey and vincristine. Turkish mad honey and/or vincristine was given in the last 4 weeks of the 13-week trial period. White blood cell and lymphocyte counts differed significantly in the CCG and VG groups. Alanine transaminase and total protein levels were higher in the CCG and VinG groups. Aspartate transaminase was higher in the CCG, HG and VG groups. Caspase-3 and Bax protein levels were in the HG and VHG groups significantly higher than CCG. In caspase-8 protein level VHG significantly higher than other groups. Caspase -9 protein level was in CG and VG groups significantly lower than other groups. Bcl-xL increased more in the CCG group. Anaplasia was reduced in the HG and VinG groups, although apoptosis and other cellular damages increased. It was concluded that mad honey and vincristine could be considered together as effective therapeutic agents in this model of DMBA-induced breast cancer.

Introduction

Breast cancer is one of the most common cancers in the world. As a progressive disease with high mortality rates, it is one of the leading causes of death in both Turkey and the world as a whole and it impairs patients' quality of life. Breast tumors are the most common type of tumor among all cancer types in women (39). In veterinary medicine, breast cancer is also frequently encountered in dogs and cats, although mammary tumors are particularly more common in unspayed female dogs. This adversely affects the quality of life of animals, similarly to humans, and it brings great economic costs. In recent years, veterinarians who have adopted the "single health care" principle have been undertaking important tasks in terms of developing effective treatment methods against neoplastic diseases with poor prognoses. For this reason, in vivo breast cancer models have become a popular subject of research for human physicians and veterinarians alike (3, 42).

Chemotherapy is a treatment method used alone or in combination treatment of breast tumors (43). However, the toxicity of chemotherapeutics used in the treatment of tumors and the resistance of tumor cells to

chemotherapeutic drugs are the main obstacles for these treatment applications. To avoid those disadvantages, studies are being carried out to develop protocols for the combination of various chemotherapeutics used in cancer treatment or for alternative therapeutic agents applied together with chemotherapeutic agents. It has been predicted that the side effects of chemotherapeutic drugs can be reduced and their anti-carcinogenic potential can be increased by using combinations that include alternative substances with antioxidant properties (8, 14, 15).

Vincristine is an important chemotherapeutic agent used in various stages of breast cancer treatment (8). However, in addition to the effectiveness of vincristine against cancerous cells, it also has toxic effects against rapidly proliferating healthy cells. In recent years, researchers have considered the use of antioxidants such as quercetin, genistein, and geraniol separately or in combination to reduce the side effects of chemotherapeutic agents without reducing the antitumoral effectiveness of those agents (43).

The honeys popularty known as “mad honey” or “rhododendron honey” are obtained from regions of the Black Sea provinces where *Rhododendron ponticum* and *R. luteum* are found in the flora. These honeys are mainly produced in the provinces of Artvin, Kastamonu, Zonguldak, Rize, Ordu, Tokat, and Sinop. Said to be a source of healing when consumed in measured amounts, these honeys have traditionally been used for health purposes and are also called “medical honey.” Grayanotoxin types I-III are most typically found in mad honey (17, 35) and these substances exert cytotoxic effects without harming healthy cells. The phenolic compounds in mad honey are the most significant group among the honey’s total compounds. They prevent mitotic catastrophe and scavenge free radicals. In this way, oxygenation is increased within the mitochondria of the cells and the microenvironment, helping to preserve cellular morphology by balancing the oxidative capacity (1, 6, 26). Many in vivo and in vitro studies have been conducted to evaluate the antioxidant properties of mad honey and Rhododendron ponticum extracts.

The main reasons for the frequent occurrence of breast cancer are inflammation, angiogenesis, the rapid cell cycle of breast tissue, and hormonal and genetic factors. The main factors that cause a predisposition to breast cancer are genetic variants in breast tissue cells, hormonal changes and the responses of breast tissue receptors to those changes, and variations in cell signaling pathways that change according to environmental factors (37).

This study aimed to evaluate whether the administration of vincristine and mad honey separately or together was an effective treatment for rats in a model of breast cancer. Changes in complete blood counts, blood serum parameters, and oxidative stress parameters were clinically assessed and regressive changes in breast tissue masses were evaluated in histopathological examinations.

Materials and Methods

Animal and Experimental Design: This study included 4 experimental and 2 control groups created using 72 inbred female Wistar albino rats that weighed approximately 195-240 g and had reached sexual maturity. A minimum of 12 animals were included in each group so as not to distort the statistical analysis with small sample sizes. The animals were cared for, housed, and fed for the duration of the experiment in the Experimental Animal Unit of the Ankara University, Türkiye. Considering animal welfare, appropriate numbers of animals were kept in each cage and food was provided ad libitum with appropriate temperature and ventilation conditions on the basis of a 12/12-hour cycle of light and darkness. Ethical permission was obtained from the relevant review board (Decision No: 2021-13-113).

A total of 72 rats were examined in six groups, 12 rats in each group. Administration of honey using gavage, and intraperitoneal administration of vincristine were performed during the last four weeks of the 13-week study period. (Figure 1).

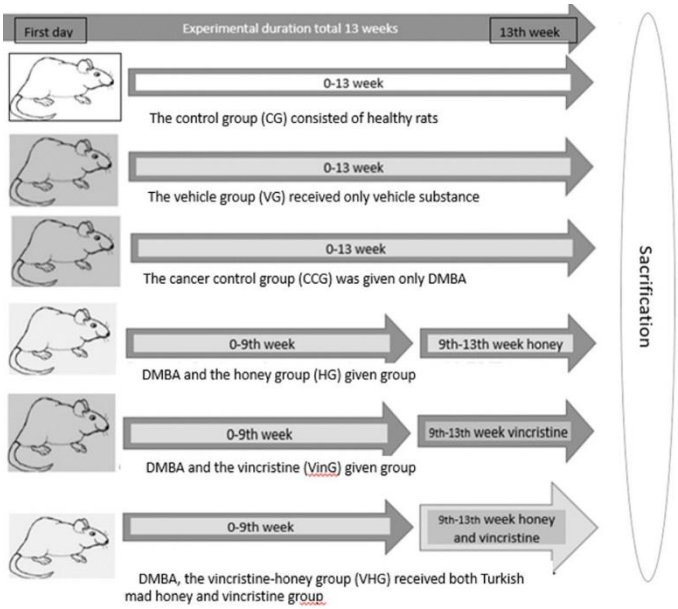

Figure 1. Experimental procedure.
1. Rat: The control group (CG) consisted of healthy rats.
2. Rat: The vehicle group (VG) received only vehicle substance
3. Rat: The cancer control group (CCG) was given only DMBA.
4. Rat: DMBA and the honey group (HG) given group.
5. Rat: DMBA and the vincristine (VinG) given group,
6. Rat: DMBA, the vincristine-honey group (VHG) received both Turkish mad honey and vincristine.

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Mammary Cancer Induction Modelling: After DMBA administration, 6 pairs of mammary glands were examined weekly in each group to monitor the experimental protocol. The presence of mass formations was monitored by inspection, palpation, and micrometer measurements. After DMBA administration, the tumors in breast tissues were considered based on a volume of 200 mm3, but the expected mass size did not develop and so the treatment protocol was started. Therefore, considering that masses developing in the mammary glands may be micromorphometric, the standard formula for calculating tumor volume of V=0.5×(W2×L) was not applied (V=tumor volume, W=length of the shorter side of the tumor; L=length of the longer side of the tumor).

Blood Sampling and Biochemical Analysis: A total of 7 mL of blood, with 5 mL collected into a tube without anticoagulant and 2 mL into a tube with EDTA, was obtained from each rat. Serum samples were separated within 3 hours of the collection of blood samples and stored at -80 °C until analysis. Hematological analyses were performed using an automated blood count device (Mindray BC-5000) within 3 hours of blood collection. Using serum samples, C-reactive protein (CRP), total protein, albumin, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and creatinine levels were measured with an automated biochemistry analyzer (Mindray BS-300).

Bcl xL level (Bioassay Technology Laboratory Cat No: E3340Ra, Zhenjiang, China) and Bax level (Bioassay Technology Laboratory Cat No: E1869Ra, Zhenjiang, China) and caspase-3 level (Bioassay Technology Laboratory, Cat No: E1648Ra Zhenjiang, China) caspase 8 level (Bioassay Technology Laboratory Cat No: E1370Ra, Zhenjiang, China) caspase 9 level (Bioassay Technology Laboratory Cat No: E1898Ra, Zhenjiang, China), and survivin level (Bioassay Technology Laboratory Cat No: E0191RA, Zhenjiang, China) ELISA test kits were measured spectrophotometrically (Sunrise RS-232, Tecan, Grödig, Austria).

Superoxide dismutase (SOD) and malondialdehyde (MDA) were also evaluated as parameters of oxidative stress. SOD activity was measured using a commercial test kit according to the method developed by Sun et al. (40). MDA level was measured using a commercial test kit according to the method developed by Ohkawa et al. (28).

End of Experiment: At the end of the experiment, the rats were anesthetized by intramuscular injections of xylazine (10 mg/kg BW) and ketamine (100 mg/kg BW), and then the rats were sacrificed by taking a large amount of blood from the heart with injectors. The suggested changes has been made. After the animals were euthanized, necropsies were performed in a species-specific manner.

Necropsy and Histopathological Examinations: Tumor development in the affected breast lobes was detected along the incised breast lines. Samples were taken from various points of the tissue to determine whether there was metastasis around the breast lobe or into the lymph nodes draining the area. Information about the size, color, shape, and appearance of the upper and sectional faces of tumoral masses in the affected area was recorded. Liver and kidney tissues were collected and fixed in 10% buffered formalin (pH 7.2) for 48 hours. Tissue samples were trimmed, passed through ethanol and xylene series and liquid paraffin series with an automatic tissue processer (Leica TP1020, Germany), and blocked in paraffin (EG1150, Thermo Shandon, Germany). Sections were taken from blocks with thicknesses of 5 µm (AS325, Shandon, Germany). Tissue sections were stained with hematoxylin and eosin (H&E) (22).

Sections were examined at magnification of ×200 to identify the presence of significant differences in the masses in breast lobes between the groups. Atypical malignancy criteria including pleomorphism, mitotic index, polychromasia, and islet formation were evaluated and scored in terms of vascularization and inflammatory cell infiltrations in anaplastic cells in a total of 10 different areas. Previous findings of relevant studies were taken into account in this process (13, 33). The findings were calculated according to equal percentiles obtained from mean calculation of all microscopic fields. The results were categorized with scores of 0 for no findings and 1, 2, 3, 4, 5, or 6 for cases of lesions according to the severity of the findings. According to this; numerics and clarification has been showed between parenthesis. (score 0): no finding 0%: (score 1) very light, 1-15%; (score 2) mild, 16-30%; (score 3) weak moderate, 31-45%, (score 4) moderate, 46-60%: (score 5) severe, 61-75%; (score 6) very severe, 76-100%.

Statistical Analysis: G*Power version 3.1.9.7 (44) was used to perform a priori power analysis to estimate the minimal sample size that is necessary to test the study hypothesis. To control Type I and Type II errors, α=0.05 and 1-β=0.8 levels were accepted and to indicate effectiveness of experimental conditions d=0.5 was determined. Thus, N=72 the obtained sample size was considered to be adequate to test the study hypothesis. The data were initially summarized with descriptive statistics and checked for whether assumptions were met. Results were evaluated by using Shapiro-Wilk test and Q-Q plot for normality and the Levene test for homogeneity of variances. One-Way Anova and Kruskal-Wallis test were used for comparisons between groups. In case of statistically significant difference between groups; Tukey, Games Howell or Dwass-Steel-Critchlow-Fligner pairwise comparisons analysis were used appropriately. Results were analyzed and expressed as mean ± standard error (M±SE).

Histopathological data were converted into equally divided percentiles and scored, they were validated with one-way ANOVA and post hoc Tukey tests and statistically compared between groups.

P value of <0.05 was considered to be statistically significant result for all analyses. The data were analysed using IBM SPSS Statistics 26.0 (SPSS®, IL, USA) and Graph-pad (8.0.1, San Diego, California, USA).

Results

Macroscopic Findings: In the rats of all groups, no macroscopic findings were observed in terms of criteria for size, color, and shape in the areas of the breast lobes.

Histopathological Findings: The tissues of each of the animals in the experimental groups and the control group of healthy animals were scored in terms of islet formation, vascularization, inflammatory cell infiltration, and apoptotic-degenerative changes in the glands as well as malignancy criteria. In the control group (CG), the cells were not anaplastic, as they were in the VG group, and islets had formed. Cystic changes and other changes such as degeneration, necrosis, and apoptosis were not observed in this group. Vascular and inflammatory changes were also not observed.

Islet proliferation was evident in the cells of the groups with cancer, especially in the cancer control group (CCG). Rather than forming a glandular structure, the glands were separated from each other and malignant cells were scattered within glands. Malignant cell islets were observed to be intense with features of adenocarcinoma. However, there were no cystic changes. In these cells, destruction was extensive in the nuclei and cytoplasm in terms of parenchymal degeneration, necrosis, and apoptotic changes. Pleomorphism and polychromasia were evident in the cells and mitotic figures were observed. However, the mitotic index was not as high as expected. Vascular changes were also prevalent in capillaries parallel to the degree of anaplasia. Arterioles and venioles, as well as capillaries, were enlarged with erythrocytes and were hyperemic. In some cases in the CCG group, free erythrocytes were observed outside of blood vessels, signifying hemorrhage. numerous eritrocytes were observed at periphery of vessels. In some cases, edema was also encountered. No inflammatory changes were seen (Figure 2).

Anaplastic changes with features of adenocarcinoma due to malignant cells, pleomorphism, and polychromasia were found at higher rates in the honey-administered group (HG) compared to the CCG group. Although the anaplastic activity in the glands was higher in this group, cystic changes were also more prevalent compared to the CCG group. The mitotic index was again higher than the value obtained for the CCG group. Inflammatory changes, apoptosis, and degenerative changes were absent (Figure 2).

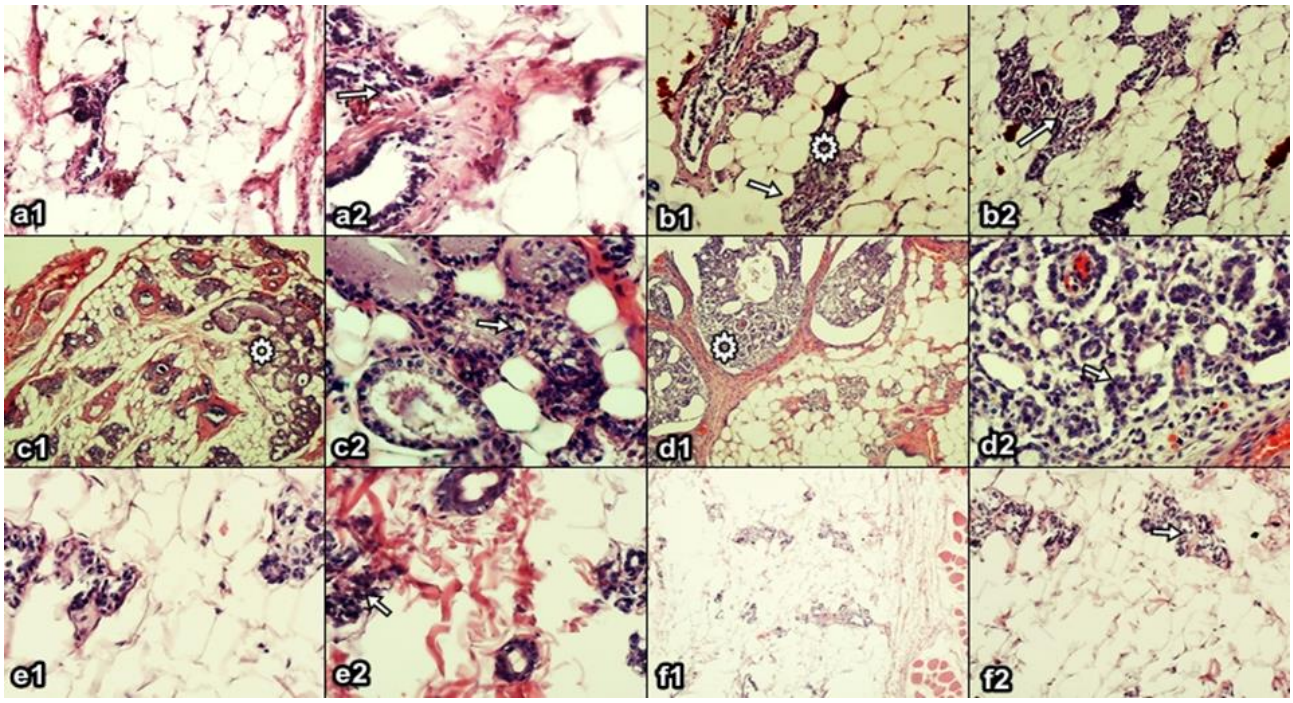

Figure 2. Histopathological changes in breast tissues of the experimental groups.
(a) CG: Mammary glands with normal appearance, ×100 (a1), ×200 (a2). (b) CCG: Anaplastic changes in the mammary glands with islets of proliferating cells (asterisk) and cells showing polychromasia and pleomorphism (arrow), ×100 (b1), ×100 (b2). (c) HG: Proliferating changes in the mammary glands (asterisk) and proliferating cells (arrow), some of which show degenerative findings, ×100 (c1), ×200 (c2). (d) VinG: Islet of prominently proliferating cells in the mammary glands (arrow), ×100 (d1), ×200 (d2). (e) VG: Normal appearance of mammary glands with degenerative changes in one or two areas (arrow), ×100 (e1), ×200 (e2). (f) VHG: Mild proliferative and marked degenerative changes in the mammary glands (arrow), ×100 (f1), ×100 (f2).

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Anaplasia in the islets formed by malignant mammary gland epithelial cells was not as intense in the vincristine group (VinG) in comparison to the CCG and HG groups. However, the cystic changes were intense. No apoptosis or degenerative changes were observed in the glands, as in the HG group. Pleomorphism, polychromasia, and vascular changes were common in many parts of the tissues, although less so than in the other group. The mitotic index was higher compared to the CCG group and lower compared to the HG group (Figure 2).

Cells in the vehicle control group (VG) were not anaplastic and cells showing mild polychromasia were found in some areas. Gland cells were smooth and formed islets. Cystic changes, degeneration, necrosis, and apoptosis were not observed in this group.

In the group of rats receiving both vincristine and honey (VHG), malignant mammary gland epithelial cells were seen in the form of islets in some cases. Pleomorphic changes were mild and polychromasia was generally mild to moderate. Gland structures were cystic in only one case. No vascular or inflammatory changes were found. However, degenerative changes in the gland epithelium were observed more intensely in both the nuclei and cytoplasm than in the other groups, except for the CCG group (Table 1, Table 2, Figure 2).

Table 1. Evaluations of histopathological lesions in breast tissue (mean ± standard error).

	Histopathological Parameters					
Groups	**CG (n=12)**	**VG (n=12)**	**CCG (n=12)**	**VinG (n=10)**	**HG (n=12)**	**VHG (n=7)**
Pleomorphism	1.51±1.51	0.00±0.00	56.23±13.33	69.41±4.95	42.39±5.20	4.5±7.24
Mitotic index	0.00±0.00	0.00±0.00	6.24±4.38	9.08±4.12	24.99±6.64	0.00±0.00
Polychromasia	18.17±5.24	11.10±4.73	64.80±11.92	36.33±5.86	76.36±5.21	28.33±5.83
Islet Anaplasia	55.55±14.26	61.10±13.34	66.63±9.20	33.31±5.49	67.42±4.16	23.33±6.18
Islet Cyst Formation	0.00±0.00	0.00±0.00	0.00±0.00	13.62±5.86	22.71±8.18	3.33±3.33
Vascular changes	0.00±0.00	0.00±0.00	22.90±11.32	15.14±8.54	27.76±4.73	3.33±2.20
Inflammation	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Apoptosis, degeneration, necrosis	20.82±6.52	0.00±0.00	75.9±6.28	0.00±0.00	0.00±0.00	33.33±6.50

Level of significance: * (mild), ** (moderate), *** (strong).

Table 2. Comparison of histopathological findings in breast tissues.

Tukey Multiple Correlation Test	Significance	Correlation	P-value
Pleomorphism vs. Mitotic index	-	-	0.3971
Pleomorphism vs. Polychromasia	-	-	0.3653
Pleomorphism vs. Islet anaplasia	-	-	0.6132
Pleomorphism vs. Islet cysts	-	-	0.4752
Pleomorphism vs. Vascular changes	-	-	0.4339
Pleomorphism vs. Inflammation	-	-	0.4260
Pleomorphism vs. Apoptosis-degeneration	-	-	0.9995
Mitotic index vs. Polychromasia	-	-	0.0700
Mitotic index vs. Islet anaplasia	Yes	*	0.0147
Mitotic index vs. Islet cysts	-	-	>0.9999
Mitotic index vs. Vascular changes	-	-	0.6051
Mitotic index vs. Inflammation	-	-	0.6982
Mitotic index vs. Apoptosis-degeneration	-	-	0.9371
Polychromasia vs. Islet anaplasia	-	-	0.9095
Polychromasia vs. Islet cyst	-		0.1140
Polychromasia vs. Vascular changes	Yes	*	0.0494
Polychromasia vs. Inflammation	-	-	0.1173
Polychromasia vs. Apoptosis-degeneration	-	-	0.8739
Islet anaplasia vs. Islet cysts	Yes	*	0.0291
Islet anaplasia vs. Vascular changes	Yes	*	0.0260
Islet anaplasia vs. Inflammation	Yes	*	0.0104
Islet anaplasia vs. Apoptosis-degeneration	-	-	0.4779
Islet cysts vs. Vascular changes	-	-	0.8551
Islet cysts vs. Inflammation	-	-	0.6896
Islet cysts vs. Apoptosis-degeneration	-	-	0.9492
Vascular changes vs. Inflammation	-	-	0.4197
Vascular changes vs. Apoptosis-degeneration	-	-	0.9824
Inflammationvs. Apoptosis-degeneration	-	-	0.6584

Histopathological Findings in the Liver: In order to evaluate changes in catabolic metabolism triggered by DMBA, livers were evaluated histopathologically. degenerating hepatocytes, the nuclei were pushed to one side of the cell and were usually pycnotic or lytic. Their cytoplasm had either swollen or several vacuoles of varying sizes. Degeneration were present in the VinG and CCG groups at same appearence. The rate of degeneration was higher in the HG group compared to the VHG and VG groups; however, it was lower compared to the VinG and CCG groups. In addition, the VHG group had a lower rate of degeneration than all other groups excluding CG and VG. Necrotic changes were more prominent in the VinG and CCG groups compared to the HG group. No signs of degeneration were found in the VHG, VG, and CG groups. There were no remarkable findings in terms of vascular changes or inflammation between control and experimental group (Figure 3). First of all, the control group and then the experimental groups seem to be better.

Histopathological Findings in the Kidneys: To evaluate the impact of DMBA on catabolic metabolism, the kidneys were evaluated histopathologically and minor changes were observed. In the kidneys, the findings were concentrated in the cortex. In tissues that showed signs of degeneration in the epithelium lining of the cortical tubules, the cytoplasm was generally pale. The nuclei were lytic and some of them were pycnotic. In cases of acute cell swelling or in more advanced stages, vacuolations of varying widths were present in the cytoplasm. The numbers of cells in the kidneys showing such findings were higher in the VG group. However, these disorders were encountered at lower rates in the HG and CCG groups. The values obtained for those two groups were found to be close to each other. There was a significant decrease in the rate of such degenerative changes in the VHG and VG groups compared to the other experimental groups. Tubules were healthy in most cases. No findings were observed in the CG group. In terms of necrotic changes, higher values were observed in the VinG group and, to a lesser extent, in the CCG group, while these values were lower in the HG group. No findings were observed in the CCG, VG, or VHG groups. There were no remarkable pathological findings in terms of vascular changes or inflammation (Figure 3, 4).

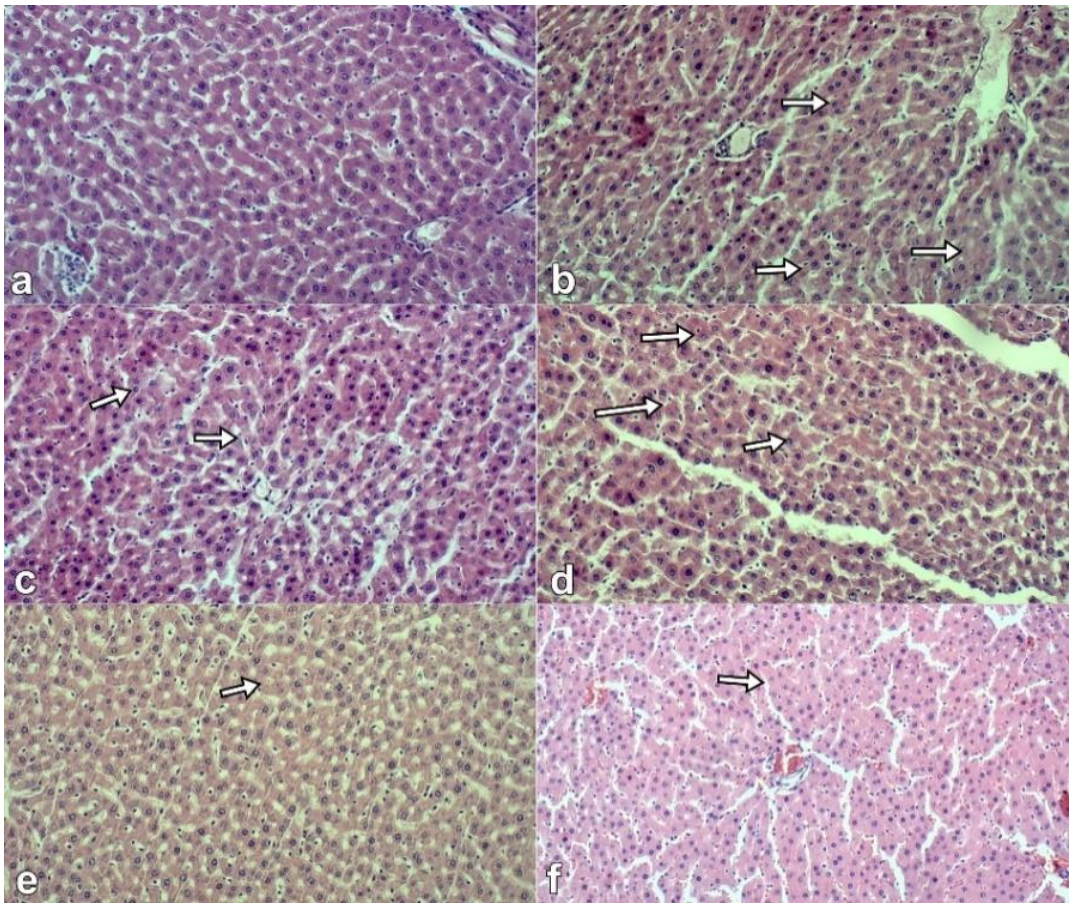

Figure 3. Histopathological findings in the liver in the experimental groups.
(a) Healthy appearance in hepatocytes in the control group (CG). (b) Intense degenerative and necrotic changes in hepatocytes in the cancer control group (CCG). (c) Moderate degenerative changes in hepatocytes in the group given honey (HG). (d) Intense degenerative and necrotic changes in hepatocytes in the group given vincristine (VinG). (e) Slight degenerative changes in hepatocytes in the group given the vehicle substance (VG). (f) Slight degenerative changes in hepatocytes in the group given vincristine and honey (VHG). ×100, H&E.

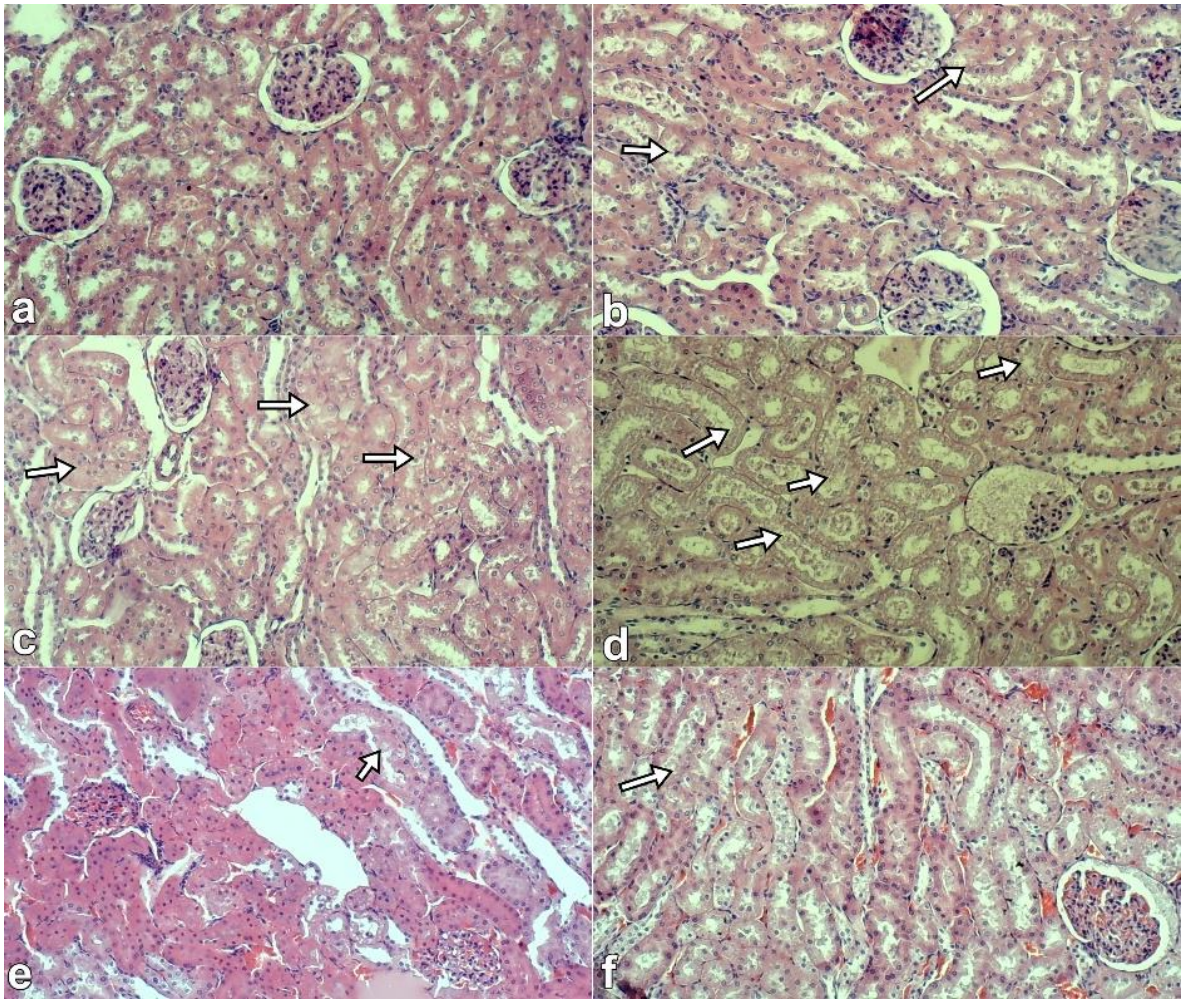

Figure 4. Histopathological findings in the kidneys in experimental groups.
(a) Healthy appearance in the kidney tubules in the control group (CG). (b) Degenerative and necrotic changes in the cortical tubules in the cancer control group (CCG). (c) Moderate degenerative changes in the cortical tubules in the honey-treated group (HG). (d) Intensive degenerative and necrotic changes in the cortical tubules in the vincristine group (VinG). (e) Degenerative changes in some cortical tubules in the group given the vehicle substance (VG). (f) Mild degenerative changes in the cortical tubules in the group given vincristine and honey (VHG) ×100, H&E.

Table 3. Classification of histopathological findings in the liver and kidneys in experimental groups.

Tukey multiple comparisons test (Liver)	Mean diff.	Significance	Adjusted P-value
Degeneration vs. Necrosis	0.2450	-	>0.9999
Degeneration vs. Vascular changes	25.32	-	0.1090
Degeneration vs. Inflammation	25.32	-	0.1090
Necrosis vs. Vascular changes	25.08	-	0.0979
Necrosis vs. Inflammation	25.08	-	0.0979
Vascular changes vs. Inflammation	0.000		
Tukey multiple comparisons test (Kidney)			
Degeneration vs. Necrosis	16.12	-	0.0968
Degeneration vs. Vascular changes	46.55	-	0.0850
Degeneration vs. Inflammation	46.55	-	0.0850
Necrosis vs. Vascular changes	30.43	-	0.2822
Necrosis vs. Inflammation	30.43	-	0.2822
Vascular changes vs. Inflammation	0.000	-	

Level of significance: * (mild), ** (moderate), *** (strong).

Table 4. Hematologic-Biochemical parameters according to experimental groups.

	Hematologic Parameters						
Groups	**CG (n=12)**	**VG (n=12)**	**CCG (n=12)**	**VinG (n=10)**	**HG (n=12)**	**VHG (n=7)**	**P**
WBC (10^9/L)	8.16±0.42^{a}	8.46±0.19^{a}	6.86±0.4^{b}	6.38±0.24^{b}	6.8±0.32^{b}	6.46±0.47^{b}	*P<0.001*
Lymphocyte (10^9/L)	5.4±0.18^{ab}	5.86±0.27^{a}	3.97±0.59^{bc}	4.43±0.2^{ac}	3.64±0.67^{cd}	2.04±0.4^{d}	*P<0.001*
Monocyte (10^9/L)	0.33±0.03	0.34±0.04	0.34±0.52	0.43±0.04	0.42±0.08	0.4±0.11	
Neutrophil(10^9/L)	2.16±0.25	2.12±0.3	2.27±0.26	1.61±0.18	1.74±0.14	1.88±0.23	
	Biochemical Parameters						
CRP (mg/L)	0.51±0.03	0.51±0.04	0.58±0.03	0.54±0.04	0.53±0.04	0.55±0.05	
TP (g/dL)	7.25±0.1^{b}	7.55±0.42^{ab}	8.17±0.19^{a}	7.68±0.14^{ab}	7.15±0.26^{ab}	6.92±0.27^{b}	*P=0.002*
Alb (g/dL)	3.15±0.13	3.29±0.19	3.55±0.09	3.5±0.06	3.09±0.11	3.58±0.1	
ALT(U/L)	44.08±4.11^{c}	47.85±2.54^{c}	83.88±1.77^{a}	71.19±5.62^{a}	60.39±3.73^{b}	53.68±2.61^{bc}	*P<0.001*
AST(U/L)	138.83±5.2^{c}	122.7±11.9^{c}	305.9±19.16^{a}	297.5±32.1^{a}	185.35±9.77^{b}	159.8±11.3^{bc}	*P<0.001*
Urea (mg/dL)	38.7±1.11	41.28±1.53	37.63±1.79	36.65±1.54	36.45±0.92	38.74±2.59	
Creatinine (mg/dL)	0,67±0.02^{ab}	0,75±0.03^{a}	0,72±0.02^{a}	0,64±0.05^{b}	0,65±0.01^{b}	0,71±0.02^{ab}	*P=0.009*
Survivin (ng/L)	38.04±0.91^{b}	38.75±1.39^{b}	46.03±1.43^{a}	35.67±1.91^{b}	39.8±1.85^{b}	36.61±1.02^{b}	*P<0.001*
Caspase-3 (ng/mL)	147.61±0.9^{a}	147.73±1.4^{a}	110.32±3.86^{c}	135.59±4.7^{ab}	129.6±2.18^{b}	126.17±1.1^{b}	*P<0.001*
Caspase-9 (ng/L)	6.72±0.06^{c}	6.22±0.08^{c}	8.87±0.27^{a}	7.92±0.2^{b}	8.03±0.09^{ab}	8.23±0.04^{a}	*P<0.001*
Caspase-8 (ng/mL)	120.95±1.1^{b}	121.09±1.6^{b}	117.38±1.57^{b}	117.65±0.8^{b}	132.59±3.18^{b}	136.15±2.5^{a}	*P<0.001*
Bax (ng/mL)	7.32±0.04^{a}	7.35±0.06^{a}	6.38±0.11^{c}	6.39±0.04^{c}	6.86±0.1^{b}	7.08±0.04^{b}	*P<0.001*
Bcl-xL (ng/mL)	7.27±0.06^{b}	6.2±0.03^{c}	8.31±0.23^{a}	7.11±0.23^{b}	7.24±0.11^{b}	7.48±0.05^{b}	*P<0.001*

Values in the table are given as arithmetic mean ± standard eror (M±SE).
*a,b,c,d indicate differences between groups in the same row.

Hematological and Biochemical Results: WBC values determined in CG and VG group rats were found to be significantly higher than other groups. The lymphocyte values determined in the CG and VG group rats were found to be significantly higherer than the values determined in the VinG, HG and VHG group.

Total protein and AST values determined in CCG and VinG groups were significantly higher than the values determined in CG and VG groups. Although the total protein and AST values determined in the HG group were not as high as CCG and VinG, they were found to be significantly higher than the values determined in the other groups. ALT levels determined in CCG and VinG groups were found to be significantly higher than the values determined in other groups. The survivin value determined in the CCG group was found to be significantly higher than the values determined in the other groups. The Caspase-3 value determined in the CCG group was found to be significantly lower than the values determined in the other groups. In addition, Caspase-3 values determined in the HG and VHG groups were found to be significantly lower than the values determined in the CG and VG groups. Caspase 9 levels determined in the CG and VG groups were found to be significantly lower than the values determined in the other groups. Caspase 8 levels determined in VHG group was found to be significantly higher than the values determined in other groups. Bax levels determined in the CG and VG groups were found to be significantly higher than the values determined in the other groups. Bax levels determined in CCG and VinG groups were found to be significantly lower than the values determined in other groups. The Bcl-xl level determined in the CCG group was found to be significantly higher than the values determined in the other groups (Table 4).

Discussion and Conclusion

Breast cancer is the second most common type of cancer in humans after lung cancer and the most common type of cancer in women (24). *In vivo* breast cancer models are important research tools for understanding both the pathogenetic mechanism of the disease and the development of treatment options (16). It is extremely crucial to evaluate disease progression by looking at levels of relevant biomarkers in serum or tissue and deciding accordingly on main or alternative treatment applications for breast tumors (18). By determining the levels of prognostic biomarkers for patients with breast tumors, critical evaluations and operational or chemotherapeutical decisions can be properly made based on knowledge of whether the cells have acquired malignant atypia, the epithelial-mesenchymal transition state, vascularization in the microenvironment, changes indicating hypoxia, or dysplasia. In this way, decisions can be made considering the likelihood of the cancer accelerating with neoplastic development, regressing, or remaining stable. The important point here is whether mitotic cells in neoplastic tissues are experiencing death or remaining alive. The

detection of cell proliferation and survival or the death of the cells via programmed cell death or apoptosis is very important in terms of making decisions about the severity of the patient's pathophysiological condition (31). While apoptosis may cause inhibition of the cancer to maintain homeostasis in tissues where cancer has developed, the inhibition of apoptosis is provided by survivin. However, there are still debates about whether the evaluation of survivin levels alone can allow an informed decision about cancer progression. Generally speaking, in the last 15 years it has been accepted that survivin is a good biomarker in terms of evaluating cell proliferation resulting from the disruption of homeostasis (27, 41).

Survivin is normally expressed in both the cytoplasm and nuclei of cells. Although survivin is expressed in the nuclei of different tumor types, it has been shown to be more intensely expressed in the cytoplasm of tissues in cases of breast cancer. Survivin has another function apart from maintaining neoplastic cells to facilitate their continuous division. In many *in vitro* studies and *in vivo* experiments, it has been shown that survivin may inhibit the promoter activity of caspase-9, an initiator of apoptosis, which is a critical step in apoptosis. It has been shown *in vivo* that apoptosis inhibits the activation process in mitochondrial pathways, and it is thought to do so by inhibiting secondary cancer initiation (21, 27, 29). Again, in studies on survivin, it was stated that the rate of survivin expression ranges between 60% and 90% according to differences in the methods used to evaluate breast cancer in humans (19, 20, 27, 34). However, survivin is accepted as a reliable marker in the early diagnosis of breast cancers and in determining treatment procedures according to expression levels. The cytoplasmic expression of survivin is stated to be highly effective in determining the prognosis of the disease (27). In the current study, the results showed that survivin levels were slightly increased in the cancer control group. The survivin levels determined in the groups given vincristine, vincristine and honey, and the vehicle substance were close to the levels obtained for the control group. However, when compared among themselves, the survivin levels in the vincristine group and the vincristine-honey group were slightly lower than the values determined in the control group or the vehicle group. These results showed that survivin levels were notably triggered in the presence of rapidly proliferating anaplastic cells. The decrease in survivin levels in the group given vincristine and the group given vincristine and honey showed that these substances suppressed the anti-neoplastic effects of the cancer by slowing down the transitions among the G0, G1, S, G2, and M phases of anaplastic cells during the division and proliferation stages. Therefore, the survivin levels began to decrease. This selected marker is also valuable for the determination of cancer prognosis and evaluations of co-treatment with grayanotoxin as well as vincristine chemotherapy.

Apoptosis, on the other hand, is an important mechanism for the determination of cell death or survival following different chemotherapy treatments. For this mechanism, there are mainly internal and external pathways. The internal pathways in apoptosis are extremely vital, particularly the one known as the mitochondrial pathway. These pathways generally act by increasing or decreasing the function of Bcl-2 protein family members (9, 11). Proteins that promote or inhibit apoptosis interact with each other and play roles in the death or survival of cells. Bax is also a member of the Bcl-2 family and it is known as a proapoptotic protein that plays a key role in promoting apoptosis. Bcl-2 is an antiapoptotic protein that inhibits Bax function in the initiation of cell death. An increase in Bax expression increases the susceptibility of cells to apoptotic stimuli and reduces tumor growth (2). In this sense, it has been suggested that the Bcl-2/Bax ratio alone may be an effective prognostic factor in determining apoptosis. Components that stimulate or inhibit apoptosis also determine the death or survival of cells. Under the influence of apoptotic stimuli, the release of cytochrome c from the mitochondria of the cell is triggered. With the help of ATP, Apaf-1, and cytochrome c a complex is formed and caspases are activated. Stimuli are commonly reported to be trigged by initiator caspases (caspase-2, -8, -9, and -10) with propagation via effector caspases (caspase-3, -6, and -7). As a result, researchers (36, 38) predict decreases in caspase-9 levels and high Bcl-2/Bax ratios in resistant cells.

In vitro studies of breast cancer have shown that dysregulated caspase activity plays a role not only in the progression or proliferation of breast tumor cells but also in chemotherapeutic resistance. It was previously shown that the MCF-7 breast cancer cell line may be susceptible to apoptosis due to restoration of caspase-3 expression and to doxorubicin in the event of caspase-3 deficiency, and this suggests that caspase-3 deficiency may be a possible mechanism for chemoresistance. In addition, activation mediated by pro-caspase-9, cytochrome c, and caspase-8 was shown to be regulated in MCF-7 cells (4). However, no association of caspase-8 with breast cancer-specific survival was found. Blazquez et al. (5) and Pu et al. (32) observed that caspase-3-dependent overexpression and apoptotic activity were more common in breast carcinomas, but in terms of prognosis, no significant relationship could be demonstrated between these findings and baseline caspase-3 expression. In the present study, in the process of apoptosis, Bax and Bcl-xL protein levels were close to each other in all groups. While the initial values of caspase-9 were close to each other in the control group, the group given honey, and the group given the vehicle substance, these values were seen to decrease in the cancer control group, the group given vincristine, and the group given vincristine and honey. Although it was

observed that these changes were triggered by caspase-3, values were relatively lower in the cancer control group, the group given honey, the group given vincristine, and the group given vincristine and honey, in contrast to expectations. Although the Bax and Bcl-xL levels remained stable in apoptosis, the decreases in caspase-9 and caspase-3 in the cancer control group and in the vincristine group were interpreted as signs of intense anaplastic changes. Therefore, it could be suggested that the apoptosis-triggering genes did not function sufficiently and did not initiate signaling as adequately as in the healthy groups. In line with the histopathological data, the cells were destroyed in the cancer control group and partially so in the group given vincristine and honey, and the changes were more apt to take the forms of degeneration and necrotic changes instead of apoptosis. Although anaplastic changes were common in the group given honey, cystic changes were observed and grayanotoxin functioned to trigger apoptosis even though degeneration was also observed. However, the induction of apoptosis in the group that received vincristine and honey was not observed at this critical level. In this groups, histopathological alterations were more prevalent than apoptosis. Furthermore, in this study, no changes related to anaplasia were found in the healthy control group; in those animals, the mammary gland cells had a regular order and gland structures were appropriately formed. It was thus thought that mild apoptotic changes may have been encountered in the cell nuclei in some areas as a result of physiological effects. In the cancer control group and the group given honey, intense anaplastic changes and islet formation due to the proliferation of cells lining the glands were detected. On the other hand, oxygenation and the feeding of the blood supply are important processes for cell survival during cancer development. Hence, we also observed capillarization and blood supply in medium-sized vessels in the mammary tissue. In addition, degenerative and necrotic changes were intense in the cancer control group. However, rather than degeneration and necrosis in the honey-administered group, cystic structures were noted, which were seen at high levels in all groups. In the vincristine group, anaplasia was reduced and islet formations were weaker, and vascular changes were also reduced compared to other groups. In addition, no degenerative or necrotic changes were found in the mammary tissue of this group. Gland structures in the vehicle group were normal, as in the control group. Apart from this, no remarkable findings were noted. In the group given vincristine and honey, anaplastic changes in mammary gland epithelial cells were considerably reduced, glandular cell proliferations were not sufficient for the formation of islets, cells were close to normal in appearance, and vascular changes were weak. On the other hand, degenerative and necrotic changes were observed, although not as much as in the cancer group. Inflammatory changes were not observed in any experimental groups.

As a result of chemical interactions, certain DMBA metabolites and excessive reactive oxygen species are produced in the cellular microenvironment. This situation creates carcinogenicity because it disturbs DNA and mitotic activities. After cumulative exposure, these metabolites create a deficit of cytochrome P450 enzymes, particularly in hepatocytes and kidney cells (10, 25). The cycles of tricarboxylic acids are also disrupted by metabolites of DMBA and free radicals. The first changes occur in biochemical parameters because the toxic compounds have catastrophic effects via cellular organelles (25). All cellular organelles, and particularly mitochondria, continue to shift toward catabolism (7, 10). As a result of these interactions, some enzymes, proteins, and macromolecules were found to be elevated in serum in the present study. At the same time, nuclear and cytoplasmic changes developed in cells, seen as degeneration, apoptosis, or necrosis. In our study, total protein levels were found to be relatively higher in the CCG, VinG, and VG groups when compared to the others. Values of ALT and AST were nearly the same. Levels were also higher in the CCG and VinG groups compared to CG, VG, and VHG for total protein. However, differences in CRP, urea, and creatinine were not significant. These findings confirm that the liver and kidneys are affected similarly by the toxicity of DMBA and vincristine. Plasma protein levels were elevated dramatically as a result of major damage in the cells. On the other hand, the groups that received honey containing grayanotoxin did not experience increases as large as expected for biochemical parameters. Hence, we believe that mad honey may be useful and effective in fighting cancer cells.

We encountered histopathological alterations as expected. Normally, the histological results obtained from the liver and kidneys provide evidence of biochemical alterations (27). We observed parallel results in our study. In previous studies of DMBA-induced liver damage, it was stated that necrosis, vascular changes, and non-purulent inflammation were observed in rat livers (12, 23, 30). In our study, degeneration and necrosis were also observed in the livers, but there was no inflammation. Vascular changes were not remarkable. The histopathological results obtained from the livers of the VinG and CCG groups were similar. Liver cells were also affected in the HG group, while the opposite was seen for VHG. In the VG and CG groups, the rates of degeneration were lowest. In the kidneys, cortical tubules were more affected by degeneration and necrosis. The cells were affected in VinG and CCG as well as the HG group. Preservation of cellular morphology in the kidneys was similar in the VG and CG groups. There were no remarkable pathological changes in terms of vascular changes or inflammation.

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In conclusion, it was understood that in the group given honey alone, degeneration and apoptosis were triggered. Thus, the development of breast tumors was reduced. Additionally, in the group given vincristine alone and in the group given vincristine and honey together, it was observed that these applications did not have significant regressive effects on breast tumors. It is clearly important to evaluate the relationship between the results obtained in the group given honey and the findings of apoptosis and degeneration in more depth, as the amount of grayanotoxin in particular honey samples may be a determining factor. It would also be useful to look at dose-response curves and Bax, Bcl-xL, caspase-9, and caspase-3 levels after selecting grayanotoxin applications in amounts close to the grayanotoxin levels in honey in mouse models and the frequently preferred human MCF-7 breast cancer line in *in vitro* trials. Evaluation of Bcl-2 antiapoptotic proteins with dose-response curves proportioned to Bax proteins, together with results obtained from literature reviews, will also be valuable. Although grayanotoxin targets the cell mitochondrial pathway with intrinsic apoptosis, it may also be effective on extrinsic pathways. Another result obtained from this study is that when cells are under the influence of cancer-inducing DMBA, vincristine, and vincristine-grayanotoxin, cellular toxicity develops and cell homeostasis is disrupted, and this affects the viability of the cells, resulting in strong effects of degeneration and necrosis instead of apoptosis. We believe that the most effective grayanotoxin types, namely I and III, which are found at high concentrations in Turkish mad honey, can be successfully applied as co-therapeutics with vincristine chemotherapy, which is classically preferred for mammary cancer therapy, after the proper therapeutic dosages are determined.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

EK, MEA, AMA, BB, NK and EG conceived and planned the experiments. EK, MEA, AMA, BB, NK and EG carried out the experiments. EK, MEA, AMA, BB, NK and EG planned and carried out the simulations. EK, MEA, AMA, BB, NK and EG contributed to sample preparation. EK, MEA, AMA, BB, NK and EG contributed to the interpretation of the results. EK, MEA, AMA, BB, NK and EG took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

Permission to conduct the study was granted by the ethics committee decision numbered 2021-13-113 of Ankara University Animal Experiments Local Ethics Committee, Türkiye.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Alzahrani HA, Boukraa L, Bellik Y, et al** (2012): *Evaluation of the Antioxidant Activity of Three Varieties of Honey from Different Botanical and Geographical Origins*. Glob J Health Sci, **4**, 192-196.
2. **Bargou RC, Wagener C, Bommert K, et al** (1996): *Overexpression of the death-promoting gene bax-alpha which is downregulated in breast cancer restores sensitivity to different apoptotic stimuli and reduces tumor growth in SCID mice*. J Clin Invest, **97**, 2651-2659.
3. **Beaudu-Lange C, Larrat S, Lange E, et al** (2021): *Prevalence of Reproductive Disorders including Mammary Tumors and Associated Mortality in Female Dogs*. Vet Sci, **8**, 184.
4. **Blanc C, Deveraux QL, Krajewski S, et al** (2000): *Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells*. Cancer Res, **60**, 4386-4390.
5. **Blazquez S, Sirvent JJ, Olona M, et al** (2006): *Caspase-3 and caspase-6 in ductal breast carcinoma: a descriptive study*. Histol Histopathol, **21**, 1321-1329.
6. **Buratti S, Benedetti S, Cosio MS** (2007): *Evaluation of the antioxidant power of honey, propolis and royal jelly by amperometric flow injection analysis*. Talanta. **71**, 1387-1392.
7. **Cavalcanti C, Costa-Lotufo LV, Moraes MO, et al** (2006): *Genotoxicity evaluation of kaurenoic acid, a bioactive diterpenoid present in Copaiba oil*. Food Chem Toxicol, **44**, 338-392.
8. **Chen J, Li S, Shen Q, et al** (2011): *Enhanced cellular uptake of folic acid-conjugated PLGA-PEG nanoparticles loaded with vincristine sulfate in human breast cancer*. Drug Dev Ind Pharm, **37**, 1339–1346.
9. **Chipuk JE, Moldoveanu T, Llambi F, et al** (2010): *The BCL-2 family reunion.* Mol Cell, **37**, 299-310.
10. **Christou M, Savas U, Schroeder S, et al** (1995) *Cytochromes CYP1a1 and CYP1B1 in the rat mammary gland: cell specific expression and regulation by polycyclic aromatic hydrocarbons and hormones*. Mol Cell Endocrinol, **115**, 41-50.
11. **Danial NN, Korsmeyer SJ** (2004): *Cell death: critical control points*. Cell, **116**, 205-219.
12. **Farombi EO, Shrotriya S, Surh YJ** (2009): *Kolaviron inhibits dimethyl nitrosamine-induced liver injury by suppressing COX-2 and iNOS expression via NF-ÍB and AP-1*. Life Sci, **84**,149-155.

13. **Goldschmidt M, Pena L, Rasotto R, et al** (2011): *Classification and grading of canine mammary tumors*. Vet Pathol, **48**, 117-131.
14. **Guillen KP, Fujita M, Butterfield AJ, et al** (2022): *A human breast cancer-derived xenograft and organoid platform for drug discovery and precision oncology*. Nat Cancer, **3**, 232–250.
15. **Gulbahce ME, Baltaci S, Oztekin M, et al** (2021): *The Effect of Zinc and Melatonin Administration on Lipid Peroxidation, IL-6 Levels, and Element Metabolism in DMBA-Induced Breast Cancer in Rats*. Biol Trace Elem Res, **199**.
16. **Holen I, Speirs V, Morrissey B, et al** (2017): *In vivo models in breast cancer research: progress, challenges and future directions*. Dis Models Mech, **10**, 359-371.
17. **Jansen SA, Kleerekooper I, Hofman ZL, et al** (2012): *Grayanotoxin poisoning: 'mad honey disease' and beyond.* Cardiovasc Toxicol, **12**, 208-215.
18. **Kabel AM** (2017): *Tumor markers of breast cancer: New prospectives*. J Oncol Sci, **3**, 5-11.
19. **Kayaselcuk F, Nursal TZ, Polat A, et al** (2004): *Expression of survivin, bcl-2, P53 and bax in breast carcinoma and ductal intraepithelial neoplasia (DIN 1a).* J Exp Clin Cancer Res, **23**, 105-112.
20. **Kennedy SM, O'Driscoll L, Purcell R, et al** (2003) *Prognostic importance of survivin in breast cancer.* Br J Cancer, **88**, 1077-1083.
21. **LaCasse EC, Baird S, Korneluk RG, et al** (1998): *The inhibitors of apoptosis (IAPs) and their emerging role in cancer*. Oncogene, **17**, 3247-3259.
22. **Luna LG** (1968): Manual of Histologic Staining Methods; Armed Forces Institute of Pathology (U.S.); Editor, Lee G. Luna; Edition, 3; Publisher, McGraw-Hill, 1968.
23. **Mohamed AM, Abdalla MS, Rizk MZ, et al** (2014): *Alleviation of Dimethylnitrosamine-induced liver injury and fibrosis by supplementation of Anabasis articulata extract in rats*. Indian J Clin Biochem, **29**, 418-429.
24. **Moulder S, Hortobagyi GN** (2008): *Advances in the treatment of breast cancer*. Clin Pharmacol Ther, **83**, 26-36.
25. **Nandakumar N, Balasubramanian MP** (2011): *Hesperidin protects renal and hepatic tissues against free radical-mediated oxidative stress during DMBA-induced experimental breast cancer.* J Environ Pathol Toxicol Oncol, **30**, 283-300.
26. **Narimane S, Demircan E, Akkal S, et al** (2017): *Correlation between antioxidant activity and phenolic acids profile and content of Algerian propolis: Influence of solvent*. Pak J Pharm Sci, **30**, 1417-1423.
27. **Oh JW, Yang WI, Lee MJ, et al** (2009): *The prognostic significance of survivin expression in breast cancer*. J Breast Cancer, **12**, 285-294.
28. **Ohkawa H, Ohishi N, Yagi K** (1979): *Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction*. Anal Biochem, **195**, 351-358.
29. **Okada E, Murai Y, Matsui K, et al** (2001): *Survivin expression in tumor cell nuclei is predictive of a favorable prognosis in gastric cancer patients*. Cancer Lett, **163**, 109-116.
30. **Ozdemir H, Oto G, Ekin S, et al** (2017): *The protective effects of Lepidium sativum l. In 7,12-dimethylbenz(a)anthracene applied rats*. Fresenius Environ Bull, **26**, 2446-2453.
31. **Pakkiri P, Lakhani SR, Smart CE** (2009): *Current and future approach to the pathologist's assessment for targeted therapy in breast cancer*. Pathology, 4189-4199.
32. **Pu X, Storr SJ, Zhang Y, et al** (2017): *Caspase-3 and caspase-8 expression in breast cancer: caspase-3 is associated with survival.* Apoptosis, **22**, 357-368.
33. **Russo J, Russo IH** (2000): *Atlas and histologic classification of tumors of the rat mammary gland*. J Mammary Gland Biol Neoplasia, **5**, 187-200.
34. **Ryan BM, Konecny GE, Kahlert S, et al** (2006): *Survivin expression in breast cancer predicts clinical outcome and is associated with HER2, VEGF, urokinase plasminogen activator and PAI-1*. Ann Oncol, **17**, 597-604.
35. **Sahin A, Türkmen S, Güzel N, et al** (2018): *A Comparison of the Effects of GrayanotoxinContaining Honey (Mad Honey), Normal Honey, and Propolis on Fracture Healing*. Med Princ Pract, **27**, 99-106.
36. **Sharifi S, Barar J, Hejazi MS, et al** (2014): *Roles of the Bcl-2/Bax ratio, caspase-8 and 9 in resistance of breast cancer cells to paclitaxel*. Asian Pac J Cancer Prev, **15**, 8617-8622.
37. **Sheikh A, Hussain SA, Ghori Q, et al** (2015): *The spectrum of genetic mutations in breast cancer*. Asian Pac J Cancer Prev, **16**, 2177-2185.
38. **Shi Y** (2002): *Mechanisms of caspase activation and inhibition during apoptosis.* Mol Cell, **9,** 459-470.
39. **Shokouh TZ, Ezatollah A, Barand P** (2015): *Interrelationships Between Ki67, HER2/neu, p53, ER, and PR Status and Their Associations With Tumor Grade and Lymph Node Involvement in Breast Carcinoma Subtypes: Retrospective-Observational Analytical Study*. Medicine (Baltimore), **94**, 1359.
40. **Sun Y, Oberley LW, Li Y** (1988): *A simple method for clinical assay of superoxide dismutase*. Clin Chem, **34**, 497-500.
41. **Tanaka K, Iwamoto S, Gon G, et al** (2000): *Expression of survivin and its relationship to loss of apoptosis in breast carcinomas*. Clin Cancer Res, **6**, 127-134.
42. **Testa U, Castelli G, Pelosi E** (2020): *Breast Cancer: A Molecularly Heterogenous Disease Needing Subtype-Specific Treatments*. Med Sci (Basel), **8**, 18.
43. **Thomas E, Holmes FA, Smith TL, et al** (2004): *The use of alternate, non-cross-resistant adjuvant chemotherapy on the basis of pathologic response to a neoadjuvant doxorubicin-based regimen in women with operable breast cancer: long-term results from a prospective randomized trial*. J Clin Oncol, **22**, 2294-2302.
44. **Wolf EJ, Harrington KM, Clark SL, et al** (2013): *Sample Size Requirements for Structural Equation Models: An Evaluation of Power, Bias, and Solution Propriety.* Educational and Psychological Measurement, **6,** 913–934.

Concurrent infection of Infectious Bronchitis Virus and *Mycoplasma gallisepticum* in a backyard poultry

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ABSTRACT

This study reports the co-existence of two S1 mutants of GI-13 (4/91-like) Infectious Bronchitis Virus (IBV) and *Mycoplasma gallisepticum* (MG) in a backyard poultry flock that had non-vaccinated 30 broiler chickens and four turkey pullets. Serum samples and tracheal swabs were taken from the chickens and turkey pullets showing respiratory signs. Serum antibody levels were measured using commercial ELISA kits against IBV, Avian Influenza Virus (AIV), Newcastle Disease Virus (NDV), Avian Metapneumovirus (AMPV), MG, *Mycoplasma synoviae* (MS), and *Ornithobacterium rhinotracheale* (ORT). Additionally, tracheal swabs were tested for AIV serotypes H5, H7, and H9, NDV, IBV, AMPV, MG, MS, *Pasteurella multocida*, *Avibacterium paragallinarum*, and *Bordetella avium* by circular amplification technology (CAT). Anti-MS, -IBV, -MG, -NDV, -AMPV, and -ORT IgG antibodies were detected in some chicken sera, while anti-NDV, -MG, -MS, and -ORT IgG antibodies were detected in turkey sera. All avian tracheal swabs were positive for MG. However, IBV was only detected in chicken tracheal samples tested by CAT. The IBV strains were genotyped by sequencing a part of the *S1* glycoprotein gene. The alignment analyses of two isolates showed 99.35% and 98.69% nucleotide similarities and 99.02% amino acid similarities with the 4/91 IBV vaccine and field strains. Two mutants showed 99.35% nucleotide and 100% amino acid sequence identity to each other. The turkeys and chickens in the flock had MG and MG/IBV co-infections, respectively. Consequently, the presence of mutants of 4/91 (GI-13) IBV genotypes and MG found in backyard poultry could be a potential epidemiological source for commercial flocks in poultry integrations.

Introduction

Infectious diseases in areas of intensive poultry production can easily spread if poultry houses encounter an infection, especially one capable of aerosol transmission. In intensive production, strict biosecurity regulations are followed, but there are numerous factors that defuse these biosecurity practices against infectious diseases, including insufficient immunity in poultry flocks, improper vaccinations, inadequate vector control, and the presence of infection sources around poultry production facilities (16). Backyard poultry can be a source of infectious diseases for chickens and turkeys in commercial poultry premises. These backyard flocks are mostly in the gardens of villagers and can easily be exposed to viruses which are generally carried by migratory birds (28).

Viral and bacterial respiratory infections and related pathological problems such as swollen head syndrome and infectious sinusitis are the most common problems in poultry (1, 24). Avian Influenza Virus (AIV), Newcastle Disease Virus (NDV), Avian Coronavirus Infectious Bronchitis Virus (IBV), Avian Metapneumovirus (AMPV), *Mycoplasma gallisepticum* (*M.gallisepticum*: MG), *Mycoplasma synoviae* (*M. synoviae*: MS), *Pasteurella multocida* (*P. multocida*), *Avibacterium*

paragallinarum (*A.paragallinarum*), and *Bordetella avium* (*B. avium*) are responsible for respiratory tract infections in both chickens and turkeys, as well as some other birds such as finches, ducks, geese, pigeons (26). Some agents such as MG and MS can infect birds without clinical signs and cause sub-clinical infections. These agents can be easily transmitted vertically or laterally from bird to bird. Sub-clinical MG infections can show clinical chronic respiratory disease in chickens and infectious sinusitis in turkeys (7, 30). Additionally, birds infected with MG and MS are more prone to getting infected with other viral and bacterial agents such as IBV and *Escherichia coli* (18). The presence of IBV genotypes in backyard chickens has been reported in some studies. For example, in the United States, IBV was found to be the most commonly detected virus in backyard poultry between 2015 and 2017 (5). Moreover, Shokri et al. (25) demonstrated the presence of 793/B, IS/1494/06, and QX IBV genotypes in backyard poultry flocks as a potential source of IBV infection in commercial chicken flocks. In Canada, Brochu et al. (4) conducted a prospective 2-year prevalence study between 2015 and 2017 and reported that IBV was detected at a rate of 39% in all samples. They also stated that the most common co-infection was a combination of IBV, MG, and MS.

Backyard poultry production should be considered a great risk for a great number of poultry stocks in intensive poultry production units and commercial poultry production. Although this issue is very important in national poultry breeding, there is rather limited information on the infection dynamics of backyard production in the literature. Moreover, few studies have focused on avian respiratory diseases at the molecular level in poultry production. In this study, we investigated the possible viral and bacterial respiratory agents and demonstrated a co-infection consisting of the 4/91 IBV genotype and MG in a non-commercial backyard poultry flock where chickens and turkeys were being bred together within the same garden. We then genetically characterised the two mutant IBVs that were detected in the 4/91 genotype of the GI-13 genetic lineage from chickens.

Materials and Methods

Sampling: The study was performed on a backyard flock located in the Bandırma district of the province of Balıkesir, Türkiye. This flock was in the center of the province of Bandırma where numerous commercial poultry production companies are located. The backyard flock consisted of 30 broiler chickens and 4 turkey pullets. All chickens and turkey pullets showed severe respiratory symptoms such as swollen head, swollen submaxillary sinus, gasping, as well as dullness, fatigue, and depression. None of these animals had been vaccinated against any poultry infections. Blood samples and tracheal swabs were taken from four turkey pullets and randomly selected 12 chickens. Blood samples (1.5-2 ml) were drawn from *Vena cutanea ulnaris* into 2 ml sterile microfuge tubes. Sera were obtained by centrifugation at 1300× g for 10 min and stored at −20 °C until analysis. Twelve chicken and four turkey pullet tracheal samples collected using sterile swabs were pooled to comprise four individual swabs each, and used as four samples for subsequent analysis. For this, tracheal swabs were pooled and placed in sterile tubes containing phosphate-buffered saline (PBS). After vortexing vigorously for 15-20 seconds, each swab was pressed against the inner wall of the tube to release the collected material into PBS and was stored at -20°C for further tests.

ELISA for antibody detection: To detect AIV-, NDV-, IBV-, AMPV-, MG-, MS-, and ORT-specific IgG, commercial ELISA kits were used. The test procedures were performed based on the instructions provided by the manufacturers (for AIV-, NDV-, IBV-, AMPV-, MG-, MS-specific IgG: BioCheck, UK, Ltd., London, United Kingdom; for ORT-specific IgG: IDEXX, Westbrook, ME, USA). Absorbance was measured at 405 nm (for AIV-, NDV-, IBV-, AMPV-, MG-, and MS-specific IgG) and 650 nm (for ORT-specific IgG) using a BioTek ELx800 ELISA Reader (BioTek Instruments Inc., Winooski, USA). The cut-off values were used as given in the kit instructions.

Nucleic acid extraction: Tracheal swabs were transferred to Molecular Transport and Lysis Reagent (MTRL) tubes (Nucleogene Biotechnology Co., Istanbul, Türkiye) and left for 30 min. All liquids were then transferred to a spin column placed in a collection tube, and they were centrifuged at 8000xg for one minute. 500 µl of 80% ethanol was added into the spin column and centrifuged at 8000xg for one minute. Next, the spin column was centrifuged for one minute at 16000xg until there was no residual ethanol left. Afterward, 50 µl of Nuclease-Free Water was added to the center of the spin column and centrifuged at 8000xg for one minute. Finally, the obtained nucleic acids were stored at -80°C.

Nucleic acid detection: To detect possible viral and bacterial etiological agents, we used circular amplification technology (CAT) (Nucleogene Biotechnology Co., Istanbul, Türkiye) based on the principles of the isothermal amplification of nucleic acids (11). We performed the CAT method to detect a specific part of the genes of AIV serotypes H5, H7, and H9, NDV, IBV, AMPV, MG, MS, *P. multocida*, *A. paragallinarum*, and *B. avium*. The working principle of the CAT test is based on the binding of 10 specific primers and 3 special

enzymes to a targeted gene region. Ten primers bind to the targeted gene region, and these primers then fold into DNA or RNA that has been translated into cDNA using3 special enzymes to form loops. By introducing primary radiation marked with a dye to these loops, they are measured with a Molecular Detection Assay device, curves are drawn on the screen, and positive samples are determined (23).

IBV genotyping: A nested reverse transcriptase polymerase chain reaction (RT-PCR) described by Worthington et al. (29) was modified to genotype the IBV isolates. The first round of amplification was carried out in a final volume of 25 µl of a mixture containing 0.5 µl of the primers SX1+ (CACCTAGAGGTTTGT/CTA/TGCAT) and SX2- (TCCACCTCTATAAACACCC/TTT), 2.5 µl of RNA, 5 µl OneStep RT-PCR Buffer (Qiagen), 1 µl of OneStep RT-PCR Enzyme Mix (Qiagen), 1 µl of dNTP mix (Qiagen), and 14.5 µl of ddH_2O. For the second nested PCR, a mixture at a volume of 20 µl [0.4 µl of the primers SX3+ (TAATACTGGC/TAATTTTTCAGA) and SX4- (AATACAGATTGCTTACAACCACC), 10 µl of Taq PCR master mix (Qiagen), 7.2 µl of ddH_2O, and 2 µl of the first amplicon] was prepared. The first thermal profile was set up as 50°C/30 min for the RT stage, 95°C/15 min, 30 cycles at 94°C/10 min, 50°C/1.5 min, and 72°C/2 min in order. The second profile was set up as 94°C/3 min, 30 cycles at 94°C/1 min, 48°C/1.5 min, and 72°C/2 min in this order using a thermal cycler (BioRad C100 Touch Thermal Cycler, BioRad Laboratories, California, USA). The amplification products were purified and sequenced using an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer). We evaluated the similarities between the detected IBV samples and previously reported IBV genotypes in the BLASTn online tool provided by NCBI based on partial *S1* sequences. Multiple sequence alignments were performed using Jalview v2.7. Next, the aligned sequences were subjected to bootstrapping (1000 replicates), and a phylogenetic tree was constructed with MEGA 7 v7.0.26 by the neighbor-joining method with a Kimura two-parameter model (17).

Results

ELISA results: Among the chicken serum samples (n=12), 83.33% (n=10) had MS-specific IgG, 50% (n=6) had IBV-specific IgG, 33.33% (n=4) had MG-specific IgG, 16.67% (n=2) had NDV-specific IgG, 16.67% (n=2) had AMPV-specific IgG, and 8.33% (n=1) had ORT-specific IgG. However, all samples were negative for AIV (Table 1). The IgG levels for each chicken sample are presented in Figure 1. The rates of dual, triple, and quadruple antibody presence were found in 33.33%, 25%, and 8.33% of the samples, respectively. All turkey samples were positive against NDV and MG. Additionally, 75% (n=3) had MS-specific IgG, and 25% (n=1) had ORT-specific IgG. None of the turkey samples had AIV-, IBV-, or AMPV-specific IgG (Table 1).

Table 1. Numbers and percentages of chicken and turkey serum samples with positive IgG OD levels for AIV, NDV, IBV, AMPV, MG, MS, and ORT.

	Chicken (n=12)		Turkey (n=4)	
	n	%	n	%
IBV	6	50	0	-
MG	4	33.33	4	100
MS	10	83.33	3	75
AMPV	2	16.67	0	-
ORT	1	8.33	1	25
NDV	2	16.67	4	100
AIV	0	-	0	-

Molecular detection of agents: The chicken samples were found to be negative for AIV serotypes H5, H7, and H9, NDV, AMPV, MS, *P. multocida*, *A. paragallinarum*, and *B. avium*, while they were positive for MG and IBV. The turkey samples were found to be positive for MG. Some examples of amplification curves for the positive and negative samples are presented in Figure 2a-2d.

We then identified the genotypes of the two IBV isolates detected according to the *S1* gene sequence analysis. The BLAST analysis revealed that two isolates (AvCoV/backyard/chicken/Bandirma1/21 and AvCoV/backyard/chicken/Bandirma2/21) were closely related to the 4/91 variant (GenBank Acc. No: AF093794). The nucleotide similarity rates of AvCoV/backyard/chicken/Bandirma1/21 and AvCoV/backyard/chicken/Bandirma2/21 to the original 4/91 IBV (AF093794) were 99.35% and 98.69%, respectively. The amino acid similarity rate between the 4/91 strain and the two isolates obtained in this study was 99.02%. Moreover, AvCoV/backyard/chicken/Bandirma1/21 and AvCoV/backyard/chicken/Bandirma2/21 showed 99.35% nucleotide and 100% amino acid sequence identity to each other (Figure 3a, 3b). Two mutations were observed at positions 801 (thymine to cytosine) and 1062 (adenine to thymine) in AvCoV/backyard/chicken/Bandirma2/21 compared to the 4/91 *S1* sequences. There were also two mutations at positions 1092 (thymine to adenine) and 1095 (thymine to adenine) in AvCoV/backyard/chicken/Bandirma1/21 and AvCoV/backyard/chicken/Bandirma2/21. The mutations in the nucleotide sequence observed in our IBV isolate led to three silent mutations (amino acid positions 267, 354, and 364). On the other hand, the mutation at amino acid position 365 was a missense mutation recognised by the phenylalanine to leucine alteration.

To determine the phylogenetic relationships among the IBV strains, we analysed the *S1* gene sequences. As shown in Figure 4, two IBV strains in our study were clustered into GI-13 genotype that includes 4/91 vaccine (KF377577), 4/91 pathogenic strain (AF093794), UK/2016/81 (MH590028), AvCoV/chicken/TR/L37/2017 (OL956527), and CR88-UPM2013 (KM067900). Two IBV strains obtained in this study were deposited to GenBank with the following access numbers: AvCoV/backyard/chicken/Bandirma1/21 (OL981643) and AvCoV/backyard/chicken/Bandirma2/21 (OL981644).

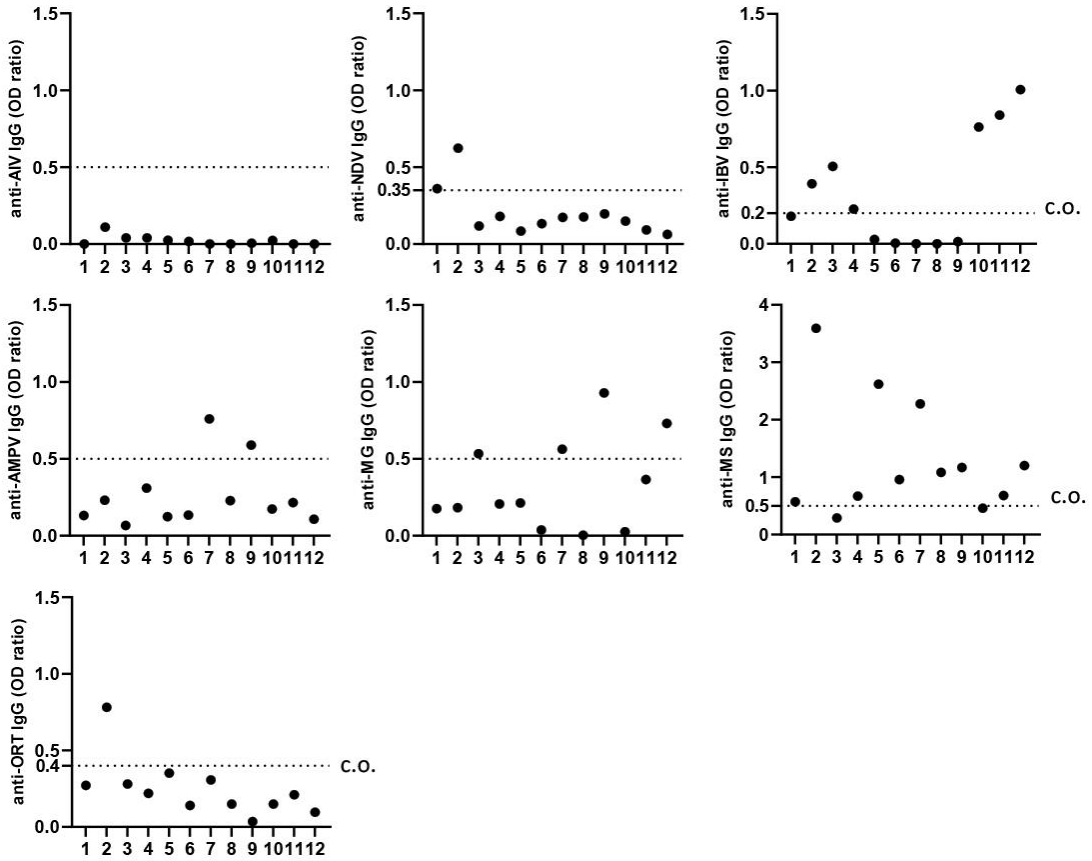

Figure 1. AIV, NDV, IBV, AMPV, MG, MS, and ORT-specific IgG levels in chicken samples. Horizontal lines indicate cut-off values (c.o.) given the instructions of the kit.

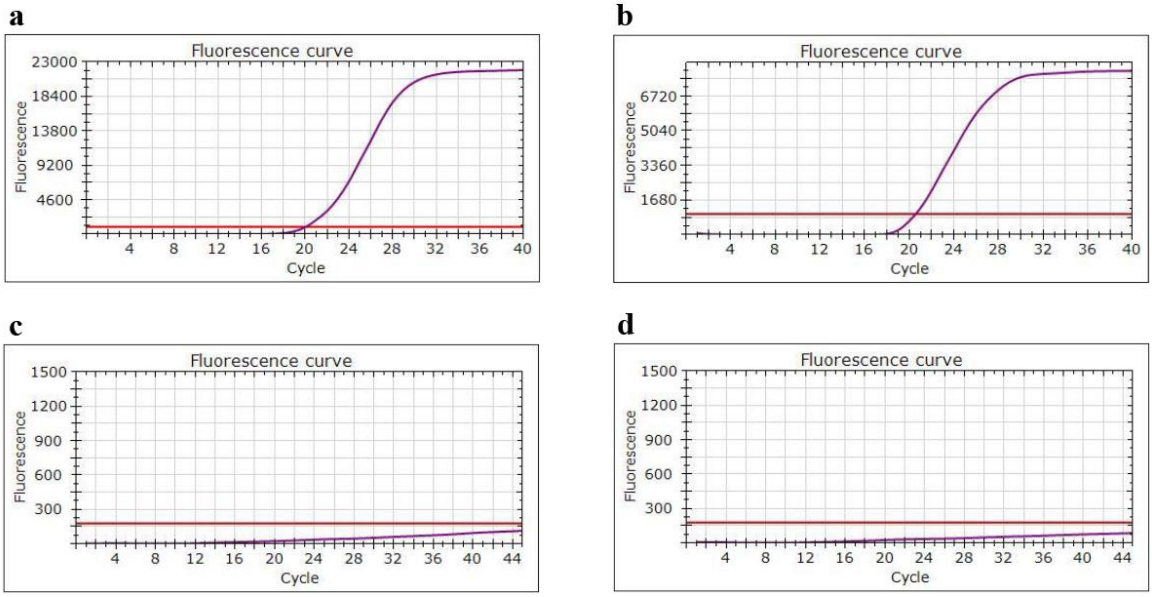

Figure 2. Results of circular amplification technology. Graphic demonstration of positive amplification curves for (**a**) MGand (**b**) IBV and negative curves for (**c**) NDV and (**d**) AMPV.

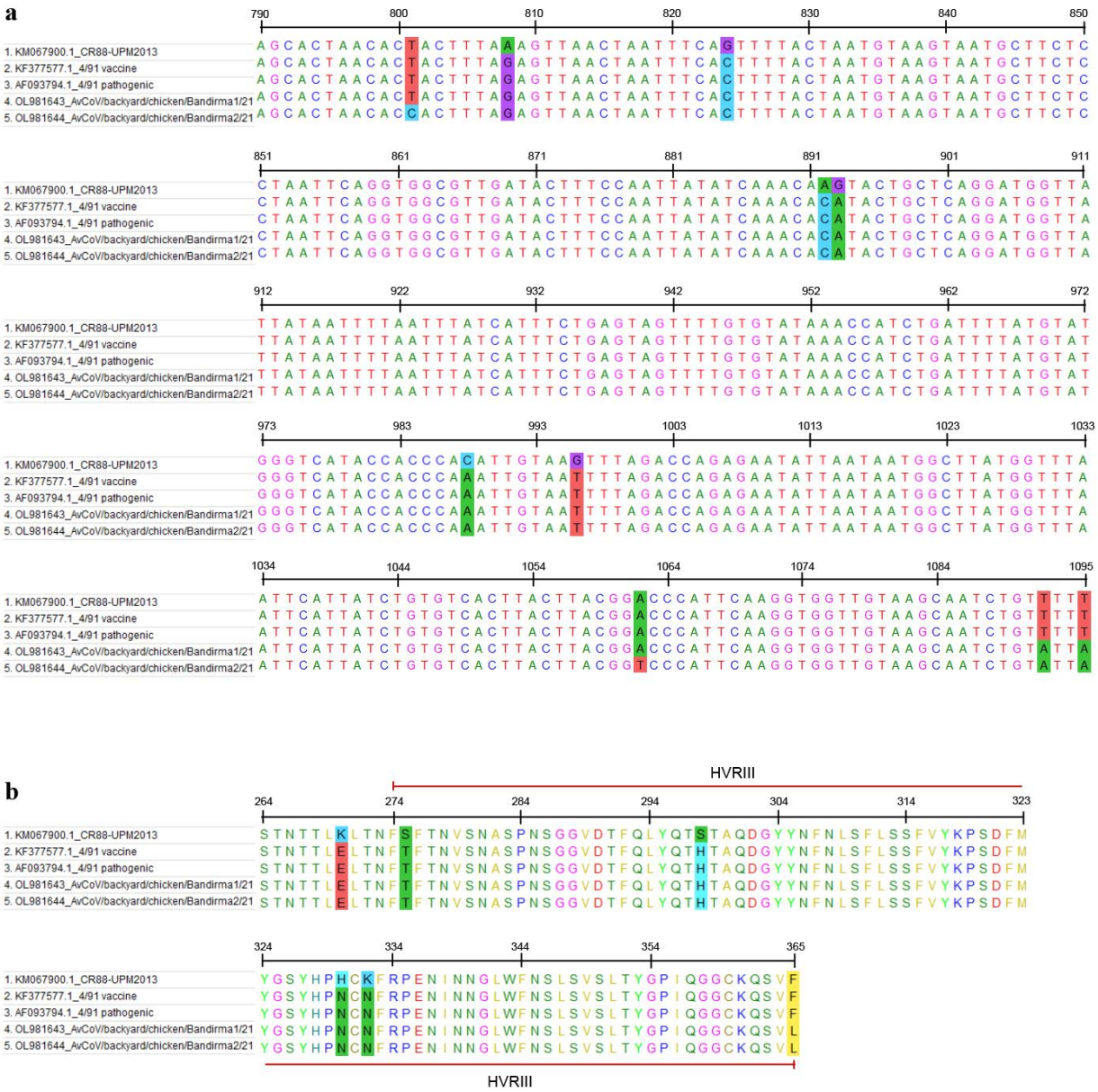

Figure 3. Alignment and comparison of a partial *S1* sequence. (**a**) Nucleotide and (**b**) amino acid sequences of *S1* gene comparison of two IBV isolates (AvCoV/backyard/chicken/Bandirma1/21 and AvCoV/backyard/chicken/Bandirma2/21) obtained in this study. HVRIII: hypervariable region III.

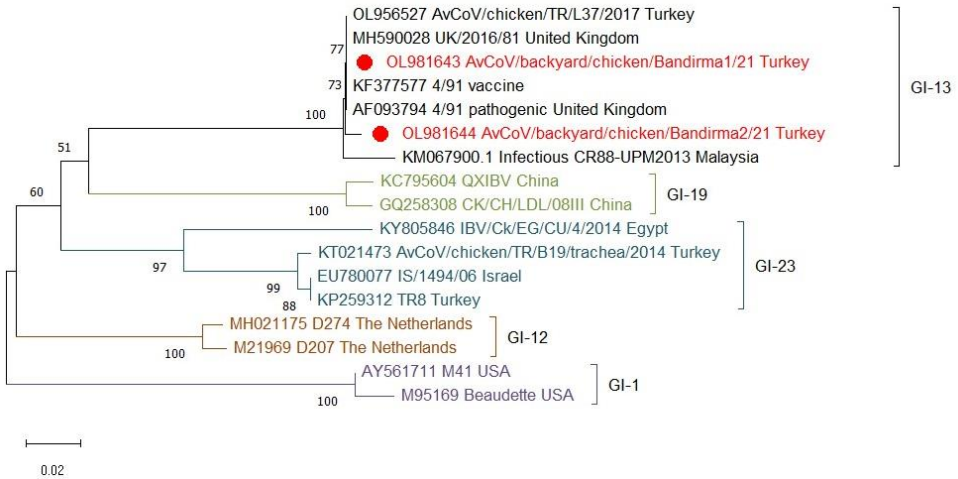

Figure 4. Neighbour-joining tree constructed using the Kimura two-parameter model based on partial sequence of the *S1* gene sequences.

Turkish isolates in the current study are indicated by red circles. The scale bar demonstrates the distance unit between sequence pairs. The sequences were acquired from GenBank.

Discussion and Conclusion

The presence of common avian viral and bacterial respiratory pathogens such as IBV, AIV, NDV, AMPV, MG, and MS in backyard poultry as single infections or co-infections may cause a great threat to commercial chicken flocks. Backyard flocks, especially those that are not immunised with live or inactivated virus vaccines, can be infected with field strains of pathogenic microorganisms more easily. Hence, they become reservoirs for pathogenic bacteria and viruses for commercial chicken flocks (4, 5, 7). Therefore, in this study, we screened the tracheal samples of chickens and turkeys showing respiratory signs for the presence of AIV serotypes H5, H7, and H9, NDV, IBV, AMPV, MG, MS, *P. multocida*, *A. paragallinarum*, and *B. avium*. We found that the samples of the chickens had MG and IBV, while the samples of the turkeys had only MG. Additionally, anti-IBV and anti-MG IgG ELISA antibody titers in the serum samples were also a supportive result, indicating active infections with IBV and MG in the chickens in the studied flock (Figure 1). We also observed that most of the serum samples had high antibody titers to MS, although MS could not be detected in the tracheal samples. This may suggest that most antibody positivity for MS infection could be possible because of cross-reactions (false positives) frequently seen in serological tests (8, 19). Anti-MS antibodies observed in this study could also be a result of the high sensitivity of the ELISA kits which may have led to cross-reactivities of other antibodies with non-pathogenic or other pathogenic mycoplasma species such as MG (9, 15). In terms of NDV, the ELISA results were positive for NDV-specific IgG, while tracheal swabs were negative based on the CAT method in our study, which was in agreement with the results reported by Marks et al. (20). This may be due to circulating NDV strains with low pathogenicity, which may induce seroconversion with inconspicuous clinical manifestations. Moreover, we could not detect IBV in the tracheal samples of the turkeys that had close contact with the IBV-infected chickens. A possible explanation is that IBV could not naturally infect turkeys because IBV and the turkey coronavirus (TCoV) are host-dependent avian coronaviruses. They only share ~34% similarity for the S protein sequence (13).

In our study, the presence of MG infection both in turkeys and chickens in the flock may have been a result of vertical or lateral transmission. In Türkiye, chicks and turkey pullets in small backyard flocks are sold in the open market for non-commercial purposes (6). These are mostly discarded unhealthy young birds, and they can be infected with pathogenic bacteria such as MG and MS (30). Additionally, the studied flock was being reared in a primitive and open-top garden which was open to exposure to humans and exotic wild birds that could be mechanical or biological carriers of MG. The co-existence of MG and IBV in commercial chickens with respiratory problems is frequently seen in the field and reported in several studies (10, 24). However, the existence of the dual infection of MG and IBVs in backyard poultry as a source of these infections for commercial poultry facilities here can be considered uncommon (4, 5) and valuable epidemiological data. These data revealed that preventive IBV and MG control attempts such as vaccinations seem to be an urgent need and should be implemented in backyard flocks grown near poultry production companies.

The genotyping of the IBV samples indicated that both isolates were assigned to the GI-13 lineage (27), and they shared nucleotide identities of 99.35% and 98.69% with both pathogenic and attenuated 4/91 IBV strains showing us that we had two different S1 mutants in these backyard chickens. Up to our knowledge, there is no previously published data regarding the detection of any genotype of IBV from backyard chickens in Türkiye although the presence of the genotypes in this genetic lineage in backyard chickens has been reported in several countries as Canada, United States, and Iran (4, 5, 25). Remarkably, IBV genotypes can vary regionally, and their prevalence in backyard poultry may differ from commercial settings. However, due to the lack of comprehensive studies on IBV genotypes particularly in backyard chickens worldwide, specific information about the prevalence and distribution of genotypes in this context is limited. On the other hand, there are a number of studies on the detection and genotyping of IBV from commercial chickens with respiratory problems in Türkiye. For example, the first genotyping and isolation study on IBVs in commercial poultry flocks was performed by Kahya et al. (14), and the isolate was designated as GI-23 (IS/1494/06). Another large-scale survey in Türkiye indicated that the heterogenicity of the IBV genotypes consisted of M41-based isolates in GI-1, 4/91, or 793/B-related isolates in GI-13 and IS/1494/06 isolates in GI-23 (31). In a recent paper, Mustak et al. (22) reported that IS/1494/06 is the most prevalent genotype in commercial broiler flocks. Moreover, these researchers indicated that H120, 793/B, and D274 genotypes are also circulating in Türkiye. Here, it should be noted that our ongoing laboratory studies and a recently published paper (2) on the genotyping of IBV isolates in broiler and layer chicken flocks showed that 793/B- or GI-13-related IBV detection rates in respiratory samples have increased, and 793/B IBVs have become the second most dominant genotype subsequent to IS/1494/06 IBVs. Taken together, the cumulating evidence has demonstrated that dominant IBV genotypes in commercial poultry are GI-13 (793/B) and GI-23 (IS/1494/06) in Türkiye (2, 22, 31). The beginning of the occurrence of 793/B IBV detections in

chicken flocks after 2016 in Türkiye might be a result of the usage of live-attenuated vaccines in chicken flocks although there were no infection problems with the same wild-type genotype in the field (31). The usage of novel vaccine strains has always been an infection source in the field worldwide because attenuated vaccine IBVs can regain their virulence (3).

In this study, we detected four mutations at the nucleotide positions of 801, 1062, 1092, and 1095 (Figure 3a). Among these, three were silent mutations. Nevertheless, the mutation at nucleotide position 1095 led to F/L alteration in amino acid position 365 (Figure 3b). This F/L mutation can be considered to cause critical conformational changes in the antigenic epitopic region located in the hypervariable region 3 of the S1 protein of IBV (12). Additionally, we examined the genetic connection of our two IBV isolates to the IBV vaccine strains of CR88, 4/91, and 4/91 field strains belonging to the GI-13 lineage based on the partial *S1* gene and amino acid sequences (Figure 3a, 3b). The phylogenetic analyses (Figure 4) of our two IBV isolates revealed that these isolates could be genetically closer to the 4/91 isolates than the CR88 isolate. A possible reason could be the widespread usage of 4/91-derived vaccines compared to CR88-derived ones in commercial chicken flocks in Türkiye.

While the sample size may be limited, it is important to emphasize that these samples are representative of a backyard setting lacking vaccines, drugs, and biosecurity measures. Additionally, the absence of similar backyards in the vicinity, coupled with the high density of commercial poultry flocks in the area, renders this sampling unique. Consequently, the present analysis was undertaken using this distinctive set of samples.

In conclusion, the IBV isolates that were examined in this study were closely related to 4/91 vaccine strains, which are commonly used in this region of Türkiye. The possible mutations of attenuated IBVs or the presence of native 4/91 field strains with mutations may have the potential to overcome the protective immunity induced by vaccine strains. Additionally, backyard chickens infected with such mutant IBV isolates can be evaluated as an important epidemiological source for the development and introduction of novel mutant IBVs for commercial chicken flocks. Apart from this, the existence of MG in the IBV-infected backyard chickens in this study could have been a result of the vertical transmission of the agent from breeder flocks or the horizontal transmission of the chickens by wild birds infected with MG, respiratory aerosols, hatchery transmission or indirect modes including environmental factors and fomites (21, 30).

We strongly recommend that backyard flocks, along with commercial chicken flocks, be monitored regularly and continuously for IBV genotypes. Furthermore, the screening of mutations in epitopic sites in the *S1* gene is a critical practice for evaluating the current knowledge about the presence of circulating IBVs and logically selecting the vaccine protectotype of IBV. Thus, the level of protective immunity against novel variants led by mutations, especially in the HVRs of the *S1* gene, can be increased in flocks.

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Conflict of interest

No potential conflict of interest was reported by the authors.

Author Contributions

ÖA, SKD, and KTC conceived and planned the experiments and took the lead in writing the manuscript. TSK, AGC, and AÖ contributed to sample preparation. TSK and SE carried out the experiments. ÖA and KTC contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Abdelmoez NS, Shawky MM, Abdelhady HA, et al** (2019): *Isolation and identification of some possible causative agents of swollen head syndrome (SHS) in broiler chickens in Egypt*. Slov Vet Res, **56**, 781–788.
2. **Ardicli O, Demirbilek SK, Coven F, et al** (2022): *A surveillance for avian coronavirus infectious bronchitis virus, infectious laryngotracheitis virus, avian metapneumovirus, and avian reovirus in poultry flocks with respiratory signs in Türkiye.* Turkish J Vet Anim Sci, **46**, 687-697.
3. **Britton P, Armesto M, Cavanagh D, et al** (2012): *Modification of the avian coronavirus infectious bronchitis virus for vaccine development.* Bioengineered, **3**, 114-119.
4. **Brochu NM, Guerin MT, Varga C, et al** (2019): *A two-year prospective study of small poultry flocks in Ontario, Canada, part 1: prevalence of viral and bacterial pathogens.* J Vet Diagn Invest, **31**, 327-335.

5. **Cadmus KJ, Mete, Harris M, et al** (2019): *Causes of mortality in backyard poultry in eight states in the United States.* J Vet Diagn Invest, **31**, 318-326.
6. **Ceylan N** (2015): *Organik ve geleneksel tavuk eti üretimi (yanlış bilinenler ve gerçekler).* Besdbir Uzman Görüşleri 6, **21**, 1-29.
7. **Derksen T, Lampron R, Hauck R, et al** (2018): *Biosecurity assessment and seroprevalence of respiratory diseases in backyard poultry flocks located close to and far from commercial premises.* Avian Dis, **62**, 1-5.
8. **Feberwee A, Mekkes D, De Wit J, et al** (2005): *Comparison of culture, PCR, and different serologic tests for detection of Mycoplasma gallisepticum and Mycoplasma synoviae infections.* Avian Dis, **49**, 260-268.
9. **Frey J** (2002): Mycoplasmas of animals. 73-90. In: S Razin, R Herrmann (Eds), Molecular Biology and Pathogenicity of Mycoplasmas. Springer, Boston, MA.
10. **Fujisawa S, Murata S, Takehara M, et al** (2019): *Molecular detection and genetic characterization of Mycoplasma gallisepticum, Mycoplama synoviae, and infectious bronchitis virus in poultry in Myanmar*. BMC Vet Res, **15**, 1-7.
11. **Gill P, Ghaemi A** (2007): *Nucleic acid isothermal amplification technologies-A review*. Nucleosi Nucleot Nucl, **27**, 224-243.
12. **Gong H, Ni R, Qiu R, et al** (2022): *Evaluation of a novel recombinant strain of infectious bronchitis virus emerged from three attenuated live vaccine strains*. Microb Pathog, **164**,105437.
13. **Jindal N, Mor SK, Goyal SM** (2014): *Enteric viruses in turkey enteritis*. VirusDisease, **25**, 173-185.
14. **Kahya S, Coven F, Temelli S, et al** (2013): *Presence of IS/1494/06 genotype-related infectious bronchitis virus in breeder and broiler flocks in Turkey*. Ankara Univ Vet Fak Derg, **60**, 27-31.
15. **Kahya S, Temelli S, Eyigor A, et al** (2010): *Real-time PCR culture and serology for the diagnosis of Mycoplasma gallisepticum in chicken breeder flocks*. Vet Microbiol, **144**, 319-324.
16. **Kim S-H** (2018): *Challenge for one health: co-circulation of zoonotic H5N1 and H9N2 avian influenza viruses in Egypt*. Viruses, **10**, 1-16.
17. **Kimura M** (1980): *A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences.* J Mol Evol, **16**, 111-120.
18. **Koutsianos D, Athanasiou LV, Mossialos D, et al** (2022): *Investigation of serotype prevalence of Escherichia coli strains isolated from layer poultry in Greece and interactions with other infectious agents.* Vet Sci, **9**, 1-14.
19. **Luciano R, Cardoso A, Stoppa G, et al** (2011): *Comparative study of serological tests for Mycoplasma synoviae diagnosis in commercial poultry breeders.* Vet Med Int, **2011**, 1-5.
20. **Marks FS, Rodenbusch CR, Okino CH, et al** (2014): *Targeted survey of Newcastle disease virus in backyard poultry flocks located in wintering site for migratory birds from Southern Brazil.* Prev Vet Med, **116**, 197-202.
21. **Mugunthan SP, Kannan G, Chandra HM, et al** (2023): *Infection, transmission, pathogenesis and vaccine development against Mycoplasma gallisepticum.* Vaccines, **11**, 469-483.
22. **Mustak IB, Mustak HK, Bilgen N** (2022): *S1 gene based phylogeny of Israel variant-2 infectious bronchitis virus isolated in Turkey in a five year period.* Pol J Vet, **25**, 45-50.
23. **NucleoGene Molecular Detection Assay (Isothermal Amplification Technology)** (2023): Available at https://www.nucleogene.com/product-nucleogene-molecular-detection-assay (Accessed July 3, 2023).
24. **Roussan D, Haddad R, Khawaldeh G, et al** (2008): *Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan*. Poult Sci, **87**, 444-448.
25. **Shokri S, Karimi V, Langeroudi AG, et al** (2018): *Seroprevalence and genotyping of avian infectious bronchitis virus detected from Iranian unvaccinated backyard chickens.* Iranian J Microbiol, **10**, 65-71.
26. **Swayne DE** (2020): Diseases of poultry. John Wiley & Sons, Hoboken, NJ.
27. **Valastro V, Holmes EC, Britton P, et al** (2016): *S1 gene-based phylogeny of infectious bronchitis virus: an attempt to harmonize virus classification.* Infec Genet Evol, **39**, 349-364.
28. **Wang Y, Jiang Z, Jin Z, et al** (2013): *Risk factors for infectious diseases in backyard poultry farms in the Poyang Lake area, China.* PloS one, **8**, e67366.
29. **Worthington KJ, Currie R, Jones RC, et al** (2008): *A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006.* Avian Pathol, **37**, 247-257.
30. **Yadav JP, Tomar P, Singh Y, et al** (2021): *Insights on Mycoplasma gallisepticum and Mycoplasma synoviae infection in poultry: A systematic review.* Anim Biotechnol, **33**, 1711-1720.
31. **Yilmaz H, Altan E, Cizmecigil UY, et al** (2016): *Phylogeny and S1 gene variation of infectious bronchitis virus detected in broilers and layers in Turkey*. Avian Dis, **60**, 596-602.

Rapid determination of chicken meat ratios in Beef Mixtures and Beef Sausages by Near Infrared Reflectance (NIR) spectroscopy

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ABSTRACT

This study aims to determine the percentage of chicken meat in beef and chicken mixtures, which is the most common form of beef adulteration. Ground beef and beef sausages were prepared with mixtures containing chicken meat, ranging from 0.0% to 100.0% with 5.0% increments, and analyzed using a near-infrared spectroscopy device. Optimal analysis conditions were determined through the examination of a wide range of regression models. The best regression model for ground beef mixtures yielded the following results: RMSEC (Root Mean Square Error of Calibration): 2.35, RMSEV (Root Mean Square Error of Validation): 3.36, R^2C (R-Value Calibration): 0.99, R^2V (R-Value Validation): 0.98. The results for beef sausages were as follows: RMSEC: 2.56, RMSEV: 3.66, R^2C: 0.99, R^2V: 0.98. As a result, the chicken meat content in beef mixtures was detected with a margin of error of 2.05%, while the chicken meat content in beef sausages was detected with a margin of error of 2.12%.

Introduction

Meat and meat products are highly sought after by consumers worldwide due to their high bioavailability, essential amino acids, and vital nutrients such as iron and B_{12}, as well as their unique taste. However, they are often less accessible than plant-based foods with similar protein content (7). Consequently, meat and meat products may be mixed with meats from different species, making them vulnerable to adulteration (6), a problem that is not limited to developing countries but also occurs in developed countries (34). The media coverage of food adulteration has created a public opinion that food needs to be analyzed more frequently and easily (28). Infrared spectrocopy used in this study, is one method for rapidly analyzing food (33).

The present study focuses on near-infrared radiation (NIR), a segment of electromagnetic radiation with wavelengths between 800 nm and 2500 nm. In the context of food analysis, NIR has demonstrated the ability to interact with the bonds linking carbon, hydrogen, oxygen, and nitrogen atoms, where these bonds store energy similar to a spring with a mass attached to the end. Thus, NIR is a promising method for food analysis owing to its capability to provide accurate and rapid results (33).

In the field of near-infrared spectroscopy, a significant focus lies on the six types of interatomic bond vibrations that can be categorized into two groups. The first group includes symmetrical and asymmetrical stretching vibrations, while the second group comprises four bending vibrations. Specifically, two of these bending

vibrations - scissoring and rocking - occur within the same plane, while the remaining two vibrations - wagging and twisting - manifest in a different plane (1).

Infrared spectroscopy has been applied to food analysis since its inception, with the Department of Agriculture of the United States (USDA) publishing the earliest known work on the subject in 1949 (26). A significant step forward was made with the publication of the first quantitative study in 1962, which employed the technique to determine seed moisture via methanol extracts (16). The routine analysis of wheat protein using infrared spectroscopy was later adopted by the U.S. Federal Grain Inspection Service (FGIS) during the 1980s, and it has since become a widely adopted method (14).

Infrared spectroscopy has become a ubiquitous technique in the meat industry for the rapid quantification of fat, moisture, and protein contents in meat and meat products (3). In the present investigation, an adapted version of this established approach was employed to ascertain the extent of beef adulteration with chicken.

In 2013, the regulation on the inclusion of poultry meat in red meat products (e.g., salami, sausages, etc.) was revised, prohibiting any such mixtures (30). This may be attributed to the impracticality of determining the proportion of poultry meat in red meat. Although the literature reports the use of Real-time PCR to quantify this ratio (23), it has not been implemented in practice. The current study presents a modified version of a standard infrared spectroscopy method, which enables rapid detection of chicken meat - one of the most commonly used adulterants - in beef, potentially offering a practical solution to this challenge.

In the scientific literature, only one study has been reported on determining the ratios of different species of meat added to cooked meat products using infrared spectroscopy (13). Hence, this study is one of the few investigations in this field, which aims to detect various types of animal meats added to heat-treated meat products by infrared spectroscopy. Previous literature on the detection of meat of different animal types added to beef by infrared spectroscopy (8, 11, 12, 21, 25, 29, 31, 32) demonstrates that these studies were conducted with laboratory equipment that is economically more costly than the device employed in this study. The percentage range of the prepared samples in those studies was limited, and the sample preparation process was laborious, particularly when using FTIR spectroscopy. The error margins reported in those studies were higher than those observed in this study. Therefore, the present study aims to address the above-mentioned issues related to detecting adulteration in beef using infrared spectroscopy.

Materials and Methods

Preparation of meat mixtures: Ground beef (modified atmosphere packaged, max fat content 20% [m/m], max collagen to protein ratio 15% [m/m]) and chicken breast (without skin) produced according to the Turkish Food Codex: Meat, Prepared Meat Mixtures and Meat Products Communiqué (30) were supplied from Meat and Milk Board's Ankara store. Meat mixtures were prepared from 0% chicken to 100% chicken in 5% increments. Therefore a total of 21 meat mixtures were prepared to contain 100.0%, 95.0%, 90.0%, 85.0%, 80.0%, 75.0%, 70.0%, 65.0%, 60.0%, 55.0%, 50.0%, 45.0%, 40.0%, 35.0%, 30.0%, 25.0%, 20.0%, 15.0%, 10.0%, 5.0%, and 0.0% ground beef, and chicken breast vice-versa. All mixtures were prepared with an accuracy of ±0.50g. Mixtures were homogenized using a food processor (600 W) for about two minutes. After the preparation of each mixture, the food processor was cleaned with a degreaser and dried with paper towels to avoid leaving any fat residue. Pre-analysis images of mixtures containing 100.0% beef, 50.0% beef – 50.0% chicken, and 100.0% chicken are presented in Figure 1.

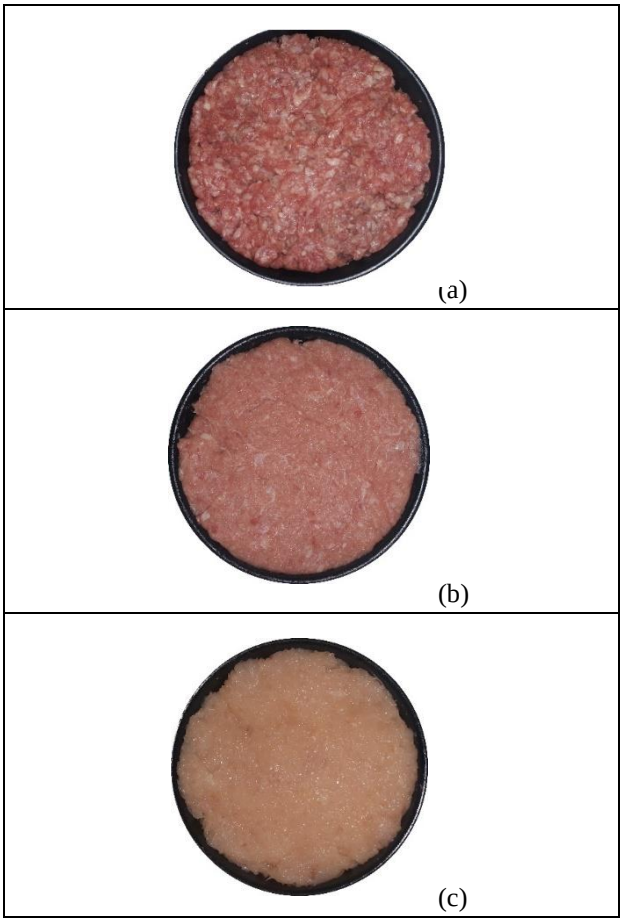

Figure 1. Pre-analyzed views of meat mixtures (a): %100.0 beef, (b): %50.0 beef, %50.0 chicken, (c): %100.0 chicken.

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Preparation of mixed sausages: The same meats and the same proportions used in the preparation of mixed meats were also used in the preparation of mixed sausages. Sausages were prepared according to the formula of: 20.0% ice, 5.0% starch (cornstarch), 3.5% salt (NaCl), 3.0% spice mixture (red sweet pepper, red chili pepper, thyme, black pepper, white pepper, paprika, garlic powder, onion powder, fennel, basil, sage, mustard, cumin, coriander, ginger), 0.25% polyphosphate (P_2O_5), 0.10% ascorbic acid (E300), 0.035% sodium nitrite (E250), [all proportions are m/m, modified from (24)]. The sausage mixes were emulsified and then filled into synthetic sausage cases. The sausages were then dried and pre-cooked in hot air at 60.0°C for 15 minutes. Then they were boil-cooked at 80.0°C until their core temperature reached 72.0°C. Their cooking finished as their core temperature was kept at 72.0°C for 15 minutes in the oven. Cooked sausages were then showered with cold water (approx. 15.0°C-17.0°C). When their core temperature dropped to room temperature, they were transferred to the cold room which was set to +4.0°C. They were kept in a cold chain until their analysis. The sausages were analyzed under the same conditions as the meat mixtures. Cross-sections of sausages made from 100.0% bef, 50.0% beef – 50.0% chicken meat, and 100.0% chicken are shown in Figure 2.

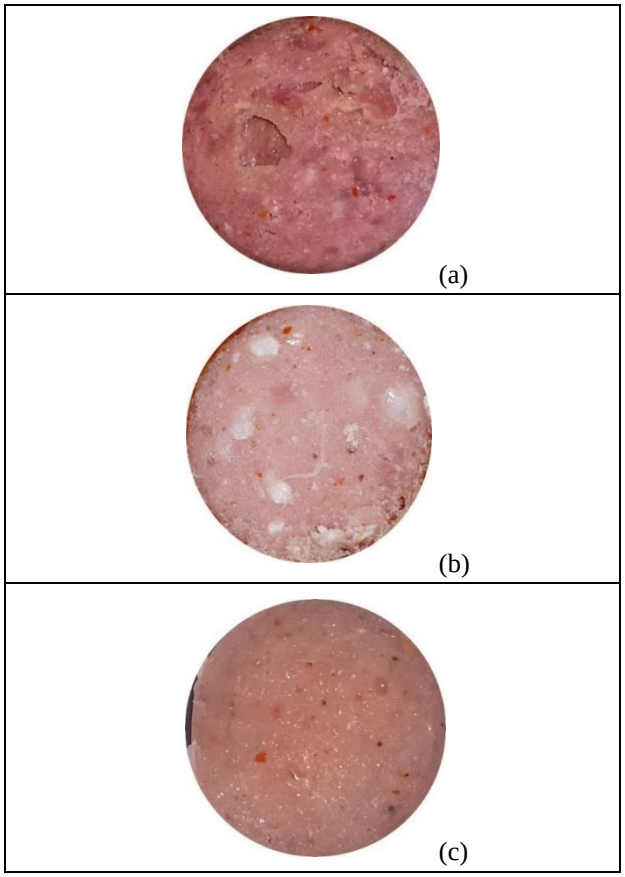

Figure 2. Cross-sections of mixed sausages (a):100.0 beef, (b): %50.0 beef, %50.0 chicken, (c)%100.0 chicken.

Obtaining near-infrared spectrums: Near-infrared reflectance (NIRS) spectra were obtained via NIR Spectrometer (Perten DA 7250, Perkin Elmer, United States of America; wavelength range: 950 nm – 1650 nm; wavelength sensitivity: better than 0.05 nm). The device has two different sample containers (500 ml and 150 ml), and both of them rotate 360° around their x-axis during the analysis. The analysis of the mixtures was performed twice in both large and small containers and the spectrums were collected at a resolution of 0.5 nm, 1 nm, 2 nm, and 5 nm. The analyses have been performed in so different ways to determine the optimal analysis conditions.

Moisture, protein, fat, collagen, ash, salt, and pH analyses: All these results were obtained using the device's (Perten DA 7250) own built-in calibration models with two significant numbers (10, 17, 27).

Statistical analysis of infrared spectrums: The obtained infrared spectra was analyzed using The Unscrambler® X program (Version 10.4, 32 bits). Spectra were examined with and without several pre-processing methods. The pre-processing methods to be applied and the calibration models to be established were chosen from (4, 15, 19, 21, 22). Normalization (Area Normalization), Saviztky – Golay Derivative (First Derivative), Gaussian Filter (Standart Deviation 2), and Multiplicative Scatter Correction methods were used for pre-processing. Then, partial least squares regression (PLSR) models were constructed from pre-processed and non-pre-processed spectra. When constructing regression models, predicted values were verified with full cross-validation.

A total of 180 statistical models were constructed for each infrared radiation resolution (0.5 nm, 1 nm, 2 nm, and 5 nm), whether the spectra were pre-processed or not, for the sample container used (large, small, or both), and for the sample's analysis count (once, twice or using both). All generated models were examined for RMSEC (Root Mean Square Error of Calibration), RMSEV (Root Mean Square Error of Validation), Slope (calibration), Slope (validation), R^2 (calibration), and R^2 (validation) values. In addition, the average error values of the estimates of the 10 models with the highest R^2 (validation) values were calculated according to the following formula:

$$Average\ margin\ of\ error\ (\%) = \frac{\sum_{i=1}^{n}|x|}{n}$$

Where:

$|x|$ = absolute value of the error rate of each predicted value (%)

n = total number of analyses.

All models with similar characteristics were then compared and the best-performing model with the lowest average margin of error was determined. It was found that

the best-performing model is achieved by scanning once using the large sample container, using the spectra with a resolution of 5 nm, and pre-processing the spectra with the normalization method. Therefore, the design of this model was chosen as optimal conditions, and the parameters in this model were used in the analysis of mixed sausages.

Results

Composition analysis of meat mixtures with varied proportions of beef and chicken: The moisture, protein, fat, collagen, ash, salt ratios, and pHs of meat mixtures are given in Table 1. Table 1 shows that as the proportion of chicken meat in the meat mixtures increased; the amount of moisture, protein, and collagen-free protein also increases, but on the other hand, the percentage of fat decreases. A graphical representation of the moisture, fat, protein, and collagen-free protein values of meat mixtures along with the chicken meat ratios is presented in Figure 3 (a, b, c, d).

Table 2 (MM) shows that chicken meats added to beef meats could be detected with an average of 2.05% margin of error. In addition, the values showing the statistical success of the model are RMSEC: 2.35, RMSEV: 3.36, Slope (calibration): 0.99, Slope (validation): 0.96, R^2 (calibration): 0.99, R^2 (validation): 0.98.

Among the wavelengths of infrared radiation used in the creation of the model, the wavelengths that contributed the most to the regression model were found to be 950 nm, 1140 nm (±20 nm), 1210 nm (±25 nm), and 1310 nm (±35 nm).

Composition analysis of mixed sausages with varied proportions of beef and chicken: The analysis of the mixed sausages was carried out according to the parameters in the optimal model (spectrum obtained at 5 nm resolution, analyzed only once in the large analysis tray, and pre-processed with normalization).

Moisture, protein, fat, collagen-free protein, ash, salt ratio,s, and pH values of mixed sausages are presented in Table 3. As shown in Table 3, as the chicken ratio increased in the mixed sausages, the moisture, protein, and collagen-free protein ratios increased; the fat ratio decreased. Graphical representations of these ratios are presented in Figure 3 (e, f, g, h).

Average error rates of predictions of beef ratios in mixed sausages are presented in Table 2 (MS). Table 2 (MS) shows that the estimation rate of beef in mixed sausages was found to be seven per ten-thousand percent worse than the estimated rate of meat mixtures'.

Indicators of the success of the statistical model in predicting the meat content of mixed sausages are found as RMSEC: 2,56, RMSEV: 3.66, Slope (calibration): 0.99, Slope (validation): 0.97, R^2 (calibration): 0.99, R^2 (validation): 0.98. While creating the regression model, the wavelengths that contributed the most to the model were found to be 1160 nm (±30 nm), 1210 nm (±15 nm), and 1390 nm (±10nm).

Table 1. Meat mixtures' moisture, protein, fat, collagen, ash, salt ratios and pH values.

Beef Ratio %	Chicken Ratio %	Moisture %	Protein %	Fat %	Collagen %	Ash %	Salt %	pH
100.0	0.0	63.6	18.6	16.4	1.75	1.32	0.35	5.59
95.0	5.0	65.0	19.0	15.1	2.50	1.19	0.28	5.56
90.0	10.0	65.3	19.0	14.7	2.38	1.04	0.32	5.54
85.0	15.0	65.7	19.2	14.0	1.96	1.09	0.45	5.52
80.0	20.0	65.5	19.3	14.3	2.28	1.48	0.31	5.7
75.0	25.0	65.9	19.2	13.9	2.51	1.19	0.07	5.56
70.0	30.0	67.1	19.1	12.0	2.16	1.97	0.32	5.63
65.0	35.0	66.2	19.1	13.0	2.16	2.23	0.29	5.69
60.0	40.0	67.8	19.6	10.7	1.83	1.1	0.30	5.57
55.0	45.0	67.2	19.4	11.7	1.66	1.17	0.29	5.61
50.0	50.0	68.6	19.2	10.6	1.68	1.84	0.19	5.73
45.0	55.0	70.1	19.1	9.26	1.72	1.22	0.35	5.62
40.0	60.0	72.2	19.4	8.02	1.34	1.06	0.22	5.77
35.0	65.0	70.9	18.9	8.46	-0.25	1.21	0.50	5.58
30.0	70.0	74.1	19.2	6.15	1.37	1.39	0.37	5.77
25.0	75.0	73.6	19.0	5.18	0.75	1.45	0.45	5.77
20.0	80.0	73.5	19.5	4.40	1.01	1.33	0.40	5.79
15.0	85.0	74.0	20.6	4.07	0.16	1.39	0.45	5.55
10.0	90.0	75.1	20.5	3.03	-0.04	1.51	0.81	5.49
5.0	95.0	74.4	20.8	3.18	0.46	1.41	0.85	5.55
0.0	100.0	76.3	20.6	1.06	-0.72	1.85	1.16	5.45

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Table 2. Average error margin of the regression model for meat mixtures (MM) and mixed sausages (MS).

Beef Ratio (%)	Predicted (MM) (%)	Difference (MM) (%)	Predicted (MS) (%)	Difference (MS) (%)
100.0	99.6	0.33	98.1	1.86
95.0	89.5	5.42	94.7	0.29
90.0	92.3	2.35	85.0	4.98
85.0	84.3	0.67	85.8	0.85
80.0	76.7	3.22	78.8	1.16
75.0	75.8	0.88	76.1	1.13
70.0	69.9	0.02	72.6	2.63
65.0	63.6	1.33	68.1	3.13
60.0	58.6	1.36	62.4	2.44
55.0	58.8	3.89	50.8	4.21
50.0	52.3	2.30	52.4	2.39
45.0	48.8	3.88	49.2	4.17
40.0	39.6	0.39	42.1	2.15
35.0	37.2	2.28	36.6	1.58
30.0	27.6	2.36	25.9	4.05
25.0	23.2	1.73	24.5	0.52
20.0	17.1	2.85	18.8	1.21
15.0	12.7	2.25	13.6	1.42
10.0	6.72	3.27	12.9	2.87
5.0	3.85	1.14	5.69	0.69
0.0	1.15	1.15	0.89	0.89
Average Error (%) :		**2.05 (MM)**	Average Error (%) :	**2.12 (MS)**

Table 3. Mixed sausages' moisture, protein, fat, collagen-free protein, ash, salt ratios and pH values.

Beef Ratio %	Chicken Ratio %	Moisture %	Protein %	Fat %	Collage-Free Protein %	Ash %	Salt %	pH
100	0	57	18.82	18.74	14.19	2.88	2.13	5.86
95	5	58.26	18.47	18.78	14.34	2.7	2.18	5.84
90	10	59.92	18.86	17.43	14.75	2.96	2.14	5.91
85	15	58.28	19.28	18.03	14.59	2.85	2.19	5.86
80	20	58.06	19.96	17.37	15.12	3.06	2.09	5.88
75	25	60.27	21.58	15.38	16.26	3.16	2.1	5.93
70	30	59.92	21.22	15.51	16.73	3.04	2.16	5.9
65	35	61.43	20.88	13.06	15.92	2.74	2.03	5.94
60	40	61.3	21.12	13.58	17.05	3.13	2.02	5.92
55	45	61.13	20.24	12.15	17.43	2.99	2.23	5.9
50	50	60.13	21.57	11.67	20.06	3.21	2.3	5.9
45	55	59.11	21.77	10.37	18.79	3.09	2.22	5.94
40	60	60.09	21.33	9.01	19.9	2.99	2.25	5.98
35	65	59.48	21.87	8.62	20.59	3.07	2.03	5.95
30	70	59.94	23.53	7.54	21.36	3.21	2.15	5.96
25	75	61.63	24.4	6.79	21.76	3.3	2.21	6
20	80	62.42	24.03	5.95	22.3	3.36	2.3	6.01
15	85	64.02	25.04	4.61	22.88	3.77	2.16	6.07
10	90	64.98	24.3	4.23	22.34	3.16	2.13	6.07
5	95	64.46	24.96	3.41	23.82	3.66	2.33	6.05
0	100	65.35	24.48	2.23	23.96	3.4	2.08	6.06

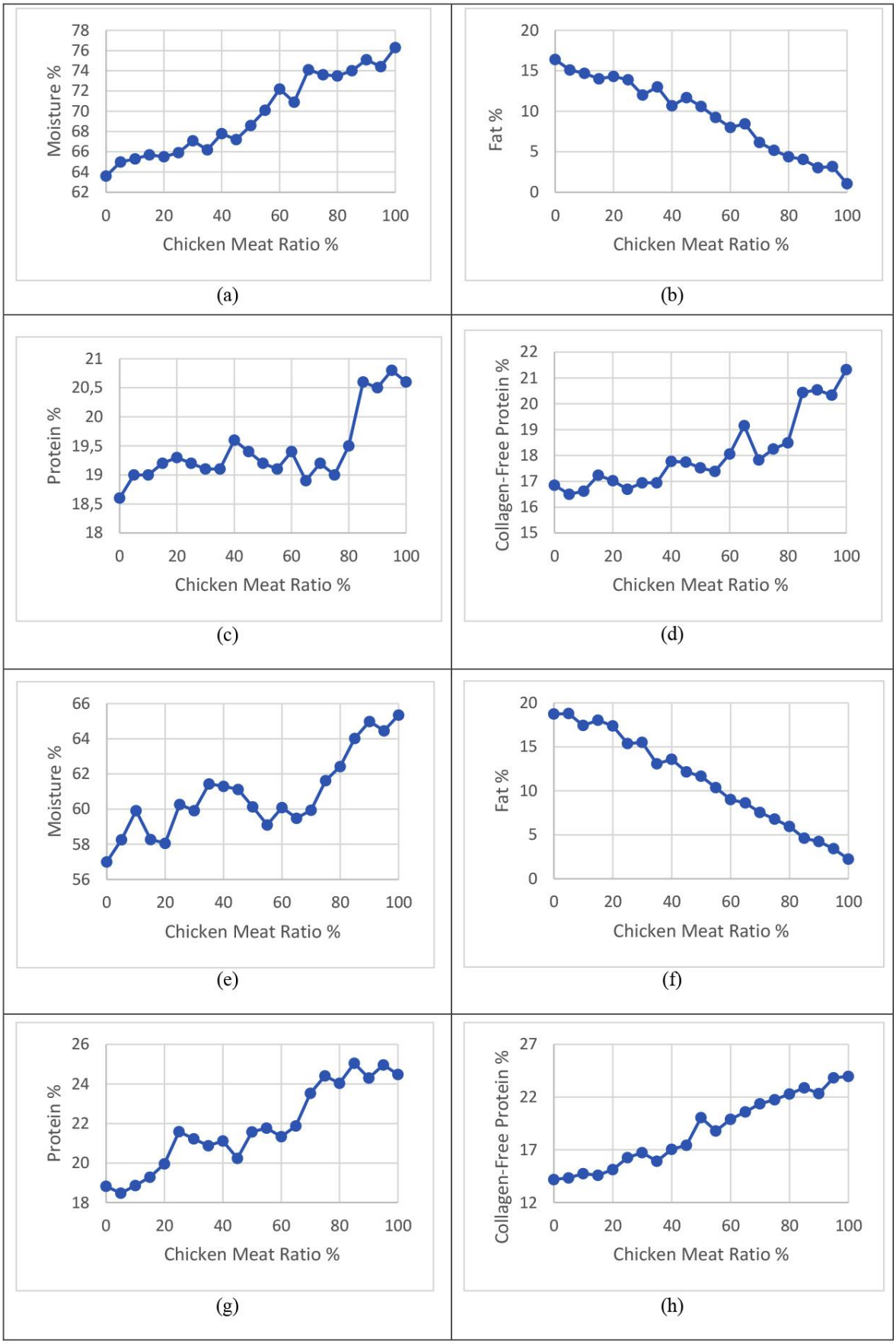

Figure 3. Graphical representations of moisture, fat, protein and collagen-free protein ratios of meat mixtures (a, b, c, d) and mixed sausages (e, f, g, h).

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Discussion and Conclusion

This study demonstrates that near-infrared spectroscopy can detect chicken added to ground beef and chicken added to beef sausages with a precision of 2.05% and 2.12%, respectively. The results indicate that the higher error rate of mixed sausages, with a discrepancy of 0.07%, is due to the presence of diverse spices and additives incorporated in the sausage formulation and the alterations that occur during heat processing. The infrared spectra of spices and additives differ from those of beef and chicken, resulting in the development of various compounds with distinctive infrared spectra during heat treatment.

Ding and Xu (13) have previously demonstrated the ability to detect adulterated beef hamburgers using infrared radiation in the 400–2500 nm region, with a resolution of 2 nm. Their study involved the preparation of adulterated hamburgers by substituting beef with minced pork and mutton at 5%, 10%, and 25% (m/m) levels, and collecting infrared spectrums from both raw and cooked samples. The R^2 values obtained in their study for mutton and pork were relatively low, at 0.87 (raw), 0.79 (cooked), 0.84 (raw), and 0.74 (cooked), respectively. In our study, we were able to achieve higher R^2 values (0.98) for both adulterated ground beef and adulterated beef sausages. The differences in infrared radiation range, meat types, sample sizes, and adulteration rates employed may account for the differences in R^2 values between the two studies.

In a previous study by Restaino et al. (31), beef and pork patés were analyzed using infrared radiation in the 1100-2500 nm region with a resolution of 2 nm in reflectance mode. While the individual beef and pork paté samples were accurately classified at 100%, binary mixtures received only 72% correct classification. Despite paté being one of the most homogenized meat products, the low classification rate was attributed to the statistical model used, namely Stepwise Linear Discriminant Analysis (SLDA). In contrast, our study employed the PLSR model and was able to achieve 100% classification accuracy for all mixtures. This suggests that the PLSR model provides superior results compared to SLDA.

The adulteration of beef with horse meat was studied by Boyacı et al. (8) using Raman spectroscopy, a variant of infrared spectroscopy. In this study, Raman spectra were obtained using a laser and infrared radiation with 200-2000 cm^{-1} wavenumber at a $2cm^{-1}$ resolution. Adulterated samples were prepared by adding horse meat to beef at ratios of 0%, 25%, 50%, 75%, and 100% by weight, and all adulterated samples were accurately classified. However, the analysis of samples using Raman spectroscopy requires long and laborious preparation steps, such as extracting the fat with hexane and centrifugation. In contrast, in our study, sample preparation only involved homogenization in a food processor for approximately two minutes, with an analysis time of approximately six seconds using NIRS. Therefore, we concluded that the NIRS setting we used in our study is more easily applicable than Raman spectroscopy for detecting food adulteration.

The study conducted by Nolasco-Perez et al. (25) aimed to compare the effectiveness of portable NIR and NIR + hyperspectral imaging (NIR-HSI) systems in detecting beef adulterated with chicken. Adulterated samples were prepared by varying the chicken content from 0% to 50% in 2% increments (w/w). The portable NIR system showed R^2 values of 0.93 (calibration) and 0.7 (validation), while the NIR-HSI system showed R^2 values of 0.98 (calibration) and 0.94 (validation). Our study, using the NIR system, produced better results with R^2 values of 0.99 (calibration) and 0.98 (validation). We attribute this to the sample size and range of adulteration rates used in our study, which outperformed the more advanced NIR-HSI system.

This study is distinctive from previous researches mentioned above, because it utilizes a cost-effective infrared spectroscopy instrument and a simple sample preparation technique. The prepared samples used in this study are 500g, which provides a more precise representation of complex meat samples. Moreover, the margin of error in this study is considerably low.

The regression model used in this study revealed that the infrared radiation with a wavelength of 950 nm was particularly significant in distinguishing mixed beef and chicken meats. This wavelength is known to interact mainly with O-H bonds, which are influenced by the water ratios in the meats. Moreover, infrared radiation with wavelengths of 1140 nm, 1210 nm, 1310 nm, and 1160 nm, 1201 nm, 1390 nm, were found to be important in identifying chicken meat in beef mixtures and mixed sausages, respectively. These wavelengths are known to interact primarily with C-H bonds, which are influenced by the proportions of fat, chromophores, and hydrocarbons in the different meats (2, 5, 9, 18, 20, 35).

In this study, the efficacy of NIRS in detecting chicken meat in ground beef and beef sausages was investigated, and accuracy rates of 2.05% and 2.12%, respectively, were achieved. This suggests that NIRS could be a viable candidate for a rapid and straightforward method of detecting adulteration in these meat products, as well as in other beef products. Moreover, as the cost of food continues to rise, there is a growing demand for affordable and high-quality protein alternatives, which may include blends of beef and chicken meat. To ensure the quality and ratio of such blends, NIRS could potentially provide an effective means of analysis.

For NIRS to gain acceptance as a reliable analysis method for determining the ratio of meat mixtures, further

research is required using whole chicken carcasses and mechanically separated meats (MSM). Moreover, in order to use NIRS in detecting the ratio of beef products such as beef sausages, salami, etc., calibration models incorporating common ingredients of such processed meats, including milk proteins, soy proteins, starch, vegetable oils, food additives, and dyes, need to be developed.

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Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

BT and ÖK designed the experiments. BT prepared the samples under the supervision of ÖK and carried out experiments. Both authors interpreted the results. BT took the lead in writing the manuscript. Both authors provided critical feedback and helped shape the research, analysis and manuscript.

References

1. **Adapa P, Karunakaran C, Table L, et al** (2009): *Potential applications of infrared and raman spectromicroscopy for agricultural biomass*. CIGR J, **11**.
2. **Alomar D, Gallo C, Castañeda M, et al** (2003): *Chemical and discriminant analysis of bovine meat by near infrared reflectance spectroscopy* (NIRS). Meat Sci, **63**, 441–450.
3. **AOAC** (2019): 21st Ed., AOAC International, Gaithersburg, MD, USA, Official Method 2007.04.
4. **Barbin DF, Badaró AT, Honorato DCB, et al** (2020): *Identification of turkey meat and processed products using near infrared spectroscopy*. Food Control, **107**, 106816.
5. **Barlocco N, Vadell A, Ballesteros F, et al** (2006): *Predicting intramuscular fat, moisture and Warner-Bratzler shear force in pork muscle using near infrared reflectance spectroscopy*. Anim Sci, **82**, 111–116.
6. **Bilge G, Velioglu HM, Sezer B, et al** (2016): *Identification of meat species by using laser-induced breakdown spectroscopy*. Meat Sci, **119**, 118–122.
7. **Bohrer BM** (2017): *Review: Nutrient density and nutritional value of meat products and non-meat foods high in protein*. Trends Food Sci Technol, **65**, 103-112.
8. **Boyacı İH, Temiz HT, Uysal RS, et al** (2014): *A novel method for discrimination of beef and horsemeat using Raman spectroscopy*. Food Chem, **148**, 37–41.
9. **Cozzolino D, Murray I** (2004): *Identification of animal meat muscles by visible and near infrared reflectance spectroscopy*. LWT, **37**, 447–452.
10. **DA7250-SD** (2022): Available at: https://www.s4science.at/wordpress/wp-content/uploads/2020/06/DA7250-SD_Brochure_EN.pdf. (Accessed June 8, 2022).
11. **Deniz E, Güneş Altuntaş E, Ayhan B, et al** (2018): *Differentiation of beef mixtures adulterated with chicken or turkey meat using FTIR spectroscopy*. J Food Process Preserv, **42**, e13767.
12. **Ding HB, Xu RJ** (1999): *Differentiation of beef and kangaroo meat by visible/near-infrared reflectance spectroscopy*. J Food Sci, **64**, 814-817.
13. **Ding HB, Xu RJ** (2000): *Near-infrared spectroscopic technique for detection of beef hamburger adulteration*. J Agri Food Chem, **48**, 2193–2198.
14. **Ertugay MF, Başlar M** (2011): *Gıdaların kalite özelliklerinin belirlenmesinde yakın kızılötesi (NIR) spektroskopisi*. Gıda, **36**, 49–54.
15. **Geniş HE** (2018): Hızlı Gıda Analizlerine Yönelik Yakın Kızılötesi Spektroskopisi (NIR) Sistemi Geliştirilmesi. PhD Thesis, Graduate Institute of Natural and Applied Sciences Hacettepe University.
16. **Hart JR, Norris KH, Golumbic C** (1962): *Determination of the moisture content of seeds by near-infrared spectrophotometry of their methanol extracts*. Cereal Chem, **39**, 94–99.
17. **ISO** (2017): *ISO 12099:2017*. Available at https://www.iso.org/cms/render/live/en/sites/isoorg/contents/data/standard/06/73/67352.html, (Accessed June 8, 2022).
18. **Jr JW, Weyer L** (2012): Practical Guide and Spectral Atlas for Interpretive Near-Infrared Spectroscopy, Second Edition. CRC Press.
19. **Kumar Y, Chandrakant Karne S** (2017): *Spectral analysis: A rapid tool for species detection in meat products.* Trends Food Sci Techno, **62**, 59-67.
20. **Liu D, Guo W, Li Q, et al** (2019): *Relationship of the bulk optical properties in 950–1650 nm wavelength range with internal quality and microstructure of kiwifruit during maturation*. Biosyst Eng, **184**, 45–54.
21. **López-Maestresalas A, Insausti K, Jarén C, et al** (2019): *Detection of minced lamb and beef fraud using NIR spectroscopy.* Food Control, **98**, 465–473.
22. **Mamani-Linares LW, Gallo C, Alomar D** (2012): *Identification of cattle, llama and horse meat by near infrared reflectance or transflectance spectroscopy.* Meat Sci, **90**, 378–385.
23. **Martín I, García T, Fajardo V, et al** (2009): *SYBR-Green real-time PCR approach for the detection and quantification of pig DNA in feedstuffs*. Meat Sci, **82**, 252–259.
24. **Nacak B** (2020): Bitkisel Yağ Kombinasyonları ve Farklı Antioksidanlar Kullanılarak Üretilen Jel ve Çoklu

Emülsiyonların Sosis Formülasyonunda Sığır Et Yağı İkamesi Olarak Kullanımının Oksidasyon Mekanizmaları Üzerine Etkisi. Master Thesis Graduate Institute of Natural and Applied Sciences, Ege University.

25. **Nolasco-Perez IM, Rocco LACM, et al** (2019): *Comparison of rapid techniques for classification of ground meat*. Biosystems Engineering, **183**, 151–159.
26. **Norris KH** (1996): *History of NIR*. J Near Infrared Spectrosc, **4**, 31-37.
27. **Perten DA** (2022): Meat and Meat products.pdf. Available at: https://www.perten.com/Global/Application%20notes/DA%2072xx/Perten%20DA%207250%20Meat%20and%20Meat%20products.pdf. (Accessed: June 8, 2022).
28. **Pico Y** (2012): Chemical Analysis of Food: Techniques and Applications. Elsevier.
29. **Rady A, Adedeji A** (2018): *Assessing different processed meats for adulterants using visible-near-infrared spectroscopy*. Meat Sci, **136**, 59–67.
30. **Resmi Gazete** (2019): Türk Gıda Kodeksi Et, Hazırlanmış Et Karışımları ve Et Ürünleri Tebliği. Available at : https://www.resmigazete.gov.tr/eskiler/2019/01/20190129-4.htm. (Accessed June 8, 2022).
31. **Restaino E, Fassio A, Cozzolino D** (2011): *Discrimination of meat patés according to the animal species by means of near infrared spectroscopy and chemometrics Discriminación de muestras de paté de carne según tipo de especie mediante el uso de la espectroscopia en el infrarrojo cercano y la quimiometria. CyTA*, J Food, **9**, 210–213.
32. **Schmutzler M, Beganovic A, Böhler G, et al** (2015): *Methods for detection of pork adulteration in veal product based on FT-NIR spectroscopy for laboratory, industrial and on-site analysis*. Food Control, **57**, 258–267.
33. **Sun DW** (2009): Infrared Spectroscopy for Food Quality Analysis and Control. Academic Press.
34. **Wikipedia** (2022): 2013 horse meat scandal (2021). Available at: Wikipedia. https://en.wikipedia.org/wiki/2013_horse_meat_scandal. (Accessed June 8, 2022).
35. **Workman Jr Jerry, Weyer L** (2007): Practical Guide to Interpretive Near-Infrared Spectroscopy (0 ed.). CRC Press.

Investigation of hereditary cholesterol deficiency (CD) in Holstein Cattle at the state farms in Türkiye

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ABSTRACT

Türkiye is exposed to international animal and semen movements. For this reason, Türkiye is very affected by diseases and hereditary disorders. State Farms is a state organization that raises and distributes breeding cattle. This study was aimed at investigating the presence and distribution of mutant alleles causing cholesterol deficiency (CD) disorder in Holstein cattle on state farms. For this purpose, blood and sperm samples were collected from 466 Holstein cattle. The real-time PCR method was used for genotyping. A total of seven cattle were found to be heterozygous. The frequency of the mutant allele was determined to be 0.75%. The kinship of four cattle carrying the mutant allele with Mauglin Storm, the bull in which this disease was first detected, was determined. The presence of cattle carrying the mutant allele in Türkiye is quite low compared to other countries. The Apolipoprotein-B (APOB) mutant allele was found at low frequencies and detected for the first time at the State Farms in Türkiye. Therefore, it is necessary to develop control programs by screening other Holstein herds.

Introduction

In intensive production, obtaining healthy herds is one of the main goals. Hereditary diseases also negatively affect the health of herds. Hereditary diseases are defects that occur in the form of structural disorders, anomalies, and syndromes that are transmitted from generation to generation depending on genes. Therefore, knowing the genetic background of calves is very important in terms of breeding strategy. Today, animals with defective genes can be detected more easily.

Artificial insemination is extensively used in cattle breeding. Therefore, as long as a hereditary disease carrier bull is used in artificial insemination, it spreads the abnormal gene, causing its frequency to increase. It has been reported that 29% of 273 Holstein bulls whose semen was imported to Türkiye in 2015 carried at least one hereditary defect (10). One of these diseases is a hereditary disorder called cholesterol deficiency (CD), which was detected in Holstein cattle by German researchers in 2015. Cholesterol deficiency is an autosomal recessive disease that results in death in calves. Affected animals show signs of hypocholesterolemia and hypolipidemia (13). It has been reported that the digestive system of calves shows normal activity, their stools vary between yellow and olive green, and the normal odor and stool density vary between soft (normal) and liquid (intermittent diarrhea) (18).

The source of the mutation is based on a bull named Mauglin Storm, born in 1991 (17, 23). The use of this bull's sons and grandchildren in artificial insemination has increased the spread of the disease (4). The major disadvantage of hereditary disorders arises when the mutation occurs in bulls that are far superior in terms of yields. It has been calculated that the economic loss due to the disease in Germany is 1.3 million euros per year (13).

It has been reported that the mutation that causes the disease in homozygous animals affected by the disease

occurs on the Apolipoprotein-B (APOB) gene (17, 22). CD is an autosomal recessive disease seen in homozygous mutant animals due to the APOB mutation, resulting in a 1299 bp insertion of the APOB gene on the 11th chromosome between the 24th and 25th nucleotides of the 5th exon in Holstein cattle (17).

Although it has been reported that this hereditary defect shows autosomal recessive inheritance, in an article (9), it was stated that some of the heterozygotes showed clinical signs, and the penetrance effect of the gene decreased in heterozygotes compared to homozygotes with full penetrance effect. Therefore, it was concluded that the disease is a metabolic disorder with incomplete dominant inheritance and incomplete penetrance in heterozygotes. In a study, it was determined that non-carriers had significantly higher serum cholesterol than carriers. It has been determined that CD carriers have shorter milking times and lower body weights than other control groups (3).

In Germany, the mutant gene frequency at which 80% of 234 homozygous calves died by the age of one was reported to be 8.7% (13). In Canada, the carrier frequency decreased from 17% in 2012 to 12% in 2016 (5). In a study conducted in Germany, 17.4% of 264 bulls were found to be carriers (17). The frequency of carriers in Holstein bulls born in Germany between 2012 and 2015 has been reported as 12.7% (22). In Poland, 9 out of 27 bulls with Mauglin STORM pedigree were found to be carriers (11). It has been reported that 10 of the 539 cows in Taiwan are carriers, and the frequency is 1.86% (16).

In China, 7 out of 138 bulls (5.07%) and only 1 out of 90 cows (1.11%) were identified as carriers. That is, the mutant allele frequency was determined to be 2.53% in bulls and 0.56% in cows. In the pedigree analysis, the carrier bulls and the cow were based on the cow named Braedale Baler Twine (HOCANF 6860888), the daughter of Mauglin Storm (15). The carrier rate of 1633 Holstein cows in China was determined to be 3.62% (59 cows) (12). The average frequency of mutant gene carriers was found to be 14.6% in 14 herds with 917 cows in Canada (24). In the study conducted in Russia, 35 of 451 cows were found to be carriers, and the carrier frequency was calculated at 7.76% (19). According to Pozovnikova et al. (20), 147 out of 1817 cows in Russia were carriers, and the carrier rate was 8.09%; 50 out of 171 cows whose fathers were carriers (29.23%); and 27 out of 160 cows and heifers whose fathers were carriers were reported to be carriers (16.87%). The carrier rate in cows of a local breed obtained with the contribution of Holstein in Russia was determined to be 4% (25). The carrier rate among Holsteins in Uruguay has been reported as 2.61% (21). In India, 1 out of 60 Holstein bulls was found to be carriers (frequency 1.67%) (14).

In this study, it was aimed to investigate the presence, distribution, and pedigree relationships of the mutant allele that causes an autosomal hereditary disease called "Cholesterol Deficiency (CD)" in Holstein cattle raised at State Farms.

Materials and Methods

A total of 466 Holstein cattle samples were taken from state farms, 25 of which were bull semen and 441 blood samples.

Genomic DNA was isolated using the "Zymo Research/D3025 Quick-gDNA™ Blood MiniPrep" DNA isolation kit. DNA samples were checked for their integrity on an agarose gel, and their amounts were measured using a spectrophotometer device (Thermo Multiscan GO). The amount and quality of the controlled DNA samples were diluted to 30-50 ng/µl and stored at -20 ºC.

"Primer 200 nM (60 bases) Primer/P200HPLC" as primer APOB.e3.WT.F_5'-CTGCAAAGCCACCTAGCCTA-3', APOB.e3.WT.R_5'-GCCTCTTCTGTTTCTGGGGG-3', and APOB.e3.Ins. R_5'-TCACGAGTGGAATGCCT CAC-3' primers from the insertion site were used.

"Bioline/BIO-92020, SensiFAST SYBR HI-ROX Kit" was used as the green master mix.

In real-time PCR, primers recommended by Schütz et al. (24), APOB.e3.WT.F_5'-CTGCAAAGCCACCT AGCCTA-3', APOB.e3.WT.R_5'-GCCTCTTCTGTTT CTGGGGG-3', and APOB.e3.Ins.R_5'-TCACGAGTG GAATGCCTCAC-3' from the insertion site, were used. For each PCR reaction, 50 ng/µL of DNA, 1x SYBR Green Master Mix (Bioline, SensiMix SYBR Hi-ROX), and 0.2 µM of each primer were added and made up to 25 µL with ultra-distilled water. The initial denaturation of the Real-Time PCR device (ThermoFisher StepOne plus Real-Time PCR) will be 10 min at 95 °C, followed by 35 cycles of 15 sec at 95 °C, 20 sec at 57 °C, and 30 sec at 72 °C. Measurements were made at the end of each cycle. Then, high-resolution melting curve (HRM) analysis was performed between 65 and 95 °C. Mutant and normal alleles were determined according to the HRM analysis.

LongRange PCR Protocol: Long-range PCR was performed to confirm the heterozygous and homozygous samples in real-time PCR analysis. For this purpose, APOB.e3.WT.F_5'-CTGCAAAGCCACCTAGCCTA-3' and APOB.e3.WT.R_5'-GCCTCTTCTGTTTCTGGGGG-3' primers were used (24). A PCR mix (20 µL) consisting of 1xPCR buffer, 3.5 mM MgCl2, 0.2 mM dNTPs, 0.3 pmol forward primer, 0.3 pmol reverse primer, 1 U Platinum Taq polymerase (Invitrogen, 10966034), and 20

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ng/µL DNA was prepared. PCR instrument (ThermoFisher, Veriti) for 2 minutes: one cycle at 95 °C, 45 sec at 94 °C, 30 sec at 57 °C, and 1 min at 72 °C, 35 cycles, and a final stage at 72 °C for 10 minutes.

DNA Sequencing Analysis: Before DNA sequencing analysis, 4 µl of PCR product, 0.5 µl Exonuclease-1 (ThermoFisher, EN0581), and 1.0 µl FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher, EF0652) were added to clean the amplified PCR products. The prepared mixture was kept in the PCR device for 15 minutes at 37 °C and 15 minutes at 85 °C. Then, for sequencing PCR, 12 µl of 1x sequencing buffer, 1 µl of BigDye solution, 5 µl (1 pmol) of F/R primer, and 2 µl of cleaned PCR product were added per sample. The PCR device was programmed for 2 minutes and 1 cycle at 96 °C, 10 seconds at 96 °C, 20 seconds at 54 °C, and 4 minutes and 30 cycles at 60 °C.

The products obtained as a result of the sequence PCR were cleaned according to the Ethanol-EDTA-Sodium acetate method. For this purpose, 1 µl of EDTA (pH 8.0), 1 µl of 3M sodium acetate (NaOAc, pH 5.07) and 50 µl of Ethanol (98%) were added to each sample. The plate was covered with aluminum sealing and turned upside down 4-5 times and kept in the dark at room temperature for 15 minutes. Then it was centrifuged at 4000 rpm at +4 °C for 30 min. After centrifugation, the sealing on it was removed and inverted on a napkin and centrifuged at +4 °C for 1 minute at 700 rpm. Then, 70 µl of 70% ethanol was added to the samples, covered with plate sealing, and centrifuged at 4000 rpm for 10 minutes at +4 °C. After centrifugation, the sealing on it was removed and inverted on a napkin and centrifuged at +4 °C for 1 minute at 700 rpm. The plate was then incubated in a dark place at room temperature for 60 min. After adding 15 µl of Hi-Di Formamide to each sample, the plate was sealed and vortexed vigorously and centrifuged briefly at 1300 rpm. After centrifugation, the sealing was removed and the plate was closed with septa and loaded into the DNA sequence analysis device (ThermoFisher, ABI 3500 Genetic Analyzer). The sequences obtained as a result of the DNA sequence analysis were arranged with the Sequencher Version 5.4.6 (Gene Codes Co) package program.

PCR Analysis: Primers proposed by Schütz et al. (24) and used in real-time PCR were used in PCR analysis. For each PCR reaction, 50 ng/µL of DNA, 5xPhusion HF Buffer (Thermo, F530L), 0.2 µM of each primer, and 1 U of Phusion Taq Polymerase were added and made up to 25 µL with ultra-distilled water. In the gradient PCR analysis to determine the Tm degree of the primers, the PCR device uses the initial denaturation at 98 °C for 1 min, followed by 40 cycles at 98 °C for 5 sec, 54–64 °C for 20 sec, and 72 °C for 20 second the last stage was programmed to last 10 minutes at 72 °C. The T_m temperature was determined to be 64 °C, and PCR of all samples was performed at this Tm temperature. After PCR, mutant and normal alleles were checked with 2% agarose gel electrophoresis.

Agarose Gel Electrophoresis: To prepare the agarose gel, 1 g of agarose and 50 ml of TAE (Tris-Acetate-EDTA) solution were mixed and heated in a microwave oven for 2 minutes. 1 µl of RedSafe (INtRON, 21141) dye solution was added to the prepared 2% agarose gel. The gel was then poured slowly into the tray. The prepared gel was kept at room temperature for 30 minutes and at 4 °C for 30 minutes for polymerization. After polymerization, the agarose gel was placed in a tank containing 1xTAE solution. The tank was filled with 1xTAE solution to cover the gel. 8.5 µl of the loading solution (1X Loading Dye) and 2.5 µl of the PCR product mixture were added to each well. After loading, 7 cm/V was applied to the gel for 30 minutes. After the run was completed, the gel was removed from the tank and visualized using the gel imaging system. A DNA ladder (ThermoFisher, SM0323) was used to determine the size of the PCR product.

Statistical Analysis: Gene frequencies were determined by the gene counting method. The frequency of gene A was found by dividing the sum of twice the number of homozygous (AA) individuals and the number of heterozygous individuals by twice the total number of individuals. The frequency of the B gene was found by doing a similar procedure for the B gene.

Gene Frequency = (Number of Homozygous Individuals x 2) + Number of Heterozygous Individuals / Total Number of Individuals x 2.

Whether the genotype frequencies fit the Hardy-Weinberg equation was checked with the chi-square test.

Results

Real-Time PCR: As a result of the real-time PCR analysis, the amplification graph of the samples is given in Figure 1. As a result of the HRM analysis performed at the end of the real-time PCR analysis, it was observed that in some samples, it gave a single peak at 85.8 ºC, and in some samples, two peaks at 85.7 and 92.6 ºC (Figure 2 and Figure 3). Samples with a single peak (Figure 2) were evaluated as not carrying the mutant allele (homozygous), while samples with two peaks (Figure 3) were considered to be carrying the mutant allele (heterozygous).

Figure 1. Real-Time PCR amplification plot of the samples.

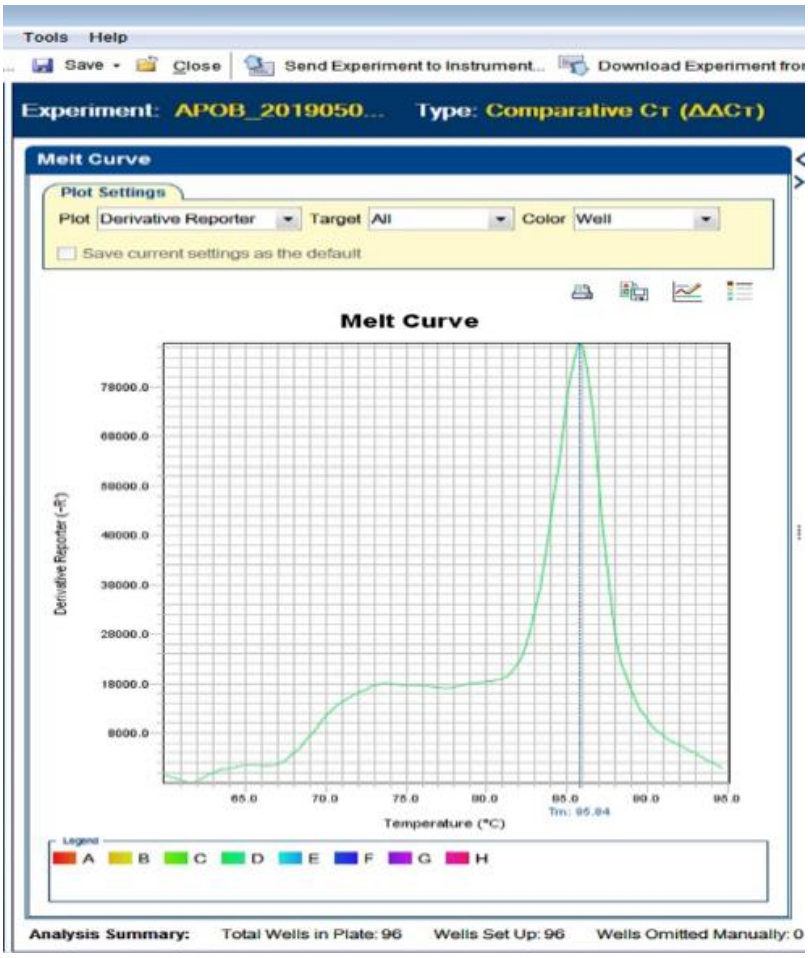

Figure 2. HRM (High Resolution Melting curve) analysis results of the samples (Homozygous).

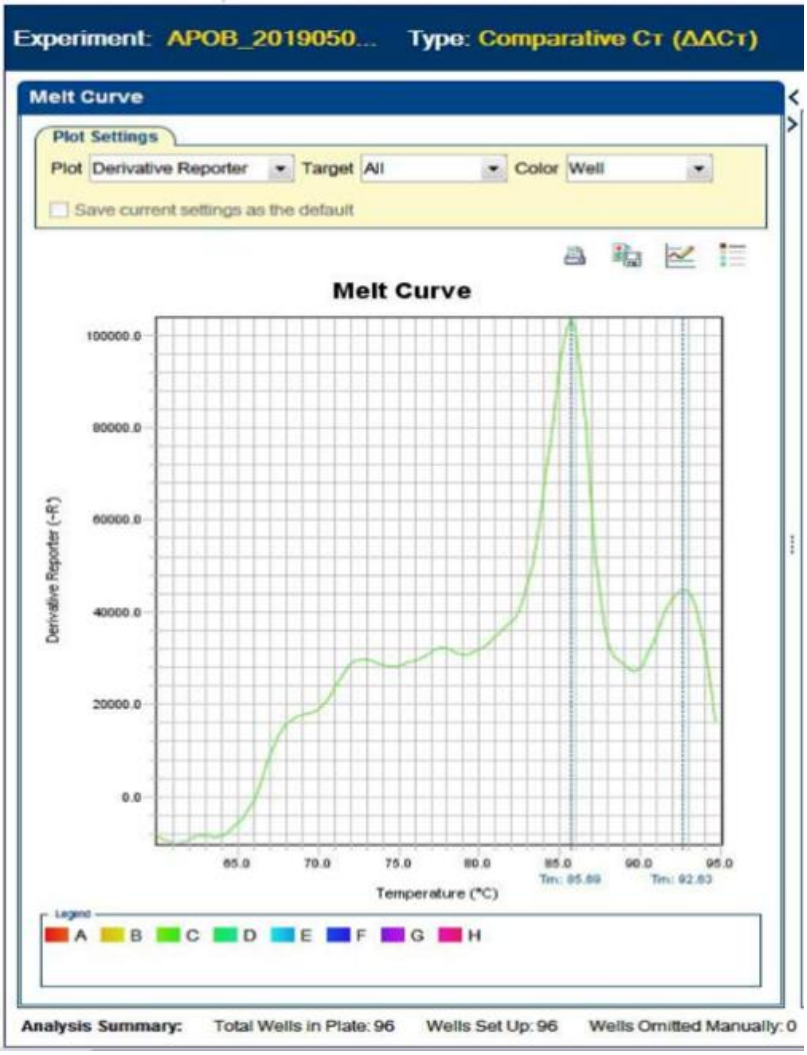

Figure 3. HRM (High Resolution Melting curve) analysis results of the samples (Heterozygous).

Sequence Analysis: DNA sequencing analysis was performed using APOB.e3.WT.F and APOB.e3.WT.R (long-range PCR) to confirm the samples evaluated as heterozygous and homozygous in real-time PCR. As a result of the sequencing analysis, no difference was observed in homozygous and heterozygous samples, and DNA sequences of 206 bp (base pair) length were obtained in all. In DNA sequencing analysis, the bases in the insertion region of the 1287 bp-long mutant allele were not found. In the agarose gel, the PCR products were determined to be 206 bp in length, and the band with bases in the insertion region could not be visualized. In other words, homozygous and heterozygous individuals could not be distinguished from each other.

PCR: In the analysis, it was determined that the heterozygous samples had two bands with a length of 206 and 132 bp.

Genotype and Allele Frequencies: Distributions of heterozygous and homozygous genotypes observed in agarose gel electrophoresis, expected genotype frequencies, and chi-square analyses are given in Table 1, and allele frequencies are given in Table 2. Of the 466 samples analyzed, 25 of which were bulls, 459 were homozygous for the wild allele. Seven cows were identified as heterozygous for the mutant allele. All 25 bulls sampled from Sultansuyu Farm were found to be homozygous for the wild allele (Table 1). It was determined that 7 cows were carriers: 3 in Çukurova Farm, 3 in Ceylanpınar Farm, and 1 in Kocas Farm. It has been demonstrated by X^2 analysis that the difference between observed and expected genotype frequencies in farms and in total is not significant (Table 1). The population appears to be in equilibrium in terms of the alleles studied. Mutant allele frequency varies between 0.00 and 1.97%, according to the farms (Table 2). When all the farms were evaluated together, the mutant gene frequency was calculated at 0.75%. The frequency of the mutant gene is quite low.

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Table 1. Distribution of genotypes for the APOB allele by farms (N=Number).

Farms	AA			AB			BB			X^2
	Obs.		Exp.	Obs.		Exp.	Obs.		Exp.	
	N	%	N	N	%	N	N	%	N	
Cukurova (Adana)	76	96.21	76.04	3	3.79	2.92	-	0.00	0.03	NS
Ceylanpinar (Urfa)	73	96.06	73.03	3	3.94	2.93	-	0.00	0.03	NS
Kocas (Aksaray)	95	98.96	95.00	1	1.04	0.99	-	0.00	0.01	NS
Polatli (Ankara)	108	100.0	108.00	-	0.00	-	-	0.00	0.00	NS
Turkgeldi (Tekirdag)	82	100.0	82.00	-	0.00	-	-	0.00	0.00	NS
Sultansuyu* (Malatya)	25	100.0	25.00	-	0.00	-	-	0.00	0.00	NS
TOTAL	**459**	**98.50**	**459.03**	**7**	**1.50**	**6.94**	**-**	**0.00**	**0.07**	**NS**

AA: Wild type homozygote, AB: Heterozygote carrier, BB: Mutant homozygote; NS: Non-significant
Obs.: Observed, Exp.: Expected, *: Bulls.

Table 2. Allele frequencies of the APOB gene by farms.

Farms	A (%)	B (%)
Cukurova (Adana)	98.11	1.89
Ceylanpinar (Urfa)	98.03	1.97
Kocas (Aksaray)	99.48	0.52
Polatli (Ankara)	100.00	0.00
Turkgeldi (Tekirdag)	100.00	0.00
Sultansuyu (Malatya) - Bulls	100.00	0.00
General	**99.25**	**0.75**

A: Wild allele, B: Mutant allele.

Discussion and Conclusion

Real-Time PCR, Sequence Analysis and PCR: Menzi et al. (17) showed that Taq polymerase used prefers the shorter wild type allele (249 bp) in animals with mutant alleles in their long-range PCR analysis to show the insertion in the 5th exon of the APOB gene. Therefore, they reported that they could not amplify the PCR product of the mutant allele with a length of 1548 bp. Based on this analysis to detect carriers; they suggested PCR by using a primer from the insertion site. Therefore, Menzi et al. (17), PCR of all samples again using Phusion Taq polymerase with APOB.e3.WT.F, APOB.e3.WT.R, and APOB.e3.Ins.R used in real-time PCR. They were made and visualized by running on a 2% agarose gel. It was determined that the majority of the samples detected in the heterozygous structure in the Real-Time PCR application were homozygous. They stated that this was due to the fact that the primers in Real-Time PCR were affected by the T_m grade used, and the affinity of Taq polymerase was different depending on the change in the $MgCl_2$ concentration in the master mix used.

Genotype and Allele Frequencies: All 25 bulls in the study were homozygous for the wild allele. That is, bulls do not inherit the mutant allele that causes the disease. When the pedigrees of the fathers of the bulls were examined, no kinship ties with Mauglin Storm were found. In a study examining bull pedigrees of imported semen in Türkiye, the carrier rate was reported as 5.5% (10). In this case, it can be said that it is possible that the mutant gene causing CD disease has spread throughout the country.

For this hereditary defect, it is seen that carrier frequencies have increased over the years in countries such as Germany and Canada, where control programs have not yet started (6, 7, 13, 17, 24). This situation shows that there is a need for eradication programs to reduce the frequency of the mutant gene. The frequency of the gene will increase if there is no intervention for eradication. The fact that the frequency of mutant genes in some countries such as China and Taiwan is lower than in some countries such as Germany and Canada can be attributed to the low import of semen whose pedigree is based on Storm.

In our study, it was determined that 7 out of 466 cattle were carriers, and the carrier frequency was 1.5% (Table 1 and Table 2). Gürses and Dere (8) reported that the rate of CD carriers was 4.67% in 450 cows in the Balıkesir region of Türkiye. Although the carrier frequency of CD disease is high on private farms in the Balıkesir region, it is quite low on state farms. This can be seen as an advantage. Therefore, it would be easier to control or prevent the spread of the ABOP gene. For the control of the disease, the pedigrees of the bulls should be paid attention to, and if they are not specified in their

pedigrees, genetic tests should be made in the laboratory to ensure that they are used.

The Presence, Distribution, and Pedigree Relationships of The Mutant Allele: After the mutation in the APOB gene, which causes cholesterol deficiency, was detected in Türkiye, it is important to associate these animals with Mauglin STORM. The inbreeding status of seven carrier cows with Mauglin STORM was investigated (Table 1). It was determined that the fathers of TR63671712 and TR63678971 ear tag carrier 2 cows in Ceylanpınar Farm were ELITE born in 2004 with HODEUM000662151690 ear tags. He is the grandson of Mauglin Storm. The pedigree information of the other carrier TR16775699 cow with ear tags could not be reached. Thus, it has been proven that two cows are related to Mauglin Storm (1, 2).

The carrier of the cow's father with ear tag number TR68677555 born in 2014 in KOCAS farm is a bull named GUNNAR with 2006 birth date and HODEUM001500838492 ear tag (Table 1). The bull named GUNNAR was revealed to be the son of Mauglin STORM's grandson, Braedale GOLDWYN, who is known to be a carrier. Therefore, it has been proven that this cow is also related to Mauglin Storm. It was determined that the father of the cow with ear tag number 1245066 from the 3 carrier cows in Cukurova Farm was also Gunnar. However, it could not be determined from the available pedigree information of the other two carrier cows from where they got the mutant gene (1, 2).

It was understood that 4 of the 7 carriers in question were related to Mauglin STORM; the other 3 could not be associated with STORM according to the available information (Table 1). These findings indicated that carrier bulls were also carried through semen import. The mutant gene was found as a result of the importation of the semen of his sons and grandchildren, who are known to be carriers of Mauglin STORM in Türkiye. As it is known, for success in dairy cattle breeding, it is necessary to obtain the highest yield and to maintain this situation for years. For this, it is important to choose the bulls and cows correctly. Hereditary diseases are more easily identified with the development of technology, and the pedigrees of bulls include information on whether a bull carries a hereditary disease as well as its productivity characteristics. Considering that proven bulls pass on their hereditary characteristics to their offspring, the use of semen from these bulls and the importation of their semen can continue to be passed on to future generations through heredity, along with traits such as milk yield, fertility, and meat yield, as a result of the use of these proven bulls in artificial insemination in many countries. Mauglin Storm is the beginning of the mutation; the mutant gene spread throughout countries with his children, grandchildren, and daughters. Carrier bulls have been found in many countries, such as Germany, the United States, the Netherlands, Canada, Belgium, England, Denmark, Luxembourg, and France. Mauglin Storm passes on both its good properties and cholesterol deficiency to its offspring. With the import of semen, this disease does not remain in a limited number of countries but spreads to more. Thanks to genetic tests, it can be determined whether the bulls are carriers of hereditary diseases, and information about the bull can be obtained when the bull catalogs and pedigrees of the bulls are examined. After the molecular detection of the disease, bulls with this disease have also become distinguishable, and in the pedigrees of the bulls of many countries, the bulls carrying the disease are shown with the CD code and those determined not to be carrying the disease are shown with the TC code. Türkiye also imports semen and female breeding cattle. For this reason, it is necessary to know whether bulls and breeding females carry hereditary diseases.

It was concluded that ABOP carrier cows were detected on state farms in Türkiye. For this reason, it is necessary to carry out controls for hereditary diseases in imported live animals and semen. The fact that clinical symptoms were observed in some of the heterozygous individuals suggests that the effect of the disease may be greater. For this reason, efforts should be made to completely eradicate the mutant gene by reducing its frequency. Cholesterol and triglyceride levels should be monitored and evaluated in individuals determined to be carriers. It is important to identify breeder cows, especially bulls, in terms of mutant genes.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

The contributions of the authors are equal.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was carried out after the animal experiment was approved by Ankara University Local Ethics Committee (Decision number: 2017-10-88).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Anonymous** (2023): STgenetics. Available at https://www.stgen.com. (Accessed Feb 15, 2023).
2. **Anonymous** (2023): Altagenetics. Available at https://bullsearch.altagenetics.com. (Accessed Feb 15, 2023).
3. **Basiel BL, Macrina AL, Dechow HCD** (2020): *Cholesterol deficiency carriers have lowered serum cholesterol and perform well at an elite cattle show*. JDS Communications, **1**, 6-9.
4. **Doormaal BV, Beavers L** (2015): Discovering genetic anomalies from genotyping. Available at https://www.HCDn.ca. (Accessed May 5, 2016).
5. **Doormaal BV, Beavers L** (2015): HCD: Haplotype associated with Cholesterol Deficiency. Available at https://www.HCDn.ca. (Accessed May 5, 2016).
6. **Doormaal BV, Beavers L** (2016): Mananing recessives & haplotypes. Available at https://www.HCDn.ca. (Accessed July 8, 2016).
7. **Doormaal BV, Beavers L** (2019): Current Status on Haplotypes and Genetic Recessives. Available at https://www.HCDn.ca. (Accessed April 1, 2019).
8. **Gurses M, Dere N** (2023): *Identification of the ERV insertion of APOB gene and deletion of TFB1M gene associated with lethal haplotypes of Holstein cattle reared in Balıkesir province, Türkiye*. Kafkas Univ Vet Fak Derg, **29**, 145-150.
9. **Hafliger IM, Hofstetter S, Mock T, et al** (2019): *APOB-associated cholesterol deficiency in Holstein cattle is not a simple recessive disease*. Anim Genet, **50,** 372-375.
10. **İnal Ş, Çam M** (2016): *Hereditary defects in bulls whose semen was imported to Türkiye in 2015.* Eurasian J Vet Sci, **4,** 278-284.
11. **Kaminski S, Rusc A** (2016): *Cholesterol Deficiency–new genetic defect transmitted to Polish Holstein-Friesian cattle.* Pol J Vet Sci, **19**, 885-887.
12. **Khan YA, Omar AI, He Y, et al** (2021): *Prevalance of nine genetic defects in Chinese Holstein Cattle.* Vet Med Sci, **7**, 1728-1735.
13. **Kipp S, Segelke D, Schierenbeck S, et al** (2015): *A new holstein haplotype affecting calf survival.* Interbull Bull, **49,** 49-53.
14. **Kumar A, Gupta ID, Kumar S, et al** (2021): *First report of cholesterol deficiency associated APOB mutation causing calf mortality in Indian Holstein Friesian population.* Indian J Anim Sci, **91,** 148-150.
15. **Li Y, Fang L, Liu L, et al** (2018): *The cholesterol-deficiency associated mutation in APOB segregates at low frequency in Chinese Holstein cattle.* Life J Anim Sci, **99,** 332–335.
16. **Liaw RB, Chen JC, Tsay SS, et al** (2017): *Genotype screening on cholesterol deficiency of dairy cattle herds in Taiwan.* Journal of Taiwan Livestock, **50,** 265-270.
17. **Menzi F, Besuchet-Schmutz N, Fragniere M, et al** (2016): *A transposable element insertion in APOB causes cholesterol deficiency in Holstein cattle*. Anim Genet, **47,** 253-257.
18. **Mock T, Mehinagic K, Menzi F, et al** (2016): *Clinicopathological phenotype of autosomal recessive cholesterol deficiency in Holstein cattle.* Journal of Veterinary Internal Medicine, **30,** 1369-1375.
19. **Pozovnikova MV, Lihacheva TE, Kudinov AA, et al** (2018): *Cholesterol deficiency mutation HCD does not impact milk productivity and blood levels of cholesterol and triglycerides in Russian Holstein black and white cattle.* Agricultural Biology, **53,** 1142-1151.
20. **Pozovnikova MV, Gladyr EA, Romanenkova OS, et al** (2020): *Screening of haplotype for cholesterol deficiency genetic defect in Russian Holstein cattle population.* Pol J Vet Sci, **23,** 313-315.
21. **Rodriguez BC, Romero A, Llambi S, et al** (2021): *Lethal and semi-lethal mutations in Holstein calves in Uruguay.* Animal Production Cienc Rural, **51**.
22. **Schutz E, Wehrhahn C, Wanjek M, et al** (2016): *The Holstein Friesian lethal haplotype 5 (HH5) results from a complete deletion of TBF1M and cholesterol deficiency (CDH) from an ERV - (LTR) insertion into the coding region of APOB.* Plos One, **11.**
23. **VanRaden P, Null D** (2015): Holstein haplotype for cholesterol deficiency (HCD). Available at https//:www.CDcb.us/reference/changes/HCD_inheritance.pdf. (Accessed May 5, 2016).
24. **Wang M, Do ND, Peignier C, et al** (2019): *Cholesterol deficiency haplotype frequency and its impact on milk production and milk cholesterol content in Canadian Holstein cows.* Can J Anim Sci, **100,** 786-791.
25. **Zimina AA, Romanenkova OS** (2020): *Main genetic defects of improving breeds in the population of Sychevsky cattle of the Smolensk region*. E3S Web of Conferences, **222,** 03017.

Total financial and avoidable losses due to lameness in Turkish dairy herds

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ABSTRACT

This study aimed to estimate the total financial (direct and indirect) and avoidable losses related to lameness in Turkish dairy herds. Official and previous published data were used for determining the mean (12.9%) and target prevalence (6.6%) values. Direct financial losses due to lameness were calculated as treatment costs, involuntary culling, and milk yield losses. Indirect losses consisted of extended calving intervals and extra insemination losses. As a result, the average financial loss of lameness per case on dairy farms was $153.8 ($116.8 for mild and $264.7 for severe forms) in Türkiye. Avoidable losses ($75.1) were calculated to be equivalent to 187 liters of cow's milk with current prices for the 2023 year. The share of direct and indirect financial losses was 49.6% and 50.4%, respectively. Total annual losses related to lameness in Türkiye are calculated at $130.5 million (326 million Lt. milk eq.), although $63.7 million (159 million Lt. milk eq.) of the losses could be avoided by farmers. The milk equivalent of avoidable losses corresponds to 0.77% of Türkiye's total annual milk production. In conclusion, lameness causes significant financial losses in Turkish dairy herds. However, almost half of these losses (48.8%) may be avoidable with better management practices which help to increase the productivity and profitability of dairy farmers.

Introduction

Lameness is an endemic disease that causes significant financial losses, particularly on intensive dairy cattle farms (25). The disease can cause direct financial losses such as loss of milk yield, involuntary culling, and treatment costs, as well as indirect losses such as extended calving intervals and additional insemination for pregnancy (18, 28, 35). Lameness is also responsible for the third most crucial monetary loss after mastitis and fertility disorders in dairy farms (34). Complete elimination of endemic diseases (mastitis, metritis, lameness, etc.) in dairy production is not feasible (32).

The total losses due to lameness can be divided into avoidable and unavoidable losses (14, 49). While unavoidable losses may be the minimum cost for eliminating the disease under current conditions, avoidable losses may be considered an extra cost and incur costs over the unavoidable losses. Thus, avoidable losses can be reduced and/or avoided with a better management system.

The prevalence of lameness in dairy cattle varies according to race, age, nutrition, breeding type, parity, genetic predisposition, disorders of digits, environmental factors, climate, and region (41, 49). In Türkiye, the prevalence of lameness was reported to range from 4.5-40 (2, 24, 26, 27, 33, 38, 45, 48, 50-52). Studies conducted in different countries about the prevalence of lameness reported 8% in New Zealand (16), 11.6-22.9% in Ireland (42), 24% in Australia (4), 30.5% in the Czech Republic (37), 17-39% in Kosovo (23), 19-40% in Hungary (34), 21.4-31.6% in the UK (20, 31), 10-37% in the USA (22, 36), and 26.6-42.8% in the Netherlands (10).

On the other hand, many studies from different countries have reported financial losses caused by lameness (9, 12, 15, 21, 23, 28, 30, 34, 40, 47). However, there is a lack of detailed loss estimation due to lameness in dairy herds in Türkiye. Also, it has been observed that studies on this topic in Türkiye are limited to the technical aspects of the disease, such as prevalence and treatment

procedures (26, 33, 48, 52). Therefore, dairy farmers and policymakers need to know all aspects of fiscal impact (total losses) and the share of unavoidable/avoidable losses to provide optimal decision support.

This study aimed to estimate the total financial (direct and indirect) and avoidable losses related to lameness in Turkish dairy herds.

Materials and Methods

Considering the results of the over 10 studies (given in Table 1) conducted in six different geographical regions of Türkiye, the weighted mean prevalence of lameness in dairy herds was calculated at 12.9%, and the target prevalence was assumed to be 6.6% (49). Financial losses were calculated for two forms (mild and severe cases) of the disease and an average case (75% mild and 25% severe) (49). In the loss calculations, digital and interdigital dermatitis, hyperplasia, and erosion were categorized as mild. In contrast, phlegmon, ungular fissure, abscess, white line disease, and sole ulcer were categorized as severe. Total, unavoidable, and avoidable losses of lameness for the Turkish economy were calculated using the losses per average case with 2023 prices.

Some technical and financial data and criteria used to estimate lameness-related losses are given in Tables 1 and 2.

Table 1. Technical and financial data used in estimating lameness-related losses.

Technical information	Value	Reference
a. No. of a total milked cow (head)	6,580,753	(44)
b. Mean prevalence of lameness (%)	12.9	*
c. No. of total infected cow (head)	848,917	Calculation (a × b)
d. Target prevalence (%)	6.6	(49)
e. No. of infected cow in target prevalence (head)	434,330	Calculation (a × d)
f. No. of infected cow over target prevalence (head)	414,587	Calculation [a × (b - d)]
g. Total milk yield (lt/lactation)	3,170	(17)
h. Daily milk yield (lt)	10.4	Calculation (g / 305)
i. Daily feed consumption (kg/head)	12	(49)
j. Consumed feed for 1 lt of milk (kg)	1.2	Calculation
k. Treatment period (min./case/day)	30	(49)
Financial information		
l. Farm-gate milk price ($/lt)	0.4	(46)
m. Concentrated feed price ($/kg)	0.4	(46)
n. Refresh heifer price ($/head)	2,000	(46)
o. Culled cow price ($/head)	1,200	(46)
p. Cost of culling ($/head)	800	Refresh heifer-culled cow
q. Cost of labour ($/hour)	2	Calculation
r. Cost of sperma ($/dose)	15	Field survey
s. Cost of extended calving interval ($/day)	5	(49)

*: (2, 24, 33, 41, 45, 48-50, 52).

Table 2. Some criteria used in estimating lameness related losses in study.

Parameters	Mild cases	Severe cases	Reference
-Intensity of cases (%)	75	25	(49)
-Possibility of vet. treatment (%)	10	30	(49)
-Possibility of farmer treatment (%)	90	70	(49)
-Treatment period (day)	5	8	(49)
-Decrease in total milk yield (%)	1.5	3	(14)
-Culling rate due to lameness (%)	1.2	1.8	(49)
-Extended calving interval (day)*	10	30	(14)
-Extra insemination (%)*	30	50	(5)
-Decrease in feed consumption (%)	15	30	(32)
-Vet. fee ($/case)	20	30	Field survey

*: Indirect losses

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Mild, severe, and total loss estimation due to lameness in Türkiye was calculated according to technical and financial parameters and data, as shown in Table 3.

In the financial analysis, a decrease in feed consumption due to lameness was also considered. Direct financial losses due to lameness were calculated as treatment costs, involuntary culling, and milk yield losses. Indirect losses consisted of extended calving intervals and extra insemination losses. Total losses were the subtraction of the decrease in feed consumption from the sum of total losses (direct and indirect losses).

Avoidable losses were calculated using target prevalence values, and over-target prevalence values were given for the avoidable losses. The calculations of losses were provided in US dollars and for the current 2023 prices.

Results

Financial losses due to mild and severe forms of lameness and average losses per infected cattle are presented in Table 4.

Total losses per mild and severe case due to lameness were calculated at $116.8 (292 lt. milk equivalent) and $264.7 (662 lt. milk eq.). Average losses per infected cow were calculated at $153.8 (385 lt. milk eq.) in lameness, and $75.1 (187 lt. milk eq.) of losses were avoidable. The highest loss item was the extended calving interval, both in mild (41.5%) and severe cases (54.3%). At the same time, the share of direct losses in mild form was 56.7% of total losses and decreased to 43.0% in severe form. Indirect losses related to the reproductive efficiency of animals were increased in extreme cases. Due to lameness, total milk losses and involuntary culling costs vary between 25.5-33.0% and 5.3-8.0%, respectively (Table 4).

Total, unavoidable, and avoidable losses of lameness in Türkiye are given in Table 5.

Total losses related to the lameness in the dairy industry for the Turkish economy were calculated at $130.5 million (326 million lt. milk eq.). However, $63.7 million (159 million lt. milk eq.) of the total losses (48.8%) could be avoided with better management of dairy farms in Türkiye. Total unavoidable losses were $66.8 million annually (Table 5).

Table 3. Loss estimation model used in the study.

Losses	Estimation model
A- Mild cases ($/case)	A = [Total milk yield (lt) × Decrease in milk yield (%) × Farm-gate milk price ($/lt)]
B- Severe cases ($/case)	B = [Total milk yield (lt) × Decrease in milk yield (%) × Farm-gate milk price ($/lt)]
C- Average losses ($/case)	C = [(A × 0.75) + (B × 0.25)]
D- Total losses ($/year)	D = [C × No. of total infected cow (head)]
E- Unavoidable losses ($/year)	E = [C × No. of infected cow in target prevalence (head)]
F- Avoidable losses ($/year)	F = [C × No. of infected cow over target prevalence (head)]

Table 4. Financial losses due to lameness in mild and severe cases in Türkiye.

Loss item	Mild form ($/case)	Severe form ($/case)	Average losses ($/case)**
A. Direct Losses (1+2+3)	65.9 (56.7%)*	118.7 (43.0%)	79.1 (49.6%)
1. Total milk losses	39.8 (33.0%)	71.3 (25.8%)	47.7 (29.9%)
a. Decreased yield	19.0	38.0	23.8
b. Discarded milk	20.8	33.3	23.9
2- Treatment costs	16.5 (13.7%)	33.0 (11.9%)	20.6 (12.9%)
a. Drug, vitamin etc.	10.0	16.0	11.5
b. Vet. med.	2.0	9.0	3.8
c. Extra labor	4.5	8.0	5.4
3- Culling cost	9.6 (8.0%)	14.4 (5.3%)	10.8 (6.8%)
B. Indirect Losses (4+5)	54.5 (45.3%)	157.5 (57.0%)	80.3 (50.4%)
4-Extended calving interval**	50.0 (41.5%)	150 (54.3%)	75.0 (47.1%)
5-Extra insemination**	4.5 (3.8%)	7.5 (2.7%)	5.3 (3.3%)
C. Decreased feed consumption	3.6	11.5	5.6
TOTAL LOSSES [(A+B)-C]	**116.8**	**264.7**	**153.8**

*The value in parenthesis indicates its share in total losses. **In total losses 75% was mild and 25% was severe form.

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Table 5. Total, unavoidable and avoidable losses of lameness in Türkiye.

Loss item	Total cost in Türkiye ($/year)	Unavoidable cost in Türkiye ($/year)	Avoidable cost in Türkiye ($/year)
A. Direct Losses (1+2+3)	67,149,335	34,355,474	32,793,861
1. Total milk losses	40,472,118	20,706,665	19,765,453
a. Decreased yield	20,161,779	10,315,329	9,846,450
b. Discarded milk	20,310,339	10,391,336	9,919,003
2- Treatment costs	17,508,913	8,958,049	8,550,865
a. Drug, vitamin etc.	9,762,546	4,994,791	4,767,755
b. Vet. med.	3,183,439	1,628,736	1,554,703
c. Extra labour	4,562,929	2,334,522	2,228,407
3- Culling cost	9,168,304	4,690,760	4,477,544
B. Indirect Losses (4+5)	68,125,589	34,854,953	33,270,637
4-Extended calving interval**	63,668,775	32,574,722	31,094,053
5-Extra insemination**	4,456,814	2,280,231	2,176,584
C. Decreased feed consumption	4,732,712	2,421,388	2,311,325
TOTAL LOSSES [(A+B)-C]	**130,542,212**	**66,789,039 (51.2%)**	**63,753,173 (48.8%)**

Discussion and Conclusion

Besides being a serious animal welfare issue, lameness is a significant endemic disease on dairy farms in Türkiye, as in many other countries (3). The reported prevalence values for lameness in Türkiye are mainly in line with the figures reported from other countries (1, 10, 20, 36, 37, 39, 42).

Lameness also causes significant financial losses not only on individual farms but also at the national level. In the current study, the mean loss per case was found to be $153.8 ($116.8 for mild and $264.7 for severe forms). In the literature, financial losses due to lameness in an infected animal were reported as £246-323 in the UK (28, 47), $95-127 in the Netherlands (6, 11, 12), €192 in Denmark (15), €100-300 (34), $120-533 in the USA (8, 21, 30, 40), $6.2-283 in Türkiye (3, 19, 49), $53-622 in Spain (9), €54.5 in Hungary (29) and $227 in Brasil (43).

Although prevalence values are effective in determining the magnitude of financial losses due to lameness, it is thought that some different methodological approaches encountered in the calculations and the severity of the disease also have a significant impact (8, 32, 34, 47). For example, while some researchers (13, 14, 47) consider losses more detailed (indirect losses, decrease in feed consumption of diseased cows, labor cost, etc.), as in this study, some other studies only consider direct losses (5, 12). Therefore, comparisons of the studies about the total losses and direct and indirect losses due to lameness are getting more complex.

In this study, besides the prevalence values due to lameness, determining the target prevalence level and calculating the avoidable losses are novel findings for Türkiye. Having knowledge about avoidable losses is significant for farmers to understand the extent of the disease problem and how much they can reduce these losses. So, this evidence-based information can be used as a decision-support tool by dairy farmers.

Contrary to this study, avoidable losses are not considered in most studies. Almost half of the avoidable losses (48.8%) can be considered a potential opportunity for dairy farmers and decision-makers in disease control. The milk equivalent of avoidable losses corresponds to 0.77% of Türkiye's total annual milk production. Similarly, in the study conducted by Esslemont and Spincer (14) in British field conditions, it was reported that 82% of the total loss was an avoidable loss. In a previous study conducted in Türkiye, it was reported that 62% an avoidable loss (49).

In conclusion, besides total losses due to disease, knowing the amount of avoidable losses is vital to ensuring economic efficiency for farmers in disease prevention and control decisions. However, to provide healthier decision support to farmers and policymakers against lameness, determining the alternative control strategies and their effects on prevalence and losses can improve their success in production.

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Ethical Statement

Ethical committee approval is not required.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

SS, designed the research, analysis, supervision, writing, and editing. MK, investigation, collecting the data, writing, and review.

References

1. **Adams A, Lombard J, Fossler C, et al** (2016): *Associations between housing and management practices and the prevalence of lameness, hock lesions, and thin cows on US dairy operations*. J Dairy Sci, **100**, 2119-2136.
2. **Atalan G** (2018): *Kayseri Develi tarımsal kalkınma kooperatifi süt sığırı çiftliğinde bulunan sığır tırnaklarının muayenesi ve problemli tırnakların sağaltımı.* Erciyes Üniv Vet Fak Derg, **15**, 222-229.
3. **Aydın R** (2017): Süt sığırcılığı işletmelerinde işletme içi kirlilik ile topallık arasındaki ilişki ve topallığa bağlı ekonomik kayıplar. Yüksek Lisans Tezi, Namık Kemal Üniversitesi Fen Bilimleri Enstitüsü, Tekirdağ.
4. **Beggs DS, Jongman EC, Hemsworth PH, et al** (2019): *Lame cows on Australian dairy farms: A comparison of farmer-identified lameness and formal lameness scoring, and the position of lame cows within the milking order*. J Dairy Sci, **102**, 1522-1529.
5. **Bennett R** (2003): The economics of mastitis. The University of reading home page. Available at www.reading.ac.uk. (Accessed May 15, 2022).
6. **Bruijnis MRN, Hogeveen H, Stassen EN** (2010): *Assessing economic consequences of foot disorders in dairy cattle using a dynamic stochastic simulation model.* J Dairy Sci, **93,** 2419-2432.
7. **Canpolat İ, Bulut S** (2003): *Elazığ ve çevresinde sığırlarda görülen ayak hastalıklarının insidansı üzerine gözlemler*. FÜ Sağ Bil Derg, **17**, 155-160.
8. **Cha E, Hertl JA, Bar D, et al** (2010): *The cost of different types of lameness in dairy cows calculated by dynamic programming*. Prev Vet Med, **97**, 1-8.
9. **Charfeddine N, Pérez-Cabal MA** (2017): *Effect of claw disorders on milk production, fertility, and longevity, and their economic impact in Spanish Holstein cows*. J Dairy Sci, **100**, 653-665.
10. **De Vries M, Bokkers EAM, Van Reenen CG, et al** (2015): *Housing and management factors associated with indicators of dairy cattle welfare.* Prev Vet Med, **118**, 80-92.
11. **Dijkhuizen AA, Morris RS** (1997): Animal health economics. (1st ed). 1-306. University of Sydney, Sydney, Australia.
12. **Enting H, Kooij D, Dijkhuizen AA, et al** (1997): *Economic losses due to clinical lameness in dairy cattle.* Livest Prod Sci, **49**, 256-267.
13. **Esslemont RJ, Kossaibati MA** (1996): *The incidence of production diseases and other health problems in a group of dairy herds in England*. Vet Rec, **139**, 486-490.
14. **Esslemont RJ, Spincer I** (1993): The incidence and costs of diseases in dairy herds. 58. Daisy report No 2, University of Reading. Department of Agriculture.
15. **Ettema JF, Østergaard S** (2006): *Economic decision making on prevention and control of clinical lameness in Danish dairy herds.* Livest Sci, **102**, 92-106.
16. **Fabian J, Laven RA, Whay HR** (2014): *The prevalence of lameness on New Zealand dairy farms: A comparison of farmer estimate and locomotion scoring*. Vet J, **201**, 31-38.
17. **FAO** (2022): FAOSTAT. Available at https://www.fao.org/faostat/en/#data; (Accessed Jan 20, 2022).
18. **Fourichon CH, Seegers N, Bareille N, et al** (1999): *Effects of disease on milk production in the dairy cow: A Review.* Prev Vet Med, **41**, 1-35.
19. **Göncü S** (2023): Süt sığırcılığı işletmelerinde topallık. Available at http://www. muratgorgulu. com. tr/altekran.asp?id=117. (Accessed May 20, 2023).
20. **Griffiths BE, Dai White G, Oikonomou G** (2018): *A cross-sectional study into the prevalence of dairy cattle lameness and associated herd-level risk factors in England and Wales.* Front Vet Sci, **5**, 65.
21. **Guard C** (2008): The costs of common diseases of dairy cattle. 695-700. In: Proceedings of the California Veterinary Conference. Lenexa, USA.
22. **Hoffman AC, Moore DA, Wenz JR, et al** (2013): *Comparison of modeled sampling strategies for estimation of dairy herd lameness prevalence and cow-level variables associated with lameness.* J Dairy Sci, **96**, 5746-5755.
23. **Ibishi L, Musliu A, Mehmedi B, et al** (2022): *Economic losses due to clinical lameness in Kosovo dairy cattle*. Vet Stanica, **53**, 295-304.
24. **İstek Ö, Han MC, Tanrısever M** (2019): *Fırat Üniversitesi Hayvan Hastanesine getirilen sığırlardakı tırnak deformasyonları ile ayak hastalıklarının retrospektif değerlendirilmesi.* Harran Univ Vet Fak Derg, **8**, 57-63.
25. **İzci C, Erdal FÇ** (2021): *Süt sığırlarında topallık: verimlilik ve çiftlik ekonomisine etkileri.* BU Ziraat Fak Derg, **35**, 451-466.
26. **Kayapınar SD, Han MC** (2021): *Malatya ve yöresinde sığır ayak hastalıklarının prevalansı.* FÜ Sağ Bil Derg, **35**, 37-42.
27. **Keskin E** (2016): Gaziantep ve yöresinde gözlenen sığır ayak hastalıklarının insidansı ve tedavileri üzerine gözlemler. Yüksek Lisans Tezi, Fırat Üniversitesi Sağlık Bilimleri Enstitüsü, Elazığ.
28. **Kossaibati MA, Esslemont RJ** (1997): *The costs of production diseases in dairy herds in England.* Vet Journal, **254**, 41-51.
29. **Lehoczky J** (2001): Maintenance hoof trimming (in Hungarian). Thesis. SZIE FVS Department of Animal Hygiene, Budapest, Hungary.
30. **Liang D, Arnold LM, Stowe CJ, et al** (2017): *Estimating US dairy clinical disease costs with a stochastic simulation model*. J Dairy Sci, **100**, 1472-1486.
31. **Main DCJ, Leach KA, Barker ZE, et al** (2012): *Evaluating an intervention to reduce lameness in dairy cattle.* J Dairy Sci, **95**, 2946-2954.
32. **McInerney JP, Howe KS, Schepers JA** (1992): *A framework for the economic analysis of disease in farm livestock.* Prev Vet Med, **13**, 137-154.
33. **Özcan S, Pamuk K** (2009): *Afyonkarahisar ve çevresinde sığır ayak hastalıklarının prevalansı.* Kocatepe Vet J, **2**, 15-19.
34. **Ózsvári L** (2017): *Economic cost of lameness in dairy cattle herds*. J Dairy Vet Anim Res, **6**, 00176.
35. **Rajala-Shultz PJ, Gröhn YT, Mcculloch CE** (1999): *Effects of milk fever, ketosis and lameness on milk yield in dairy cows.* J Dairy Sci, **82**, 288-294.
36. **Salfer JA, Siewert JM, Endres MI** (2018): *Housing, management characteristics, and factors associated with lameness, hock lesion, and hygiene of lactating dairy cattle*

on Upper Midwest United States dairy farms using automatic milking systems. J Dairy Sci, **101**, 8586-8594.

37. **Šárová R, Stehulova I, Kratinová P, et al** (2011): *Farm managers underestimate lameness prevalence in Czech dairy herds.* Animal Welfare, **20**, 201-204.
38. **Saruhan A** (2015): Mardin yöresinde bazı süt sığırcılığı işletmelerinde ayak hastalıklarının prevalansı. Yüksek Lisans Tezi, Harran Üniversitesi Sağlık Bilimleri Enstitüsü, Şanlıurfa.
39. **Sharma A, Phillips CJC** (2019): *Lameness in sheltered cows and its association with cow and shelter attributes*. Animal, **9**, 360.
40. **Shearer JK, Van Amstel SR** (2013): Manual of foot care in cattle (2nd ed). 132. WD. Hoard and Sons Company, USA.
41. **Şındak N, Keskin O, Biricik HS, et al** (2003): *Şanlıurfa ve yöresinde sığır ayak hastalıklarının prevalansı.* YYÜ Vet Fak Derg, **14**, 14-18.
42. **Somers JR, Huxley J, Lorenz I, et al** (2015): *The effect of lameness before and during the breeding season on fertility in 10 pasture-based Irish dairy herds.* Irish Vet Journal, **68**, 1-7.
43. **Souza RC, Ferreira PM, Molina LR, et al** (2006): *Economic losses caused by sequels of lameness in free-stall-housed dairy cows.* Arq Bras Med Vet Zootec, **58**, 982-987.
44. **TUIK** (2022): TURKSTAT. Available at https://data.tuik.gov.tr/Kategori/GetKategori?p=tarim-111&dil=1; (Accessed Jan 15, 2022).
45. **Tutuş D** (2020): Van Muradiye Ilçesinde ruminantlarda görülen ekstremite ve ayak hastalıklarının insidansı. Yüksek Lisans Tezi, Van Yüzüncü Yıl Üniversitesi Sağlık Bilimleri Enstitüsü, Van.
46. **USK** (2023): Fiyatlar. Available at https://ulusalsutkonseyi.org.tr/ (Accessed May 25, 2023).
47. **Willshire J, Bell N** (2009): *An economic review of cattle lameness*. Cattle Pract, **17**, 136-141.
48. **Yakan S** (2018): *Ağrı ilinde sığırlarda ayak hastalıkları prevalansının belirlenmesi.* Harran Univ Vet Fak Derg, **7**, 207-212.
49. **Yalçın C, Sarıözkan S, Yıldız AŞ, et al** (2010): *Financial losses due to lameness in dairy herds in Burdur, Kırklareli and Konya provinces of Turkey.* Ankara Univ Vet Fak Derg, **57**, 99-104.
50. **Yayla S, Aksoy Ö, Kılıç E, et al** (2012): *Kars ve yöresinde sığırların bakım ve barındırma koşulları ile ayak hastalıkları arasındakı ilişkinin değerlendirilmesi.* Harran Univ Vet Fak Derg, **1**, 22-27.
51. **Yaylak E, Konca Y, Koyubenbe N** (2016): *İzmir İli Ödemiş ilçesindeki damızlık sığır yetiştiricileri birliği üyesi işletmelerde sağlık koruma uygulamaları ve sağlık sorunları üzerine bir araştırma.* J Anim Prod, **57**, 28-40.
52. **Yurdakul İ, Şen İ** (2018): *Sivas ve yöresinde sığır ayak hastalıkları prevalansının belirlenmesi.* Harran Univ Vet Fak Derg, **7**, 51-55.

Evaluation of infrared thermography, arterial Doppler ultrasound, and Doppler echocardiography in healthy adult dogs exposed to a single session of Whole-body vibration at different frequencies

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ABSTRACT

This study aimed to evaluate the infrared thermography, arterial Doppler ultrasound, and Doppler echocardiography in healthy adult dogs exposed to a single Whole-body vibration (WBV) at different frequencies. Sixteen (16) healthy crossbreed dogs males of ages 1 to 5 years, weighing 16.3 to 24.5 kg were enrolled in the study. The dogs were exposed to a single WBV session at frequencies of 30 Hz (5 min), 40 Hz (5 min), and 50 Hz (5 min) with intervals between each frequency exposure of 10 min. The cutaneous temperature, arterial Doppler ultrasound, and Doppler echocardiography were evaluated 10 min before the WBV session, between each frequency, and 1 min after the last frequency. The cutaneous temperature of the regions of the superficial gluteal muscle and biceps femoris muscle of both hind limbs was obtained with an infrared camera. Resistive indexes of carotid and femoral arteries were determined using Doppler ultrasound, and Doppler echocardiography was used to assess the end-systolic volume and end-diastolic volume, heart rate, aortic blood flow velocity, and pulmonary artery flow velocity. Clinical parameters, complete blood count, and serum biochemical (alanine aminotransferase, creatinine, and creatine phosphokinase) were evaluated 10 min before and 60 min after the end of the WBV session. Statistically significant differences were not found in any of the variables among the time points. In conclusion, the protocol of increasing vibration frequencies (30, 40, and 50 Hz) at short-period WBV can be considered appropriate since no change occurred in the parameters evaluated.

Introduction

Vibrating platforms have been used frequently in humans to promote whole-body vibration (WBV), aiming for aspects of health, physiotherapy, sports, and exercises (3, 4, 22). Several studies have been performed to understand the acute and chronic responses to WBV on cardiovascular indices (3, 4, 12). Although, it is necessary to elucidate the effects of WBV in animals, particularly in dogs, since this type of exercise using vibrating platforms is already used in small animals (6, 10, 16-19, 23). Differences can occur among the different vibrating platforms (12, 13, 15), however, the vibrating frequency, amplitude, acceleration, and duration are variables that determine the intensity of the mechanical stimulus, being

the body position and posture also a critical elements, and is an absence of consensus on optimal parameters for humans (4, 5, 13). The authors have previously evaluated WBV in dogs considering its applicability potential in several disorders (6, 10, 16-19, 23), and to define safe protocols based on responses to vibration stimuli, the authors used the same device and kept the dog in a standing position over the vibrating platform.

Thus, this study aimed to evaluate the infrared thermography, arterial Doppler ultrasound, and Doppler echocardiography in healthy adult dogs exposed to a single WBV at different frequencies. The hypothesis was that the effects of increasing vibration frequencies would be reflected by an increase in echocardiographic and thermographic parameters and a decrease in the resistivity index. The clinical relevance of WBV programs is associated with physical condition improvement without joint damage, and muscle hypertrophy as report in human patients. Dogs diagnosed with muscle atrophy regrading to hip dysplasia can benefit from this modality as an additional form of conservative treatment for muscle atrophy and possible improvement of quality of life in dogs.

Materials and Methods

***Animal selection*:** The methodology adopted in this study was approved by the Institutional Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (Unesp), Botucatu, São Paulo, Brazil (nº. 0120/2018-CEUA). A written consent form was obtained from the dog's owner before starting the study. Sixteen (16) healthy crossbreed dogs males of ages from 1 to 5 years (mean±SD, 3.5±2.5 years), and weighing 16.3 to 24.5 kg (mean±SD, 20.4±4.1 kg) were enrolled in the study. The dogs were considered healthy based on their clinical history, physical examination, complete blood count (CBC), and serum biochemical [alanine aminotransferase (ALT), creatinine, creatine phosphokinase (CK)].

The inclusion criteria were dogs that had not been submitted to any surgical procedure for at least six months and had not taking medications for three months. Exclusion criteria included concerns raised by findings in the history and changes detected in the physical examination and/or laboratory tests.

Whole-body vibration protocol: No training was necessary for the dogs to get used to the vibrating platform. Before the WBV session, 6-hour fasting and 1-hour water restriction were stipulated. In addition, 30 min of rest was determined to acclimatize in the room environment with a controlled temperature (22º C) and humidity between 40 and 45%. The room had artificial light by LED lamps and blackout curtains to prevent sunlight entrance. The WBV session was performed at 02:00 p.m. by the same operator.

The vibrating platform (92 cm length, 62 cm width, and 16 cm height) delivered a vortex wave circulation (TheraPlate Revolution, Texas, USA) that was used to promote the WBV. During the sessions, dogs remained on the top of the vibrating platform in a standing position without any sedation, and a leash was used to prevent them from moving or sitting (Figure 1). The dogs were exposed to single WBV at frequencies of 30 Hz [peak displacement (Dpeak)=3.10 mm; peak acceleration (Apeak)=11.16 m s-2) for 5 min, 40 Hz for 5 min (Dpeak=3.37 mm; Apeak=21.59 m s-2 peak acceleration), and 50 Hz for 5 min Dpeak=3.98 mm; Apeak=39.75 m s-2). The mean interval between each frequency exposure was 10 min. The frequency was determined by the manufacturer and checked by a digital oscilloscope (Mustool MDS120M, Mustool, California, USA), and for peak acceleration acquisition was used a 3-axis digital accelerometer sensor (STMicroelectronics, São Paulo, Brazil) was placed at the center of the vibrating platform. The peak displacement was calculated by using: Dpeak=Apeak/(2.f)2, f – frequency (4, 13).

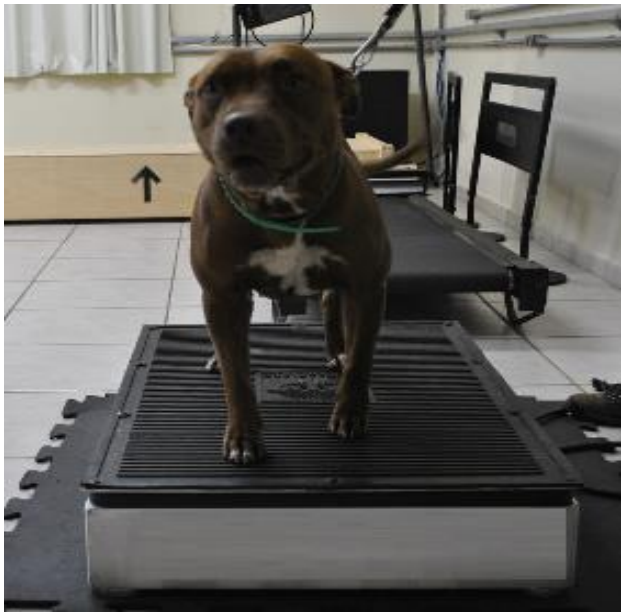

Figure 1. Dog standing on the top of the vibrating platform which delivered a vortex wave circulation, and with a leash to prevent from moving or sitting.

Infrared thermography: The cutaneous temperature of the regions of the superficial gluteal muscle and biceps femoris muscle of both hind limbs was obtained with an infrared camera (FLIR Model E4, FLIR Systems, Boston, USA) with image resolution 256×256 pixels and thermal sensitivity of 0.07° C (Figure 2). The camera was positioned at 1 meter from the assessed regions with an angle of 90º while the dogs standing on the top of the vibration

platform. The room where the infrared thermography exams were performed and the acclimatization time in the room environment, temperature, and humidity were the same as the WBV sessions. During the exams, the room was maintained with the light on (led lamps) and blackout curtains, and only three persons were allowed in the room, to prevent variations in the room environment.

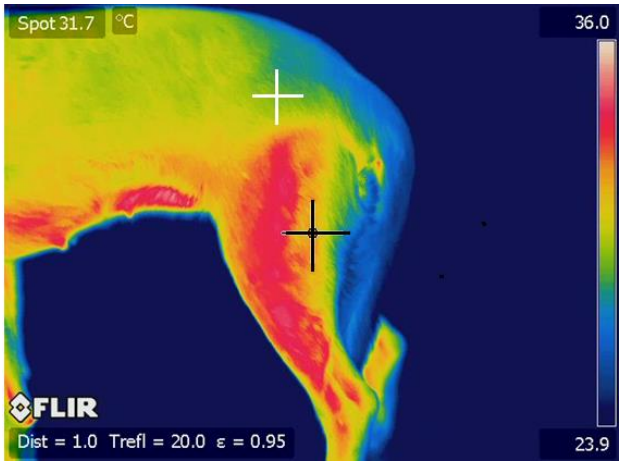

Figure 2. Cutaneous temperature of the regions of the superficial gluteal muscle (white spot) and biceps femoris muscle (black spot) of left hind limbs obtained with an infrared camera (image resolution=256×256 pixels, thermal sensitivity=0.07° C, distance from the assessed regions=1 meter, angle=90º).

The images were analyzed as spots in thermography software (Flir Systems v.1.2 software, FLIR Systems; Boston, USA). The same investigator carried out all thermographic examinations. The infrared thermography was performed at the following time points: 10 min before the WBV session, 2 min after starting, and 1 min after finishing with a frequency of 30 Hz; 2 min after starting and 1 min after finishing with a frequency of 40 Hz; 2 min after starting and 1 min after finishing with a frequency of 50 Hz.

Arterial Doppler ultrasound: Doppler ultrasonography of the common carotid and femoral arteries, on both the right and left sides, was performed using an ultrasound machine (MyLab™Five, Esaote Healthcare; São Paulo, Brazil) with a 13 MHz multi-frequency linear transducer. The Resistivity Index (RI) of the common carotid artery was assessed in the mid-cervical region with the dog placed in lateral recumbency. Spectral Doppler sampling was performed with angles between 52° and 60° in the longitudinal plane. The RI of the femoral artery was evaluated with the dog on the top of the vibrating platform (turned off). After the femoral artery was identified in the femoral triangle area, the transducer was placed in the transverse plane. In both common carotid and femoral arteries, ultrasound gel was used at the time of scanning. The RI was measured when three similar consecutive waveforms were obtained using triplex Doppler sonography. The ultrasound machine software calculated the RI automatically.

Doppler echocardiography: Doppler echocardiography assesses the end-systolic volume, end-diastolic volume, heart rate, aortic blood flow velocity, and pulmonary artery flow velocity. An ultrasound device (MyLab™Five, Esaote Healthcare, São Paulo, Brazil) mode Triplex Doppler with an 8 MHz convex transducer was used.

The first evaluation was done between the fourth and fifth right intercostal spaces with the dog positioned in left lateral recumbency. Then, the evaluation was performed between the sixth and seventh left intercostal spaces with the dog positioned in the right lateral recumbency. Before ultrasound scanning, the acoustic gel was applied. The Arterial Doppler ultrasound and Doppler echocardiography parameters were measured 10 min before the WBV session and 1 min after finishing the WBV session in the frequencies of 30 Hz, 40 Hz, and 50 Hz. All sonographic examinations were performed by a single experienced operator with the dogs on top of the vibrating platform and in the same room of WBC sessions.

***Clinical parameters*:** Clinical parameters included in the study were respiratory rate (RR), heart rate (HR), and body temperature (BT). All these parameters were measured with the dogs on top of the vibrating platform and assessed at 10-min before the WBV session, 1-min, 10 min, 30 min, and 60 min after the WBV session.

The RR was measured by counting the number of times the chest rose and falls, which combination counts were considered one breathes. The breaths were counted for 15 seconds and multiplied by four to get the respiratory rate in breaths per minute. The HR was measured using a veterinarian pulse oximeter (Prevtech®, São Paulo, Brazil) which connected to the left dog's ear. For the BT parameters, a digital thermometer (G-Tech®, Accumed Laboratory, São Paulo, Brazil) was inserted and placed in dog´s rectum mucosa.

Blood analysis: Complete blood count (CBC) and serum biochemical (ALT, creatinine, CK) parameters were evaluated 10 min before and 60 min after the end of the WBV session. All blood samples were collected using a 21-gauge needle on a 10-mL syringe with the dog standing on the vibrating platform (turned off). Each blood sample (4 mL) for CBC was placed in a 5-mL plastic tube containing lithium heparin, and a blood sample (3 mL) for serum biochemical analysis was placed in a 5-mL plastic serum separator tube and centrifuged. Red blood cell

(RBC) values were determined by using an electronic cell counter (Ebram 18 hemascreen, Ebram; São Paulo, Brazil), and the white blood cell (WBC) values were verified by using a Wright-stained blood smear and examined under an optical microscope (X100). Alanine aminotransferase and CK values were determined by using a commercial kit (Labtest; São Paulo, Brazil). Creatinine values were performed by applying a colorimetric method (Spectronic 21, Merck KGaA; Darmstadt, Germany).

Statistical analysis: Data normality was examined using the Shapiro-Wilk test. Friedman test was used to compare the values of infrared surface thermography, arterial Doppler ultrasonography, and echocardiography among the time points. Wilcox test compared clinical parameters, CBC values, and serum biochemical parameters between time points. Differences were considered significant at $P<0.05$.

Results

The dogs maintained the standing position during the WBV session. However, they tried to sit while the vibration frequency was changed from 30 to 50 Hz. Signs of gastrointestinal conditions were not detected during and after the WBV session. No statistical differences were identified in the cutaneous temperature of regions of the superficial gluteal muscle and biceps femoris muscle of both hind limbs among time points (Table 1) ($P>0.05$). On both the right and left sides, the RI values of the common carotid and femoral arteries did not show statistical variations among the time points (Table 2) ($P>0.05$).

The values of end-systolic volume, end-diastolic volume, heart rate, aortic blood flow velocity, and pulmonary artery flow velocity did not show statistical differences among the time points (Table 3) ($P>0.05$). No significant differences were found for clinical parameters (RR, HR, and BT) (Table 4), CBC values, and ALT, creatinine, and CK levels (Table 5) ($P>0.05$).

Table 1. Cutaneous temperature (º C) measured by infrared thermography (CT – IR) of regions of the superficial gluteal and biceps femoris muscles at different time-points (TP).

CT – IR (º C)	TP0 (Mean ± SD)	TP1 (Mean ± SD)	TP2 (Mean ± SD)	TP3 (Mean ± SD)	TP4 (Mean ± SD)	TP5 (Mean ± SD)	TP6 (Mean ± SD)	P value
SGM_{right}	32.37 ± 0.92	33.68 ± 1.31	33.50 ± 1.33	33.80 ± 1.59	33.69 ± 2.14	33.69 ± 2.14	33.36 ± 1.81	0.2189
SGM_{left}	32.32 ± 1.10	33.78 ± 1.30	33.38 ± 1.22	33.83 ± 1.50	33.90 ± 1.98	33.90 ± 1.98	33.30 ± 1.91	0.1372
BFM_{righ}	36.01 ± 0.50	36.17 ± 0.40	36.22 ± 0.50	36.23 ± 0.38	36.48 ± 0.77	36.48 ± 0.77	35.67 ± 0.92	0.1472
BFM_{left}	35.92 ± 0.65	35.93 ± 0.49	36.19 ± 0.44	36.20 ± 0.46	36.82 ± 0.87	36.82 ± 0.87	35.89 ± 0.99	0.1243

SGMright - Right superficial gluteal muscles, SGMleft – Left superficial gluteal muscles, BFMrigh – Right biceps femoris muscles, BFMleft – Left superficial biceps femoris muscles.TP0 - 10 min before WBV session; TP1 - 2 min after starting WBV (30 Hz); TP2 - 1 min after finishing WBV (30 Hz); TP3 - 2 min after starting WBV (40 Hz); TP4 - 1 min after finishing WBV (40 Hz); TP5 - 2 min after starting WBV (50 Hz); TP6 - 1 min after finishing WBV (50 Hz).

Table 2. Arterial Doppler ultrasound values at different time-points (TP).

RI	TP0 (Mean ± SD)	TP1 (Mean ± SD)	TP2 (Mean ± SD)	TP3 (Mean ± SD)	P value
CA_{right}	0.87 ± 0.04	0.86 ± 0.05	0.85 ± 0.06	0.85 ± 0.07	0.2108
CA_{left}	0.87 ± 0.04	0.87 ± 0.05	0.86 ± 0.07	0.86 ± 0.05	0.6489
FA_{right}	0.94 ± 0.02	0.92 ± 0.02	0.92 ± 0.05	0.92 ± 0.03	0.1865
FA_{left}	0.94 ± 0.01	0.90 ± 0.07	0.93 ± 0.03	0.90 ± 0.02	0.1928

RI - Resistive index, CAright – Right common carotid artery, CAleft – Left common carotid artery, FAright – Right femoral artery, FAleft – Left femoral artery. TP0 - 10 min before WBV session; TP1 - 1 min after finishing WBV (30 Hz); TP2 - 1 min after finishing WBV (40 Hz); TP3 - 1 min after finishing WBV (50 Hz). Reference values - RI of common carotid artery: 0.83 – 0.91; RI of common femoral artery: 0.92 – 0.96.

Table 3. Doppler echocardiography values at different time-points (TP).

Parameters	TP0 (Mean ± SD)	TP1 (Mean ± SD)	TP2 (Mean ± SD)	TP4 (Mean ± SD)	P value
ESV (mL)	17.22 ± 16.98	25.62 ± 19.39	45.28 ± 56.88	45.94 ± 59.62	0.2590
EDV (mL)	53.00 ± 28.85	68.05 ± 36.48	81.96 ± 57.29	79.69 ± 61.72	0.3420
HR (bpm)	117.44 ± 18.56	115.22 ± 15.67	127.22 ± 23.78	113.44 ± 14.49	0.0858
AoV (cm/sec)	146.33 ± 36.92	147.00 ± 44.37	146.44 ± 31.80	148.22 ± 33.57	0.9816
PulmV (cm/sec)	124.22 ± 33.03	110.11 ± 29.91	106.00 ± 43.69	109.00 ± 37.99	0.2185

ESV - end-systolic volume; EDV - end-diastolic volume; HR - heart rate; AoV - aortic blood flow velocity; PulmV - pulmonary artery flow velocity. TP0 - 10 min before WBV session; TP1 - 1 min after finishing WBV (30 Hz); TP2 - 1 min after finishing WBV (40 Hz); TP3 - 1 min after finishing WBV (50 Hz). reference values – ESV: 0.22 – 34.22 mL; EDV: 24.00 – 82.00 mL; HR: 60 – 160 bpm; AoV: 109.00 – 183.00 cm/sec; PulmV: 91 – 157.00 cm/sec.

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Table 4. Clinical parameters at different time-points (TP).

Parameters	TP0 (Mean ± SD)	TP1 (Mean ± SD)	TP2 (Mean ± SD)	TP3 (Mean ± SD)	TP4 (Mean ± SD)	P value
RR (mpm)	19.7 ± 2.1	23.7 ± 1.4	24.8 ± 1.3	18.9 ± 1.8	17.6 ± 2.3	0.120
HR (bpm)	100.4 ± 21.2	110.9 ± 21.6	106.9 ±19.1	91.9 ± 11.4	90.4 ± 20.1	0.222
BT (º C)	38.5 ± 0.4	38.8 ± 0.3	38.7 ± 0.6	38.4 ± 1.1	38.6 ± 1.12	0.066

RR – Respiratory rate; CK; HR – Heart rate; BT – Body temperature. TP0 - 10 min before WBV session, TP1 – 1- min after a WBV session, TP2 – 10 min after WBV session, TP3 - 30 min after WBV session, TP4 - 60 min after WBV session. Reference values – RR - [18 – 36 movements per minute (mpm)]; HR - [60 – 160 beats per minute (bpm)]; BT: 37.5 – 39.2º C.

Table 5. Complete blood count and serum biochemistry values at different time-points (TP).

Parameters	TP0 (Mean ± SD)	TP1 (Mean ± SD)	P value
Erythrocytes (x10^6/µ)	7.42 ± 0.36	7.16 ± 0.29	0.1225
Hemoglobin (g/dL)	16.98 ± 0.99	16.21 ± 0.86	0.1156
Hematocrit (%)	50.00 ± 2.74	48.44 ± 1.13	0.1206
Total protein (g/dL)	7.08 ± 0.60	7.12 ± 0.32	0.1071
Leukocytes (x10^3/µL)	10.27 ± 0.98	10.36 ± 0.24	0.9052
Neutrophils (x10^3/µL)	7.12 ± 0.15	7.53 ± 0.23	0.5519
Eosinophils (x10^3/µL)	0.92 ± 0.44	1.03 ± 0.13	0.6341
Lymphocytes (x10^3/µL)	1.66 ± 0.59	1.46 ± 0.80	0.4753
Monocytes (x10^3/µL)	0.56 ± 0.41	0.31 ± 0.12	0.3411
ALT (UI/L)	43.78 ± 4.97	37.33 ± 6.33	0.2340
Creatinine (mg/dL)	1.13 ± 0.21	1.10 ± 0.13	0.3398
CK (UI/L)	159.33 ± 111.46	188.778 ± 106.99	0.9050

ALT - Alanina aminotransferase; CK - Creatine phosphokinase. TP0 - 5 min before WBV session, TP1 - 60 min after a WBV session. Reference values - Erythrocytes: 5.60 – 8.70 x106/µ; Hemoglobin: 14.0 – 20.0 g/dL; Hematocrit: 40.0 – 59.0%; Total protein: 5.40 – 7.10 g/dL; Leukocytes: 6.00 – 17.00 x10^3/µL; Eosinophils: 0.00 – 1.90 x10^3/µL; Lymphocytes: 0.53 – 4.80 x10^3/µL; Monocytes: 0.10 – 1.80 x10^3/µL; ALT: 10.00 – 88.00 UI/L; Creatinine: 0.50 – 1.50 mg/dL; CK: 20.00 – 200.00 UI/L.

Discussion and Conclusion

The present study evaluated infrared thermography, arterial Doppler ultrasound, and Doppler echocardiography in healthy adult dogs exposed to a single whole-body vibration (WBV) at different frequencies. Recently, vibrating platforms that produce mechanical vibrations and spread throughout the whole body have been researched for various purposes, including rehabilitation and exercise in small animals (1, 6, 10, 16-19, 23). The hypothesis was not confirmed in this study, as all parameters evaluated did not change despite the increasing vibration frequencies of WBV in a short time. The dogs showed good adaptation to the vibrating platform, as verified in other studies using the same equipment to assess short- and long-term responses (6, 17). However, changes in the dogs' behavior due to an increase in vibration frequency were also described in other studies. These changes were attributed to the pressure exerted on the paws (18) or possible paraesthesia (23). In a study involving healthy adult humans, a reduction in touch-pressure sensitivity of the feet was noted 10 minutes after WBV exposure (20).

No signs of gastrointestinal conditions were identified in the dogs in the present study. This finding is consistent with a study involving adult and elderly dogs exposed to 30 Hz and 50 Hz in a single WBV session (18). Considering that the suppression of gastric smooth muscle activity and contractions has been observed in humans exposed to short-term WBV (9), it is essential to evaluate the influence of food intake during WBV in dogs.

Although infrared thermography is considered a non-invasive technology used in sports and exercise science in humans, the analysis can be affected by the selection of regions of interest in anatomical areas (11). In the present study, infrared thermography of both the superficial gluteus muscle and biceps femoris muscle did not show changes in mean cutaneous temperature values during the evaluation time points. This indicates that the tested vibration frequencies in a single session were not sufficient to promote significant superficial vascular changes in these muscle regions. Notably, healthy Beagle dogs exposed to WBV sessions for five days, despite differences in vibration frequencies, also did not exhibit thermographic changes in the biceps femoris and vastus

lateralis muscles (17). In contrast, different results were observed in Beagle dogs subjected to high-speed physical exercise on a treadmill, which showed an increase in surface temperature, with the thigh being one of the most influenced sites (21). On the other hand, a decrease in lower limb temperature was found in humans during 15 minutes of WBV exposure at frequencies of 31, 35, 40, and 44 Hz, which was attributed to vasoconstriction (20). As reported by these authors, although WBV exercise has been compared to aerobic exercises, the responses to the same variables can be different.

In this study, no differences were detected in the Resistance Index (RI) of the femoral and common carotid arteries at frequencies of 30 Hz, 40 Hz, and 50 Hz ($P>0.05$). A study conducted in healthy dogs subjected to a single WBV session, using frequencies of 30 Hz, 50 Hz, and 30 Hz with exposures of five minutes each, also demonstrated no changes in renal RI (6). In contrast, healthy dogs exposed to the same frequencies and duration of WBV, as reported by Freire et al. (6), exhibited an increase in RI of the femoral artery from the second to the fifth day, suggesting reduced blood flow in the region of the quadriceps femoris muscles (17).

The absence of variations in end-systolic and end-diastolic volumes, heart rate, aortic blood flow velocity, and pulmonary artery flow velocity can be considered strong indicators that the vibration frequencies used in the present study were safe. Heart rate, systolic blood pressure, and ambulatory electrocardiography evaluated in neutered young and aged dogs were not modified at frequencies of 30 Hz (5 min), 50 Hz (5 min), and 30 Hz (5 min) during 15 minutes of WBV (19). Additionally, systolic blood pressure and mean heart rate did not show changes in healthy humans exposed to 30 minutes of vibration at 60 Hz (2). In contrast, healthy humans submitted to exercise until exhaustion on a vibration platform exhibited elevated heart rate and increased systolic blood pressure; however, these effects were considered mild and returned to normal within 15 minutes of recovery (14).

No significant variations ($P>0.05$) were identified regarding the values of clinical parameters, including RR, HR, and BT, which is similar to a study conducted in healthy younger and older adult male non-athletic dogs (19) and human patients (2). Nevertheless, it was observed that WBV encouraged increases in RR, HR, and BT after the WBV session. On the other hand, a study that used long-term WBV demonstrated an increase in HR of up to 30% after the WBV session (14). This suggests that short-term WBV does not induce significant changes in cardiac function. However, it would be important to evaluate cardiac troponin I (cTnI) to identify possible deleterious effects on the myocardium. In the present study, cTnI was not evaluated due to the absence of significant variations in healthy dogs (both younger and adult) who used the same vibrating platform and short-term WBV protocols as those in the present study (19).

The CBC values did not show differences over time ($P>0.05$). However, a study with healthy Beagles subjected to WBV for five days showed a decrease in erythrocyte and hemoglobin values within the reference intervals. This decrease was attributed to hemolysis due to the action of mechanical vibration. In the same study, an increase in leukocyte, neutrophil, and eosinophil values was identified in these animals, which was associated with the release of epinephrine and norepinephrine as a result of acute stress (16).

Regarding serum biochemical values (ALT, creatinine, and CK), differences over time were also not detected ($P>0.05$). However, a study in humans during an ultramarathon race showed an increase in CK values, which was used as a marker of muscle injury (7, 8, 24). As previously discussed, the responses to WBV as exercise and traditional aerobic exercises may not be the same (20). This data is important since the use of WBV in dogs may help prevent severe muscle damage.

Lactate dehydrogenase and aspartate aminotransferase are important parameters when evaluating muscle functions in animals and human patients. However, the present study did not evaluate these parameters due to budget limitations. Therefore, it is important to include these parameters in future studies.

In conclusion, the protocol of increasing vibration frequencies (30, 40, and 50 Hz) in short-duration WBV, using a vibrating platform that delivered a vortex wave circulation, can be considered appropriate since no changes occurred in the evaluated parameters.

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Ethical Statement

This study was approved by the Institutional Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (Unesp), Botucatu, Brazil (nº. 0120/2018-CEUA).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Batista MAB, Wallerstein LF, Dias RM, et al** (2007): *Effects of training with vibratory platforms*. Rev Bras Cienc Mov, **15**, 103-113.
2. **Button C, Anderson N, Bradford C, et al** (2007): *The effect of multidirectional mechanical vibration on peripheral circulation of humans.* Clin Physiol Funct Imaging, **27**, 211-216.
3. **Cardinale M, Wakeling J** (2005): *Whole body vibration exercise: are vibrations good for you?* Br J Sports Med, **39**, 585-589.
4. **Cochrane DJ** (2011): *Vibration exercise: the potential benefits.* Int J Sports Med, **32**, 75-99.
5. **Dolny DG, Reyes GF** (2008): *Whole-body vibration exercise: training and benefits.* Curr Sports Med Rep, **7**, 152-157.
6. **Freire LF, Rahal SC, Santos IFC, et al** (2015): *Renal resistive index of adult healthy dogs submitted to short-term Whole-body vibration exercise*. Asian J Anim Vet Adv, **10**, 797-802.
7. **Kaneko JJ, Harvey JW, Bruss ML** (2008): Clinical Biochemistry of Domestic Animals. 6th ed. Academic Press, New York.
8. **Kim HJ, Lee YH, Kim CK** (2007): *Biomarkers of muscle and cartilage damage and inflammation during a 200 km run*. Eur J Appl Physiol, **99**, 443-447.
9. **Miyazaki Y** (2000): *Adverse effects of whole-body vibration on gastric motility.* Kurume Med J, **47**, 79-86.
10. **Nagai LR, Rahal SC, Dadalto C, et al** (2020): *Acute effects of Whole-body vibration on dopplerfluxometry of the common carotid artery parameters among adult and elderly nonathletes healthy dogs.* Acta Sci Vet, **48**, e1764.
11. **Perpetuini D, Formenti D, Cardone D, et al** (2021): *Regions of interest selection and thermal imaging data analysis in sports and exercise science: a narrative review.* Physiol Meas, **42**, 1088–1098.
12. **Rauch F** (2009): *Vibration therapy*. Dev Med Child Neurol, **51**, 166-168.
13. **Rauch F, Sievanen H, Boonen S, et al** (2010): *Reporting Whole-body vibration intervention studies: recommendations of the International Society of Musculoskeletal and Neuronal Interactions.* J Musculoskelet Neuronal Interact, **10**, 193-198.
14. **Rittweger J, Beller G, Felsenberg D** (2000): *Acute physiological effects of exhaustive Whole-body vibration exercise in man.* Clin Physiol, **20**, 134-142.
15. **Rittweger J** (2010): *Vibration as an exercise modality: how it may work, and what its potential might be*. Eur J Appl Physiol, **108**, 877-904.
16. **Santos IFC, Rahal SC, Shimono J, et al** (2017): *Whole-Body Vibration exercise on hematology and serum biochemistry in healthy dogs*. Top Companion Anim Med, **32**, 86-90.
17. **Santos I, Rahal S, Santos A, et al** (2019): *Whole-body vibration on leg muscles thermography and femoral resistive index of in adult healthy dogs*. Res Vet Sci, **122**, 118–123.
18. **Silva BM, Santos IFC, Rahal SC, et al** (2020): *Effects of Whole-body vibration in hematobiochemical and hemogasometric parameters in adult and elderly healthy dogs.* Acta Sci Vet, **48**, e.1777.
19. **Silva BM, Rahal SC, Filippi MG, et al** (2022): *Short-term effects of Whole-Body Vibration on clinical, cardiac, and ambulatory electrocardiographic (Holter) parameters of healthy younger and older adult male non-athletic crossbreed*. Arq Bras Med Vet Zootec, **74**, 33-42.
20. **Sonza A, Robinson CC, Achaval M, et al** (2015): *Whole-body vibration at different exposure frequencies: infrared thermography and physiological effects*. Sci World J, **33**, e452657.
21. **Soroko M, Górniak W, Howell K, et al** (2021): *Changes in body surface temperature associated with high-speed treadmill exercise in beagle dogs measured by infrared thermography*. Animals, **11**, e2982.
22. **Stania M, Juras G, Słomka K, et al** (2016): *The application of Whole-body vibration in physiotherapy - A narrative review.* Physiol Int, **103**, 133-145.
23. **Tannus FCI, Rahal SC, Oba E, et al** (2021): *Serum cortisol and clinical response to a single session of Whole-body vibration in healthy adult dogs*. Front Vet Sci, **8**, e563898.
24. **Thrall MA, Weiser G, Allison RW, et al** (2021): Veterinary hematology and clinical chemistry. 2nd ed. Willey Blackwell, Philadelphia.

Outcomes of oxytocin treatment on intestinal ischemia-reperfusion injury in rats

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ABSTRACT

Ischemia-reperfusion injury is a clinical condition that poses life-threatening risks and can be caused by diseases or operations such as trauma, shock, and gastric dilatation volvulus. The objective of this study was to examine the effect of oxytocin on intestinal damage in rats induced by experimental ischemia-reperfusion injury. Three groups of Wistar albino rats were established: a control group (CTR, n=6), an intestinal ischemia-reperfusion group (I-IR, n=6), and an intestinal ischemia-reperfusion with oxytocin group (I-IR+Oxt, n=6). The I-IR+Oxt group received an intraperitoneal injection of 1 mg/kg oxytocin 30 minutes before anesthesia. In the I-IR and I-IR+Oxt groups, the superior mesenteric artery was ligated for 1 hour to induce ischemia-reperfusion injury, followed by one hour of reperfusion by opening the ligatures. At the end of the reperfusion period, the rats were euthanized, and blood and intestinal tissue samples were collected. From the blood samples, ALT, ALP, AST, LDH, BUN, creatinine, IL-1β, and TNF-α concentrations were evaluated. Tissue samples were analyzed for IL-1β, TNF-α, and MDA activity. Serum and tissue IL-1β and TNF-α concentrations were higher in both the I-IR and I-IR+Oxt groups compared to the CTR group. However, these levels were found to be lower in the I-IR+Oxt group compared to the I-IR group. The histopathological analysis showed that the I-IR+Oxt group had better epithelial regeneration and less inflammatory cell infiltration compared to the I-I/R group. In conclusion, oxytocin inhibited the release of IL-1β and TNF-α and the harmful effect of I/R on intestinal cells.

Introduction

Numerous diseases and operations can result in intestinal ischemia-reperfusion (I-I/R) injury, including neonatal necrotizing enterocolitis, mesenteric ischemia, embolism or volvulus, trauma, shock, aortic aneurysm repair, and rejection of intestinal transplantation in human medicine (13, 18). In veterinary medicine, ischemic reperfusion injury is observed in cases such as gastric dilatation-volvulus (GDV), mesenteric torsion, intestinal incarceration, and colic in the gastrointestinal system or abdominal compartment syndrome (11, 15, 17). Intestinal epithelial cells are susceptible to the effects of ischemia, and correcting the impact of ischemia is essential for restoring blood flow and preserving the structure of single-layer epithelial cells (8).

The nonapeptide neurohypophysial hormone oxytocin (Oxt), produced by the hypothalamic supraoptic and paraventricular nuclei, binds to the Oxt receptor. The effects of Oxt on various vascular beds can act as either vasoconstrictors or vasodilators, which stimulate uterine contractions during sexual activity and labor as well as myoepithelial contractions in the mammary gland during lactation (5). In experimentally induced duodenal and gastric ulcers, Oxt exerts an antiulcer effect primarily due to its moderate anti-secretory activity (2). Additionally, Oxt has been reported to protect the colon from acetic acid-induced and stress-induced colitis (7). And has also been shown that the release of Oxt decreases macrophage activity, tumor necrosis factor-alpha (TNF-α), and interleukin-1beta (IL-1β) levels (20). The rats, due to their

structural similarities, are often used in experimental-based modeling of diseases and metabolic or inflammatory reactions that may also occur in humans and other mammals. For this reason, we selected rats as our animal of choice for our study on I-I/R injury. Additionally, recently there have been numerous studies in both veterinary and human medicine to test different therapeutic strategies for preventing or treating ischemia-reperfusion injury. Therefore, in this study, it was investigated whether Oxt administration has an anti-inflammatory and protective effect against experimentally induced I-I/RI.

Materials and Methods

Experimental Protocol and Animals: The local animal ethics committee approved our study protocol (2021-130). Eighteen outbred male Wistar albino rats, weighing between 200 and 250 g, were used in this investigation. All rats were housed in a room with a regulated temperature (22±2°C), humidity (60%), and a 12-hour light/dark cycle. Rats had unrestricted access to water and food pellets. Rats were allocated into three groups: control group (CTR, n=6), intestinal ischemia-reperfusion group (I-I/R, n=6), and intestinal ischemia-reperfusion+ Oxytocin group (I-I/R+Oxt, n=6). In I-I/R+Oxt groups, 1 mg/kg of Oxt was given intraperitoneally 30 minutes before the anesthesia (9). All groups were fixed in the supine position after receiving intraperitoneal injections of a combination of Xylazine (2% Vetaxyl 20 mg, Vetagro ®) and Ketamine (2% Ketamine, Dutchfarm®, 100 mg/kg) to induce anesthesia. A ventral midline incision was used to access the abdominal cavity. The abdominal cavities of the rats in the CTR group were opened, but no interventions or applications were applied. By ligating the superior mesenteric artery for 1 hour, ischemia was established in the I-I/R and I-I/R+Oxt groups, and the small intestine color alterations were noticed. The intestines were reperfused for one hour by opening the ligature (21). The rats were euthanized using an overdose of Xylazine+Ketamine anesthesia at the end of the reperfusion periods. The small intestines were excised, and blood samples were collected from all groups.

Sampling: Serum was isolated following complete coagulation of blood samples taken from rats and placed into serum separator tubes. Fresh intestinal tissue samples were initially homogenized, and the homogenates were centrifuged at +4ºC 10000 g x 10 minutes, after which the supernatant was collected.

Blood analysis: Alanine aminotransferase (ALT, U/L), alkaline phosphatase (ALP, U/L), aspartate aminotransferase (AST, U/L), lactate dehydrogenase (LDH, U/L) enzyme activities, and blood urea nitrogen (BUN, mg/dL) and creatinine (Crea, mg/dL) levels were detected in sera using commercial clinical chemistry assay kits.

Rat-specific enzyme immunoassay kits were assayed to measure concentrations of IL-1β (pg/mL) and TNF-α (pg/mL) in sera and tissue homogenizates (Rat TNF-α ELISA Kit Catalog No: E-EL-R0019; Rat IL-1β Catalog No: E-EL-R001, Elabscience Biotechnology Inc., TX, USA). To assess lipid peroxidation levels, malondialdehyde (MDA) levels were evaluated in intestinal tissue homogenizates using commercially available test kits (TBARS Assay Kit, Item No. 10009055, Batch No. 0510196 and 0502129, Cayman Chemicals, Michigan, USA) (19). Tissue TNF- α, IL-1β, and MDA concentrations were detected performing Coomassie brilliant blue method (3).

Histopathological analysis: Intestine samples were fixed in a formaldehyde solution. Tissues were routinely treated with alcohol and toluene, fixed to paraffin blocks, and stained with hematoxylin-eosin. The light microscope's bright field mode (Zeiss-Axio Scope A1, Carl Zeiss, Gottingen, Germany) was used to examine histopathological sections. Each criterion was graded using a semi-quantitative system as 0: no, 1: mild, 2: moderate, and 3: severe.

Statistical analysis: GraphPad Prism 9 was used for the statistical analysis (GraphPad Software, San Diego, CA, USA). Means (±1 SD) were used to describe all results. The Shapiro-Wilk test was used to evaluate the statistically normal distribution of data. IL-1β and TNF-α levels in intestines and serum were compared using a one-way analysis of variance (ANOVA). Further analysis for binary comparisons was conducted using Tukey's test. P values less than 0.05 were considered significant.

Results

Serum ALT, AST, ALP, LDH, BUN, and Crea levels were examined in rats' experimentally developed I-I/R model to ascertain the effects of ischemia-reperfusion injury (Table 1). While there were significant increases in ALT, AST, ALP, and LDH activities, Crea, and BUN levels in the I-I/R group compared to the CTR group ($P<0.001$ and $P<0.01$). The increased values were less in the I-I/R+Oxt group compared to the I-I/R group.

In the blood samples, IL-1β and TNF-α levels as cytokines were evaluated in measuring the response to ischemia-reperfusion injury (Table 1). IL-1β ($P<0.01$) and TNF-α ($P<0.001$) levels were significantly higher in the I-I/R group compared to the CTR group. However, it was observed that IL-1β and TNF-α levels were significantly reduced in the I-I/R+Oxt group in comparison to the I-I/R group, with $P<0.05$ and $P<0.01$, respectively. Similarly, it

was determined that IL-1β and TNF-α levels obtained from tissue samples were significantly higher in the I-IR group compared to the CTR group, respectively P<0.0001 and P<0.5. Alternatively, IL-1β and TNF-α levels were found to be lower in the I-IR+Oxt group compared to the I-IR group, respectively P<0.05 and P<0.0001 (Figure 1 a, 1b).

Lipid peroxidation and oxidative tissue damage were assessed in the tissue samples using MDA analysis (Figure 1c). Comparing the I-I/R group to the CTR group, MDA levels were much higher in the I-I/R group (P<0.01). However, it was observed that MDA levels decreased in the I-I/R+Oxt group compared to the I-I/R group (P<0.001).

According to histopathological examination and semi-quantitative scoring system, we observe that the CTR group had a regular epithelial and glandular structure, and almost no inflammatory cell infiltration (Figure 2a, Table 2). However, epithelial and glandular structures in the I-I/R group had a large number of inflammatory cells, and destructive morphological degeneration (Figure 2b, Table 2). Alternatively, in the I-I/R+Oxt group, it was observed that epithelial regeneration of both epithelium and glands structure and decreased inflammatory cell infiltration compared to the I-I/R group (Figure 2c, Table 2).

Table 1. Plasma ALP, ALT, AST, LDH, BUN, Creatinine, IL-1β and TNF-α activities in the control (CTR), intestinal ischemia/reperfusion (I-I/R) and intestinal ischemia/reperfusion +Oxytocin (I-I/R+Oxt) groups.

Parameters (n=6)	CTR	I-I/R	I-I/R+Oxt
ALP (U/L)	66.6 ± 12.4	143.3 ± 4.2 ****	100.4 ± 9.9 $^{+}$
ALT (U/L)	40.1 ± 5.79	1375 ± 281 ****	105.1 ± 20.9 $^{+++}$
AST (U/L)	99.1 ± 14.1	2692 ± 542 ****	192.7 ± 24.9 $^{++++}$
LDH (U/L)	1532 ± 223	7131 ± 1021 ****	1832 ± 157 $^{++++}$
BUN (mg/dL)	34.1 ± 3.5	81.5 ± 5.1 ****	58.4 ± 5.5 **$^{, ++}$
Creatinine (mg/dL)	0.47 ± 0.05	0.95 ± 0.06 ***	0.54 ± 0.09 $^{++}$
IL-1β (pg/mL)	391.3 ± 26.8	830.9 ± 30.2****	679.8 ± 21****$^{,++}$
TNF-α (pg/mL)	260.6 ± 30.9	553.4 ± 40.5****	371 ± 37.6^{++}

** P<0.01; *** P<0.001, **** P<0.0001 compared with the control (CTR) group. + P < 0.05, ++ P<0.01, +++ P<0.001, ++++ P<0.0001 compared with the I-I/R group.

Table 2. Histopathological scoring results of control (CTR), intestinal ischemia/reperfusion (I-I/R), and intestinal ischemia/reperfusion +Oxytocin (I-I/R+Oxt) groups.

Parameters (n=6)	CTR	I-I/R	I-I/R+ Oxt
Desquamation of villus tip	0.43 ± 0.07	2.76 ± 0.07****	1.63 ± 0.03$^{****,++++}$
Hyperplasia of intestinal glands	0.36 ± 0.06	2.48 ± 0.09****	1.55 ± 0.07^{++++}
Inflammatory cell infiltration	0.48 ± 0.04	2.53 ± 0.07****	1.67 ± 0.07^{++++}

Each of the criteria was scored semi-quantitatively as 0: none, 1: mild, 2: moderate, 3: severe. **** P<0.0001 compared with the control (CTR) group. ++++ P<0.0001 compared with the I-I/R group.

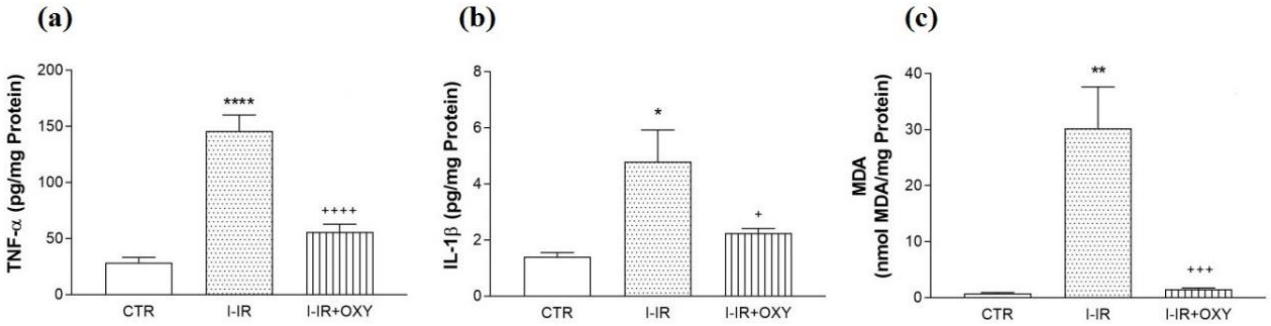

Figure 1. Intestinal tissue TNF-α (A), IL-1β (B), and MDA (C) activities in the control (CTR), intestinal ischemia/reperfusion (I-I/R), and intestinal ischemia/reperfusion +Oxytocin (I-I/R+Oxt) groups.*P<0.05, ** P<0.01; *** P<0.001, **** P<0.0001 compared with the control (CTR) group.+ P< 0.05, ++ P<0.01, +++ P<0.001, ++++ P<0.0001 compared with the I-I/R group.

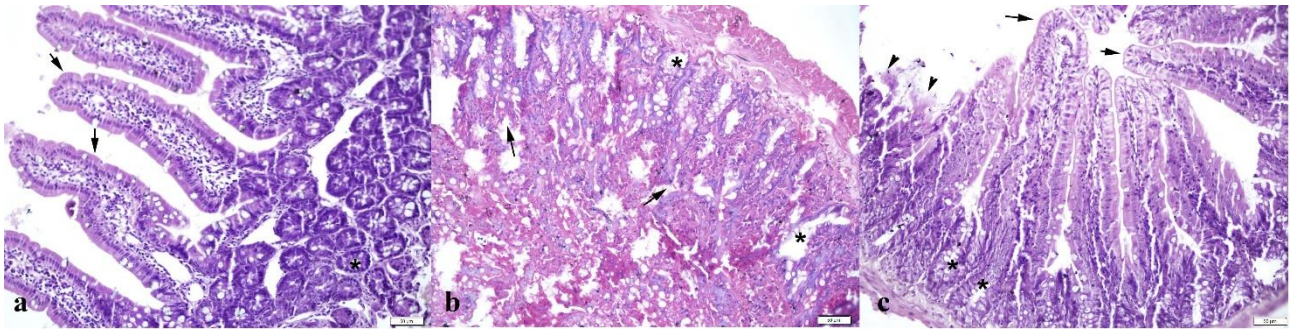

Figure 2. (a) Regular layout of epithelium (arrows) and gland (*) in the control group, (b) Severe desquamation of epithelium (arrows) and degeneration of glands (*) note the congestion of interstitium in the intestinal ischemia/reperfusion group, (c) Regeneration of epithelium (arrows) and renewal of intestinal glands (*) in the intestinal ischemia/reperfusion +Oxytocin group, inset (H&Ex200, 50μm).

Discussion and Conclusion

The primary function of the intestines is the digestion and absorption of nutrients, the removal of waste products, elimination of bacterial formations and toxins. One of the most important structures that enable it to perform these functions is the mucosal barrier of the intestine. After I-I/RI, the integrity of the mucosal barrier is impaired, and bacterial toxins pass into the systemic circulation, causing the release of cytokines with the inflammatory response that develops in the intestinal mucosa. At the same time, tissue damage occurs due to toxins, bacteria, and released cytokines in the systemic circulation (18). To defend against the adverse effects of ischemia-reperfusion injury or to reduce or prevent the production of components that contribute to the damage, numerous strategies are being developed (6, 27, 30). We investigated the effects of Oxt, an effective agent in ischemia-reperfusion injury, on important intestinal pro-inflammatory cytokines IL-1β and TNF-α, and thus its preservation impact on intestinal tissues in the experimentally induced I-I/R injury model in rats.

I-I/R injury is a complex pathophysiological cascade that includes the destruction of intestinal epithelial cells and enterocytes with high oxygen activity due to oxygen deficiency, causing the release of IL-1 and TNF-α, stimulation of inflammatory cells and the disruption of the mucosal barrier, leading to bacterial endotoxins entering systemic circulation (12). IL-1β and TNF-α, produced in the intestinal mucosal inflammatory cells and mediate the aforementioned metabolic changes, which cause local cell damage and trigger systemic inflammation in the early inflammatory response after I-I/R (1, 14). In this study, IL-1β and TNF-α values were found to be higher in I-I/R ($P<0.001$ and $P<0.01$) and I-I/R+Oxt ($P<0.01$ and $P<0.05$) groups, who underwent 1-hour ischemia and 1-hour reperfusion, compared to the CTR group. Histopathologically, villous desquamation, glandular and epithelial damage, and infiltration of the inflammatory cell scores were higher in the I-I/R and I-I/R+Oxt groups in comparison to the CTR group. Conversely, both histopathological findings and biochemical values were lower in the I-I/R+Oxt group compared to the I-I/R group. According to these results, a one-hour ischemia-reperfusion injury caused an increase in IL-1β and TNF-α levels, destructive morphological changes, and inflammatory cell infiltration of the intestine. In contrast, the low values in the Oxt administered group indicated that Oxt protected the intestinal mucosa through the reduction of inflammatory cell infiltration and suppressing pro-inflammatory cytokines IL-1β and TNF-α.

I-I/R injury not only causes anatomical deformity and local inflammatory response in the intestine (1). Alterations in ALP, ALT, AST, LDH, BUN, and Crea levels are used to determine the degree of tissue damage (4, 28). However, the effects of Oxt on both kidney damage in hepatic ischemia-reperfusion injury and hepatic damage in kidney ischemia-reperfusion injury have previously been investigated. In these studies, a significant decrease in ALT, AST, LDH, BUN, and Crea levels were found using Oxt. Similarly, in the same studies, pro-inflammatory cytokine values were established to be low Oxt treated groups (10, 23, 29). In this study, ALP, ALT, AST, LDH, BUN, and Crea levels were also measured to determine tissue damage. While ALP, ALT, AST, LDH, BUN, and Crea enzyme activities were significantly increased in both I-I/R and I-I/R+Oxt groups compared to the CTR group, it was determined that Oxt administration caused a remarkable reduction in these values. Studies indicate that these parameters, which are frequently used in the determination of clinical ischemia-reperfusion damage, are more specific in the more advanced stages of the disease, and it has been stated that the increase in serum values is due to the inflammatory response (16, 22). An I-I/R injury study conducted by Alexandropoulos et al. (1) concluded that the levels of pro-inflammatory cytokines increase in the ischemia-reperfusion and cause damage to the tissue (1). In this study, IL-1β and TNF-α levels addressed that the damage in the intestine is due to

the formation of cytokines and concur with the findings of the mentioned studies. Therefore, we have shown that Oxt administration reduces the effects of ischemia-reperfusion injury by decreasing enzyme activity and reducing cytokine levels, which are both organ damage markers.

Any tissue damage, such as ischemia-reperfusion, is detected by macrophages and monocytes, causing the release of cytokines such as IL-1β and TNF-α. Cytokines activate inflammatory cells that trigger the peroxidation of membrane lipids. Lipid peroxidation affects the permeability of cell membranes, which ultimately results in cell lysis (26). MDA, a marker of lipid peroxidation, is widely used because of its sensitivity and reliability. Previous studies have established that MDA is a good marker of oxidative damage to lipids (24, 25). MDA, IL-1β, and TNF-α results obtained from intestinal tissue in the study show that the values of the I-IR+Oxt group are lower than the I-IR group. Both cytokine and MDA levels obtained in blood or tissue samples and histopathological examinations show that oxytocin is a protective agent against cytokines and reactive oxygen radicals that cause destructive effects in ischemia-reperfusion injury of the intestinal tissue.

In conclusion, local and systemic inflammatory responses that develop with I-I/R injury depend on the early diagnosis of the developing problem together with the success of the medical and/or operative treatment used to alleviate or eliminate the problem. A method that can be used as a medical treatment together with early diagnosis is Oxt. The efficacy of Oxt has previously been demonstrated in models of liver and kidney ischemia-reperfusion injury or stress colitis and colonic burn, but its efficacy has yet to be directly investigated with the experimentally established intestinal ischemia-reperfusion model. In this sense, our study was an initial design to establish the effect of Oxt on pro-inflammatory cytokines in I-I/R injury and its protective or preventive effect on damage after ischemia-perfusion. However, all aspects of biochemical pathways need to be investigated to clarify the full impact of Oxt in I-I/R injury, and future studies should be focused on this direction.

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Ethical Statement

The local animal ethics committee approved the study protocol (2021-130).

Conflict of Interest

The authors declare that there is no conflict of interest.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Author Contributions

ÇG, AÖŞ, and SS provided study conception and design. ÇG and AÖŞ performed experiments. SS, ŞÇ, and AÖŞ analyzed data. ÇG, AÖŞ, and SS interpreted the results and wrote the manuscript.

References

1. **Alexandropoulos D, Bazigos GV, Doulamis IP, et al** (2017): *Protective effects of N-acetylcysteine and atorvastatin against renal and hepatic injury in a rat model of intestinal ischemia-reperfusion*. Biomed Pharmacother, **89**, 673-680.
2. **Asad M, Shewade DG, Koumaravelou K, et al** (2001): *Gastric antisecretory and antiulcer activity of oxytocin in rats and guinea pigs*. Life Sci, **70**, 17–24.
3. **Bradford MM** (1976): *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, **72**, 248-254.
4. **Braun JP, Lefebvre HP** (2008): Kidney function and damage. 485-529. In: JJ Kaneko, JW Harvey, ML Bruss (Eds.), Clinical Biochemistry of Domestic Animals. Elsevier Academic Press, Burlington.
5. **Carter CS, Kenkel WM, MacLean EL, et al** (2020): *Is oxytocin "nature's medicine"?* Pharmacol Rev, **72**, 829-861.
6. **Cassutto BH, Gfeller RW** (2003): *Use of intravenous lidocaine to prevent reperfusion injury and subsequent multiple organ dysfunction syndrome*. J Vet Emerg Crit Care, **13**, 137-148.
7. **Çetinel Ş, Hancıoğlu S, Şener E, et al** (2010): *Oxytocin treatment alleviates stress-aggravated colitis by a receptor-dependent mechanism*. Regul Pept, **160**, 146-152.
8. **Deng F, Lin ZB, Sun QS, et al** (2022): *The role of intestinal microbiota and its metabolites in intestinal and extraintestinal organ injury induced by intestinal ischemia-reperfusion injury*. Int J Biol Sci, **18**, 3981-3992.
9. **Düşünceli F, İşeri SÖ, Ercan F, et al** (2008): *Oxytocin alleviates hepatic ischemia–reperfusion injury in rats*. Peptides, **29**, 1216-1222.
10. **ELKady AH, Elkafoury BM, Saad DA, et al** (2021): *Hepatic ischemia-reperfusion injury: effect of moderate-intensity exercise and oxytocin compared to l-arginine in a rat model*. Egypt Liver J, **11**, 1-15.
11. **Gardner AK, Schroeder EL** (2022): *Pathophysiology of intraabdominal hypertension and abdominal compartment syndrome and relevance to veterinary critical care*. J Vet Emerg Crit Care, **32**, 48-56.
12. **Gharishvandi F, Abdollahi A, Shafaroodi H, et al** (2020): *Involvement of 5-HT1B/1D receptors in the inflammatory response and oxidative stress in intestinal ischemia/reperfusion in rats*. Eur J Pharmacol, **882**, 173265.

13. **Gonzalez LM, Moeser AJ, Blikslager AT** (2015): *Animal models of ischemia-reperfusion-induced intestinal injury: Progress and promise for translational research*. Am J Physiol Gastrointest Liver Physiol, **308**, G63–G75.
14. **Gregová K, Číkoš Š, Bilecová-Rabajdová M, et al** (2015): *Intestinal ischemia-reperfusion injury mediates expression of inflammatory cytokines in rats*. Gen Physiol Biophys, **34**, 95-99.
15. **Hamilton TR, Thacher CW, Forsee KM, et al** (2010): *Trauma-associated acute mesenteric ischemia in a dog*. J Vet Emerg Crit Care, **20**, 595-600.
16. **Lapsekili E, Menteş Ö, Balkan M, et al** (2016): *Role of alkaline phosphatase intestine-isomerase in acute mesenteric ischemia diagnosis*. TJTES, **22**, 115-120.
17. **McMichael M, Moore RM** (2004): *Ischemia–reperfusion injury pathophysiology, part I*. J Vet Emerg Crit Care, **14**, 231-241.
18. **Nadatani Y, Watanabe T, Shimada S, et al** (2018): *Microbiome and intestinal ischemia/reperfusion injury*. J Clin Biochem Nutr, **63**, 26-32.
19. **Ohkawa H, Ohishi N, Yagi K** (1979): *Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction*. Anal Biochem, **95**, 351–358.
20. **Oliveira-Pelegrin GR, Saia RS, Cárnio EC, et al** (2013): *Oxytocin affects nitric oxide and cytokine production by sepsis-sensitized macrophages.* Neuroimmunomodulation, **20**, 65-71.
21. **Pergel A, Kanter M, Yucel AF, et al** (2012): *Anti-inflammatory and antioxidant effects of infliximab in a rat model of intestinal ischemia/reperfusion injury*. Toxicol Ind Health, **28**, 923-932.
22. **Powell A, Armstrong P** (2014): *Plasma biomarkers for early diagnosis of acute intestinal ischemia*. Semin Vasc Surg, **27**, 170-175.
23. **Ragy MM, Aziz NM** (2017): *Prevention of renal ischemia/perfusion-induced renal and hepatic injury in adult male Albino rats by oxytocin: Role of nitric oxide*. J Basic Clin Physiol Pharmacol, **28**, 615-621.
24. **Sayıner S, Gülmez N, Sabit ZA, et al** (2019): *Effects of Deep-Frying Sunflower Oil on Sperm Parameters In A Mouse Model: Do Probiotics Have A Protective Effect?* Kafkas Univ Vet Fak Derg, **25**, 857-863.
25. **Şehirli AÖ, Sayiner S, Savtekin G, et al** (2021): *Protective effect of bromelain on corrosive burn in rats*. Burns, **47**, 1352-1358.
26. **Senturk GE, Erkanli K, Aydin U, et al** (2013): *The protective effect of oxytocin on ischemia/reperfusion injury in rat urinary bladder*. Peptides, **40,** 82-88.
27. **Soares RO, Losada DM, Jordan MC, et al** (2019): *Ischemia/reperfusion injury revisited: an overview of the latest pharmacological strategies*. Int J Mol Sci, **20**, 5034.
28. **Sookoian S, Pirola CJ** (2015): *Liver enzymes, metabolomics, and genome-wide association studies: from systems biology to the personalized medicine*. World J Gastroenterol, **21**, 711-725.
29. **Tuğtepe H, Şener G, Bıyıklı NK, et al** (2007): *The protective effect of oxytocin on renal ischemia/reperfusion injury in rats*. Regul Pept, **140**, 101-108.
30. **VanderBroek AR, Engiles JB, Kästner SB, et al** (2021): *Protective effects of dexmedetomidine on small intestinal ischemia-reperfusion injury in horses*. Equine Vet J, **53**, 569-578.

First dose optimization study on freezing Anatolian buffalo semen

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ABSTRACT

The main objective of sperm production centers is to produce as many straws as possible from the obtained ejaculates using the optimal dilution rate. To this end, this study is the first to evaluate the effect of different semen extender rates on Anatolian buffalo semen quality. Ejaculates were collected by artificial vagina from three Anatolian buffalo bulls. These ejaculates were divided into three aliquots and filled into 0.25 ml straws with soy-based extenders at concentrations of 35, 25, and 15 million sperm/straw (n=105). The straw samples of different sperm concentrations were frozen. The quality of sperm was evaluated after thawing (37 °C, 30 sec) and following the thermoresistance test (37 °C, 3 h). The post-thaw total motility and progressive motility values were similar between the groups. However, following the thermoresistance test, there was a significant decrease in total motility in the 35 million sperm/straw group, and the progressive motility was significantly higher in the 25 million sperm/straw group. There was no statistically significant difference between the groups in terms of sperm kinetic parameters, except for VSL after thawing, as well as VAP and LIN values following the thermoresistance test. The overall mean PMAI and STR values were the highest in the 25 million sperm/straw group. In conclusion, it is recommended to dilute the Anatolian buffalo semen at a concentration of 25 million/0.25 ml when freezing it with a soy-based semen extender. In addition, it is considered that soy-based extenders compensate for cryo-damage to sperm motility for a short time, and the thermoresistance test should be applied for objective evaluation in dose optimization studies.

Introduction

The frozen bull semen industry has become a global market share with high economic potential with the advances in the safe transportation of frozen semen in liquid nitrogen and acceptable conception rates with artificial insemination. The main purpose of frozen sperm production centers is to produce as many straws of frozen semen as possible from the obtained ejaculates while maintaining high quality (8, 34).

Since the discovery of the cryoprotectant property of glycerol in 1949 by Polge et al. (25) studies on semen extenders and diluent rate optimization are still ongoing to minimize the biochemical and mechanical stress to which spermatozoa are exposed during the cryopreservation process (32).

Currently, the optimal dilution rate of semen extenders in mammalian semen varies according to breed and species, and even the concentration of sex-sorted sperm of the same breed differs considerably. For instance, the dilution rates to the final concentration of some mammalian species have been reported as 400 million (M) sperm/ml for rams, 400 M sperm/ml for goats, 300 M sperm/ml for boars, 100 M sperm/ml for stallions, and 60 M sperm/ml for bulls. If bull semen is sexed, dilution rates for the final concentration range from 8 M to 20 M sperm/ml (4, 6, 13, 14, 22, 26, 31).

The first buffalo semen was frozen by Roy et al. (27). Previously, many semen extenders and dilution rate optimization studies were carried out for quality semen freezing in river and swamp buffalo breeds (2, 9, 29). However, our study is the first to investigate the optimal dilution rate using soybean-based extenders for the cryopreservation of semen collected from Anatolian buffalo bulls in Türkiye, which originated from the Mediterranean subgroup of river buffaloes (30). To investigate the ideal dilution rate for Anatolian buffaloes, three different dilution rates were considered. These are 15, 25 M sperm/0.25 ml, and 35 M/0.25 ml, which are commonly used in the freezing of bull and buffalo semen. (1, 13, 22). Therefore, the objective of the current study was to compare the spermatozoa total motility and progressive motility, spermatozoa movement parameters, plasma membrane, and acrosome integrity at hours, 0, and 3 after thawing using frozen semen diluted at different rates ranging from 15, 25, and 35 M spermatozoa/straw.

Materials and Methods

This study was conducted at the Artificial Insemination Laboratory, Biotechnology Division, International Center for Livestock Research and Training Institute (located at an altitude de 1,080 meters above the mean sea level at 39.96°N latitude and 33.10°E longitude).

Preparation of artificial vagina (AV): A bovine AV was prepared by filling the water jacket with water hot enough to achieve a final AV internal temperature of 40-42 °C (IMV, France). The internal surface of the AV was coated with a thin layer of sterile and non-spermicidal petroleum jelly to lubricate it. A 15-ml sterile glass cylindrical tube was placed at the end of the AV to collect the sperm samples and carry them to the laboratory. The AV and the collection tube were placed into a protective felt sheath to maintain the optimal temperature during the collection process (2, 5).

Semen collection and dilution: The semen was collected using a teaser female buffalo (Lalahan Model). Each bull came to the semen collection area from its pen without a handler and exhibited mounting. Ejaculates were collected from three Anatolian buffalo bulls (at least 4 years old and housed in individual boxes) using the AV twice per week (5). After sample collection, each tube containing the fresh ejaculate was kept in a water bath at 36 °C. Only ejaculates with more than 80% total motility were used for semen processing and cryopreservation. Each ejaculate was divided into three aliquots to form different sperm concentration groups: 15 M sperm/0.25 ml, 25 M sperm/0.25 ml, and 35 M sperm/0.25 ml (n=105). The sperm concentrations of each group were determined using the Accucell photometer (IMV, L'Aigle, France). A commercial soybean-based semen extender (Andromed, Minitube, Germany) was used to dilute the semen samples. The semen samples of the three groups were diluted with the extender to the final concentrations of 15, 25, and 35 M sperm/0.25 ml, respectively.

Semen freezing process: The extended semen samples were cooled slowly to +4 °C for equilibration in a cold handling cabinet for three hours. After equilibration, the semen samples were loaded into 0.25-ml French mini straws (IMV, L'Aigle, France) using an automatic straw filling and sealing machine (MX4, IMV, L'Aigle, France). Subsequently, the straws were frozen to -140 °C (a programmed rate of -3 °C/min from +4 to -10 °C; -40 °C/min from 10 to 100 °C; and -20 °C/min from -100 to -140 °C) using a digital freezing machine (Digital cool 5300ZB 250, IMV, L'Aigle, France). Following the freezing stage, the straws were plunged into liquid nitrogen for storage at -196 °C (2, 5).

Computer-aided sperm analysis: A computer-aided semen analyzer system (CASA; IVOS I, Hamilton Thorne, USA) was used to examine various sperm motion characteristics after thawing (37 °C, 30 sec) and incubation (thermoresistance test; 37 °C, 3 h). After thawing, two-straw pairs were pooled from each straw batch in a microcentrifuge tube before analysis. Then, a 3-µl sample of semen was placed onto a prewarmed four-chamber slide and inserted into CASA with a 10× objective (IMV, L'Aigle, France), and the analysis was performed at 37 °C. Motile spermatozoa with an average path velocity (VAP) of 50 µm/s and a straight-line velocity (VSL) of 70% were evaluated as progressively motile (%). Other kinematics values, namely VAP (µm/s), VSL (µm/s), curvilinear velocity (VCL, µm/s), linearity [LIN=(VSL/VCL)×100], straightness [STR=(VSL/VAP) ×100], the amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz), were also evaluated and expressed with their units. Approximately 400 spermatozoa per sample were evaluated in five microscopic fields (5, 28).

Flow cytometric analysis: Plasma membrane and acrosome integrity (PMAI) in frozen-thawed semen were assessed with a CytoFlex flow cytometer (Beckman Coulter, USA). The semen samples were analyzed using an argon laser at a 488 nm excitation wavelength and a laser optical output power of 50 mW. Fluorescein isothiocyanate–conjugated peanut agglutinin (FITC-PNA; green fluorescence)/propidium iodide (PI)-combined staining procedures were followed as described by Korkmaz et al. (16). Accordingly, 2.5 µL of FITC-PNA (100 µg/mL) and 1.5 µL of PI (2.99 mM) were added into 5 µL of semen previously diluted in 246 µL of phosphate-buffered saline solution. Then, the diluted semen samples

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containing the dyes and the solution were assessed using flow cytometry after a 15-minute equilibration at 37 °C. The structural integrity of the plasma membrane and acrosome were evaluated in four subgroups: a) FITC-PNA label- and PI label-negative sperms with an intact plasma membrane and acrosome (accepted as viable), b) FITC-PNA label-negative and PI label-positive sperms with an intact acrosome and non-intact plasma membrane, c) FITC-PNA label-positive and PI label-negative sperms with a non-intact acrosome and intact plasma membrane, and d) FITC-PNA label- and PI label-positive sperms with a non-intact acrosome and plasma membrane after except for non-cellular events (Figure 1).

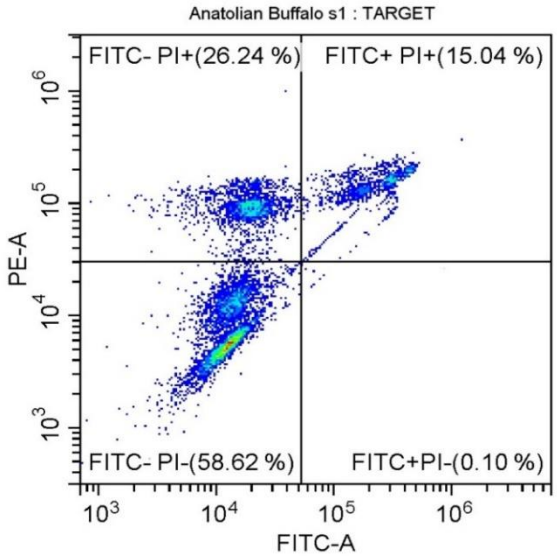

Figure 1. Flow cytometric evaluation of frozen-thawed Anatolian buffalo semen stained with FITC-PNA/PI for the analysis of the plasma membrane and acrosome integrity (FITC-, PI- live sperm with an intact acrosome population (p.); FITC-, PI+=dead sperm with an intact acrosome p.; FITC+, PI-=live sperm with a reacted acrosome p.; FITC+, PI+=dead sperm with a reacted acrosome p.).

Statistical Analysis: Descriptive statistics for the data were calculated and presented as mean ± standard error. A mixed model was utilized to determine the effect of sperm concentration, the effect of incubation time, and their interaction on CASA and flow cytometry parameters. The following repeated measures model was used:

$$Y_{ijk} = \mu + D_i + T_j + (D \times T)_{ij} + e_{ijk}$$

where Y_{ijk} is the dependent variable, μ is the overall mean, D_i is the sperm concentration group (i=15, 25, and 35), T_j, is the effect of incubation time (j=hour 0 and hour 3), $(D \times T)_{ij}$ is the interactions between the sperm concentration groups i and the effect of incubation time j, and e_{ijk} is residual error. The intra-group assessment of the animals was undertaken as a random effect, while the sperm concentration group, incubation time effect of sampling, and their interaction were assessed as fixed effects. Any significant difference was compared using simple effect analysis with the Bonferroni adjustment. The criterion of P<0.05 was considered significant in all the analyses. All the data were analyzed using IBM SPSS Statistics for Windows, version 23.0.

Results

Table 1 presents the dose, time, and dose x time interactions of the sperm total motility, progressive motility, and PMAI values of the semen samples after thawing and following the three-hour incubation period.

The total motility value after thawing was statistically similar between the groups, while the total motility values following the thermoresistance test were similar in the 15 M sperm/0.25 ml group and the 25 M sperm/0.25 ml group (41.65±1.73 and 44.14±1.73, respectively), but significantly higher compared to the 35 M sperm/0.25 ml group (P<0.05).

Table 1. Effect of different dilution rates on the post-thaw spermatozoon total motility, progressive motility, and PMAI values of the groups at hours 0 and 3 (mean±pooled SEM).

		Time			P-Value		
	Group	**0. h**	**3. h**	**Overall mean**	**Dose**	**Time**	**D*T**
Total motility (%)	15M sperm/0.25 ml	52.94 ± 1.73^{a}	41.65 ± 1.73^{b,A}	47.30 ± 1.22	<0.001	<0.001	0.048
	25M sperm/0.25 ml	58.71 ± 1.73^{a}	44.14 ± 1.73^{b,A}	51.42 ± 1.22			
	35M sperm/0.25 ml	53.34 ± 1.73^{a}	33.51 ± 1.73^{b,B}	43.42 ± 1.22			
Overall mean		55 ± 1	39.77 ± 1				
Progressive motility (%)	15M sperm/0.25 ml	22.10 ± 0.89^{a}	9.45 ± 0.89^{b,B}	15.77 ± 0.67	<0.001	<0.001	0.042
	25M sperm/0.25 ml	23.87 ± 0.89^{a}	16.30 ± 0.89^{b,A}	18.09 ± 0.67			
	35M sperm/0.25 ml	20.67 ± 0.89^{a}	6.90 ± 0.89^{b,B}	13.79 ± 0.67			
Overall mean		22.22 ± 0.58	9.55 ± 0.58				
PMAI (%)	15M sperm/0.25 ml	41.90 ± 1.46	33.20 ± 1.46	37.55 ± 1.03^{C}	<0.001	<0.001	0.073
	25M sperm/0.25 ml	57.00 ± 1.46	42.10 ± 1.46	49.55 ± 1.03^{A}			
	35M sperm/0.25 ml	52.26 ± 1.46	38.20 ± 1.46	45.23 ± 1.03^{B}			
Overall mean		50.38 ± 0.84^{a}	37.83 ± 0.84^{b}				

SEM: Standard error of the mean, M: million, PMAI: Plasma membrane and acrosome integrity.
^{a,b} Different letters on the same row for each parameter represent statistically significant differences (P<0.05).
$^{A, B}$ Different letters on the same column in different groups for each parameter represent statistically significant differences (P<0.05).

Table 2. Effect of different dilution rates on the post-thaw spermatozoon kinetic values of the groups at hours 0 and 3 (mean±pooled SEM).

		Time			P-Value		
	Group	**0. h**	**3. h**	**Overall mean**	**Dose**	**Time**	**D*T**
VCL (µm/s)	15M sperm/0.25 ml	169.76 ± 4.80	132.80 ± 4.80	151.28 ± 3.39			
	25M sperm/0.25 ml	161.24 ± 4.80	129.64 ± 4.80	145.44 ± 3.39	0.469	<0.001	0.822
	35M sperm/0.25 ml	167.56 ± 4.80	130.90 ± 4.80	149.23 ± 3.39			
Overall mean		166.19 ± 2.77^{a}	131.11 ± 2.77^{b}				
VAP (µm/s)	15M sperm/0.25 ml	101.59 ± 2.29^{a}	69.36 ± 2.29^{b,A}	85,47 ± 1.62			
	25M sperm/0.25 ml	94.68 ± 2.29^{a}	65.19 ± 2.29^{b,AB}	79.93 ± 1.62	0.033	<0.001	0.021
	35M sperm/0.25 ml	101.48 ± 2.29^{a}	59.67 ± 2.29^{b,B}	80.57 ± 1.62			
Overall mean		99.25 ± 1.32	64.74 ± 1.32				
VSL (µm/s)	15M sperm/0.25 ml	79.88 ± 1.54^{a,A}	50.92 ± 1.54^{b}	65.40 ± 1.13			
	25M sperm/0.25 ml	74.32 ± 1.54^{a,B}	48.77 ± 1.54^{b}	61.54 ± 1.13	<0.001	<0.001	0.002
	35M sperm/0.25 ml	68.69 ± 1.54^{a,C}	50.19 ± 1.54^{b}	59.44 ± 1.13			
Overall mean		74.29 ± 0.96	49.96 ± 0.96				
ALH (µm)	15M sperm/0.25 ml	7.25 ± 0.12	6.60 ± 0.12	6.92 ± 0.09			
	25M sperm/0.25 ml	7.21 ± 0.12	6.75 ± 0.12	6.98 ± 0.09	0.316	<0.001	0.681
	35M sperm/0.25 ml	7.34 ± 0.12	6.87 ± 0.12	7.10 ± 0.09			
Overall mean		7.27 ± 0.08^{a}	6.74 ± 0.08^{b}				
STR (%)	15M sperm/0.25 ml	75.63 ± 0.83	73.83 ± 0.83	74.73 ± 0.64^{B}			
	25M sperm/0.25 ml	77.35 ± 0.83	75.83 ± 0.83	76.59 ± 0.64^{A}	0.039	0.012	0.942
	35M sperm/0.25 ml	75.92 ± 0.83	74.63 ± 0.83	75.7 ± 0.64^{AB}			
Overall mean		76.3 ± 0.56^{a}	74.76 ± 0.56^{b}				
LIN (%)	15M sperm/0.25 ml	46.95 ± 0.92^{a}	42.51 ± 0.92^{b,AB}	44.73 ± 0.71			
	25M sperm/0.25 ml	46.36 ± 0.92	44.39 ± 0.92^{A}	45.38 ± 0.71	0.238	<0.001	0.005
	35M sperm/0.25 ml	47.68 ± 0.92^{a}	40.31 ± 0.92^{b,B}	43.99 ± 0.71			
Overall mean		47 ± 0.63	42.40 ± 0.63				
BCF (Hz)	15M sperm/0.25 ml	28.46 ± 0.39	28.31 ± 0.39	28.39 ± 0.28			
	25M sperm/0.25 ml	29.21 ± 0.39	28.50 ± 0.39	28.86 ± 0.28	0.485	0.079	0.643
	35M sperm/0.25 ml	28.97 ± 0.39	28.11 ± 0.39	28.54 ± 0.28			
Overall mean		28.88 ± 0.23	28.31 ± 0.23				

The progressive motility value after thawing was statistically similar between the groups (P>0.05). Following the thermoresistance test, the 15 M sperm/0.25 ml and 35 M sperm/0.25 ml groups had similar progressive motility values, while the 25 M sperm/0.25 ml group had a statistically significantly higher value (16.30±0.89) (P<0.05).

The overall mean PMAI values of the 15, 25, and 35 M sperm/0.25 ml groups were 37.55±1.03, 49.55±1.03, and 45.23±1.03, respectively (P<0.001). The highest PMAI value was observed in the 25 M sperm/0.25 ml group, with the values being determined as 57.00±1.46 at hour 0 and 42.10±1.46 at hour 3 after thawing (P<0.001). However, the dose x time interaction for the PMAI value was statistically non-significant (P>0.05).

The dose, time, and dose x time interaction values of the sperm kinetic parameter variables are shown in Table 2. The changes in the VCL values over time were statistically significant (P<0.001); however, there was no statistically significant difference between the three groups regarding the dose and dose x time interaction values (P>0.05). In contrast, the dose, time, and dose x time interactions statistically significantly differed between the groups for the VAP value (P<0.05). Although the VAP value measured at hour 0 after thawing was similar between the groups, it was significantly higher in the 15 M sperm/0.25 ml group (69.36±2.29) at hour 3 (P<0.05). Concerning the VSL value, the difference between the groups after thawing was statistically significant, and this value was found to be higher in the 15 sperm/0.25 ml group (79.88±1.54) (P<0.05). However, the VSL value obtained following the three-hour incubation period was statistically similar between the groups (P>0.05). When the ALH values measured at hours 0 and 3 were compared, statistically significant differences were observed (P<0.05). However, there was

no significant difference between the groups in terms of dose x time interaction (P>0.05). The statistical evaluation of the STR value was significant for dose and time (P<0.05) and non-significant for the dose x time interaction (P>0.05). While the LIN value was similar between the groups after thawing, it was significantly higher in the 25 M sperm/0.25 ml group after the three-hour incubation period (44.39±0.92) (P<0.05). There was no statistically significant difference in the evaluation of the BCF value in terms of dose, time, and dose x time interaction (P>0.05).

Discussion and Conclusion

In buffalo breeds, the estrus phase varies between 5 and 72 h, and the time of ovulation varies between 26 and 33 h after estrus, which is considerably longer than the estrus and ovulation times observed in cattle breeds (21). Therefore, to determine the fertilization ability of buffalo semen, it is important to repeat sperm quality analyses not only after thawing but also following the thermoresistance test applied at a certain time and temperature or after an incubation period.

In this study, no statistically significant difference was observed in the 15 M, 25 M, and 35 M sperm/0.25 ml groups in terms of total motility after thawing (hour 0), but there was a statistically significant decrease in the total motility value in the 35 M sperm/0.25 ml group after three hours of incubation at 37 °C. Similarly, in terms of the progressive motility value, there was no statistically significant difference between the groups after thawing, but a smaller decrease was detected in the 25 M sperm/0.25 ml group after the three-hour thermoresistance test. In a study conducted on Sahiwal bull semen using the tris egg yolk extender, Lone et al. (17) found that the 20 M and 15 M sperm/0.25 ml groups had similar total and progressive motility values after thawing and it was higher compared to the 10 and 5 M sperm/0.25 ml groups. In another study examining crossbred bull (*Bos Taurus* × *Bos indicus*) semen diluted with the Bioxcell® and Triladyl® extenders, Vera-Munoz et al. (33) formed three groups of 30, 15, and 5 M sperm/0.25 ml and reported that the total motility value was higher in the 30 M sperm/0.25 ml group. Patil et al. (23), who used tris egg-yolk and liposome-based extenders in Murrah breed buffaloes, determined that the total motility and progressive motility values of the 20 and 12 M sperm/0.25 ml groups after thawing were similar and higher than the 2 M sperm/0.25 ml group. The post-thaw total motility and progressive motility values reported in the studies mentioned above are consistent with our findings (30 M, 20 M, 15 M, and 12 M sperm/0.25 ml) except for the high dilution rate. In the literature, it has been observed that high dilution rates (2 M, 5 M, and 10 M sperm/0.25 ml) adversely affected the post-thaw total motility and progressive motility values. This could be because the high dilution of semen reduces the amount of proteins, antioxidants, and other beneficial compounds in the seminal plasma, which are necessary for spermatozoa to maintain membrane integrity and function (3, 10, 12, 17).

In this study, after the three-hour thermoresistance test, statistically significant differences were detected between the groups in terms of total motility and progressive motility values. In terms of the third-hour total motility value, the highest decrease was seen in the 35 M sperm/0.25 ml group, while the highest third-hour progressive motility value belonged to the 25 M sperm/0.25 ml group. According to previous research, the cryotolorence of buffalo sperm is lower than that of cattle breeds due to the low membrane phospholipid content in the sperm membrane structure and seminal plasma (3, 9, 11, 12, 15, 18). Unlike the studies mentioned above, we used a soybean-based extender containing phospholipids. This extender is considered to have compensated for the short-term cryodamage to sperm motility (total and progressive motility) at hour 0 after thawing (22). This hypothesis is supported by the results of another study that examined Murrah buffalo semen frozen with the tris egg egg-yolk-based tender and showed a decrease of approximately 70% in motility after the two-hour thermoresistance test compared to the post-thaw (hour 0) motility value (3).

In this study, the PMAI value was the highest in the 25 M sperm/0.25 ml group at hours 0 and 3 after thawing. In some studies evaluating the plasma membrane of sperm with the hypoosmotic swelling (HOS) test, it has been suggested that a high dilution rate adversely affects the plasma membrane of sperm. Consistent with this idea, Patil et al. (23) found that the plasma membrane integrity of Murrah semen samples at the 20 M sperm/0.25 ml dose was higher than that of the semen samples at the 12 and 2 M sperm/0.25 ml doses. Similarly, Vera-Munoz et al. (33) reported that the plasma membrane integrity of crossbred bull (*Bos taurus* × *Bos indicus*) semen samples at the 30 M sperm/0.25 ml freezing dose was higher than that of the 15 and 5 M sperm/0.25 ml groups. Lone et al. (17) showed that the plasma membrane values of the Sahiwal bulls' semen samples in the 20, 15, and 10 M sperm/0.25 ml groups were similar but higher than the 5 M sperm/0.25 ml group. In another crossbred bull (*Holstein-Friesian x Tharparkar*) study using the same freezing doses, the 10 and 5 M sperm/0.25 ml groups were found to have much lower plasma membrane integrity than the 20 and 15 M sperm/0.25 ml groups (13). In addition, in these studies, sperm acrosome integrity was manually evaluated with the FITC-PNA- and PI-modified staining protocols. Lone et al. (17) determined the sperm acrosome integrity of the 20

and 15 M sperm/0.25 ml groups be similar and higher than the 10 and 5 M sperm/0.25 ml groups, while Karan et al. (13) reported that the acrosome integrity of the 20 M sperm/0.25 ml group was higher compared to the 15, 10, and 5 M sperm/0.25 ml groups. Unlike the studies mentioned above, in the current study, plasma membrane integrity was evaluated using flow cytometry with FITC-PNA and PI staining, along with acrosome integrity, after thawing and following the three-hour incubation period. Although flow cytometry, a more objective method than the HOS test or manual staining methods, allows for the examination of a considerably higher number of sperm populations (10 thousand to 20 thousand spermatozoa), the data we obtained were parallel to the above-mentioned studies.

In this study, no statistically significant difference was found between the groups in terms of sperm kinetic parameters, except for the VSL value at hour 0 after thawing, which was significantly higher in the 15 M sperm/0.25 ml group. These results are generally consistent with those reported by Lone et al. (17) and Patil et al. (23). After the three-hour incubation period, significant differences were determined between the groups in terms of the mean VAP and linearity LIN values. At hour 3, the mean VAP value was higher in the 15 M sperm/0.25 ml group, while the mean LIN value was higher in the 25 M sperm/0.25 ml group. In addition to the total motility value, linear motility is important in the progression of the sperm toward the oocyte and the penetration of the zona pellucida (19, 20). The high LIN value in the 25 M sperm/0.25 ml group indicates that the rate of circularly moving sperms was lower in this group. This is also supported by the progressive motility value obtained from this group at hour 3.

Studies on the optimal dilution rate of semen generally focus on bull (*Bos taurus and Bos indicus*) semen. In this regard, there is limited literature on buffalo semen. Previous studies examining buffalo and bovine semen indicate that an excessive dilution rate (2 to 5 M sperm/0.25 ml) reduces sperm quality after thawing. This is considered to be related to the partial or complete removal of the components in the seminal plasma that are protective of the sperm (3, 10, 12, 13).

In general, the optimal dilution rate for cattle breeds is specified as 10 or 15 M spermatozoa per 0.25-ml straw (13, 22). However, in the current study, in which the optimal dilution rate was investigated for the first time in Anatolian buffalo semen, it was determined that the 25 M sperm/straw dose was more suitable for higher semen quality after thawing. This may be due to the biochemical differences between bovine and buffalo semen (7, 12, 24).

The conception rate is the most important indicator in determining a bull's fertilization ability. However, we were not able to evaluate the conception rate in this study, mainly because Anatolian water buffalo is widely grown in small-scale enterprises with different management and feeding systems. Patil et al. (23) reported a higher conception rate in the 20 M sperm/straw group than in the 15, 10, and 5 M sperm straw groups, which is parallel to their spermatological analysis results. Accordingly, it is predicted that a higher conception rate can be achieved at a dose of 25 M sperm/straw.

In conclusion, this study aimed to determine the optimal dilution rate for the cryopreservation of Anatolian buffalo semen by applying the main spermatological analyses, such as sperm kinematic, morphological, and thermoresistance tests. The findings of this study suggest that dosing the semen at 25 M sperm per straw hence, a final concentration of 100 M sperm/ml is considered effective when a soy-based semen extender is used. However, there is a need for both in vitro and in vivo studies to determine the optimal dilution rate for different cryopreservation protocols.

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Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Animal Experiments Local Ethics Committee of the International Center for Livestock Research and Training Institute, Ankara (Decision Number: 26.12.2018-160).

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

IB, DŞ, FK, SŞ, and MŞ conceived and planned the experiments. IB, DŞ, FK, SŞ performed spermatological experiments. The UK carried out a statistical analysis. IB

took the lead in writing the manuscript. All authors provided critical feedback and helped shape the manuscript.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Ahmed H, Jahan S, Khan A, et al** (2020): *Supplementation of l-tryptophan (an aromatic amino acid) in tris citric acid extender enhances post-thaw progressive motility, plasmalemma, mitochondrial membrane potential, acrosome, and DNA integrities, and in vivo fertility rate of buffalo (Bubalus bubalis) bull spermatozoa.* Cryobiology, **92,** 117-123.
2. **Ansari MS, Rakha BA, Akhter S** (2017): *Cryopreservation of Nili-Ravi buffalo (Bubalus bubalis) semen in AndroMed® extender; in vitro and in vivo evaluation.* Reprod Domest Anim, **52**, 992-997.
3. **Arjun V, Kumar P, Dutt R, et al** (2021): *Is addition or removal of seminal plasma able to compensate for the dilution effect of buffalo semen?* Andrologia**, 53**, e14123.
4. **Basioura A, Tsakmakidis IA, Martinez E, et al** (2020): *Effect of astaxanthin in extenders on sperm quality and functional variables of frozen-thawed boar semen.* Anim Reprod Sci, **218**, 106478.
5. **Bastan I, Korkmaz F, Sahin D, et al** (2021): *First commercial semen cryopreservation and main spermatological features of Anatolian buffalo.* Livestock Studies**, 61,** 060-065.
6. **Dejarnette JM, Nebel RL, Marshall CE, et al** (2008): *Effect of sex-sorted sperm dosage on conception rates in Holstein heifers and lactating cows*. J Dairy Sci, **91,** 1778-1785.
7. **Evans HC, Dinh TT, Ugur MR, et al** (2020): *Lipidomic markers of sperm cry tolerance in cattle*. Sci Rep, **10,** 1-9.
8. **Funk DA** (2006): M*ajor advances in globalization and consolidation of the artificial insemination industry.* J Dairy Sci, **89,** 1362-1368.
9. **Gaviraghi, A, Puglisi R, Balduzzi D, et al** (2013): *A minimum number of spermatozoa per dose in Mediterranean Italian buffalo (Bubalus bubalis) using sexed frozen semen and conventional artificial insemination*. Theriogenology, **79**, 1171-1176.
10. **Gaitskell-Phillips G, Martín-Cano FE, Ortiz-Rodríguez JM, et al** (2022): *Seminal plasma proteins as potential biomarkers for sperm motility and velocities.* Theriogenology, **177**, 34-41.
11. **Gurupriya VS, Divyashree BC, Roy SC** (2014): *Cryogenic changes in proteases and antiprotease activities of buffalo (Bubalus bubalis) and cattle (Bos taurus) semen*. Theriogenology, **81**, 396-402.
12. **Juyena NS, Stelletta C** (2012): *Seminal plasma: an essential attribute to spermatozoa.* J Androl, **33,** 536-551.
13. **Karan P, Mohanty TK, Kumaresan A, et al** (2018): *Improvement in sperm functional competence through modified low-dose packaging in French mini straws of bull semen.* Andrologia, **50,** e13003.
14. **Kulaksız R, Çebi Ç, Akçay E, et al** (2010): *The protective effect of egg yolk from different avian species during the cryopreservation of Karayaka ram semen.* Small Rumin Res, **88,** 12-15.
15. **Kogan T, Grossman Dahan D, Laor R, et al** (2021): *Association between fatty acid composition, cryotolerance, and fertility competence of progressively motile bovine spermatozoa.* Animals, **11,** 2948.
16. **Korkmaz F, Malama E, Siuda M, et al** (2017): *Effects of sodium pyruvate on viability, synthesis of reactive oxygen species, lipid peroxidation, and DNA integrity of cryopreserved bovine sperm.* Anim Reprod Sci, **185**, 18-27.
17. **Lone SA, Mohanty TK, Bhakat M, et al** (2020): *Modification of French mini-straw plug position for cryopreservation of small doses of bull sperm.* Anim Reprod Sci, **218**, 106485.
18. **Minervini F, Guastamacchia R, Pizzi F, et al** (2013): *Assessment of different functional parameters of frozen–thawed buffalo spermatozoa by using cytofluorimetric determinations.* Reprod Domest Anim, **48,** 317-324.
19. **Mortimer ST, Swan MA** (1999): *Effect of image sampling frequency on established and smoothing-independent kinematic values of capacitating human spermatozoa.* Hum Reprod, **14,** 997-1004.
20. **Nagy Á, Polichronopoulos T, Gáspárdy A, et al** (2015): *Correlation between bull fertility and sperm cell velocity parameters generated by computer-assisted semen analysis.* Acta Vet Hung, **63,** 370-381.
21. **Neglia G, Nicola D, Esposito L, et al** (2020): *Reproductive management in buffalo by artificial insemination.* Theriogenology, **150**,166-172.
22. **Ntemka A, Tsousis G, Brozos C, et al** (2016): *Breed differences of bull frozen-thawed semen.* Reprod Domest Anim, **51,** 945-952.
23. **Patil S, Kumar P, Singh G, et al** (2020): *Semen dilution effect on sperm variables and conception rate in buffalo.* Anim Reprod Sci, **214,** 106304.
24. **Peddinti D, Nanduri B, Kaya A, et al** (2008): *Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility*. BMC Syst Biol, **2,** 1-13.
25. **Polge C, Smith A, Parkes A,** (1949): *Revival of spermatozoa after vitrification and dehydration at low temperatures.* Nature, **164**, 666.
26. **Restrepo G, Varela E, Duque JE, et al** (2019): *Freezing, vitrification, and freeze-drying of equine spermatozoa: Impact on mitochondrial membrane potential, lipid peroxidation, and DNA integrity.* J Equine Vet Sci, **72**, 8-15.
27. **Roy A, Srivastava RK, Pandey MD** (1956): *Deep freezing of buffalo semen diluted and preserved in glycine–egg yolk medium.* Ind J Dairy Sci, **9,** 61-62.
28. **Sahin D, Bastan I, Cil B, et al** (2020): *The number of false mountings affects the quality of semen in bulls.* Livestock Studies, **60**, 9-14.
29. **Singh AK, Kumar A, Honparkhe M, et al** (2018): *Comparison of in vitro and in vivo fertilizing potential of buffalo bull semen frozen in egg yolk, soya bean lecithin- and liposome-based extenders.* Reprod Domest Anim, **53**, 195-202.

30. **Soysal M, Ozkan E, Kok S, et al** (2007): *Genetic characterization of indigenous Anatolian water buffalo breed using microsatellite DNA markers.* Ital J Anim Sci, **6**, 409-412.
31. **Tekin K, Daşkın A** (2019): *Effect of polyvinyl alcohol on survival and function of angora buck spermatozoa following cryopreservation*. Cryobiology, **89,** 60-67.
32. **Ugur MR, Saber Abdelrahman A, Evans HC, et al** (2019): *Advances in cryopreservation of bull sperm.* Front Vet Sci, **6,** 268.
33. **Vera-Munoz O, Amirat-Briand L, Diaz T, et al** (2009): *Effect of semen dilution to low-sperm number per dose on motility and functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender: Comparison to Triladyl® and Bioxcell®.* Theriogenology, **71,** 895-900.
34. **Vincent P, Underwood SL, Dolbec C, et al** (2014): Bovine semen quality control in artificial insemination centers. 1019-1031. In: Richard M. Hopper (Ed), Bovine Reproduction.

Polycythemia, emperipolesis and extramedullary haematopoiesis caused by acute shock: the first record in the Northern white-breasted hedgehog *Erinaceus roumanicus* Barrett-Hamilton, 1900

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ABSTRACT

An adult female hedgehog *Erinaceus roumanicus*, was found in a city traffic zone, exhibiting severe injuries consistent with a vehicular collision. Rapid diagnostics, including X-ray imaging, were performed at the veterinarian clinic to rescue the animal. Due to multiple traumatic injuries and poor prognosis, the animal was anesthetized for blood sampling and then euthanized. Polycythaemia, platelet aggregation, rare megakaryoblasts, neutrophilia, lymphocytosis, and a high red blood cell (RBC) and white blood cell (WBC) count were found in peripheral blood. Eosinophilia and the physiological phenomenon of emperipolesis were detected in the femur bone marrow, while the liver biopsy confirmed the extramedullary haematopoiesis (EMH). Acute hypovolemic shock results in rapid changes in hematological and biochemical parameters, endangering the patient's life. Treatment of hypovolemic shock and intensive care of small and exotic animals is further complicated by their small size, physiological diversity, lack of research and clinical data on their response to therapy. Given the increased vulnerability of the small mammal fauna as a result of expanding urbanization, this case study aims to facilitate the recognition of this life-threatening condition, the possible physiological response in hedgehogs and the adequate care of injured animals. Raising awareness of the fragmentation and decline of their populations in the habitats they share with humans can contribute to finding solutions to mitigate their road mortality, and improve their well-being and conservation.

Shock is generally considered a syndrome caused by systemic tissue hypoperfusion leading to widespread cellular dysoxia and vital organ dysfunction (7). Following severe trauma, increased production of catecholamines, particularly norepinephrine, has been associated with bone marrow dysfunction due to prolonged mobilization of haematopoietic progenitor cells and decreased proliferation of erythroid progenitor cells in the bone marrow (3). After a traumatic injury, mainly caused by haemorrhagia, anaemia occurs very early and often lasts for months (16). However, bleeding can increase bone marrow activity and lead to accelerated erythropoiesis up to several times the usual rate, and if blood loss continues, anaemia develops. While the JAK2/STAT5 pathway has been shown to activate genes fundamental for erythroid progenitor survival, proliferation and differentiation (8), STAT5 phosphorylation is essential for erythropoiesis acceleration during the hypoxic stress.

Emperipolesis is an extraordinary biological process in which one cell invades another living cell. A penetrated cell is maintained inside another and can come out anytime without structural and functional abnormalities (17). Moreover, emperipolesis is now understood as a mechanism to improve cell survival and help prevent apoptosis of cells within the host cell (12). This

phenomenon also occurs in certain pathological conditions such as autoimmune haemolytic anaemia, leukemia, myeloma (9) and inbreeding (18). More detailed research is needed to determine whether emperipolesis is the result of an existing pathological process or acute shock, which was not feasible in this case. Furthermore, it is already known that various haematopoietic stressors, such as infection, can induce extramedullary haematopoiesis (EMH) (2). In contrast, hypoxia can induce EMH in the presence of anaemia, particularly in the spleen. Splenic erythropoiesis secondary to hypoxia, also known as "stress EMH," has been thoroughly studied in mice (4, 10).

To the best of our knowledge, there are no previous studies on the effect of acute haemorrhagic shock on the change of haematological and biochemical parameters of the northern white-breasted hedgehog *Erinaceus roumanicus* Barrett-Hamilton, 1900. While only a few studies have reported average reference values for several haematological and biochemical parameters on few species of family Erinaceidae such as European hedgehog *E. europaus* (13, 26), desert hedgehog *Paraechinus aethiopicus* (19), and African pygmy hedgehog *Atelerix albiventris* (22), there are no data on hepatocytes and haematopoietic cell alterations in the hedgehog bone marrow as the output of severe injuries. In this study, it has been noted for the first-time different features as a result of the acute hypovolemic shock in the northern white-breasted hedgehog *Erinaceus roumanicus*, such as polycythaemia in peripheral blood, the physiological phenomenon of emperipolesis in the bone marrow as well as extramedullary haematopoiesis in the liver.

An adult female hedgehog *Erinaceus roumanicus*, weighing 2300 g, was found at the end of May 2022 in a city traffic zone with serious injuries, most likely caused by being struck by a vehicle. The animal hid in the nearby vegetation, but it is unknown how much time passed from being wounded to being found. External examination of the animal revealed a massive separation of the ventral fur integument from the dorsal skin with quills along the left flank, showing fully exposed muscle fascia in the lower abdominal region, partially torn. The skin of the left hind leg was wholly peeled off, which in addition to the pathological mobility of the femur, made movement extremely difficult. The exhausted and dehydrated hedgehog was transported to the veterinary clinic for examination assessment. External examination revealed a large deep purple ecchymotic area, of the left ventral part. The abdominal bleeding was immediately assumed based on the findings of a swollen, tight abdomen, accompanied by tachycardia and rapid, shallow breathing. A skeletal X-ray of the skeleton showed an open pelvic fracture with a large sacroiliac (SI) dislocation followed by a left hip dislocation. The caudal vertebrae were mostly bruised, even crushed, while the damage to the internal organs was difficult to assess (Fig. S1A). Due to multiple traumatic injuries and poor prognosis, the animal was anesthetized for blood sampling and then euthanized.

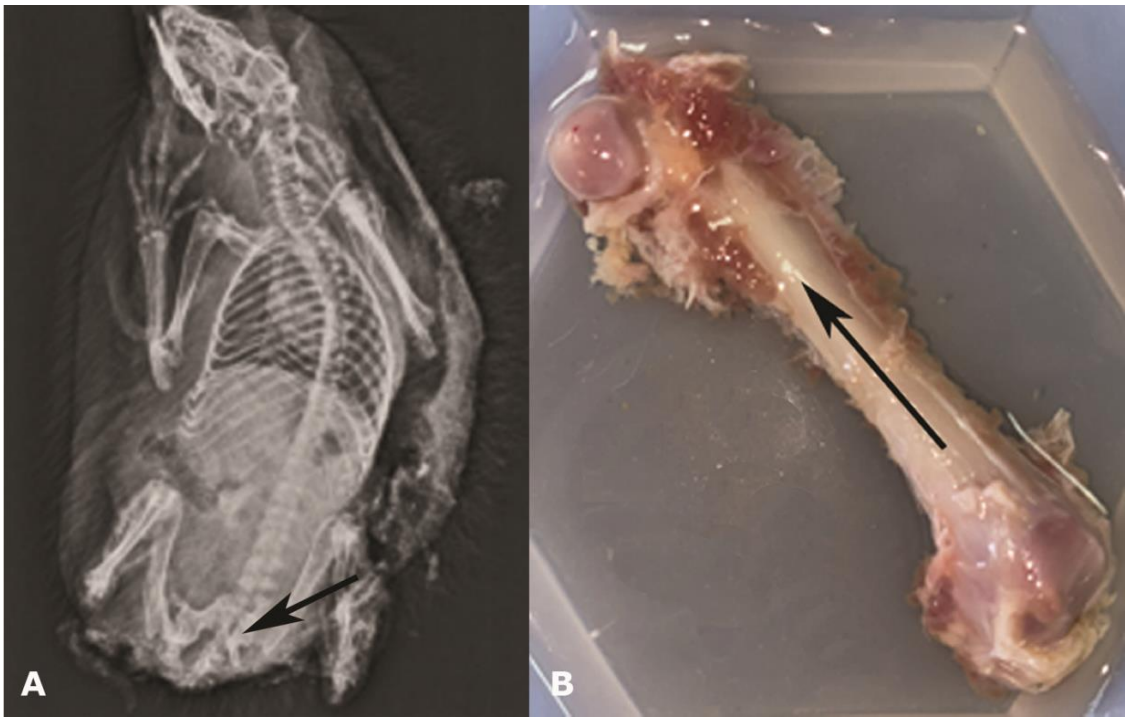

Figure S1. A - X-ray imaging of the skeleton of the northern white-breasted hedgehog (*Erinaceus roumanicus*) exhibited open pelvis fracture with massive sacroiliac (SI) dislocation with crushed caudal vertebrae (marked with black arrow). **B -** Presentation of longitudinal dissection of the femur for the purpose of bone marrow biopsy. The length of the dissection is indicated by a black arrow.

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A combination of 60 mg/kg ketamine (Intervet International) and 7 mg/kg diazepam (Alkaloid), intraperitoneally (i.p.) has been used for long-lasting analgesia prior to cardiac puncture, according to the University of Minnesota Research Animal Resources; repeated dose administrated for euthanasia, as recommended by the American Veterinary Medical Association (AVMA). After sedation, blood obtained by cardiac puncture immediately before euthanasia was centrifuged and serum was separated for spectrophotometric analysis of biochemical parameters, such as total protein, albumin, globulin, A/G ratio, glucose, creatinine and urea. A drop of fresh blood with EDTA (100:1) for the May-Gruenwald-Giemsa stain of blood smear was taken for microscopic examination with manual blood count/percentage of peripheral cells. In parallel, the total number of erythrocytes and leukocytes was confirmed by a haemocytometer.

Necropsy and partial dissection (excluding head region) showed that the massive abdominal bleeding was most likely caused by rupture and crushing of the spleen, which therefore could not be sampled. Pelvic fracture with severe SI dislocation, along with severe injuries to the surrounding structures, contributed to blood loss and the onset of acute hypovolemic shock. Apart from ectoparasites common in hedgehogs (mites), no endoparasites, obvious pathological formations or neoplasms were found during dissection. A bone marrow (BM) biopsy was performed from the middle part of the femur (Fig. S1B) and smeared with the touch technique (28). The longitudinal dissection of the femur showed that the central region contains most of the BM, while the *caput femoris* and *collum femoris* lack a sufficient BM amount for analysis. A liver biopsy was performed, and hepatocytes were analyzed by the imprint method (28).

Although there is no information on time since injury, significant erythrocytosis and mild leukocytosis followed by lymphocytosis and neutrophilia are most likely caused by the onset of acute hypovolemic shock. The haematological and biochemical parameters of the northern white-breasted hedgehog *E. roumanicus* from this case study, compared to healthy specimens of the same and related species of the family Erinaceidae, are shown in Table 1. Given that differences between biochemical parameters may be due to physiological conditions, sex differences and a season, obtaining values for *E. roumanicus* should be taken with reservation because the values are changed as a consequence of acute hypovolemic shock and dehydration, as indicated by the high value of urea (28.30 mmol/L) and mild hypoglicemia (4.07 mmol/L). Table 2 contains morphological observations focusing on the percentage of different forms of erythrocytes and platelets in the peripheral blood. In addition to normal erythrocytes, rare macrocytes and a large proportion of microcytes (38%) were observed. The appearance of different stages of erythrocytes may indicate anaemia or accelerated erythropoiesis, with the latter being the more likely scenario. However, rare megakaryoblasts present in peripheral blood, app. 1% is a phenomenon that has not been described so far. It remains unknown how such large cells can reach the circulation (Figure 1A). The platelets were shown different morphological changes with 90% average sized, 10% extra small and 9% large forms, and their notable aggregation (Table 2; Figure 1B). The total number of platelets could not be calculated due to pronounced aggregations.

Table 1. Obtained haematological and biochemical values of northern white-breasted hedgehog *Erinaceus roumanicus* in acute hypovolemic shock, in comparison to the healthy specimens of related species from the family Erinaceidae (except for 6. *E. europaeus* # (24) with notably tick-induced blood loss and regenerative anaemia).

	1. ***Er****	**2.** ***Ec/Er***(23)	**3.** ***Ee***^{a} (29)	**4.** ***Ee***^{b} (13)	**5.** ***Ee***^{c} (14)	**6.** ***Ee***^{d}# (24)	**7.** ***Ee***^{e} (26)	**8.** ***Aa*** (22)	**9.** ***Pe*** (19)
RBC (10^{12}/L)	16.41	-	5.1	9.64	8.06	6.39-8.02	8.1	5.13	5.90
WBC (10^{9}/L)	10.96	-	4.7	8.20	7.69	10-11	8.4	14.35	6.00
Neut %	62	23.9	65	45	-	39-43	35.1	64.08	51.36
segs %	51	-	-	-	-	-	-		-
bands %	11	-	-	-	-	-	-		-
LYM %	29	65.5	29	51	-	38-46	50	33.17	41.9
MONO %	4	1.80	4	0	-	4-5	2.7	1.08	6.88
BASO %	2	1	-	3	-	2-4	3.5	0.42	-
EOS %	3	9	2	2	-	6	7.5	0.75	-
PLT (10^{9}/L)	-	-	-	-	135	301-334	230.7	-	150
Albumin (g/L)	33.67	-	-	-	-	-	35.00	34.3	18.30
Globulin (g/L)	29.55	-	-	-	-	-	33.80	26.30	47.70
Total protein (g/L)	63.22	-	-	-	-	-	68.80	59.80	66.10
A/G ratio	1.13	-	-	-	-	-	1.07	1.30	0.38
Glucose (mmol/L)	4.07	-	-	-	-	-	5.9	8.60	7.15
Urea (mmol/L)	28.30	-		-	-	-	-	4.7	14.40
Creatinine (µmol/L)	59.53	-		-	-	-	-	67	44.20

Table 2. Percentage and morphological characteristics of erythrocytes and platelets in peripheral blood of northern white-breasted hedgehog *E. roumanicus* as a result of acute shock.

	Cell types	Surface area (µm^2)	%	Description
Erythrocytes	Macrocytes	28.16-33.26	2%	Very large, round, often with a central pallor
	Normal erythrocytes	19.04-21.36	50 %	Round, rarely with a central pallor
	Microcytes (spherocytes)	11.20-14.40	38%	Very small, round, without central pallor
Platelets	Extra small	8.39-9.01	10%	Single, small and round
	Normal	7.16-9.90	90%	Standard size, irregular round shape, often in clusters
	Large	20.46-29.56	9%	Large than normal, the membrane often regular round
	Megakaryoblasts (Shown in Fig 1. A1)	70.23-77.45	1%	Largest cells, rarely present, significantly smaller than bone marrow megakaryocytes, regular round shape with few visible nuclei

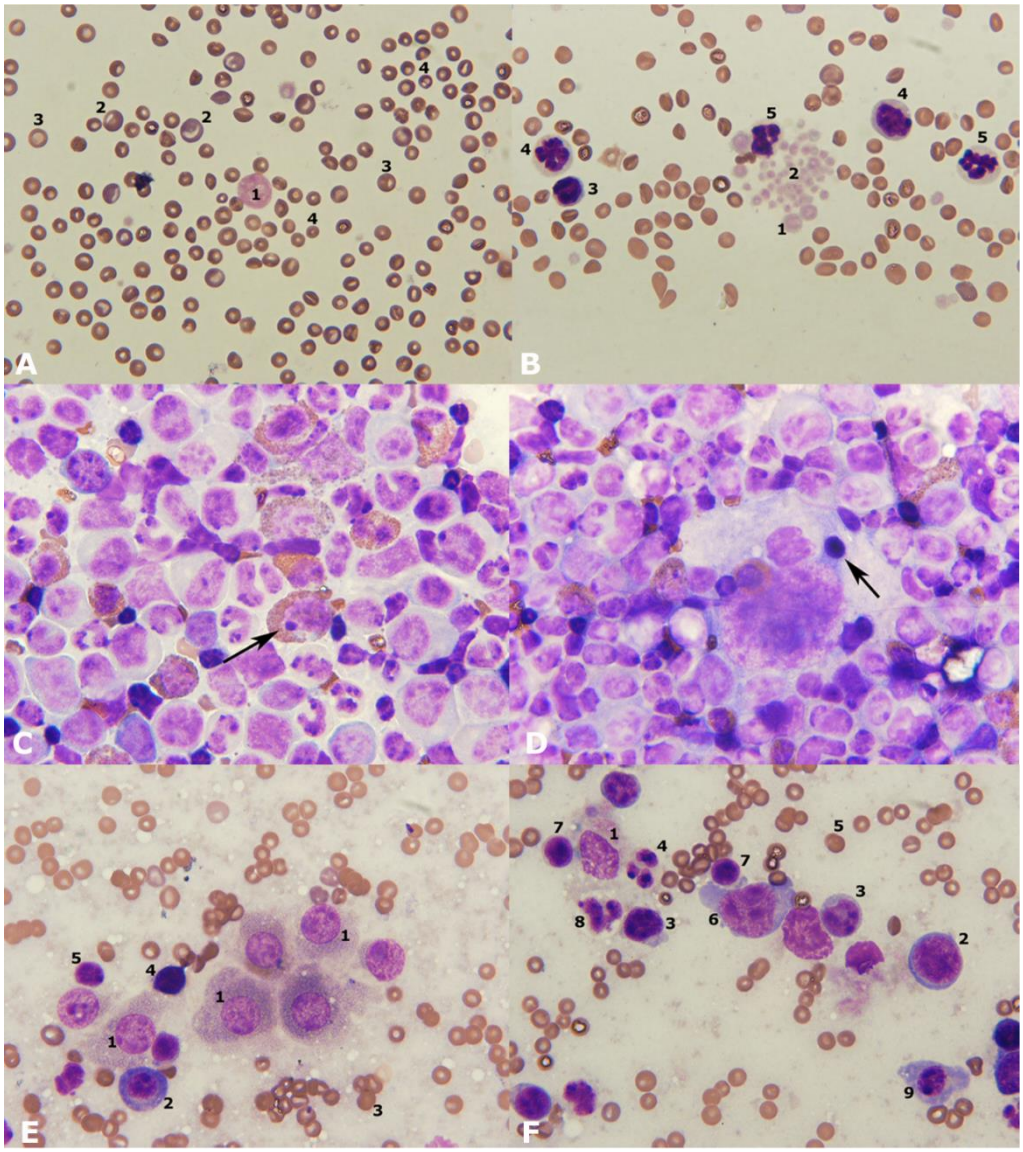

Figure 1. A-B Overview of hedgehog blood cells features. A – Polycythaemia; 1. megakaryoblast, 2. macrocyte, 3. normal erythrocyte, 4. microcyte. **B -** Leukocytes and platelets aggregation; 1. – large platelets, 2 – normal platelets, 3. lymphocyte, 4. non-segmented granulocyte, 5. segmented granulocyte; **C-D Overview of hedgehog bone marrow features. C -** The arrow indicates eosinophilic blasts; **D -** The arrow indicates emperipolesis. Inside the active megakaryocyte are basophilic erythroblast, undifferentiated blast, lymphocyte, and eosinophilic granulocyte; **E-F Overview of hedgehog liver features. E –** 1. hepatocytes, 2. erythroblast, 3. mature erythrocytes, 4. lymphoblast, 5. mature lymphocyte; **F -** extramedullary haematopoiesis in liver: 1. macrophage, 2. proerythroblast, 3. lymphoblast, 4. acidophilic erythroblast, 5. mature erythrocyte, 6. myeloblast, 7. prolymphocyte, 8 neutrophilic granulocyte (immature), 9. basophilic erythroblast.

Increased production of eosinophilic blasts was found in the bone marrow (Figure 1C). Emperipolesis (Figure 1D) detected in all types of megakaryocytes: acidophilic, basophilic and thrombocytopenic, involved engulfment of immature and mature blood cells of the erythrocyte and leukocyte line in the bone marrow. Cunin et al. (5) recently established that emperipolesis accelerates platelet production both *in vitro* and *in vivo* which is common to several conditions associated with high platelet demand, such as blood loss and hemorrhagic shock.

Hedgehog hepatocytes (Figure 1E) are large, usually with 1-2 nucleoli, structurally very similar to the hepatocytes of other mammals. Numerous hepatoblasts have been observed in the liver indicating EMH (Figure 1F). Present EMH in the liver can be associated with acute shock and a pronounced emperipolesis phenomenon in the bone marrow. In addition to blasts, immature erythrocytes and leukocytes were also observed during different developmental stages. Although a recent study reported the presence of megakaryocytes indicating EMH in the liver in two specimens of the African small hedgehog (27), megakaryocytes or megakaryoblasts in the liver of *E. roumanicus* were not identified in our study. Future studies should elucidate whether EMH in the liver is possible for all types of leukocytes and platelets.

The most crucial aspect of acute shock appears to be the presence of relative polycythemia, probably caused by hypovolemia followed by prolonged dehydration, haemoconcentration and stress. The blood smears (Figure 1A and Figure B) showed mild poikilocytosis, while present polychromasia indicates that RBCs are being released prematurely from the BM during formation. Therefore, in this case study we hypothesized that different forms of erythrocytes in peripheral blood are associated with their high production, indicating extramedullary erythropoiesis with increased production of erythrocytes in the bone marrow. Acute hypovolemic shock leads to polycythaemia with significantly increasing RBC and WBC count. We identified different forms of platelets and the presence of megakaryoblasts in the peripheral blood, eosinophilia, as well as the emperipolesis observed in the BM. Moreover, the finding of a robust BM response in protecting immature blood cells (emperipolesis) with the aim of preserving future mature cells crucial for blood loss replacement is another novelty of this study that has not been previously reported in response to acute hypovolemic shock in small mammals. It seems *E. roumanicus* appears to possess robust physiological and biochemical mechanisms of defence against acute shock at several levels, ranging from blood cells and variations in biochemical parameters in the blood to significant hematopoietic processes in the bone marrow, aided by extramedullary haematopoiesis detected in liver. Although data on extramedullary erythropoiesis after severe injury and/or disease are very scarce, a recent study on male Sprague-Dawley rats exposed to chronic stress, lung contusion and haemorrhagic shock showed the occurrence of extramedullary erythropoiesis in spleen (1). EMH of unknown origin, common in hedgehogs and other small mammals, can also appear during systemic infections and active immune response, most often occurring in the spleen and liver (11). Thus, the presence of EMH in *A. albiventris* has been reported for the spleen (25) and liver (27). Although the studies provide data for EMH, mainly in the spleen, detailed data for this phenomenon in the liver have been reported for the toad *Bufo bufo* (28). Since, in our study, the location of tissue sampling in the liver was chosen randomly, the authors tentatively conclude that EMH occurs only in certain liver regions. Future research should determine whether this mechanism is prevalent throughout the liver tissue and whether it also occurs in the spleen. As noted above, the spleen was not analyzed in this study due to tissue damage and profuse bleeding.

The results presented here are based on a case report of single animal specimen and therefore the obtained values cannot be generalized to other animals of the same or different species. Nevertheless, we strongly believe that EMH is a consequence of acute hypovolemic shock, although possible existing pathological conditions cannot be completely excluded. Distinguishing EMH from chronic myeloid and erythroid leukemia is a rare diagnostic problem due to the low frequency of such neoplasia in animals (10), and possible only using immunochemical techniques. However, the existence of a malignant tumor cannot be completely excluded, especially as neoplasia is the most common pathological finding of *A. albiventris* (21).

Hedgehogs are nocturnal animals with maximum activity after midnight. They prefer forest edges, hedgerows, suburban, but they also inhabit urban habitats. In recent decades, they are certainly the most prominent accidental victims of human activities (6), with thousands of them killed on our roads each year. The season from April to July is thought to have the highest rate of road fatalities for hedgehogs because of increased activity and reduced visibility at night (30). Considering the increased vulnerability of small mammals in urban areas, recognition of acute shock and rapid response is necessary to avoid animal suffering and mortality (20). Treatment of hypovolemic shock and monitoring of intensive care in exotic pets is hampered by their fragility, physiological diversity and lack of clinical data on their response to

therapy (15). Veterinarians who work with exotic animals or practice emergency care need to be well-versed in the pathophysiology of shock since many exotic pets appear with an acute crisis or an acute manifestation of a chronic condition that results in poor organ perfusion.

This case report also aims to raise awareness of the importance of hedgehogs and other small mammals in the ecosystem, and the problem of fragmentation and/or loss of their habitats, accompanied by the decline of their populations, especially in suburban and urban areas. Therefore, improving the care of injured animals and applying timely therapy can significantly contribute to their preservation and well-being.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

DS, LLB and MF conceived and planned the experiments. DS and MF carried out the experiments. DS and MF contributed to sample preparation. DS and LLB contributed to the interpretation of the results. LLB took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Alamo IG, Kannan KB, Loftus TJ, et al** (2017): *Severe trauma and chronic stress activates extramedullary erythropoiesis*. J Trauma Acute Care Surg, **83**, 144-150.
2. **Baldridge MT, King KY, Boles NC, et al** (2010): *Quiescent haematopoietic stem cells are activated by IFN-γ in response to chronic infection*. Nature, **465**, 793–797.
3. **Bible LE, Pasupuleti LV, Gore AV, et al** (2015): *Daily propranolol prevents prolonged mobilization of hematopoietic progenitor cells in a rat model of lung contusion, hemorrhagic shock, and chronic stress*. Surgery, **158**, 595–601.
4. **Bozzini CE, Barrio Rendo ME, Devoto FC, et al** (1970): *Studies on medullary and extramedullary erythropoiesis in the adult mouse.* Am J Physiol, **219**, 724–728.
5. **Cunin P, Bouslama R, Machlus KR, et al** (2019): *Megakaryocyte emperipolesis mediates membrane transfer from intracytoplasmic neutrophils to platelets*. Elife, **8**, e44031.
6. **Garcês A, Soeiro V, Lóio S, et al** (2020): *Outcomes, Mortality Causes, and Pathological Findings in European Hedgehogs (Erinaceus europeus, Linnaeus 1758): A Seventeen Year Retrospective Analysis in the North of Portugal*. Animals, **10**, 1305.
7. **Glazer JM, Rivers EP, Gunnerson KJ** (2016): Shock BT - Surgical Intensive Care Medicine. 61-79. In: O'Donnell JM, Nácul FE (Eds), Springer International Publishing.
8. **Grebien F, Kerenyi MA, Kovacic B, et al** (2008): *Stat5 activation enables erythropoiesis in the absence of EpoR and Jak2*. Blood, **111**, 4511–4522.
9. **Gupta N, Jadhav K, Shah V** (2017): *Emperipolesis, entosis and cell cannibalism: Demystifying the cloud*. J Oral Maxillofac Pathol, **21**, 92-98.
10. **Johns J, Christopher M** (2012): *Extramedullary Hematopoiesis: A New Look at the Underlying Stem Cell Niche, Theories of Development, and Occurrence in Animals*. Vet Pathol, **49**, 508–523.
11. **Kim C** (2010): *Homeostatic and pathogenic extramedullary hematopoiesis*. J Blood Med, **1**, 13–19.
12. **Lee WB, Erm SK, Kim KY, et al** (1999): *Emperipolesis of erythroblasts within Kupffer cells during hepatic hemopoiesis in human fetus*. Anat Rec, **256**, 158–164.
13. **Lewis JH** (1976): *Comparative hematology—Studies on hedgehogs (Erinaceus europaeus)*. Comp Biochem Physiol Part A Physiol, **53**, 237–240.
14. **Lewis J, Norcott M, Frost L, et al** (2002): *Normal haematological values of European hedgehogs (Erinaceus europaeus) from an English rehabilitation centre*. Vet Rec, **15**, 567–569.
15. **Lichtenberger M** (2004): *Principles of shock and fluid therapy in special species*. Semin Avian Exot Pet Med, **13**, 142–153.
16. **Livingston DH, Anjaria D, Wu J, et al** (2003): *Bone Marrow Failure Following Severe Injury in Humans*. Ann Surg, **238**, 748-753.
17. **Rastogi V, Sharma R, Misra S, et al** (2014): *Emperipolesis – A Review*. J Clin Diagn Res, **8**, ZM01–2.
18. **Suljević D, Filipić F, Islamagić E** (2019): *Emperipolesis: Sternal and femoral microenvironment induces*

megakaryiocyte emperipolesis in the Wistar strain. Mac Vet Rev, **42**, 71–77.

19. **Mohammed OB, Amor NMS, Omer SA, et al** (2021): *Haematology and biochemistry panels in the Ethiopian hedgehog, Paraechinus aethiopicus (Ehrenberg, 1833) from central Saudi Arabia: Establishing reference intervals and assessing variability across sex and hibernation*. J King Saud Univ – Sci, **33**, 101228.
20. **Moore LJ, Petrovan SO, Baker PJ, et al** (2020): *Impacts and Potential Mitigation of Road Mortality for Hedgehogs in Europe*. Animals, **10**, 1523.
21. **Okada K, Kondo H, Sumi A, et al** (2018): *A retrospective study of disease incidence in African pygmy hedgehogs (Atelerix albiventris)*. J Vet Med Sci, **80**, 1504–1510.
22. **Okorie-Kanu CO, Onoja RI, Achegbulu EE, et al** (2015): *Normal haematological and serum biochemistry values of African hedgehog (Atelerix albiventris)*. Comp Clin Path, **24**, 127–132.
23. **Ozparlak H, Celik I, Sur E, et al** (2011): *A study of peripheral blood in hedgehogs in Turkey*. J Zoo Wildl Med, **42**, 392–398.
24. **Pfäffle M, Petney T, Elgas M, et al** (2009): *Tick-induced blood loss leads to regenerative anaemia in the European hedgehog (Erinaceus europaeus).* Parasitology, **136**, 443–452.
25. **Raymond J, White M** (1999): *Necropsy and histopathologic findings in 14 African hedgehogs (Atelerix albiventris): a retrospective study*. J Zoo Wildl Med, **30**, 273–277.
26. **Rossi G, Mangiagalli G, Paracchini G, et al** (2014): *Hematologic and biochemical variables of hedgehogs (Erinaceus europaeus) after overwintering in rehabilitation centers*. Vet Clin Pathol, **43**, 6–14.
27. **Silva GF, Rêma A, Teixeira S, et al** (2022): *Pathological Findings in African Pygmy Hedgehogs Admitted into a Portuguese Rehabilitation Center*. Animals, **12**, 1361.
28. **Suljević D, Fočak M, Filipić F, et al** (2018): *Haematopoiesis in the European common toad Bufo bufo (Linnaeus, 1758): new methodological insights to study general, seasonal, and sexual haematopoietic distribution and maturation pattern*. Turkish J Zool, **42**, 198–206.
29. **Quilliam TA, Clarke JA, Salsbury AJ** (1971): *The ecological significance of certain new haematological findings in the mole and hedgehog*. Comp Biochem Physiol Part A Physiol, **40**, 89–102.
30. **Vadonlesõ G, Berndt M, Lukács A, et al** (2019): *Urban hedgehogs (Erinaceus roumanicus) in Budapest: Live or let die*. Hungarian Agric Res, **3**, 20–26.

Treatment of traumatic elbow luxation and radius fracture with non-rigid transarticular external fixation and paraosseous clamp cerclage in a cat

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ABSTRACT

An 8-year-old, female, neutered mixed-breed cat presented with traumatic elbow luxation and radius fracture due to high-rise syndrome. According to the anamnesis, the trauma had occurred 10 days prior. Therefore, the patient was treated with an open reduction technique. The reduction of the elbow joint was performed with the cerclage transarticular external fixation (CTEF) method. Fixation of the radius fracture was performed using the paraosseous clamp cerclage method. The follow-up time for the patient was 1 year, and the functional outcome was considered fair due to humero-ulnar ankylosis of the elbow joint with 110° of extension. In this case report, post-operative long-term clinical and radiographic results were not as desired. This can be explained by the inability to perform surgery at the desired time, the severity of the trauma, and cartilage damage. However, further cases are required to determine whether this procedure is optimal for the treatment of traumatic elbow luxation in cats.

Traumatic elbow dislocation is a relatively rare orthopedic disorder observed in cats and dogs (1, 6, 8, 13). The strong collateral ligaments and the presence of the anconeal process which interlocks into the olecranon fossa ensure the inherent stability of the elbow joint while the elbow joint is in extension (2, 5, 8, 13). Elbow dislocation occurs when the elbow is flexed to 45° or less at the time of trauma (2). Luxation occurs usually in the lateral direction because the medial epicondyle of the humerus is larger than the lateral, and the medial articular surface slopes distally (1-3, 6, 8). Also, the medial collateral ligament is weaker than the lateral collateral ligament and has a thin insertion; All those causes result in this predisposes to lateral luxation (1, 8).

Elbow dislocation can be treated using either closed or open reduction technique. Closed reduction usually gives successful results in acute cases. It has been reported that open reduction is indicated in cases where closed reduction cannot be performed or instability is present despite the reduction (1, 3, 5, 6, 8, 13).

Many methods, such as rigid external fixation, have been described in the operative treatment of elbow dislocation. Rigid external fixators provide joint alignment and necessary stability during the repair of supporting structures. However, rigid joint immobilization causes decrease in synovial fluid production, cartilage stiffness, and range of motion (ROM). It also causes to muscle atrophy, the development of degenerative joint disease, and intra-articular adhesions (9, 12). The non-rigid transarticular external fixation method promotes the healing process while minimizing the effect of joint immobilization on joint homeostasis and

cartilage metabolism (12). In this study, the non-rigid transarticular external fixation method and its results are reported.

An 8-year-old female, neutered mixed-breed cat, weighing 4 kg, was referred for lameness in the left front limb due to high-rise syndrome. After clinical and radiographic evaluation, luxation of the elbow joint and transverse fracture of the proximal diaphyseal radius were detected (Figure 1). According to the anamnesis, the trauma had occurred 10 days prior with a concurrent fracture of the radius. Therefore, the patient was treated with an open reduction technique.

Cefazolin sodium 20 mg/kg i.v (Eqizolin®, Tüm-Ekip, Turkey) was administered for antibiotherapy. For premedication, 0.1 mg/kg diazepam (Diazem®, Deva, Turkey) and 6 mg/kg propofol (Propofol® 1%, Fresenius, Turkey) were administered for induction. Anesthesia was maintained with 2% isoflurane (Isoflurane USP®, Piramal Critical Care, USA) in oxygen. For analgesia, before the surgery, sc injection of 0.1 mg/kg morphine HCl (Morphine®, Galen, Turkey) and 0.1 mg/kg sc meloxicam (Bavet Meloxicam®, Bavet, Turkey) were administered for the first 3 days after the surgery. Intraoperative analgesia was managed with a constant-rate infusion (CRI) using a combination of saline, ketamine (Ketasol® 10%, Richter Pharma AG, Austria), butorphanol (Butomidor® Sanovel, Turkey), medetomidine (Domitor®, Pfizer, Finland) (100 ml + 12 mg + 4.8 mg + 0.04 mg, respectively).

During the surgery, the elbow joint was exposed with the caudo-lateral surgical approach. Complete rupture of the medial collateral ligament (MCL) was detected. A 1.5 mm Kirschner wire was placed transcondylar to the humerus and bilaterally in the olecranon. After the reduction of humeroulnar articulation, the elbow joint was extended to 140° and Kirschner wires were fixed together with a 0.4 mm cerclage wire. The cerclage wires were adjusted in tension to prevent re-luxation of the elbow joint. The radius fracture was fixed with the paraosseous clamp cerclage method using 1 mm Kirschner wires and 0.4 mm cerclage wire, and a reduction of caput radii was performed. Since a tear of the annular ligament was detected, the ulna was fixed to the radius with a 2.0 x 16 mm cortical screw then the surgical field was closed in a routine manner. After the surgery, the hemogram tube's rubber stoppers were placed with the cerclage wire in the middle of the Kirshner wires. Post-operatively, the elbow joint flexion degree was measured as 30 ± 5°, and the extension angle was 150 ± 5° (Figure 2).

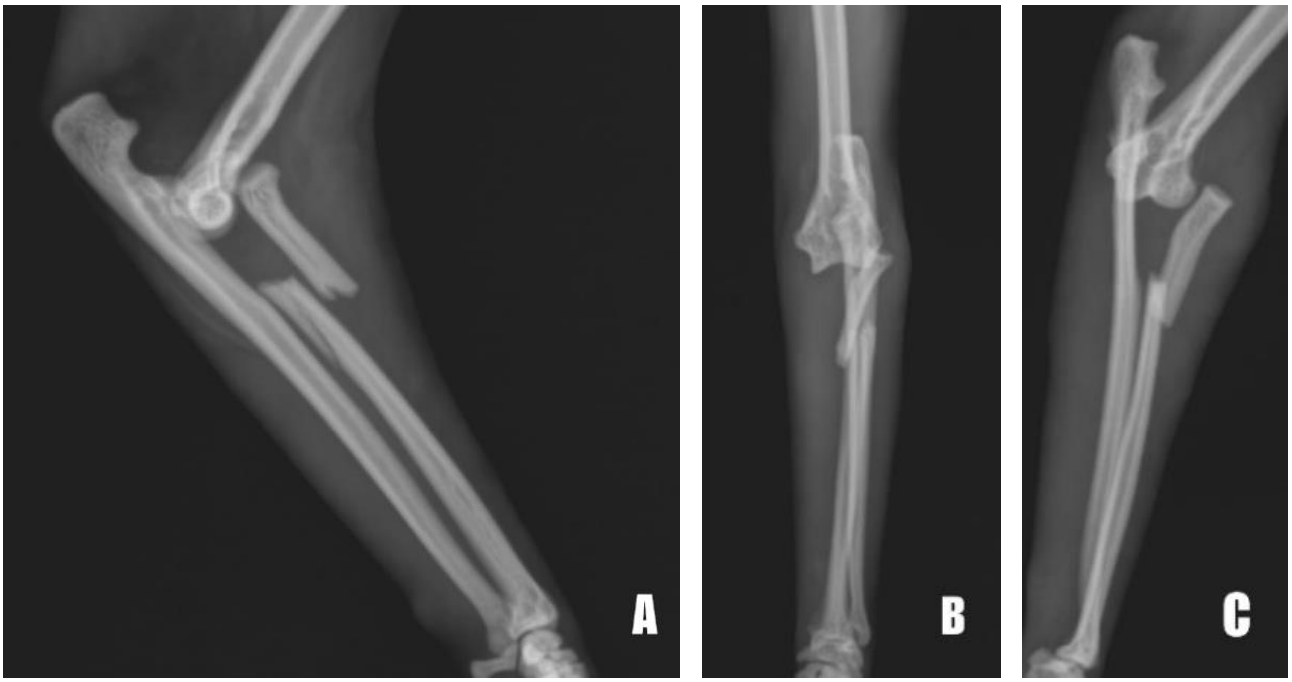

Figure 1. Preoperative radiographs of the left elbow joint.
A. M/L (Medio/lateral) radiography
B. Cr\Ca (Cranio/Caudal) radiography
C. Cr-M \ Ca-L (Cranio-medial / Caudo-lateral oblique) radiography.

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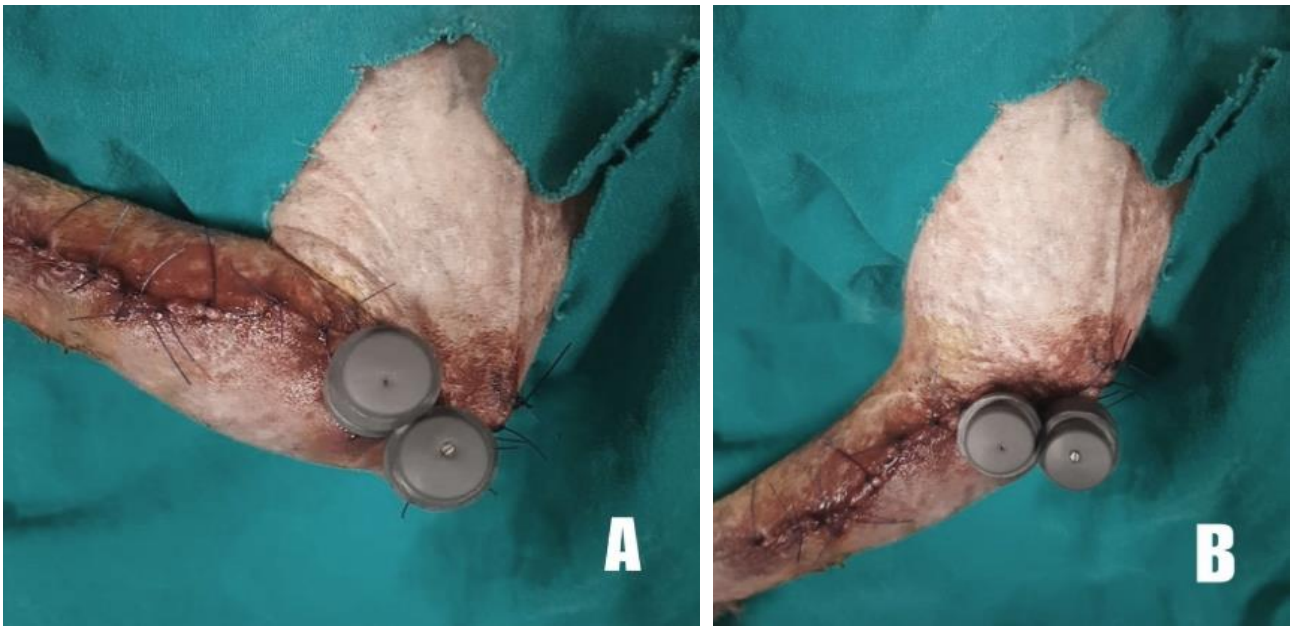

Figure 2. Postoperative view of the elbow joint.
A. 30 ± 5° flexion
B. 150 ± 5° extension.

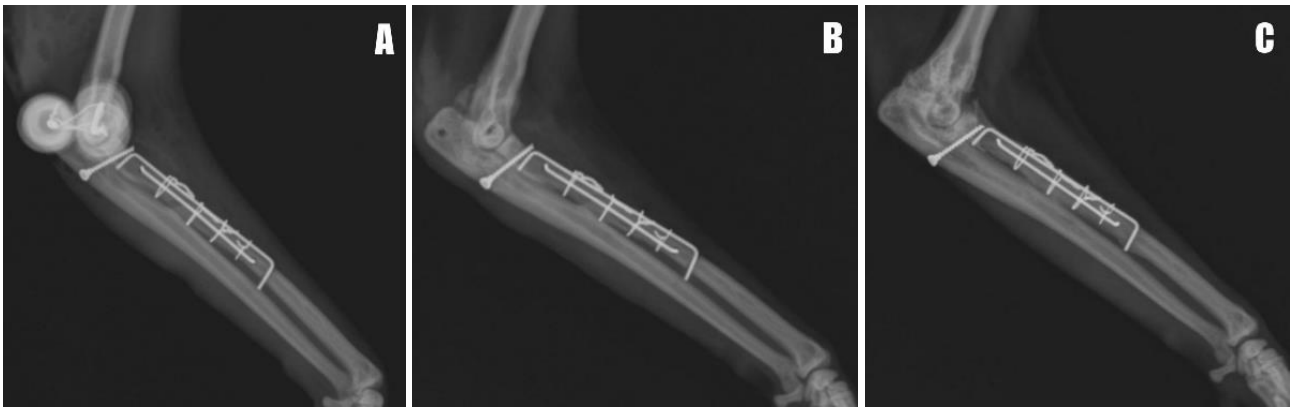

Figure 3. Long-term radiographic follow-up at 1 year after surgery, radiographs show a bony ankylosis of the left elbow involving the humero-ulnar joint.
A. Immediately post-operative M/L radiography
B. 10 days postoperatively M/L radiography
C. 1 year postoperatively M/L radiography.

The fixator was removed on the 10th postoperative day, and the flexion angle of the elbow joint was measured as 45 ± 5° and the extension angle 140 ± 5°. The patient was reassessed 4 weeks postoperatively, and the flexion angle was measured as 65 ± 5°, the extension angle as 125 ± 5°. The last postoperative follow-up examination occurred 1 year after surgery. A humero-ulnar ankylosis of the elbow joint with 110° of the extension was observed (Figure 3 and Figure 4).

Traumatic elbow dislocation is an uncommon orthopedic disorder observed in cats and dogs (1, 3, 6, 8, 13). Direct and indirect forces applied to the elbow typically cause articular or periarticular fractures rather than luxations. Elbow dislocation is frequently the result of vehicle trauma and is thought to result from the indirect effect of rotational forces (3). Even though luxation can occur in the lateral or medial direction, more than 90% of luxations occur laterally (1-3, 6, 8).

Various open techniques have been described for the treatment of elbow dislocations. However, better results are obtained if the joint is stable after closed reduction (1). A dislocated elbow should be reduced as soon as possible under general anesthesia. The Campel test should be performed to assess collateral stability after closed reduction. Open reduction should be considered if the joint cannot be reduced or if instability is evident after closed reduction (1, 3-6, 8, 13). However, the requirement for surgical intervention when only mild instability follows reduction is also controversial (11). In this case report, open reduction was preferred since the patient was

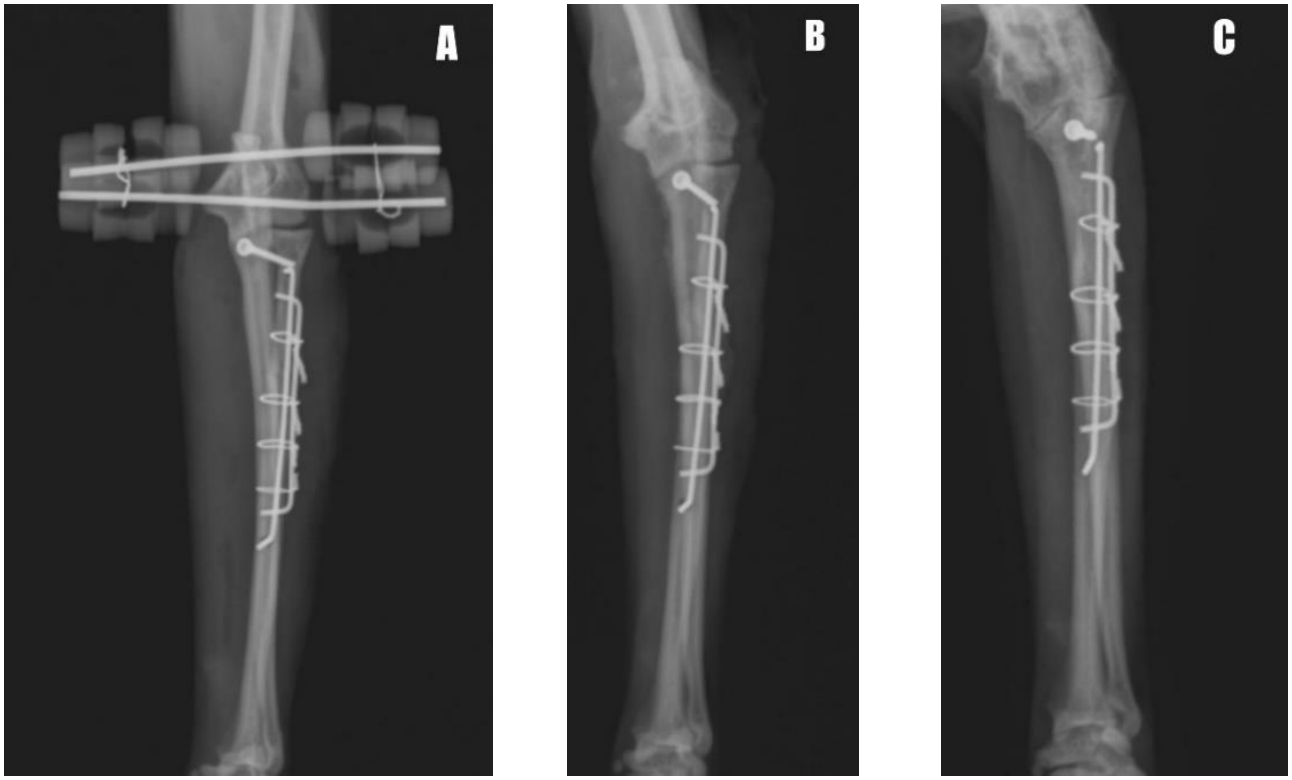

Figure 4. Long-term radiographic follow-up at 1 year after surgery, radiographs show a bony ankylosis of the left elbow involving the humero-ulnar joint.

A. Immediately post-operative Cr/Ca radiography
B. 10 days postoperatively Cr/Ca radiography
C. 1 year postoperatively Cr/Ca radiography.

presented 10 days after trauma and radius fracture detected accompanying the elbow dislocation.

Treatment options for traumatic elbow dislocation include closed and open reduction. Immobilization of the joints for at least 2 weeks is recommended to prevent reluxation, and therefore external coaptation is often used. When joint immobilization is applied for more than 2 weeks, it causes to joint stiffness and degenerative joint disease (7, 9). On the other hand, continuous passive movement is recommended during the postoperative care period (9, 10). In studies using elastic transarticular external fixator (ETEF) in the treatment of elbow dislocation, rigid bars were used in the first days until the post-operative swelling decreased, then the bars were replaced with elastic bands (9, 12). In this study, only cerclage wires were used as a bar until the fixator was removed and no complication had occurred, such as reluxation.

Although cats and dogs have a similar collateral ligament anatomy, the cat's anconeal process is relatively smaller than that of dogs. In spite of this, one study reported that after transection of the collateral ligaments (CLs), feline elbows luxated significantly less consistently than canine elbows (3, 11). A biomechanical study of canine elbows reported that elbows retain their original stiffness after MCL section. The same study reported that suture repair failed in all elbows after MCL section. Therefore, external coaptation or external fixation is recommended for the elbow joint after MCL repair (3, 11). Nonetheless, the importance of CL deficiency in traumatic elbow dislocations is still discussed (11). In this case report, a complete rupture of MCL was detected, but it has not been repaired. After the reduction of the articulatio humeroulnaris, CTEF was applied and the ulna was fixed to the radius with a cortical screw. Pin tract drainage or pin loosening, which is frequently encountered in the external fixators, was not observed in this study due to the short-term use of the external fixator.

Recent studies have reported varying degrees of osteoarthritis as a result of open or closed reduction. Although ROM decreased in these patients, it was reported that patients tolerated it well (1, 4). The patient was reassessed 10 days postoperatively. Mild osteoarthritis and decresed ROM were observed. The last postoperative follow-up examination occurred 1 year after surgery. A humero-ulnar ankylosis of the elbow joint with 110° of the extension was revealed. There was a obvious lameness on the left thoracic limb, especially while running. However, the elbow joint was pain free, and the

patient continued with its daily activities without any obvious limitation.

In this case report, the post-operative long-term clinical and radiographic results were not as expected. To the best of our knowledge, the long-term fair clinical outcome with the development of elbow osteoarthritis, could be explained by the inability to perform surgery at the desired time, the severity of the trauma, and cartilage damage. However, further cases are required to determine whether this procedure is optimal for the treatment of traumatic elbow luxation in cats.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

SU is the primary author who planned, designed the writing of the work, and supervised all procedures. FQ, MY-D, and AD contributed to case management and manuscript preparation. AB contributed to manuscript review and editing.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Abrescia P, Cinti F, Pisani G** (2019): *Traumatic caudal elbow luxation in two cats.* Open Vet J, **9**, 361-365.
2. **Bongartz A, Carofiglio F, Piaia T, et al** (2008): *Traumatic partial elbow luxation in a dog.* J Small Anim Pract, **49**, 359-362.
3. **Farrell M, Draffan D, Gemmill T, et al** (2007): *In vitro validation of a technique for assessment of canine and feline elbow joint collateral ligament integrity and description of a new method for collateral ligament prosthetic replacement.* Vet Surg, **36**, 548-556.
4. **Farrell M, Thomson DG, Carmichael S** (2009): *Surgical management of traumatic elbow luxation in two cats using circumferential suture prostheses.* Vet Comp Orthop Traumatol, **22**, 66-69.
5. **Hamilton K, Langley-Hobbs S, Warren-Smith C, et al** (2014): *Caudal elbow luxation in a dog managed by temporary transarticular external skeletal fixation.* Case Reports in Vet Med, **2014**, 1-7.
6. **Istrate A, Peteoaca A, Tanase A, et al** (2018): *Open reduction methods of luxations in dogs and cats: a comparatıve study*. Scientific Works Series C Vet Med, **64**, 48-53.
7. **Jaeger GH, Wosar MA, Marcellin-Little DJ, et al** (2005): *Use of hinged transarticular external fixation for adjunctive joint stabilization in dogs and cats: 14 cases (1999–2003).* J Am Vet Med Assoc, **227**, 586-591.
8. **Mitchell KE** (2011): *Traumatic elbow luxation in 14 dogs and 11 cats*. Aust Vet J, **89**, 213–216.
9. **Schwartz Z, Griffon D** (2008): *Nonrigid external fixation of the elbow, coxofemoral, and tarsal joints in dogs.* Compend Contin Educ Pract Vet, **30**, 648-653.
10. **Slater RB, Simmonds DF, Malcolm BW, et al** (1980): *The biological effect of continuous passive motion on the healing of full-thickness defects in articular cartilage. An experimental investigation in the rabbit.* J Bone Joint Surg Am, **62**, 1232-1251.
11. **Suess Jr RP, Trotter EJ, Konieczynski D, et al** (1994): *Exposure and postoperative stability of three medial surgical approaches to the canine elbow*. Vet Surg, **23**, 87-93.
12. **Vedrine B** (2017): *Use of an elastic transarticular external fixator construct for immobilization of the elbow joint*. Can Vet J, **58**, 353-359.
13. **Williams H, Calvo I, Gaines A, et al** (2020): *Multi-Centre retrospective study of the long-term outcome following suspected traumatic elbow luxation in 32 cats.* J Small Anim Pract, **61**, 354-362.

Sustainable Livestock Farming with Oil Seed Crops and Their By-Products

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Abstract

The increasing human population and food shortage are fueling the demand for alternative feed resources for animals not meant for human consumption. Oil seeds and their derivatives are suitable options to meet the escalating global demand for animal feed proteins; camelina is one of them. Camelina sativa (CS), an ancient oilseed crop belonging to the Brassicaceae family, is known for its resistance to drought and cold, as well as its various uses for meal, oil, and other products. However, it also has some anti-nutritional factors (ANF) that can limit its use as animal feed. These ANFs can be reduced by various methods, such as enzyme addition, heat treatment, fermentation, or genetic engineering. CS and its by-products can affect animal metabolism, especially lipid metabolism and hormone levels, and can also improve the fat profile of meat and milk products, making them more suitable for human consumption and health. CS and its by-products achieved weight gain and protected dietary PUFAs, but decreased bio-hydrogenation intermediates. Small ruminants fed CS-supplemented diets produced meat with a suitable fat profile for human consumption. Feeding with CS seeds and derivatives decreased milk fat concentration, yield, and fat-corrected milk. Camelina forage, however, increased the milk fat percentage. The effects of CS and its by-products on milk fatty acid composition were contradictory. CS meals may improve the composition of milk products, making them healthier for humans. Researchers need to determine how CS meals can be used in dairy ewe and goat diets at different life stages.

Introduction

The world population is steadily increasing, which has led to increasing concerns about food security for human beings (31). Critics have targeted the ruminant production system for competing for feed supplies that could be used for human consumption. Ruminants have a lower feed conversion ratio than monogastric animals, which, combined with their higher environmental footprint, raises concerns (36, 68, 105). Ruminants, on the other hand, are important for livestock sustainability because they can consume crop residues and by-products that are not suitable for human consumption while also producing high-quality milk and meat (64). Milk and milk products, meat, and meat products have been reported to provide 25% of the total dietary energy intake and account for half of the saturated fatty acids (SFA), the primary source of monounsaturated fatty acids (MUFA), n-3 polyunsaturated fatty acids (PUFA), and trans-fatty acids for humans (107).

Enriching the animal diet could improve animal health and increase the contribution of milk and meat to the dietary intake of beneficial FAs (6, 90, 107). However, in several countries, forages have low protein levels, and they import high-protein resources, such as soybeans, which is costly. To address these issues, research on alternative feed resources has gained attention. The use of alternative feed resources is determined by their nutritional composition, animals, price, and environmental impact (1, 18, 81, 95). Agro-industrial byproducts are a promising source of alternative feed.

Soybean, rapeseed, canola, sunflower, cottonseed, groundnut, linseed, chia, palm kernel, and hempseed are commonly used oil- seeds, and their by-products after processing during oil extraction contain high volumes of nutrients and bioactive compounds. During the production process, manufacturers obtain a substantial byproduct in the form of oilseed cakes and meals. The nutritional composition of these byproducts varies a lot and can affect the health, production, and performance of animals. The nutritional makeup of these byproducts varies considerably, which can have a significant impact on the health, productivity, and performance of animals.

Camelina sativa (L.) Crantz has garnered attention from scientists worldwide as a potential source of healthy and nutritious ingredients for use in both food and feed. The positive agronomic traits, such as excellent environmental adaptability, drought resistance, cold tolerance, pest and pathogen resistance, a shorter life cycle, and tolerance to other environmental stresses, result in a reduced need for inputs, making it advantageous for farming (65, 72).

Camelina sativa (CS) seeds, oil, and cake are exceptional sources of beneficial fatty acids (FA), particularly long-chain PUFA. In addition to being an energy source for high-producing animals, CS seeds and their derivatives have the ability to decrease methane emissions, which is a major contributor to environmental pollution as a greenhouse gas (94, 100). Unlike other oilseed plants, CS also contains anti-nutritional factors (ANF) that can impair the performance of animals if included in large amounts in their diets (75). However, after oil extraction, CS meal showed a lower amount of crude fat and an increased amount of crude protein and amino acids (23).

The amino acid profile of the supplemented SC shows a more significant effect on the mRNA expression levels of the selected genes that are relevant to ewes' immune systems (20). Moreover, new CS varieties and the application of technologies for their processing have lowered the antinutritional content (35, 75). Researchers have discussed the effects of CS seeds, oil, and their by-products in ruminants, swine, poultry, and other animals (7, 70, 83, 98, 110). Nevertheless, the effects of CS seeds and their derivatives on feed intake, rumen digestion, fermentation, milk production, meat production, and composition are unclear because of conflicting results and the limited availability of research on small ruminants.

The present review aims to provide a comprehensive overview of the available literature on the general characteristics and nutritional composition of CS seeds and their derivatives as alternative feed sources, along with their use in the feeding of small ruminants, including their impact on the overall health and performance of small ruminants, such as feed intake, digestion, metabolism, milk production, composition, and milk by-product quality.

Use of Oil Seeds and Its By-Products in Animal Nutrition

There is a growing interest in identifying locally generated alternative protein feed sources to replace soybean meal in livestock production due to issues such as resource depletion, population growth, unsustainable consumption habits, rising demand for animal-sourced food, and climate change (4, 78).

The world's top cultivated seeds are soybeans, sunflowers, rapeseed and canola (102). Additionally, the world market has camelina, linseed, cotton, coconut, hempseed and pumpkin as noteworthy oilseeds (85). In animal nutrition, oilseeds are primarily utilized to provide various vegetable oils. The oil industry now provides protein-rich byproducts for livestock feed, in addition to oil. This is due to the abundance of byproducts accessible after extraction of oil. Cakes and meals are byproducts produced after the majority of the oil has been extracted from oilseeds. Oilseed cakes and meals may serve as an alternate protein source to meet the growing demand for protein-rich foods. Indeed, the worldwide need for animal protein is predicted to double by 2050 (73).

Soybean meal (SBM), a significant protein source, is commonly included in feed mixer rations (16) to boost the protein composition of diets. SBM has a CP concentration of 42-50%, which contains a major part as rumen-degradable protein (43). Nonetheless, as SBM is in popular sources, the price has risen, resulting in increased total feed costs. Furthermore, expanded soybean cultivation and enhanced commercial crop production are typically connected with negative environmental outcomes (54).

Rapeseed meal (RSM), which is the post-pressing leftover, is generated at 39 million metric tonnes per year (48), mostly used as animal protein feed (104). However, other dietary components (phenolics, glucosinolates, lignocellulosic fibre and phytates) impede the direct utilisation of RSM. This might harm protein solubility, digestion, and the production of toxic compounds. This has restricted both the species of animals that can be offered RSM and the percentage of RSM in overall diet. Ruminants, for example, may tolerate RSM due to their complicated digestive systems (97), It can only be used in up to 50% of swine feed and is not suggested for poultry (106). These limits have reduced the price of RSM low as compared to the more desirable SBM.

Canola meal (CM), a byproduct of canola oil extraction, has tremendous potential as animal feed since it comprises 35-40% protein, somewhat less than SBM (25), but much more B-vitamins and minerals. However,

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CM is a significantly lower-valued feed than SBM due to its high fibre and anti-nutrient concentration. Its use is confined to ruminant animals, and in some markets, it is applied straight to the soil as fertiliser (32). Anti-nutrients found in CM, such as phenolic chemicals and glucosinolates, might impair cattle growth performance (58). The overall fiber content in CM is on average 31.7 percent of dry biomass, which is greater than in SBM (61). Non-ruminant animals digest fibres poorly, particularly hemicellulose.

Sunflower meal (SFM) contains a significant protein level (about 30% to 50%) (27) e.g. albumins (17-30%), globulins (mostly helianthin protein), and other small proteins, such as oleosins (38). SFM peptide isolates are free of harmful ingredients and have a lower level of anti-nutritional components than other protein-rich alternatives such as mustard meal (glycosylates), SBM (trypsin inhibitors), or cotton meal (gossypol) (39).

Camelina is sometimes called false flax or gold of delight, is a Brassicaceae family oilseed crop. This crop's enormous potential is also being used to produce a stable feed for its variety of applications and to enhance dryland agriculture (49). Camelina has a comparable nutritious profile as CM, with high amounts of protein and fibre, but it is not as excellent a source as SBM, which contains more protein and less fibre. The oil extraction process yields identical meals, but the ejected camelina meal has significantly more fat and less protein, whereas the solvent-extracted meal contains less fat and more protein. This is particularly notable since the high quantities of glucosinolates in camelina meal are a major obstacle to its use in animal feeds, particularly for pigs. Lowering the glucosinolates by thermal processing, fungus fermentation, or genetic manipulation to develop low-glucosinolate variants may increase the nutritional value of camelina meal (29). In this review we will discuss the details of camelina usage in animal nutrition.

Discovery and Distribution

Bu In Auvervier and Switzerland, CS was cultivated as far back as 4000 BCE (41) the Iron Age (100 CE-250 BCE) (52) and evidence of extensive planting across northern Europe from Southern Scandinavia (103) to central Asia (eastern Turkey). The CS was grown for food and oil production, and it was widely accessible by the late Bronze Age (1200 BCE), according to archaeological sites (15). The cultivation of CS decreased throughout the Middle Ages but increased during the past century in northern, central, and eastern Europe, and the Balkans (51). False flax name was given to CS because it was probably brought to the Americas as a weed with flax (76). The CS is successfully farmed in the USA (34), and Canada (42). It tolerates heat stress with the following mechanism; increased root prospection and changes in the organic acid exudation are signs that camelina adopts a more acquisitive strategy (28).

General Characteristics

The CS is a heavily branching plant that is 20–80–100 cm in length morphologically. Its basal leaves form a rosette, and it has a taproot and whole or dentate leaves (62). The blooms have four nectaries and yellow petals, and terminal inflorescences. The fruits are tiny silicles with many seeds (11, 82), each of which has a high oil content (36 to 47 % (55). The positive agronomic traits of this oilseed can be very useful in agriculture, which is now developing into a significant issue for the environment and ecosystems (82). Due to their excellent environmental adaptability, CS crops can help to mitigate this problem. They can withstand drought, cold, pest and pathogen attacks, and other environmental stresses, which reduces the number of inputs needed for their maintenance, particularly irrigation, fertilizer, and pesticides (5, 7). In dry and semi-arid environments, its short life cycle (85–100 days), robust root structure, and resistance to cold weather make it a suitable choice for crop rotation, reducing fallow time and offering an alternative to monoculture (111).

Crop rotation and intercropping are said to have positive effects on the environment, including reducing weed growth, increasing soil organic matter, and reducing erosion (10, 22, 53). The CS possesses nectareous blooms (112). Cultivating CS increases agricultural productivity by increasing the diversity of insects and by giving them a healthy environment (forage, nectar, nesting, etc.) (62). The CS stands out for its wide range of applications in addition to its advantages for ecology and its agronomic traits. Due to the high protein content of the seed meal (60, 83) and the excellent nutritional value of the seed oil (80), studies are also being conducted to investigate its potential application in animal and human feed. Finally, in this context of climate and global change, CS crops can play a significant role in lowering the consumption of fossil fuels, the use of land, and the production of greenhouse gases, thereby assisting in the development of circular and sustainable agriculture that does not harm ecosystems or biodiversity (66, 93).

Factors Affecting *Camelina Sativa*

Factors affecting the production of CS include biotic (weed, insects, disease) and abiotic (temperature, water, salt (NaCl and KCl). The findings of a study (108) show that salt (NaCl and KCl) stress significantly lowers the speed, percentage and index of germination, shoot length, root length, vigor index, root shoot ratio, and seedling fresh weight of the salt-treated CS seeds. At the highest levels of salt concentration (5g/L), the fresh weight of seedlings dropped as seedling length fell with increasing

salinity levels. Although CS is resistant to various external factors compared to other plants still some factors need to be considered, which are shown in figure 1.

The CS seed vegetable oil is extracted by different methods which has been displayed in Figure 2.

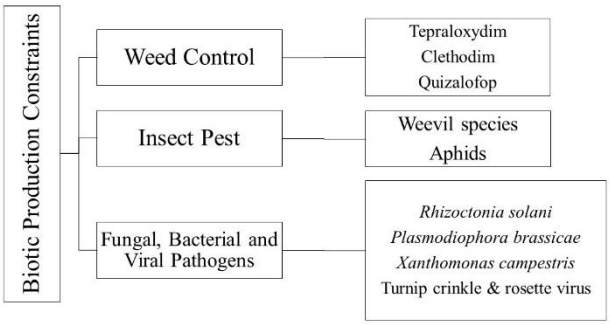

Figure 1. Biotic production constraints for Camelina Sativa Extraction of oil from *Camelina sativa*.

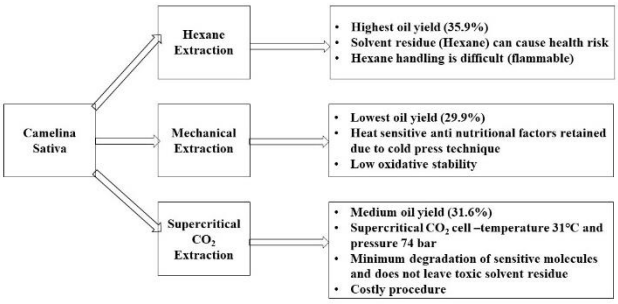

Figure 2. Different methods of oil extraction from *Camelina Sativa.*

Combinations of both methods are most often used for economic reasons since the pressing process leaves a significant amount of residual oil in the oil cakes and meals, which can be extracted by solvent extraction. There is another method of oil extraction which is instant controlled pressure drop (DIC), when using solvent extraction, DIC pre-treatment combined with Accelerated Solvent Extraction (ASE) enabled the extraction of 10.8% more oil from CS seeds compared with untreated seeds. Bouallegue et al. (9) stated that the best way to extract CS oil is by DIC pretreatment since it increased CS oil yields, speeds up the extraction process, and valorized pressing meals. Indeed, CS and its byproducts contain moisture (6-11.4%), dry matter (88.2-94%), crude protein (19.35-41.1%), NE_L (2.20-2.58 Mcal/kg), ADF (11.1-22.53%) and NDF (22.7-39.9%) based on dry matter (83).

Nutritional Characteristics of *Camelina Sativa*

The CS stands out from other oilseed crops thanks to its appealing nutritional profile. Understanding this profile is essential to comprehending both the promise of this crop in terms of human and animal nutrition, as well as the difficulties experienced in increasing its nutritional features. The CS seeds have a high amount of oil, with a weight percentage of oil content ranging from 38% to 43% (113). Particularly interesting is the fatty acid makeup of this oil. Alpha-linolenic acid (ALA), an omega-3 fatty acid, is the major form of polyunsaturated fatty acid (PUFA) found in abundance in it (63). CS oil contains PUFAs in a larger proportion than many other oilseed crops, at around 35% of the total fatty acids (44).

Because the human body is unable to produce omega-3 fatty acids, it is necessary to consume them in the diet (92). They have been associated with several health advantages, including lowering heart disease risk and inflammation, enhancing mental health, and promoting growth and development (14, 84). CS oil is a good plant-based source of these vital nutrients due to its high omega-3 level.

In addition to having a lot of oil, CS also has a lot of protein. It has been discovered that the leftover seed meal contains up to 40% protein after oil extraction (46). This protein-rich meal might be used as a healthy ingredient in livestock feed, creating sustainable animal farming techniques (33). Additionally, the oil of CS is high in tocopherols, a class of chemicals sometimes called vitamin E (50). Strong antioxidants like vitamin E shield cells from the harm that free radicals may do (69). Additionally, it is essential for immunological performance, cell communication, and other metabolic activities (87). The CS oil's high tocopherol concentration increases its nutritional value and extends both the stability and shelf life of the oil (86).

The CS's high plant sterol content is another beneficial nutritional characteristic. These substances, often referred to as phytosterols, have been demonstrated to prevent cholesterol absorption in the stomach, hence assisting in the reduction of blood cholesterol levels (56). Consuming meals high in plant sterols may improve heart health since eating high cholesterol-free diets lowers the chance of developing heart disease (45).

The CS is distinguished for its higher nutritional profile when compared to other oilseed crops. For example, CS oil, one of the most popular vegetable oils, has a much greater omega-3 concentration than canola oil (59). Furthermore, its protein level is equivalent to that of soybean meal, a key ingredient in livestock feed (47).

The CS is a desirable choice for initiatives to improve the quality and quantity of oilseed crops because of its overall nutritional characteristics. Nevertheless, despite its promise, there are still obstacles standing in the way of completely reaching this potential, such as genetic restrictions, agronomic problems, and legal barriers (79). Along with its positive aspect, some anti-nutritional factors are also present in it, which are described in the anti-nutritional factor part.

Anti-Nutritional Factors

There is a significant difference in protein content between different types of CS. In contrast to the Alba variety, whose autumn and spring values did not surpass 39 g 100 g of DM, some of them had values greater than 45 g 100 g of DM in both sowing times (75). With the high protein content of CS, some ANF also come with them to reduce their digestibility which has been elaborated below.

Trypsin Inhibitor: The presence of trypsin inhibitor (TI) in CS seeds has not received much attention up to this point, even though it is known that they negatively impact protein digestion by blocking the proteinase enzymes (2, 12). This calls for careful consideration when selecting the types to be used as a source of protein in the diets of fish and mammals (17, 40). Like, Budin et al. (12) and Almeida et al. (2), Pozzo et al. (75) likewise found a broad range in TI content. The mean results, however, were lower than those mentioned in the earlier investigations. Regarding Luna, the greatest value observed for the spring Spanish CCE29 was about three times higher than the lowest. The figures for Luna and Przybrodzka, on the other hand, that were lower than 7.0 TIU mg^{1} during spring sowing indicated that their addition to feed, at the proper proportion, would be safe (75). The lowest level of TI was likewise found in the Luna and Przybrodzka meals, and the planting season does not appear to have an impact on their quantity.

Glucosinolates: Secondary metabolites called glucosinolates (GLSs), which include sulphur, are mostly found in the Brassicaceae family of plant species. They are the primary cause of CS meals' exclusion from animal feed. Genetic and environmental variables have been implicated in variations in the quantity and pattern of GLSs (91, 101). The GLSs were between 25.66 to 38.94 µmol/g. The cultivars CCE36, CCE26, and Pearl (in both sowing seasons) had the maximum GLS content (> 36 µmol/g), but Luna and Przybrodzka's meal had the lowest amount (26.5 µmol/g). Between the two planting dates, all varieties showed equivalent levels of GLSs, however only the Spanish varieties CCE26, CCE29, CCE32, and CCE40 and Celine showed discernible changes (75). The GLS pattern showed variations amongst the kinds as well. The literature found three primary GLSs: GLS9 (glucoarabin), GLS10 (glucocamelinin), and GLS11 (91). With a content of around 65 per cent of the total glucosinolates, GLS10 was as predicted the most prevalent glucosinolate in all kinds (data not shown), and following Russo and Reggiani (89), the quantity of GLS9 was frequently greater than GLS11. Only Przybrodzka (75), one of the other types examined in this study, had a significant aliphatic GLS chain elongation that resulted in a greater concentration of GLS11 than GLS9. Despite being less common, several genotypes have previously been shown to have more GLS11 than GLS9 (3, 88). Some scientists linked this unusual pattern to winter biotypes, hybrids, or wild species of CS (3, 91). Brassicaceae are known for their tendency to create GLSs with elongated chains, however longer aliphatic chains are thought to reduce the likelihood of hazardous chemicals being produced during the breakdown process.

Sinapine: A meal may acquire an unpleasant odour and flavour when sinapine (96), a choline ester of sinapic acid, is present in large proportions. Which, in turn, may have an impact on its flavour and the standard of animal products. With average values of 3.65 and 3.88 µg/mg for the two sowing dates, the sinapine in this study varied from 2.92 g µm/g (Ligena in spring sowing) to 5.03 µg/mg (Pearl in autumn sowing) (75). Sinapine was found at a level comparable to that reported for CS by Amyot et al. (3) and Juodka et al. (47), although it was much lower than that of other Brassicaceae species, whose median values ranged from 12 to 15 µg/mg (57, 114). Therefore, it might be concluded that sinapine side effects should not arise in the tested CS varieties.

Phytic acid: Due to its potent chelating abilities with nutritionally significant cations (Ca, Fe, Mg, Zn), phytic acid (PA), another ANF present in CS meal, can be a cause of the problem. The PA quantity in the different cultivars under investigation varied from 24.96 to 33.62 µg/mg in the fall planting (75). The amount of PA reported by Zubr and Matthäus (114) is consistent with the quantity and significant heterogeneity. In general, the amount of PA was a little bit lower in spring sowing than in fall sowing, with Alba, Cypress, and Celine being the exceptions. Different methods to reduce ANF have been summarized in Figure 3.

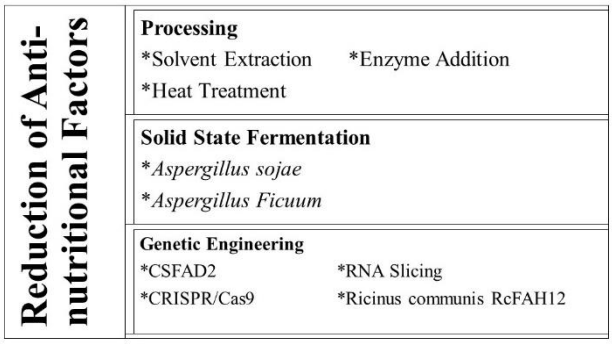

Figure 3. Reduction of Anti-Nutritional Factors of *Camelina Sativa*.

Use of *Camelina Sativa* and Its by-products in Small Ruminants

Effect of Camelina sativa and by-products on Feed Intake, rumen digestion and metabolism in small Ruminants: Dry matter (DM) is the primary component in the preparation of animals' rations, and the DM intake and digestibility are crucial factors influencing animal performance. In high-producing animals, sufficient DM intake (DMI) is critical to provide adequate nutrients. Additionaly, the use of natural, safe, and sustainable intervention options, such as the incorporation of oils, oil-seed plant, and by-products has the potential to contribute to the safe production of animal products. Current evidence regarding the effects of CS and its by-products on the DMI of small ruminants is inconclusive. Noci et al. (67) studied the effects of various plant seeds, including CS seeds, linseed (LS), and NaOH-soaked CS, NaOH-soaked linseed, as well as oils such as camelina sativa oil (CO), linseed oil, and ethanolamine-reacted CO, and rumen-protected saturated fats (SF), on lambs. The DMI increased with the oil-supplemented diets compared to the seeds supplementation. However, the CS-supplemented diets reduced DMI compared to the linseed diets. The DMI was higher in the CS diet than in the NaOH-treated seeds. Similarly, DM, organic matter (OM), neutral detergent fiber (NDF), and fat digestibility were similar among the treatments compared to the control; however, crude protein (CP) digestibility increased, while ash digestibility increased in all treatments compared to the control. Similar to large ruminants (78), DM and OM digestibility was higher in CS-supplemented diets than in linseed-supplemented diets. Compared with the oil-supplemented diets, DM, OM, and CP digestibility decreased in seed-supplemented diets.

Studies also reported the effect of CS seeds cake (CSC) (12% in concentrate) and dried distiller grains (DDGS) (12% in concentrate) on blood serum metabolic, hormonal, and FA profile in the lactating ewes. Compared to the control, CSC supplementation reduced the levels of triglycerides, glucose, free fatty acids, and insulin. Blood urea nitrogen, alanine transaminase (ALT), aspartate transaminase (AST), leptin, and T3 levels remained unaltered among all treatments. However, T4 concentration increased with CSC and DGGS diets, thus reducing the T3/T4 ratio. This increase in insulin and T4 concentrations could be the result of increased metabolism and oxidation of lipids in the liver and muscles (83). Similarly, the serum fatty acid profile showed no change in saturated fatty acid (SFA), unsaturated fatty acid (UFA), MUFA, PUFA, n-3, n-6, n-6/n-3, medium-chain FA, or long-chain FA compared to the control diet. However, DGGS resulted in higher levels of MUFA, PUFA, and n-6 FA. Based on the available literature, the effects of CS seeds on the DMI in small ruminants are unclear. Studies have shown that supplementing the diet with CS and its by-products can cause changes in metabolism, particularly lipid metabolism and hormone levels. More research is needed to fully understand and optimize DM intake, digestion, and metabolism of the animals fed diets containing CS and its by-products.

Effect of the Camelina products on meat production, carcass traits, and tissue composition: Diet composition and supplementation can lead to changes in the rumen environment, microbial community and fermentation (30, 109). The presence of GSLs in CS is a concern when used as a replacement for protein and fat sources in ruminant diets, because they can have toxic effects on thyroid function and cause metabolic imbalances. However, Noci et al. (67) reported similar average daily gain (ADG), total intramuscular adipose tissues in the muscles of animals fed plant seeds or oils. Carcass weight and perirenal fat increased with oil supplementation compared with in those seeds. However, the results remained similar for the CS products (seeds vs oil). However, CS amides increased liver and kidney weights compared to the LS diets fed animals. Ramírez et al. (77) also reported no change in the average weight gain and ADG when compared the diet containing 50% CS meal (CM) in replacement of soybean to the fibrous diet containing a small amount of CM and other fibrous diets without grains and soybean, and control diet containing soybean and grains. The fibrous diet resulted in higher DMI in comparison to the CM and the control group. However, the lower feed intake compensated for the price in the CM and control diets. This higher feed intake in fibrous diet fed group could be the result of less energy supply by the fibrous diet, which was 20% lower than that of the other two diets.

Carcass traits such as hot carcass weight, cold carcass weight, dressing percentage, chilling loss, and pH at the time of slaughter and 24 h after slaughtering remained similar with diet having 50% soymeal-replaced with CM and fibrous diet (containing CS husk) compared to the control diet. The total fat, lean meat, and bone percentage remained similar among all diets. Similarly, after seven days of storage of meat, muscle color, cooking loss, dripping loss, Warner-Bratzler shear force (WBSF), and thiobarbituric acid reacting substances (TBARS) did not change among the treatments; however, pH was slightly higher in fibrous diet meat, which may be owing to the lower glycogen content of the muscle (74, 77). While studying the effect of CM (8% inclusion in the ration) and CS hay (45% inclusion in diet) reported an increase in fatty acid (n-3 FA, MUFA, PUFA) in the muscles from yearling and lamb meat compared to the control diet. Both animal type and storage period affected the oxidative stability of lipids and meat color. Researchers have reported a lower amount of vitamin E in

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the muscles of animals supplemented with CM and CS hay. Therefore, fortification with vitamin E is recommended to prevent color change during display and change in cooked meat color to off-white when long-term storage under semi-frozen conditions is required. The CS diet supplementation results in higher muscle fat; therefore, vitamin E supplementation is required to protect essential fats from oxidation.

Partial replacement (50%) of soybean meal (SBM) with CM and concentrates without grain and SBM offered to lightweight lambs for fattening resulted in altered FA composition in the muscles. Collectively, total SFA increased with partial replacement of CM and decreased with the fibrous diet compared to the control. However, CM replacement resulted in higher cis-MUFA compared to the fibrous diet, which produced higher MUFA trans-isomers, mainly the 18:1 isomer. Total CLA increased with a fibrous diet and remained similar for CM supplementation compared to the control (37). Noci et al. (67) reported a decrease in SFA with the supplementation of NaOH-treated CS, however, SFA remained unchanged with all the other treatments in intramuscular fat. The CS also resulted in higher MUFA content. The NaOH-treated seeds resulted in higher PUFA and n-3 FA compared to oil supplementation, whereas n-6 remained similar among all treatments. The PUFA/SFA ratio was similar for CM and CO and higher for LS. Seed supplementation resulted in a lower n3/n-6 ratio, which was significantly lower in the LS than in the CS seeds. NaOH-treated CS seeds supplementation resulted in an increase in cis-9 trans-11 CLA levels in subcutaneous adipose tissue and intramuscular fat. Thus, the health-beneficial FA can be increased efficiently within the muscles with NaOH-treated CS seeds or LS seeds, which can easily be processed on-farm. The use of CS and its by-products resulted in comparable outcomes for weight gain and a more substantial protection of dietary PUFA, but it decreased the incorporation of bio-hydrogenation intermediates. However, meat from animals fed diets supplemented with CS and its by-products showed a fat profile that is suitable for human consumption.

Effect of the Camelina products on the milk yield and Composition in Small Ruminants: The utilization of dietary oilseeds or their by-products affects milk production and composition, which is dependent on the inclusion level, derivative type, concentration of USFA, and composition of the basic diet (Table 1). Supplementation of dairy ewes with CS seeds at three different inclusion levels (6%, 11%, and 16%) did not change the milk yield, energy-corrected milk yield, fat-corrected milk yield, milk fat, or milk protein yield. In chemical analysis, the fat percentage decreased at the higher inclusion rate (16%), which also resulted in a decrease in the total solids percentage, while protein, lactose, and solid not fat remained similar among all treatments compared to the control (19). Similar results were reported by Szumacher-Strabel et al. (99), who studied the inclusion of CSC at rates of 10% and 20%, respectively, in the feed compared to the control (0%). The addition of 12% CSC resulted in similar results (26). Dairy ewes supplemented with CS forage (CF) had higher milk DM, fat, and lactose percentages than the control, while protein and ash contents remained unaltered (24).

Table 1. Effect of the Camelina sativa and its by-products on the production and composition of milk in small ruminant's.

Treatment	Inclusion rate (%, DM Basis)	Milk Production (g/d)	Lactose (g/d)	Protein (g/d)	Fat (g/d)	Lactose (%)	Protein (%)	Fat (%)	References
Control	0%[x]	1181	58.64	71.91	64.89	4.89	5.99	5.41[a]	
CSC	10%	1316	58.23	70.65	61.22	16.13	5.89	5.10[ab]	(99)
CSC	20%	1272	58.19	71.30	55.68	15.75	5.94	4.64[b]	
Control	0%[x]	1705		89.15	99.76	4.94	5.21	5.89[a]*	
CSS	6%	1857		97.76	105.74	5.00	5.28	5.71[ab]	(19)
CSS	11%	1874		101.26	107.1	5.04	5.43	5.85[a]	
CSS	16%	1887		98.60	99.75	5.02	5.23	5.35[b]	
CFD						4.62[a]	3.72	4.33[a]	(24)
Control						4.54[b]	3.61	4.11[b]	
CS seeds	12%		5.75[y]	3.92	3.93[b]				(26)
Control	0%		5.98	4.08	5.28[a]				

CSC= Camelina seed cake; CSS= Camelina Sativa Seeds; CFD = Camelina Forage Diet
[x] = % in concentrate (DM basis)
[y]= Chemical composition presented as g/kg.
[a,b,c,]= Values with superscripts describe the significant difference (P<0.05).

Table 2. Effect of the Camelina sativa and its by-products on the composition of milk and meat fatty acids in small ruminants.

Treatment	Inclusion rate (%, DM Basis)	SFA (%)	MUFA (%)	PUFA (%)	CLA Cis-9, trans-11 (%)	Total CLA (%)	n-3 (%)	n-6 (%)	n-6/n-3	Reference
Meat Fatty acids Composition										
Control	0%[x]	0.26	0.23	0.10[b]	0.71 [b, y**]		22.60[b,y**]	81.41[y]	1.22[y**]	
CSC	10%	0.28	0.26	0.14[a]	4.12[a]		33.97[a]	109.22	0.95	(21)
CSC	20%	0.30	0.28	0.14[a]	6.14[a]		38.07[a]	107.27	0.83	
CSM	12%	33.34[a]**		15.44[b]	0.28[b]	0.57[b]				
FIBD	6%	31.12[b]		18.29[a]	0.79[a]	1.07[a]**				(37)
Control	0%	32.60[ab]		17.27[ab]	0.18[b]	0.46[b]				
Milk Fatty acids Composition										
Control	0%[z]	76.01[a]	19.78[d]	4.12[d]	0.45[d]		0.77d	2.91[d]	3.78[a]	
CSS	6%	69.93[b]	24.68[c]	5.19[c]	0.68[c]		1.05[c]	3.46[c]	3.30[b]	(19)
CSS	11%	66.69[c]	26.68[b]	6.44[b]	1.03[b]		1.24[b]	4.14[b]	3.34[b]	
CSS	16%	60.75[d]	30.90[a]	8.11[a]	1.65[a]		1.44[a]	4.91[a]	3.41[b]	
Control	0%[x]	71.88	20.93[c]	4.02[b]	0.62[b]		1.05[c]	2.76	0.26[a]	
CSC	10%	66.86	23.43[b]	4.96[a]	1.07[a]		1.53[b]	3.08	0.20[b]	(99)
CSC	20%	60.85	28.59[a]	5.43[a]	1.10[a]		1.87[a]	3.06	0.16[c]	
Control	0%	68.36[b]	27.53[a]	4.11[a]		0.53[a]	0.99	2.33		(26)
CSC	12%	65.48[a]	29.57[b]	4.95[b]		1.53[b]	0.96	2.15		
Control		62.41	16.26	5.34[b]	0.71[b]		0.94	3.40[b]		(24)
CFD		61.55	16.29	5.35[a]	0.91[a]		0.93	3.42[a]		

CSM=Camelina Sativa Meal; FIBD= Fibrous Diet; CH=Camelina Hay; CSS=Camelina Sativa Seeds; CSC= Camelina Seed Cake; CFD= Camelina sativa Forage Diet; FA=Fatty Acids; SFA= Total Saturated FA; MUFA= Mono-Unsaturated FA; PUFA= Poly-Unsaturated FA; n-3=Total n-3 FA, n-6=Total n-6 FA; CLA= Conjugated linoleic Acid

[1]= % on DM basis otherwise stated; x=%age of concentrate; [y]= Values presented as mg/100g; [z] = As fed basis

[a,b,c,*,**]= Values with superscripts describe the significant difference (* = P<0.05, ** = P<0.001)

Increasing levels of CS seeds resulted in a linear decrease in short-chain FA, SFA, and SFA/USFA and a linear increase in long-chain FA, MUFA, PUFA, n-6, and n-3 FAs. The n-6/n-3 ratio decreased with CS seeds supplementation (Table 2). Similarly, α-linolenic acid cis-9, trans-11 (C18:2), and trans-10, cis-12 (C18:2) also increased with CS seeds supplementation. Overall, when the health-promoting index was evaluated, it increased in all treatments. Similar to the supplementation of CS seeds, the antioxidant enzymes and total oxidant capacity of milk increased, indicating the stability of milk for a longer duration. In addition, biomarkers for oxidative stress also remained similar among the 6% and 11% treatments, with a slight increase in blood and a decrease in milk with 16% group (19). Dairy ewes supplemented with CF had similar milk fat SFA, MUFA, short-chain SFA, and n-3 fatty acids. Total MUFA, CLA, total n-6, and total n-3 FA increased and long-chain SFA decreased with CS seeds' inclusion in diet. Compared to other CS seeds and cake, CS forage inclusion in diets resulted in a higher increase in total n-6 FA and n-6/n-3, however, their content remained at favorable levels. Overall, forage supplementation resulted in higher quality fatty acid production.

Szumacher-Strabel et al. (99) also reported an increase in all the health-beneficial fatty acids in ewe's milk with supplementation of 10% and 20% CSC compared to the control. In addition, thrombogenic and atherogenicity indices increased. However, dairy ewes supplemented with CF did not show any change in the atherogenic index, thrombogenic index, hypocholesterolemic FA, or hypercholesterolemic FA. Similar to previous results in other ruminants, CSC supplementation resulted in increased trans-MUFA, which should be considered when supplementing CSC.When CSC is used in amounts of 10–20%, it causes considerable alterations in the fragrance of ewe milk. Sheep fed CSC milk lost the general dairy aroma. It has a distinct lack of freshness in its scents. Regardless of the quantity of CSC supplied in the feed, pasteurization of the tested milk intensified the dairy fat, and cooked odors. With an increase in the quantity of CSC in the feed and after pasteurization, the level of total volatiles increased significantly (13).

At present, only a single study is available on goats, where 12% supplementation of CSC in dairy goats resulted in no difference in DM, protein, fat, and mineral content of milk compared to the control. Compared to the control, goats fed the diet supplemented with CSC showed a change in the FA composition of the milk. The MUFA, PUFA, cis-9, trans-11 (C18:2), and CLA levels significantly increased, and SFA decreased in milk from CSC supplementation compared to the control (71). Feeding with CS seeds and its derivatives reduced milk fat concentration and yield, as well as the production of fat-corrected milk. However, camelina forage led to an increase in the milk fat percentage. Nevertheless, all other milk production and composition parameters remained unchanged in small ruminants fed diets containing CS and its by-products. However, the effects of CS and its by-products on the fatty acid composition of milk are unclear. Further studies are needed to evaluate the impact of different doses of SC and its derivative doses on milk production and composition.

Effect of the Camelina products on the milk by-products in small ruminants: Supplementation with 12% CSC was advantageous in terms of the FA profile of milk fat, leading to a greater proportion of MUFA, trans-MUFAs, and PUFA, including CLA. Milk from sheep with higher concentrations of bioactive ingredients is beneficial for the creation of yoghurt. Both immediately and 21 days after storage, yoghurt generated from CSC-supplemented milk showed the same beneficial variations in FA content. In addition, these bioactive components did not alter the color, acidity, and consistency or sensory characteristics (consistency, taste, and smell) of yogurt produced from CSC-supplemented milk compared to the control (26).

Cheese composition changes with the milk source and its composition (8). Caciotta cheese produced from the milk of ewes supplemented with CF showed no change in texture or color between the two treatments. Additionally, cheese produced from this milk did not show any difference in caciotta cheese DM composition and fatty composition, except for n-6 and n6/n-3, which remained higher in cheese similar to that of milk. Sensory properties, such as goat hardness, solubility, odor, taste, and overall liking, increased with the CF diet. However, all other studied sensory parameters remained similar among treatments (24). Similarly, the kefir produced from the milk of dairy goats supplemented with 12% CSC (% of DM in concentrate) showed no difference compared to the control diet milk kefir. However, similar to the milk composition, the incorporation of MUFA, PUFA, and CLA increased in kefir. Sensory parameters such as the taste, consistency, and aroma of kefir also remained similar between the two treatments (71). In a nutshell, CS and its by-products improve the composition of milk products, making them more suitable for human consumption and health.

Conclusion

In conclusion, to improve animal nutrition and overall farm sustainability, incorporating oilseed plants into livestock production systems presents a promising strategy. This review has revealed the numerous advantages of incorporating oilseed-based products, such as camelina, soybeans, sunflower seeds, and canola into the diets of livestock. These feedstuffs supply all the important nutrients to the animals and they also demonstrate the potential for reducing environmental impact by decreasing the use of conventional protein sources. Due to its nutritional advantages, environmental flexibility, and potential to enhance general health and performance, *Camelina sativa* has promise as a great resource for small ruminant feed. It has outstanding agronomic characteristics, a high oil content, and beneficial nutritional qualities, making it a good choice for sustainable agriculture and nutrition improvement. It is a potential oilseed that is a strong option for sustainable agriculture and nutrition improvement due to its high amounts of tocopherols, high levels of protein, and beneficial plant sterols. In order to fully realize its potential, one must take into account some anti-nutritional variables that are present in it. Small ruminants may benefit from the usage of CS and its byproducts in terms of dry matter intake, metabolism, meat output, and carcass characteristics. The addition of CS and its byproducts to the diets of small ruminants had no discernible impact on milk basic composition. Nevertheless, it does have favorable effects on fatty acid profiles, perhaps improving the quality of dairy products for ingestion without significant sensory changes. Additional research is required to examine the best doses, how they affect animal performance, the standard of animal products, and long-term impacts. Smilarly, to identify how to utilize CS meal as an alternative feed ingredient in the diets of developing dairy ewes and dairy goats at various life phases, further studies are required.

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Author Contributions

All authors contributed equally in the curation of data, organization, evaluation of data, and writing and reviewing of the manuscript.

Data Availability Statement

Upon request, the corresponding author will provide the numerical data that were needed to support the study's conclusions.

Ethical Statement

Not applicable.

Animal Welfare

Not applicatble.

References

1. **Akin AC, Polat M, Burak M, et al** (2023): *Determining the variables affecting the prices of animal products by the network analysis in Türkiye.* Ankara Univ Vet Fak Derg, **70**, 359-366.
2. **Almeida F, Htoo J, Thomson J, et al** (2013): *Amino acid digestibility in camelina products fed to growing pigs.* Can J Anim Sci, **93**, 335-343.
3. **Amyot L, McDowell T, Martin SL, et al** (2018): *Assessment of antinutritional compounds and chemotaxonomic relationships between Camelina sativa and its wild relatives.* J Agric Food Chem, **67**, 796-806.
4. **Ancuța PSonia A** (2020): *Oil press-cakes and meals valorization through circular economy approaches: A review.* J Agric Food Chem, **67**, 796-806.
5. **Bacenetti J, Restuccia A, Schillaci G, et al** (2017): *Biodiesel production from unconventional oilseed crops (Linum usitatissimum L. and Camelina sativa L.) in Mediterranean conditions: Environmental sustainability assessment.* Renew Energy, **112**, 444-456.
6. **Bayat A, Kairenius P, Stefański T, et al** (2015): *Effect of camelina oil or live yeasts (Saccharomyces cerevisiae) on ruminal methane production, rumen fermentation, and milk fatty acid composition in lactating cows fed grass silage diets.* J Dairy Sci, **98**, 3166-3181.
7. **Berti M, Gesch R, Eynck C, et al** (2016): *Camelina uses, genetics, genomics, production, and management.* Ind Crops Prod, **94**, 690-710.
8. **Bhat AR, Shah AH, Ayoob M, et al** (2022): *Chemical, rheological, and organoleptic analysis of cow and buffalo milk mozzarella cheese.* Ankara Univ Vet Fak Derg, **69**, 51-60.
9. **Bouallegue K, Allaf T, Younes RB, et al** (2020): *Pressure, temperature and processing time in enhancing Camelina sativa oil extraction by Instant Controlled Pressure-Drop (DIC) texturing pre-treatment.* Grasas Y Aceites, **71**, e365-e365.
10. **Bowles TM, Mooshammer M, Socolar Y, et al** (2020): *Long-term evidence shows that crop-rotation diversification increases agricultural resilience to adverse growing conditions in North America.* One Earth, **2**, 284-293.
11. **Brock JR, Ritchey MM, Olsen KM** (2022): *Molecular and archaeological evidence on the geographical origin of domestication for Camelina sativa.* Am J Bot, **109**, 1177-1190.
12. **Budin JT, Breene WM, Putnam DH** (1995): *Some compositional properties of camelina (Camelina sativa L. Crantz) seeds and oils.* J Am Oil Chem Soc, **72**, 309-315.
13. **Cais-Sokolińska D, Majcher M, Pikul J, et al** (2011): *The effect of Camelina sativa cake diet supplementation on sensory and volatile profiles of ewe's milk.* Afr J Biotechnol, **10**, 7245-7252.
14. **Candela CG, López LB, Kohen VL** (2011): *Importance of a balanced omega 6/omega 3 ratio for the maintenance of health. Nutritional recommendations.* Nutr Hosp, **26**, 323-329.
15. **Carciumaru M** (2007): *Cultivarea plantelor in Dacia (Plants cultivation in Dacia).* Thraco-Dacica, **8**, 1-6.
16. **Cherdthong A, Wanapat M, Saenkamsorn A, et al** (2014): *Effects of replacing soybean meal with dried rumen digesta on feed intake, digestibility of nutrients, rumen fermentation and nitrogen use efficiency in Thai cattle fed on rice straw.* Livest Sci, **169**, 71-77.
17. **Cherian G** (2012): *Camelina sativa in poultry diets: opportunities and challenges.* Biofuel co-products as livestock feed: opportunities and challenges. Rome: FAO, 303-310.
18. **Christodoulou C, Mavrommatis A, Loukovitis D, et al** (2023): *Inclusion of Camelina sativa Seeds in Ewes' Diet Modifies Rumen Microbiota.* Animals, **13**, 377.
19. **Christodoulou C, Mavrommatis A, Mitsiopoulou C, et al** (2021): *Assessing the Optimum Level of Supplementation with Camelina Seeds in Ewes' Diets to Improve Milk Quality.* Foods, **10**, 2076.
20. **Christodoulou C, Mavrommatis A, Simoni M, et al** (2022): *The amino acid profile of Camelina sativa seeds correlates with the strongest immune response in dairy ewes.* Animal, **16**, 100621.
21. **Cieslak A, Stanisz M, Wojtowski J, et al** (2013): *Camelina sativa affects the fatty acid contents in M. longissimus muscle of lambs.* Europ J Lipid Sci Technol, **115**, 1258-1265.
22. **Codina-Pascual N, Torra J, Baraibar B, et al** (2022): *Weed suppression capacity of camelina (Camelina sativa) against winter weeds: The example of corn-poppy (Papaver rhoeas).* Ind Crops Prod, **184**, 115063.
23. **Colombini S, Broderick GA, Galasso I, et al** (2014): *Evaluation of Camelina sativa (L.) Crantz meal as an alternative protein source in ruminant rations.* J Sci Food Agric, **94**, 736-743.
24. **Colonna MA, Giannico F, Tufarelli V, et al** (2021): *Dietary Supplementation with Camelina sativa (L. Crantz) Forage in Autochthonous Ionica Goats: Effects on Milk and Caciotta Cheese Chemical, Fatty Acid Composition and Sensory Properties.* Animals (Basel), **11**, 1589.
25. **Croat JR, Berhow M, Karki B, et al** (2016): *Conversion of canola meal into a high-protein feed additive via solid-state fungal incubation process.* J Am Oil Chem Soc, **93**, 499-507.
26. **Danków R, Pikul J, Wójtowski J, et al** (2015): *The effect of supplementation with gold of pleasure (Camelina sativa) cake on the fatty acid profile of ewe milk and yoghurt produced from it.* J Anim Feed Sci, **24**, 193-202.
27. **de Oliveira Filho JG, Egea MB** (2021): *Sunflower seed byproduct and its fractions for food application: An attempt*

to improve the sustainability of the oil process. J Food Sci, **86**, 1497-1510.

28. **Delamare J, Brunel-Muguet S, Morvan-Bertrand A, et al** (2023): *Thermopriming effects on root morphological traits and root exudation during the reproductive phase in two species with contrasting strategies: Brassica napus (L.) and Camelina sativa (L.) Crantz.* Environ Exp Bot, **210**, 105318.
29. **Delver JJ, Smith ZK** (2024): *Opportunities for Camelina Meal as a Livestock Feed Ingredient.* Agriculture, **14**, 116.
30. **Ekin S, Sonat FA** (2023): *Effects of algae derived pure β–Glucan on In vitro rumen fermentation*. Ankara Univ Vet Fak Derg, **70**, 447-452.
31. **FAO** (2017): The Future of Food and Agriculture—Trends and Challenges. *Rome.*
32. **Ferguson J** (2014): Food Recovery Hierarchy: Quantifying Food Recovery for the End Users.
33. **Fröhlich A, Rice B** (2005): *Evaluation of Camelina sativa oil as a feedstock for biodiesel production.* Ind Crops Prod, **21**, 25-31.
34. **Gesch RW** (2014): *Influence of genotype and sowing date on camelina growth and yield in the north central US.* Ind Crops Prod, **54**, 209-215.
35. **Ghidoli M, Ponzoni E, Araniti F, et al** (2023): *Genetic Improvement of Camelina sativa (L.) Crantz: Opportunities and Challenges.* Plants, **12**, 570.
36. **Gökdai A, Sakarya E** (2022): *Determination of goat milk cost and assessment of factors affecting the profitability of Saanen goat farms in Canakkale province, Turkey.* Ankara Univ Vet Fak Derg, **69**, 123-125.
37. **Gómez-Cortés P, Galisteo OO, Ramírez CA, et al** (2019): *Intramuscular fatty acid profile of feedlot lambs fed concentrates with alternative ingredients.* Anim Prod Sci, **59**, 914-920.
38. **González-Pérez S,** (2015): Sunflower proteins, in Sunflower. Elsevier. 331-393.
39. **González-Pérez S, Vereijken JM** (2007): *Sunflower proteins: overview of their physicochemical, structural and functional properties.* J Sci Food Agric, **87**, 2173-2191.
40. **Hixson SM, Parrish CC, Anderson DM** (2014): *Changes in tissue lipid and fatty acid composition of farmed rainbow trout in response to dietary camelina oil as a replacement of fish oil.* Lipids, **49**, 97-111.
41. **Huang P, He L, Abbas A, et al** (2021): *Seed priming with sorghum water extract improves the performance of camelina (Camelina sativa (L.) crantz.) under salt stress.* Plants, **10**, 749.
42. **Hutcheon C, Ditt RF, Beilstein M, et al** (2010): *Polyploid genome of Camelina sativarevealed by isolation of fatty acid synthesis genes.* BMC Plant Biol, **10**, 1-15.
43. **Ibáñez M, De Blas C, Cámara L, et al** (2020): *Chemical composition, protein quality and nutritive value of commercial soybean meals produced from beans from different countries: A meta-analytical study.* Anim Feed Sci Technol, **267**, 114531.
44. **Iskandarov U, Kim HJ, Cahoon EB** (2014): *Camelina: an emerging oilseed platform for advanced biofuels and bio-based materials.* Plants Bioenergy, 131-140.
45. **Jenkins DJ, Kendall CW, Marchie A, et al** (2003): *The Garden of Eden—plant based diets, the genetic drive to conserve cholesterol and its implications for heart disease in the 21st century.* Comp Biochem Physiol Part A: Mol Integr Physiol, **136**, 141-151.
46. **Jewett FG** (2015): *Camelina sativa: For biofuels and bioproducts.* Industrial Crops: Breeding for BioEnergy and Bioproducts, 157-170.
47. **Juodka R, Nainienė R, Juškienė V, et al** (2022): *Camelina (Camelina sativa (L.) Crantz) as feedstuffs in meat type poultry diet: A source of protein and n-3 fatty acids.* Animals, **12**, 295.
48. **Kastakova E, Bato V, Dubenova D** (2023): *Competitiveness on the Global Oilseeds Market.* J Econ Res Bus Administ, **144**, 3.
49. **King K, Li H, Kang J, et al** (2019): *Mapping quantitative trait loci for seed traits in Camelina sativa.* Theor Appl Genet, **132**, 2567-2577.
50. **Kirkhus B, Lundon AR, Haugen J-E, et al** (2013): *Effects of environmental factors on edible oil quality of organically grown Camelina sativa.* J Agric Food Chem, **61**, 3179-3185.
51. **Knorzer K** (1978): *Evolution and spreading of gold of pleasure (Camelina-sativa S1).* Ber Deutsch Bot Ges, **91**, 187-195.
52. **Larsson M** (2013): *Cultivation and processing of Linum usitatissimum and Camelina sativa in southern Scandinavia during the Roman Iron Age.* Veg Hist Archaeobot, **22**, 509-520.
53. **Leclère M, Jeuffroy M-H, Butier A, et al** (2019): *Controlling weeds in camelina with innovative herbicide-free crop management routes across various environments.* Ind Crops Prod, **140**, 111605.
54. **Lin BB** (2011): *Resilience in agriculture through crop diversification: adaptive management for environmental change.* Biosci, **61**, 183-193.
55. **Luo Z, Brock J, Dyer JM, et al** (2019): *Genetic diversity and population structure of a Camelina sativa spring panel.* Front Plant Sci, **10**, 184.
56. **MacKay DS, Jones PJ** (2011): *Phytosterols in human nutrition: Type, formulation, delivery, and physiological function.* Eur J Lipid Sci Technol, **113**, 1427-1432.
57. **Mailer RJ, McFadden A, Ayton J, et al** (2008): *Anti-nutritional components, fibre, sinapine and glucosinolate content, in Australian canola (Brassica napus L.) meal.* J Am Oil Chem Soc, **85**, 937-944.
58. **Makkar H** (1993): *Antinutritional factors in foods for livestock.* BSAP Occas Publ, **16**, 69-85.
59. **Mansour MP, Shrestha P, Belide S, et al** (2014): *Characterization of oilseed lipids from "DHA-producing Camelina sativa": A new transformed land plant containing long-chain omega-3 oils.* Nutrients, **6**, 776-789.
60. **Martinez S, Alvarez S, Capuano A, et al** (2020): *Environmental performance of animal feed production from Camelina sativa (L.) Crantz: Influence of crop management practices under Mediterranean conditions.* Agric Syst, **177**, 102717.
61. **Mejicanos G, Sanjayan N, Kim I, et al** (2016): *Recent advances in canola meal utilization in swine nutrition.* J Anim Sci Technol, **58**, 1-13.
62. **Montero-Muñoz I, Mostaza-Colado D, Capuano A, et al** (2023): *Seed and Straw Characterization of Nine New Varieties of Camelina sativa (L.) Crantz.* Land, **12**, 328.
63. **Moser BR, Vaughn SF** (2010): *Evaluation of alkyl esters from Camelina sativa oil as biodiesel and as blend*

components in ultra low-sulfur diesel fuel. Bioresour Technol, **101**, 646-653.

64. **Mottet A, de Haan C, Falcucci A, et al** (2017): *Livestock: On our plates or eating at our table? A new analysis of the feed/food debate.* Glob Food Sec, **14**, 1-8.
65. **Murphy EJ** (2016): Camelina (Camelina sativa), in Industrial oil crops. Elsevier. 207-230.
66. **Neupane D, Lohaus RH, Solomon JK, et al** (2022): *Realizing the potential of Camelina sativa as a bioenergy crop for a changing global climate.* Plants, **11**, 772.
67. **Noci F, Monahan F, Moloney A** (2011): *The fatty acid profile of muscle and adipose tissue of lambs fed camelina or linseed as oil or seeds.* Animal, **5**, 134-147.
68. **Opio C, Gerber P, Mottet A, et al** (2013): Greenhouse gas emissions from ruminant supply chains–A global life cycle assessment. Food and agriculture organization of the United Nations.
69. **Packer L, Weber SU, Rimbach G** (2001): *Molecular aspects of α-tocotrienol antioxidant action and cell signalling.* J Nutr, **131**, 369S-373S.
70. **Paula EM, da Silva LG, Brandao VLN, et al** (2019): *Feeding canola, camelina, and carinata meals to ruminants.* Animals, **9**, 704.
71. **Pikul J, Wójtowski J, Danków R, et al** (2014): *The effect of false flax (Camelina sativa) cake dietary supplementation in dairy goats on fatty acid profile of kefir.* Small Rumin Res, **122**, 44-49.
72. **Pîrvan A-G, Jurcoane Șmatei F** (2020): *Life Cycle Assessment of Camelina Sativa Crop In a Circular Economy Approach-A Minireview.* Scientific Bull Series F Biotechnol, **24**, 189-193.
73. **Pojić M, Mišan A, Tiwari B** (2018): *Eco-innovative technologies for extraction of proteins for human consumption from renewable protein sources of plant origin.* Trends Food Sci Technol, **75**, 93-104.
74. **Ponnampalam EN, Butler KL, Muir SK, et al** (2021): *Lipid Oxidation and Colour Stability of Lamb and Yearling Meat (Muscle Longissimus Lumborum) from SheepSupplemented with Camelina-Based Diets after Short-,Medium-, and Long-Term Storage.* Antioxidants (Basel), **10**, 166.
75. **Pozzo S, Piergiovanni AR, Ponzoni E, et al** (2023): *Evaluation of nutritional and antinutritional compounds in a collection of Camelina sativa varieties.* J Crop Improv, **37**, 934-952.
76. **Putnam D, Budin J, Field L, et al** (1993): *Camelina: a promising low-input oilseed.* New Crops, **314**, 322.
77. **Ramírez CA, Blanco FP, Ibáñez AH, et al** (2019): *Effects of concentrates rich in by-products on growth performance, carcass characteristics and meat quality traits of light lambs.* Anim Prod Sci, **59**, 593-599.
78. **Rao M, Bast A, de Boer A** (2021): *Valorized food processing by-products in the EU: Finding the balance between safety, nutrition, and sustainability.* Sustainability, **13**, 4428.
79. **Rashid F, Razzaq H** (2023): *Challenges in Improving Nutritional Traits of Camelina sativa: A Review.* Int J Res Adv Agric Sci, **2**, 56-65.
80. **Ratusz K, Symoniuk E, Wroniak M, et al** (2018): *Bioactive compounds, nutritional quality and oxidative stability of cold-pressed camelina (Camelina sativa L.) oils.* App Sci, **8**, 2606.
81. **Ren Y, Zhaxi Y, Liu M, et al** (2023): *Revealing the Fungi Microbiome Difference of Suffolk Cross with Tibetan Sheep on Plateau.* Pak Vet J, **43**, 748-756.
82. **Reyes-Palomino SE, Cano Ccoa DM** (2022): *Efectos de la agricultura intensiva y el cambio climático sobre la biodiversidad.* Rev Investig Altoandin, **24**, 53-64.
83. **Riaz R, Ahmed I, Sizmaz O, et al** (2022): *Use of Camelina sativa and by-products in diets for dairy cows: A Review.* Animals, **12**, 1082.
84. **Riediger ND, Othman RA, Suh M, et al** (2009): *A systemic review of the roles of n-3 fatty acids in health and disease.* J Am Diet Assoc, **109**, 668-679.
85. **Rodríguez-Roque MJ, Sánchez-Vega R, Pérez-Leal R, et al** (2021): *By-Products from Oilseed Processing and Their Potential Applications.* Oil and Oilseed Processing: Opportunities and Challenges, 183-201.
86. **Rojas M, Brewer M** (2007): *Effect of natural antioxidants on oxidative stability of cooked, refrigerated beef and pork.* J Food Sci, **72**, S282-S288.
87. **Rooke J, Robinson J, Arthur J** (2004): *Effects of vitamin E and selenium on the performance and immune status of ewes and lambs.* J Agric Sci, **142**, 253-262.
88. **Russo R, Galasso I, Reggiani R** (2014): *Variability in glucosinolate content among Camelina species.* Am J Plant Sci, **5**, 294-298.
89. **Russo R, Reggiani R** (2017): *Glucosinolates and Sinapine in camelina meal.* Food Nutr Sci, **8**, 1063-1073.
90. **Salsabil SS, Ardana VP, Larastiyasa RRPB, et al** (2022): *Nanoparticles of Kirinyuh (Chromolaena odorata (L.) RM King & H. Rob.) Leaves Extract as a Candidate for Natural Remedies Lowering Hypercholesterol: In Silico and In vivo Study.* Pak Vet J, **42**, 397-403.
91. **Schuster A, Friedt W** (1998): *Glucosinolate content and composition as parameters of quality of Camelina seed.* Ind Crops Prod, **7**, 297-302.
92. **Simopoulos AP** (2008): *The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases.* Exp Biol Med, **233**, 674-688.
93. **Sindelar AJ, Schmer MR, Gesch RW, et al** (2017): *Winter oilseed production for biofuel in the US Corn Belt: Opportunities and limitations.* Gcb Bioenergy, **9**, 508-524.
94. **Sizmaz Ö, Çalik ABundur A** (2021): *In Vitro Fermentation Characteristics of Camelina Meal Comparison with Soybean Meal.* Livest Stud, **61**, 9-13.
95. **Sizmaz O, Calik A, Sizmaz S, et al** (2016): *A comparison of camelina meal and soybean meal degradation during incubation with rumen fluid as tested in vitro.* Ank Univ Vet Fak Derg, **63**, 157-161.
96. **Smyk B** (2015): *Spectral properties of sinapine in water environment.* J Mol Liq, **208**, 1-4.
97. **Steingass H, Kneer G, Wischer G, et al** (2013): *Variation of in situ rumen degradation of crude protein and amino acids and in vitro digestibility of undegraded feed protein in rapeseed meals.* Animal, **7**, 1119-1127.
98. **Sydor M, Kurasiak-Popowska D, Stuper-Szablewska K, et al** (2022): *Camelina sativa. Status quo and future perspectives.* Ind Crops Prod, **187**, 115531.

99. **Szumacher-Strabel M, Cieslak A, Zmora P, et al** (2011): *Camelina sativa cake improved unsaturated fatty acids in ewe's milk.* J Sci Food Agric, **91**, 2031-2037.
100. **Tahir MN, Riaz R, Bilal M, et al** (2019): Current standing and future challenges of dairying in Pakistan: a status update. Milk Production, Processing and Marketing. 1-24.
101. **Velasco P, Cartea ME, González C, et al** (2007): *Factors affecting the glucosinolate content of kale (Brassica oleracea acephala group).* J Agric Food Chem, **55**, 955-962.
102. **Vidal NP, Roman L, Swaraj VS, et al** (2022): *Enhancing the nutritional value of cold-pressed oilseed cakes through extrusion cooking.* Innov Food Sci Emerg Technol, **77**, 102956.
103. **Vollmann J, Eynck C** (2015): *Camelina as a sustainable oilseed crop: Contributions of plant breeding and genetic engineering.* Biotechnol J, **10**, 525-535.
104. **Wanasundara J, Tan S, Alashi A, et al** (2017): Proteins from canola/rapeseed: Current status, in Sustainable protein sources. Elsevier. 285-304.
105. **Wilk M, Król B, Słupczyńska M, et al** (2021): *In vitro rumen methanogenesis and fermentation profile of sorghum whole crop cereal and bagasse ensilaged with inoculum Lactobacillus buchneri.* Pak Vet J, **42**, 41-46.
106. **Wongsirichot P, Gonzalez-Miquel M, Winterburn J** (2022): *Recent advances in rapeseed meal as alternative feedstock for industrial biotechnology.* Biochem Eng J, **180**, 108373.
107. **Woods VB, Fearon AM** (2009): *Dietary sources of unsaturated fatty acids for animals and their transfer into meat, milk and eggs: A review.* Livest Sci, **126**, 1-20.
108. **Yohannes G, Kidane L, Abraha B, et al** (2020): *Effect of Salt Stresses on Seed Germination and Early Seedling Growth of Camelina sativa L.* Momona Ethiop J Sci, **12**, 1-19.
109. **Yue S, Li X, Qian J, et al** (2023): *Impact of Enzymatic Hydrolyzed Protein Feeding on Rumen Microbial Population, Blood Metabolites and Performance Parameters of Lactating Dairy Cows.* Pak Vet J, **43**, 804-808.
110. **Zanetti F, Alberghini B, Marjanović Jeromela A, et al** (2021): *Camelina, an ancient oilseed crop actively contributing to the rural renaissance in Europe. A review.* Agron Sustain Dev, **41**, 1-18.
111. **Zanetti F, Eynck C, Christou M, et al** (2017): *Agronomic performance and seed quality attributes of Camelina (Camelina sativa L. crantz) in multi-environment trials across Europe and Canada.* Ind Crops Prod, **107**, 602-608.
112. **Zhang C-J, Kim D-S, Jiang C, et al** (2021): *Hourly pollen dispersal of Camelina sativa (L.) Crantz under different weather conditions and mitigation of wind-blown pollen dispersal using maize barrier.* Ind Crops Prod, **162**, 113318.
113. **Zubr J** (1997): *Oil-seed crop: Camelina sativa.* Ind Crops Prod, **6**, 113-119.
114. **Zubr J, Matthäus B** (2002): *Effects of growth conditions on fatty acids and tocopherols in Camelina sativa oil.* Ind Crops Prod, **15**, 155-162.

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