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Seroprevalence of *Schmallenberg virus* infection in sheep in Kars province

Research Article

ABSTRACT

Schmallenberg virus (SBV) infection is an infectious disease transmitted by species vectors as *Culicoides sp.* and characterised by fever, anorexia, decreased milk yield, loss of condition, abortion and birth of offspring with arthrogryposis hydranencephaly syndrome. The aim of this study was to determine the prevalence and presence of SBV infection in sheep in private farms in Kars province. For this purpose, blood serum samples were taken from 376 (301 females + 75 males) healthy-looking Akkaraman sheep raised in small-scale family-type farms in five central villages of Kars province (Kümbetli, Çakmak, Dikme, Subatan, Cumhuriyet) and evaluated for SBV-specific antibodies with a commercial ELISA kit. The animals included in the study were 1-4 years old. Of the sampled animals, 1.1% (4/376) were positive and 1.1% (4/376) were suspected. Seropositivity rates were determined between 0% and 2.53% according to the settlements. 1.33% (4/301) of ewes were antibody positive and all rams were antibody negative (0/75). Of the four animals with suspected antibodies, two were sheep (2/301, 0.66%) and two were rams (2/75, 2.66%). It was shown that there was no statistically significant variation in seropositivity rates amongst age groups, genders, and villages (P>0.05). This study examined the seroprevalence of SBV in sheep grown in the province of Kars. The presence of the infection was serologically demonstrated for the first time, and it was determined that the seroprevalence rate of SBV infection was low.

Keywords: ELISA, Kars, *Schmallenberg virus*, seroprevalence, sheep, Türkiye

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INTRODUCTION

Schmallenberg virus (SBV) is a member of the *Peribunyaviridae* family and belongs to the genus *Orthobunyavirus.* (ICTV, 2018) and there are three serogroups in this genus including animal and human viruses. As a result of genetic analysis, SBV was found to be similar to *Aino*, *Akabane* and *Shamonda viruses* and was classified in the Simbu serogroup (Garigliany et al., 2012b; Goller et al., 2012; Hoffmann et al., 2012). SBV, as other Simbu serogroup viruses, spreads mainly by biting Culicoides midges. Additionally, SBV is transmitted vertically as well. (De Regge et al., 2014; Garigliany et al., 2012b; Lehmann et al., 2012). There is no data showing that there is transmission from animal to animal by direct contact (Pawaiya & Gupta, 2013; Wernike et al., 2014). SBV was found in cattle, sheep, goats and bison. In addition to these animals, the presence of antibodies has been demonstrated in roe deer and fallow deer and natural infection was thought to have occurred. Dogs and wild boars have also been found to have SBV antibodies. (Brülisauer et al., 2017; Kauffold et al., 2021; Kęsik-Maliszewska et al., 2018; Sailleau et al., 2013; Wensman et al., 2013). The disease in cattle progresses with fever, loss of appetite, decrease in milk yield, loss of

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condition, miscarriage and calf birth with arthrogryposis hydranencephaly syndrome, and congenital malformations characterized by stillbirth in sheep and goats. (Bilk et al., 2012; Hoffmann et al., 2012; Muskens et al., 2012; Van den Brom et al., 2012).

SBV has been reported on the European continent so far. Cases that started first in Germany and the Netherlands were later seen in establishments in Belgium, England, France, Italy, Spain and Luxembourg (Conraths et al., 2013). As a result, SBV seriously impairs domestic ruminant productivity. SBV is also observed in several locations of Türkiye, and seroprevalence tests in goats, sheep, cattle, and Anatolian buffaloes have shown low levels of SBV (Azkur et al., 2013; Elmas et al., 2018; Macun et al., 2017; Tonbak et al., 2016; Varol, 2022). The seroprevalence of SBV has not been investigated in any ruminant species in Kars region of Türkiye until now. Reverse transcription-polymerase chain reaction (RT-PCR), virus neutralization test (VNT), immunofluorescence assay test (IFAT), and enzyme-linked immunosorbent assay (ELISA) are frequently utilized for SBV diagnosis (Garigliany et al., 2012a; Garigliany et al., 2012b; Garigliany et al., 2012c; Kessell et al., 2011; Tarlinton et al., 2012). The SBV nucleocapsid protein formed the basis for the development of the currently available ELISA kit. This test is known to be rapid and simple to run, but it can also identify antibodies to other orthobunyaviruses (Breard et al., 2013). VNT is regarded as the SBV gold standard test (Van der Poel et al., 2014).

In this study, SBV antibodies were detected, and Indirect ELISA (I-ELISA) was used to measure SBV seroprevalence in sheep for the first time in the Kars region of Türkiye. The seroprevalence rate of SBV infection in sheep in the Kars province is low, according to these seroepidemiological data.

MATERIALS AND METHODS

Serum Samples

Blood samples were randomly gathered from 376 Akkaraman sheep (301 sheep + 75 rams) without clinical signs from small-scale family-type farms in five villages of Kars province (Kümbetli, Çakmak, Dikme, Subatan, Cumhuriyet). The samples were collected randomly from small-scale family type farms (less than twenty sheep), not on a herd basis. There is no information on the case history of abortions or births with congenital malformations in the sampled animals. The ages of the animals range from 1 to 4 years old (age of 1, n= 52; age of 2, n= 129; age of 3, n= 152; age of 4, n=43), and the samples were collected between November 2022 and March 2023 (Table 1, Figure 1). Blood samples were taken from the animals' jugular veins. Blood samples taken into silicone tubes were centrifuged at 2000 rpm for 10 minutes and serums were separated. Then serum samples taken into stock tubes were preserved at -20^{o}C until tested.

Table 1. Distribution of the number of samples in the study according to gender and location.

Location	Number of animals sampled (n)		
	Sheep	Ram	Total
Kümbetli	48	9	57
Çakmak	68	11	79
Dikme	50	10	60
Subatan	65	20	85
Cumhuriyet	70	25	95
Total	301	75	376

Figure 1. Geographical positioning of the Kars province in which the study was performed.

Indirect Enzyme Linked Immunosorbent Assay (I-ELISA)

The commercial ELISA method (IDEXX Schmallenberg Ab Test®, IDEXX, Switzerland Cat No: 99-412-59) applied to identify antibodies against SBV was carried out in accordance with the manufacturer's instructions. For each tested sample and the positive serum, the percent inhibition was calculated (%inhib) by means of the following formula:

% S/P = [(ODsample – ODneg) ÷ (ODpos - ODneg)] x100

S/P percentages were evaluated as follows; < 30 % negative, ≥30 % and <40 % suspect, ≥40 % positive

Statistical Analysis

The Statistical Package for Social Sciences software (IBM SPSS 21 Software, USA) was applied to do statistical analysis. The statistical significance of the differences between SBV seropositivity rates determined according to sampling regions, sex (rams and ewes) and age groups was evaluated using the chi-square (chi-square -χ^2) test. A significant difference was defined as a p-value less than 0.05 (P<0.05).

RESULTS

According to the results, four (1.1%, 4/376) blood samples from the study's animals contained antibodies specific to SBV. In 4 animals (1.1%, 4/376), the test result was suspectable for antibody positivity. Two of the four positive animals were Çakmak (2.53%); while two of them were detected in Cumhuriyet villages (2.10%); One of the four suspicious animals was found in Kümbetli (1.75%), one in Subatan (1.17%) and two in Çakmak (2.53%). No positivity was detected in Dikme village (Table 2).

Table 2. SBV seroprevalence rates according to sampling locations.

Sampled Locations	Number of samples (n)	Number of positive samples (%)	Number of suspicious samples (%)	X^2	P-value
Kümbetli	57	0 (0)	1 (1.75%)	8.338	0.401
Çakmak	79	2 (2.53%)	2 (2.53%)		
Dikme	60	0 (0)	0 (0)		
Subatan	85	0 (0)	1 (1.17%)		
Cumhuriyet	95	2 (2.10%)	0 (0)		
Total	376	4 (1.1%)	4 (1.1%)		

In this study, 1.33% (4/301) of the ewes were antibody positive and all of the rams were antibody negative (0/75). Of the four animals with suspicious antibodies, two were ewes (2/301, 0.66%) and two were rams (2/75, 2.66%) (Table 3).

Table 3. SBV seroprevalence rates by gender.

Gender	n	Positive (%)	Suspicious (%)	Negative (%)	X^2	p-value
Ram	75	0 (0)	2 (2.66%)	73 (97.33)	0.140	0.588
Sheep	301	4 (1.33%)	2 (0.66%)	295 (98.0)		
Total	376	4 (1.1%)	4 (1.1%)	368 (97.8)		

Two of the animals determined as positive for SBV specific antibodies were in the age group of 2 and two of them were in the age group of 3, while two of the animals determined as suspicious were in the age group of 2 and two of them were in the age group of 3 (Table 4). In this study, the data on SBV positivity collected from villages in Kars province were statistically analysed. It was found that the differences in SBV positivity rates among different age groups (p>0.05) (age groups χ^2= 0.544, p=0.762), females and males (p>0.05) (Male/female χ^2= 0.140, p=0.588), and villages (p>0.05) (χ^2= 8.338, p= 0.401) were statistically insignificant.

Table 4. SBV seroprevalence rates according to age groups.

Age (years)	n	Positive (%)	Suspicious (%)	Negative (%)	X^2	p-value
1	52	0 (0)	0 (0)	52 (100)		
2	129	2 (1.55%)	2 (1.55%)	125 (96.89%)		
3	152	2 (1.31%)	2 (1.31%)	148 (97.89%)	0.544	0.762
4	43	0 (0)	0 (0)	43 (100)		
Total	376	4 (1.1%)	4 (1.1%)	368 (97.8%)		

DISCUSSION

SBV, one of the arboviruses carried by Culicoides biting midges, causes serious economic losses in sheep, goats and cattle due to factors such as death, abortion and abnormal offspring births, as well as animal movements, restriction of the use of embryos, semen and other animal products in regions where this infection is endemic (Lievaart-Peterson et al., 2015).

There are few studies on SBV in sheep raised in Türkiye. In one of these studies, ELISA test was performed on 307 serum samples collected between 2006-2012 and the seroprevalence of the disease in sheep was found to be 1.6% (5/307) (Azkur et al., 2013). In another study, Kızıltepe et al. (2023) detected 4.4% SBV positivity in blood samples taken from 180 sheep in the province of Iğdır. In a study conducted in Kırıkkale province, a total of 1038 sheep were sampled and 0.38% (4/1038) of the sampled animals were found positive for SBV-specific antibodies (Macun et al., 2017). In a study in Sivas province, a total of 368 animals (250 sheep, 118 rams) belonging to the Kangal Akkaraman breed were sampled and seropositivity was detected in only 1 sheep (0.27%) (Elmas et al., 2018). In a seroprevalence study performed in Hatay, the seropositivity rate in sheep was found to be 23.60% (Doğan, 2018). However, in this investigation, only 1.1% seropositivity was detected in blood samples collected from 376 Akkaraman sheep aged between one and four years, which were raised in small-scale production units located in the villages of Kars province. The rate observed in our study is higher than the rates reported in previous studies (Elmas et al., 2018; Macun et al., 2017) and lower than the rates reported by Azkur et al., (2013), Kızıltepe et al., (2023) and Dogan et al., (2022).

Seropositivity rates were especially high in animals in the 2 and 3 age groups. According to this result, it is thought that there may be an age-related difference in susceptibility to infection in animals. In their study, Wernike et al., (2018) reported that the presence of seronegative young animals in the herd may decrease herd immunity, potentially leading to higher yield losses as a result of increased virus circulation and susceptibility to infection.

In our study, statistical comparison of antibody positivity and negativity according to the sex of the animals did not reveal any significance ($P>0.05$). Based on these results, it can be concluded that sex does not play a significant role in the distribution and susceptibility to infection, as indicated by statistical comparisons made according to the sex of the animals.

According to the study's findings, which indicate the presence of SBV infection, the infection holds significance for the Northeastern Anatolia Region, and preventive measures should be taken. In this study, a low seropositivity rate was detected in sheep raised in private family farms in Kars province. It is thought that this may be due to the sampling time when the vector population was low. Additionally, the low number of animals sampled may have also contributed to this. Kars

province has a harsh continental climate, so the population of Culicoides vector insects is low in this region, which may have led to a low SBV seroprevalence rate. Considering the current climate of the Kars region, the high prevalence of SBV in studies conducted in Hatay (Dogan et al., 2022) and Samsun (Azkur et al., 2013) provinces, which have a warmer and more humid climate, supports this situation.

On the other hand, the construction of dams and irrigation areas in the Kars region in recent years has led to milder winter months compared to previous years and an increase in humidity rates. This is thought to result in an increase in both mosquito population and flight activity. The expressed changes have a significant impact on the epidemiology of SBV infection.

In their publication, Doğan et al. (2022) reported that measures such as controlling breeding animals and semen, managing vectors, and implementing early warning systems may be important in combating SBV infection. Currently, there is no vaccine for SBV in our country. However, given that this disease has posed a risk in recent years, the development of a vaccine against SBV may be under consideration.

Vector control and vaccination should be applied together to control SBV infection. There are various methods of prevention and control of SBV infection. These methods include control strategies to reduce larval/adult habitat, drainage of swamps and dirty water ponds (Carpenter, 2008); various insecticides, biorational pesticides (the use of hormones or microbial agents such as Bacillus thuringiensis that limit the development of Culicoides), repellent applications in animals and fly traps can be considered as alternative methods (Mullens et al., 2004).

CONCLUSION

As a result of this study, the prevalence of SBV infection in Kars province was determined and the presence/prevalence of the infection was revealed serologically. According to the results of this study, SBV appears to be circulating in the Kars region. It should be considered that SBV may play a role as an etiological agent in abortions, premature births, and congenital malformations, which are common in the region, similar to infections such as *Bovine Viral Diarrhoea Virus* (BVDV), *Border Disease Virus* (BDV), *Bluetongue Virus* (BTV), and *Akabane Virus* (AKAV), which cause abortions and congenital malformations in pregnant sheep. We believe that testing sheep for SBV antibodies can be helpful in determining whether the disease is present in the region, identifying potential financial losses and selecting the best control programme for disease prevention. It is also thought that this study may shed light on epidemiological studies that can be conducted across the region/country from a broader perspective. In our study, only serum samples were tested and it is a preliminary study. In future studies, the aim is to determine antigen positivity by screening samples taken from animals exhibiting clinical findings, such as whole blood, semen, and abortion material.

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Consent: Serum samples were obtained from herds in small-scale family-type enterprises in five villages of Kars province. This research was conducted after the

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Assessment of diaphyseal long bone fracture healing in cats using radiography, ultrasonography and doppler impedance index

ABSTRACT

Color Doppler ultrasonography evaluates blood flow and neovascularization in vessels, assessing Pulsatility Index (PI) and Resistive Index (RI) values. The primary objective of this study is to monitor the healing periods of fractures using color Doppler ultrasonography index values, thereby preventing complications through objective data. The emphasis is particularly on establishing the foundation for studies aimed at early detection of delays in vascularization associated with fracture healing. The research involved 20 cats of various breeds and genders, aged 1 to 4 years, diagnosed with femur and tibia diaphyseal fractures. Mediolateral and anteroposterior radiographs were taken on postoperative days 0, 10, 20, and 30. The fracture site was examined using color Doppler ultrasonography. As a result of the study, using Doppler ultrasonography and radiography, neovascular areas from fracture formation to callus formation were converted into objective data through vascular indices. Particularly noteworthy was a significant increase in RI and PI values observed on the postoperative 10th day. It was deduced that the detection of fracture healing could occur earlier with the assistance of these index values when compared to radiographs taken on the 10th and 30th days. In conclusion, PI and RI values may be important parameters in evaluating the healing process in complex fractures, especially in determining complications and monitoring the progress of healing. Moreover, Doppler indices might be employed to complement traditional diagnostic approaches in the early detection of pathological bone conditions

Keywords: Color doppler, femur, pulsatility index, resistive index

INTRODUCTION

Radiography, commonly favored for diagnosing and treating fractures, proves highly advantageous in imaging bone anatomy, evaluating postoperative periosteal reactions, and assessing changes in bone mineral density (Kealy et al., 2011; Thrall, 2013). In the early stages of pathologies such as periosteal reactions or avascular necrosis, where bone opacity is minimal, changes in opacity may only become apparent through radiography approximately 7 to 10 days later (Hammond, 2016). Postoperative radiographs taken during the fracture healing process are established as a standard procedure. However, the periosteal reactions obtained through radiography are quantitative assessments. The evaluation of mineralization and callus formation is subjective and may vary depending on the assessor. To enhance objectivity in evaluations, diverse imaging techniques should be employed. Ultrasonography is one of the diagnostic methods utilized for soft tissue monitoring of extremities and the assessment of fracture healing. Bone tissue appears hyperechoic and is visualized as a straight line in ultrasound imaging. In fractures, the imaging of surrounding soft tissues allows for the evaluation of muscle tears, hematomas, and the healing processes of soft tissue (Wawrzyket al., 2015).

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Research Article

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The process of bone formation varies depending on the vascular supply to the region. Color Doppler ultrasonography can be employed to monitor the neoangiogenesis process surrounding the fracture site and the formation of callus (Caruso et al., 2000). This diagnostic method is also valuable for assessing secondary fracture healing in simple fractures, cases of non-union, and situations of delayed union (Kealy et al., 2011; Kramer, 2011). Doppler spectrum contributes to the evaluation of vascular flow characteristics (Kim, 2020; Varshney et al., 2022). Parameters used to assess the resistance of blood flow in the vascular lumen and the perfusion of organs are available. Quantitative information obtained from these parameters aids in diagnosis (Chung et al., 2020; River et al., 1997). The pulsed wave waveform concurrently displays blood flow velocity and changes, facilitating the calculation of indices (Gorgas, 2011; Kim et al., 2020; Varshney et al., 2022). Doppler waveforms, specifically Pulsatility Index (PI) and Resistive Index (RI) values obtained from them, are utilized in assessing blood flow resistance in the vascular lumen and perfusion of blood circulation in organs (Rawashdeh et al., 2001; River et al., 1997). While RI examines the negative relationship between vascular resistance and vascular perfusion, PI is a parameter related to the flexibility of arteries (Bude et al.,1999; Ginther, 2007; Gosling et al., 1974).

This study aims to evaluate the healing process of diaphyseal fractures of the long bones of the hind limb of cats by radiography, ultrasonography and Doppler ultrasonography. It is aimed to increase the usability of the index results obtained from neovascular areas especially with Doppler ultrasonography in the evaluation of healing periods in clinical practice. With the objective data to be obtained, standardisation of neovascularisation occurring in the fracture line will be established, thus early detection of vascularisation-induced healing delays will be provided in the future.

MATERIALS AND METHODS

Selection of cases

The study material comprised a total of 20 cats, aged between 1 and 4 years, admitted to the Ankara University Faculty of Veterinary Medicine Animal Hospital and Aksaray University Faculty of Veterinary Medicine Animal Hospital due to single-piece fractures along the mid-diaphyseal line of the femur and tibia bones. Ethical approval for the study was obtained from the Ankara University Local Ethics Committee for Animal Experiments (Approval no: 2020-5-36). Moreover, the owners of the animals were adequately informed about the study, and their consent was obtained prior to their participation in the research.

Operation method

General anesthesia was provided with Medetomidine HCl (80 micrograms/kg, intramuscularly, Domitor®, Finland) and Ketamine HCl (5 mg/kg, intramuscularly, Keta-Control®, Türkiye). Isoflurane (with 100% oxygen, Isoflurane®, USA) was used for anesthesia maintenance. Butorphanol tartrate (0.1mg/kg subcutaneously, Butomidor®, Austria) was administered for analgesia. Balanced electrolyte solutions (Ringer's lactate, 50 ml/kg/h, intravenously) and cefazolin (25 mg/kg, intravenously, Eqizolin®, Türkiye) were administered intraoperatively. In the postoperative period, antibiotic treatment was provided with amoxicillin/clavulanic acid (25 mg/kg, oral, Augmentin®, Türkiye) for one week.

In all cases, reduction of the fractures was achieved using the retrograde intramedullary pinning method. After complete anatomical reduction, fracture fixation was performed by a routine surgical approach using a Steimann pin or a Schanz pin in a retrograde technique according to the diameter of the medullary cavity (to fill approximately 70 % of the diameter of the medullary canal) (Roe, 2005**)**. In addition to the fracture fixation method, cerclage wire was

applied to prevent rotation of the fracture ends. In the postoperative days, the affected extremity was supported with a bandage for 2 weeks to minimise movement, and the bandage was renewed on control days. Postoperatively, area restriction was applied during the recovery period.

Radiographic examination

Radiographic and ultrasonographic assessments were performed on postoperative days 0, 10, 20, and 30. Direct radiographs were obtained in mediolateral and anteroposterior positions for radiographic evaluations. The assessment included the examination of fracture healing, fracture reduction, intramedullary pin position, and callus formation. These evaluations were based on the radiodensity of the bone cortex and the condition of the cortical bone.

Ultrasonographic examination

Fracture line assessment in all cases was conducted using B-mode ultrasonography and color Doppler ultrasonography with 5 and 7.5 MHz sector and linear probes. In pulse Doppler ultrasonographic examination, Doppler color gain setting (G) was set between 046-050, wall filter mean (WF) 100 Hz, sampling rate (PRF) mean 2.2-2.6 KHz, sampling interval 1-1.5 mm and Doppler angle ≤ 60°. Evaluation involved a lateral approach for the femur and a medial approach for the tibia. The ultrasound probe was positioned parallel to the bone axis of the relevant extremity, with ultrasound beams oriented perpendicular (Figure 1). During ultrasound evaluation, any anesthetic agent was not administered to the patients.

Color Doppler ultrasonography was used to evaluate the vascular space surrounding the fracture line (the vascular network formed by the periosteal arteries in the area for fracture healing). The spectral waveforms obtained from these regions in pulsed wave mode provided pulsatility and resistance indices by automatically measuring their values through the instrument software. Measurements were taken from the surrounding tissues and neovascular areas of the periosteal vessels during fracture healing, with samples obtained from the fracture line for each case.

Figure 1. Placement of the linear probe along the axis of the tibia.

Statistical analysis

The SPSS 21 (Version 28.0, IBM Corp., NewYork, USA) package program was employed for the statistical analysis of the acquired data. The normal distribution of the data was assessed using the Shapiro-Wilk test, and the assumption of sphericity was examined through Mauchly's Sphericity Test. The Resistive Index and Pulsatility Index data were subjected to analysis using the Repeated Measures Analysis of Variance Test with respect to time groups. A significance level of $p < 0.05$ was considered to determine statistical significance.

RESULTS

The study included a total of 20 cats, consisting of 6 females and 14 males. The average age of the cases was determined to be 1.35 ± 0.17 years, with an average weight of 3.24 ± 0.24 kg (ranging from 2 kg to 6.5 kg) (Table 1). Trauma

(n=10) emerged as the predominant cause of fractures based on anamnesis. Fractures were evenly distributed between femurs and tibias, each accounting for 50% of the cases. Specifically, among femoral fractures, 2 were spiral and 4 were oblique, while tibial fractures included 4 oblique and 6 transverse fractures. Cerclage wire was applied in all cases except case 2.

Table 1. Signalment and postoperative index values for the cases.

Case	Fracture Bone	Signalment				Day 0		Day 10		Day 20		Day 30	
		Breed	Age (year)	Weight (kg)	Gender	RI	PI	RI	PI	RI	PI	RI	PI
1	T	Tabby	1	3.0	M	0.59	0.89	0.54	0.82	0.75	1.66	0.76	1.73
2	T	Bombay	4	6.5	M	0.49	0.86	0.68	1.23	0.35	0.42	0.42	0.57
3	F	Tabby	1	4.0	M	0.58	0.89	0.62	1.18	0.55	0.92	0.56	1.01
4	F	Tabby	2	3.6	M	0.51	0.77	0.67	1.23	0.53	1.00	0.72	1.83
5	T	Tabby	1	2.8	M	0.63	0.97	0.45	0.63	0.59	1.20	0.39	0.52
6	F	Tabby	1	4.0	M	0.47	0.68	0.52	0.75	0.73	1.25	0.49	0.79
7	F	Tabby	1	2.7	M	0.48	0.99	0.60	1.02	0.45	0.98	0.68	1.22
8	F	Tabby	1	2.9	M	0.42	0.64	0.48	0.64	0.51	0.71	0.55	0.85
9	T	Bombay	1	4.1	M	0.59	0.98	0.60	1.03	0.62	1.04	0.62	1.09
10	T	Tabby	1	3.0	M	0.43	0.56	0.52	1.01	0.68	1.22	0.63	1.13
11	F	Tabby	1	2.0	M	0.58	0.96	0.59	0.95	0.57	0.92	0.59	1.01
12	T	Tabby	2	3.2	Fe	0.40	0.51	0.45	0.61	0.48	1.20	0.52	0.89
13	T	Tabby	2	3.2	Fe	0.43	0.66	0.62	1.13	0.48	1.20	0.54	0.86
14	T	Tabby	2	5.0	M	0.51	0.82	0.65	1.16	0.38	0.91	0.70	1.48
15	T	Tabby	1	2.2	Fe	0.55	0.87	0.67	1.13	0.53	0.83	0.50	0.68
16	F	Angora	1	2.0	Fe	0.49	0.70	0.65	1.10	0.62	1.19	0.65	1.18
17	F	Tabby	1	2.5	Fe	0.63	1.08	0.66	1.12	0.66	1.28	0.57	0.94
18	F	Tabby	1	3.0	M	0.52	0.75	0.64	1.05	0.63	1.18	0.59	1.02
19	T	Van	1	2.8	Fe	0.37	0.64	0.55	1.05	0.60	1.13	0.62	1.07
20	F	Bombay	1	2.3	M	0.51	0.73	0.69	1.16	0.56	1.02	0.66	1.15

F: Femur, T: Tibia, M: Male, Fe: Female, RI: Resistive index, PI: Pulsatility index.

Radiographic examination findings

In all cases, it was observed that appropriate anatomical reduction was achieved at the fracture site on postoperative day 0, and the intramedullary pin was correctly positioned with the appropriate diameter. By postoperative day 10, it was noted that the fracture ends lost their sharpness and became rounded. On the 20th postoperative day, a reduction in the distance between fragments was observed, and periosteal callus formation around the fracture site was evident. Furthermore, periosteal reactions in regions away from the fracture line were noted in three cases (Case no: 1, 4, and 8): periosteal reaction was observed 10 mm proximal to the fracture line in case 1, 6 mm distal to the fracture line in case 4, and towards the distal region of the bone in case 8 (Figure 2). Cases 3, 5, and 18 exhibited pin migration, and in case 6, an exuberant callus formed.

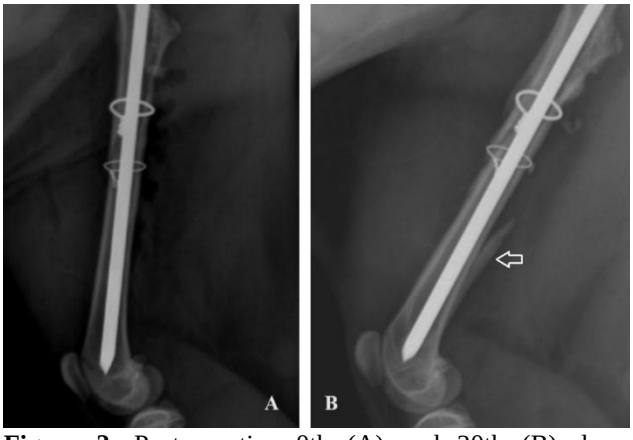

Figure 2. Postoperative 0th (A) and 20th (B) days radiographic images of Case 8. White arrow: callus.

By postoperative day 30, it was observed that the fracture line became indistinct, and the amount of callus tissue increased, becoming more prominent compared to the previous checkup. Specifically in case 2, it was noted that the fracture ends rounded and closed, yet the fracture line remained distinct, and cortical integrity was not maintained. Consequently, treatment for this case continued for some time due to the lack of union. On postoperative day 50, union finally formed, leading to a delayed union diagnosis for Case 2 (Figure 3). In case 2, cerclage wire application was not performed and it was thought that delayed union may have occurred due to the inhibition of rotation movement, which is the disadvantage of intramedullary pin, and movement of the fracture ends. None of the other cases had any complications during the recovery period.

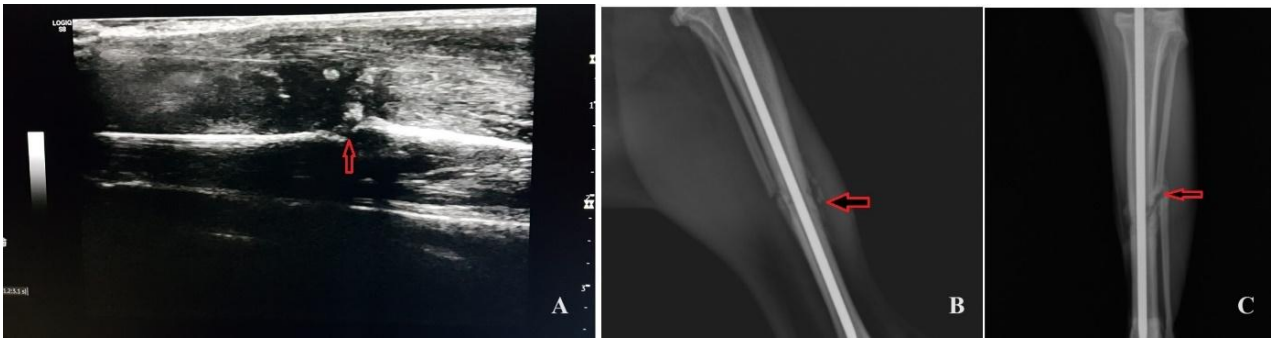

Figure 3. A. Postoperative 20th day B-mode ultrasonography image of Case 2. Red arrow: Fracture line. B. Postoperative 20th day mediolateral radiographic image. Red arrow: callus C. Postoperative 30th day anteroposterior radiographic image. Red arrow: Fracture line.

Ultrasonographic examination findings

On the postoperative 0th day, B-mode ultrasonography of all cases revealed a hyperechoic, longitudinal bone structure. The bone line, visualized as a hyperechoic straight line, was followed, and the interruption of the line was identified as the anechoic region, representing the fracture site. When the area around the fracture line was examined with color Doppler ultrasonography, the presence of small-diameter vessels was identified as a neovascular area, with the signal coming towards the probe appearing in red and the signal moving away from the probe appearing in blue. While a high-resistance spectral waveform was observed in most cases, a low-resistance spectral waveform was detected in Case 2. A decrease in downstroke was observed in late systole.

On the 10th day postoperatively, there was an observed increase in small-diameter vessels displaying both blue and red color flow, signifying an increase in neovascular areas around the callus and its surroundings (Figure 4). On postoperative days 20 and 30, a decrease in neovascular areas was observed, and challenges in pulsed wave blood flow acquisition persisted. In Case 6, the presence of exuberant callus irregularly appeared as a hyperechoic bone line on days 20 and 30. A high-resistance and turbulent flow were noted in the spectral waveform.

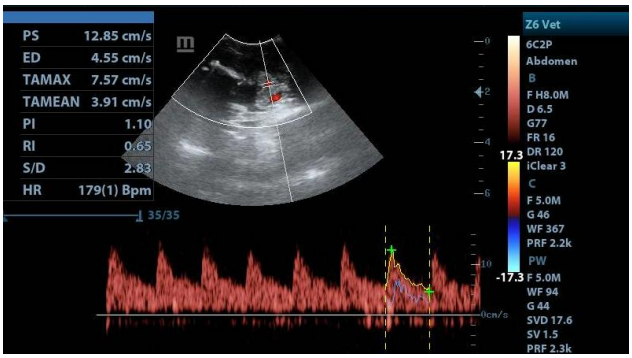

Figure 4. Postoperative 10th day doppler ultrasonography image of case 16

In Case 2, on postoperative day 20, it was observed that the fracture ends became more distinct, and the fracture line was anechoic.

During the 30th-day follow-up, it was noted that cortical bone integrity was not maintained. Consequently, on the 50th day, a reduction in the fracture line interval and a shift to a high-resistance spectral waveform were notable.

Index findings

According to the index analysis results, on postoperative day 0, the mean RI value was determined to be 0.509 ± 0.017, and the mean PI value was 0.798 ± 0.035. The data indicated similar results among the cases on postoperative day 0. On postoperative day 10, a statistically significant increase was observed in the arithmetic mean of RI values by 0.1 units compared to day 0, along with a 0.255-unit increase in PI values. On postoperative day 20, it was observed that, compared to day 10, there was a decrease of 0.05 units in the arithmetic mean of RI values for all cases, and an increase of 0.04 units in PI values. Examining the postoperative 30th-day RI analysis results, a 0.05 unit increase was noted compared to the postoperative 20th day. The variance analysis of RI-time intra-group factors has been indicated in the table for repeated measurements (Table 2). Upon general examination of the cases, it was observed that the RI values showed a statistically significant increase on the 10th and 30th days. According to the data, it was observed that the increase in PI value between the 0th and 10th days is more significant compared to the rate of increase on other days, while the difference in PI between the postoperative 20th and 30th days was found to be less pronounced (Table 3).

Table 2. Change in Resistive Index values over time.

Resistive index	n	Art.Mean ± Std. Deviation	Median (Min-Max)	P value
RI (t0)	20	0.509 ± 0.017[a]	0.510 (0.370 – 0.630)	0.010
RI (t10)	20	0.593 ± 0.017[b]	0.610 (0.450 – 0.690)	
RI (t20)	20	0.564 ± 0.023[ab]	0.565 (0.350 – 0.750)	
RI (t30)	20	0.588 ± 0.021[ab]	0.590 (0.390 – 0.760)	

[a,b]: Different letters in the same column indicate statistically significant differences (p<0.05). Art.Mean: Arithmetic mean value, Std. Deviation: Standard deviation, Min: Minimum, Max: Maximum, RI: Resistive index.

Table 3. Change in Pulsatility Index values over time.

Pulsatility index	n	Art.Mean ± Std. Deviation	Median (Min-Max)	P value
PI (t0)	20	0.798 ± 0.035[a]	0.795 (0.51 – 1.08)	0.002
PI (t10)	20	1.000 ± 0.045[b]	1.050 (0.61 – 1.23)	
PI (t20)	20	1.063 ± 0.057[b]	1.085 (0.42 – 1.66)	
PI (t30)	20	1.051 ± 0.075[b]	1.015 (0.52 – 1.83)	

[a,b]: Different letters in the same column indicate statistically significant differences (p<0.05). Art.Mean: Arithmetic mean value, Std. Deviation: Standard deviation, Min: Minimum, Max: Maximum, PI: Pulsatility index.

Upon individual examination of cases, it was noted that on the 20th and 30th days of Case 2, on the 10th and 30th days of Case 5, on the 30th day of Case 6, and on the 10th day of Case 8, both RI and PI values were lower compared to other cases (Table 1). The statistical significance level was determined to be $p < 0.01$ for the RI value and $p < 0.002$ for the PI value, and a significant change in the data obtained on control days was observed.

DISCUSSION

Fractures can result from various factors such as trauma, metabolic diseases, age, and others. The diagnosis of a fracture, the methods employed in treatment, and postoperative follow-up processes are typically determined through radiography. Following the trauma period, the evaluation of images termed "callus" on radiographs, which appear adjacent to the bone opacity around the fracture, plays a crucial role in determining the process of fracture healing. However, this assessment is subjective and relies on the knowledge and expertise of the physician interpreting the radiographic images. Ultrasonography, as an imaging method, offers

more detailed results in the early stages of fracture healing compared to radiography. Ultrasonography not only enables the visualization of the fracture line and bone tissue but also provides information about the soft tissues affected by trauma (Maffulli, 1995).

Color Doppler ultrasonography is a significant diagnostic method that identifies the presence and direction of blood flow in tissues, along with the normal and abnormal vascularization structure in organs. While widely utilized in various fields, it is commonly employed in the physiological and pathological evaluations of bone tissue (Kealy et al., 2011; Kramer, 2011). In a study assessing fracture healing, Doppler signals were identified until the 50th day, but a decrease in the signal was observed after the 30th day. Concurrently, reductions in the vascular area also occurred during this process (Risselada, 2006). Similar findings were obtained in the conducted study, where an increase in neovascularization was observed in the fracture area on the 10th day after surgery, while a decrease was noted on the 20th and 30th days. The increased angiogenesis during the inflammatory phase is thought to contribute to the proliferation of osteoprogenitor cells, leading to more neovascular area signals on the 10th day compared to other days in color Doppler ultrasonography.

Su et al. (2013), reported that bone union was inadequate in cases with decreased index values. In the research, periosteal reaction was identified in the radiographic images of case 2 on the 10th and 20th days, indicating that the fracture line was beginning to blur. Based on the radiographic examination results, the healing process of the fracture was considered to be progressing normally. However, during the assessment on the 30th day, it was observed that the fracture ends started to close, and the fracture line became more pronounced. In the radiographic findings, the ultrasound images of the case with complete anatomical reduction were notable for the anechoic appearance of the fracture line. On the postoperative 20th day, the anechoic appearance of the fracture line became even more apparent. Considering the ultrasound evaluation of the fracture line, it is suggested that delayed union could be diagnosed earlier compared to radiographic images. In the index assessments of the same case, it is noteworthy that the PI and RI values were lower on the postoperative 20th and 30th days compared to other cases. Taking into account the existing neovascular areas and index values, it is believed, similar to the findings of a previous study (Caruso, 2000; Su et al., 2013), that these assessments are crucial for the early diagnosis of callus complications.

In a human study reported an inverse relationship between the RI value and the amount of callus, although no significant difference was observed within the group (Su et al., 2013). Conversely, another study concluded a direct proportionality between bone integrity and the RI value (Wawrzyk et al., 2015). Discrepancies in the study results have been interpreted as potentially arising from variables such as the assessment of different extremities, the inclusion of middle-aged adults and children in the study, and variations in vessel diameters. Upon reviewing the conducted studies, it is evident that cases achieving bone integrity, particularly by the 10th day, exhibit a notable increase in the RI value (Wawrzyk et al., 2015). The direct proportionality between the development of bone integrity and the increase in the RI value aligns with the findings of the current study. This observed proportional increase in the RI value with callus formation can be attributed to heightened vascular resistance, especially in microvascular areas where vascular walls are shaped. This is particularly evident on the 10th day when osteoblastic activity and neoangiogenesis are

intense. The subsequent decrease in the RI value on the postoperative 20th day can be interpreted as a reduction in resistance due to a decrease in cell density compared to the 10th day. In the healing process on the postoperative 30th day, there was a decrease in extraosseous blood supply compared to other days. Nevertheless, the diminished neovascular area lead to an increase in flow in other small vessels. Ongoing cellular activity contributed to an increase in resistance in the vascular wall. Consequently, there was an observed increase in the RI value on the 30th day compared to the value on the 20th day.

The pulsatilite index is associated with the flexibility of arteries and provides information about the cardiovascular system. The flexibility of large arteries plays a role in tolerating vibrations caused by left ventricular contractions. The expansion of the aorta during each heartbeat and the storage of a portion of its volume allow smaller vessels to be less exposed to pulsatile stress (Michel and Zernikow, 1998; Wielicka et al., 2020). In our study, an increase in the pulsatility index was recorded throughout the fracture healing process. Especially on the 10th postoperative day, microvascular structures were exposed to pulsatile stress due to the increase in blood flow. The increase observed in the PI index on this day, when osteoblastic activity and inflammation are active, can be interpreted as the presence of high blood flow and perfusion in the distal tissues. The increase in neoangiogenesis in proportion to the fracture healing process leads to a significant increase in the vascular network formed by the main artery and a decrease in the flow per vessel. This was interpreted as a decrease in pulsatile stress in the vessels and a decrease in the pulsatility index on the 30th postoperative day.

Intramedullary pin application minimally traumatises the periosteal circulation compared to plate application because it is placed in the medullary cavity (Decamp et al., 2016). In this study, the microvascular structures that provide extraosseous blood supply and callus formation are investigated by imaging methods during the healing process. The fracture fixation method to be applied was decided in line with this purpose, therefore intramedullary pin application was preferred. In addition the cerclage wire affected the periosteum from a single site and was ignored because it was thought that the extraosseous blood supply would not be affected as much as the plate.

Fracture fixators utilized in reduction are typically made from inert materials. However, in certain instances, it becomes necessary to remove these fixators. Based on the data obtained in this study, normal values for index values during fracture healing have been established. According to the data, it was determined that the average unit value of RI on the 30th day for cases is 0.588 ± 0.0021, and the average unit value of PI is 1.051 ± 0.075. If both indices exceed the normal values on the 30th postoperative day, early consideration may be given to the removal of fixators. This is particularly crucial in cases where the growth plate is affected or to alleviate the stress factor created by the bandage. Early detection of a decrease in index values in cases where fracture healing is delayed, possibly due to hormonal imbalances or inadequate nutrition, can prompt consideration for nutritional supplementation with fortified foods.

CONCLUSION

The study produced statistically significant results regarding vascular index values throughout the period from the emergence of neovascular areas to callus development in the healing stages of long bones. The conclusion drawn was that PI and RI values could serve as crucial parameters for evaluating the healing process in complex fractures, especially in promptly identifying complications and monitoring recovery progress. Furthermore, it has been suggested that alternative Doppler indices could be utilized to supplement

traditional diagnostic approaches for the early detection of pathological bone conditions.

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Evaluation of pregnancy-associated glycoprotein (PAG) test in early pregnancy diagnosis in dairy cows

Research Article

ABSTRACT

In this study, it was aimed to evaluate and compare the effectiveness of pregnancy-associated glycoprotein (PAG) levels in the milk samples of dairy cows in their early pregnancy diagnosis. A total of 229 Holstein cows, aged between 3 and 7 years old, inseminated in the first natural estrus after completing the voluntary waiting period and not returning to their estrus at 18-24 days after insemination, were used as animal material. Ultrasound examination was performed on the 29th day after insemination to diagnose early pregnancy, and milk samples were collected on the same day to determine PAG levels. The ultrasound examination was used as a reference test. The sensitivity, specificity, predictive values, and accuracy rates of the milk PAG test were found to be 92%, 100%, 100%, 91.7%, 95.8%, respectively, and the agreement between the ultrasound and milk test results was excellent according to the Kappa value (κ=0.919). The overall validity of the milk test was found to be 95.75%. The false negative rate of the milk PAG test was found as 8%. The false negative results were thought to be caused by embryonic deaths. In conclusion, the milk PAG test is a reliable test for early pregnancy diagnosis in dairy cows.

Keywords: Dairy cows, diagnosis, early pregnancy, milk PAG

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INTRODUCTION

Correct pregnancy diagnosis is very important for field conditions. It is important to determine pregnancy as early as possible and by the most reliable method. This importance lies in identifying non-pregnant cows as soon as possible and reinseminating them to ensure conception in order to shorten the calving interval. This way, infertile animals in the herd can be detected and treated in a shorter time, or economic losses can be reduced by removing them from the herd. Sound reproductive management depends on establishing and maintaining pregnancy as soon as possible after the end of the voluntary waiting period after birth or after the breeding season in seasonal systems (Pohler et al., 2016).

Pregnancy detection can be performed by using both direct and indirect methods. Direct methods include transrectal palpation and transrectal ultrasound. Indirect methods include chemical pregnancy markers, such as milk or plasma progestogen concentrations or pregnancy-associated protein and pregnancy-associated glycoprotein (PAG) measurements (Bekele et al., 2016; Doğan and Köse, 2022; Fosgate et al., 2017; Gajewski et al., 2008; Purohit, 2010).

Bovine PAGs are produced by the binuclear trophoblastic cells of the placenta immediately after implantation. PAGs can be detected in maternal circulation from the 22nd day of pregnancy to 2-3 months after delivery (Çiplak, 2024; Schlafer et al., 2000).

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Evaluation of pregnancy-associated glycoprotein (PAG) test is important in the control of caruncles and uterine gland morphogenesis, differentiation, and functions, which are necessary for fetal nutrition and the maintenance of intrauterine viability (Igwebuike, 2006). The sensitivity and specificity of the milk PAG testing method used as a pregnancy test are very high. As a stress-free method in pregnancy management in ruminant animals, it is particularly useful in the diagnosis of pregnancy and the determination of embryonic deaths (Gajewski et al., 2008).

This study aimed to evaluate diagnostic power of the milk PAG test used in the early diagnosis of pregnancy in of cows after insemination.

MATERIALS AND METHODS

Animal material

The study was carried out in a dairy farm with 3,000 dairy cows in Aydın, where intensive feeding and herd management systems are used.

The animal material of the study included healthy and clinically normal 229 cows without reproductive disorders and aged from 3 to 7 years that were artificially inseminated by the farm's technical staff after 60 days of calving and whose estrus did not return 18-24 days after insemination. The average daily milk production per cow was about 55 kg during the study period. Efforts were made to reduce variation in the general condition of cows so that milk PAG changes could be attributed to factors other than the clinical condition of the cows at the time of the study.

The cows were housed in a closed barn with a red-light system and a barn air conditioning system. In the farm, cows were milked 3 times a day and fed a Total Mixed Ration (TMR) prepared with feed ingredients calculated according to their needs (Ethics committee approval 2016/1-2).

Method

Estrus detection and insemination

Artificial insemination was performed by the technicians in the first natural estrus of the cows, and after the voluntary waiting period, a herd management program (activity meter, decrease in milk yield) was applied, and the results were evaluated in combination with their observations.

Collection of milk samples

On the 29th day after insemination, following the expression of a few shots of foremilk from any of the healthy udder lobes from the cows whose estrus did not return, milk samples were taken into a tube in an amount of approximately 20 ml.

Performing ultrasound examinations

Ultrasound examination was accepted as a reference test in the study. In the examination, device (Shenzhen Well. D Wed-3000v, China) has a B mode, 7.5 MHz frequency, and with a transrectal linear probe was used. Ultrasound examinations and milk sampling were performed on the same day (29th day). In cases where the pregnancy diagnosis was uncertain, a second examination was performed within the first week after the first examination (up to the 35th day after insemination) to confirm the diagnosis.In the ultrasonic examination, the quality of the ovary note CL (>22 mm, luteal tissue density, compactness) found in rectal examination was determined. Likewise, the other ovary was also evaluated. This way, when ultrasound-guided pregnancy diagnosis emerged while scanning the uterus, the corn where CL was found was revealed. The animal was considered pregnant when at least 3 of the following findings were observed while scanning the corn uterus: amniotic fluid, fetal membranes, umbilical cord, fetal heartbeat, fetus itself, and compartmental structures in the uterus. In animals without quality CL in one of the ovaries (not in sufficient size), one of the cornu uteri was scanned in detail in terms of possible pregnancy in longitudinal

and transverse sections starting from the bifurcation area. The animals in which these symptoms were not observed were considered non-pregnant.

PAG Determination in milk samples

A commercial ELISA kit for measuring PAG levels (IDEXX Milk Pregnancy Test-Diagen, Türkiye) was used to test the collected milk samples. The test was performed by experienced technical personnel, in line with the instructions of the manufacturer.

Interpretation of the results

According to the value obtained from the reader: <0.100 was considered empty or non-pregnant (negative result), 0.100-0.250 was considered suspicious, and >0.250 was considered pregnant (positive result). The test result was considered as a false positive result for the test when a non-pregnant cow was diagnosed as pregnant on ultrasound, and as a false negative result for the test when a pregnant cow was diagnosed as not pregnant on ultrasound. Among the cows diagnosed as suspicious as a result of the test, the positive suspicious result was accepted for pregnant animals according to the ultrasound examination, and the negative suspicious result was accepted for non-pregnant animals (Akkose, 2023).

Statistical analysis

In the early pregnancy diagnosis process, the results of ultrasound examinations, whose validity was previously determined and was quite high, were used as reference values. In the validity and reliability analyses of the milk PAG test, sensitivity and specificity values were determined by identifying positives, negatives, and their accuracy rates. The agreement between the ultrasound and milk PAG test results was evaluated using the Kappa test.

The Kappa coefficient takes values between 0 and 1. Accordingly, values of 0.93-1 are considered excellent, values of 0.81-0.92 are considered very good, values of 0.61-0.80 are considered good, values of 0.41-0.60 are considered moderate, values of 0.21-0.40 are considered somewhat poor, and values of 0.01-0.20 are considered poor (Altman, 1991).

The general strength or validity of the milk PAG test was found using the following formules:

Sensitivity: True positive /(true positive + false negative) x 100

Specificity: True negative /(false positive + true negative) x 100

Positive predictive value: True positive /(true positive + false positive) x 100

Negative predictive value: True negative /(false negative + true negative) x 100

Accuracy ratio: (True positive+true negative)/(true positive + true negative + false positive + false negative) x 100

RESULTS

Pregnancy findings obtained by two methods from 229 cows whose pregnancy statuses were examined on the 29th day after insemination by ultrasound and the milk PAG test are given in Table 1.

Table 1: Pregnancy findings detected by ultrasound and milk PAG test on the 29th day after insemination.

Parameters	Ultrasound	Milk PAG
Pregnant	104	103
Not pregnant	125	100
False positive	0	9
False negative	0	0
Positive suspect	0	1
Negative suspect	0	16
Total	229	229

In the ultrasound examination, which was accepted as the reference method in the study, it was determined that 104 cows out of the 229 cows included in the study were pregnant, and 125 cows were not pregnant.

In the milk PAG tests of the same 229 cows, it was found that 103 cows were pregnant, 100 cows were not pregnant, and 17 cows were suspicious.

Nine out of the 125 cows diagnosed as not pregnant by ultrasound were diagnosed as pregnant with the milk PAG test, and 16 were diagnosed as suspicious. Similarly, while none of the 104 cows diagnosed as pregnant by ultrasound were found to be not pregnant, 1 cow was diagnosed as suspicious with the milk PAG test. While 9 cows were diagnosed as false positive by the milk PAG test, no false negative diagnosis was made.

The overall strength or validity of the milk PAG test (103 + 100/103 + 100 + 0 + 9=) was found to be 95.75%.

The sensitivity, specificity, indication of pregnancy and non-pregnancy, accuracy, and compatibility rates of the milk PAG tests were found as shown in Table 2.

Table 2: Sensitivity, specificity, predictive values, accuracy, and agreement rates of the milk PAG test.

	Sensitivity %	Specificity %	Positive Predictive Value %	Negative Predictive Value %	Accuracy %	Kappa
Milk PAG	92	100	100	91.7	95.8	0.919*

* Kappa value> 0.8: excellent agreement.

The accuracy rate of the milk PAG test was found to be 95.8%, and the agreement between the two tests was excellent (kappa value>0.8). The false positive rate of the milk PAG test was found as 8.03%.

DISCUSSION

In this study, which aimed to evaluate the effectiveness of PAG testing that has the potential to be used in early pregnancy diagnosis and has attracted the attention of researchers in recent years, ultrasound examination, whose validity was previously determined and was quite high, was accepted as the reference test method.

Dairy farms should determine whether their animals are pregnant as early as possible, using a method that is low-cost, highly accurate, and easily applied in field conditions (Balhara et al., 2013). Determining pregnancy early and with high accuracy plays a key role in establishing the appropriate delivery interval.

Early pregnancy can be observed by performing daily ultrasound examinations. Additionally, it is reported that chemical analyses can be used in cows. In chemical methods used in pregnancy diagnosis, the presence or quantity of reproductive hormones is measured at a specific time after insemination. Another approach is to investigate the existence of a living embryo based on offspring-specific structures in the mother's blood. Progesterone analysis was the first to come to mind and the most researched method about pregnancy diagnosis with chemical methods. In recent years, PAG, which was found to be synthesized only from the pregnant uterus, has attracted attention. Moreover, PAG testing has advantages over progesterone-based pregnancy diagnosis. One of these advantages is that PAG can always be checked after an appropriate number of days following insemination, while the timing of progesterone testing is limited to 19-23 days after insemination. Secondly, the reliability of positive results in progesterone testing was reported as 80% by the best estimates by Sasser and Ruder (1987), while the reliability of positive results in PAG testing was determined to be 95-100% (Sasser et al., 1986; Zoli et al., 1992).

Routine pregnancy examination by ultrasound is recommended in cows on the 28th day after insemination (Dinç, 2008). Based on extensive studies today, ultrasound has been widely accepted as a valid method for the diagnosis of pregnancy. In addition to the

detection of a viable embryo in the uterus, ultrasound can quickly detect pregnancy loss, embryonic deaths, and embryonic degeneration (Moharrami et al., 2013). It also provides additional information about the non-pregnant animal. Furthermore, it allows the determination, direction, and rapid implementation of synchronization or resynchronization protocols according to the existence of functional structures in the ovaries (Dinç, 2008).

From an economic point of view, the sensitivity of the early non-pregnancy test (correct diagnosis of the pregnant animal) was found to be more important than its specificity (correct determination of the non-pregnant animal). It was reported that the sensitivity of a test should be higher than 96% if the test is performed 31 days after insemination and higher than 94% if it is performed after 24 days to gain potential economic benefit from the early diagnosis of the non-pregnant animals with chemical tests (Fricke and Giordano., 2011; Giordano at al., 2013).

Le Blanc (2013) found a sensitivity value of 99.2% and a specificity value of 95.5% in a study using 683 animals in 8 dairy farms. Lawson et al. (2014) reported that they found these values as 100% and 97.9% in 112 cows tested on the 33rd and 52nd days after insemination.

In this study, similar to previous studies, the sensitivity of the milk PAG test in determining pregnancy was determined as 92% (true positive rate). Similarly, the milk PAG test was found to have 100% specificity in revealing non-pregnant cows (true negative rate). In this study, the accuracy rate of the milk PAG test was found to be 95.8%. It was concluded that the milk PAG testing method performed above the economic gain limits (Lavon et al., 2022).

While none of the 104 cows diagnosed to be pregnant with ultrasound were diagnosed to be not pregnant by milk PAG testing, 1 cow was diagnosed with suspected pregnancy. While 9 cows (8.3/7.2%) were diagnosed as false positive in the milk PAG tests, no false positive diagnosis was made. Embryonic deaths indicative of false negative results in a test are important in terms of economic losses (Fosgate et al., 2017). Akkose (2018; 2023), determined that 95% of cattle with PAG values below 1.4 ng/ml on the 31st day of gestation experienced embryonic death until the 60th day of gestation. It was stated as a result that this test can be used as a good marker between the 31st and 59th days to identify embryonic deaths (Ask-Gullstrand et al.,2023; Yang et al.,2024).

In the milk PAG tests in this study, 1 cow was diagnosed with suspected pregnancy (positive suspicion), and 16 were diagnosed with suspected pregnancy (negative suspicion). When the value read in the evaluation made according to the milk PAG levels was in the range of 300-1,000 pg/ml, the result was considered suspicious. According to this assumption, the reading values of 17 cows (7.42%) were found to be suspicious. One of these 17 animals was evaluated as a positive (pregnant) suspect, and the remaining 16 were evaluated as negative (non-pregnant).

CONCLUSION

As a result, in this study, it was determined that the milk PAG test performed on the 29th day after insemination is a reliable and practical method for early pregnancy diagnosis.

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Author contributions: HA carried out the created the records and examinations, HA and DAD conceived the idea and prepared the original draft of the article. HA did the writing and DAD helped to finalize the manuscript. All authors made corrections and approved the final version of the manuscript.

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Investigation of diamine oxidase as a biomarker of intestinal mucosal injury in calves exposed to antibacterial treatment

ABSTRACT

Diamine oxidase (DAO) (alternatively old-fashioned name histaminases), as being an enzyme in high concentrations, support the integrity and maturation of small intestine. Histamine intoxication and related issues are suggested to exist lacking enzyme diamine oxidase. Enrollment of the gastrointestinal mucosa in several diseases, along with influence of some medications seemed to diminish gastrointestinal DAO activity. The aim of this study was to investigate intestinal mucosal injury in sick calves exposed to antibiotic treatment. In accordance with the inclusion criteria, 20 sick calves with gastroenterologic or respiratory system problems exposed to antibiotic treatment for >3 weeks and 10 other healthy calves were evaluated. Commercially available DAO ELISA kit: Bovine Diamine Oxidase ELISA Kit and Sandwich ELISA-mediated DAO assays were performed. In this study, the mean DAO (ng/mL) level was determined as 5.552 in sick calves exposed to antibiotic treatment, while the mean value was determined as 16.48 in healthy calves in the comparative evaluation ($p<0.001$). The data obtained suggest that DAO activity may be affected in calves exposed to antibiotic treatment for at least 3 weeks.

Keywords: Antibiotics, calves, diamine oxidase, mucosal injury

INTRODUCTION

Diamine oxidase (DAO) is of great importance intracellular enzyme with antihistaminic property exhibited within the small intestinal mucosae for prevention of enterocytes against battling histamine (Kovacova-Hanuskova et al., 2015). DAO is the foremost biomarker for interpretation of gut mechanical barrier and the proportion of mucosal villus injury (Fukudome et al., 2014). The latter enzyme is exhibited at the apical border of mature villous cells with selectively increased activation and its activation denotes the rectitude and full growth of the small intestinal mucosa (Honzawa et al., 2011). In case of injured mucosa or underdeveloped gut wall rectitude, DAO leaks from the jejunal villus tips through central circulatoric vehicle (Zhang et al., 2016). DAO concentration through the circulation might denote intestinal permeability biomarker (Alizadeh et al., 2022). In addition, small intestinal mucosal injury can reduce diamine oxidase activity (Alizadeh et al., 2022). Elevated DAO serum levels and decreased DAO activity are associated with increased intestinal permeability and thus lower intestinal development (Alizadeh et al., 2022; Song et al., 2017). In Turkey, only one doctoral dissertation on intestinal mucosal injury in calves (Türk, 2023) and the author of this article served as the second thesis advisor within the scope of that thesis. Since this thesis work is at

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Research Article

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the publication stage and no other article has been encountered, it is necessary to better understand why this study was carried out in order to better understand the original value of the subject. Many different drugs reduce DAO activity (Finazzi-agro et al., 1979; Leitner et al., 2014; Maintz and Natalija, 2007; Tobajas et al., 2023) yet random drug use continues without this issue being on the agenda and possibly ignored. Regarding the hypothesis that intestinal mucosal injury should be related to drug usage among calves, in this study, we aimed to determine the intestinal mucosal injury mediated by DAO enzyme activity in calves with diarrhea exposed to antibiotic treatment for more than 3 weeks.

MATERIALS AND METHODS

This retrospective field study was conducted in commercial calf facilities located in the Aegean Region of Turkey. HADYEK certificate holders took an active role in the collection of blood samples in the field study. The study was approved by the local ethics committee of Aydın Adnan Menderes University Ethics Committee on 27/10/21 with the reference number 64583101/2021/146. All participating calves were included in the current study with the written consent of the owner.

In accordance with the inclusion criteria, 20 sick calves with gastroenterologic or respiratory system problems exposed to antibiotic treatment for >3 weeks and 10 other healthy calves were evaluated. Anticoagulated tubes were used to collect 0.5 ml of blood from the *V. jugularis*. Plasma was separated after centrifugation. Commercially available DAO ELISA kit: Bovine Diamine Oxidase ELISA Kit (My Biosource, San Diego, United States of America) (Alic Ural et al., 2023) was purchased and made available by RDA Group, Istanbul. The corresponding Sandwich ELISA exhibits high sensitivity and excellent specificity for detecting DAO. As previously described, there is no known cross-reactivity/interaction between DAO and analogs. Plasma samples were analyzed with an available Quantitative Competitive assay via Sandwich ELISA. The sensitivity was 1.0 ng/mL and the detection range was 0.312-20 ng/mL. All samples were stored at the appropriate temperature prior to analyses and all reagents were kept at 2-8°C. The stool scoring system has been described previously (Graham et al., 2018). Sera samples were picked up into relevant tubes and then forwarded to storage in -80°C freezer until analysis were performed. The samples were then analyzed by Bovine Diamine Oxidase ELISA assay. The Diamine Oxidase ELISA kit is a test based on the competitive enzyme immunoassay technique. In this method, polyclonal anti-dAo antibody and dAo-HRP conjugate were used. The detection range was between 0.312 ng/ml and 20 ng/ml. Serum samples and buffer were added to pre-coated plates and incubated with dAo-HRP conjugate for one hour. Following incubation era, relevant pores were emptied and subjected to washing 5 times. The pores were then incubated with a substrate for the HRP enzyme. The enzyme-substrate reaction leads to the formation of a blue colored complex. A final liquid was added to finalize the reaction, which turns the blue colored enzyme-substrate complex yellow. The color vigour was measured spectrophotometrically at 450 nm in a microplate reader. For statistical interpretation, SPSS 22.00 (IBM, America) program was used and Kruskal Wallis one-way ANOVA test was applied to rank the non-parametric analytes. P value was set as 0.01.

RESULTS

It was learned that at least one antibiotic option was used for digestive system problems or suspected infectious diseases for more than 3 weeks in the anamnesis taken clearly under the control of a veterinarian under field conditions. For enrollment at ths study the calves were

selected to those of solely antibiotic prescribed, otherwise no other drug usage was deemed available. During blood collection, it was ensured that at least 21 days of treatment had been completed under the supervision of a veterinarian with the relevant certificate.

Table 1 shows the relevant data together with statistical values. During the ELISA analysis, there were no false readings that could be recorded, and all samples could be analyzed. Mean DAO (ng/mL) values showed statistical significance ($p<0.01$) in antibiotic treated calves compared to healthy control group.

Table 1. Circulating serum diamine oxidase levels among diseased and healthy calves.

DAO (ng/mL)	Sick calves exposed to antibiotic treatment	Healthy calves	P value
Average	5.552	16.48	0.001
Standard Error	0.577	0.683	
95% CI	(4.34 - 6.77)	(14.93 - 18.02)	

DISCUSSION

In the intestine, single-layered epithelial cells separating the microorganism-dense lumen and separated through the vast majority immune complex function as a protective barrier, preventing microorganisms, toxins, inflammatory metabolites and antigens from entering the systemic circulation (Mani et al., 2012). Even if the intestinal integrity is unsettled, permeation of toxic intraluminal foreign compounds and microorganisms could elucidate fired immune respond prone to low grade systemic inflammation. Such inflammation accompany alterations within the functioning of the tissue changing the animal's metabolic load to support the elevated energy requirement of the immune respond, in turns unwantedly influence productivity and growth (Kvidera et al., 2017; Liehr et al., 2017). In the present study the author investigated DAO, as a biomarker of intestinal mucosal injury to those of calves subjected to prior antibiotic treatment by field veterinarians.

Intestinal penetrability is frequently preferred as a biomarker for assessing intestinal barrier function. Given inflammation of intestines, penetrability is a vital condition for interpretation of the health or disease status for the gastrointestinal tract (Usuda et al., 2021) Several studies in humans and animals reveal that increased intestinal permeability is positively correlated with plasma DAO concentration and negatively correlated with DAO activity (Lackner et al., 2019). In this study, DAO analysis was performed to investigate intestinal mucosal injury in calves exposed to antibacterial treatment.

In a study in which calves with diarrhea were classified through fecal consistency and blood pH (Fukuda et al., 2019a), circulatory DAO concentration was markedly diminished in calves with severe/moderate diarrhea compared to the control group and in the severe group compared to the moderate group. Quanz (2022) reported that plasma DAO activity was clearly decreased in weeks when clinical signs coinciding with markers of dysbiosis indicated gastrointestinal distress in cows in the study group. In a study examining the influence of untimely pathogenic *Escherichia coli* (*E. coli*) invasion at gut barrier and immune functioning of newborn calves (He et al., 2022); increased DAO and IL-6 levels are shown. Although cytokine analysis was not possible in this study, it is planned to conduct these studies in the next study by establishing a consensus and multidisciplinary team with broad participation.

Heat stress is also a factor that impairs intestinal function by inducing overproduction of reactive oxygen species (ROS) and proinflammatory cytokines along with increased intestinal permeability (Cheng et al., 2019; Song et al., 2017). It was revealed that serum DAO

activity increased in heat-stressed broilers (Lan et al., 2020). As a result of the induction of permeable intestine by *E. coli in* a mouse model, in addition to the observation of villus injury in histologic examination; DAO and zonulin levels were found to be significantly higher compared to control group mice (Ren et al., 2022). Increased DAO and endotoxin levels are associated with an increase in intestinal permeability after the administration of methotrexate, which is used for antitumoral activity but is likely to be toxic to other cells, for chemotherapy in children (Meng et al., 2016). In a study in 69 humans with inflammatory bowel disease, increased levels of DAO and D-Lactate, an intestinal bacterial metabolite associated with intestinal permeability and intestinal injury, were found after treatment (Song et al., 2009). In a porcine model, DAO activity, which decreased after induction of intestinal mucosal injury by LPS, increased after administration of fish oil to pigs due to an increase in the villus height-to-crypt depth ratio (Liu et al., 2012). In Crohn's disease patients, decreased DAO activity in the intestine has been reported to correlate with the severity of histologic changes (Thompson et al., 1988). Takimoto and colleagues (2014) found decreased DAO activity in patients with anorexia nervosa, suggesting the existence of intestinal morphological disorder related to malnutrition. Since etiologically based assessment was not possible in this study, the presence/absence of infectious agents was not evaluated. Nevertheless, since the analyzes were collected in a temperate climate, it does not seem possible to mention heat stress.

In a previous study, DAO activity was measured in diarrheic calves to detect intestinal mucosal disorders. Based on stool composition (between 0-3) and blood pH (acidemia: blood pH<7.25) in 36/50 calves with diarrhea, DAO activity was lower ($p < 0.05$) in severely or moderately diarrheic calves compared to the control group; plasma DAO activity was significantly and negatively correlated with stool scores (Fukudo et al 2019a). Fukuda et al. (2019b) investigated whether probiotic administration could be an alternative to antibiotics in diarrheic calves and measured DAO activity related to diarrhea as a second objective in their study. Twenty-two evenly divided Japanese black calves with diarrhea were treated with probiotics (n=11) or antibiotics (n=11) limited to 8 days; serum DAO activity was found to be significantly elevated only in probiotic-treated calves. In the light of the data obtained, it was suggested that probiotics can affect serum DAO activity in diarrheic calves (Fukuda et al., 2019b). Very recently, another study comparable to the studies in the above paragraph aimed to establish even if plasma concentrations of DAO in calves indicate mucosal injury during diarrhea, against which rectal enema probiotic treatment was used. Following acceptance to commercial facility, calves were scored on a scale from 0 to 3 according to fecal consistency. Calves exhibiting a stool score of 2 (loose stools) or 3 (watery stools) were considered to have diarrhea and only calves with diarrhea were recorded. All calves with diarrhea were treated with rectal enema with multi-strain probiotics. The mean DAO levels (ng/mL) of diarrhea calves prior to and thereafter probiotic enema therapy were 8.48±1.67 and 28.06±3.51, respectively, which showed statistically significant changes ($p<0.001$). In summary, it was stated that plasma DAO activity was lowered in respond to intestinal mucosal injury associated with diarrhea and this was reversed by rectal enema probiotic treatment for 10 days and it was possible to draw a preliminary conclusion that DAO activity reflects a feedback regulation associated with mucosal healing as suggested (Alıç Ural et al., 2023).

It has been well postulated that even if physiological activity entire organs have been modified by the microbiota (Goyal et al., 2015; Marsland and Salami, 2015), gut mucosal lining and its immune accompanying components, are influenced by this symbiosis (Caballero et al., 2015). Moreover, microbiome manage intestinal developmental stages by modifying vascularization, thickening of villi, widening of mucosal location, existence of mucus, proliferation of cells and epithelial junctioning (Kelly et al., 2015; Sommer and Backhed, 2011; Reinhaedt et al., 2012). On the other hand, antibiotics, to the present author's knowledge are increasingly used at field conditions, rattle the balance between commensal microecology and could accompany to a diminished or changed interaction between the microbiota and the underlying mucosa (Becattini et al., 2016). In the present study DAO activity was analyzed for directly measuring intestinal mucosal injury and thus related mechanisms aforementioned above. In the present study although etiological interpretation was not deemed available, available data should help veterinary surgeons for planning treatment protocols.

CONCLUSION

In the present study, the mean DAO levels (ng/mL) in calves exposed to antibiotic treatment for at least 3 weeks were 5.552 vs. 16.48, suggesting that antibacterial treatment may contribute to decreased DAO activity and thus intestinal mucosal injury. Further prospective studies with larger populations and comparative analyses with other biomarkers are warranted.

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Conflict of interest: There is no conflict of interest in this study.

Ethical statement or informed consent: This study was approved by the Aydın Adnan Menderes University-HADYEK local ethics committee on 27.10.2021 with number 64583101/2021/146.

Author contributions: Motivation / Concept: DAU; Design: DAU; Control/Supervision: DAU; Data Collection and / or Processing: DAU; Analysis and / or Interpretation: DAU.

Availability of data and materials: Data and materials may be used subject to the author's permission.

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Determination of the effects of vitamin D and Nettle (*Urtica dioica* L.) extract administration on SOD-2 and TNF-α levels in liver tissue of TNBS (2,4,6 trinitrobenzene sulfonic acid)-induced rats

ABSTRACT

The aim of this study was to investigate the effects of vitamin D and nettle (*Urtica dioica* L.) extract on SOD-2 and TNF-α levels in liver tissue of rats induced with TNBS (2,4,6 Trinitrobenzene Sulfonic Acid) by immunohistochemical methods. All rats used in the study were weighed and randomly divided into four groups. Liver tissue samples were taken at the end of the experiment. They were blocked in paraffin by applying routine tissue tracking procedure. Histologic and immunohistochemical methods were applied to the sections taken from the paraffin blocks. It was determined that histopathologic changes were intense in the TNBS group and less in the TNBSD and TNBSI groups. Strong SOD-2 immunoreactivity was detected in the cytoplasm of hepatocytes in control and TNBSD groups, weak in TNBS group and moderate in TNBSI group. TNF-α immunoreactivity was weak in the cytoplasm of hepatocytes in control group, strong in TNBS group, and moderate in TNBSD and TNBSI groups. In conclusion, it is thought that vitamin D and nettle extract may have positive effects on liver tissue and both substances may be protective against liver damage due to their antioxidant and anti-inflammatory effects.

Keywords: Liver, nettle, SOD-2, TNF-α, vitamin D

Research Article

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INTRODUCTION

Inflammatory bowel diseases (IBD) affect the liver as well as the intestines, leading to the development of different diseases such as hepatobiliary disorders (HD) or nonalcoholic fatty liver disease (Gizard et al., 2014; Larsen et al., 2010). It has been reported that hepatobiliary disorders may occur in both ulcerative colitis (UC) and Crohn's disease (CD). However, hepatic symptoms occur more commonly in ulcerative colitis (Gizard et al., 2014). Although hepatic disorders are associated with IBD, the clinical picture is usually independent of IBD. It is important to perform screening for HD in IBD patients because it is predicted that approximately 5% of adults with IBD will develop liver disease. Chronic liver disease has also been reported in IBD patients with normal liver biochemical diagnostic tests. The most specific hepatobiliary complication associated with IBD is primary sclerosing cholangitis (PSC). Development of cholangiocarcinoma and colon cancer is observed in these patients (Mendes et al., 2007).

Tumor necrosis factor alpha (TNF-α) is a cytokine with effects known to be involved in the pathogenesis of some inflammatory and autoimmune diseases (Bradley, 2008). Superoxide dismutase (SOD), one of the enzymatic antioxidants, is the primary enzyme for the antioxidant,

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Yediel Aras Ş., (2024). Determination of the effects of vitamin D and Nettle (*Urtica dioica* L.) extract administration on SOD-2 and TNF-α levels in liver tissue of TNBS (2,4,6 trinitrobenzene sulfonic acid)-induced rats. *Journal of Advances in VetBio Science and Techniques, 9*(3), 197-205. https://doi.org/10.31797/vetbio.1428195

is the primary enzyme for the antioxidant defense system (Fridovich, 1975). SOD has been shown to have important contributions in protecting the host from intracellular pathogens (Beaman and Beaman, 1990; Özel and Birdane, 2014).

Vitamin D is an important vitamin that regulates calcium and phosphorus metabolism in the body. It also has a role in the regulation of immune system functions. It has been reported that the incidence of Crohn's disease is high in patients with vitamin D deficiency (Del Pinto et al., 2015; Suibhne et al., 2012). Vitamin D deficiency has been suggested in patients with inflammatory bowel disease. However, it is not yet certain whether vitamin D deficiency is the cause or the result of the disease (Palmer and Weaver, 2013; Sentongo et al., 2002). Nettle (*Urtica dioica* L.) is a wild plant belonging to the Urticaceae family. It has been used in traditional medicine for many years in the treatment of diseases such as rheumatism and arthritis. In addition, its hemostatic and diuretic effects have also been utilized. Nettle has the ability to increase the level of vitamin B12, folate and iron binding in the blood. Therefore, it has been suggested that brewing nettle and using it as detox may have positive effects in the treatment of many diseases (Upton, 2013).

This study aims to reveal the effects of vitamin D and nettle (*Urtica dioica* L.) extract administration on SOD-2 and TNF-α levels in liver tissue by immunohistochemical methods in rats induced with TNBS.

MATERIALS AND METHODS

Material

Thirty-two male *Sprague-Dawley* rats weighing 200-250 g were used for the study. The rats were housed in standard cages at an ambient temperature of 22 ± 2°C, 12 hours of light and 12 hours of darkness, and fed *ad-libitum* with tap water.

Method

All rats were weighed and randomly divided into four groups.

1. Control group (C, n=8): No treatment was given to the rats in this group.

2. TNBS group (TNBS, n=8): Rats in this group were administered 150 mg/kg TNBS (2,4,6 trinitrobenzene sulfonic acid) rectally in a single dose. Then, 1ml physiologic saline solution was given by oral gavage at the same time every day for 10 days (Xia et al., 2019).

3. TNBS + vitamin D group (TNBSD, n=8): In this group, 150 mg/kg TNBS (2,4,6 trinitrobenzene sulfonic acid) was administered rectally in a single dose. Then, 7,500 IU vitamin D was administered by oral gavage once a day at the same time every day for 10 days (Xia et al., 2019).

4. TNBS + Nettle group (TNBSI, n=8): In this group, 150 mg/kg TNBS (2,4,6 trinitrobenzene sulfonic acid) was administered rectally in a single dose. Then, 2.5 ml/kg nettle extract was given by oral gavage once a day at the same time every day for 10 days (Genc et al., 2011).

Histopathological Examinations

At the end of 10 days, liver tissue samples were taken. They were blocked in paraffin by applying routine tissue follow-up procedure. Hematoxylin & Eosin staining was applied to examine the general structure of the liver tissue.

Immunohistochemical Investigations

Sections of 5 µm were taken on slides coated with chromium alum gelatin and Streptavidin-biotin peroxidase method was applied. After deparaffinization and rehydration, the sections were rinsed in PBS (0.1 M, pH, 7.2). Then they were kept in 3% H_2O_2 prepared in 0.1 M PBS for 15 min. Boiled in citrate buffer solution for 10 min in a microwave oven at maximum temperature. Incubated with Large Volume Ultra V Block solution for 10 min. SOD-2 (B-1): sc-133254, 1/500 dilution) and TNFα (52B83) (sc-52746, 1/500 dilution) primary antibodies were added to the sections and kept at room temperature and humidified for 1 hour. The sections were washed with PBS and Biotinylated

Goat Anti B Polyvalent and Streptavidin Peroxidase solutions were added and incubated at room temperature for 15 min each. DAB-H_2O_2 (Diaminobenzidine hydrogen peroxide) Substrate Solution was added for chromogen application. Modified Gill III hematoxylin solution was used for counterstaining. The preparations were examined under a research microscope and photographed. To determine whether the immunoreactivity was specific or not, the sections were kept in PBS without the addition of primary antibody (negative control) and the other procedures were applied exactly the same. Immunohistochemical evaluation was performed by looking at the staining characteristics of the target cells and the staining intensity in the stained target cells. In the evaluation, two independent observers assigned values from 0 to 3 for no staining (0), weak staining (1), moderate staining (2), and strong staining (3). For each group, 20 regions were determined and immunohistochemical scoring was performed from these regions (Yediel Aras and Karadağ Sarı, 2021).

Statistical analysis

One-way analysis of variance was performed to compare SOD-2 and TNF-α immunoreactivity scores. Before analysis of variance, normality and homogeneity assumptions were examined. The kurtosis and skewness coefficients of SOD-2 (kurtosis=-0.48, skewness=-1.33) and TNF-α (kurtosis=-0.04, skewness=-0.93) scores were found to meet the normality assumption. Levene's test results of SOD-2 (Levene's statistic=1.379, $p>0.05$) and TNF- α (Levene's statistic=0.814, $p>0.05$) scores also showed that the variances were homogeneous. Therefore, Scheffe test was used for post-hoc comparisons between groups after analysis of variance. Analyses were performed in SPSS 22 package program and the significance level was set as 0.05.

RESULTS

Histopathologic results

Control group liver tissues were found to have normal histologic structure. Microvesicular fat droplets, shrinkage in hepatocyte nuclei and apopitotic areas were detected in TNBS group. Histopathologic changes were found to be less in TNBSD and TNBSI groups. In both groups, hepatocyte nuclei were similar in size to the control group and remark cords were more regular. While lymphocyte foci were observed in both TNBSD and TNBSI groups, microvesicular fat droplets were observed at a small level in TNBSI group (Figure 1).

Immunohistochemical Results

SOD-2 immunoreactivity

Strong immunoreactivity was detected in the cytoplasm of hepatocytes in control and TNBSD groups, weak in TNBS group and moderate in TNBSI group. In addition, moderate immunoreactivity was observed in lymphocyte foci in TNBSI group (Figure 2).

TNF-α immunoreactivity

Weak TNF-α immunoreactivity was detected in the cytoplasm of hepatocytes in Control group, strong in TNBS group, and moderate in TNBSD and TNBSI (Figure 3).

As a result of the analysis, it was determined that there was a significant difference between the groups in SOD-2 ($F=1043.442$, $p<0.001$) and TNF-α ($F=778.898$, $p<0.001$) immunoreactivity scores. In SOD-2 immunoreactivity scores, Control and TNBSD groups had higher mean scores than the other groups and TNBSI group had higher mean scores than TNBS group (Table 1).

In TNF-α immunoreactivity scores, TNBS group had a higher mean score than the other groups and TNBSD and TNBSI groups had a higher mean score than Control group (Table 1).

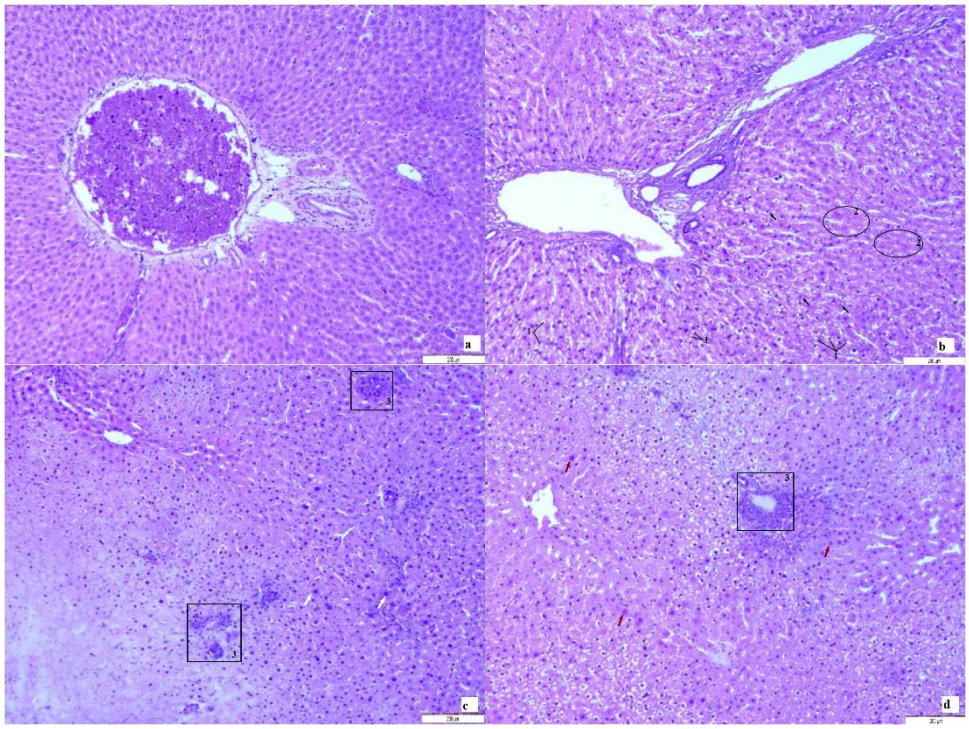

Figure 1. Rat liver tissue. a: Control group. b: TNBS group, 1: microvesicular fat droplets, 2: apopitotic areas, arrow: hepatocyte nuclei shrinkage. c: TNBSD group, white arrow: hepatocyte nuclei close to control, 3: lymphocyte follicle. d: TNBSI group, red arrow: hepatocyte nuclei close to control, 3: lymphocyte follicle. H-E staining.

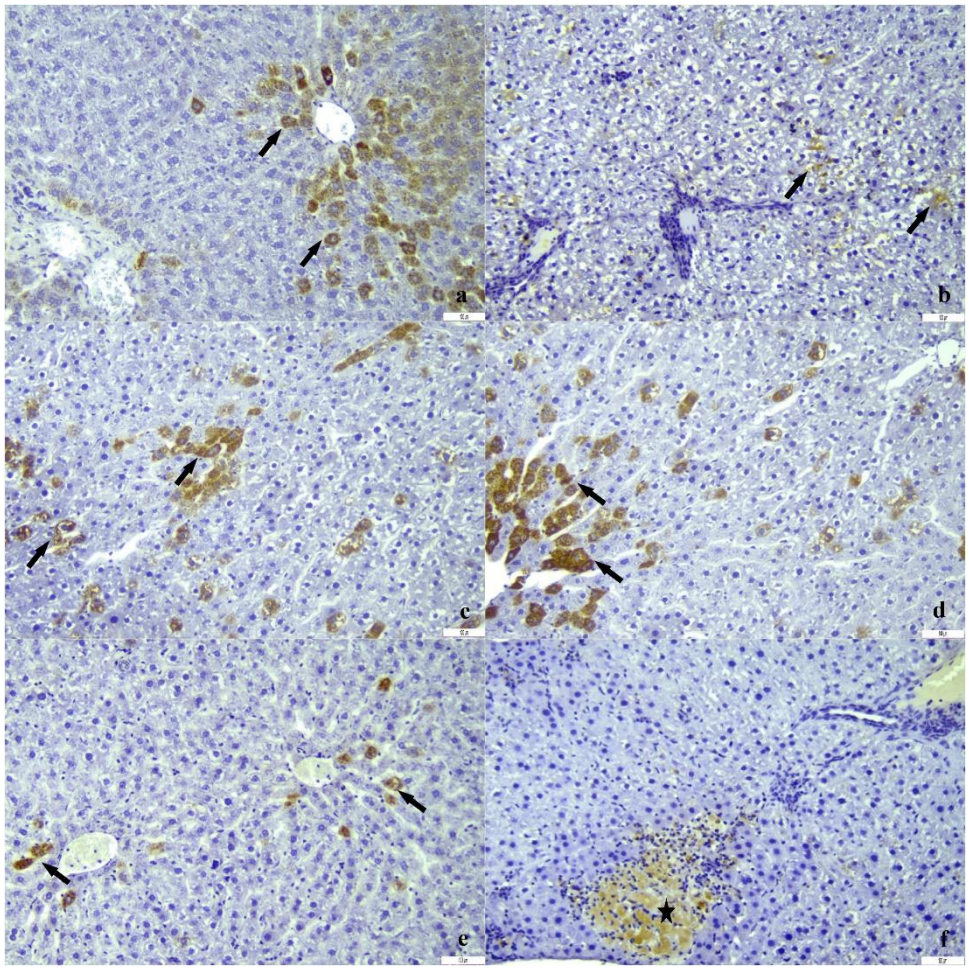

Figure 2. SOD-2 immunoreactivity in rat liver tissue. a: Control group, b: TNBS group, c, d: TNBSD group, e, f: TNBSI group. Hepatocytes (arrow), lymphocyte follicle (asterisk). a, c, d: strong immunoreactivity; weak immunoreactivity. e, f: moderate immunoreactivity.

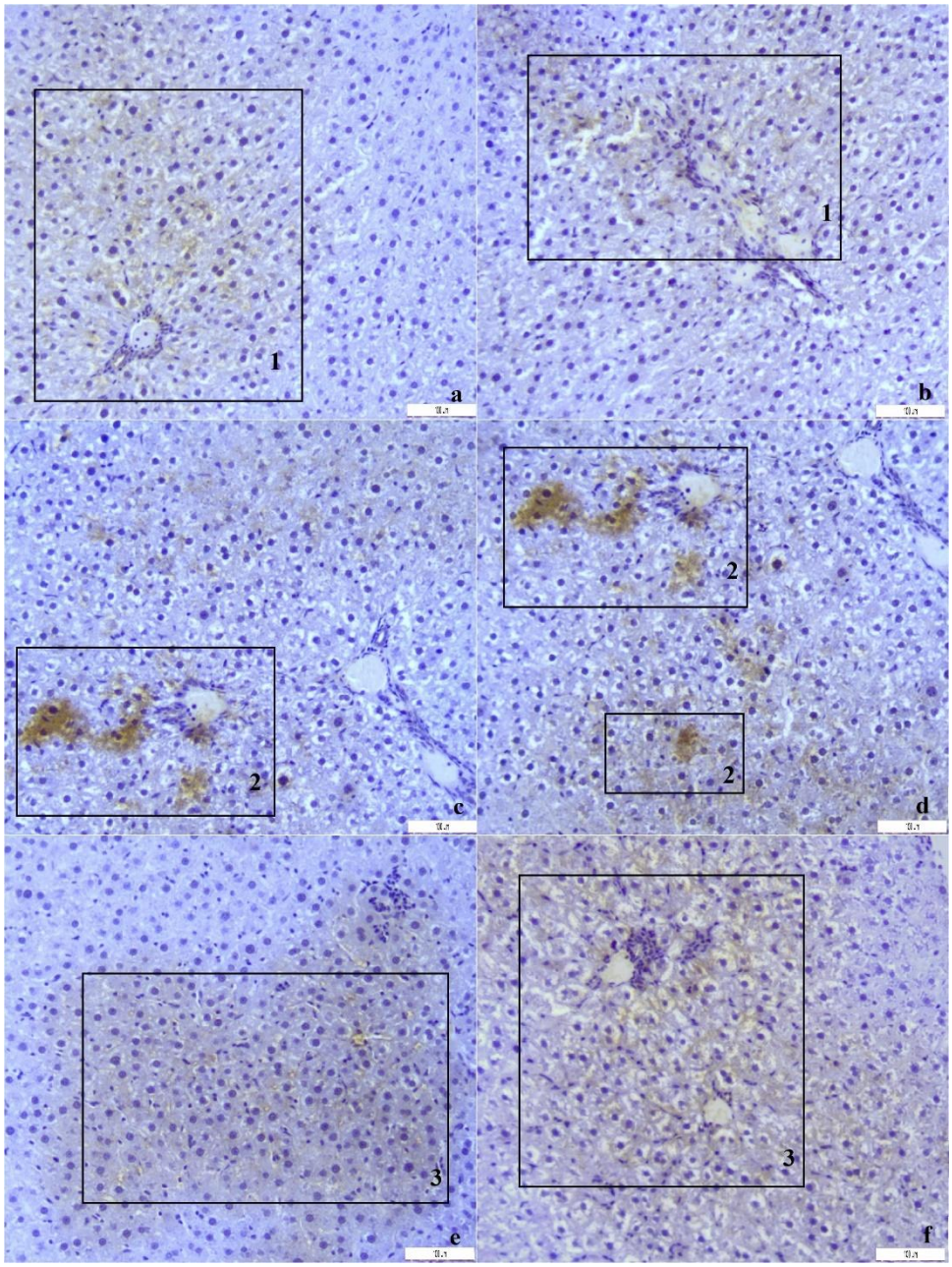

Figure 3. TNF-α immunoreactivity in rat liver tissue. a,b: Control group, c,d: TNBS group, e: TNBSI group, f: TNBSD group. 1: Weak immunoreactivity in hepatocytes. 2: Strong immunoreactivity in hepatocytes. 3: Moderate immunoreactivity in hepatocytes.

Table 1. Comparison of SOD-2 and TNF-α immunoreactivity scores between groups.

		n	Ort	SS	F	p	post-hoc
SOD-2 immunoreactivity	C (a)	20	2.95	0.13	1043.442	0.000	a, c > d > b
	TNBS (b)	20	1.05	0.10			
	TNBSD (c)	20	2.94	0.16			
	TNBSI (d)	20	2.01	0.10			
TNF-α immunoreactivity	C (a)	20	1.05	0.10	778.898	0.000	b > c, d > a
	TNBS (b)	20	2.94	0.16			
	TNBSD (c)	20	2.03	0.11			
	TNBSI (d)	20	2.03	0.11			

DISCUSSION

Inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease (CD) are chronic diseases that, like cardiovascular disease, can reduce a patient's life expectancy. Its global prevalence is predicted to increase up to 1% in many regions by 2030 (Kaplan and Windsor, 2021). Non-alcoholic fatty liver disease (NAFLD), a disease characterized by fatty liver and determined by liver fat content (LFC) measurements, is among the most important causes of liver diseases even in lean patients. Its global prevalence is estimated to be over 24% (Younossi et al., 2018). It has been suggested that IBD is associated with NAFLD and these two diseases usually coexist (Lin et al., 2021). However, another study compared IBD patients with and without NAFLD and found no significant difference in the characteristics of IBD disease in both groups (Magrì et al., 2019). However, it has been suggested that a high-fat diet has an effect on the quality of the intestinal barrier and the composition of the gut microbiome, affects the pathogenesis of IBD, and LFC may also be associated with UC (Jamali et al., 2017). The development of hepatic fat accumulation (steatosis) is a common finding in CD, but the pathophysiologic processes leading to steatosis have not yet been fully elucidated. It has been suggested that insulin resistance, lipotoxicity, immune cell response and inflammation may be involved in the pathogenesis of steatosis, along with strong environmental and genetic influences. It has even been reported that steatosis may progress and lead to nonalcoholic steatohepatitis (NASH) and liver fibrosis (Bechmann et al., 2012). Studies have emphasized that changes in bile acid metabolism and intestinal microbiota dysbiosis may also be effective on the development and progression of steatosis (Sydor et al., 2020). On the other hand, it has been reported that intestinal microbiota is also impaired in CD and has a favorable course in relation with the treatment of the disease (Connors et al., 2020). Determination of microvesicular fat droplets, shrinkage in hepatocyte nuclei and apopitotic areas in the TNBS group suggested that inflammatory bowel diseases may have pathologic effects in the liver tissue and may cause dysfunction of the liver and other digestive system organs related to the liver.

In studies conducted to investigate the effectiveness of vitamin D as an immunomodulator in preventing liver tissue damage, vitamin D deficiency was found in 91% of HBV-infected patients and low vitamin D levels were found to be significantly associated with high viral replication (Ilkovska et al., 2019). It has been suggested that vitamin D deficiency is associated with disease severity in patients with chronic hepatitis (Rahman and Branch, 2013). In another study, a significant relationship was found between low vitamin D levels and increased levels of inflammation in the liver (Hoan et al., 2016). Vitamin D is an anti-inflammatory vitamin that inhibits the expression of tumor necrosis factor alpha and interleukin-1, which are key inflammatory markers of NAFLD-related liver injury. It has been suggested that vitamin D deficiency leads to severe liver inflammation and oxidative stress (Abe et al., 2021). Vitamin D treatment has been reported to be effective in improving hepatic lesions in NAFLD (Bingül et al., 2021). In addition to its direct antioxidant effect, vitamin D has also been reported to act by increasing the gene expression of proteins/enzymes in the antioxidant system (Mokhtari et al., 2017). It was reported that mRNA expressions and activities of SOD and GSH-Px increased in the liver of vitamin D-treated rats (Bingül et al., 2021). The decrease in SOD-2 immunoreactivity and increase in TNF-α immunoreactivity in the liver tissues of the TNBS group suggested that inflammation may have a negative effect on antioxidant and anti-inflammatory cytokines and cause impairment in liver function. In TNBSD group, an increase in SOD-2 immunoreactivity

and a decrease in TNF-α immunoreactivity were determined.

Nettle is a plant that should be taken into the body because of its rich nutrient content and being a good antioxidant (Hoşbaş, 2008). It was observed that nettle seed extract decreased the levels of liver enzymes and liver lipid peroxidation levels which increased due to carbon tetrachloride (CCl4). The extract shows a protective effect on the liver by reducing the hepatoxic effect of CCl4 (Şener et al., 2010). It was reported that nettle treatment effectively protected the liver against aflatoxin-induced hepatotoxicity, decreased AST, ALT and GGT levels and increased antioxidant levels (Yener et al., 2009). In another study, it was suggested that nettle significantly decreased serum lipid hydroperoxide and ceruloplasmin levels and increased catalase, paraoxonase and arylesterase levels. It has also been reported to significantly reduce liver tissue damage and to have a protective effect on the liver (Kandis et al., 2010). In the TNBSI group, an increase in SOD-2 immunoreactivity and a decrease in TNF-α immunoreactivity were determined.

CONCLUSION

Inflammatory bowel diseases negatively affect the quality of life of patients and cause many complications in the long term. The main involvement of the intestines, but it can also affect other organs, especially the liver. Inflammatory bowel diseases often cause non-alcoholic fatty liver disease in the liver. The results of the study suggested that vitamin D and nettle extract may have positive effects in alleviating the complications of the disease in the liver and that both substances may be protective against liver damage due to their antioxidant and anti-inflammatory effects. However, it is thought that many more studies are needed to elucidate the protective mechanisms of vitamin D and nettle.

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Author contributions: Concept, Design, Control, Data Collection, Analysis: ŞYA.

Availability of data and materials: Data and materials may be used subject to the author's permission.

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The effects of cetuximab with agomelatine on gene expression in colon cancer cells

ABSTRACT

This study investigated the combined effects of agomelatine, a melatonergic antidepressant, and cetuximab, an EGFR inhibitor, on the colorectal cancer cell line (Caco-2). Caco-2 cells were treated with agomelatine (0.3 µg/ml and 3 µg/ml) and cetuximab (50 µg/ml), individually and in combination, for 24 and 48 hours. Cell viability was assessed using the MTT assay. Gene expression analysis of *EGFR*, *BCL2*, *PIK3CA*, *BAX*, *mTOR*, and *AKT3* was performed using real-time PCR. All treatment groups showed significant decreases in cell viability compared to the control ($p<0.05$), with enhanced effects in combined treatments. *EGFR* expression was significantly reduced in drug-treated groups, particularly with cetuximab ($p<0.05$). While changes were observed in *BCL2*, *PIK3CA*, *BAX*, *mTOR*, and *AKT3* expression, these were not statistically significant ($p>0.05$). This study demonstrates the potential synergistic cytotoxic effects of agomelatine and cetuximab on Caco-2 colorectal cancer cells. The significant reduction in *EGFR* expression suggests a potential mechanism of action. These findings provide insights into combining chemotherapeutic agents with drugs addressing circadian rhythm disorders in CRC treatment strategies. Further research is warranted to elucidate the clinical implications of these observations.

Keywords: Agomelatine, cetuximab, colorectal cancer, gene expression, chemotherapy

INTRODUCTION

Colorectal cancer (CRC) remains one of the most prevalent and lethal malignancies worldwide, demonstrating a substantial burden on global public health (Xi and Xu, 2021). Characterized by unregulated cell growth in the colon or rectum, CRC has been intricately associated with genetic mutations, environmental influences, and various pathophysiological mechanisms (Alharbi et al., 2022). A significant percentage of CRC cases have been identified to exhibit overexpression of the Epidermal growth factor receptor (EGFR), which is intricately involved in cellular proliferation, apoptosis, and differentiation (Han et al., 2022; Ogrodnik, 2021).

Targeted therapies, particularly employing monoclonal antibodies, have emerged as a pivotal approach in CRC treatment. Cetuximab (CTX), an EGFR antagonist, has been widely utilized due to its ability to inhibit ligand-induced phosphorylation and activation of receptor-associated kinases, thus interfering with downstream signaling pathways implicated in cancer progression (Moreno-SanJuan et al., 2023). However, despite the initial response, resistance to cetuximab invariably develops, necessitating alternative or adjunctive therapeutic strategies.

Agomelatine, primarily recognized for its utility in managing depressive disorders by modulating circadian rhythms, has recently garnered attention in oncology (Fekry and Eckel-Mahan, 2022).

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Research Article

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Intriguingly, disturbances in circadian rhythms have been implicated in the pathogenesis and progression of several cancers, including CRC, through mechanisms involving cell cycle regulation, DNA damage response, and metabolism (Aghamiri et al., 2019; Hossain et al., 2022; Wathoni et al., 2020). The potential impact of agomelatine on CRC, particularly in conjunction with cetuximab, remains a fertile ground for exploration, potentially unraveling novel insights into the intricate web of CRC pathophysiology and therapeutic resistance.

This research seeks to illuminate the effects of cetuximab and/or agomelatine on cell proliferation and apoptosis in Caco-2 colorectal cancer cells. Through a meticulous investigation of cellular and molecular responses to these agents, this study endeavors to elucidate the underlying mechanisms and pathways, thereby contributing to the burgeoning field of targeted cancer therapy and paving the way toward more effective and sustainable therapeutic strategies in CRC management.

MATERIALS AND METHODS

Cell culture

The colorectal cancer cell line (Caco-2) used in this study is preserved at -196°C in a nitrogen storage tank at the Molecular Cancer Biology Laboratory, Erzurum Technical University. Cell culture medium was prepared by adding 10% fetal bovine serum (FBS), 1% ml of penicillin-streptomycin (Pen-Strep) to DMEM. Parenteral Caco-2 monolayer cell lines were incubated in a cell culture medium at 37°C with 5% carbon dioxide (CO2) and 95% humidity in an incubator (Esco Co., Korea) in 25 cm² flasks under sterile conditions to facilitate cell proliferation.

Drug treatment

Cetuximab and agomelatine treatments

Caco-2 colorectal cancer cells were exposed to treatments with cetuximab (IMC-C225, Erbitux) and/or agomelatine (Valdoxan, Thymanax, AG0178) to evaluate their effects on cellular proliferation and apoptosis related genes. Specifically, agomelatine was prepared in cell medium and administered at dosages of 0.3 µg/ml and 3 µg/ml, whereas cetuximab was utilized at a dosage of 50 µg/ml, both independently and in combination with agomelatine.

For viability analyses, 1500 cells were seeded into each well of a 96-well plate, and interventions were administered as outlined in Table 1 of the original study. Subsequent to the treatments, cells were incubated for 24 and 48 hours in a 37°C, 5% CO_2 incubator, followed by an MTT cytotoxicity analysis utilizing an appropriate MTT assay kit.

Table 1. Primer sequences used in RT-PCR analysis

Gene	Forward Primer	Reverse Primer
BAX	CGCATCCTGAGGCACCGG	TTTCATCCAGGATCGAGCAGGG
EGFR	TCGTTGGACAGCCTTCAAGACC	AACACCCTGGTCTGGAAGTACG
BCL2	CGCATCCTGAGGCACCG	TTTCATCCAGGATCGAGCAGGG
AKT3	GGAAGAATGGACAGAAGCTATTCCA	TCCACTTGCCTTTCTCTCGAAC
MTOR	GTCAGTGGGACAGCATGGAAG	CCCATATGCCCGACTGTAACTC
PIK3CA	TGGATGCTTCACAGGGCTTTCT	TATCTTGCCGTAAATCATCCCCCA

MTT assay for cell viability

Assay procedure

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to assess cell viability post-drug treatments. Cells were seeded at a density optimized for the detection of cellular metabolic activity and subsequently treated with the drugs. Post-treatment, MTT solution was added, and cells were incubated to facilitate the formation of

formazan crystals. The crystals were dissolved, and the absorbance was measured at 570 nm spectrophotometrically. Data was normalized and analyzed to determine the effects of treatments on cellular viability.

Quantitative real-time PCR (qRT-PCR) analysis

RNA isolation

Total RNA was isolated from the Caco-2 cells treated with the drugs using a commercial kit, adhering to the manufacturer's guidelines (Ambion RNA Mini Kit, USA). The RNA isolation procedure involved several steps including cell lysis, homogenization, and purification, following a detailed protocol to ensure the integrity and purity of the isolated RNA. The concentration and purity of the extracted RNA were determined spectrophotometrically using a Nanodrop device (EPOCH Take3 Plate, Biotek), and RNA samples were stored at -20°C until further use.

cDNA synthesis

Following RNA extraction, cDNA synthesis was conducted using the Maxime RT Premix kit. The synthesis protocol involved combining 5 µl of RNA with 15 µl of RNase-free water, and the reaction was performed using a Veriti 96 Well Thermal Cycler (Applied Biosystem) with the temperature settings set at 45°C for 60 minutes and 95°C for 5 minutes. Subsequent to synthesis, cDNA samples were quantified spectrophotometrically and stored at -20°C.

qRT-PCR analysis

Gene expression analyses of *AKT3*, *PIK3CA*, *EGFR*, *Bcl-2*, *Bax*, *MTOR*, and *GAPDH* genes were performed using qRT-PCR with specific primers designed for each gene (Table 1). The amplification, detection, and data analysis were conducted using the Qiagen Rotor-Gene Real Time PCR System (Rotor-Gene Q 5plex HRM System), ensuring specificity, efficiency, and reproducibility of the results. The amplification conditions were set at 95°C for 3 minutes for enzyme activation, 95°C for 5 seconds for denaturation (40 cycles), and 60°C for 10 seconds for amplification (40 cycles). Relative gene expression was calculated using the ΔΔCT method, providing insights into the molecular mechanisms underlying the cellular responses to drug treatments (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis of the cell viability data, obtained from four replicates, was performed using Microsoft Office Excel. Data were presented as mean ± standard deviation. IBM SPSS Statistics 22.0 software was used for significance analysis. Differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. Statistical significance was set at $p<0.05$.

RESULTS

Cytotoxicity analysis

The cytotoxic effects of agomelatine and cetuximab, individually and in combination, were evaluated on Caco-2 colorectal cancer cell lines through comprehensive analyses. Table 2 presents the cytotoxicity results for Caco-2 cells incubated for 24 and 48 hours, following treatment with various dosages of agomelatine and cetuximab.

Table 2. Cytotoxicity results for CACO-2 colorectal cancer cells treated with different doses of agomelatine and cetuximab and incubated for 24 and 48 hours.

Groups	24h	48h
Control	0.250± 0.022	0.281± 0.023
Ago-0.3	0.139± 0.011	0.190 ± 0.020
Ago-3	0.143± 0.011	0.205± 0.053
Cet-50	0.143± 0.012	0.183± 0.007
Ago-0.3+Cet-50	0.152 ± 0.008	0.173± 0.009
Ago-3+Cet-50	0.142 ± 0.005	0.182± 0.012

The results indicate a discernible decrease in cell viability across all treatment groups compared to the control, with distinct dose-dependent and time-dependent variations ($p<0.05$). Particularly, the administration of agomelatine at dosages of 0.3 µg/ml (Ago-0.3) and 3 µg/ml (Ago-3) demonstrated a significant

reduction in cell viability after 24 and 48 hours of incubation ($p<0.05$). Likewise, cetuximab at a dosage of 50 µg/ml (Cet-50) exhibited potent cytotoxic effects, further pronounced when combined with agomelatine at both aforementioned dosages ($p<0.05$).

The combined application of agomelatine and cetuximab indicated a notable synergistic effect, particularly pronounced in the 48-hour incubation period ($p<0.05$). The cytotoxicity was not merely additive but exhibited an enhanced effect, suggesting an interaction in the apoptotic and proliferative pathways influenced by the two drugs.

MTT assay results

The viability of Caco-2 cells, subsequent to treatment with varying concentrations of agomelatine and cetuximab, was scrutinized utilizing the MTT assay. Results manifested discernible alterations in cell viability in response to both singular and combined drug treatments across the distinct incubation periods 24h and 48h (Figure 1).

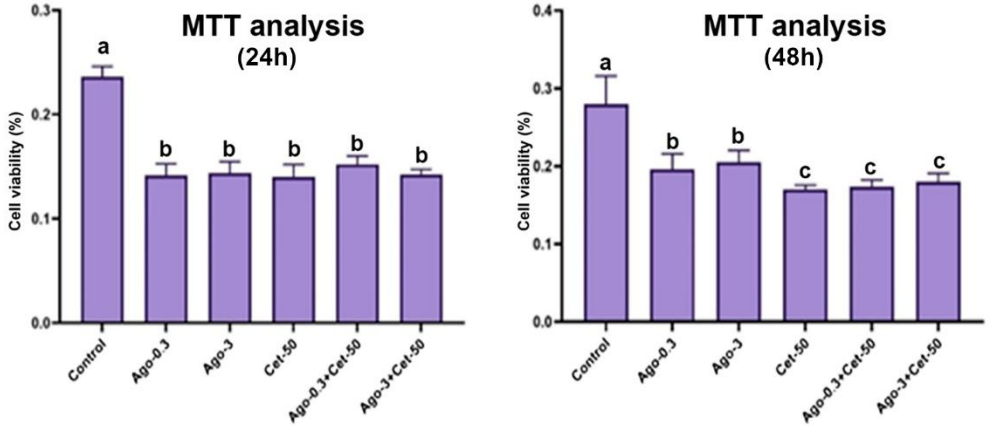

Figure 1. Cytotoxicity results of CACO-2 colorectal cancer cell lines incubated for 24 and 48-hours following treatment with different doses of agomelatine and/or cetuximab. Different letters (a, b, c) indicate statistically significant differences between groups ($p<0.05$). Data is presented as mean ± SD of four independent experiments.

Invert microscopic analysis

Subsequent observations at 24 and 48h further elucidated the cellular dynamics and morphological changes induced by the drug treatments, as depicted in Figure 2. A detailed analysis revealed a significant reduction in viability in groups subjected to agomelatine and cetuximab treatments, with pronounced effects observed at specific dosage levels ($p<0.05$). Furthermore, the results elucidated potential dose-dependent and time-dependent cytotoxic effects of the administered drugs.

Real time-PCR analysis results

This study investigated the effects of agomelatine and cetuximab on gene expression in Caco-2 colon cancer cells. Real-time PCR analysis was conducted to examine the expression of *EGFR*, *BCL2*, *PIK3CA*, *BAX*, *mTOR*, and *AKT3* genes under various treatment conditions and incubation periods of 24 and 48 hours. The results are presented in Figure 3.

***EGFR* gene expressions**

EGFR gene expression analysis revealed significant differences between the control group and various treatment conditions (Figure 3). The control group exhibited the highest *EGFR* expression levels across both 24-hour and 48-hour incubation periods ($p<0.05$). Groups treated with drug combinations, particularly Cet-50 and Ago-0.3+Cet-50, showed reduced *EGFR* mRNA expression, with ratios approaching 1.01. The inhibitory effect on *EGFR* expression was most pronounced in the Cet-50 group during the 48-hour incubation period ($p<0.05$).

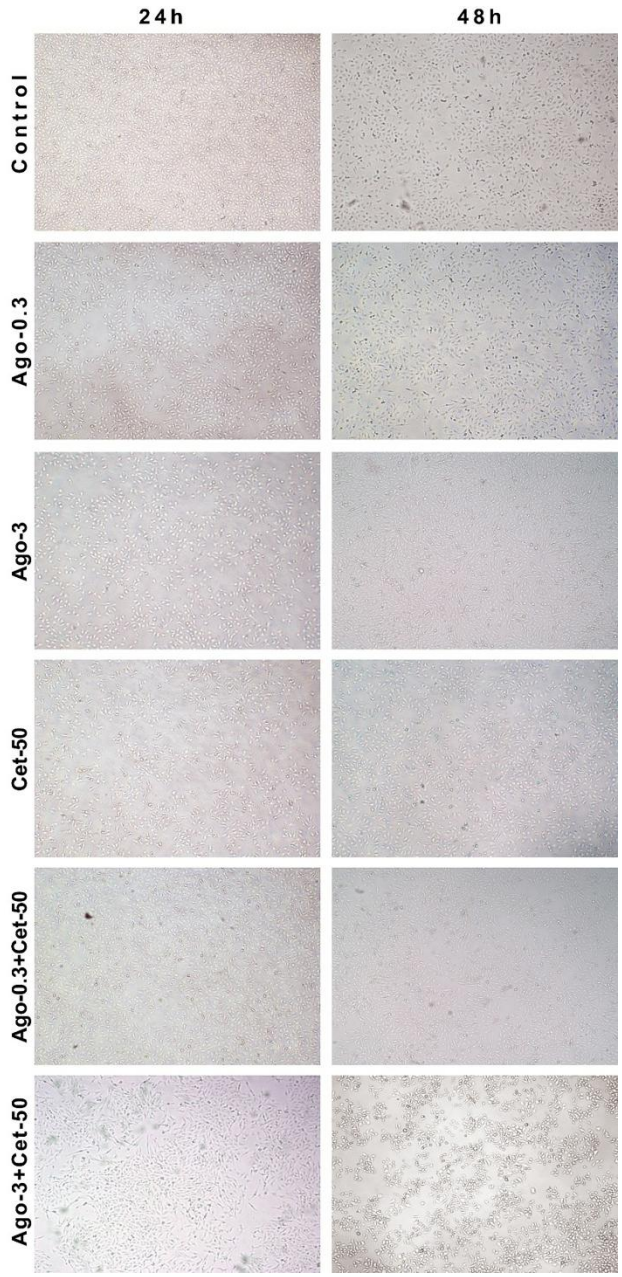

Figure 2. Inverted microscope images of Caco-2 cells for various groups after drug application at 24h and 48h.

BCL gene expression

BCL2 expression patterns, as shown in Figure 3, remained relatively stable across treatment groups and incubation periods. At 24 hours, the Cet-50 group showed a slight increase in *BCL2* expression compared to the control, but this difference was not statistically significant ($p>0.05$). At 48 hours, all groups exhibited similar *BCL2* expression levels.

PIK3CA gene expression

PIK3CA expression, depicted in Figure 3, showed some variability among groups. At 24 hours, Ago-3 and Cet-50 groups showed slightly higher expression compared to the control. At 48 hours, Ago-3 and Ago-3+Cet-50 groups exhibited a minor increase in *PIK3CA* expression compared to other groups. However, these differences did not reach statistical significance ($p>0.05$).

BAX gene expression

BAX gene expression results are presented in Figure 3. A trend towards upregulation was observe in drug-treated groups at 24 hours, with Ago-3 and Ago-0.3+Cet-50 groups showing slight elevations. At 48 hours, the Ago-0.3 group demonstrated a minor increase in BAX expression. Despite these observations, the differences were not statistically significant when compared to the control group ($p>0.05$).

mTOR gene expression

mTOR gene expression, as illustrated in Figure 3, showed some fluctuations across treatment groups and time points. The Ago-3 group showed a slight elevation in *mTOR* expression at 24 hours. At 48 hours, the Cet-50 group displayed a minor increase in *mTOR* expression. However, these changes were not statistically significant relative to the control group ($p>0.05$).

AKT3 gene expression

AKT3 expression levels, presented in Figure 3F, remained relatively consistent across most treatment conditions. A slight increase was observed in the Ago-0.3+Cet-50 group at 48 hours, but this change did not reach statistical significance ($p>0.05$).

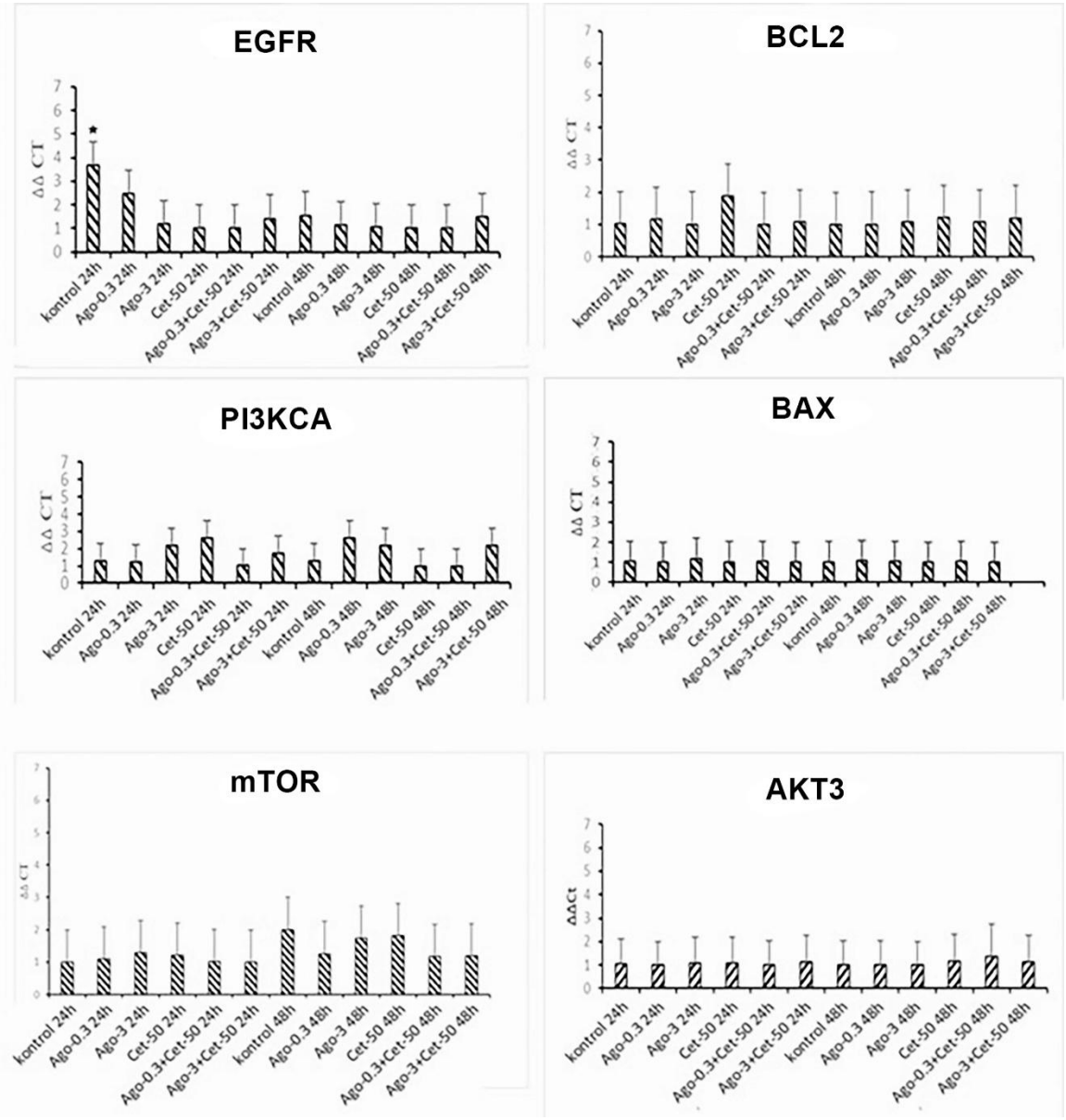

Figure 3. Gene expression values of *EGFR*, *BCL*, *PIK3CA*, *BAX*, *PIK*3CA, *mTOR*, and *AKT3* in Caco-2 colon cancer cell lines Incubated for 24 and 48 hours with various doses of agomelatine and cetuximab. The astarix indicate the Statistical significance between the groups. Statistical differences were determined with $p<0.05$ compared to the other groups. Data is presented as mean ± SD of four independent experiments.

DISCUSSION

CRC ranks as the third most common type of cancer worldwide, witnessing a continual rise in new case rates year after year. The treatment of colorectal cancer is contingent upon the stage of the cancer and the patient's overall condition, offering a range of chemotherapy drugs and treatment modalities (Li et al., 2020). Cetuximab, employed in this study, acts as an antagonist of EGF and a monoclonal antibody, finding pervasive use in CRC treatment (Giordano et al., 2019). Nevertheless, cancer patients often face significant challenges in treatment progression due to circadian rhythm and mood disorders, necessitating, at times, the incorporation of antidepressant agents into the cancer treatment regimen (Kılıç and Erbaş, 2021) . In this context, recent studies advocate the use of agomelatine, a melatoninergic agonistic analog, renowned for its chronobiotic, anxiolytic, and antidepressant effects and its capacity to expedite the resynchronization of

fundamental biological circadian rhythms (Moreno-SanJuan et al., 2023).

In essence, individuals diagnosed with cancer are commonly administered chemotherapy drugs and/or medications like chronobiotics, anxiolytics, and antidepressants for treatment purposes and to manage psychopathological disorders stemming from the disease, respectively (Chang and Shen, 2019; Naser et al., 2021). Consequently, the combined use of a chemotherapeutic agent and an antidepressant in this study explores a potential treatment approach and examines the effects of cetuximab and agomelatine in cancer treatment, an area that has not been extensively studied. This research protocol aims to contribute to the literature by assessing the effects of a chemotherapeutic agent along with agomelatine, a melatonin analog, in a model that aligns with the pathophysiology of the disease.

This study investigated the effects of agomelatine and/or cetuximab on cell cytotoxicity, apoptotic cell death, and the expression levels of regions related to genes associated with proliferation in the Caco-2 cancer cell line under in vitro conditions. To this end, Caco-2 cells were incubated for 24 and 48 hours with agomelatine at concentrations of 3 µg/ml and 0.3 µg/ml, and cetuximab at a concentration of 50 µg/ml. Cell cytotoxicity was determined using the MTT method, while the expression levels of *BAX*, *EGFR*, *BCL2*, *AKT3*, *mTOR*, and *PIK3CA* genes were analyzed through quantitative real-time PCR.

EGFR plays a pivotal role in cancer development as a key protein, its overexpression being prevalent in numerous tumors. It has been targeted for treatment via small molecule inhibitors and monoclonal antibodies, with the latter playing a role in the treatment of metastatic disease (Alharbi et al., 2022; Amodio et al., 2020; Giordano et al., 2019). CTX, being an EGFR antagonist and a monoclonal antibody, is widely employed in CRC treatment. It can reduce receptor activation in some cancer cells by inhibiting the binding of the ligand to the respective receptor and can activate apoptosis (Cho et al., 2010). The combination of anti-EGFR therapy and cytostatics, which cause DNA damage, has been shown to exert an anti-tumor effect by inhibiting cell cycle progression and activating apoptosis. Cetuximab augments apoptosis while suppressing cell proliferation, angiogenesis, and metastasis by blocking downstream signaling (Hanck-Silva et al., 2020). In our study, we observed changes in EGFR expression in Caco-2 cells treated with CTX and agomelatine. The control group exhibited the highest EGFR expression levels, while groups treated with drug combinations, particularly Cet-50 and Ago-0.3+Cet-50, showed reduced EGFR mRNA expression. These observations align with existing research that suggests CTX's ability to affect receptor activation (Cunningham et al., 2004). The simultaneous application of CTX and agomelatine appeared to influence EGFR expression, which may be related to the approach of combining anti-EGFR therapy with other agents. Previous studies have shown that such combinations can affect cell cycle progression and apoptosis (Sartore-Bianchi et al., 2016). These findings may contribute to our understanding of EGFR-focused interventions in CRC and suggest potential areas for further research into combined therapeutic approaches in oncology.

The PI3K/Akt/mTOR pathway, a central signaling stream system, plays a crucial role in vital physiological events such as the cell cycle, cell life, protein synthesis, growth, metabolism, and angiogenesis (Miricescu et al., 2020). AKT, a serine/threonine kinase and a central mediator in the PI3K pathway, governs key cellular events, stimulating protein synthesis and cell growth by activating mTOR (Revathidevi and Munirajan, 2019). 24-hour application of drugs at concentrations of 0.3 µg/ml and 0.3 µg/ml+50 µg/ml suppressed the proliferation of *PIK3CA*, *EGFR*, and *AKT* genes, but not *mTOR*. This

finding is interesting when compared with existing literature, which often highlights the central role of mTOR in driving cell growth and proliferation in the context of the PI3K/Akt pathway. The selective suppression of upstream components like *PIK3CA* and *EGFR* may indicate potential for targeted inhibition strategies in colorectal cancer. For example, studies have shown that targeting *EGFR* can effectively disrupt cancer progression (Ayati et al., 2020; Li et al., 2022), and our findings seem to support this strategy, particularly in the early stages of drug treatment. The results showing that a 48-hour application of drugs at concentrations of 50 µg/ml and 0.3 µg/ml+50 µg/ml suppressed *mTOR*, *PIK3CA*, and *EGFR* genes, but not *AKT*, suggest a time-dependent response in the pathway's components. This aligns with the understanding that prolonged drug exposure can lead to different cellular responses. In the context of colorectal cancer, studies have indicated that sustained inhibition of mTOR can be more effective over time (Faivre et al., 2006; Fasolo and Sessa, 2008).

On the apoptosis mechanism, proteins like caspase-3, caspase-9, bax, bcl-2, and p53 play a key role. Some members of the Bcl-2 protein family are pro-apoptotic, while others are anti-apoptotic (Choudhury et al., 2012). This protein, localized on the mitochondrial membrane, controls the permeability of mitochondrial pores and has an anti-apoptotic effect. BAX protein, a proapoptotic protein localized on the mitochondria, plays a crucial role in facilitating apoptosis (Dadsena et al., 2021). In our study, we examined *BAX* and *BCL2* gene expressions in colorectal cancer cells. We observed changes in gene expression in groups treated with 3 µg/ml and 0.3 µg/ml+50 µg/ml drug combinations for 24 hours, particularly noting changes in *BAX* expression in the Ago-3 and Ago-0.3+Cet-50 groups. These observations may be considered in the context of recent literature exploring the impact of melatonin on cancer cells. Studies have demonstrated that melatonin can affect stress-induced insulin resistance and cellular responses to apoptotic signals by influencing COX expression and the Bax/Bcl-2 ratio (Bu et al., 2017). While our study used agomelatine rather than melatonin, these findings may suggest areas for further investigation. The effects of MEL on apoptosis and autophagy appear to be cell-type dependent. For instance, Tran et al. (2021) reported that melatonin synergizes with doxorubicin to activate apoptosis in breast cancer cells and enhances the therapeutic effect of doxorubicin by inducing autophagy. In the context of colorectal cancer, Zhao et al. (2022) found a synergistic anti-tumor effect of melatonin and *Andrographis paniculata* in reducing the viability of colon cancer cells and stimulating apoptosis. They also noted that this combination inhibited autophagy by affecting the expression of autophagy-related genes such as NR4A1, CTSL, and Atg12 (Ma et al., 2020). Similarly, Chok et al. (2021) observed that melatonin increased colorectal cancer cell death, oxidative stress, and autophagic vacuole formation in a dose-dependent manner. These studies highlight the complex interplay between pro-apoptotic and anti-apoptotic mechanisms in cancer cells, and the role of external agents like melatonin in modulating these processes. The parallels between these findings and our own suggest that the regulation of *BAX* and *BCL2* expression is a critical factor in the effectiveness of cancer therapies and underscore the potential of targeting these pathways in colorectal cancer treatment.

CONCLUSION

In conclusion, this study demonstrates the potential cytotoxic effects of agomelatine and cetuximab, both individually and in combination, on Caco-2 colorectal cancer cells. The MTT assay revealed significant reductions in cell viability across treatment groups, with

pronounced synergistic effects observed in combined treatments, particularly after 48 hours of incubation. Gene expression analysis showed a significant decrease in *EGFR* expression in drug-treated groups, especially with cetuximab, suggesting a potential mechanism of action. While changes were observed in the expression of *BCL2*, *PIK3CA*, *BAX*, *mTOR*, and *AKT3* genes, these were not statistically significant. These findings provide insights into the molecular effects of agomelatine and cetuximab on colorectal cancer cells and suggest potential avenues for further research in combining chemotherapeutic agents with drugs addressing circadian rhythm disorders in cancer treatment strategies.

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Author contributions: Concept - RK, AK; Supervision –AK, HÜ; Materials- RK, KA, AK; Data Collection and/or Processing- RK, HÜ, EE, KA, AK; Analysis and/or Interpretation - RK, EE, KA, AK; Writing – RK, HÜ, AK.

Availability of data and materials: Data and materials related to this study are available from the corresponding author upon reasonable request.

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Effects of increasing doses of enrofloxacin on biochemical parameters in ducks

ABSTRACT

This research aims to determine the effect of oral single-dose administration of 10, 50, and 100 mg/kg of enrofloxacin on ducks on biochemical parameters. The research was carried out on eighteen ducks. Ducks were divided into 3 equal groups to receive 10, 50, and 100 mg/kg doses. Blood samples were taken at 0, 6, 12, 24 and 48 hours. No clinical side effects were observed in ducks after enrofloxacin administration. When dose groups were compared, significant differences were observed in aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), albumin (ALB), cholesterol (CHOL), total protein (TP) and creatinine (CRE) values ($p<0.05$). However, these differences returned to normal at the 48 hour. When the dose groups were evaluated within themselves, ALT, GGT, CHOL, triglyceride, and urea values did not differ ($p>0.05$). However, there were significant differences in AST, ALP, ALB, and CRE values at 10 mg/kg, AST at 50 mg/kg, and TP at 100 mg/kg ($p<0.05$). In conclusion, it was determined that oral administration of enrofloxacin to ducks at doses of 10, 50, and 100 mg/kg caused temporary changes in biochemical parameters. In this study, enrofloxacin was administered as a single dose. However, considering the repeated use of enrofloxacin in case of bacterial infection, attention should be paid to possible adverse effects that may occur in ducks.

Keywords: Ascending dose, biochemical, duck, enrofloxacin.

INTRODUCTION

Enrofloxacin is a fluoroquinolone group antibiotic approved for the treatment of infections caused by susceptible microorganisms in many animal species including cattle, pigs, cats, dogs, and poultry (EMA, 2001). It exhibits its antibacterial effect by inhibiting the bacterial DNA gyrase and topoisomerase IV enzymes. Enrofloxacin is extensively utilized in veterinary medicine because of its broad spectrum of activity, superior pharmacokinetic properties, and few adverse effects (Corum et al., 2019; Uney et al., 2021). Enrofloxacin is used in the treatment of respiratory, digestive, and soft tissue infections caused by Gram-negative and Gram-positive bacteria as well as mycoplasma-type bacteria and secondary bacterial infections accompanying viral infections (EMA, 2001). Enrofloxacin is used in the treatment of respiratory and digestive system diseases caused by *Mycoplasma gallisepticum, M. synoviae, Avibacterium paragallinarum, Clostridium perfringens, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella spp.* in poultry (Bonassa et al., 2021; Cerda et al., 2002).

Duck is an important poultry species cultivated in many parts of the world for its meat and eggs (Corum et al., 2024; Coskun et al., 2023). The use of enrofloxacin is recommended for infections caused by Gram-negative bacteria such as *Salmonella spp., Campylobacter spp., E. coli, Vibrio spp.*, and *Yersinia spp.* in ducks (Aggad et al., 2010; EMA, 2001;

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Research Article

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Patil et al., 2021). It has also been reported that enrofloxacin can be used in the prevention and treatment of secondary infections that may occur during viral infection in ducks (EMA, 2001; Patil et al., 2021).

Fluoroquinolone group antibiotics show their effect in a concentration-dependent manner and their efficacy increases as the dose increases (Corum et al., 2019; Coskun et al., 2020). The traditional dose of enrofloxacin in poultry is 10 mg/kg. However, recent efficacy studies have recommended a dose of 50-100 mg/kg against *Salmonella* and *Clostridial* spp. bacteria in chickens (Kang et al., 2019; Li et al., 2017a; Li et al., 2017b; Maślanka et al., 2009). However, increasing the dose of drugs may cause undesirable effects on the body and these undesirable effects may differ between poultry species (Coskun et al., 2023). Side effects of drugs can be classified as pharmacological, biochemical, pathological, genotoxic, and allergic reactions. Therefore, biochemical parameters can be utilized in the evaluation of adverse effects in organs and tissues (Corum et al., 2015; Coskun et al., 2018). There is no information on the safety of enrofloxacin in ducks. This study aimed to determine the effect of enrofloxacin on biochemical parameters following oral administration of 10, 50, and 100 mg/kg single dose to ducks.

MATERIALS AND METHODS

Animals

The study was carried out on eighteen male ducks (8-12 months old) with a body weight of 1.8-2.5 kg. Ducks that were determined to be healthy by general clinical examination and had not received any medication in the last eight weeks before the study were included in the study. Ten days before the start of the study, the animals were taken to the pens where the study would be conducted and acclimatized to the environment. The animals were given ad-libitum access to water and fed concentrate feed twice a day. Animals were fasted 6 hours before the drug administration to prevent the effect of nutrients on the absorption of enrofloxacin. All procedures were approved by the ethics committee of Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Experimental Animal Production and Research Center (2022/06-03).

Experimental design

Eighteen ducks used in the study were divided into three equal dose groups. A single dose of enrofloxacin (Enrocure 10% oral solution, Teknovet, Istanbul/Türkiye) was administered orally to the first group (n=6) 10 mg/kg, to the second group (n=6) 50 mg/kg, and to the third group (n=6) 100 mg/kg. Drug administration was performed by gastric gavage. Blood samples (3 mL) were collected in anticoagulant-free tubes from the the brachial vein by venepuncture for biochemical analyses before (0 hour, control) and at 6, 12, 24, and 48 hours after enrofloxacin administration. Blood samples were centrifuged at 4000 x g for 10 minutes and the serum samples were stored at -80 ºC until analysis.

Analysis of biochemical parameters

Albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), cholesterol (CHOL), alkaline phosphatase (ALP), total protein (TP), triglycerides (TG), creatinine (CRE) and urea levels were measured in serum samples using an autoanalyzer (Abbott architect c8000, Abbott Core Laboratory, USA).

Statistical analysis

Biochemical parameters were presented as mean ± standard deviation (SD). The homogeneity of variance was evaluated using Levene's test, and the normality of the data distribution was assessed using the Shapiro-Wilk criterion. Intra- and inter-group statistical analysis of the obtained data was performed using one-way analysis of variance (ANOVA) and post-hoc Tukey tests in SPSS software (22.0 software; IBM). $p < 0.05$ was accepted as the limit of statistical significance.

RESULTS

Oral administration of enrofloxacin at doses of 10, 50, and 100 mg/kg did not cause any abnormalities in the duck's behavior, feed and water consumption, frequency, and consistency of defecation. Changes in biochemical parameters after oral administration of 10, 50, and 100 mg/kg single dose of enrofloxacin to ducks are presented in Table 1.

Table 1. Biochemical parameters in ducks following oral single-dose administration of enrofloxacin 10, 50, and 100 mg/kg (n=6, Mean ± SD).

Parameters	Group	Sampling time (hour)				
		0	6	12	24	48
AST	10 mg/kg	14.33 ± 5.89^{b}	$^{x}35.33\pm12.75^{a}$	22.67 ± 15.68^{ab}	11.67 ± 3.14^{b}	16.83 ± 5.23^{b}
	50 mg/kg	15.00 ± 4.38^{b}	$^{xy}23.83\pm5.81^{a}$	16.33 ± 5.24^{ab}	11.67 ± 3.83^{b}	10.67 ± 4.18^{b}
	100 mg/kg	14.50 ± 6.28	$^{y}14.00\pm6.51$	11.17 ± 5.74	8.67 ± 4.46	11.00 ± 5.37
ALT	10 mg/kg	26.33 ± 8.29	32.83 ± 10.61	33.83 ± 9.91	30.83 ± 8.91	25.83 ± 8.16
	50 mg/kg	28.17 ± 6.52	31.33 ± 10.97	33.17 ± 11.62	26.50 ± 8.41	21.50 ± 8.64
	100 mg/kg	26.00 ± 8.10	29.33 ± 7.15	28.50 ± 10.60	22.33 ± 5.50	20.17 ± 4.12
ALP	10 mg/kg	28.50 ± 8.31^{b}	$^{x}60.67\pm24.55^{ab}$	63.83 ± 22.41^{ab}	$^{x}83.33\pm29.21^{a}$	94.83 ± 32.26^{a}
	50 mg/kg	27.33 ± 6.98^{c}	$^{xy}35.00\pm15.47^{bc}$	64.83 ± 25.10^{ab}	$^{xy}60.17\pm18.65^{ab}$	72.17 ± 24.03^{a}
	100 mg/kg	28.33 ± 9.52	$^{y}32.50\pm10.01$	38.67 ± 17.21	$^{y}47.83\pm14.77$	61.67 ± 35.31
GGT	10 mg/kg	1.00 ± 0.63	$^{x}2.00\pm1.10$	$^{x}2.33\pm1.37$	$^{x}2.33\pm1.03$	2.00 ± 0.63
	50 mg/kg	1.33 ± 0.52	$^{y}0.83\pm0.41$	$^{y}0.67\pm0.52$	$^{y}0.83\pm0.75$	1.00 ± 0.89
	100 mg/kg	1.50 ± 0.55	$^{y}0.50\pm0.55$	$^{y}0.67\pm0.52$	$^{y}0.67\pm0.82$	1.33 ± 1.03
ALB	10 mg/kg	12.33 ± 1.37^{b}	$^{x}15.67\pm3.39^{a}$	12.17 ± 1.47^{b}	$^{x}14.00\pm1.41^{ab}$	11.67 ± 1.03^{b}
	50 mg/kg	12.83 ± 1.33	$^{y}11.50\pm1.97$	13.33 ± 2.66	$^{xy}11.67\pm1.37$	12.33 ± 1.37
	100 mg/kg	12.67 ± 1.63	$^{y}11.33\pm0.82$	11.83 ± 1.33	$^{y}9.17\pm4.45$	11.67 ± 1.37
TP	10 mg/kg	40.33 ± 2.76	40.65 ± 7.32	39.23 ± 4.41	$^{x}42.82\pm3.39$	39.92 ± 3.03
	50 mg/kg	40.70 ± 2.84	34.48 ± 3.27	40.08 ± 10.01	$^{y}35.52\pm2.83$	36.23 ± 4.48
	100 mg/kg	40.08 ± 3.83^{a}	33.95 ± 3.74^{b}	35.68 ± 1.80^{ab}	$^{y}36.24\pm3.34^{ab}$	36.48 ± 4.42^{ab}
CHOL	10 mg/kg	127.78 ± 19.00	$^{x}147.20\pm40.47$	134.67 ± 24.34	$^{x}152.94\pm28.93$	141.20 ± 27.00
	50 mg/kg	129.62 ± 18.50	$^{y}109.01\pm7.44$	127.07 ± 19.39	$^{y}120.15\pm11.13$	122.78 ± 17.18
	100 mg/kg	127.83 ± 28.92	$^{xy}110.84\pm11.29$	124.54 ± 10.09	$^{xy}127.80\pm15.62$	122.62 ± 18.66
TG	10 mg/kg	113.35 ± 20.66	101.80 ± 33.81	118.43 ± 44.99	117.66 ± 26.84	132.00 ± 57.52
	50 mg/kg	116.24 ± 18.96	96.83 ± 22.74	133.52 ± 31.29	102.55 ± 31.09	122.95 ± 25.61
	100 mg/kg	115.55 ± 17.65^{ab}	82.88 ± 8.68^{b}	137.56 ± 38.93^{a}	112.95 ± 35.47^{ab}	117.95 ± 27.65^{ab}
Urea	10 mg/kg	3.53 ± 1.68	4.33 ± 1.09	2.61 ± 0.91	3.37 ± 1.62	2.61 ± 1.34
	50 mg/kg	3.56 ± 1.44	4.15 ± 1.29	2.55 ± 1.34	2.51 ± 0.91	2.35 ± 0.83
	100 mg/kg	3.60 ± 1.70	3.59 ± 0.98	2.35 ± 0.75	2.45 ± 0.67	1.89 ± 0.56
CRE	10 mg/kg	0.06 ± 0.03^{b}	$^{x}0.20\pm0.08^{a}$	0.08 ± 0.02^{b}	0.09 ± 0.05^{b}	0.13 ± 0.06^{ab}
	50 mg/kg	0.06 ± 0.02	$^{xy}0.13\pm0.10$	0.15 ± 0.06	0.12 ± 0.08	0.13 ± 0.09
	100 mg/kg	0.07 ± 0.02	$^{y}0.07\pm0.04$	0.10 ± 0.06	0.11 ± 0.06	0.16 ± 0.09

^{x,y}; Indicates statistical difference between groups ($p<0.05$). ^{a,b,c}; Indicates statistical difference within the group ($p<0.05$)
AST; aspartate aminotransferase, ALT; alanine aminotransferase, ALP; alkaline phosphatase, GGT; gamma glutamyltransferase, ALB; albumin, TP; total protein, CHOL; cholesterol, TG; triglyceride, CRE; creatinine.

When the different dose groups were compared, significant changes were observed in AST, ALP, GGT, ALB, CHOL, and CRE at 6 hour, GGT at 12 hour, and ALP, GGT, ALB, CHOL, and TP at 24 hour ($p<0.05$). However, no difference was observed between dose groups at 48 hour ($p>0.05$). When the dose groups were evaluated within themselves, no difference was observed in ALT, GGT, CHOL, TG, and urea levels ($p>0.05$). In comparison to the 0 hour, significant increases in AST levels were observed at the 6 hour in the 10 mg/kg and 50

mg/kg dose groups. ALP levels showed significant increases at 24 and 48 hours in the 10 mg/kg dose group, and at 12, 24, and 48 hours in the 50 mg/kg dose group. Additionally, ALB and CRE levels increased significantly at the 6 hour in the 10 mg/kg dose group, while TP levels decreased at the 6 hour in the 100 mg/kg dose group ($p<0.05$).

DISCUSSION

Duck farming is an important part of industrial and rural poultry farming. Duck meat and eggs are crucial economic resources for the rural economy. Duck farming has increased significantly worldwide in recent years. It is very important to reduce the losses and deaths that will occur with the effective treatment of bacterial infections that occur with this increase (Adzitey & Adzitey, 2011). Enrofloxacin is widely used in the treatment of bacterial infections in poultry. Enrofloxacin is more effective than amoxicillin, colistin, erythromycin, oxytetracycline, and chlortetracycline against agents causing salmonellosis and colibacillosis in ducks (Aggad et al., 2010), and *Staphylococcus aureus* isolated from ducks was resistant to erythromycin, streptomycin and chloramphenicol, while it showed high sensitivity to enrofloxacin and ciprofloxacin (Amen et al., 2019). The antibacterial effect of enrofloxacin is concentration dependent, and increasing the dose increases its effectiveness (Bonassa et al., 2021; Coskun et al., 2020; Riviere & Papich, 2018). Although the traditional dose of enrofloxacin in bacterial infections is 10 mg/kg, it is recommended to use higher doses in infections caused by microorganisms such as *Salmonella enteritidis* and *Clostridium* spp. (Kang et al., 2019; Temmerman et al., 2021). Use of medicines in high doses may cause adverse effects (Coskun et al., 2018). In this study, the effect of increasing doses of enrofloxacin on biochemical parameters in ducks was demonstrated for the first time. Although some significant changes were observed in biochemical parameters due to the increase in enrofloxacin dose, it was determined that these changes were transient and that enrofloxacin was generally well tolerated in ducks.

In this study, after oral administration of enrofloxacin to ducks at doses of 10, 50 and 100 mg/kg, significant changes were observed in AST, ALP, GGT, ALB, CHOL and CRE at 6 hour, GGT at 12 hour and ALP, GGT, ALB, CHOL, and TP at 24 hour. However, no difference was detected between dose groups in biochemical parameters at the 48 hour. In the intra-group evaluation, changes were observed in AST and ALP levels at a dose of 10 mg/kg, in AST, ALB, and CRE levels at a dose of 50 mg/kg, and in TP levels at a dose of 100 mg/kg. After oral administration of enrofloxacin to broiler chickens at doses of 10 and 100 mg/kg for 5 days, an increase in serum ALT and AST levels and histopathological changes in the liver associated with these increases were observed in the high-dose group (Ellakany et al., 2008); in another study, administration of 100 and 200 mg/kg doses to broiler chickens for 30 days caused anemia and leukopenia by changing hematological parameters (Ibrahim et al., 2011). It was stated that it did not cause any changes in cartilage formation and structure when applied to chickens at doses of 10, 50, and 100 mg/kg, while it caused significant changes at doses of 300 and 600 mg/kg (Maślanka et al., 2009). It has been reported that it does not affect biochemical parameters (AST, creatinine kinase, LDH, ALB, ALT, glucose, TP, uric acid, and CHOL) when given intramuscularly (15 mg/kg) and orally (3, 15, and 30 mg/kg) to African gray parrots (Flammer et al., 1991).

The effect of enrofloxacin on biochemical parameters has been demonstrated in other animal species as well as poultry. Administration of enrofloxacin to lambs at a dose of 35 mg/kg for 15 days (Khazaeil et al., 2012) and to rams at a dose of 10 mg/kg for 14 days did not cause chondrotoxic effects (Coskun et al., 2018). It has also been reported that it caused significant

changes in ALP, ALT, AST, TP, BUN, and CRE values in rams, but these changes were within reference limits (Coskun et al., 2018). Enrofloxacin was administered orally to dogs for 3 days (18-20 mg/kg) and intramuscularly for 5 days (2.5 mg/kg) and administered to cats intramuscularly for 7 days (5, 15, and 25 mg/kg) not cause any changes in biochemical parameters (Shoorijeh et al., 2012; Vinay et al., 2017; Westropp et al., 2012). However, it has been stated that oral administration to rats at doses of 5 and 10 mg/kg for 28 days increased ALT, AST, GGT, ALP, ALB, bilirubin, BUN, and CRE levels and that these effects were due to oxidative stress in the kidney and liver (Khan et al., 2017; Mishra et al., 2021; Srinivasu et al., 2022).

CONCLUSION

It was determined that oral administration of enrofloxacin at 10, 50, and 100 mg/kg single doses to ducks caused temporary changes in AST, ALP, GGT, CHOL, TP, ALB, and CRE values. Although enrofloxacin was administered as a single dose in this study, repeated use is recommended in case of bacterial infection. Therefore, the safety of enrofloxacin after repeated use in ducks needs to be demonstrated hematologically.

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Conflict of interest: The authors declare no conflicts of interest.

Ethical statement or informed consent: The experiment was approved (2022/06-03) by the Local Ethics Committee for Animal Research Studies at Hatay Mustafa Kemal University (Hatay/Türkiye) and carried out by the European Directive (2010/63/EU).

Author contributions: *Conceptualization; investigation; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing*: Duygu Durna Corum, Devran Coskun, Orhan Corum. *Writing – review and editing; project administration:* Feray Altan, Zafer Bulut.

Availability of data and materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Antimicrobial activity of *Lactobacillus* cell free supernatant against *Salmonella* Enteritidis and Infantis

Research Article

ABSTRACT

Cell-free supernatants (CFS) produced by lactic acid bacteria (LAB) have been characterized as natural antagonists of important pathogens, including *Salmonella*. Their bacteriostatic or bactericidal properties have been reported to serve as an alternative to antibiotics by minimizing problems related to antimicrobial resistance. This study aimed to evaluate the antimicrobial activity of CFS of 4 selected LAB strains belonging to *Lacticaseibacillus paracasei* (2 strain), *Limosilactobacillus reuteri* (1 strain), and *Lacticaseibacillus rhamnosus* (1 strain) species against *Salmonella* Enteritidis and *S.* Infantis serovars by the agar-well diffusion method. Cell-free culture media of lactic acid bacteria were used in either crude CFS (acidic) and neutralized form (NCFS) to also understand non-pH-dependent antimicrobial potential. All crude CFSs were found to exhibit antimicrobial activity against pathogens, ranging from moderate to strong. After pH neutralization, the crude CFS of *L. paracasei* (2 strains) lost their antimicrobial activity, except for the crude CFS produced by *L. reuteri* and *L. rhamnosus*. However, there was a significant decrease in the level of anti-*Salmonella* activity of *L. rhamnosus* NCFS. It was determined that *L. reuteri* NCFS continued to show antimicrobial activity at levels similar to the effects of crude CFS. It is thought that the antimicrobial activity of *L. reuteri* and *L. rhamnosus* CFS determined in the research does not depend only on their acidity and that the chemical characterization of the postbiotics, which is the source of this antimicrobial activity, should be evaluated.

Keywords: Antimicrobial activity, cell-free supernatant**,** lactic acid bacteria, *Salmonella* Infantis, *Salmonella* Enteritidis.

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INTRODUCTION

Postbiotics are formulations containing non-living microbes or their elements that offer health benefits to the host (Zołkiewicz et al., 2020). Exopolysaccharides and peptidoglycans are examples of extracellular postbiotics secreted by lactic acid bacteria (LAB), while intracellular postbiotics include organic acids, short-chain fatty acids (SCFAs), indole from amino acids, and peptides like acidophilin, bifidin, reuterin, and lactocepin (Thorakkattu et al., 2022). Cell-free supernatant (CFS) is a sort of postbiotic fluid that remains after living cells are removed from bacteria cultured in a culture medium via centrifugation and filtering, and it contains the bacteria's biological metabolites. Studies show that these metabolites can be isolated and used independently (Żółkiewicz et al., 2020). Although the mechanisms underlying their health advantages are not entirely understood, investigations have indicated that CFS has bacteriostatic or bactericidal properties, competing with harmful microorganisms and reducing their growth (Thorakkattu et al., 2022).

Salmonella is one of the most important zoonotic bacteria, ranking second globally in terms of foodborne gastrointestinal illnesses. It is predicted that the

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eating of *Salmonella*-contaminated food causes 86% of these infections (EFSA,2022). Salmonellosis in humans is caused by contaminated items such as dairy, seafood, poultry, eggs, and meat. *Salmonella* Typhimurium and *Salmonella* Enteritidis are known to be among the most frequently isolated serotypes in human salmonellosis cases, but the prevalence of *Salmonella* Infantis in salmonellosis cases has been reported to be rapidly increasing in recent years (Alzahrani et al., 2023).

Probiotics, prebiotics, essential oils, and bacteriophages have all been developed in recent years as alternatives to antimicrobials for *Salmonella* infection control. According to studies, CFS could be used as a feed addition to manage *Salmonella* in livestock farming, providing an alternative to antibiotics and alleviating concerns about antimicrobial resistance (Abramov et al., 2023; Dobreva et al., 2022; Evangelista et al., 2021).

Many LAB produce CFS, which have been identified as natural antagonists of key pathogens such as *Salmonella*, highlighting the need to research their anti-pathogenic activities (Dobreva et al., 2022). In this context, the present study focuses on the antimicrobial effects of CFS from selected LAB against *Salmonella* Enteritidis and Infantis serovars.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The study used LAB strains that had previously been isolated from conventional fermented dairy items (Yilmaz and Turkyilmaz, 2022). *Limosilactobacillus reuteri* (I-3) (n:1), *Lacticaseibacillus rhamnosus* (I-4) (n:1) and two strains of *Lacticaseibacillus paracasei* (I-2 and I-7) were selected for the study. *S.* Enteritidis (n:3; Lab. Code: N12, N13, N24) and *S.* Infantis (n:3; Lab. Code: N9, N10, N11) serovars, which were previously isolated from poultry litter and were found multidrug-resistant (MDR) bacteria in antimicrobial screening tests, were used as test microorganisms. All bacterial strains were used among the bacterial culture collection of the Microbiology Department, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Aydın, Türkiye.

Prior to preparing the CFS, LAB strains were cultivated for 24 hours at 37°C on De Man, Rogosa, and Sharpe Agar (MRS Agar, Merck, Darmstadt, Germany). Before conducting antimicrobial assays, *Salmonella* strains were cultured on Tryptic Soy Agar (TSA, Merck, Darmstadt, Germany), and Tryptic Soy Broth (TSB, Merck, Darmstadt, Germany). Nutrient Agar (NA, Thermo Fisher Scientific) was employed for the agar well diffusion technique. Pathogenic strains were revitalized in TSB with a 10% inoculum at 37°C overnight. Each bacterial strain underwent two subcultures before experimentation and was stored at -20°C in Brain Heart Infusion broth (BHI, Merck, Darmstadt, Germany) supplemented with 20% glycerol.

CFS preparation

CFSs were generated utilizing the agar well diffusion technique, following the method outlined by Shokryazdan et al., 2014, with slight adjustments. Briefly, LAB strains from overnight cultures were introduced into MRS Broth at a concentration of 1% (v/v) and were left to grow at 37°C for 24 hours. Following the overnight incubation period, CFSs were prepared by centrifuging the broth at 4000 × g for 20 min at 4°C. The supernatant was sterilized using membrane filters (0.22 µm pore size, Sartorius, Göttingen, Germany) and were named as crude CFS (acidic CFS). To test the acid-dependent antimicrobial activities of CFS, pH-adjusted CFS-free media; MRS broth were used as negative controls. Neutralized CFS samples were prepared with 2 M NaOH and was called neutralized cell-free supernatant (NCFS) to test acid-independent antimicrobial activity. The pH

of the crude CFSs and NCFSs were measured with a pH meter.

Antimicrobial screening assay

Each strain of pathogen, initially cultured on TSA and left to grow overnight at 37°C, was adjusted to a concentration corresponding to 0.5 McFarland standard (~108 CFU/mL). These pathogens were then evenly spread onto NA plates using a cotton swab, and the plates were allowed to air-dry at room temperature. Subsequently, wells of 6 mm in diameter were created on the plates using a sterile cork borer, into which 100 µL of the CFS was carefully pipetted. Following this, the petri dishes were placed in an incubator at 37°C overnight. The effectiveness of both crude CFS and NCFS were assessed separately to determine their antimicrobial properties.

Statistical analysis

The quantitative data of the experimental results were presented as mean ± standard deviation (SD) of two independent experiments, tested in triplicate. The significance of differences ($p<0.05$) was determined using one-way ANOVA with the statistical package for Social Sciences (SPSS) software (Version 21, SPSS Inc., Chicago, IL. USA).

RESULTS

Anti-Salmonella activity of crude CFSs

The investigation revealed that the pH levels of the CFSs ranged from 4 to 5 prior to undergoing neutralization. The pH values of CFSs *L. paracasei* (I-2), *L. reuteri* (I-3), *L. rhamnosus* (I-4), and *L. paracasei* (I-7) were 4.07, 4.13, 4.43 and 4.07, respectively. Table 1 displays the pH values of the media containing varying concentrations of crude CFSs.

Table 1: The pH values of crude CFSs.

CFS-producing strains	Before pH neutralization Crude CFS
	Mean±SD
Lacticaseibacillus paracasei (I-2)	4.07±0.12
Limosilactobacillus reuteri (I-3)	4.13±0.12
Lacticaseibacillus rhamnosus (I-4)	4.43±0.12
Lacticaseibacillus paracasei (I-7)	4.07±0.12

Mean: Arithmetic mean of the CFSs, SD: Standard Deviation

In the current study, significantly different antimicrobial activities were found in all CFSs at acidic pH (Crude CFS) ($p<0.05$) (Table 1-4). The *L. paracasei* (I-2) CFS exhibited strong antimicrobial activity against *S.* Infantis (N9, N10 and N11) and *S.* Enteritidis N24, with mean inhibition zone diameters of 22.17, 20.10, 20.27 and 25.13mm, respectively. CFS showed moderate activity against the pathogens *S.* Enteritidis (N12 and N13) with zones of inhibition that varied between 18.20 and 18.13mm, respectively (Table 2). The *L. reuteri* (I-3) CFS exhibited moderate antimicrobial activity against *S.* Enteritidis (N12, N13 and N24) and *S.* Infantis (N9, N10 and N11), with mean inhibition zone diameters of 10.13, 10.50, 11.23, 13.73, 10.13 and 14.03mm, respectively (Table 3). The *L. rhamnosus* (I-4) CFS exhibited strong antimicrobial activity against *S.* Enteritidis (N24) and *S.* Infantis (N11), with mean inhibition zone diameters of 26.03 and 21.17mm, respectively. CFS showed moderate activity against the pathogens *S.* Enteritidis (N12 and N13) and *S.* Infantis (N9 and N10) with zones of inhibition that varied between 17.03, 19.10, 18.17 and 18.23mm, respectively (Table 4). The *L. paracasei* (I-7) CFS exhibited strong antimicrobial activity against *S.* Enteritidis (N24) and *S.* Infantis (N11), with mean inhibition zone diameters of 25 and 21.03mm, respectively. CFS showed moderate activity against the pathogens *S.* Enteritidis (N12 and N13) and *S.* Infantis (N9 and N10) with zones of inhibition that varied between 16.23, 18.27,

18.03 and 15.10mm, respectively (Table 5). CFS-free media: MRS used as a negative control did not show any antimicrobial activities as expected.

Table 2: Anti-*Salmonella* activity of crude CFS of *Lacticaseibacillus paracasei* I-2.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)	
	Crude CFS	**Inhibition effect**
***S.* Enteritidis N12**	18.20±0.26*	Moderate
***S.* Enteritidis N13**	18.13±0.15*	Moderate
***S.* Enteritidis N24**	25.13±0.15*	Strong
***S.* Infantis N9**	22.17±0.15*	Strong
***S.* Infantis N10**	20.10±0.10*	Strong
***S.* Infantis N11**	20.27±0.15*	Strong
Control: CFS-free media	0.00	Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by crude CFS

Table 3: Anti-*Salmonella* activity of crude CFS of *Limosilactobacillus reuteri* I-3.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)	
	Crude CFS	**Inhibition effect**
***S.* EnteritidisN12**	10.13±0.15*	Moderate
***S.* EnteritidisN13**	10.50±0.50*	Moderate
***S.* EnteritidisN24**	11.23±0.25*	Moderate
***S.* Infantis N9**	13.73±0.25*	Moderate
***S.* Infantis N10**	10.13±0.15*	Moderate
***S.* Infantis N11**	14.03±0.15*	Moderate
Control: CFS-free media	0.00	Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by crude CFS.

Table 4: Anti-*Salmonella* activity of crude CFS of *Lacticaseibacillus rhamnosus* I-4.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)	
	Crude CFS	**Inhibition effect**
***S.* EnteritidisN12**	17.03±0.15*	Moderate
***S.* EnteritidisN13**	19.10±0.10*	Moderate
***S.* EnteritidisN24**	26.03±0.15*	Strong
***S.* Infantis N9**	18.17±0.15*	Moderate
***S.* Infantis N10**	18.23±0.21*	Moderate
***S.* Infantis N11**	21.17±0.15*	Strong
Control: CFS-free media	0.00	Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by crude CFS.

Table 5: Anti-*Salmonella* activity of crude CFS of *Lacticaseibacillus paracasei* I-7.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)	
	Crude CFS	**Inhibition effect**
***S.* EnteritidisN12**	16.23±0.25*	Moderate
***S.* EnteritidisN13**	18.27±0.21*	Moderate
***S.* EnteritidisN24**	25.00±0.10*	Strong
***S.* Infantis N9**	18.03±0.15*	Moderate
***S.* Infantis N10**	15.10±0.10*	Moderate
***S.* Infantis N11**	21.03±0.15*	Strong
Control: CFS-free media	0.00	Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by crude CFS.

Anti-Salmonella activity of NCFS

To mitigate the influence of organic acid compounds such as lactic, acetic, and formic acid, as well as bacteriocins present in crude CFSs that hinder pathogen growth, neutralization was performed. After pH neutralization, crude CFSs from the *L. paracasei* I-7 and *L. paracasei* I-2 lost their antimicrobial activity (Table 6-7).

Table 6: Anti-*Salmonella* activity of NCFS of *Lacticaseibacillus paracasei* I-7.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)		
	NCFS	NCFS (Neutralized by NaOH)	Inhibition effect
***S.* EnteritidisN12**	-*	6.93±0.06	Negative
***S.* EnteritidisN13**	-*	6.83±0.06	Negative
***S.* EnteritidisN24**	-*	6.87±0.06	Negative
***S.* Infantis N9**	-*	6.83±0.06	Negative
***S.* Infantis N10**	-*	6.93±0.06	Negative
***S.* Infantis N11**	-*	6.93±0.06	Negative
Control: CFS-free media	-		Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by NCFS. The inhibition zone is not determined: - (Negative).

Table 7: Anti-*Salmonella* activity of NCFS of *Lacticaseibacillus paracasei* I-2.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)		
	NCFS	NCFS (Neutralized by NaOH)	Inhibition effect
***S.* Enteritidis N12**	-*	6.80±0.10	Negative
***S.* Enteritidis N13**	-*	6.53±0.06	Negative
***S.* Enteritidis N24**	-*	6.67±0.15	Negative
***S.* Infantis N9**	-*	6.80±0.10	Negative
***S.* Infantis N10**	-*	6.87±0.06	Negative
***S.* Infantis N11**	-*	6.87±0.06	Negative
Control: CFS-free media	-		Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by NCFS. The inhibition zone is not determined: - (Negative).

The *L. rhamnosus* (I-4) NCFS exhibited moderate antimicrobial activity against *S.* Enteritidis (N12, N13 and N24) and *S.* Infantis (N9, N10 and N11) strains, with mean inhibition zone diameters of 15.34, 16.17, 14.17, 16.20, 15.23 and 14.27mm, respectively (Table 8). The *L. reuteri* (I-3) NCFS exhibited moderate antimicrobial activity against *S.* Enteritidis (N12, N13 and N24) and *S.* Infantis (N9, N10 and N11), with mean inhibition zone diameters of 10.00, 10.17, 10.27, 12.10, 10.07 and 12.17mm, respectively (Table 9). Neutralizing CFSs from the *L. rhamnosus* (I-4) and *L. reuteri* (I-3) strains with NaOH lowered anti-*Salmonella* activity, but it was not totally abolished (Table 8-9). There was a significant decrease in the level of anti-*Salmonella* activity of *L. rhamnosus* NCFS ($p<0.05$). It was determined that *L. reuteri* NCFS continued to show antimicrobial activity at levels similar to the effects of crude CFS ($p<0.05$).

Table 8: Anti-*Salmonella* activity of NCFS of *Lacticaseibacillus rhamnosus* I-4.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)		
	NCFS	NCFS (Neutralized by NaOH)	Inhibition effect
***S.* EnteritidisN12**	15.34±0.28*	6.80±0.10	Moderate
***S.* EnteritidisN13**	16.17±0.15*	6.53±0.06	Moderate
***S.* EnteritidisN24**	14.17±0.15*	6.67±0.15	Moderate
***S.* Infantis N9**	16.20±0.17*	6.80±0.10	Moderate
***S.* Infantis N10**	15.23±0.21*	6.87±0.06	Moderate
***S.* Infantis N11**	14.27±0.12*	6.87±0.06	Moderate
Control: CFS-free media	-		Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by NCFS. Moderate inhibition effect: 10 mm ≤ Inhibition zone < 20 mm.

Table 9: Anti-*Salmonella* activity of NCFS of *Limosilactobacillus reuteri* I-3.

***Salmonella* Strain**	***Salmonella* Strain Growth Inhibition Zone (mm)**		
	NCFS	**NCFS (Neutralized by NaOH)**	**Inhibition effect**
***S.* EnteritidisN12**	10.00±0.00*	6.77±0.06	Moderate
***S.* EnteritidisN13**	10.17±0.15*	6.63±0.06	Moderate
***S.* EnteritidisN24**	10.27±0.25*	6.77±0.06	Moderate
***S.* Infantis N9**	12.10±0.10*	6.83±0.06	Moderate
***S.* Infantis N10**	10.07±0.12*	6.83±0.06	Moderate
***S.* Infantis N11**	12.17±0.29*	6.87±0.06	Moderate
Control: CFS-free media	-		Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by NCFS. Moderate inhibition effect: 10 mm ≤ Inhibition zone < 20 mm.

DISCUSSION

Salmonella Enteritidis and Infantis serovars are reported as the most commonly reported *Salmonella* serovars in broilers, implicated in cases of salmonellosis in humans (Alzahrani et al., 2023). Given the presence of strains exhibiting multidrug resistance in these scenarios, it is evident that a serious public health issue may arise. LAB and their metabolites have recently been regarded as important possibilities for managing *Salmonella* pathogens due to their proven bio-preservative and antimicrobial properties (Evangelista et al., 2021; Yilmaz and Turkyilmaz, 2022).

CFS produced by LAB is fluid containing byproducts of microbial proliferation and residual nutrients from the cultivation medium that were not absorbed (Thorakkattu et al., 2022). The number of studies studying CFS's anti-infective, antimicrobial, and antioxidant capabilities, such as their function in pathogen inhibition, has increased in recent years (Lee et al., 2022). It is expected that additional research into CFS characteristics and mechanisms of action will lead to a better knowledge of their bioactivities (Cuevas-Gonzalez et al., 2020). The antimicrobial activities of LAB CFS were evaluated in this research using strains of *S.* Enteritidis and *S.* Infantis serovars.

The research discovered that the pH levels of CFSs ranged from 4 to 5 before neutralization. The pH values of the CFSs *L. paracasei* (I-2), *L. reuteri* (I-3), *L. rhamnosus* (I-4), and *L. paracasei* (I-7) were 4.07, 4.13, 4.43, and 4.07. Although pH values fluctuate depending on the LAB strains utilized, pH values of around 4 have been reported in the literature (Abramov et al., 2023).

The research indicated that all crude CFSs had moderate to strong antimicrobial efficacy against microorganisms. All LAB strains' crude CFSs had high antimicrobial activity against *S.* Infantis N11 and *S.* Enteritidis N24, with the exception of *L. reuteri* (I-3). In addition, crude CFS of *L. paracasei* (I-2) demonstrated strong antimicrobial action against *S.* Infantis N9 and N10. The inhibitory impact of *L. reuteri* (I-3) crude CFS on all pathogens was found to be moderate, with no strong activity identified.

There are various studies in the literature demonstrating the antimicrobial activity of CFS against *Salmonella* strains (Arrioja-Bretón et al., 2020; Divyashree et al., 2021; Goa et al., 2022; Lando et al., 2023). A study reported that the CFSs of *Limosilactobacillus fermentum* LBF 233, *Limosilactobacillus fermentum* LBF 433, and *Lacticaseibacillus casei* LBC237 strains exhibited antimicrobial activity against *S.* Enteritidis and *S.* Typhimurium pathogens at concentrations of 10% and 20% (Lando et al., 2023). Another study examined the antimicrobial activity of LAB isolates against clinical isolates of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* spp.

using the agar well diffusion method. It was determined that all 12 LAB isolates used, including *Lactococcus lactis* subsp. *lactis* (6), *Lactobacillus acidophilus* (2), *Lactiplantibacillus plantarum* (1), *L. fermentum* (2), and *Leuconostoc lactis* (1), exhibited antimicrobial activity against the tested bacterial strains (Goa et al., 2022). Shi et al. (2022) reported that the CFS and cells of *L. rhamnosus* SQ511 exhibited antagonistic activity against *S.* Enteritidis ATCC13076 and were able to inhibit the growth of the pathogen. The researchers determined the average diameters of the inhibition zones to be 21.82, 18.45, and 12.34mm. In line with the literature, the *L. rhamnosus* (I-4) crude CFS utilized in this study has strong antimicrobial activity against *S.* Enteritidis (N24) and *S.* Infantis (N11), with mean inhibition zone widths of 26.03 and 21.17mm, respectively. Crude CFS shown moderate effectiveness against the pathogens *S.* Enteritidis (N12 and N13) and S. Infantis (N9 and N10). The zones of inhibition measured 17.03, 19.10, 18.17, and 18.23mm, respectively.

Abramov et al., (2023) found that the low pH of the environment enhanced the antimicrobial efficacy of *Ligilactobacillus salivarius* CFSs against the *S.* Typhimurium pathogen. Additionally, it was discovered that the antimicrobial activity of the neutralized CFS diminished following the neutralizing treatment. Evangelista et al., (2021) investigated the in vitro efficacy of LAB CFS against *Salmonella* and discovered that all acidic CFS suppressed the pathogen's development. The researchers performed pH neutralization on the crude CFS. After pH neutralization, they reported that the neutralized CFS of *L. acidophilus* Llorente, *L. fermentum* CCT 1629, *L. plantarum* PUCPR44, *L. reuteri* BioGaia, *L. rhamnosus* ATCC 7469, and *Pediococcus pentosaceus* UM116 strains partially retained their antimicrobial activities. In line with this research, crude CFSs that hinder pathogen growth, such as lactic, acetic, and formic acid, or bacteriocins, were neutralized. PH neutralization assays were important to determine whether the antimicrobial activity of CFS was acidity dependent. In this study, it was also determined that the CFSs of *L. reuteri* and *L. rhamnosus* continued to exhibit activity after neutralization. The *L. rhamnosus* (I-4) NCFS exhibited moderate antimicrobial activity against *S.* Enteritidis (N12, N13 and N24) and *S.* Infantis (N9, N10 and N11) strains, with mean inhibition zone diameters of 15.34, 16.17, 14.17, 16.20, 15.23 and 14.27mm, respectively. The *L. reuteri* (I-3) NCFS exhibited moderate antimicrobial activity against *S.* Enteritidis (N12, N13 and N24) and *S.* Infantis (N9, N10 and N11), with mean inhibition zone diameters of 10.00, 10.17, 10.27, 12.10, 10.07 and 12.17mm, respectively. However, after pH neutralization, crude CFSs from the *L. paracasei* (I-7) and *L. paracasei* (I-2) lost their antimicrobial activity.

It is known that the CFS produced by LAB mostly contains hydrogen peroxide, organic acids (mainly lactic acid), fatty acids, and proteins/peptides, and has mildly to strongly acidic profiles (ranging from pH 2.2 to 6.0) (Lim et al., 2018). Studies have determined that the production of hydrogen peroxide, organic acids, and bacteriocins are the main strategies by which *Lactobacillus* inhibits the growth of *Salmonella* (Ayeni et al., 2019). In the study, pH neutralization led to a decrease in the efficacy of some CFSs and resulted in the loss of antimicrobial activity in others. This finding underscores the importance of pH for pathogen inhibition. It is also known that pH neutralization reduces the activity of fatty acids because fatty acids at neutral pH are ionized, preventing them from penetrating bacterial cells (Shehata et al., 2019).

According to certain research, the presence of non-acidic antimicrobial substances such as bacteriocins causes the CFS of certain LAB strains to retain some of their antimicrobial activities following pH neutralization. Although these chemicals prefer low pH for best action, they can still be active at neutral pH (Prudêncio

et al., 2016; Shi et al., 2022). The evaluation suggests that the antimicrobial impact is predominantly derived from lactic acid and acetic acid, with additional metabolites such as organic acids, bacteriocins, and short and long-chain fatty acids all contributing to antimicrobial activity (Mani-Lopez et al., 2022).

In the research where moderate/good and strong activity against *S.* Typhimurium was determined for CFS of some LAB strains, it was reported that the identified antimicrobial activity might be attributed to the formation of organic acids. Researchers determined that the neutralized and catalase-treated supernatants had no effect on the tested Gram-negative pathogenic bacteria (Bahri et al., 2014). Parallel to this research, it was found that the antimicrobial effects of *L. paracasei* (I-2 and I-7) CFSs disappeared after the neutralization process. The disappearance of this effect suggests that the antimicrobial activities of these CFSs may stem from organic acids.

CONCLUSION

The antimicrobial activities of neutralized CFSs from *L. rhamnosus* (I-4) and *L. reuteri* (I-3) strains against pathogens might be associated with the presence of other antimicrobial agents, different from the predominant acidic components. The disappearance of the antimicrobial effects of *L. paracasei* (I-2 and I-7) CFSs after the neutralization process suggests that the antimicrobial activity may originate from organic acids. Analysis of the compound responsible for antimicrobial activity may provide information on the potential uses of CFS.

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The absence of the Bluetongue virus in abortion cases in cattle, sheep, and goats in Türkiye: 2012-2017

Research Article

ABSTRACT

Bluetongue (BT) is a disease that affects domestic and wild ruminants, and it is caused by a virus called bluetongue virus (BTV) that is transmitted by *Culicoides* midges. Although clinical signs of BT are most apparent in sheep, BTV could induce abortion and birth defects in cattle, sheep and goats. BTV infection has been reported in Türkiye, but the role of BTV in cattle and small ruminant abortion cases in Türkiye remains uncertain. Therefore, this research aimed to fill this research gap by investigating the prevalence of BTV in cattle and small ruminant abortion cases. To investigate the frequency of BTV in ovine, caprine, and bovine foetuses, a total of 1718 foetuses were collected from different farms between 2012 and 2017. A one-step real-time reverse transcription polymerase chain reaction (RT-PCR) assay was used to detect BTV RNA in aborted foetuses. BTV specific RNA was not detected in the analysed foetuses. To the best of my knowledge, this is the longest study that has investigated whether BTV infection has a role in cattle and small ruminant abortion cases in Türkiye. The results of this study are limited only to the regions studied. Therefore, further epidemiological studies are needed to confirm the findings of this study.

Keywords: Abortion, bluetongue virus, cattle, goats, sheep.

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INTRODUCTION

Bluetongue (BT), one of the reportable diseases by the World Organization for Animal Health (WOAH), is an arboviral disease of sheep, goats, cattle, and wild ruminants, including pronghorn antelope, bighorn sheep, and white-tailed deer (Backx et al., 2007; Falconi et al., 2011; Johnson et al., 2006; Maclachlan, 1994; Niedbalski, 2015). Furthermore, bluetongue virus (BTV) infection has been reported in camelids (Schulz et al., 2012), and BTV was isolated from aborted foetuses of dogs (Dubovi et al., 2013).

The causative agent of the disease, BTV, is a non-enveloped RNA virus of the genus *Orbivirus* within the family *Sedoreoviridae* (ICTV, 2022). The viral genome consists of 10 segments that encode five non-structural proteins (NS5, NS4, NS3/NS3A, NS2, and NS1) and seven structural proteins (VP-1 to VP-7) (Belhouchet et al., 2011; Mertens et al., 1984; Ratinier et al., 2011; Roy, 1992). Until now, a total of 29 serotypes of BTV have been recognised worldwide based on sequence analysis of the segment 2 nucleotide sequences and serum neutralisation tests (Bumbarov et al., 2020; Thota et al., 2020; Yang et al., 2021). Serotypes 1, 2, 3, 4, 6, and 10 have the potential to cause epidemics (Dungu et al., 2004). Furthermore, pathogenicity may change within a serotype due to antigenic drift (i.e. point mutations) and antigenic shift (i.e. reassortment of BTV gene segments) in BTV (Bonneau et al., 2001; Van Schalkwyk et al., 2023).

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The signs of BT vary from mild to severe; the disease is more severe in sheep than in cattle and goats (Maclachlan, 1994). Although fever, cyanosis of the tongue, oedema of the face and lips, mucopurulent nasal discharge, excessive salivation, and coronitis are the main clinical signs of the disease (Darpel et al., 2007; Elbers et al., 2008), BTV infection may lead to abortion and teratogenic defects in vertically infected foetuses, depending on the foetal age at infection (Maclachlan and Osburn, 2017). The morbidity rate of the disease ranges from 2 to 48% (Calistri et al., 2004; Conraths et al., 2009), whereas the mortality rate ranges from 1 to 67% (Conraths et al., 2009; Gambles, 1949).

BTV is mainly transmitted by species of *Culicoides* midges, especially *C. imicola*, *C. pulicaris*, *C. chiopterus*, *C. dewulfi*, and *C. obsoletus* (Goffredo et al., 2015; Mellor et al., 2000; Purse et al., 2015). Furthermore, direct contact, vertical transmission of BTV (Wouda et al., 2009; Bréard et al., 2018) and its transmission through semen have been reported (De clercq et al., 2021).

BTV infection was first reported in southern Africa in the late 18th century, and then it was reported in other regions of Africa, Asia, Australia, the Americas, the Indian subcontinent, the Middle East, and Europe (Gestier et al., 2023; Khanal et al., 2016; Mellor and Wittmann, 2002; Sreenivasulu et al., 2004; Wilson and Mellor, 2009). In Türkiye, the first BT cases were reported in 1944, and later outbreaks caused by serotypes 4, 8, 9, and 16 were observed at different times (Rajko-nenow et al., 2020; Saegerman et al., 2008; Taylor and Mellor, 1994). Although previous serological and molecular studies have reported the presence of BTV infection in Türkiye (Ertürk et al., 2004; Ozkul et al., 2009), the role of BTV in cattle and small ruminant abortion cases in Türkiye remains uncertain. Therefore, this research aimed to fill this research gap by investigating the prevalence of BTV in cattle and small ruminant abortion cases.

MATERIALS AND METHODS

Study location

A total of 1718 foetuses (from 1144 sheep, 82 goats, and 492 cattle) were submitted to the Veterinary Control Institute (Konya, Türkiye) from different farms between 2012 and 2017 (Table 1). Farms included in this study were located in three geographical regions of Türkiye, including the Mediterranean (Isparta, Burdur, and Antalya Provinces), Aegean (Afyonkarahisar Province), and Central Anatolian (Konya, Karaman, Aksaray, and Niğde Provinces) regions with elevations of 30-1229 m (Figure 1).

Table 1. Distribution of the foetal samples by provinces and years.

Province	Years																	
	2012			2013			2014			2015			2016			2017		
	O	C	B	O	C	B	O	C	B	O	C	B	O	C	B	O	C	B
Afyonkarahisar	9	-	7	15	-	11	14	-	10	15	-	11	14	-	6	6	-	7
Aksaray	6	-	7	17	-	8	16	2	9	15	21	-	10	2	3	3	2	3
Karaman	6	-	2	4	-	-	17	-	1	4	-	-	7	-	-	1	-	3
Konya	30	3	24	36	-	8	55	1	7	82	3	14	40	11	17	25	5	21
Niğde	75	-	20	38	2	22	42	-	15	79	3	11	27	1	12	21	2	24
Antalya	20	-	25	17	1	8	54	1	9	134	4	5	11	10	10	1	4	13
Burdur	4	-	20	14	-	9	23	-	8	-	-	9	6	-	8	3	-	20
Isparta	12	-	30	18	1	14	77	-	4	17	-	5	2	3	11	2	-	1
Total	**162**	**3**	**135**	**159**	**4**	**80**	**298**	**4**	**63**	**346**	**31**	**55**	**117**	**27**	**67**	**62**	**13**	**92**

O: Ovine, C: Caprine, B: Bovine

Figure 1. Geographic location of the study area (pink colour) where the study was conducted

Among the studied provinces, Antalya Province has the lowest elevation (30 m), whereas Niğde Province has the highest elevation (1229 m). The climatic conditions in the Mediterranean, Aegean, and Central Anatolian regions are characterised by high temperatures and humidity, cold winters and hot summers, hot and dry summers, and cold and snowy winters, respectively. Average temperatures in the Mediterranean, Aegean, and Central Anatolian regions are 19°C, 15°C, and 16°C, respectively (Turkish state meteorological service, 2020). Domestic ruminant production plays a crucial role in the rural economic development of the studied regions.

Tissue collection

Necropsy was performed on each foetus under aseptic conditions to prevent contamination. During necropsy, tissue samples from the brain, spleen, lung, kidney, and liver were collected, placed into sterile tubes, and stored at -20°C until nucleic acid extraction. Furthermore, information related to abortions, the age of the pregnant animals, the clinical signs, and date of abortion was obtained from farmers using a semi-structured questionnaire.

Viral RNA extraction

The tissues of each foetus were pooled (30 mg) and homogenized in sterile phosphate-buffered saline by using a tissue homogenizer (Qiagen, Germany). The supernatant of tissue homogenates (200 µl) was collected and used for viral RNA extraction. The extraction was performed with a commercially available kit (QIAamp Cador Pathogen Mini Kit, Qiagen, Germany) according to the manufacturer's instructions, and nuclease-free water was used as a control sample to verify the absence of contamination. Viral RNA measurement was performed using a spectrophotometer (DeNovix DS-C, DeNovix Inc., USA), and viral RNA was kept at -85°C until analysis.

One-step real-time RT-PCR assay

Viral RNAs were denatured at 98°C for 5 min on a heating block (Stuart Scientific, UK), then placed at -20°C for 5 min. The PCR reaction mix for a one-step real-time RT-PCR assay was prepared with a OneStep RT-PCR kit (Qiagen, Germany) in a final volume of 25 µl, containing 5 µl 5 X RT-

PCR buffer, 0.8 µM of each primer, 0.1 µM probe, and 6 µl RNA of the sample. The primers and probe used in one-step real-time RT-PCR assay are listed in Table 2. One-step real-time RT-PCR assay was performed using a real-time PCR machine (Rotor-Gene Q, Qiagen, Germany) with the following amplification conditions: 55 °C for 30 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 sec, and 60 °C for 1 min. In the current study, samples with cycle threshold (Ct) values > 35 were considered negative, whereas samples with Ct values < 35 were considered positive (Shaw et al., 2007). BTV-4 RNA obtained from the Central Veterinary Control and Research Institute (Ankara, Türkiye) was used as a positive control, and nuclease-free water was used as a negative control in one-step real-time RT-PCR assay.

Table 2. Primers and probe sequences used for one-step real-time RT-PCR assay for the detection of BTV segment 1.

Primer	Sequence (5'-3')	Reference
BTVrsa 291–311F	GCGTTCGAAGTTTACATCAAT	SHAW et al. (2007)
BTVuni 291–311F	GCTTTTGAGGTGTACGTGAAC	
BTVrsa 387–357R	CAGTCATCTCTCTAGACACTCTATAATTACG	
BTVuni 381–357R	TCTCCCTTGAAACTCTATAATTACG	
BTV-Probe	CYG GAT CAA GTT CAC TCC AYG GC	

RESULTS

Semi-structured questionnaire survey results

Flock owners reported that depression, anorexia, and nasal discharge were the common signs in ewes, whereas nanny goats showed anorexia before abortion. Abortions occurred in ewes and nanny goats at one to five months of gestation. No congenital defects were observed in caprine and ovine foetuses.

Herd owners reported that pregnant cattle did not show any clinical signs, and abortion occurred at 2 to 9 months of gestation. Congenital malformations were not observed in the examined bovine foetuses.

Detection of BTV-specific RNA by one-step real-time RT-PCR assay

BTV RNA was not detected in the examined foetuses. The Ct value of the positive control sample was 29.56 (Figure 2).

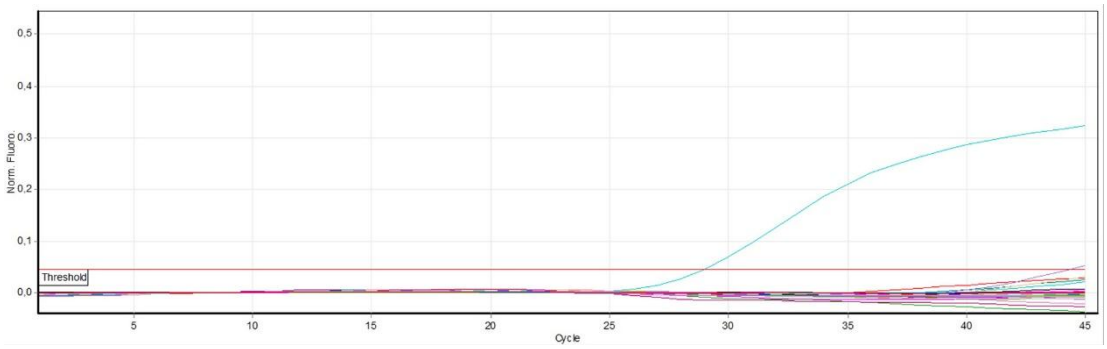

Figure 2. One step real time RT-PCR assay based on Segment 1 of BTV. Light-green line: positive control, red line: negative control.

DISCUSSION

Infectious agents have a significant impact on the livestock abortion (Alzuguren et al., 2023; Dorsch et al., 2022). BTV is one of the important viral abortion agents in cattle and small ruminants (Chauhan et al., 2014; Osburn, 1994). Although BTV infection has been reported in Türkiye, the role of BTV in cattle and small ruminant abortion cases in Türkiye remains uncertain. Therefore, this research aimed to fill this research gap by investigating the prevalence of BTV in cattle and small ruminant abortion cases. To the best of my knowledge, this is the longest study that has investigated the question

of whether BTV infection has a role in cattle and small ruminant abortion cases in Türkiye.

Real-time RT-PCR assays are more specific and sensitive when compared with conventional methods (Batten et al., 2008). Furthermore, WOAH (2021) recommended real-time RT-PCR assays for the detection of BTV in clinical cases. Therefore, in this study, a one-step real-time RT-PCR assay described by Shaw et al. (2007) was used to detect BTV genome segment 1 in ovine, caprine, and bovine foetuses.

BTV is one of the teratogenic infectious agents and can cause central nervous system defects that range from severe hydranencephaly to porencephaly (Maclachlan and Osburn, 2017). The disease is more severe in sheep than in cattle and goats, and central nervous system defects are mostly seen in lambs (Maclachlan, 1994; Maclachlan and Osburn, 2017). Furthermore, BTV can cause abortions without congenital abnormalities (Luedke, 1985). Therefore, in this study, all submitted foetuses without congenital abnormalities were analysed for the presence of BTV.

Although isolation of the BTV and serological evidence of BTV infection in Türkiye has been reported (Ertürk et al., 2004; Yavru et al., 2015), there are currently no reports of detection of BTV in ovine, caprine, or bovine foetuses in Türkiye. In the present study, BTV was not also detected in any of the foetuses examined. A possible explanation for the lack of detection of BTV in foetuses could be related to vaccine-mediated immunity, since there is an official vaccine programme against BTV in Türkiye.

Another possible explanation could be that the insect vectors that play a role in the transmission of the BTV are not available in the research area. *C. obsoletus*, *C. imicola*, *C. dewulfi*, *C. bolitinos*, *C. scoticus*, *C. pulicaris*, *C. insignis*, *C. sonorensis*, and *C. brevitarsis* have a major role in the biological transmission of BTV (Duan et al., 2019; Hudson et al., 2023; Purse et al., 2015). *C. imicola*, *C. pulicaris*, and *C. circumscriptus* were detected in a field study in the Antalya Province (Dik et al., 2006). In the present study, Antalya is the only city where climatic conditions are suitable for the introduction and distribution of *Culicoides* biting midges throughout the entire season, whereas climatic conditions in other studied provinces are suitable for the abundance and distribution of *Culicoides* spp. only between spring and autumn. A possible explanation for the lack of detection of BTV in foetuses could be related to the sampling period, since the majority of aborted foetuses were collected in the winter period, when vector activity is not observed in the Aegean and Central Anatolia Regions.

BTV infection is one of the endemic viral diseases in Türkiye (Ertürk et al., 2004; Ozkul et al., 2009; Yavru et al., 2015), and it has been reported that immunity in animals infected with BTV is lifelong. Lifelong immunity has been linked to protection from severe disease (WOAH, 2021). This could explain why BTV was not detected in the foetuses examined.

It has been reported that BTV infection during the early stages of pregnancy can lead to abortion in infected pregnant animals (Saegerman et al., 2011). In the present study, foetuses were in different stages of pregnancy; the majority of the caprine and ovine foetuses were between 3- and 5-month gestations, whereas bovine foetuses were between 4- and 7-month gestations. The foetal immune response in a bovine foetus develops between days 125 and 150 of gestation (Baker, 1995), whereas in caprine and ovine foetuses it develops after day 70 of gestation (Lopez et al., 2012). Therefore, this study could not detect BTV in foetuses, which may be due to the presence of immune responses in foetuses to BTV, which could contribute to virus clearance in aborted foetuses (Maclachlan et al., 1984).

Contrary to the results of this study, previous studies carried out by Anderson et al. (1989) in the United States and Chauhan et al. (2014) in India detected BTV in bovine and caprine foetuses, respectively. Furthermore, an experimental study carried out by Van der sluijs et al. (2011) also detected BTV in ovine foetuses. This difference in the results of studies may be related to the sample type (malformed or aborted foetuses), the number of animals sampled, the sampling period, the virulence of the virus strain, the immune status of animals, the presence of biological vectors in investigated areas, and the differences in farm management.

CONCLUSION

In this study, results were obtained only from the studied regions. Climate change can affect the global distribution of *Culicoides* biting midges and aid their introduction into new geographic locations. Therefore, further epidemiological studies are needed to confirm the findings of this study.

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Estimating the production losses related to fasciolosis in water buffaloes in Türkiye

Research Article

ABSTRACT

This study aimed to estimate the annual production losses related to fasciolosis in water buffaloes in Türkiye. Some official data and prices were used in the analysis and the mean prevalence of the disease in water buffaloes was calculated as 6.9% in Türkiye. Estimated loss analysis was performed for meat losses, milk losses, liver losses, and extended calving intervals. As a result, the total production losses were estimated as US$ 1,007,918 in 2024 year at current prices. The highest loss was estimated for the extended calving interval (US$ 492,658) and the lowest was for the condemned liver (US$ 48,021). In conclusion, the magnitude of the losses may provide producers and policymakers with quantitative decision support for preventing and eradicating fasciolosis in water buffaloes in Türkiye.

Keywords: Cost, fasciolosis, losses, Türkiye, water buffalo.

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INTRODUCTION

The water buffalo is a species in the Bovidae family, which is mostly rare in Asia (98%), and is raised particularly for milk, meat, leather, and labor. Buffaloes are adaptable to diverse environmental conditions and can efficiently utilize inexpensive and low-quality fodder. Water buffalo breeding is most common in India (54%), Pakistan (20%), and China (13%) in the world. They are also breeding in Europe, especially in Italy, and are called Italian Buffaloes (FAO, 2022). The water buffaloes in Türkiye originate from the Mediterranean water buffaloes, which is a subgroup of the river buffaloes and are called Anatolian Buffaloes (Soysal et al., 2005).

Last fifty years, the world buffalo population has nearly doubled and increased more than 3 times in the EU. However, in the same period, a dramatic decrease has been observed in Türkiye. Thus, the share of the Anatolian water buffalo population in the world has seriously fallen (Türkyılmaz, 2010).

In Türkiye, water buffalo breeding is carried out for the production of milk (milk cream, yogurt, cheese, ice cream) and meat (sausage, salami, pastrami). However, the buffalo breeding enterprises are of the traditional family type, 83% of which are small-scale (1-5 heads) and the remaining 17% are medium-sized enterprises with an average of 8-10 buffaloes in Türkiye (Sarıözkan, 2011).

In Türkiye, livestock diseases, particularly parasitic originated, are encountered frequently. One of them is fasciolosis (=liver fluke), which is commonly caused by F. *hepatica* and/or F. *gigantica* worldwide (Soulsby, 1968). Previous studies have demonstrated that the disease causes remarkable economic losses (Abdel-Fatah et al., 2022; Arbabi et

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al., 2018; Kadir et al., 2012; Karshima et al., 2016; Wamae et al., 1998). The main production losses (PL) caused by fasciolosis in water buffaloes are summarized as; a reduction in carcass weight, milk yield losses, condemned liver, and extended calving intervals.

In the literature, there are many studies which estimated the losses due to fasciolosis in various species such as cattle, sheep, and goats (Abdel-Fatah et al., 2022; Arbabi et al., 2018; Arias-Pacheco et al., 2020; Hossain et al., 2011; Mehmood et al., 2017; Oljira et al., 2022). However, studies on water buffalos are rare (Abdel-Fatah et al., 2022; Tum et al., 2007). Due to the lower population and nervous temperament of the buffaloes, they have not been studied as much as cattle both in the world and in Türkiye. Therefore, there are limited studies on the presence of parasites/helminths in water buffaloes in Türkiye (Yılmaz et al., 2012).

The authors couldn't find any attempt about the production losses of fasciolosis in water buffaloes at the national scale in Türkiye. Therefore, this study is the first to estimate the annual (2024-year) production losses due to fasciolosis in water buffaloes in Türkiye.

MATERIALS AND METHODS

In the study, some technical and economic parameters used in the analysis were given in Table 1.

Table 1. Technical and economic parameters used in the analysis

Parameters	Value	References
Technical parameters		
-Total number of buffaloes	161,749	MAF, 2023
-Number of slaughtered buffaloes	69,597	MAF, 2023
-Number of milked buffaloes	79,333	MAF, 2019
-Reduction in weight gain (kg/year/head)*	5.2	Sarıözkan and Küçükoflaz, 2022
-Reduction in milk yield (kg/year/cow)*	44.3	Sarıözkan and Küçükoflaz, 2022
-Extended calving interval (day)	20	Sarıözkan and Küçükoflaz, 2022
-Weight of condemned liver (kg)	2	Sarıözkan and Küçükoflaz, 2022
-Mean prevalence (%)	6.9	Celep et al., 1990; Güzel and Kozan, 2013
*Financial parameters***		
-Price of meat (US$/kg)	9	ATB, 2024
-Price of milk (US$/kg)	1.0	DMYMB, 2024
-Cost of extended calving interval (US$/day)	4.5	Sarıözkan and Yalçın, 2009
-Price of whole liver (US$)	10	Calculated value

*2.5% in carcass weight and 5% in milk yield loss were considered due to disease. **34 TL=1 US$ in September 2024

Some official data and prices were used in the analysis and mean prevalence was calculated from previous limited published studies (Celep et al., 1990; Güzel and Kozan, 2013). Estimated loss analysis was performed for meat losses, milk losses, liver losses, and extended calving intervals (Since artificial insemination is not widely practiced in buffaloes in Türkiye, extra service cost could not be calculated). The calculation methods are given in detail in Table 2.

Table 2. Calculation method for estimating the total production losses due to fasciolosis in water buffaloes in Türkiye

Loss Items	Calculation Method
1. Meat losses	No. of slaughtered water buffaloes × prevalence of disease × reduction in carcass weight × price of meat
2. Milk losses	No. of milked water buffaloes × prevalence of disease × reduction in milk yield × price of milk
3. Liver losses	No. of slaughtered water buffaloes × prevalence of disease × price of liver
4. Extended calving interval	No. of slaughtered water buffaloes × prevalence of disease × extended day for calving interval × cost of extended calving
Total losses	(1+2+3+4)

Similar to Sariozkan and Yalcin (2009), a deterministic method was used to estimate annual losses. A spreadsheet model was designed in Microsoft Excel to estimate the annual loss (in 2024 current prices) caused by fasciolosis in water buffalo in Türkiye.

RESULTS

Estimated annual total production losses due to fasciolosis in water buffaloes in Türkiye are given in Table 3.

Table 3. Total production losses due to fasciolosis in water buffaloes in Türkiye

Loss Items	Quantity of Losses (US$)	%
1. Meat losses	69,597× 0.069 × 5.2 × 9 = 224,742	23.7
2. Milk losses	79,333 × 0.069 × 44.3 × 1 = 242,497	25.6
3. Liver losses	69,597 × 0.069 × 10 = 48,021	5.1
4. Extended calving interval	69,597 × 0.069 × 20 × 4.5 = 432,197	45.6
Total losses	947,457	100.0

The highest loss was estimated for the extended calving interval (US$ 492,658). Condemned liver losses due to disease got the lowest (4.8%) share in total losses. Milk losses and meat losses were 24.1% and 22.3% respectively (Table 3).

DISCUSSION

Fasciolosis is more common in Asian and African countries, and the disease is seen more in developing countries compared to developed countries in the world (Mehmood et al., 2017). Many studies have reported the prevalence of disease in water buffaloes in the world (Abdel-Fatah et al., 2022; Garg et al., 2009; Kadir et al., 2012; Yadav et al., 2015). Disease prevalence varies amongst different countries of the world. It has been reported to be 13.9% in India (Garg et al., 2009), 2.08% in Iraq (Kadir et al., 2012), 68.0% in Nepal (Yadav et al., 2015), 30.5% in Pakistan (Khan et al., 2009), 62.0% in Vietnam (Linh et al., 2003), 44.7% in China (Liu et al., 2009) and 4.2% in İran (Soosaraei et al., 2020).

The possible reasons for variations of disease prevalence in different countries and regions may be due to; environmental and climatic conditions, snail population, buffalo age, gender, diversity in management systems, and pasture pollution. The high prevalence is related to poor management practices and farmers' lack of information on its control.

Countries that have a higher prevalence may be a potential source of infection spread to other regions and a risk for possible future outbreaks. Hence, countries with a high prevalence need to pay attention to strategic points such as preventive medicine practices, and control of intermediate hosts and pastures.

The financial impact of the disease should not be ignored due to a decrease in production and profitability. Therefore, with this study, the total cost of fasciolosis was estimated in water buffaloes in Türkiye.

In Türkiye, previous reports showed that the total cost of the bovine fasciolosis for the Turkish economy could range between 29.5-43.3 million US$ annually (Sarıözkan and Yalcin, 2011). This equates the 1.1-1.7% of the reported total losses due to fasciolosis in the world (Zerna et al., 2021). The estimated cost of the disease for dairy cows varied between 23.1-34.1 million US$. The present study demonstrated that the cost of disease in water buffaloes (US$ 1,007,918) equates to 3-4% of the total losses of bovine fasciolosis in Türkiye.

There are few studies investigating the economic losses of disease in buffaloes worldwide. Additionally, in some of these studies, only losses attributed to condemned liver, live weight loss, and/or reduction in milk yield have been taken into account. For example, in India/Uttarakhand, Bardhan et al. (2014)

estimated the milk losses due to fasciolosis in buffaloes as 5.3 million US$. In Iran, losses of the condemned liver due to infected buffaloes were estimated between US$ 81,000-113,000 annually (Khaniki et al., 2013). Rehman et al. (2013) estimated the condemned liver losses per infected buffalo as US$ 1.1 in Pakistan. In this study, total annual losses were firstly estimated due to fasciolosis in water buffaloes in Türkiye. Moreover, this estimation may also allow the calculation of the worldwide losses in buffaloes.

CONCLUSION

In the future some measures might be implemented to reduce the financial impact of the disease;

- Farmers need to be informed about the quantity of losses and total cost of the disease,

- To prevent resistance, drugs should be used at the correct time and dose,

- Disease control and eradication expenditures must be increased,

- Pastures must be controlled and ameliorated.

- The buffaloes should be systematically dewormed and included helminth control programme.

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