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CONTENTS

Effect of ohmic heating application on *Salmonella* Enteritidis in liquid whole egg

Research Article

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ABSTRACT

Liquid eggs have high nutrient levels and are prone to spoilage. Although pasteurization can reduce the risk of pathogenic microorganisms in liquid eggs, high temperatures can damage the egg's basic components and structure. Ohmic heating (OH) is a food processing technique that is applied to inactivate food pathogens and causes less change in the nutritional profile. This study aimed to investigate the inactivation level of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) in liquid eggs by different voltage gradients of ohmic heating. Inoculated liquid egg samples with *S.* Enteritidis PT4 (NCTC 13349) were exposed to OH at 5V/cm, 10V/cm, and 20 V/cm for 5 minutes. The results showed that OH at 20 V/cm reduced *S.* Enteritidis counts by about 4 logs (65.6% reduction) in 4 minutes without coagulation, while OH at 5V/cm and 10 V/cm had no significant impact. In conclusion, the effectiveness of OH for inactivating pathogens in liquid eggs depends on the electric field intensity and the duration of treatment. Therefore, the best OH conditions should be chosen carefully to ensure food safety and quality.

Keywords: Foodborne pathogens, inactivation, liquid egg, ohmic heating, *Salmonella* Enteritidis

INTRODUCTION

The egg is a food with high biological value due to the exogenous amino acids, vitamins, and minerals it contains, and it has an essential place in human nutrition. Egg and egg products are used in the production of pasta, mayonnaise, confectionery, and ice cream for functions such as coagulation, emulsification, adding flavor color to the product, and increasing its nutritional value (Yüceer, 2019). Since eggs are widely used in producing of many food products and provide a suitable environment for the growth of microorganisms due to their high biological value, they can be contaminated with various pathogens during production and processing (Doğruer et al.,2015). Due to the significant health risks, eggs should not be consumed raw and should be subjected to heat treatment before use. It should be noted that especially dirty, cracked, broken, or insufficiently heat-treated eggs and egg products can cause Salmonella infection (Anonymous 2001; Braden 2006). The most common serotype isolated from egg, eggshell, and egg-borne outbreaks is *S.* Enteritidis (Martelli and Davies, 2012, Keyvan et al., 2023). Since the early 1980s, *S.* Enteritidis phage type 4 (PT4, multidrug-resistant forms) has grown significantly more common in poultry and humans (Głośnicka and Dera-Tomaszewska, 1999).

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Due to the role of eggs in foodborne illnesses, pasteurized liquid egg products that are microbiologically safer and easier to use are demanded by food producers (Nemeth et al., 2011). The pasteurization aims to reduce the risk of pathogenic microorganisms in the product and preserve liquid eggs' physical and functional properties. In the heat treatments applied for pasteurization, the highest possible temperature is tried to be used to reduce the risk of pathogens. These temperature degrees are 58–65.5°C for 2.5–5.0 min for a liquid whole egg, 58–63°C for 2.5–4.0 min for a liquid egg yolk, and 55–57.2°C for 1.0–8.0 min for egg white (Liang, 2007).

Nowadays, alternative processing methods that allow safe products to be obtained by preventing all the negative effects caused by heat treatment are becoming increasingly important. In addition, with the increasing demand for healthy and safe food by conscious consumers, the food industry has entered into new searches in food processing technologies. Ohmic heating (OH), an innovative food processing technique, is based on the principle that the food placed between two electrodes heats up due to the resistance it shows against the applied electric current (Baysal et al., 2011). Although there are many publications on the applicability of OH in different solid and liquid media (Balpetek and Gürbüz, 2015; İçier and Bozkurt, 2011; Kim and Kang 2015; Park and Kang, 2013; Tian et al., 2019), there is no study has been found on the inactivation of *Salmonella* Enteritidis in liquid eggs by OH. This study aimed to evaluate the effectiveness of OH applied at different electric field intensities on the inactivation of *S.* Enteritidis, which poses a health hazard in liquid eggs.

MATERIALS AND METHODS

Sample preparation

In the research, pasteurized liquid whole eggs obtained from the local markets of Burdur province were used**.** For sterility control of the liquid eggs used, 25 mL sample was taken and placed in 225 mL buffered peptone water (Oxoid, CM0509) and incubated at 35 ± 2°C for 24 ± 2 hours (Berrang et al., 1991). Then, 0.1 mL bacterial culture was incubated in 10 mL Rappaport-Vassiliadis broth (Oxoid, CM0669) at 42 ± 0.2°C for 24 ± 2 hours. After that, it was checked whether it was contaminated with *Salmonella* by spreading it on XLD agar (Merck 1.05287) using the spread plate method (Andrews et al.,2016). The *S.* Enteritidis PT4 (NCTC 13349) was inoculated into a 10 mL Tryptic Soy Broth (TSB) (Merck 1.05459) and incubated at 37°C for 18 hours. Then, it was centrifuged at 5000 g for 20 minutes to remove the supernatant. Pellets were washed twice with sterile 0.9 % NaCl and the pellet suspend into a 0.1% peptone suspension. To enumerate bacterial cells, they were serially diluted in 0.1% peptone water and sprouted on XLD agar (Merck, Germany). The plates were then incubated at 37°C for 24-48 hours. The final concentration of *S.* Enteritidis cells in liquid egg was confirmed to be approximately 10^7 CFU/mL using the spreading plate technique.

Experimental equipment

The OH unit used in the experiment was based on a previous study by Özkale and Kahraman (2023). The OH device consisted of 304 L stainless steel electrodes, a K-type thermocouple, a microprocessor, a personal computer, a power supply (AC, 50Hz, 10 A, 0-250 V), a magnetic stirrer and a heating unit. During the heating process, time and temperature changes were recorded using a microprocessor connected to a personal computer. Two hundred mL liquid whole egg samples inoculated with *S.* Enteritidis PT4 (1 mL) were subjected to 5V/cm, 10V/cm and 20 V/cm in the OH treatment. All experiments began at 23.5°C and were continued until the temperature at the core of the liquid egg reached 57°C. This temperature was regarded as the end of the heating.

Enumeration of cells

A mL of liquid egg sample cooled with ice was serially diluted in 0.1% peptone water. The dilutions were plated onto XLD agar to count the viable ones and onto XLD+TSA (XLD-TSA) to count both injured and uninjured bacterial cells. All plates were incubated at 37°C for 24-48 hours before counting the colonies. The sub-lethal rate (%) was calculated according to Tian et al. (2019).

Statistical analysis

The experiments were carried out in triplicate. The data was analyzed using one-way ANOVA and the T-test with SPSS software (Version 21.0; SPSS Inc., IBM Corporation, USA). Duncan's multiple range test ($p<0.05$) was used to determine significant differences.

RESULTS

This study investigated the effect of different electric field intensities of OH on the inactivation of *S.* Enteritidis in pasteurized liquid egg. Pasteurized liquid egg samples in 200 mL sterile glass beakers were inoculated with 1 mL (10^7 log CFU/mL) bacteria. OH was applied at 5 V/cm, 10 V/cm and 20 V/cm electric field intensities using stainless steel electrodes. The OH process began when the liquid egg temperature was around 23.5 °C and samples were collected with a sterile syringe at 0, 1., 2., 3., 4. and 5. minutes of heating. After the five-minute process, the liquid egg temperature reached 57 °C in about 2 minutes in the group with 20 V/cm electric field intensity, while this temperature was not achieved in the groups with 5V/cm and 10 V/cm electric field intensity.

Table 1. Colony counts of *S.* Enteritidis (log CFU/mL) in liquid egg samples treated with 5V/cm, 10V/cm and 20V/cm voltage gradient

Experiment time (min)	5 V/cm	10 V/cm	20 V/cm
0	6.69±0.16	6.69±0.23	$6.74±0.10^{A}$
1	6.55±0.22	6.94±0.04	$6.59±0.13^{A}$
2	$6.74±0.29^{a}$	$6.68±0.13^{a}$	$4.86±0.96^{Bb}$
3	$6.60±0.22^{a}$	$6.58±0.24^{a}$	$3.65±0.11^{Cb}$
4	$6.76±0.18^{a}$	$6.60±0.13^{a}$	$2.77±0.02^{Db}$
5	$6.65±0.09^{a}$	$6.67±1.11^{a}$	$2.32±0.01^{Db}$

Values were means ± standard deviation of three replicates. $^{a-b}$: Values with different superscripts within rows differ significantly ($p<0.05$) $^{A-D}$: Values within a column with different letters are significantly different ($p<0.05$)

Table 1 presents the change in *S.* Enteritidis count in liquid egg after OH with different electric field intensities (5V/cm, 10V/cm and 20 V/cm). There was a significant difference in *S.* Enteritidis count between the groups after 2 minutes of OH ($p<0.05$). The group with 20 V/cm electric current had a greater reduction of *S.* Enteritidis count than the other two groups ($p<0.05$). Moreover, the group with 20 V/cm electric field intensity showed a significant decrease in *S.* Enteritidis count from the initial level from the 2nd minute of the process and this decrease lasted until the 4th minute ($p<0.05$). The groups with 5 V/cm and 10 V/cm electric field intensity did not significantly decrease the *S.* Enteritidis count from the initial level. The group with 20 V/cm electric field intensity reached a *S.* Enteritidis count of 2.32±0.01 log CFU/mL at the 5th minute of the process, which was about 4.42 log lower (65.6 % reduction) than the initial *S.* Enteritidis count (Table.1; $p<0.05$).

Figure 1 shows the percentage of sub-lethally injured cells during the OH process. The number of sub-lethally injured cells increased progressively in all three groups as the processing time increased. This increase was statistically significant for all groups except for the one with 10 V/cm electric field intensity

(p<0.05). The percentage of sub-lethally injured cells in a group with 20 V/cm was significantly higher than after the 2nd minute of the OH process. The groups with 20 V/cm, 10 V/cm, and 5 V/cm electric field intensity had the highest and lowest percentage of injured *S.* Enteritidis cells (%) during the process, respectively (p<0.05).

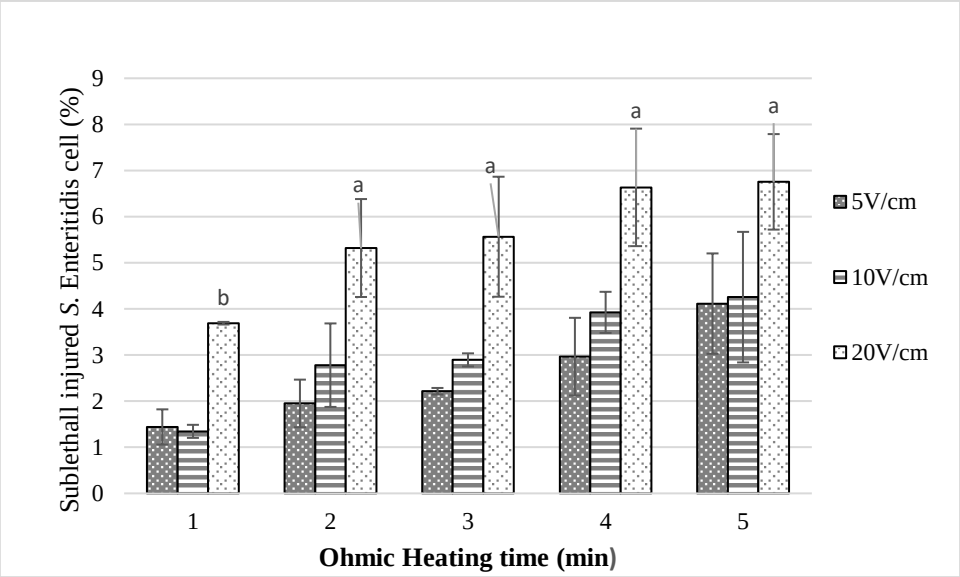

Figure 1. Sublethal injury levels (%) of *Salmonella* Enteritidis in liquid egg samples by 5V/cm, 10 V/cm and 20V/cm OH treatments. Values with different superscripts (a-b) differ significantly (p<0.05).

DISCUSSION

The mechanism of microbial inactivation in OH is due to the thermal effect generated during the process and the electroporation caused by the alternating current in the bacterial cells (Jeager et al., 2016). The electric current increases the membrane permeability of microbial cells, causing the formation of pores in the cell membrane and reducing the resistance of bacteria to heat (Cappato et al., 2017; Yıldız-Turp et al., 2013). In a study investigating the inactivation of *Streptococcus thermophilus* inoculated into milk by ohmic heating, Sun et al. (2011) stated that OH caused non-thermal damage to *S. thermophilus* cell membrane by increasing the permeability of bacterial cell membrane. According to our results, we assume that this electroporation effect generated during the OH process is the reason for the inactivation of the *Salmonella* Enteritidis PT4.

Alamprese et al. (2019) stated that OH is a suitable alternative to conventional pasteurization in eggs, and that low temperature applications should be preferred to avoid rheological problems caused by protein denaturation. Furthermore, due to low processing temperatures, the standard pasteurization procedure may not entirely eliminate *Salmonella* in products (Nemeth et al., 2011). *Salmonella* cells that survive the low-temperature pasteurization process can still cause infection. (Braden, 2006; Noble et al., 2012; Sasaki et al., 2011). However, high temperatures reduce the foaming ability of liquid eggs and cause protein coagulation. (Hamid-Samimiet al., 1984). In general, methods used at low temperatures to eradicate pathogens take more processing time, but processes used at high temperatures can harm food quality. As a result, balancing the processing time and temperature of classical pasteurization is difficult. For this reason, the results we obtained from our study show that OH provides pathogen inactivation at lower temperature and in a shorter time without

damaging the general properties of the liquid egg.

Although there are many studies on the OH application in liquid eggs (Alamprese et al., 2019; Darvishi et al., 2012; İçier and Bozkurt, 2011), the number of studies on the inactivation of pathogens in liquid eggs is quite few. Martín-Belloso et al., (1997) conducted research on the inactivation of *E. coli* in a liquid egg with a pulsed electric field which resulted that a 60% reduction for viable *E. coli* using a pulsed electric field without coagulation. The results of this study demonstrate the similarity to our results in the reduction rate of bacteria. In another study investigating the inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* inoculated into orange and tomato juice with OH at 10-20 V/cm electric field strength and 540 s exposure to electric current, Sagong et al. (2011) reported that *L. monocytogenes* population decreased to 3.76 log CFU/mL after 180 s application of 15 V/cm current in orange juice. They also reported that the same electric field strength decreased *L. monocytogenes* count below the detection limit (1 log) after 210 s application. They observed that 20 V/cm electric field strength applied for 90 s reduced three pathogens below the detection limit (<1 log) of all in tomato juice, while 15 V/cm electric field strength applied for 180 s and 150 s reduced the detection limit of all three pathogens They also reported that the same pathogens were reduced below the detection limit after applying 10 V/cm electric field strength for 480 s and 420 s. These findings indicate that OH can be used to inactivate *E. coli* O157:H7, *S.*Typhimurium, and *L. monocytogenes*, and that the effectiveness of inactivation is dependent on the applied electric field intensity, application time, pathogen, and type of food.

Lee et al., (2013) investigated the inactivation level of *Escherichia coli* O157: H7 and *Salmonella* Typhimurium in salsa by OH. They concluded that when the frequency increased, the time required to reduce *Escherichia coli* O157:H7 and *Salmonella* Typhimurium to below the detection limit (1 log CFU/g) decreased. Our results confirm that the effectiveness of OH in pathogen inactivation depends on the applied electric field intensity and the application time and are in a line with the results of Lee et al, (2013), Martin-Belloso et al., (1997), and Sagong et al. (2011).

CONCLUSION

Salmonella is an important burden for the egg industry. Alternative methods are required to ensure food safety with the least detrimental impact on the nutritional content of egg products. Due to the short processing time and rapid microbial inactivation provided by ohmic processing technology, reliable products requiring less processing can be produced for the egg industry by selecting appropriate electric field strength and processing time.

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Conflict of interest: The authors declared that there is no conflict of interest.

Ethical statement: In studies that do not require ethics committee approval, authors must declare this situation with justification.

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Concentrations of serum amyloid A, haptoglobin and some cytokines in calves with Cryptosporidiosis in the pre- and post-treatment stage

Research Article

ABSTRACT

This study aims to determine serum amyloid A (SAA), haptoglobin (Hp), interleukin-1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) concentration in cases of Cryptosporidiosis that are frequently encountered in calves in veterinary medicine. Although many experimental studies have been conducted in this field, studies of naturally infected calves are quite a few. In this study, 10 neonatal Holstein calves diagnosed with *Cryptosporidium* were included. Stool samples were taken from calves with diarrhea using a rapid test kit. Blood samples were obtained from the jugular vein pre- and post-treatment for biochemical measurements. SAA, Hp, IL-1β, IL-6 and TNF-α concentration measurements were conducted with ELISA reader using commercial kits. Calves with Cryptosporidiosis presenting with diarrhea showed a statistical difference in SAA, Hp, IL-1β, IL-6, white blood cell and hemoglobin values before and after treatment, whereas hematocrit, red blood cell and TNF-α concentrations did not show any statistical difference before and after treatment. According to these findings, to follow up the treatment process of calves with Cryptosporidiosis, it is thought that measuring the concentration of SAA, Hp, IL-1β and IL-6 will be useful for determining disease severity, selecting appropriate treatment, following treatment efficacy and determining subclinical diseases.

Keywords: Haptoglobin, interleukin 1, interleukin 6, serum amyloid A, tumor necrosis factor-α

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INTRODUCTION

Cryptosporidiosis causes severe diarrhea in humans and farm animals (Cho et al., 2013; Kotloff et al. 2013). Cryptosporidium species commonly occur in ruminants and include *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium ryanae* and *Cryptosporidium andersoni* (Ryan et al., 2014). Among these, *C. parvum* is zoonotic and leads to *Cryptosporidium* infection in humans (Chalmers & Giles, 2010; Taylan-Özkan et al., 2016; Xiao 2010). Suler et al., (2016) reported that gastrointestinal diseases with diarrhea are highly common in calves, and the major cause is *Cryptosporidium*, which is transmitted to humans through the fecal–oral route. Physiological responses to infections and injuries, including the onset of episodes that will emerge as inflammation and systemic response, are also known as acute phase reactions (APR). These changes, occurring in a remote location from the inflammation area, are also characterized by fever, leukocytosis, and qualitative and quantitative modifications of a specific group of proteins, excluding structural proteins, functioning in blood and other body fluids. These proteins are called acute phase proteins (APPs) and are promising biomarkers in veterinary medicine (Ceciliani et al., 2012; Zhang et al., 2019).

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Proteomic research on serum/plasma trailing natural or experimental infections found that APPs underwent significant changes and were found in very high levels in these fluids in a wide variety of ways (Ceciliani et al., 2012; Zhang et al., 2019). Today, APPs are routinely used for the diagnosis and prognosis of many diseases (Eckersall & Bell, 2010; Petersen et al., 2004; Zhang et al., 2019). APPs have been demonstrated to have a different level of importance for each animal species; however, due to lack of sufficient research in this area, these proteins have not been fully utilized in the field of veterinary medicine (Eckersall & Bell, 2010; Gruys et al., 2005). SAA is used to determine the activity and prevalence of inflammatory cases, to distinguish inflammatory diseases from non-inflammatory diseases, to monitor the course of diseases and to evaluate the success of the treatment applied (Petersen et al., 2004; Sack, 2018). In addition, it has been reported that high C-reactive protein (CRP) CRP and SAA levels can be considered indicators of latent infection or malignancy even in the absence of fever and neutrophilia (Ceciliani et al., 2012; Eckersall & Bell 2010; Germolec et al., 2018; Sack, 2018).

Proinflammatory cytokines such as IL-6, TNF-α and IL-1β are the main mediators for APPs synthesized from the liver. While IL-6 is more effective in hepatic acute-phase response, IL-1β and TNF-α are effective in extrahepatic cases. Basically, these cytokines are released from macrophages; however, they can also be released from other cells as a result of internal or external stimuli. IL-1β is produced by activated monocytes and macrophages. TNF-α is a polypeptide released from macrophages stimulated by lipopolysaccharides (Murata et al., 2004). It is defined as an effective biologically active mediator or primary cytokine in response to gram-negative bacterial septicemia or endotoxemia. IL-6 can be synthesized from the liver's Kupffer cells, from keratinocytes, from hypophysis or from mucosal epithelia. In the event of inflammation, infection or tissue damage, the release of cytokines is stimulated by the cells that organize the defence. Hence, the synthesis of APPs is also stimulated (Murata et al., 2004; Nukina et al., 2001; Slaats et al., 2016; Yoshioka et al., 2002).

In this study, it was aimed to determine the concentrations of SAA, Hp, IL-1β and IL-6 and TNF-α before and after treatment in calves with Cryptosporidiosis. It also aims to reveal the changes in acute phase proteins (APPs) and cytokines of the applied treatment protocol.

MATERIALS AND METHODS

Calves aged between 1-30 days with clinical complaints of diarrhea constitute the study. Agent isolation was performed with rapid test kits in calves with diarrhea complaints. Cryptosporidium positive animals were included in the study routine clinical examinations were performed on calves with the disease to eliminate other diseases. Care was taken that the calves included in the study had never been treated before. Thus, the interaction of different treatment protocols and their effect on values were eliminated. Calves constituting the patient group were selected to be included in the study at the latest two days after the onset of symptoms. Calves with signs of disease for three days or more were not included in the study. For the treatment of Cryptosporidium, 100 µg halofuginone base/kg ca/day was applied to the calves. The current study was performed Afyon Kocatepe University, Türkiye after the approval of the Local Ethics Committee of Faculty of Veterinary Medicine under approval No: AKÜHADYEK-121-16, on 08.11.2016.

Detecting Cryptosporidium by clinical examination and rapid test kits

In the study, stool samples were taken from calves with diarrhea using a measuring spoon; the sample was diluted with the liquid mixture in the test kit (BIO-K 313 rapid test kit), and then the test kit was soaked in the mixture for 10

minutes. A total of 10 calves diagnosed with *Cryptosporidium* were included in the study.

Treatment procedure

The calves with Cryptosporidiosis diarrhea were treated with 100 µg halofuginon base/kg ca/day administered orally for 7 days. In the pre- and post-treatment stages, blood samples were collected from the jugular vein in tubes without anticoagulant and transferred to EDTA tubes for plasma and hematological measurements.

Hematological procedure

For hematological examination, blood samples were measured for white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB) and hematocrit (HCT) (Compteur Analyseur d'Hematologie MS9-3).

SAA, Hp, IL-1, IL-6 and TNF-α measurements

Anticoagulant-free blood samples taken for biochemical parameters were centrifuged at 5000 rpm for 5 min at room temperature. Serums were stored at –20°C until measurement. In the serums obtained from the blood samples, the concentrations of SAA (Tridelta Development LTD, Ireland), Hp (Life Diagnostics Inc. Bovine Haptoglobin Test Kit), IL-1β (Cusabio Biotech Co. LTD, China), IL-6 (Cusabio Biotech Co. LTD, China), TNF-α (Cusabio Biotech Co. LTD, China) were measured via ELISA reader.

Statistical analysis

After applying ANOVA using SPSS software for Windows (version 18.0) in an electronic environment, the statistical difference was determined via the Tukey's test and the significance of the difference between pre- and post-treatment was determined via Duncan's test. Differences were considered significant when p values were less than 0.05; table values are given as mean ± standard error.

RESULTS

SAA and Hp concentrations in calves with Cryptosporidiosis presenting with diarrhea were higher in the pre-treatment stage at a statistically significant level ($P<0.05$) in comparison to the post-treatment stage (Table 1). IL-1β and IL-6 levels were higher in pre-treatment compared to post-treatment ($P<0.05$), TNF-α concentrations were not found to be different before and after treatment in calves (Table 1).

WBC and HGB concentrations in calves with Cryptosporidiosis presenting with diarrhea showed statistically significant differences ($P<0.05$) before and after treatment. RBC and HCT values were lower in the pre-treatment stage than in the post-treatment stage (Table 2).

Table 1. SAA, Hp, IL-1β, IL-6, TNF-α concentrations (mean ± SE) in pre- and post-treatment stages

Parameters	Pre-treatment	Post-treatment	P value
SAA (mg/L)	2.43 ± 1.11	1.69 ± 0.83	0.045
Hp (µg/mL)	1.01 ± 0.52	0.09 ± 0.02	<0.001
IL-1 (pg/mL)	2.83 ± 0.42	2.38 ± 0.34	0.030
IL-6 (pg/mL)	3.89 ± 2.15	3.07 ± 2.67	0.019
TNF-α (ng/mL)	1.01 ± 0.14	1.03 ± 0.18	0.597

SAA: Serum Amyloid A; Hp: Haptoglobulin; IL-1: Interleukin 1; IL-6: Interleukin 6; TNF-α: Tumor necrosis factor- α

Table 2. WBC, RBC, HGB, HCT concentrations (mean ± SE) in pre- and post-treatment stages.

Parameters	Pre-treatment	Post-treatment	P value
WBC x10^9/L	13.97 ± 3.77	9.91 ± 1.04	0.007
RBC x10^{12}/L	7.02 ± 0.87	7.70 ± 1.23	0.139
HGB g/dL	8.81 ± 1.07	9.98 ± 1.25	0.033
HCT %	27.54 ± 3.72	30.08 ± 4.05	0.636

WBC: White Blood Cell; RBC: Red Blood Cell; HGB: Hemoglobin; HCT: Hematocrit

DISCUSSION

Cryptosporidium is an obligate intracellular protozoan parasite that infects a wide variety of vertebrate hosts including humans and poses a significant threat to public health (Bouzid et al., 2013; Santin, 2020). *Cryptosporidium* infections are noted as an important problem for animal health, mostly in newborn livestock, and lead to economic losses associated with reduced growth rate and increased mortality in infected animals. Moreover, Cryptosporidiosis increases the costs of animal health maintenance and veterinary services (Santin, 2020). Our study was conducted on calves and pre- and post-treatment stage APPs and cytokines, and several hematological parameters were monitored in calves with Cryptosporidiosis presenting with diarrhea. Thus, while determining the effect of Cryptosporidiosis on APPs and cytokines in calves, it was possible to evaluate the efficacy of the applied treatment through the changes in APPs and cytokines.

SAA and Hp concentrations in calves with Cryptosporidiosis presenting with diarrhea were higher in the pre-treatment stage at a statistically significant level ($P<0.05$) in comparison to the post-treatment stage (Table 1). In their study of calves, Kabu et al. (2016) found that SAA concentrations in calves with enteritis were higher than in the control group. In some studies, SAA and Hp concentrations were reported to have remained within normal ranges in experimentally created bacterial and aseptic infections (Abdallah et al., 2016; Chae et al., 2019; Horadagoda et al., 1999). In addition, SAA and Hp concentrations have been reported to increase after natural or experimentally induced infection or inflammation in cattle (Abdallah et al., 2016; Alsemgeest et al., 1994; Fisher et al., 2001; Heegard et al., 2000). Alsemgeest et al., (1993) and Nazifi et al., (2008) reported that they could not detect Hp in the blood of healthy cattle; however, Hp levels were found to be high during inflammatory infections (enteritis, pneumonia, pleuropneumonia, peritonitis, traumatic reticuloperitonitis, endocarditis, abscess, abomasal ulcer, trauma, endometritis, myocarditis, digestive tract diseases). Ganheim et al., (2007) experimentally administered endotoxin lipopolysaccharide intravenously to calves and reported that serum Hp concentrations were higher than in healthy calves after administration. In their study, Albayrak & Kabu, (2016) reported that there was a statistical increase in serum Hp concentration in calves with enteritis compared to the control group. In our study, serum Hp concentration in calves with Cryptosporidiosis presenting with diarrhea was higher in the pre-treatment stage and was statistically significant ($P<0.05$) compared to the post-treatment stage (Table 1). Similar to the studies reporting that SAA and Hp concentration increased during infectious and inflammatory diseases (Abdallah et al., 2016; Alsemgeest et al., 1994; Risalde et al., 2011; Skinner & Roberts 1994), in our study SAA and Hp levels were also noted to be high in calves with enteritis presenting with diarrhea. It is known that Hp and SAA are the major APPs in ruminants and circulating concentrations of these APPs have generally been associated with severity of inflammation and degree of tissue damage (Ceciliani et al., 2012; Murata et al.,

2004). Therefore, measuring serum Hp and SAA concentrations in ruminants can provide diagnostic and prognostic information and evaluation of the response to the trigger event (Ceciliani et al., 2012; Gruys et al., 2005; Iliev & Georgieva, 2019; Murata et al., 2004; Tothova et al., 2014). In their study of lambs, Carroll et al., (2009) reported that with Cryptosporidiosis, SAA and Hp concentrations were high; there was a correlation between oocyte excretion and Hp; however, that was not the case for SAA. Al-Zubaidi, (2015) reported higher SAA and Hp values in calves with Cryptosporidiosis than in healthy ones. The fact that the Cryptosporidiosis agent damages the intestinal lumen and causes microvillus atrophy and mononuclear cell infiltration in the lamina propria has been suggested as the reason APPs increase (Al-Zubaidi, 2015). In our study, serum SAA and Hp concentration were high in the pre-treatment stage, and these values demonstrated a tendency to decrease during treatment, which signalled the cellular response to treatment efficacy.

IL-1β, IL-6 and TNF-α are known to play a key role in APRs (Ceciliani et al., 2012). It has been reported that they initiate the production of APPs by activating and modifying hepatocyte receptors. IL-6 is reported to be the most important of the cytokines that mediate the hepatocytic secretion of APPs (Heinrich et al., 1998). Furthermore, it has been reported that the synthesis of APPs from liver cells is initiated by pro-inflammatory cytokines (TNFα, IL-1β and IL-6) released from monocytes and macrophages during inflammation (Baumann & Gauldie, 1994).

In the presented study, while IL-1β and IL-6 levels were higher in pre- compared to post-treatment ($P<0.05$), TNF-α concentrations were not found to be different before and after treatment in calves (Table 1). In several studies of calves with diarrhea that were diagnosed with enteritis, IL-1β, IL-6 and TNF-α concentrations were found to be statistically significantly higher ($P<0.001$) in comparison to the control group (Albayrak & Kabu, 2016; Kabu et al., 2016). In another study, calves were experimentally administered intravenous endotoxin; following administration, serum IL-1β, IL-6 and TNF-α concentrations were found to be higher in comparison to the control group (Carroll et al., 2009). Other studies have demonstrated that IL-1β and TNF-α concentrations were higher in calves with diarrhea than in control groups (Risalde et al., 2011). Al-Zubaidi, (2015) reported no difference in TNF-α concentrations between calves with Cryptosporidiosis and healthy ones. In our study, whereas serum IL-1β and IL-6 and their concentrations were higher in the pre-treatment stage than the post-treatment stage, TNF-α concentrations did not present a difference. Our study results are consistent with the literature.

WBC and HGB concentrations in calves with Cryptosporidiosis presenting with diarrhea showed statistically significant differences ($P<0.05$) before and after) treatment. RBC and HCT values were lower in the pre-treatment stage than in the post-treatment stage (Table 2). WBC count has been reported to increase significantly in calves with diarrhea, and leukocytosis caused by the relative increase of neutrophil granulocytes occurs as a result of the body's reaction to gastrointestinal infection (Merk Manual, 2013). In our study, the increase in WBC values pre-treatment can be explained as such. In calves with diarrhea, the HCT value would be high due to plasma fluid loss; however, RBC and HGB values may increase relatively. In our study, HGB values were found to be higher in the post-treatment stage than in the pre-treatment stage, and it was concluded that this situation could be associated with excessive fluid loss. Although changes in WBC, RBC, HGB, HCT values were detected in calves with diarrhea due to Cryptosporidiosis, all

hematological findings were within the reference ranges (Aiello, 2016; Brun-Hansen et al., 2006).

CONCLUSION

In light of these findings, it is thought that measuring the concentrations of SAA, Hp, IL-1β and IL-6 routinely would be beneficial for determining infection, for choosing the most appropriate treatment and for monitoring the efficacy of the selected treatment. In addition, it would be useful for detecting animals that show no clinical symptoms and have a subclinical course during herd health screening in terms of veterinary medicine. Further research is needed to determine the SAA, Hp, IL-1β, IL-6 and TNF-α concentrations for the diagnosis of viral, bacterial, parasitic, and other diseases in animals and to better understand the efficiency of this parameter in the control of treatment protocols.

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Effects of propolis-containing nanofibers on corneal wound in rats

ABSTRACT

The cornea is the outermost layer of the eye and is constantly exposed to trauma due to its anatomical location. Propolis is a substance produced by honeybees by mixing the extracts they collect from plants with their secretions. Studies have shown that propolis contains essential biological active substances for the life of organisms, which enhance epithelialization and have strong analgesic, anti-inflammatory, immunomodulatory, antioxidant, antitumor, antibacterial, antifungal, and antiviral effects. In our study, we created a corneal wound with a diameter of 3 mm using a corneal blade. In the experimentally induced corneal wound, no treated the first group, the second group was treated with nanofibers containing propolis produced by the electrospinning method, and the third group treated water-based topical propolis application. Topical propolis was applied once a day for 3 days, while nanofibers containing propolis were applied once following wound formation. Fluorescein staining was performed on the rats eyes every day throughout the study, and photographs were taken to measure the wound sizes. On the third day, the rats were euthanized under general anesthesia, and histopathological examination was performed on their corneas. In terms of bleeding, no significant difference was observed between the propolis and control groups, while a lower level of bleeding was detected in the nanopropolis group. Propolis and nanofibers containing propolis groups showed a significantly positive effect on wound healing compared to the control group.

Keywords: Bee product, experimental, electrospinning, nanopropolis

INTRODUCTION

The cornea is the outermost layer of the eye and is constantly exposed to external factors due to its anatomical location (Ljubimov and Saghizadeh, 2015). Under normal conditions, the cornea is important for maintaining transparency and visual acuity (Khosravimelal et al., 2021). The cornea epithelium, Bowman's layer, corneal stroma, Descemet membrane, and corneal endothelium constitute the five layers of the cornea, with the corneal epithelium being the outermost layer (Nagai et al., 2018). Many corneal injuries involve damage to the epithelial layer (Wilson, 2020).

Normal corneal keratocytes are stable and assist in maintaining corneal transparency (Yi and Zou, 2019). In response to corneal injuries, an healing process occurs, involving the migration of surrounding cells to fill the defect, cell proliferation, cell differentiation, and remodeling. Failure to timely and properly close the defect can lead to corneal ulceration, perforation, or opacification (Wilson, 2020; Nagai et al., 2018).

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Research Article

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Increasing topical lubricants, reducing evaporative tear loss, applying topical antibiotics, shielding the corneal surface with a bandage contact lens, and surgery are all part of the standard treatment for complicated corneal wounds. These measures, however, are frequently ineffective even when taken together (Chandler et al., 2019).

Propolis is a product created by bees through the mixing of plant materials they collect with their own enzymes in order to defend their hives. The word "propolis" is derived from the Greek words "pro" (before) and "polis" (city), referring to its association with hive protection (Forma ve Brys, 2021; Przybyłek and Karpiński, 2019). Bees use propolis for various reasons such as filling gaps in the hive walls, reducing the entrance during cold days, mummifying foreign intruders, and disinfecting the hive (Przybyłek and Karpiński, 2019).

Bees collect resins from buds, exudates, and other parts of plants, mix them with their salivary enzymes and beeswax, and create propolis (Almuhayavi, 2020; Forma ve Brys, 2021; Przybyłek and Karpiński, 2019). The composition of propolis varies depending on the vegetation, season, and climatic zone of the region it is sourced from (Marquez et al., 2023; Przybyłek and Karpiński, 2019). Despite variations in composition, propolis possesses antibacterial, antiviral, antioxidant, anti-inflammatory, immunomodulatory, and antitumor properties (Hossain et al, 2022). Due to these effects, propolis has been used in cases of gastrointestinal disorders, wound healing, dermatitis, corneal wounds, and conjunctivitis (Mele, 2023).

Electrospinning is an electrohydrodynamic process used for the production of polymeric fibers with diameters in the range of a few microns. The properties of polymeric fibers produced by electrospinning can be tailored to various applications, including tissue regeneration and repair in the medical wound (Mele, 2023).

In recent years, various agents have been tried for corneal ulcers, and propolis is one of them. The concept of enhancing the adhesive properties of nanofibers on surfaces through chemical coatings or surface modifications is widely acknowledged. It has been documented in the literature that natural components such as propolis can be combined with nanofibers to create a biologically compatible material. However, nanopropolis obtained through the electrospinning method has never been tested before in corneal wounds. The aim of this study is to investigate the effectiveness of nanopropolis obtained by the electrospinning method in experimentally induced corneal wounds.

MATERIALS AND METHODS

Animals

A total of 24 female Wistar albino rats, with 8 rats in each group, were used in the study. The rats were obtained from the Bursa Uludağ University Experimental Animals Application and Research Center. Throughout the study, the animals were maintained on a 12-hour light-dark cycle and provided with *ad libitum* food and water.

The prepation propolis

The propolis used in the study was collected from an apiary located in the Muğla region through a propolis trap. The collected propolis had a reddish color. All the propolis was first frozen at -20°C and then ground into a powder using a grinder (Lavion grain mill) to achieve a homogeneous consistency. Ultra-pure water was used for the extraction of propolis. 400 g of propolis were mixed with 1 liter of ultra-pure water and left to stand in an orbital shaker for 10 days. During the time it spent in the orbital shaker, it was immersed twice a day in an ultrasonic bath for 30 min each time. The obtained mixture was filtered through Whatman No.1 filter paper to obtain the extract. The extract was sent to the Department of Textile Engineering at Bursa Uludağ University for obtaining the extract tissue.

Electrospinning methods and nanopropolis

In this study, polyethylene oxide (PEO) (Mw: 900 kDa, Sigma Aldrich, USA) was used as the polymer, and propolis was used as the active substance to be added to the polymer solution. No pre-treatment was applied to the materials used in the study. Firstly, a polymer solution was prepared with a weight ratio of 3% PEO in the polymer solution. For this purpose, the PEO polymer was mixed with ethanol and water (1:1) using a magnetic stirrer until completely dissolved. Then, a propolis: ethanol solution was added to the prepared solution, with the propolis ratio in the total solution being 1% of the weight of the PEO solution. The final solution was mixed on a magnetic stirrer for approximately 24 hours to obtain a homogeneous solution.

An electrospinning system from Inovenso was used for the electrospinning process. During electrospinning, the prepared PEO/propolis solution was fed to the system at a rate of 1.5 mL/hour. A voltage of 17 kV was applied to the polymer solution. The distance between the spinneret and the collector was maintained at 15 cm. PEO/propolis nanofibers were collected on a rotating cylinder at a speed of 300 rpm.

Corneal wound model and measurements

The animals were anesthetized with intramuscular injections of 10 mg/kg Xylazine hydrochloride (Rompun®, Bayer, Germany) followed by 70 mg/kg Ketamin hydrochloride (Ketalar®, Parke-Davis, USA). A 3 mm punch biopsy was used to determine the boundaries of the wound to be created under anesthesia. After determining the boundaries, the first two layers of the cornea were removed using a corneal knife. Following the surgery, fluorescein staining was performed daily, and photographs were taken for measurements. The obtained photographs were evaluated using the "ImageJ" (Wayne Rasband National Institutes of Health, USA) program in digital format.

Experimental design

The animals were divided into three groups, with 8 animals in each group. The applications in the groups were performed as follows:

Control Group: No application was performed following wound formation.

Nanopropolis Group: Nanopropolis was applied as a single application following wound formation.

Water-based propolis Group: Water-based propolis was applied one drop (50 µL) once a day for three days following wound formation.

Histopathologic analyses

Three days after creating the corneal wound, euthanasia was performed on the rats under anesthesia by cervical dislocation. Subsequently, enucleation surgery was carried out to collect eye samples. The collected samples were fixed in a 10% formaldehyde solution for histopathological examination. After fixation, the samples were dehydrated through alcohol and xylene series and then transferred to paraffin blocks. The obtained samples were stained with hematoxylin-eosin and examined under a microscope at magnifications of 4x, 20x, and 40x. Based on the examinations, the findings were evaluated as follows: 0; absent, 1; mild, 2; moderate, and 3; severe.

Statistical analysis

All the data was presented as mean ± standard error of mean (SEM). Statistical Package for Social Sciences (SPSS) version 22.0 for Windows (SPSS Inc., Chicago, IL) was used to compare data across groups using one-way Analysis of Variance (ANOVA) and post hoc Tukey honestly significant difference (HSD) tests were conducted. The histopathology results were evaluated using the non-parametric Kruskal-Wallis test. Pairwise comparisons for non-normally distributed variables were performed using the Mann-Whitney U test and evaluated

with Bonferroni correction. P values equal or less than 0.05 were considered statistically significant.

RESULTS

Corneal wound sizes

The eye samples, which were stained daily with fluorescein and photographed, were transferred to digital format. Subsequently, using the ImageJ (Wayne Rasband National Institutes of Health, USA) program, the boundaries of the area with dye retention were determined, and the area calculations (mm^2) were performed. The results are shown in Table 1.

Table 1. Fluorescein staining results 0, 24, 48, and 72 hours after wound formation

Time	Control	Nanopropolis	Propolis	P value
0.hours	10.054±0.242	9.827±0.126	9.962±0.203	0.716
24.hours	5.154±0.213[a]	1.074±0.196[c]	2.403±0.24[b]	<0.001
48.hours	1.653±0.201[a]	0±0[b]	0±0[b]	<0.001
72.hours	0±0	0±0	0±0	-

[a-c]: Different letters in the same line are statistically significant (P<0.001). The values are given in mm^2(square millimeters)

When the average healing areas at 24, 48, and 72 hours were calculated among the groups, in the control group, at the end of 24 hours, 49% of the total wound area had healed, 82% had healed at 48 hours, and 100% had healed at 72 hours. In the nano-propolis group, 89% of the total wound area had healed at 24 hours, and 100% had healed at 48 hours. In the propolis group, 75% of the total wound area had healed at 24 hours, and 100% had healed at 48 hours (Figure 1).

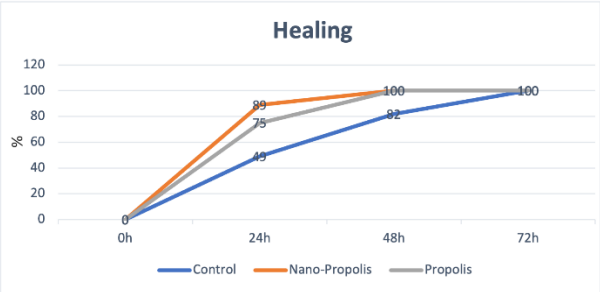

Figure 1. Percentages of corneal improvement at 0, 24, 48, and 72 hours.

The obtained values were subjected to an ANOVA test, and the statistical analysis revealed that there was no significant difference among the groups at 0 hours (P>0.05). At 24 hours, a significant difference was observed among the three groups, with the nano-propolis group showing the best healing (P<0.001). At 48 hours, there was no significant difference between the nano-propolis and propolis groups, but a significant difference was observed between the control group and the study groups (P<0.001). Since all corneas had healed by 72 hours, no statistical analysis could be performed (Table 1). After creating corneal wounds, the healing process was monitored for 72 hours and recorded in a digital format. When fluorescein staining was applied to the recorded photographs, the wounded areas were visually observed (Figure 2).

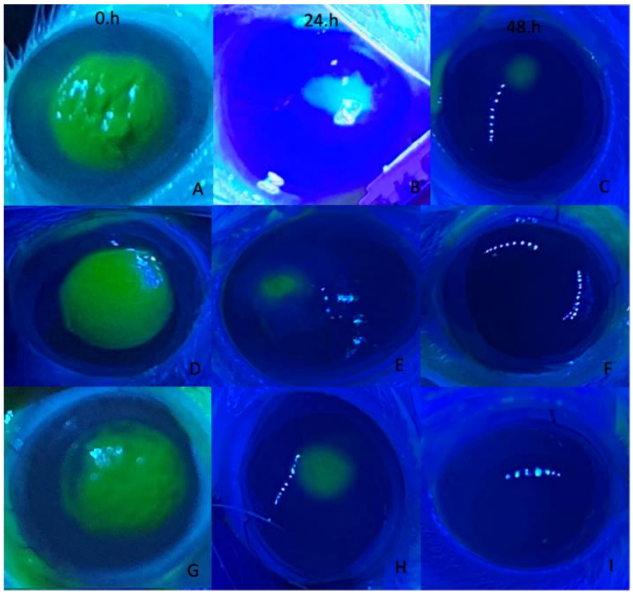

Figure 2. The digital images of the wounded areas. A: 0-hour control group. B: 24-hour control group. C: 48-hour control group. D: 0-hour nano-propolis group. E: 24-hour nano-propolis group. F: 48-hour nano-propolis group. G: 0-hour propolis group. H: 24-hour propolis group. I: 48-hour propolis group.

Histopathologic results

The findings were determined to be concentrated in the regions close to the epithelial layer. Figure 3 (A) illustrates the area of ulceration in the epithelial layer. Figure 3 (B) demonstrates cell infiltration and corneal edema in the region near the epithelial layer.

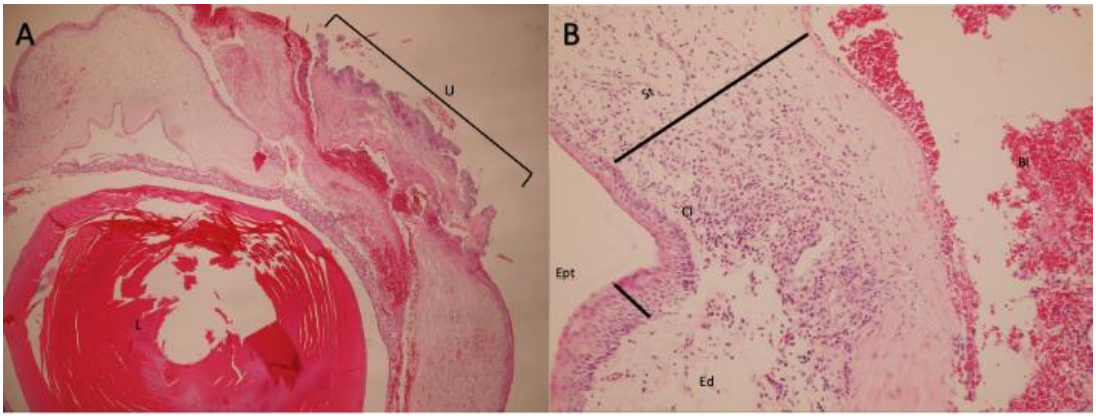

Figure 3. Histopathological images. A: Observation of ulcerated area in the control group under 4x magnification. (L: Lens, U: Ulceration). B: Figure of the propolis group under 20x magnification. (St: Stroma, Bl: Bleeding, Ed: Edema, Ept: Epithelium, Cl: Cell infiltration).

Moderate corneal edema was observed in all groups, but there was no histopathologically significant difference between the groups (P>0.05). The groups were evaluated for bleeding, and varying degrees of findings ranging from moderate to severe were observed in the control and propolis group, while the nano-propolis group showed a significantly lower occurrence (P<0.05). The groups were assessed for vascularization and cell infiltration, and findings ranging from mild to moderate were observed in all groups, with no significant difference between the groups (P>0.05). Furthermore, the groups were evaluated for ulceration, and mild ulceration was observed in all groups, with no significant difference between the groups (P>0.05). The results are shown in Table 2.

Table 2. Histopathological examination results. The findings were evaluated as follows: 0; absent, 1; mild, 2; moderate, and 3; severe.

Findings	Control	Nanopropolis	Propolis	P value
Corneal Edema	2.000±0.327	1.750±0,313	1.500±0,327	0.598
Bleeding	2.125±0.295^{b}	1.125±0.295^{a}	2.375±0.263^{b}	0.024
Vascularization	2.000±0.267	1.750±0.250	1.875±0.350	0.777
Cell infiltration	2.125±0.226	1.625±0.323	1.875±0.295	0.512
Ulceration	0.625±0.182	0.250±0.163	0.500±0.188	0.324

$^{a-b}$: Different letters in the same line are statistically significant (P<0.05).

DISCUSSION

The transparent cornea serves as the eye's frontal layer, refracting incoming light for vision and shielding the underlying ocular tissues from harm. It is crucial to have a well-coordinated corneal wound healing response after injury to avoid loss of transparency and subsequently vision as well as to keep the barrier of protection in place (Reins et al., 2016). The complex process of healing a corneal wound includes the

migration and division of epithelial cells, the death of stromal keratocytes, the regeneration of nerve tissue, and a localized inflammatory response that results in the infiltration of immune cells and the extravasation of platelets from limbal vessels (Nagai et al., 2018). In this study, we investigate the healing effect of nanopropolis on corneal wounds in rat.

Propolis is a well-known resinous material collected by bees from bud and exudates of the plants, mixed with bee enzymes, pollen and wax (Zullkiflee et al., 2022). Propolis can be used in many medical fields. Propolis has been extensively used by several civilizations to treat colds, wounds and ulcers because of its antiseptic and local anaesthetic properties (Sforcin, 2016). Propolis contains a wide variety of phenolic compounds, typically phenolic acids and flavonoids with biological effects. Several pharmacological effects have been attributed to propolis, especially ethanolic propolis extracts, such as antibacterial, antioxidant, antiviral, fungicidal, anti-inflammatory, anticarcinogenic, antiapoptotic, immunomodulatory and gastric protective (antiulcer) effects (Oruç et al., 2017). In veterinary medicine, propolis is reportedly active against fungal otitis and dermatomycosis in dogs (Cruz Sánchez et al., 2014), and may also use for treatment of bovine dermatophytosis (Çam et al., 2009). Propolis extract has been shown to have anti-inflammatory effects in the treatment of canine anal sacculitis (Durgun and Durmuş, 2004). Propolis appears to have a positive impact on the healing of wounds (Yang et al., 2022). More frequent dressing changes would have improved the antimicrobial and wound healing effects. Similar to these findings, the current study's findings demonstrated that propolis in the healing of burn wounds due to its antioxidant, anti-inflammatory, and antimicrobial properties. The wound healing effect of propolis is attributed to its contained aminoacids, phenolic acids, phenolic acids esters, flavanoids, cinnamic acid, terpens and caffeic acid (Kasote et al., 2022). According to our results propolis and nanoprolis showed an improvement corneal wound, for the first 2 days after the injury. However, since the composition of propolis was not examined in this study, it is unclear which specific propolis characteristic led to this improvement.

Re-epithelialization of the rat cornea takes roughly 72 hours after debridement of a 3 mm diameter circular area, leading to complete covering of the injured area with basal epithelial cells (Nagai et al., 2018). Before proliferating to help with re-stratification, epithelial cells must first migrate to cover the wound during re-epithelialization. Cell division is inhibited in the wound area right away to facilitate effective migration (Terai et al., 2011). When the average healing areas over the course of 24, 48, and 72 hours were calculated for each group, the control group had a wound that was 49% healed after 24 hours, 82% healed after 48 hours, and 100% healed after 72 hours. At 24 hours, 89% of the total wound area in the nanopropolis group had healed, and at 48 hours, 100% had. In the propolis group, 100% of the wound area had healed after 48 hours, and 75% of the overall wound area had.

In a study conducted, topically applied propolis has been reported to reduce cell infiltration at 24 and 48 hours (Martin et al., 2013). In our study, at the end of 72 hours, there was no significant difference among the groups regarding cell infiltration. Based on the results of our study, we believe that longer term studies in terms of vascularization should be conducted. Currently, there is no existing study on the use of nanopropolis in the treatment of corneal wounds; therefore, histopathological findings take precedence.

The use of nanofiber structures in wound healing has gained popularity in recent times. The aim is to accelerate healing by incorporating known substances that promote healing into the production of nanofiber structures (Cavalu et al., 2019). Morais and colleagues (2022) reported in

their study that collagen nanofibers obtained through the electrospinning method accelerated wound healing. Shi and colleagues (2023) also conducted a study where they produced nanofibers using hydrogel not propolis through the electrospinning method and used them in corneal wounds (Morais et al., 2022; Shi et al., 2023). In their study, they found that edema and inflammation in the corneal stroma significantly decreased. They discovered that nanofibers with hydrogel components accelerated corneal healing in acute alkali wounds. It is speculated that the use of nanofiber structures in corneal wounds will increase in the current era. One advantage of using these nanofibers is that it eliminates the difficulty of continuous application and provides successful results with a single application.

Nanopropolis has emerged as an expanding field with a wide range of applications and effectiveness, similar to propolis. Nanopropolis has become a subject of study in the field of antibacterial, antifungal, and antitumoral properties (Tatli Seven et al., 2018).

CONCLUSION

In our study, nanopropolis prepared using the Electrospinning method was tested for corneal wounds. As a result of our study, it has been concluded that the application of nanopropolis in superficial corneal wounds as a single administration would be beneficial. However, further research is needed in this field. Especially for faster and uncomplicated wound healing, advanced studies are needed to use nanofibers containing propolis in different bases, and to compare these bases, with the aim of particularly investigating their efficacy.

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

Ethical statement: The study was conducted with the approval of the Bursa Uludağ University Animal Experiments Local Ethics Committee under permit number: 2019-06/03.

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Sonorous plunge of serum magnesium levels among pre-diabetic and diabetic cats

Research Article

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ABSTRACT

Diabetes mellitus is a frequently occurring endocrine disorder in felines, and the impact of magnesium deficiency on the incidence of diabetes and related complications has garnered significant interest in the realm of human research. Our objective was to investigate the levels of magnesium present in the serum of cats with pre-diabetes and diabetes. Although magnesium levels in pre-diabetic and healthy cats did not exhibit a significant statistical difference, a notable contrast was evident in diabetic cats in this study. Specifically, diabetic cats exhibited considerably lower magnesium levels in comparison to non-diabetic felines. In conclusion it should not be unwise to draw preliminary conclusion that magnesium levels should have helped better understanding molecular insight and consequences in both pre-diabetic and diabetic cats. If necessary, as evidenced by hypomagnesemia also at this study, dietary supplementation should be considered.

Keywords: Diabetes mellitus, feline, hypomagnesemia, insulin

INTRODUCTION

Diabetes is in general practice, frequently been detected among cats even if clinical signs are existed (O'Neill et al., 2016). Diagnostic criteria for prediabetic status among cats have not been explored. On the other hand, as aforementioned previously (Gottlieb and Rand, 2018) hence, there are no longitudinal research searching for nondiabetic cats with elevated glucose concentrations, cats experiencing diabetic remission through lightly elevated glucose levels (7.5 to <9 mmol/L or >135 to <162 mg/dL) are under high risk of switching to diabetes within 270 days (Gottlieb et al., 2015). Thus, so far detection of probable prediabetic and subclinical diabetic conditions among cats should be of beneficial for suitable measurements for delaying/preventing clinical diabetes (Gottlieb and Rand, 2018). For better understanding of readers Table 1 showed useful glossary for feline diabetes, adopted from well-reviewed literature data by Gottlieb and Rand (2018).

To the best of the current authors' knowledge, there has been no documented scientific investigation conducted thus far that has examined and compared the occurrence and prevalence of hypomagnesemia among healthy cats, as well as those with diabetes and pre-diabetes. For this purpose, we aimed to determine the serum magnesium (Mg) levels among pre-diabetic and diabetic cats.

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Table 1. Useful glossary for feline diabetes. Adopted from well-reviewed literature data by Gottlieb and Rand (2018).

Factors	Glucose levels	Caution
Fasted blood glucose concentration*	~3.0–6.5 mmol/L (117 mg/dL)	By use of portable glucose meter (calibrated for feline blood)
Monitorization of blood glucose	Upper limit for cut point of 166 mg/dL (9.2 mmol/L),	Denoting probable influence of stress on diagnosing diabetes among cats (Reeve-Johnson et al., 2012)
Acute stress	-	-
Struggling	Might be responsible for elevated glucose levels on average by 74 mg/dL (4.1 mmol/L) and up to 195 mg/dL (10.8 mmol/L) even in 10 minutes	Due to elevated lactate and norepinephrine concentrations (Rand et al., 2002)
Cats ≥8 years of age	Presenting an initial blood glucose >117 mg/dL (6.5 mmol/L) should be subjected to retesting 4 hours later	If not <117 mg/dL (6.5 mmol/L) could thus be subjected to retesting after 24 hours (Gootlieb and Rand, 2018; Rand et al., 2002)

*Withdrawal of food for 18–24 hours (Gootlieb et al., 2015; Reeve-Johnson et al., 2013).

MATERIALS AND METHODS

Animals

A total of 20 cats from different breeds, ages, and both genders were enrolled. The diseased cats, comprising the subjects of this research, were chosen from among those presenting at the small animal clinics of the faculty that had received a routine diagnosis of diabetes. In contrast, the healthy cats were selected from individuals brought to the clinic for general health check-up or vaccination, having been deemed healthy based on clinical and laboratory assessments. Written consent was obtained from the owners of both the afflicted and healthy group animals for their participation in the study.

Classification to groups were healthy cats (n=10) and diseased cats with pre-diabetes (n=8) and diabetes mellitus (n=10). Inclusion criteria was partially shown in Table 1. Whether if blood glucose was >9.8 mmol/L, the cat was diagnosed as diabetic. Whether if blood glucose was between 6.5 to 9.8 mmol/L the case was considered pre-diabetic (Reeve-Johnson et al., 2016). Exclusion criteria includes diabetic ketoacidosis, thyroidal illness, renal failure or liver failure that may have interfere with blood glucose levels.

Laboratory analysis

Following an 18/24-h fast, sera samples were withdrawn gently from either ear/paw for blood glucose measurement via a portable glucose meter.

Magnesium levels were measured using the xylidyl blue colorimetric method in an autoanalyzer (Randox Daytona Plus®, Randox Laboratories Ltd, UK), utilizing serum samples obtained after blood collection.

Statistical analysis

Descriptive statistics were conducted on the levels of magnesium in healthy, pre-diabetic, and diabetic cats, and the data were presented in the form of mean and standard error. For comparisons between groups, the non-parametric test technique Kruskal-Wallis-H was used. Dunn's test were used for post hoc comparisons. Cases where the obtained p-values were less than 0.05 were considered statistically significant. All analyses were performed using the GraphPad Prism® 9.5.1 software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

All cats enrolled herein (at pre-diabetes group) existed a pre-diabetic state with a fairly elevated blood glucose levels, reaching up to 200 mg/dL

(11 mmol/L), but show no symptoms of the disease. All of them were not requiring an insulin supportive therapy, whether we suggested modification of nutrition [i.e. low carbohydrate diet (17%), low glycemic home prepared functional foods with lifestyle changes.

Magnesium levels in prediabetic and healthy cats did not change statistically, but they were shown to be substantially different in diabetic cats. Also, it was shown that diabetic cats had much lower Mg levels than non-diabetic cats, as shown in Table 2 and Figure 1 below.

Tablo 2. Mean Mg (mg/dL) concentrations among cats enrolled at this study

Parameter	Healthy cats (n=10)	Pre-diabetic cats (n=8)	Diabetic cats (n=10)
Mg (mg/dL)	2.11±0.15[a]	1.73±0.13[a]	0.90±0.16[b]
P value	0.002		

[a-b]: Values indicated by different letters on the same line are statistically significantly different.

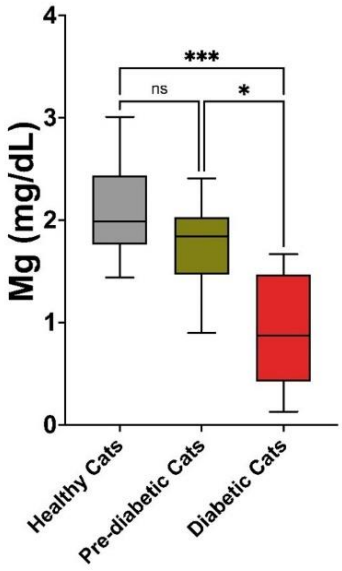

Figure 1. Boxplot analytes of magnesium median (min-max.) Mg (mg/dL) levels among healthy, pre-diabetic and diabetic cats. ns: Non-significant, *: p<0.05, ***: p<0.01.

DISCUSSION

In this study, we aimed to determine the serum Mg levels of pre-diabetic and diabetic cats. In this context we observed that the levels of magnesium in diabetic cats were less than those observed in both healthy and prediabetic cats. Beginning with 1940s, it has been well recognized that type 2 diabetes mellitus has been related to hypomagnesemia (Martin and Wertman, 1947; Pham et al., 2007). Diminished serum magnesium (Mg^{2+}) levels in relationship with type 2 diabetes mellitus in cohort studies have been explored (Chaudhary et al., 2010). In human being type 2 diabetes mellitus, the prevalence of hypomagnesemia varied between 14 and 48% in comparison to 2.5-15% detected among healthy controls (Pham et al., 2007). To the present authors knowledge herein, there has been no reported research detecting the incidence/prevalence of hypomagnesemia in diabetic, pre-diabetic and healthy cats in comparison up to date. The results that we obtained at this study, therefore, would highlight this era of endocrinology. On the other hand, a previous study reported magnesium status in cats with diabetes mellitus (n=21) and diabetic ketoacidosis (n=7), in comparison to 12 healthy cats (controls), 21 cats with DM, and 7 cats with DKA. Cats with diabetes mellitus (n=2 vs 13) and diabetic ketoacidosis (n=1 vs. 4) presented serum total Mg and ionized Mg lower than the reference ranges, in which in which hypomagnesemia was reported as a common finding (Norris et al., 1999). In the present study considering available results mean Mg (mg/dL) values among healthy cats vs. pre-diabetic and diabetic cats were 2.11, 1.73 and 0.90, respectively. Furthermore, diabetic cats presented statistically lower Mg values in contrast to both pre-diabetic and healthy control cats, (p=0.0002) (Figure 1 and Table 2).

Hypomagnesemia in relationship with a severely quick, diminishing renal function in type 2 diabetes mellitus patients (Pham et al., 2005), through a contrary correlation among oral Mg^{2+} intake and diabetes mellitus risk (Dong et al., 2011). Mg^{2+} supplementation on glucose metabolism and insulin sensitivity (Guerrero-

Romero and Rodríguez-Morán, 2014; Guerrero-Romero et al., 2015; Rodríguez-Morán and Guerrero-Romero, 2003) has been well reported.

Given endocrine/metabolic disease conditions in relationship with Mg deficiency, diabetes mellitus is quite common. Several research dedicated that mean plasma concentrations are diminished both in type 1 and type 2 diabetes in comparison to nondiabetic controls along with marked negative correlations between Mg and fasting plasma glucose (Kim et al., 2010; Sales and Pedrosa, 2006). Several different causes of hypomagnesemia among diabetics comprised i) dietary intake of low magnesium (Schulze et al., 2007), ii) osmotic diuresis related to elevated renal excretion of magnesium, iii) imperceptive relation with insulin influencing intracellular magnesium transportation and thereof resulting with elevated extracellular Mg loss (Paolisso et al., 1986), iv) uncontrollable usage of loop/thiazide diuretics resulting with Mg wasting, v) diabetic autonomic neuropathies (Pham et al., 2007) and diminished tubular reabsorption because of insulin resistance (Barbagallo and Dominguez, 2007). Herein at the present study we did not investigate underlying etiology, other than diabetes control, however there were no usage of diuretics, nor osmotic diuresis. Although we did not evaluate low dietary intake of Mg, as all cats were fed with different commercially available foods, enabling us to investigate such kind of variety, this might be a causing factor especially among pre-diabetic cats herein involved. On the other hand, probable insulin resistance might participate as a co-factor among both diabetic and pre-diabetic cats herein comprised.

CONCLUSION

In conclusion, taking into account data obtained at this research, it should not be unwise to draw preliminary conclusion that magnesium levels should have helped better understanding molecular insight and consequences in both pre-diabetic and diabetic cats. This is because Mg has a pivotal role for utilization and transportation of carbohydrates whereas if necessary, as evidenced by hypomagnesemia also at this study, dietary supplementation should be considered.

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Ethical statement: In the Regulation on the Working Procedure and Principles of the Animal Experiments Ethics Committee published in the newspaper numbered 28914 dated 15.02.2014, in the second clause's sub-clause b, it is clearly stated that there is no need for ethics committee approval for non-experimental clinical veterinary practices. In this study, since a commercial product presented as a feed additive and not considered as a drug was used both for health and for the control of the disease picture, and additionally, an informed owner consent form was obtained, there was no need for ethics committee approval.

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Synergistic gastroprotective and antioxidative effects of natural olive oil and usnic acid isolated from *Usnea longissima*, a lichen species in Anatolia (Türkiye), in the indomethacin ulcer model created in rats

ABSTRACT

Usnea longissima, a medically important lichen growing up in forests in Anatolia (Türkiye). In this study, the gastroprotective effect of usnic acid (UA) was investigated using an indomethacin (IND)-induced gastric ulcer model in rats. While 25, 50, 100 and 200 mg/kg UA doses were dissolved in 2 ml of olive oil (OO) and administered to rats, only OO was given to one group. In addition, lansoprazole (LAN) and ranitidine (RAN) and IND were dissolved in water and administered to rat groups. IND administration caused very high levels of damage to rat stomachs. On the other hand, when four doses of UA, OO, RAN and LAN were administered, it was determined that hyperaemia's in the stomach of rats was significantly reduced. After macroscopic analysis of gastric tissues, the activities of superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO) and nitric oxide synthase (iNOS and cNOS) enzymes as well as glutathione (GSH) and lipid peroxidation (LPO) levels were determined in these tissues. After IND application, it was detected increases in MPO, CAT and iNOS activities in gastric tissues and decreases in SOD, cNOS and GSH amounts. Four doses of UA, OO, RAN and LAN applications reversed the trend, bringing them closer to healthy levels.

Keywords: Indomethacin, iNOS, gastroprotective effect, myeloperoxidase, usnic acid, olive oil

INTRODUCTION

The ulcer is a condition that results in damage to the gastric mucosa, caused by various factors such as anti-inflammatory drugs, stress, alcohol, *Helicobacter pylori*, etc. (Dejban et al., 2020). Factors such as inhibition of bicarbonate secretion, decrease in gastric blood flow, loss of protection of gastric mucosal cells and stress play an important role in the pathogenesis of ulcer, which consists of various factors (Atalay et al., 2015). Two cyclooxygenase enzymes (COX-1 and COX-2) converts arachidonic acid into prostanoids in definite ratios. Non steroid anti-inflammatory drugs (NSAIDs) used to reduce inflammation, pain and fever inhibit COX enzymes and ultimately prostaglandin synthesis is inhibited (Atalay et al., 2015; Atalay et al., 2016; Dejban et al., 2020; Odabasoglu et al., 2008). Generally, the imbalance between the defence mechanism of the gastric mucosal cells and the factors causing toxicity causes the formation of this condition. One of the main aggressive factors is the NSAIDs. The use of NSAIDs is increasing due to diseases that occur throughout the life period. The risk of peptic ulcer complications is four times higher in NSAID users (Lanas et al., 2015).

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Research Article

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The indomethacin-induced ulcer model in the rat is a widely used experimental model to investigate the gastric protective potential of various agents (Ahmed et al., 2021; Berktas et al., 2021; Halici et al., 2011; Karakus et al., 2009). The ulcer induced by oxidative stress, inflammation and especially NSAIDs; Today, it has become widely used in therapy thanks to newly discovered molecules. Therefore, these molecules can be an alternative to drugs used mainly in therapy (Bi et al., 2014). Many lichen species and molecules isolated from them have been shown to have a pronounced gastric protective effect. On the other hand, there are records that olive oil provides gastric protection (Odabasoglu et al., 2008). Lichens are symbiotic organisms that live in ecosystems ranging from mountains to deserts and consist of a fungus, an algae, and/or a cyanobacterial partner. Some lichen species have been traditionally used to treat various diseases. In some societies, it is also consumed as food. On the other hand, lichens are also widely used in the manufacture of perfumes and paints (Atalay et al., 2016; Halici et al., 2005; Huneck, 1999; Odabasoglu, 2001). The fungus forms a thallus or lichenified stroma, which may contain characteristic secondary metabolites in all lichens (Atalay et al., 2015; Ahmadjian, 1993; Bayir et al., 2006; Odabasoglu et al., 2006). It has been reported that secondary metabolites isolated from lichens exhibit a wide variety of biological activities, including gastroprotective, antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative, proapoptotic, anticarsinogenic and cytotoxic effects (Atalay et al., 2015; Halici et al., 2006; Huneck, 1999; Odabasoglu et al., 2006, 2012, 2019; Solárová et al., 2020; Suleyman et al., 2003; Yingshu et al., 2021).

Usnea longissima usually lives on trees (epiphytic) and has a hanging thallus. This species of lichen is very sensitive to air pollution. *Usnea longissima* is widely used in folk medicine of different countries. It has also been noted in some studies that this lichen species is used in ulcers, expectoration, wound healing, and skin disorders (Ahmadjian, 1993; Brij, 1995; Halici et al., 2005; Odabasoglu et al., 2019; Yazici and Aslan, 2003). In addition, it was reported that *Usnea longissima* extracts have gastric protective effects and antioxidant properties (Halici et al., 2005).

Usnic acid, one of the most common lichen metabolites; It is a dibenzofuran derivative molecule and has been identified in many lichen genera such as Usnea, Cladonia, Hypotrachyna, Lecanora and Evernia. The characteristics of this lichen species have been recorded in many literatures (such as antiviral, antibiotic, antiprotozoal, antitumoral, anti-inflammatory, antipyretic, analgesic…) (Odabasoglu et al., 2006, 2019; Solárová et al., 2020; Yingshu et al., 2021). In addition, Odabasoglu et al (2006) noted that UA has gastroprotective and antioxidative effects (Odabasoglu et al., 2006).

The OO is consumed more in Mediterranean countries. Its use, which has become widespread in our country in recent years, has been supported by some studies on cancer and heart diseases. It has also been found to be protective against various diseases such as skin diseases, autoimmune disorders, ulcers and cholelithiasis. (Miraj et al., 2020). In addition, Odabasoglu et al. (2012) demonstrated a pro-apoptotic effect of olive oil in peri-implant tissues of Ti-implanted rabbit (Odabasoglu et al., 2012), and its gastroprotective effect in IND-induced rat' stomach (Odabasoglu et al., 2008).

However, since there was no study evaluating usnic acid in combination with olive oil, its gastroprotective effect was also discussed in this study. The effect was expressed through biochemical enzymes.

MATERIALS AND METHODS

Plant material

Usnea longissima Ach. was collected from the Giresun region (northern Anatolia) of Turkey and identified by Dr. Ali Aslan. Voucher specimen (KKEF-374) has been deposited in the herbarium of Kazım Karabekir Education Faculty, Ataturk University, Erzurum-Türkiye.

General analytical procedures

The chemical products used in the experiments were obtained from Sigma. Olive oil is bought from any grocery store. Column chromatography and thin layer chromatography were performed. UV-visible spectra and biochemical analyses were recorded by spectrophotometer.

Extraction of Usnea longissima and isolation of usnic acid (UA)

The dried Usnea lichen species was extracted and subjected to column chromatography after filtration and washing with solvent system. The pure usnic acid obtained was tested. Its chemical structure was determined by UV, IR, 1HNMRand 13CNMRmethods and also confirmed by comparing previously reported spectral data (Huneck and Yoshimura, 1996). The spectral information of UA: m.p. 194 0C; IR (KBr) υ cm-1: 2920, 1690, 1630, 1530, 1450, 1370, 1350, 1280, 1190, 1140, 1110, 1060, 1030, 960, 830, 810; 1H and 13C NMR data (see Table 1).

Table 1. ^{1}H and ^{13}C-NMR spectral data of Usnic acid in $CDCl_3$[a]

Position	δ_H (ppm)	δ_C (ppm)
1	-	157.2
2	-	107.2
3	-	159.4
4	-	111.2
5	-	165.8
6	-	103.4
1'	-	193.7
2'	596	100.2
3'	-	181.3
4'	-	61.1
5'	-	200.0
6'	-	105.9
4-Me	2.07	9.5
5-OH	11	-
6-COMe	-	202.2
6-COMe	2.65	33.2
4'-Me	1.74	34.1
6'-COMe	-	203.7
6'-COMe	2.65	29.8

[a] Chemical shift (δ) in ppm relative to TMS

Animals

Experiments were carried out with a total of 54 male Albino-Wistar rats weighing 180-200 g. The rats were obtained from Atatürk University, Faculty of Medicine, Department of Pharmacology and Atatürk University-Experimental Animal Teaching and Researcher Center Laboratory (ATADEM). Experiments were performed in accordance with ethical norms approved by the Ethical Committee of the Center for Experimental Animal Education and Research Center (No: 36643897-47). Animals were grouped and kept under standard conditions prior to experiments.

Indomethacin-induced gastric damages

Experimental animals were divided into nine groups. There are six animals in each group. All groups were fasted for 24 hours before being included in the experiment, and the experimental animals were treated appropriately. Experimental groups were compared with FAM and RAN groups. (Table 2-Experimental groups).

Table 2. Experimental treatments, each consisting of 6 rats

Groups	Treatment	Dose/kg body weight
Grup I	IND + UA + OO	25 mg + 25 mg + 2 ml
Grup II	IND + UA + OO	25 mg + 50 mg + 2 ml
Grup III	IND + UA + OO	25 mg + 100 mg + 2 ml
Grup IV	IND + UA + OO	25 mg + 200 mg + 2 ml
Grup V	OO	2 ml
Grup VI (Positive control)	IND + RAN	25 mg + 50 mg
Grup VII (Positive control)	IND + LAN	25 mg + 50 mg
Grup VIII (Negative control)	IND	50 mg
Grup IX (Healthy)	Healthy	-

Olive oil and olive oil and usnic acid were given orally to the animals at the indicated doses. After 10 minutes, IND was given orally to all animals. The healthy group received no treatment. At the end of the treatments, the animals were sacrificed with high-dose anesthesia and their stomachs were removed. Ulcer areas were evaluated macroscopically with millimetric paper. The protective effects were determined biochemically (Berktas et al., 2021; Suleyman et al., 2003).

Biochemical inspection of stomach tissues

After macroscopic analysis of gastric tissues, the activities of SOD, CAT, MPO and NOS enzymes as well as GSH and LPO levels were determined in these tissues. Stomach tissues were ground in a mortar with liquid nitrogen to prepare tissue homogenates. Approximately 0.5 g was weighed for each group and 4.5 ml of the appropriate buffer was then added. After mixing at low speed with the aid of a vortex, this mixture was homogenized for 20 minutes using an ultra-turrax homogenizer. All procedures were performed on ice. The homogenates were filtered and centrifuged at 4°C using a refrigerator centrifuge. All measurements were performed at room temperature using these supernatants.

Biochemical estimations

Superoxide dismutase activity

Superoxide dismutase enzyme activity in gastric tissues was determined according to the appropriate method (Sun et al., 1988). The degree of inhibition of this reaction was measured at 560 nm, and SOD activity was expressed as millimole per minute per milligram tissue (mmol/min/mg tissue).

Catalase activity

Catalase enzyme activity was determined according to the appropriate method and the decrease in absorbance was expressed as millimoles / minute (mmol/min/mg tissue) / milligram tissue (Aebi, 1984).

Total glutathione determination

The amount of glutathione in the stomach tissues was measured according to the method determined in the literature. It was measured spectrophotometrically at 412 nm after treatment with appropriate homogenates. The level of GSH in each sample was expressed as nanomoles per

milligram of tissue (nmol/mg tissue) (Sedlak and Lindsay, 1968).

Determination of lipid peroxidation

Lipid peroxidation levels were determined by estimating malondialdehyde (MDA) using the thiobarbituric acid test. Tissues treated with appropriate solvents were measured spectrophotometrically at 532 nm. Results are expressed as nmol MDA per gram tissue (nmol/g tissue) (Ohkawa et al., 1979).

Myeloperoxidase activity

Myeloperoxidase enzyme activity was determined according to the Bradley method. Changes in absorbance were recorded spectrophotometrically at 450 nm. MPO activity of tissues was expressed as µmol/min/mg tissue (Bradley et al., 1982).

Nitric oxide synthase activity

Nitric oxide synthase activity in tissues was determined according to the appropriate method. The absorption difference between 401 and 421 nm wavelengths was monitored spectrophotometrically. iNOS activity was calculated by subtracting cNOS activity from total NOS activity (Knowles et al., 1990).

Statistical analyses

Statistical calculations were made using the SPSS 22.0 program. The results are expressed as the mean ± Standard Deviation (SD). Firstly, to see whether all data are normally distributed or not, they were analysed by Kolmogorov-Smirnov tests and then the differences in variance were analysed statistically using a one-way analysis of variance (ANOVA) test. Differences between groups were reached using the Duncan option, and significance was reported at $p<0.05$.

RESULTS

Usnic acid (UA) was isolated from the diethyl ether extract obtained from the lichen *Usnea longissima*. The structure of usnic acid (Figure 1) was characterized by IR, MS, 1H- and 13C-NMR (Table 1-spectral data) and 2D-NMR spectroscopic methods and also confirmed by comparison of previously reported spectral data.

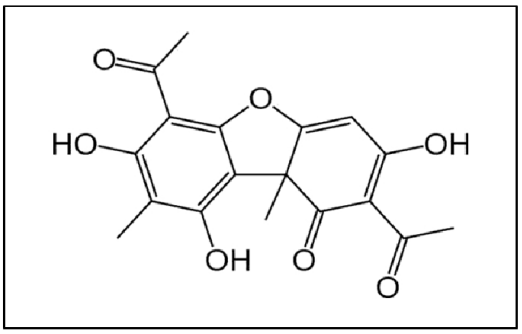

Figure 1. Structure of usnic acid.

The IND-induced gastric ulcer model is a useful and highly preferred experimental model to induce severe ulceration in rats. In this study, the IND-induced ulcer model was preferred to create an ulcer model in rats. 25, 50, 100 and 200 mg/kg UA doses dissolved in 2 ml olive oil and OO alone were administered to the experimental groups (Table 2- experimental groups). Positive controls (RAN and LAN) were dissolved in water and administered to rat groups. One group was given 25 mg/kg dose of IND dissolved in water (negative control). The resulting damages were measured in mm^2 with the help of millimetric paper. According to the results presented in Table 3 and Figure 2, administration of IND caused a very high level of damage in rat stomachs. On the other hand, it was determined that hyperaemia's were significantly reduced in stomachs of rats treated with four doses of UA, RAN, LAN and OO.

The doses of 25, 50, 100 and 200 mg/kg of UA prepared by dissolving in OO reduced ulcer areas by more than 95% compared to the IND group ($p<0.05$). UA at a dose of 100 mg/kg completely inhibited gastric lesions (Table 3 and Figure 2). On the other hand, OO, LAN and RAN reduced their mean damage areas by 70.3%, 92.9% and 72.7%, respectively. These results demonstrated that all doses of UA, LAN (50 mg/kg), RAN (50 mg/kg), and pure OO had significant ($p<0.05$) gastric protective effects against IND-induced gastric injury. While OO

prevented the formation of gastric ulcer by 70.3% when administered alone, this effect was found to be much higher when applied together with UA doses (25, 50, 100 and 200 mg/kg). These research results reveal that OO and UA have a synergistically effect when applied together. This synergistic effect is quite high compared to positive controls (LAN and RAN).

Table 3. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on damages in rat's indomethacin (IND)-induced gastric tissues

Treatment	N	Dose (mg/kg body weight)	Ulcer area (mm^2)[§]	% Inhibition[β]
IND + UA + OO	6	25 + 25 + 2 mL	0.63±0.01[d]	98.0
	6	25 + 50 + 2 mL	0.50±0.01[c]	98.4
	6	25 + 100 + 2 mL	0.00±0.00[a]	100.0
	6	25 + 200 + 2 mL	0.25±0.01[b]	99.2
IND + OO	6	25 + 2 mL	9.13±0.01[g]	70.3
IND + RAN	6	25 + 50	8.38±0.05[f]	72.7
IND + LAN	6	25 + 50	2.20±0.1[e]	92.9
IND	6	25	30.75±0.0 [h]	-
HEALTHY	6	-	0.00±0.00a	-

N: the number of rats. [§]Mean damage area±S.E.M. of six animals in each group. [B] % Inhibition in ulcer area in relation to indomethacin group. Indomethacin group was statistically compared with untreated groups. Other treated groups were statistically compared with indomethacin group. Means in the same column by the same letter are not significantly different to the Duncan test (=0.05). Results are means ± SE of three measurements [a-h]: statistical difference in the same column.

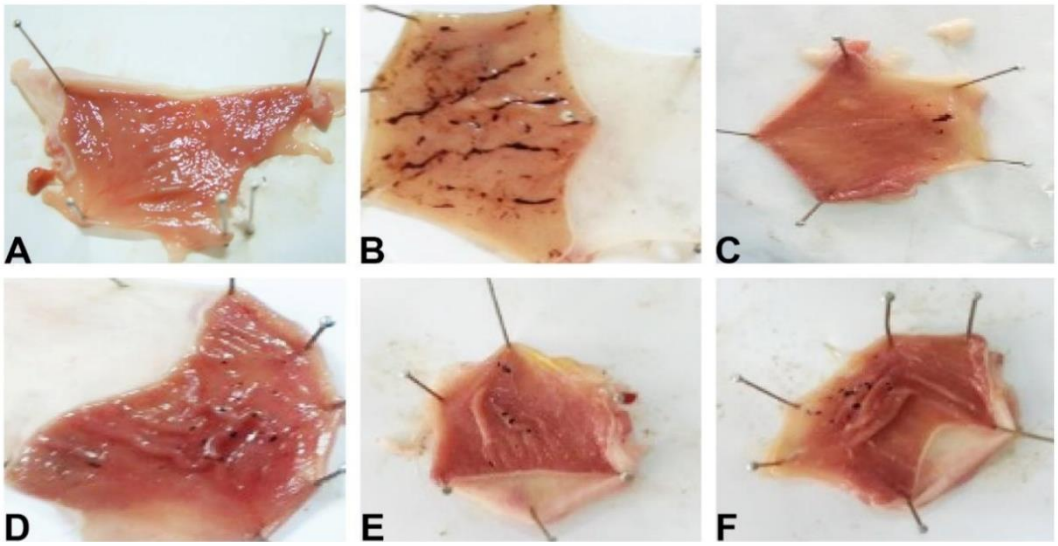

Figure 2. Ulcerous areas in indomethacin (IND)-induced rat's gastric tissues of orally administrated usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN). Sections of the gastric tissues after administrations were obtained from some experimental groups. The **A**-**F** sections show some ulcerative areas: **A**, untreated group (healthy); **B**, the negative control group (IND, 25 mg/kg body wt.); **C**, IND-administrated plus OO (2 ml) + UA (200 mg/kg body wt.) group; **D**, IND-administrated plus OO (2 ml) group; **E**, IND-administrated plus LAN (50 mg/kg body wt.) group (positive control 1); **F**, IND-administrated plus RAN (50 mg/kg body wt.) group (positive control 2).

The enzyme activities in rat tissues were measured to determine their effects on antioxidant defence systems. The amounts of LPO and GSH in determining the synergistic effect of OO and UA are expressed in Table 4. Contrary to the applied IND, it appears to be almost as protective as positive controls. Likewise, MPO, SOD and CAT enzyme activities are also expressed in the Figures (Figure 3-4-5). Compared to healthy tissues, a significant protection was determined by the enzyme activities reduced by the application of IND ($p<0.05$).

Table 4. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on changes in levels of lipid peroxidation (LPO) and total glutathione (GSH) in rat's indomethacin (IND)-induced gastric tissues

Treatment	N	Dose (mg/kg body weight)	Amount of LPO (nmol/g tissue)	Amount of GSH (nmol/g tissue)
IND+UA+OO	6	25 + 25 + 2 mL	13.54±0.34^{d}	2.99±0.01^{b}
	6	25 + 50 + 2 mL	11.77±0.02^{b}	3.23±0.01^{e}
	6	25 + 100 + 2 mL	7.37±0.01^{a}	3.46±0.01f
	6	25 + 200 + 2 mL	7.31±0.02^{a}	3.48±0.01^{f,g}
IND+OO	6	25 + 2 ml	14.15±0.04^{e}	3.10±0.01^{c}
IND+RAN	6	25 + 50	12.00±0.01^{b,c}	3.10±0.01^{c}
IND+LAN	6	25 + 50	18.40±0.10^{f}	3.15±0.01^{d}
IND	6	25	35.09±0.40^{g}	2.10±0.01^{a}
HEALTHY	6	-	12.42±0.01^{c}	3.50±0.01^{g}

Means in the same column by the same letter are not significantly different to the Duncan test (P<0.05). Results are means ± SE of three measurements. N: The number of rats. $^{a-g}$: statistical difference in the same column.

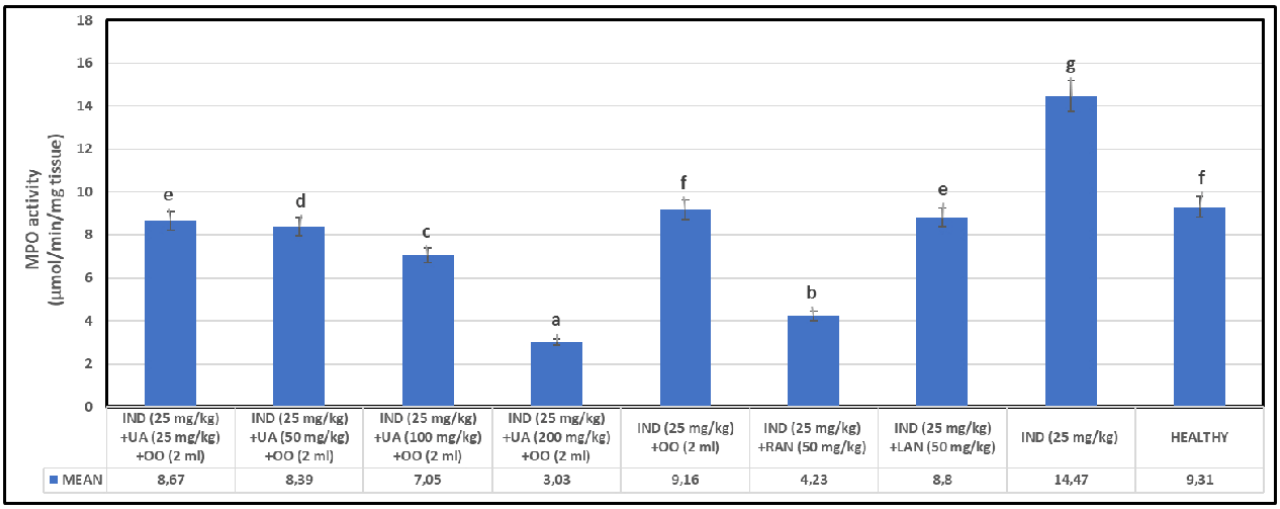

Figure 3. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on changes in enzymatic activity of catalase (CAT) in rat's indomethacin (IND)-induced gastric tissues. Means in the same column by the same letter are not significantly different to the Duncan test (P<0.05). Results are means ± SE of three measurements. N: The number of rats.

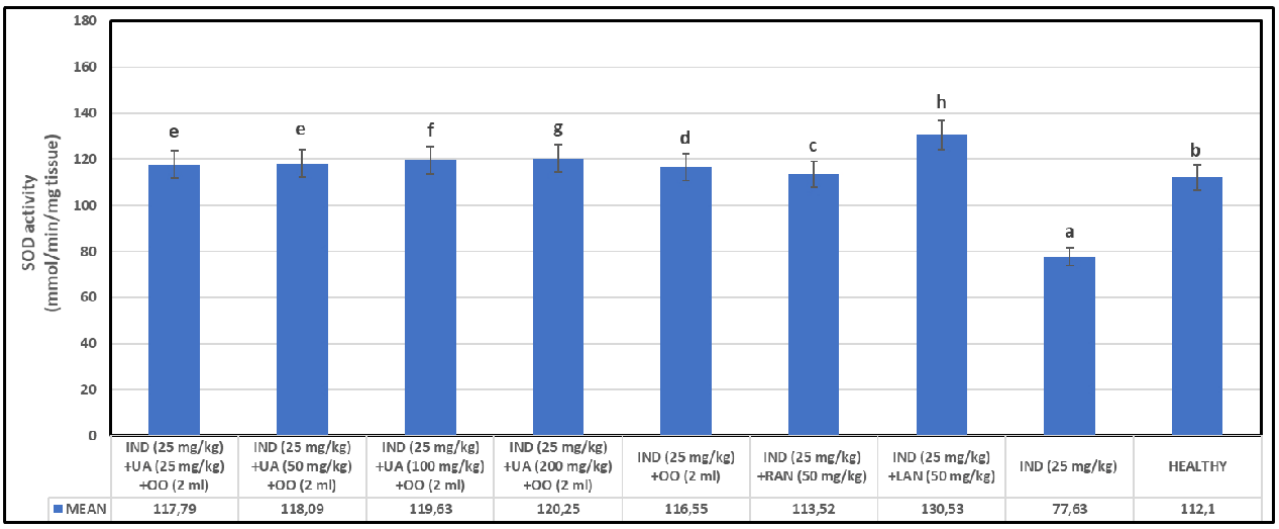

Figure 4. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on changes in enzymatic activity of superoxide dismutase (SOD) in rat's indomethacin (IND)-induced gastric tissues. Means in the same column by the same letter are not significantly different to the Duncan test (P<0.05). Results are means ± SE of three measurements. N: The number of rats.

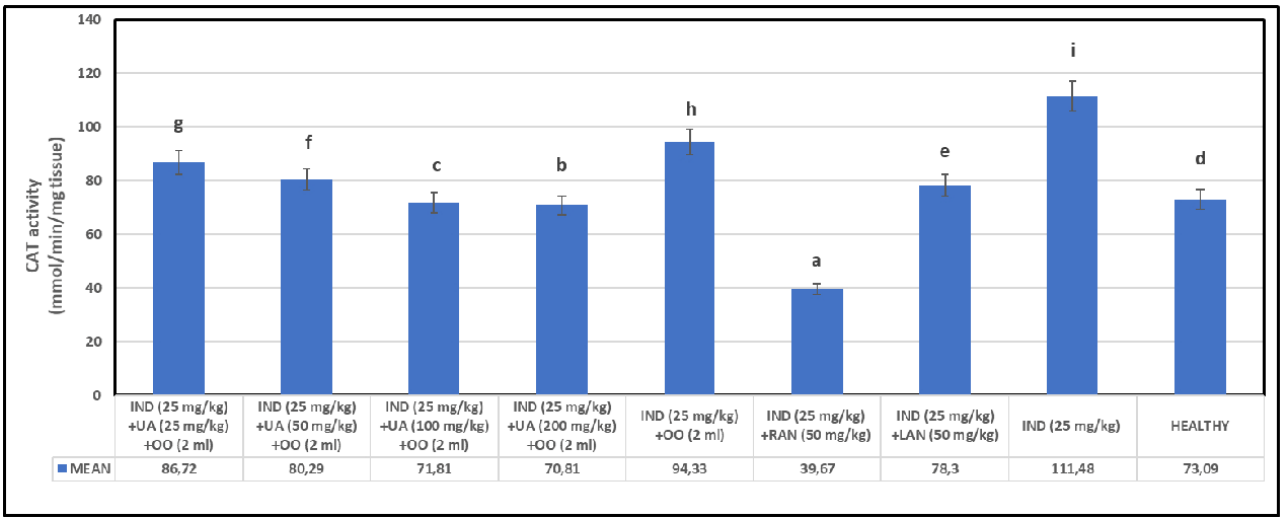

Figure 5. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on changes in enzymatic activity of myeloperoxidase (MPO) in rat's indomethacin (IND)-induced gastric tissues. Means in the same column by the same letter are not significantly different to the Duncan test (P<0.05). Results are means ± SE of three measurements. N: The number of rats.

In addition, levels of nitric oxide produced from the amino acid arginine, which is an important part of the antioxidant enzyme system, were also determined. cNOS, iNOS and tNOS activities produced in three different structures are shown in Table 5.

Table 5. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on changes in activities of inducible-nitric oxide synthase (iNOS), constitutive-nitric oxide synthase (cNOS) and total-nitric oxide synthase (tNOS) in rat's indomethacin (IND)-induced gastric tissue

Treatment	N	Dose (mg/kg body weight)	cNOS (nmol/min/mg tissue)	iNOS (nmol/min/mg tissue)	tNOS (nmol/min/mg tissue)
IND+UA+OO	6	25 + 100 + 2 mL	2.4±0.001^{e}	0.29±0.010^{b}	2.64±0.01^{d}
IND+OO	6	25 + 2 mL	1.96±0.003^{d}	0.41±0.003^{c}	2.36±0.01^{c}
IND+RAN	6	25 + 50	1.73±0.001^{b}	0.50±0.010^{d}	2.23±0.01^{b}
IND+LAN	6	25 + 50	1.84±0.003^{c}	0.50±0.003^{d}	2.32±0.01^{c}
IND	6	25	1.13±0.001^{a}	0.63±0.010^{e}	1.77±0.01^{a}
HEALTHY	6	-	2.41±0.001^{f}	0.26±0.010^{a}	2.68±0.01^{d}

Means in the same column by the same letter are not significantly different to the Duncan test (P<0.05). Results are means ± SE of three measurements. N: The number of rats. $^{a\text{-}f}$: statistical difference in the same column.

DISCUSSION

The protective effects of usnic acid in the formation of gastric ulcers have been noted in *H. pylori* ulcers (Safak et al., 2009) and IND-induced gastric lesions in rats (Odabasoglu et al., 2006). On the other hand, it has been reported in the literature that OO has protective effects against gastric damage (De la Lastra et al., 2001; Odabasoglu et al., 2008). All data obtained from the current study are consistent with the data in the literature. The results from the present study include data on the "synergistic gastro protective effect" between OO and UA, which will be presented for the first time in the literature.

The role of many factors (COX enzymes, TNF alpha, interleukins etc) in the pathogenesis of gastric mucosal damage caused by indomethacin, ethanol and other agents has been demonstrated. In recent years, besides the functions of these factors, attention has been drawn to the roles of reactive oxygen species (ROS) and antioxidants in the formation of IND

induced-stomach injury (Atalay et al., 2016; Dejban et al., 2020; Odabasoglu et al., 2006).

The present results (Figure 3-MPO enzyme activity) showed that administration of IND significantly increased the activities of myeloperoxidase enzyme (MPO) in gastric tissues ($p<0.05$). In addition, in the present experiments, it was determined that the activity of MPO in gastric tissues increased by the IND was reduced to the levels of the healthy control group after treatment with all UA doses, OO, LAN and RAN ($p<0.05$). The increase in activity of MPO as a result of IND administration may indicate that it is a marker of increased level of neutrophil infiltration into gastric damaged tissues. Similar results have been reported by various researchers (Atalay et al., 2016; Dejban et al., 2020; Nishida et al., 1998; Odabasoglu et al., 2006, 2008,).

The neutrophils and phagocytose cells are the most specialized cells of the immune system. Their activation and infiltration into tissues leads to inflammation and formation of lesions. MPO is a hem peroxidase enzyme stored in neutrophil granules. MPO activity increases, indicates the accumulation of neutrophils in tissues. Gastric ulcers and lesions that occur after treatment with IND and similar NSAIDs are a form of inflammation. MPO enzyme can be considered as control mechanisms for neutrophil infiltration into gastric mucosal tissues (Coskun et al., 1996; Dejban et al., 2020; Takeuchi et al., 1998; Wei et al., 2021). When there is an increase in the level of neutrophil infiltration into gastric damaged tissues, an increase in these enzyme activities is also observed. The peroxidases activated in damaged tissues increase the production of superoxides. Increasing superoxide trigger lipid peroxidation in membranes. As a result, the membranes are damaged and ulcers occur in the stomach tissues (Atalay et al., 2016; Kuzmanova et al., 2019; Odabasoglu et al., 2006; Ogaly et al., 2021; Tahir et al., 2022; Takeuchi et al., 1998, Valcheva et al 2019).

In the present study, lipid peroxidation levels (LPO) in gastric tissues were determined for all treatment groups on the ulcer formation process. Compared with the healthy rat group, it was determined that IND administration increased LPO levels ($p<0.05$). Contrary to IND, it was determined that all doses of UA, OO, LAN and RAN treatments decreased LPO levels ($p<0.05$). These results are in agreement with the literature (Atalay et al., 2016; Kuzmanova et al., 2019; Odabasoglu et al., 2006; Ogaly et al., 2021; Takeuchi et al., 1998; Valcheva et al., 2009). The results obtained from the present study indicate that the activated MPO enzyme in gastric damaged tissues increases the level of lipid peroxidation via superoxides. In the light of these data, it can be said that the excessive level of LPO causes damage to the gastric tissue membranes and ulcers occur in the stomach tissues.

After the ulcer formation process, enzymatic (SOD and CAT activities) and non-enzymatic (GSH amounts) antioxidant levels were determined in gastric tissues for all treatment groups. It was determined that IND application decreased SOD activities and GSH amounts, and increased CAT activities ($p<0.05$). Contrary to IND, it was determined that all doses of UA, OO, LAN and RAN treatments increased SOD and GSH levels while decreasing CAT amounts ($p<0.05$).

In previous literature, it has been reported that organisms have antioxidative defense systems against lipid peroxidation caused by ROS in cell membranes. These antioxidative systems can be enzymatic (such as SOD and CAT enzyme activities) or nonenzymatic (such as the amount of GSH) (Anvar et al., 2021; Atalay et al., 2016; Berktas et al., 2021; Bozkurt et al., 2017; Hanci et al., 2018; Odabasoglu et al., 2006; Ogaly et al., 2021; Takeuchi et al., 1998; Valcheva-Kuzmanova et al., 2019). In many previous studies, it has been shown that the administration of IND, an NSAID, reduces the amount of GSH and SOD activities in the stomach tissues (Anvar

et al., 2021; Atalay et al., 2016; Berktas et al., 2021; Bozkurt et al., 2017, Hanci et al., 2018, Ogaly et al., 2021; Valcheva-Kuzmanova et al., 2019). In contrast, administration of all doses of UA, OO, LAN and RAN resulted in a significant increase in SOD activities and GSH levels ($p<0.05$).

The amount of GSH is very important for the dissociation of thiol groups. Regular support of radicals and maintenance of this situation will be of vital importance for the cell. The current publication results reveal that all doses of UA, OO, LAN, and RAN increased the amount of GSH in the stomach of the rat. In previous studies, it has been reported that UA, OO, LAN and RAN have a protective effect by reducing oxidative stress and preventing intracellular GSH depletion against IND-induced gastric ulcer. Our findings are in agreement with previously recorded results. Previously reported studies have shown that both UA and OO stimulate both enzymatic and non-enzymatic antioxidant defence systems and have the potential to be used as a natural antioxidant. This also is true for our positive controls, LAN and RAN.

It has been reported that SOD activity in rat stomach tissues is inhibited when NSAIDs such as IND are used (Anvar et al., 2021; Berktas et al., 2021; Karakus et al., 2009, Ogaly et al., 2021; Valcheva-Kuzmanova et al., 2019). Our results are consistent with these reports and confirm previous results. The SOD enzyme plays an important role in preventing gastric damage by partially preventing oxidative damage caused by radicals. SOD enzyme converts the reactive superoxide radical ($O2^{-\bullet}$) to less reactive hydrogen peroxide (H2O2•). Formed H_2O_2 is used as substrate by CAT and MPO enzymes. In the present study, it was found that SOD activity was reduced by IND treatment. On the other hand, it was observed that inhibited SOD activity was activated to healthy tissue levels by UA, OO, LAN and RAN (Figure 4). These findings indicate that increased SOD activity is very important in gastric protection.

According to the results obtained in this study, CAT activity was found to be increased in IND-treated rat tissues compared to healthy rat tissues (Figure 5). On the other hand, all treatments reduced the trend to CAT levels in healthy tissue. Our findings are in agreement with the results reported in previous studies (Anvar et al., 2021; Atalay et al., 2016; Berktas et al., 2021; Bozkurt et al., 2017; Hanci et al., 2018; Ogaly et al., 2021; Valcheva-Kuzmanova et al., 2019).

The current research findings indicate that ROS production increases due to the elevated MPO activity in gastric tissues following IND application, and increased ROS initiate lipid peroxidation (LPO) and ultimately ulcers are formed. In other words, ROS formation and increased LPO level play a key role in the development of gastric mucosal lesions caused by IND. In parallel with the increase in ROS, the levels of H_2O_2 and OH• in the stomach tissues also increase. In response, the MPO and CAT enzymes increase and try to reduce the damage by consuming H_2O_2. After treatment with OO, LAN, RAN and all UA doses, H_2O_2 and all other ROS levels are reduced. In other words, the activities of MPO and CAT enzymes, which use H_2O_2 as a substrate, also decrease to healthy tissue levels.

It shows that indomethacin administration causes inhibition of cytoprotective NOS (cNOS) activities in gastric tissues, while also leading to significant activation of inducible NOS (iNOS) activities ($p<0.05$). In addition, in the current experiments, it was determined that after treatment with all UA doses, OO, LAN and RAN, increased iNOS and decreased cNOS activities with IND were brought to the levels of the healthy control group ($p<0.05$). In addition, in the current experiments, it was determined

that increased iNOS and decreased cNOS activities with IND after treatment with all UA doses, OO, LAN and RAN were brought to the levels of the healthy control group ($p<0.05$). Similar results have been reported by various researchers (Dejban et al., 2020; Nishida et al., 1998; Odabasoglu et al., 2006).

The nitric oxide (NO) is a biologically molecule with very important tasks. It is produced from the amino acid L-arginine by the nitric oxide synthase (NOS) enzyme. There are three isoforms of NOS: cytoprotective NOS (cNOS) produced in endothelial and wholebody tissues, inducible NOS (iNOS) produced by phagocytes and leukocytes and neuronal NOS (nNOS) produced in neuronal tissues. Many reports have been recorded that NO has a very important role in the whole organism, as well as a protective role against gastric erosions and ulcers. Rojas-Martínez et al (2013) reported that NO modulates the healing process of gastric ulcer. According to them, NO maintains the blood flow of the stomach as a vasodilator. On the other hand, when NO production is insufficient, it can lead to gastric mucosal damage, erosions and ulcers (Konturek et al., 1993). For these reasons, the amount of NO produced by cNOS in organisms must be stable. The organisms need extra NO in the inflammatory process and in case of tissue damage. Thus, it is tried to keep the NO amount stable by increasing the iNOS activity. In this case, it tells us that there has been a trauma or damage (Dejban et al., 2020).

It has also been widely accepted that NO produced by cNOS in digestive systems is cytoprotective and NO produced by iNOS is cytotoxic (Nishida et al., 1998; Odabasoglu et al., 2006). As one of the remarkable results of the present study, it was determined that cytotoxic-inducible iNOS activity was activated while cytoprotective-cNOS activity was inhibited by the IND ($p<0.05$). On the other hand, it was measured that all UA doses, OO, LAN and RAN treatments brought cNOS and iNOS activities closer to the healthy group level (Table 5-iNOS, cNOS, tNOS levels). According to the available data, the increase in the amount of LPO due to the increase in the activity of the MPO enzyme following the application of IND indicates membrane damage. Increases in MPO, CAT and iNOS activities and decreases in SOD, GSH and cNOS amounts seem to be effective in further increasing the level of damage in gastric tissues.

According to the results of the present experimental study, it can be said that UA and OO can restore and strengthen non-enzymatic and enzymatic antioxidative defence systems due to their ability to reduce lipid peroxidation. This ability may be the source of the gastroprotective effects of both UA and OO. Moreover, when UA and OO are applied together, these abilities increase synergistically. Taking these properties into account, drug preparations containing UA and OO together can be developed.

CONCLUSION

In conclusion, biochemical damages caused by IND in gastric tissues were approximated or eliminated to healthy groups with all UA doses, OO, LAN and RAN treatments. Among all these positive effects, the most striking thing is that UA showed a synergistic effect when applied together with OO.

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Conflict of interest: The authors have no commercial interest, financial interest and/or other relationship with manufacturers of drugs, laboratory supplies and/or medical devices, or commercial providers of medically related services.

Ethical statement: The rats were obteined from Atatürk University, Faculty of Medicine, Department of Pharmacology and Atatürk University-Experimental Animal Teaching and Researcher Center Laboratory (ATADEM). Experiments were performed in accordance with ethical norms

approved by the Ethical Committee of the Center for Experimental Animal Education and Research Center (No: 36643897-47).

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Clinical success of clarithromycin, amoxicillin-clavulanic acid, enrofloxacin and doxycycline in dogs with infectious tracheobronchitis

ABSTRACT

Canine infectious tracheobronchitis (ITB) is a highly contagious disease of dogs expressed with remarkable respiratory signs. Therapy with antibiotics in canine ITB still remains questionable. The purpose of this study was to compare the clinical success of clarithromycin, amoxicillin-clavulanic acid, enrofloxacin and doxycycline in the treatment of canine ITB. Client-owned dogs with canine ITB signs (n=60) were enrolled in this prospective, controlled, and randomized blinded clinical trial. A computer-generated list randomization was employed to assign the dogs equally into the Group CLA: clarithromycin (25 mg/kg, PO), the Group AMX: amoxicillin and clavulanic acid (25 mg/kg, PO), the Group ENR: enrofloxacin (2.5 mg/kg, PO), and the Group DOX: doxycycline (5 mg/kg, PO). The administration of CLA alleviated the cough sign earlier than DOX. The recovery time of oculonasal discharge in group CLA were also earlier compared to the other groups. The tracheal sensitivity also disappeared earlier in the Groups CLA and AMX. In conclusion although there is no always statistically significance between groups, clarithromycin appears to be superior to other antibiotics suggesting that it can be the first antibiotic choice to alleviate the ITB signs in dogs.

Keywords: Antibiotics, dog, infection, tracheobronchitis

INTRODUCTION

Canine infectious tracheobronchitis (ITB) is a highly contagious disease, associated with the respiratory signs, in dogs (Ford, 2012; Hawkins, 2009). In recent literatures, the disease is called as canine respiratory disease complex including multifactorial etiological pathogens such as viruses (adenovirus 2, parainfluenza, coronavirus, influenza, distemper, coronavirus, herpes and reovirus) and bacteria (Mycoplasma spp and Bordetella bronchiseptica) (Hawkins, 2009, Reagan et al., 2020), alone or in combination (Ford, 2012; Kaczorek et al., 2016; Priestnall et al., 2014). Severe clinical signs of the disease can be especially associated with coinfections secondary to multiple pathogens or bacterial agents. While adult dogs may overcome the disease, puppies and young dogs have more severe clinical signs (Hawkins, 2009). Acute honking cough elicited on gently tracheal palpation in canine ITB is remarkable. When the presence of multifactorial pathogens oculonasal discharges also accompany the coughing (Petersen and Kutzler, 2012). Infectious tracheobronchitis is generally self-limiting and outpatient treatment is usually recommended. However, ITB can progress to bronchopneumonia when the immune system is compromised (Hawkings, 2009; Petersen and Kutzler, 2012).

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Research Article

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Antimicrobiotherapy for canine ITB is controversial (Reagan et al., 2020; Köse et al., 2022). Although some reports state that antimicrobials fail to resolve the disease (Hawkings, 2009) several antibiotics including doxycycline, chloramphenicol, enrofloxacine, trimethoprim/sulpha and amoxicillin/clavulanate are often empirically prescribed (Ford, 2012). Empiric antibiotherapy should be based on the most likely agent to be present (Lappin, 2017). Studies cultured the etiological pathogens in dogs with ITB revealed often Bordetella spp. This controlled clinical study was conducted to evaluate clinical responses to various antibiotics in dogs with ITB.

MATERIALS AND METHODS

Study population

Data were collected from 69 dogs including Terrier type (n = 7), German Shepherd (n = 9), Golden Retriever (n = 9), Kangal (n = 4), Cocker (n = 2), Rotweiler (n = 9), Boxer (n = 5) and mixed breed (n = 24). Nine dogs were excluded from the study because of previous antibiotic therapy (Rotweiler, n = 2; mixed breed, n = 2) and concurrent systemic disease including diarrhea and cardiomyopathy (German Shepherd, n = 4; Boxer, n = 1). A total of 60 dogs continued on the study. All dogs had received essential antiparasitic therapy and routine vaccination against infections such as rabies, distemper, hepatitis, parvoviral enteritis and leptospirosis. They were fully vaccinated against these infectious agents every year. The dogs were not in contact with each other and only one dog resided in each household. The dogs did not receive any medication at the time of referral and during diagnostic applications. The inclusion criteria were the presence of ITB signs including spontaneous cough, oculonasal discharge, and retching induced by gentle palpation of the laryngeal and tracheal regions (Ford, 1995; Ford, 1998; Thrusfield et al., 1991). Dogs on antibiotic therapy within 7 days and have exhibited the signs of concurrent systemic disease in their medical history, during clinical examination, or based on their complete blood count were excluded from the study. Written owner consent was also obtained in the study.

Study design

In this prospective randomized trial, all dogs were examined by a standardized protocol including clinical examination (fever, appetite, weight, mucosal color, capillary refill time, lung and heart auscultation, lymph nodes, abdominal palpation), blood analysis (complete blood count and, serum profiles including urea, creatinine, total bilirubin, albumin, alanine aminotranspherase, aspartat aminotranspherase, alkaline phosphatase, gama glutamil transpherase and creatine kinase using Exigo Eos Vet Hematology Analyzer USA and Erba XL 600 Biochemical Analyzer, Russia) , and thoracic radiography (VD/LL). Blood samples were collected from the cephalic vein into EDTA tubes. Complete laryngopharyngeal diagnostic workup using a video endoscope (Video endoscopy system, Eickemeyer, Germany) was also performed in all dogs. Using a computer-generated list randomization (Microsoft Excell, 2016), dogs were divided into 4 treatment groups: Group CLA (n = 15): clarithromycin (25 mg/kg, PO), Group AMX (n = 15): amoxicillin and clavulanic acid (25 mg/kg, PO), Group ENR (n = 15): enrofloxacin (2.5 mg/kg, PO) and Group DOX (n = 15): doxycycline (5 mg/kg, PO). All prescribed therapies are administered twice daily for one week. Alleviation in clinical signs (cough, oculonasal discharge and tracheal sensitivity) was evaluated daily by a physician who was blind to the treatments. Termination of oculonasal discharge and tracheal sensitivity confirmed upon physical examination. Termination of cough was also based on the owner's information.

Statistical analysis

Kaplan Meier survival analysis was used to calculate mean recovery times and life curves. To

test the difference between the life curves, Breslow test was used. Pairwise comparisons were done using Wilcoxon test. P<0.05 was considered as significant in all analyses. All statistical analysis were performed by using SPSS 14.01 (SPSS Inc., Chicago) package programme for Windows.

RESULTS

The mean age of the dogs enrolled in the study was 3.03 ± 0.13. The mean age as well as gender and breed distributions did not vary among the groups. The cough recovery times for dogs in Group CLA, ENR, AMX and DOX were 3.73 ± 0.48 d, 4.33 ± 0.39 d, 4.60 ± 0.45 d and 5.66 ± 0.37 d, respectively (Table 1, Figure 1).

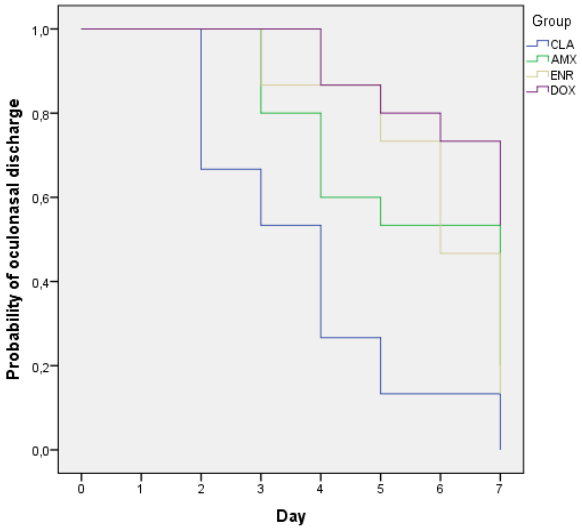

Figure 1. Probability of coughing after treatment of each antibiotics.

Table 1. Means for recovery time of coughing

Groups	Mean			
	Day	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
CLA [a]	3.733	0.485	2.783	4.684
AMX [ab]	4.600	0.450	3.718	5.482
ENR [a]	4.333	0.396	3.557	5.110
DOX [b]	5.667	0.373	4.935	6.398

[a,b]: Different lower cases indicate statistical differences (Wilcoxon test). (Kaplan-Meier, Breslow test, P=0.032) Significance levels are P<0.05. DOX: Doxycycline, CLA: Clarithromycin; AMX: Amoxicillin clavulanic acid; ENR: Enrofloxacin.

The recovery times of oculonasal discharge and tracheal sensitivity were also shown in Table 2 and Table 3 (Figure 2, Figure 3), respectively.

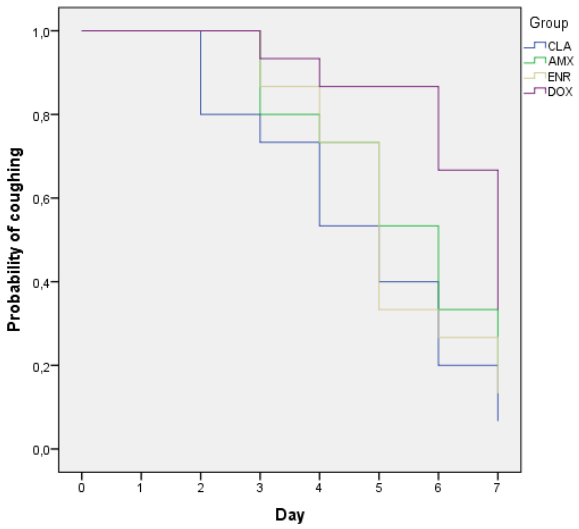

Figure 2. Probability of oculonasal discharge after treatment of each antibiotics.

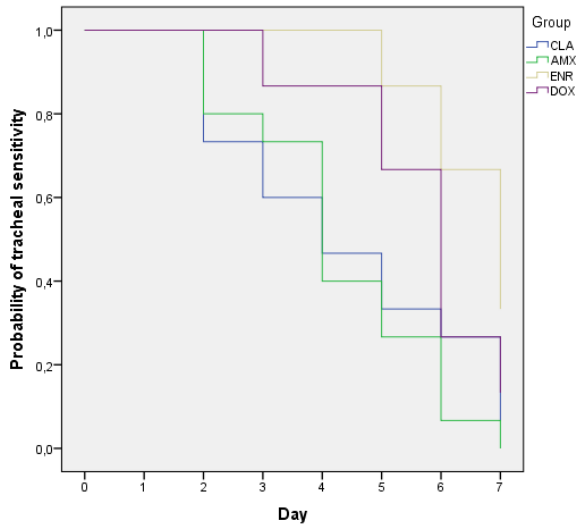

Figure 3. Probability of tracheal sensitivity after treatment of each antibiotics.

DISCUSSION

Affected dogs with ITB have the clinical signs of oculonasal discharge and paroxysmal cough induced by gentle palpation of the tracheal region.

Although ITB in adult dogs considered as self-limiting and non-systemic disease, it can progress to more severe systemic clinical signs including weight loss, persistent anorexia, fever, diarrhea, retinitis or seizures in puppies and immunocompromised dogs (Hawkins, 2009; Nelson and Couto, 2009). Dogs with only non-systemic clinical signs of ITB and normal reference ranges in complete blood counts enrolled in the study. They had been immunized against the infections of rabies, distemper, hepatitis, parvoviral enteritis and leptospirosis. The diagnosis of ITB in dogs is based on typically clinical signs and the history of the exposure to other dogs (Ford, 2012; Petersen and Kutzler, 2012). Although it has been reported that additional diagnostic applications including thoracic radiography and culture of the airway fluids are not indicated, remarkable and necessary in uncomplicated cases (Ettinger and Kantrowitz, 2012; Petersen and Kutzler, 2012), possible role of congenital respiratory problems were also ruled out by the thoracic radiography in this study. The culture of the airway fluids has not been performed. The usage of antimicrobials in dogs with ITB is still questionable. While some literatures (Hawkins, 2009) regarding the usage of antibiotics have described the resolving of the ITB spontaneously regardless of any specific treatment implemented, some of them (Ford, 2012) have justified the usage of empiric antimicrobial therapy before the complication by overt bacterial pneumonia in dogs with ITB. In various studies considering the antimicrobial sensitivities of *Bordetella bronchiseptica*, isolates have been reported as susceptible to tetracycline, DOX, AMX, and ENR (Angus et al., 1997; Speakman et al., 2000). However, the usage of CLA to alleviate the clinical signs in dogs with ITB have not been previously defined. The purpose of the current study was to compare the effectiveness of CLA, AMX, ENR and DOX in treatment of canine ITB. CLA is a broad-spectrum macrolide using in respiratory tract infections in human medicine (Evangelos et al., 2008; Langtry and Brogden, 1997). It is also safe and suitable for therapeutic use in dogs (Vilmgnyi et al., 1995). In this study CLA was well tolerated and no side effects were noted in dogs with ITB as well.

Table 2. Means for recovery time of oculonasal discharge

Groups	Mean			
	Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
CLA [a]	2.733	0.441	1.868	3.599
AMX [b]	4.667	0.496	3.695	5.639
ENR [bc]	5.067	0.383	4.317	5.817
DOX [c]	5.933	0.371	5.207	6.660

[a,b,c]**:** Different lower cases indicate statistical differences. (Wilcoxon test). (Kaplan-Meier, Breslow test, P<0.001), DOX: Doxycycline, CLA: Clarithromycin; AMX: Amoxicillin clavulanic acid; ENR: Enrofloxacin.

Table 3. Means for recovery time of tracheal sensitivity

Grup	Mean			
	Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
CLA [a]	3.467	0.532	2.424	4.510
AMX [a]	3.267	0.408	2.467	4.066
ENR [c]	5.867	0.264	5.348	6.385
DOX [b]	4.800	0.368	4.080	5.520

[a,b,c]**:** Different lower cases indicate statistical differences. (Wilcoxon test). (Kaplan-Meier, Breslow test, P<0.001), DOX: Doxycycline, CLA: Clarithromycin; AMX: Amoxicillin clavulanic acid; ENR: Enrofloxacin.

If fever, lethargy and anorexia without clinical evidence of pneumonia are present, the working group about the antimicrobial use guidelines for treatment of canine ITB recommends administration of DOX for 7-10 d as the first-line antibiotic (Lappin et al., 2017). Although it is difficult to compare this study with the present one, contrary to suggestions of working group (Lappin et al., 2017) cough recovery time at the end of the treatment was earlier in group CLA compared to other groups. Oculonasal discharge recovery time was also very late in group DOX at the end of the treatment. The same working group has also recommended the usage of AMX to be alternate first-line antibiotic in failure of DOX. Compared to group CLA, cough and oculonasal discharge recovery times were later at the end of the treatment in groups AMX. Working group (Hawkins, 2009; Lappin et al., 2017) has not also recommended the usage of fluoroquinolones even if the choice of DOX and AMX is ineffective. In this study, treatment with ENR was more effective to alleviate the signs of cough and oculonasal discharge compared to DOX. This may be related to the number of cases in the presented study. In a study from England revealed significant decrease in coughing in canine ITB with the use of AMX (Thrusfield, 1991). They (Thrusfield, 1991) also used some other drugs including ampicillin/amoxicillin-corticosteroid or trimethoprim/sulphonamide-corticosteroid combinations. Although comparison of the studies are difficult, in the study presented here cough recovery times were later in Groups AMX (4.6±0.45), ENR (4.33±0.39) and DOX (5.6±0.37) compared to Group CLA (3.7±0.48). In this study, we use only relevant antibiotics without other medications including corticosteroids. So significant decrease of coughing in the study of Thrusfield, (1991) could be associated the effect of corticosteroids. The lack of pre- and post-treatment culture results of pathogens isolated from airway fluids was a limitation of the current study. We did not also detect antibiotic resistance in the study.

CONCLUSION

In conclusion, it appears that CLA is considered as the first-line antibiotic to alleviate the ITB signs in dogs.

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Conflict of interest: The authors declared that there is no conflict of interest.

Ethical statement: Written owner consent was obtained from the owners.

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Immunohistochemical evaluation of IFN-γ levels in sheep verminous pneumonia

ABSTRACT

Lungworms, a group of parasitic nematodes, are recognized as one of the major and most common parasitic pneumonia agents in ruminants worldwide. In this study, the expressions of interferon gamma (IFN-γ), which is an important pro-inflammatory cytokine, were evaluated by immunohistochemical methods in order to evaluate the immune response against parasitic agents in sheep naturally infected with different types of lungworms. The material for this study consisted of lung tissue samples obtained from 40 dead sheep brought for routine histopathological diagnosis to the Department of Pathology. In order to reveal the histopathological changes in the tissues, Hematoxylin and Eosin (H&E) staining was applied to the sections. Lung tissues were stained with IFN-γ commercial antibody using the Avidin-Biotin Peroxidase Technique (ABC) following the procedures of the manufacturer. Subpleural multifocal nodules of several mm in diameter were detected in the dorsal regions of the lung, especially in the caudal lobes. In the histopathological examination of the lungs, it was observed that the alveoli, bronchi, and bronchiole lumens were filled with adult forms, larvae, and eggs of the parasitic agents. Compared to the control group, the expressions of IFN-γ were significantly increased in the verminous pneumonia group. Overall, the study suggests that the Th1 response, as represented by increased IFN-γ expression, appears to play an active role in the immunity developed against lungworms in ruminants.

Keywords: Cytokine, IFN-γ, lungworms, sheep, verminous pneumonia

INTRODUCTION

Sheep pneumonia is one of the multifactorial problems that can be seen in sheep of all ages around the world and causes very important economic losses. Among the causes of sheep pneumonia, in addition to viral, bacterial, and fungal agents, verminous pneumonia caused by lungworms has remarkable importance (Can et al., 2018). Lungworms, a group of parasitic nematodes, are recognized as one of the major and common parasitic pneumonia agents for ruminants worldwide (Asmare et al., 2018; Fesseha et al., 2021).

Several species belonging to the superfamily Metastrongyloidea (*Muellerius capillaris*, *Prostrongylus rufescens*, *Cysocualus ocreatus*, *Neostrongylus linearis*, etc.) and Trichostrongyloidea (*Dictyocaulus viviparus* and *Dictyocaulus filaria*, etc.) can infect domestic animals such as cattle and small ruminants (de Macedo et al., 2021; Zafari et al., 2022). Small lungworms infect sheep by damaging the lung parenchyma and bronchioles. Species such as *Muellerius capillaris* and *Prostrongylus rufescens* cause a wide range of inflammatory responses and chronic eosinophilic granulomatous pneumonia (Hanks et al., 2021; Jabbar et al., 2013).

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Research Article

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Dictyocaulus filaria lives in the trachea, bronchi, and bronchioles and causes bronchitis (dictyocaulosis, also known as husk) in young animals (Mangiola et al., 2014). Infections involving multiple agents rather than a single species in the same lung are more common (Rehbein et al., 2022; Rehbein and Visser, 2002).

Clinical symptoms such as mucus discharge, cough, and tachypnea are seen in sheep infected with lungworms. Death occurs mostly as a result of severe infections. In addition, secondary complications in the lung cause bacterial pneumonia, weight loss, and low milk yield, especially in young animals (Kırcalı Sevimli et al., 2011).

Lungworms are found in regions with cold temperatures and heavy rainfall (Papadopoulos et al., 2004). Season, species, age, gender, irrigation, and grazing management are important factors affecting the risk of lungworm infection (Hanks et al., 2022).

It is known that cytokine responses that occur when a pathogen is encountered are very important in inducing and regulating immune reactions (Holmgren et al., 2014). Interferon Gamma (IFN-γ) produced by macrophages, dendritic cells, natural killer cells (NK), $CD4^+$ T helper cells (Th1) and a subset of CD8 + T cells, is a highly active pro-inflammatory cytokine (Kak et al., 2018; Samar et al., 2017). IFN-γ plays a central role in the inflammation and the induction of cell-autonomous immune responses (Beytut et al., 2011; Sasai and Yamamoto, 2019). IFN-γ acts by activating macrophages and increasing their phagocytic capacity (Fleury et al., 2018).

In this study, the expressions of IFN-γ, which is an important pro-inflammatory cytokine, were evaluated by immunohistochemical methods in order to evaluate the immune response against parasitic agents in sheep naturally infected with different types of lungworms.

MATERIALS AND METHODS

Animals

The material for this study consisted of lung tissue samples obtained from 40 dead sheep (average 4-5 years old, female, Morkaraman breed) brought for routine histopathological diagnosis to the Department of Pathology, Faculty of Veterinary Medicine of Kafkas University. According to the anamnesis information obtained from the owners, it was observed that the animals exhibited various clinical signs such as cough, nasal discharge, and loss of appetite and weight. The lung tissue samples from 10 healthy sheep (average 4-5 years old, female, Morkaraman breed) without any pathological lesions were also used for control purposes.

Histopathological examinations

Lung tissue samples from the sheep were fixed in a 10% formaldehyde solution. Serial sections of 5 µ thickness were taken from the paraffin blocks prepared following the routine tissue follow-up procedures. In order to reveal the histopathological changes in the tissues, Hematoxylin and Eosin (H&E) Staining was applied to the sections. The prepared sections were evaluated in detail by at least two pathologists under the light microscope, and the detected lesions were photographed.

Immunohistochemical examinations

Serial sections of 4 µ thickness taken from the paraffin blocks prepared from lung tissues were stained with IFN-γ commercial antibody using the Avidin-Biotin Peroxidase-Technique (ABC) following the procedures of the manufacturer. Information about the primary antibody used in the study is detailed in Table 1. Thermo Scientific Histostain IHC Kit (HRP, broadspectrum, REF: TP-125-HL) was used to

conduct all immunostainings. The color-revealing substrate, 3.3-diaminobenzidine tetrahydrochloride (DAB) solution (Thermo Scientific, REF: TA-125-HD), was dropped onto the sections and incubated for 15 minutes. The sections were washed with distilled water for 5 minutes, stained with Mayer Hematoxylin, and coated with Immu-mount.

Table 1. Information on primary antibodies used in immunohistochemical studies

Primary Antibodies	Pretreatment	Company and Catalog Numbers	Dilution	Incubation Condition
IFN-γ	Open Microwave	MyBioSource, MBS2091397, Polyclonal	1/100	Overnight, 4°C

After bloating, the prepared sections were evaluated under a light microscope (Olympus Bx53) and photographed using the Cell ^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Detailed analyses of the captured photographs were made with the Image J program (1.51j8, Public Domain, Maryland, USA).

The evaluation of IFN-γ staining results was made with a grading system based on the number of positive cells in the areas examined with immune-positive reactions and reflecting most strongly the staining character. Three different fields from each case were examined with a 40x objective. The numbers of positively stained cells were recorded separately, and the average of these 3 fields was considered the average positive cell number of that animal. Scoring: (-) no immunoreactivity; (+) weak, 1-10% positivity; (++) moderate, 11–59% positivity; and (+++) severe positivity over 60% (Karakurt et al., 2023).

Statistical analysis

The program SPSS® (SPSS 26.0, Chicago, IL, USA) was used for the statistical analysis of the results. The Mann-Whitney U Test was used for the pairwise comparison of the control group and the verminosis group. The obtained results were given as the mean ± standard error (SE). In the evaluation of the results, the expression $P<0.05$ was considered statistically significant.

RESULTS

Macroscopic findings

Subpleural multifocal nodules of several mm in diameter were detected in the dorsal regions of the lung, especially in the caudal lobes. It was observed that the consistency of the nodules was soft in some cases and quite hard in others. The nodules were mostly gray-greenish. In addition, foci of intense bleeding were also found (Figure 1).

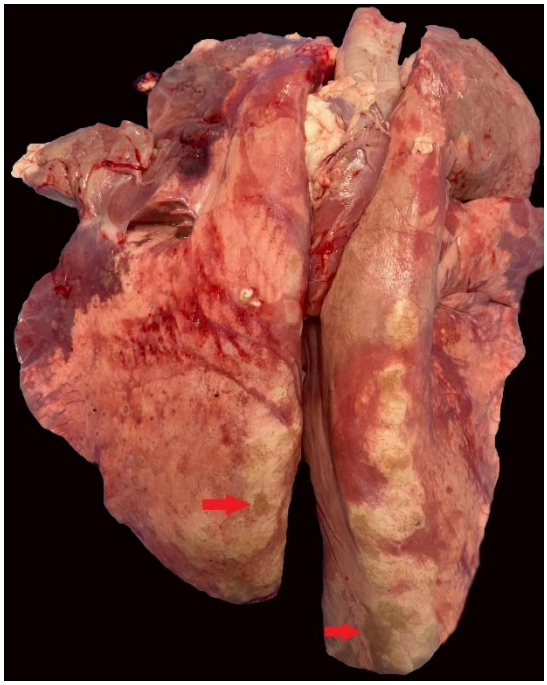

Figure 1. Lung, numerous grey-greenish-appearing nodules of varying size in the caudal lobes (red arrows).

Microscopic findings

No significant pathological changes were detected in the lung tissues of the control group (Figure 2a). In the histopathological examination of the lungs with verminous pneumonia, it was observed that the alveoli, bronchi, and bronchiole lumens were filled with adult forms, larvae, and eggs of the parasitic agents. Although a single type of parasitic agent was found in some cases, mixed infections were dominant in

most cases. In the lungs, parasitic granulomas consisting of agents and necrosis in the middle and foreign body giant cells, eosinophils, mononuclear cells, and a fibrous capsule localized around them were detected. It was observed that interalveolar septa were thickened due to connective tissue and inflammatory cell infiltration. Hyperplasia was observed in the bronchial and bronchiolar epithelium. The muscle layer around the bronchi and bronchioles was found to be hypertrophic (Figures 3-4-5).

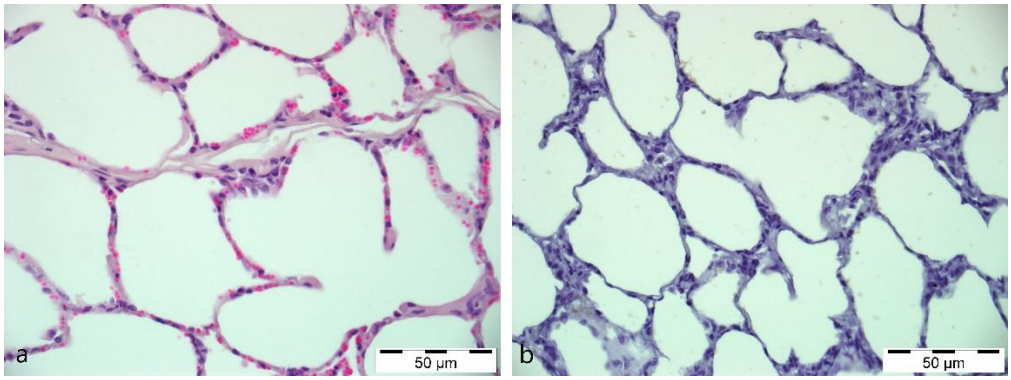

Figure 2. a: Lung, H&E, Control group, **b:** Lung, Immunohistochemistry, Control group.

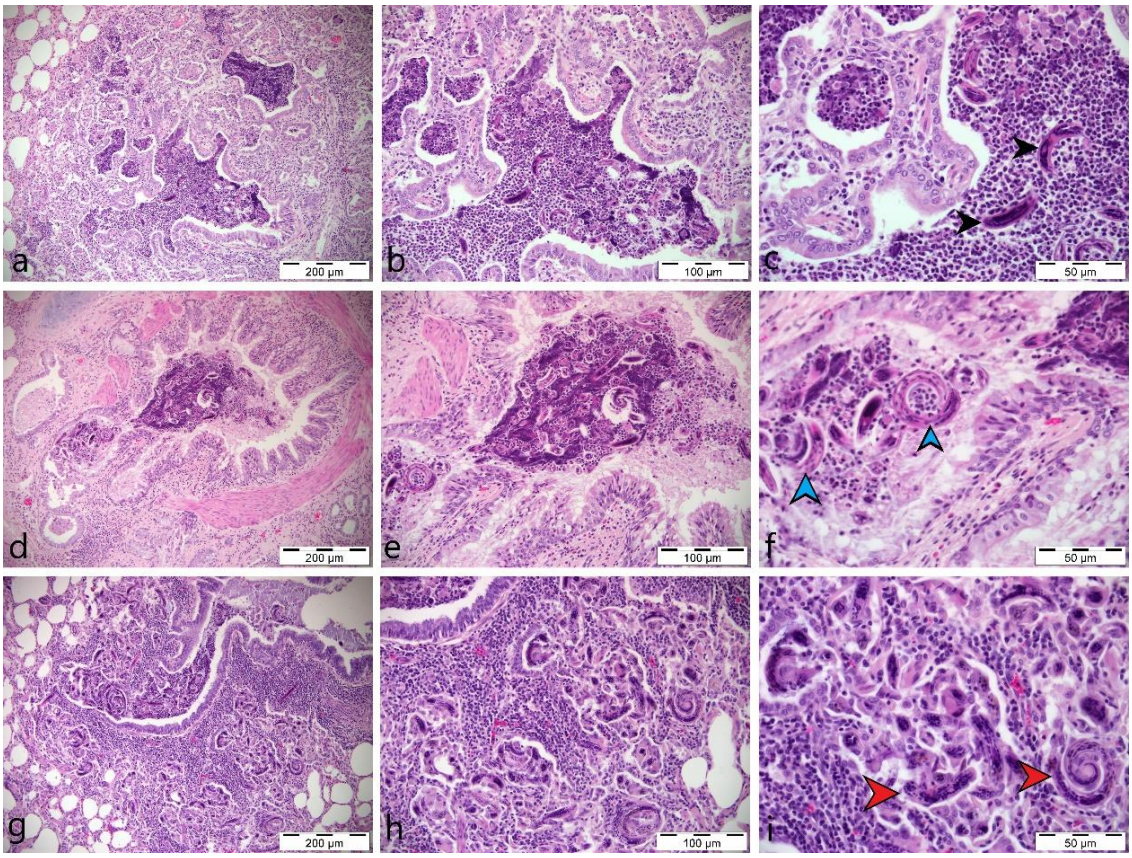

Figure 3. Lung, H&E, **a-b-c:** Appearance of the parasitic agents in the alveolar lumens (black arrowheads) in different magnifications, **d-e-f:** Appearance of parasitic larvae and eggs in the bronchial lumen (blue arrowheads) in different magnifications, **g-h-i:** Appearance of intense parasitic infiltration in the bronchiole lumens (red arrowheads) in different magnifications.

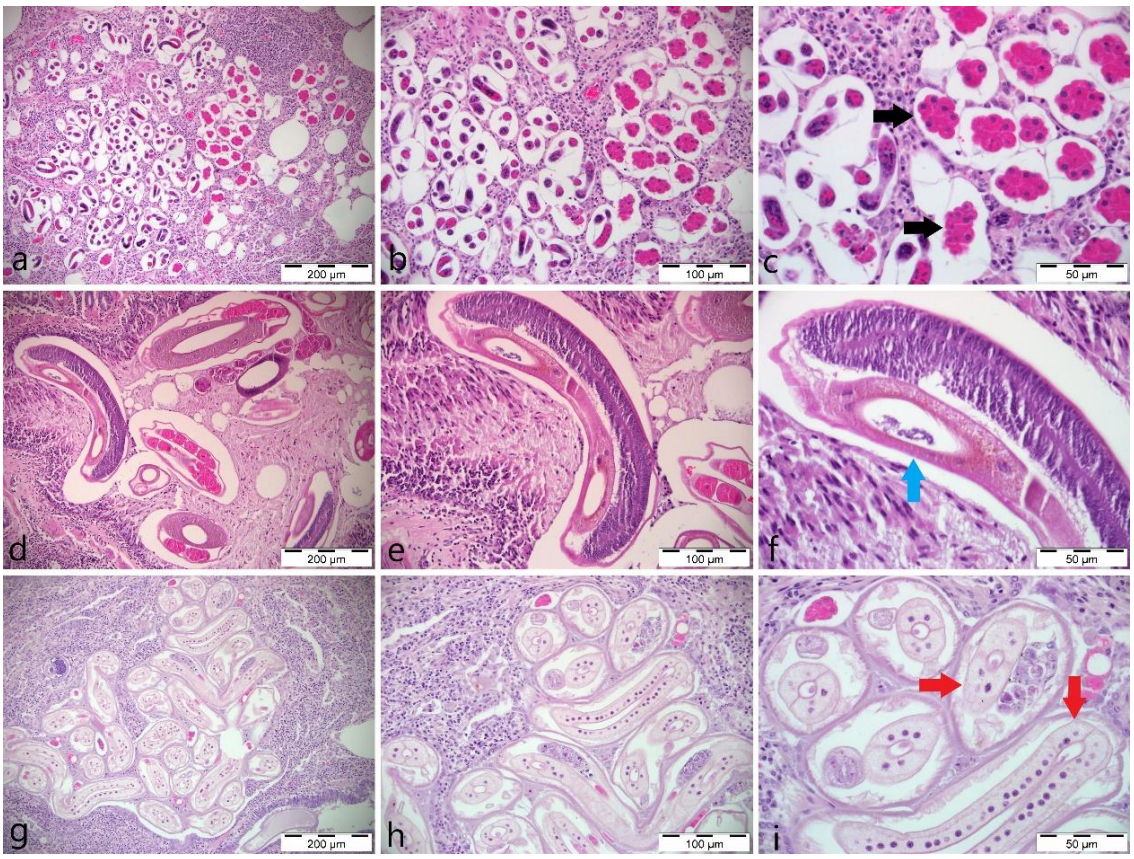

Figure 4. Lung, H&E, **a-b-c:** Appearance of the parasites located in the alveoli (black arrows) at different developmental stages and in different magnifications, **d-e-f:** Appearance of quite large parasites in the bronchial lumen (blue arrow) in different magnifications, **g-h-i:** Appearance of a large number and size of parasitic agents localized in the bronchiole lumens (red arrows) in different magnifications.

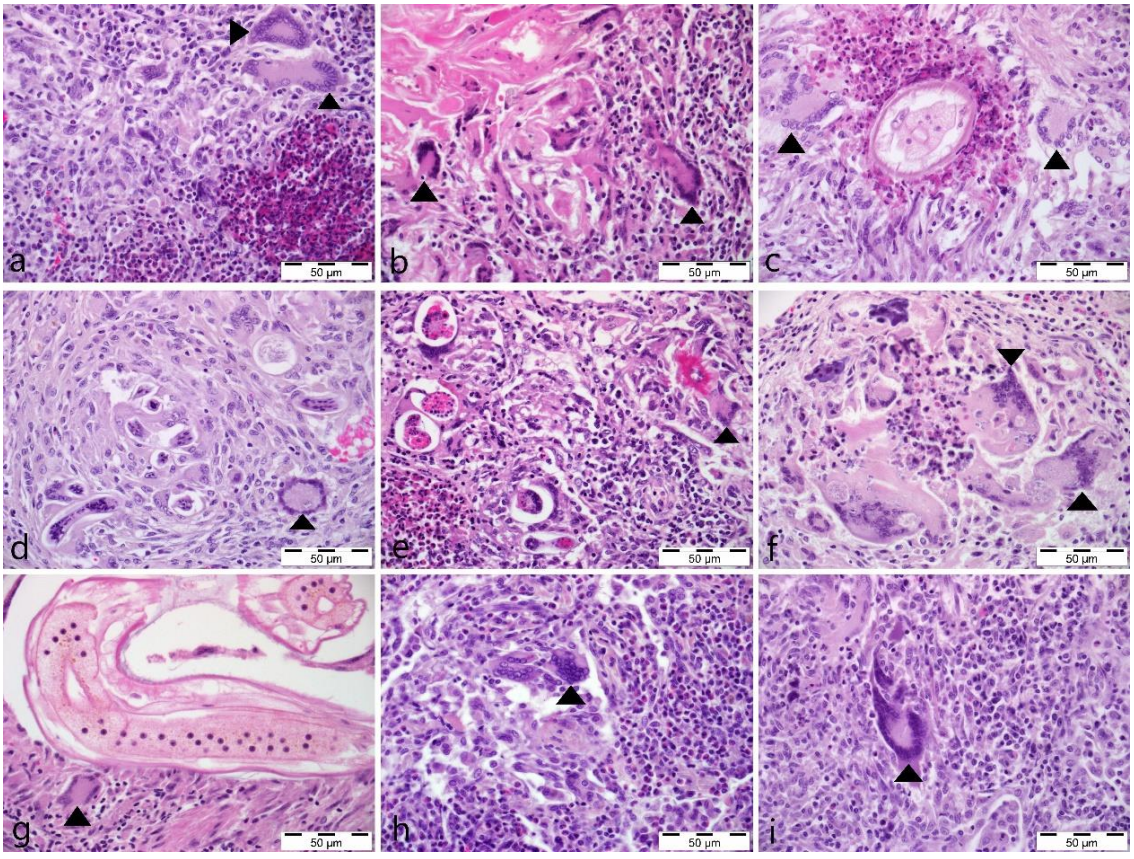

Figure 5. Lung, H&E, a-i: Multinucleated giant cells (arrowheads) localized around different parasitic agents.

Immunohistochemical findings

The immunopositivity scores of the control and verminous pneumonia groups are detailed in Table 2.

Table 2. IFN-γ immunopositivity scores of the groups

Groups	IFN-γ
Control	0.20±0.13
Verminous pneumonia	2.78±0.07
P value	<0.001

Compared to the control group (Figure 2b), the expressions of IFN-γ were significantly increased in the verminous pneumonia group. The IFN-γ positive stainings were mostly concentrated in the multinucleated giant cells localized around the parasitic agents and in the cytoplasm of the mononuclear cells around these giant cells. In addition to these cells, intracytoplasmic IFN-γ expressions were also present in the alveolar macrophages. The IFN-γ positive reactions were granular in form. It was remarkable that IFN-γ immunoreactivity was much more severe in cases with mixed infections compared to cases with a single type of parasitic agent. In cases where parasitic granulomas and the inflammatory cell infiltration were larger, IFN-γ immune-positive staining was equally intense (Figure 6).

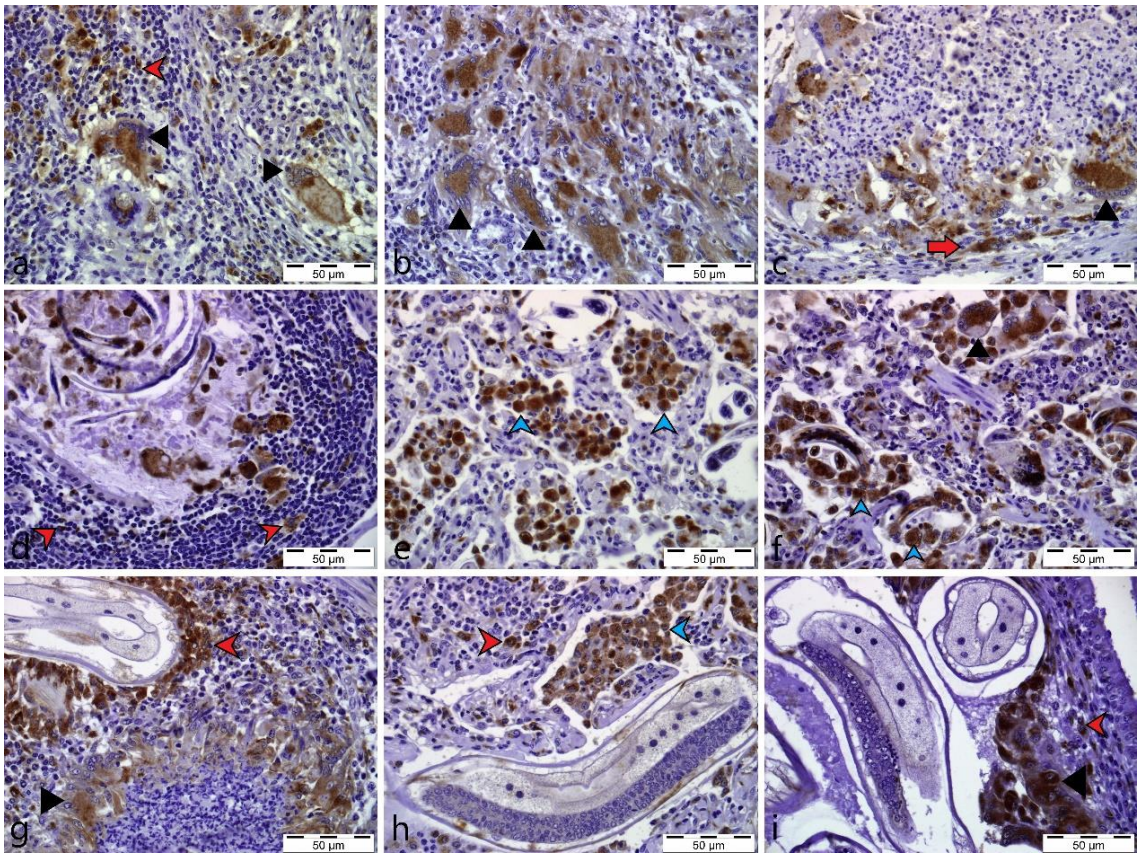

Figure 6. Lung, Immunohistochemistry, IFN-γ, **a-i:** Immune positive staining in multinucleated giant cells (black arrowheads), alveolar macrophages (blue arrows), and mononuclear cells (red arrows).

DISCUSSION

Lungworms cause significant economic losses, which emphasizes the importance of correct diagnosis methods, treatment, and control (Zafari et al., 2022). The clinical symptoms, the post-mortem lesions in the lung, the detection by the Baermann technique of agents in the first stage from fecal samples taken from animals, the FLOTAC and Mini-FLOTAC techniques, ELISA tests and bronchoalveolar lavage are

effectively used in the diagnosis of the disease (de Macedo et al., 2021; Fesseha and Mathewos, 2021; Hanks et al., 2021). In the current study, the macroscopic (Bouljihad et al., 1995; Gulbahar et al., 2009; Hashemnia et al., 2019; Panayotova-Pencheva and Alexandrov, 2010) and microscopic findings (Beytut et al., 2002; Kıran et al., 1993; Sağlam et al., 1998; Ülgen et al., 1997) were consistent with the literature data.

The cellular reaction in sheep to lungworms at various developmental stages includes the mobilization of eosinophils, mast cells, macrophages, and multinucleated giant cells that fill the alveoli, alveolar septa, and bronchioles (Gulbahar et al., 2009; Panuska 2006). Small lungworms cause long-term infections in their hosts, suggesting that the immunity to the disease is not efficiently stimulated (Rehbein and Visser, 2002). There are very few studies evaluating the immune response to lungworms in cattle (Holmgren et al., 2014; Johnson et al., 2005) and sheep (Gulbahar et al., 2009). Johnson et al., (2005) evaluated the mRNA expression of various cytokines such as IL-4, IL-5, IL-10, and IFN-γ in the lung parenchyma, tracheal rings in the bronchial bifurcation, and bronchial and caudal mediastinal lymph nodes on days 15, 22, and 43 post-infection with *D. viviparus*. They reported that the expression of IFN-γ increased due to the infection. Contrary to this study, Holmgren et al., (2014) reported that there was no significant increase in the levels of IFN-γ. Although there are indications that a mixed Th1/Th2 immune response is given with high (Th2-dependent) IgE and eosinophil levels against *D. viviparus* in cattle, detailed information is not available about the immune response against *D. filaria* (Mangiola et al., 2014). In the current study, it was observed that the IFN-γ levels were statistically increased in lung tissue samples from naturally infected sheep with various lungworm types compared to lung tissue samples obtained from uninfected healthy animals. This shows that, in addition to the Th2 immune response, in the immune response against lungworms, the Th1 response is also effective.

The control of parasitic diseases depends on the production of cytokines; the production of cytokines triggers cascade mechanisms, limiting the proliferation, survival, and invasion of parasites (Ram et al., 2020). It is known that the IFN family has important roles in the host immune response against infections, pathogens and various diseases. Parasitic infections in mammals are characterized by increased IFN-γ levels (Kim et al., 2019). In the literature reviews, IFN-γ has been found to have a protective effect against various parasitic infections such as Fasciolosis (Attia et al., 2022), Histomoniasis (Kidane et al., 2018), Coccidiosis (Kim et al., 2019), Leishmaniasis (Kak et al., 2018), Taeniasis (Fleury et al., 2018), Theileriosis (Ram et al., 2020), Toxocariasis (Samar et al., 2017) and Toxoplasmosis (Sasai and Yamamoto, 2019). In this study, it was determined that IFN-γ expressions increased directly proportional to the severity of the inflammatory response, especially against single or multiple types of lungworms. In addition, it was remarkable that IFN-γ immune-positive staining was intensified parallel to the size of the granulomas demarking the parasitic agents. This data also supported the pro-inflammatory effects of IFN-γ.

Interferon gamma is produced by professional antigen-presenting cells such as CD4+ T helper cell type lymphocytes (Th1), CD8+ cytotoxic lymphocytes, NK, monocytes and macrophages, dendritic cells, and B lymphocytes. IFN-γ is an important pro-inflammatory cytokine that plays a major role in the fight against viruses, intracellular bacteria, parasites, and tumors (Manirarora et al., 2022). It is known that IFN-γ supports cell-mediated immunity by activating the phagocytosis capacity of the macrophages (Kidane et al., 2018). The inducible nitric oxide synthetase (iNOS) isoform primarily activates lung macrophages, resulting in the production of nitric oxide (NO) from these cells. iNOS

expression is also induced by several cytokines, such as IFN-γ, IL-1, or TNF-α. In this study, IFN-γ positive staining was predominantly observed in the cytoplasm of multinucleated giant cells and alveolar macrophages. These immunohistochemical findings show that IFN-γ increases the phagocytosis capacity of giant cells and alveolar macrophages in the cellular defense developed against parasitic agents.

CONCLUSION

As a result, this remarkable increase in the levels of IFN-γ in sheep with parasitic infections compared to uninfected sheep reveals that IFN-γ is a very useful marker in the diagnosis of the disease and in determining the severity of the infection. Besides this, as a contribution to the literature data on the immunity developed against lungworms in ruminants, it is observed that, in addition to the Th2 response, the Th1 response plays an active role in IFN-γ, which is an important pro-inflammatory cytokine. In this respect, it has been concluded that the data obtained from the current study will contribute to the immunology of lungworms in sheep.

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Conflict of interest: The authors declared that there is no conflict of interest.

Ethical statement: The study was approved by the Local Ethics Committee on Animal Experiments of Kafkas University (KAU-HADYEK-2022/205).

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Ağrı ili parklarında askarit kontaminasyonunun araştırılması

Investigation of ascarid contamination in parks of Ağrı, Türkiye

Research Article

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ÖZET

Bu çalışmada Ağrı il merkezinde bulunan 18 çocuk parkının askarit yumurtaları ile kontaminasyonunun belirlenmesi amaçlanmıştır. Bu amaçla Mart-Mayıs 2023 tarihleri arasında her bir parktan tekniğine uygun bir şekilde toplanan 16 dışkı, 28 toprak ve 42 kum örneği laboratuvarda helmint yumurtaları yönünden mikroskobik olarak incelenmiştir. Dışkı örneklerinin 6'sının *Toxocara* spp., 3'ünün ise *Ancylostoma caninum* yumurtaları ile kontamine olduğu bulunmuştur. Toprak örneklerinin 5'inde *Toxocara* spp. ve 2'sinde *Dipylidium caninum* yumurtaları tespit edilmiştir. Kum örneklerinin 5'inde *Toxocara* spp. ve 1'inde *Dipylidium caninum* yumurtaları belirlenmiştir. Bu çalışma neticesinde Ağrı ilindeki çocuk parklarının zoonotik kedi-köpek askaritleri ve bazı cestod türleri ile kontamine olduğu görülmüştür.

Anahtar Kelimeler: Ağrı, helmint, kontaminasyon, park, Türkiye

ABSTRACT

In this study, it was aimed to determine the contamination of 18 parks with ascarid eggs in Ağrı. For this aim, in accordance with the technique 16 stool, 28 soil, and 42 sand samples were collected from each park between March-May 2023 and examined microscopically for the presence of helminth eggs in the laboratory. While 6 stool samples were found to be contaminated with *Toxocara* spp., *Ancylostoma caninum* eggs were found in 3 stool samples. *Toxocara* spp. eggs were seen in 5 and *Dipylidium caninum* eggs were seen in 2 soil samples. In 5 *Toxocara* spp. and 1 *Dipylidium caninum* eggs were determined in sand samples. As a result of this study, it was seen that the playgrounds in the Ağrı region were contaminated with zoonotic cat-dog ascarid and some cestode species.

Keywords: Agrı, contamination, helminth, park, Türkiye

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GİRİŞ

Zoonoz hastalıklar; hayvanlardan insanlara bulaşan ve her iki gruba dahil bireylerde ortak olarak şekillenen hastalıklar diye tanımlanabilir. Hayvanlardan insanlara çok sayıda enfeksiyon etkeni bulaşmaktadır (Bozkurt vd., 2012; Kassai, 1999; Soulsby, 1986). Bu zoonozların yayılışında kedi ve köpek gibi evcil hayvanlar da rol üstlenmektedir. Dünya'nın birçok ülkesinde olduğu gibi Türkiye'de de sayıları her geçen gün artan sahipsiz ve bakım-beslenme gibi zorluklarla karşılaşması nedeniyle sahiplerinin sokaklara bıraktığı kedi ve köpekler şehirlerde başıboş dolaşarak taşıdıkları zoonotik karakterdeki paraziter enfeksiyon etkenlerinin insanlara, özellikle de çocuklara bulaşmasına neden olmaktadırlar (Aydın, 2020; Bozkurt vd., 2012; Erol vd., 2021). Bu nedenle parklar ve çocuk oyun alanlarında bulunan kum havuzları önem arz etmektedir. Genellikle bu alanların çevrelerinde sokak hayvanlarının girmesine engel olabilecek sınırlamalar bulunmamaktadır. Bu alanlara başıboş kedi ve köpekler rahatça girip dışkılamakta veya evlerinde köpek besleyenler hayvanlarının dışkılama ihtiyaçlarını gidermeleri için bu parklara götürmektedir (Bozkurt vd., 2012). Bu hayvanların dışkıları hayvan sahipleri tarafından toplanmamakta, parklarda dışkı kutuları veya özel tuvaletler de bulunmamaktadır. Bu hayvanlar dışkılarıyla başta askaritler olmak üzere çok sayıda parazit türünün yumurtalarıyla çevreyi kontamine etmektedir. Bu durum parklarda oynayan çocuklar açısından risk taşımaktadır (Overgaauw ve van Knapen, 2013). Bunların yanı sıra piknik yerlerinde bulunan çocuk oyun alanları başıboş hayvanlar için tercih edilen bölgeler olarak ortaya çıkmaktadır. Dolayısıyla böyle alanlarda oynayan çocuklar için kedi ve köpeklerin dışkılarından etrafa saçılan çeşitli parazitlerin yumurtaları, kum veya toprağa karışarak zoonoz paraziter hastalıklar yönüyle önemli derecede risk oluşturmaktadır (Bozkurt vd., 2012). Zira önemli paraziter zoonozlar arasında yer alan ancak Amerika Birleşik Devletleri Hastalık Kontrol ve Önleme Merkezi (CDC) tarafından özellikle insanlarda göz ardı edilen hastalık etkenleri arasında sınıflandırılan, kedi ve köpeklerin ince bağırsaklarında yaşayan askaritler (*Toxocara canis, T. cati, Toxoscaris leonina*) insanlarda iç organ ve oküler (göz) larva migransa neden olabilmektedir. Ayrıca tipik bir pika belirtisi olan toprak yeme alışkanlığı çocuklarda toxocariosis riskini artırmaktadır (Akdemir, 2010; Avcıoğlu ve Burgu, 2008; Aydın, 2020; Bozkurt vd., 2012; Erol vd., 2021; Kassai, 1999; Kleine vd., 2017; Raissi vd., 2020).

Dünya genelinde halka açık yerlerde kedi ve köpek dışkılarıyla dışarı atılan askarit yumurtalarıyla kontaminasyon durumunun meta analiz sonuçlarına göre *Toxocara* spp. oranı Dünya'da %21, Türkiye'de ise %16 (Fakhri vd., 2018) olarak ifade edilmiştir. Bu konuda Dünya'da ve Türkiye'de bir çok çalışma yapılmıştır (Dada ve Lindquist, 1979; Güçlü ve Aydenizöz, 1998; Köchle vd., 2022; Lorenzo-Rebenaque vd., 2023; Şahin, 2022). Dünya'da *Toxocara* spp. oranı en düşük %0.55 oranıyla Melbörn'de (Carden vd., 2003), en yüksek %95.7 oranıyla ise Malezya'da (Zain vd., 2015), Türkiye'de ise en düşük %3.2 olarak Elazığ (Kaplan vd., 2002) ve Aydın'da (Şahin, 2022), en yüksek ise %59.3 oranında Ordu'da (Karaman vd., 2022) bildirilmiştir.

Ağrı ilinde sığırlarda (Afshar vd., 2023) ve sokak köpeklerinde (Afshar vd., 2022) askarit türlerinin yaygınlığının araştırıldığı çalışmalar bulunmakta, ancak çocuk parklarında askarit enfeksiyon yoğunluğu hakkında herhangi bir veri bulunmamaktadır. Bu çalışmada, Ağrı il merkezinde bulunan çocuk parklarından toplanan dışkı, kum ve toprak örneklerinde kedi ve/veya köpek askarit yumurtalarının tespit

edilmesi ve bu parkların toxocariosis yönünden taşıdığı riskin belirlenmesi amaçlanmıştır.

MATERYAL VE METHOD

Saha çalışması

Çalışma Mart-Mayıs 2023 tarihleri arasında Ağrı il merkezinde bulunan parklarda yürütülmüştür. Ağrı il merkezinde toplam 18 park bulunmakta olup, tüm parklardan örnek toplanmıştır. Toplanan örnek sayıları bu parkların büyüklüğüne ve fiziki yapısına istinaden değişiklik göstermiştir. Bu parkların her birinin farklı yerlerinden 42 kum, 28 toprak ve 16 dışkı örneği toplanabilmiş, her bir örnek ayrı poşetlere konulmuş ve numaralandırılmıştır. Kum, dışkı ve toprak örnekleri alınırken aralarında 5 metre kadar mesafe olmasına özen gösterilmiştir. Örnekler 10 cm kadar derinden ve 250-300 g olacak şekilde toplanmıştır. Her bir örnek alındıktan sonra kürekler dezenfekte edilmiştir (Bozkurt vd. 2012; Erol vd., 2021).

Kum, toprak ve dışkı örneklerinin incelenmesi

Parklardan toplanan kum ve toprak örnekleri, Kazacos, (1983)' un önerdiği yöntemin modifiye edilmesi ile incelenmiştir. Yaklaşık 50 g kadar alınan her bir kum ve toprak örneğinin üzerine 60 ml distile su ve 0.5 ml Tween 40 eklenmiş, iyice karışması için çalkalanmış ve gözenek genişliği 242 µm olan elekten geçirilmiştir. Falkon tüplere aktarılan örneklerin üzeri distile su ile tamamlanmış ve 2000 devirde 3 dakika süreyle santrifüj edilmiştir. Üstteki sıvının dökülüp sedimentin üzerine tekrar distile su eklenerek santrifüj edilmesi işlemi 2 kez daha tekrarlanmıştır. Sonraki aşamada kalan tortunun üzerine doymuş çinko sülfat ($ZnSO_4$, Özgül ağırlık: 1.364) eklenmiş, vortekslenmiş ve 2000 devirde 10 dakika süreyle santrifüj edilmiştir. Tüplerin üzeri lamel ile kapatılmış, 15 dakika sonra lamel alınarak lam üzerine konulmuş, x10 ve x40 büyütmede ışık mikroskobunda (Olympus DP72) incelenmiştir. Dışkı örneklerine ise doymuş çinko sülfat solüsyonu kullanılarak santrifüj flotasyon yöntemi (Bozkurt vd., 2012; Erol vd., 2021; Kassai, 1999) uygulanmış, preparatlar mikroskop altında helmint yumurtaları yönünden incelenmiştir.

BULGULAR

Çalışmada dışkı, toprak ve kum örneği toplanan 18 parkın 11 (%61.11)'inde helmint yumurtalarına rastlanmıştır. İncelenen 16 dışkı örneğinin 6'sında (6/16, %37.50) *Toxocara* spp., 3'ünde (3/16, %18.75) *Ancylostoma caninum,* toprak örneklerinin 5'inde (5/28, %17.85) *Toxocara* spp., ve 2'sinde (2/28, %7.14) *Dipylidium caninum,* kum örneklerinin ise 5'inde (5/42, %11.90) *Toxocara* spp. ve 1'inde (1/42, %2.38) *Dipylidium caninum* yumurtaları görülmüştür. Dışkı, kum ve toprak örneklerinde en yaygın parazitin %18.60 (16/86) ile *Toxocara* spp. olduğu tespit edilmiş, bunu %3.48 (3/86) *Dipylidium caninum* ve *Ancylostoma caninum* takip etmiştir.

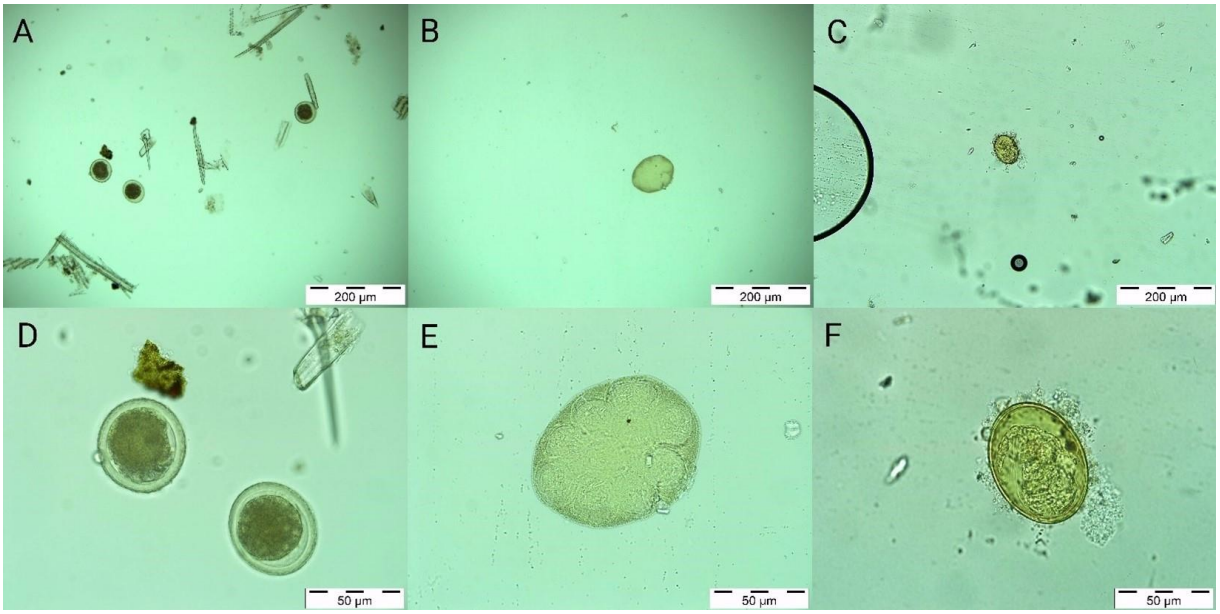
Helmint yumurtası görülen 11 parktan 7'sinin sadece *Toxocara* türleri ile, 5'ininde *Toxocara* spp. + *Dipylidium caninum, Toxocara* spp. + *Ancylostoma caninum* ile kontamine olduğu belirlenmiştir. Kum, toprak ve dışkı örneklerinde saptanan helmint yumurtalarının dağılımı Tablo 1'de ve tespit edilen türlerin mikroskobik görüntüleri Şekil 1'de verilmiştir.

TARTIŞMA

Kedi ve köpek dışkılarıyla dışarı atılan *Toxocara* spp. yumurtalarıyla kontaminasyonun meta analiz sonuçlarına göre oranı Dünya genelinde %21, Türkiye'de ise %16 (Fakhri vd., 2018) olarak tespit edilmiştir. Farklı ülkelerdeki çocuk oyun alanları/parklar ve kamusal alanların *Toxocara* spp. türü ile kontaminasyonu kıtalara göre en düşük-en yüksek oranla değerlendirildiğinde; Avrupa kıtasında İspanya'da %1.24 (de Ybxkáñez vd., 2001), Almanya'da %87.1 (Duwell, 1984), Asya kıtasında Kazakistan'da %2.3 (Shaikenov vd., 2004), Malezya'da %95.7 (Zain vd., 2015), Amerika kıtasında Kanada'da %2.3 (Gualazzi vd., 1986), Brezilya'da %68.1 (Marques vd.,

Tablo 1. Dışkı, toprak ve kum örneklerinde saptanan helmint yumurtalarının dağılımı

Park Adı	Dışkı			Toprak			Kum		
	Toxocara spp.	*Dipylidium caninum*	*Ancylostoma caninum*	*Toxocara* spp.	*Dipylidium caninum*	*Ancylostoma caninum*	*Toxocara* spp.	*Dipylidium caninum*	*Ancylostoma caninum*
Nadir Ağa Parkı			1						
Bulut Mahallesi Parkı				1			1		
Toki Park Alanı	1			1	1				
Abide Parkı									
Çevik Kuvvet Parkı	1		1						
Karaca Parkı							1		
Dudayev Parkı	1			1	1		1	1	
Kahramanmaraş Parkı									
Ağrı Milletler Bahçesi						1	1		
Silivri Parkı	1			1					
Boncuklu Parkı	1								
Selahattin Eyyübi Parkı			1	1					
Cumhuriyet Mahallesi Parkı									
Orman İşletme Fidanlığı									
Belediye Yanı Parkı									
100. yıl Parkı									
Kazım Karabekir Parkı									
Belediye Konuk Evi Parkı	1						1		
Toplam	**6**		**3**	**5**	**2**		**5**	**1**	



Şekil 1. Dışkı, kum ve toprak örneklerinde saptanan helmint yumurtalarının mikroskobik görüntüsü**;** A: *Toxocara* spp. yumurtaları 10x (Dışkı), B: *Dipylidium caninum* yumurtası 10x (Kum), C: *Ancylostoma caninum* yumurtası 10x (Toprak), D: *Toxocara* spp. yumurtaları 40x (Dışkı), E: *Dipylidium caninum* yumurtası 40x (Kum), F: *Ancylostoma caninum* yumurtası 40x (Toprak).

2012), Avustralya kıtasında Melbörn'de %0.55 (Carden vd., 2003), Afrika kıtasında Nijerya'da %8 (Umeche, 1989), Libya'da %59 (Belhage vd., 2016) olarak tespit edilmiştir. Türkiye'de yapilan çalişmalarda bu oranlar Elazığ'da %3.2-23 (Kaplan vd., 2002, Şimşek vd., 2005), Konya'da %4.16 (Güçlü ve Aydenizöz, 1998), Kütahya %10 (Akdemir, 2010), Ankara'da %11.3 ve %30.6 (Avcıoğlu ve Burgu, 2008; Öge ve Öge, 2000), Kırıkkale'de 15.6 (Aydenizöz Özkayhan, 2006), İstanbul'da %10.10-15.9 (Toparlak vd., 2002; Şengür ve Öner, 2005), Aydın'da %3.2-18.9 (Gürel vd., 2005; Şahin, 2022), Samsun %19.9 (Gürler vd., 2020), Burdur %21 (Akbaş, 2019) Kayseri'de %50 (Bozkurt vd., 2012), Karaman'da %55 (Aydın, 2020) olarak bildirilmiştir. Ordu'da 16 ilköğretim (%59.30) ve 2 orta öğretim (%18.20) okulunun bahçesinin *Toxocara* spp. yumurtaları ile kontamine olduğu görülmüştür (Karaman vd., 2022). Ağrı ilinde hem bizim yaptığımız bu çalışmada (%33.3) hem de kedi-köpeklerde Afshar vd., (2022) tarafından yapılan başka bir çalışmada (%28.7) elde edilen kontaminasyon oranının hem Türkiye (%16), hem de dünya geneli (%21) ortalamasının üzerinde olduğu tespit edilmiştir.

Ağrı ilinde bu çalışmanın yapıldığı mevsim itibariyle sıcaklığın yavaşça artmasıyla beraber parklarda belirlenen kontaminasyon oranı (%33.3); Kayseri (%50), Karaman (%55), Ordu (%59.3) ve Erzurum (%64.28)'da yapılan çalışmalardan düşük (Avcıoğlu ve Balkaya, 2011; Aydın, 2020; Bozkurt vd., 2012; Karaman vd., 2022), Elazığ (%3.2), Konya (%4.16), Kütahya (%10), Ankara (%11.3 ve %30.6), Kırıkkale (%15.6), İstanbul (%10.10-15.9), Aydın (%3.2-18.9), Samsun (%19.9) ve Burdur'da (%21) yapılan çalışmalardan yüksek bulunmuştur (Akbaş, 2019; Akdemir, 2010; Avcıoğlu ve Burgu, 2008; Aydenizöz Özkayhan, 2006; Güçlü ve Aydenizöz, 1998; Gürel vd., 2005, Gürler vd., 2020; Kaplan vd., 2002; Öge ve Öge, 2000; Şahin 2022; Şengür ve Öner, 2005; Toparlak vd., 2002). Ağrı'da yapılan bu çalışmada elde edilen prevalans değerinin bazı çalışmalardan yüksek, bazılarından ise düşük çıkması ildeki çok sayıda sokak hayvanının bulunması, iklim koşulları, yetersiz bilinçlendirme gibi faktörlerle ilişkilendirilmiştir.

Askarit yumurtaları dış ortamda gelişimini sürdürebilmek için yüksek nem ve sıcaklık (25-30°C) değerlerine ihtiyaç duymaktadır (Anderson, 2000). Ağrı yöresinde yıllık sıcaklık ortalaması 6.2-9.2°C arasında olduğundan, bu sıcaklık ortalamasının karnivor askaritlerinin gelişimini olumsuz yönde etkileyebileceği ve enfektif döneme ulaşamayabileceği ve bu nedenle Ağrı ilinde belirlenen kontaminasyon oranının düşük çıktığı kanaati oluşmuştur. Ayrıca askarit kontaminasyon oranları arasındaki farklılıkların, çalışılan örneklem büyüklüğü, uygulanan teşhis yöntemi, sosyo-ekonomik durum, nem, sıcaklık, şehir düzenlemesi, sonkonak popülasyonu, sonkonakların parklara girişini engelleyen çitlerin olmaması, hayvan dışkılarının ortamdan uzaklaştırılmaması gibi faktörlerden kaynaklanabileceği ileri sürülmektedir (Avcıoğlu ve Balkaya, 2011; Avcıoğlu ve Burgu, 2008; Bozkurt vd., 2012).

Çocuk parklarının kedi ve köpek helmintlerinin yumurtalarıyla kontaminasyonun araştırıldığı çalışmalarda Ankara'da (Avcıoğlu ve Burgu, 2008; Öge ve Öge, 2000) *Toxocara* spp. %82.4, *Toxascaris leonina* %1.9, *Ancylostoma* sp. %17.62, *Taenia* spp. %1.82-4.6, *Trichuris* sp. %2.4, *Enterobius vermicularis* %1.2, Erzurum'da (Avcıoğlu ve Balkaya, 2011) *Toxocara* spp. %64.28, *T. leonina* %1.25, *Taenia* spp. %3.12, Van'da (Ayaz vd., 2003) *Toxocara* spp. %25.97, *T. leonina* %11.25, Kırıkkale'de (Aydenizöz Özkayhan, 2006) *Toxocara* spp. %15.6, *T. leonina* %1.5, *Taenia* spp. %1 ve *Isospora* sp. %0.2, Kütahya'da (Akdemir, 2010) *Toxocara* spp. %10, Sivas'ta (Erol vd., 2021) *Toxocara* spp. %5.9, Karaman'da (Aydın, 2020) *Toxocara* spp. %19.4, *Taenia* spp. %6.8, *Ancylostoma* sp. %0.97 ve Kayseri'de (Bozkurt vd., 2012) *Toxocara* spp. %7.3, *T. leonina* %4, *Spirocerca lupi* %0.8, *Taenia* sp.-*Echinococcus*

sp. %0.8 ve *A. caninum* %0.4 oranlarında bildirilmiştir. Ağrı'da yapılan bu çalışmada ise dışkı, kum ve toprak örneklerinde *Toxocara* spp. %18.6 (16/86), *Ancylostoma caninum* %3.5 (3/86) ve *Dipylidium caninum* %3.5 (3/86) oranlarında tespit edilmiştir. Yapılan çalışmalarda elde edilen prevalans oranlarındaki farklılıkların çalışmaların yapıldığı alanlardaki kedi ve köpek populasyonu, yörenin ortalama sıcaklık ve nem değerleri, yağış rejimi, örneklerin toplandığı parktaki toprak ve kumun özelliğinin yanı sıra, sosyo-ekonomik durum, belediyelerin sokak hayvanları ile ilgili çalışmaları gibi faktörlerden kaynaklandığı düşünülmektedir (Bozkurt vd., 2012). Ağrı'da yapılan bu çalışmada elde edilen prevalans değerinin yüksek çıkması da ildeki başıboş kedi ve köpek populasyonunun fazlalığına, ilin ortalama sıcaklık değerlerinin düşük ve yağışların az olmasına, sosyo-ekonomik durumun bozuk olmasına, belediyelerin sokak hayvanları ile ilgili çalışmalarının olmamasına bağlanmıştır.

Toxocara canis, T. cati, T. leonina gibi karnivor askaritlerinin sonkonakları olan kedi ve köpeklerin çitle çevrilmiş parklara girmeleri ve dolayısıyla enfeksiyonu bulaştırmaları zor olacağı için bu konuda belediyelere görev düşmektedir (Avcıoğlu ve Balkaya, 2011). Sivas'ta yapılan çalışmada (Erol vd., 2021) kum örneklerinde *Toxocara* spp. prevalansının düşük bulunması çocuk oyun alanlarının çitle çevrili olmasına bağlanmıştır. Bu araştırmanın yapıldığı parkların tamamı şehir merkezinde yer almakta olup, parkların fiziksel koşullarının da birbirinden çok farklı olmadığı görülmüştür. Bu parklarda kedi ve köpek girişlerine sınırlama getiren uygulamalar da bulunmamaktadır. Ağrı ilinde yapılan bu çalışmada örnek toplanan çocuk parklarının etrafının çitle çevrili olmaması nedeniyle kontaminasyon oranının yüksek (%33.3) çıktığı kanısına varılmıştır.

Karaman'da yapılan bir çalışmada bir toprak örneğinde *Toxocara* spp. ve *Taenia* spp. ile (Aydın, 2020), Ankara'da yapılan bir çalışmada (Avcıoğlu ve Burgu, 2008) %0.9 oranında *Toxocara* spp. ile *Taenia* spp. ve *Toxocara* spp. ile *Toxascaris leonina* miks kontaminasyon tespit edilmiştir. Ağrı ilindeki bu çalışmada *Toxocara* spp. dışında *Ancylostoma caninum* ve *Dipylidium caninum* yumurtalarına bazı parklarda rastlanmış ve bu etkenler *Toxocara* spp. ile miks enfeksiyon şeklinde de görülmüş olup, dışkı, kum ve toprak örneklerinde *Toxocara* spp. + *Ancylostoma caninum* ve *Toxocara* spp. + *Dipylidium caninum* miks kontaminasyonları %3.5 olarak tespit edilmiştir. Miks enfeksiyon oranının tek başına askarit enfeksiyon oranından düşük çıkmasının nedeninin askarit yumurtaların kabuğunun kalın ve dolayısıyla dış ortam koşullara daha dayanıklı olmasından kaynaklandığı (Soulsby 1986) düşünülmektedir.

SONUÇ

Paraziter zoonozlar arasında yer alan ancak özellikle insanlarda göz ardı edilen hastalıklardan biri olarak değerlendirilen, insanlarda iç organ ve oküler (göz) larva migransa neden olan enfeksiyonlardan biri olan toxocariosis, Türkiye'de ve dünyanın birçok ülkesinde hijyen eksikliği, sokak hayvanlarının sayısının kontrolsüz bir şekilde artması, korunma ve kontrol tedbirlerinin yetersizliği gibi nedenlerle hızla yayılmaktadır.

İnsanların sosyal yaşam alanı olarak kullandığı, ancak taşıdıkları askaritlerin yumurtalarını dışkılarıyla dışarı atan kedi ve köpeklerin kontamine ettikleri parklar özellikle çocuklar açısından büyük risk taşımaktadır. Bu çalışmanın sonuçları, Ağrı'da parkların kontaminasyonunda köpek ve kedilerin rolünün önemli olduğunu ve bu hayvanların dışkılarıyla dışarı atılan zoonoz parazit yumurtalarıyla kontaminasyonuna karşı gerekli önlemlerin

alınmadığını, bu durumun özellikle çocuklar açısından ciddi anlamda risk oluşturduğunu göstermektedir. Bu nedenle söz konusu enfeksiyonların insanlardaki durumunun belirlenmesi, sokak hayvanlarına ilaç uygulamalarının ve periyodik kontrollerinin yapılması, hayvan sayılarının azaltılması amacıyla kısırlaştırılması, parkların etrafının çitle çevrilmesi gibi kontrol ve mücadele yöntemlerinin uygulanması, parazitlerin ildeki yaygınlığı konusunda eğitim programları ile özellikle çocukların bilinçlendirilmesi, hijyen kurallarına dikkat edilmesinin sağlanması ve toplumsal farkındalık oluşturulması gerekmektedir.

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Fire-associated bear mortalities - Histopathological study

Research Article

ABSTRACT

In the study, it was aimed to describe the histopathological findings in bears that died as a result of smoke poisoning in natural fire deaths, unlike model studies. Himalayan (n=3) and brown bears (n=7) that died due to smoke in the fire were brought for necropsy. Macroscopically, there were no burns or injuries on the bearskins. The lumens of the trachea were filled with edema and had petechial to ecchymotic hemorrhages. There were hemorrhage areas ranging from the size of a pinhead to large areas of ecchymosis, spreading diffusely in all lung lobes. Pulmonary emphysemas were found in varying sizes, especially in the distal lobes. In all bronchi, bronchioles, most alveoli, and under the pleura were detected filled areas with erythrocytes. The interalveolar septal regions were noted to be thickened by erythrocyte/inflammatory cell infiltration. Desquamation of lamina epithelialis, edema, emphysema, and carbon pigment in alveolar macrophages and/or free were observed. Since, many studies on smoke inhalation are experimental, in this respect, the death findings noted in this study are thought to be very valuable since the bears died naturally. Additionally, symptoms caused by acute smoke inhalation in bears have been described. To the authors' knowledge, this is the first fire-related study in bears, and it is considered that bears have died from carbon monoxide inhalation.

Keywords: Bear, fire, histopathology, mortality, smoke inhalation

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INTRODUCTION

As it is known, many animals die in fires, especially wildfires. While some animals die from smoke poisoning, some die from burning in the fire (Jordaan et al., 2020) Inhalation of smoke can cause various acute and chronic lung diseases in surviving animals. In addition to experimental animals (Dong et al., 2015), various animal models have been developed, including both small and large animals (Reczyńska et al., 2018). Thus, these models have enabled a better understanding of the mechanism, pathogenesis and pathophysiology of damage caused by smoke inhalation and the development of new treatments. However, unfortunately, these models do not fully reflect the natural and real consequences of fire.

Animals can tolerate a maximum body temperature of approximately 50°C. As the temperature increases, cells denature proteins and become inactive rapidly, thus disrupting the membrane structure (Schmidt-Nielson, 1979). The length of time that an animal is exposed to high temperature, anoxia, or smoke is crucial (Engstrom, 2010; Whelan, 1995). Increased carbon dioxide and carbon monoxide resulting from smoke inhalation cause decrease in oxygen binding to hemoglobin, thus improving oxygenation in the tissue. This causes lung damage (Rutter et al., 2013). Early and late complications occur in the respiratory system due to smoke inhalation. Pneumonia and airway obstruction are from early complications. Bronchiolitis obliterans, tracheal stenosis, sepsis, acute lung injury and acute

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respiratory distress syndrome are also delayed complications (Reczyńska et al, 2018; Wohlsein et al., 2016).

Early and late complications occur in the respiratory system due to smoke inhalation. Pneumonia and airway obstruction are from early complications. Bronchiolitis obliterans, tracheal stenosis, sepsis, acute lung injury and acute respiratory distress syndrome are also delayed complications (Reczyńska et al, 2018; Wohlsein et al., 2016). The thermal damage occurring here activates the inflammatory response. The functions of ciliary cells and alveolar macrophages decrease, and surfactant production is impaired. Thus, predisposing those affected by smoke inhalation to develop respiratory infections (Dries and Endorf, 2013; Wohlsein et al., 2016).

The aim of the study is to describe the histopathological findings in bears that died as a result of smoke poisoning in natural fire deaths, unlike model studies.

MATERIALS AND METHODS

It is unknown how the fire started, but it was reported that 10-bears died due to smoke inhalation in the fire that broke out in the municipal shelter where circus animals were kept. The chipboards in the shelter where the bears stayed caught fire, and the animals inside were affected by the smoke. According to the information given by the authorities, it was reported that 9 bears died there due to smoke intoxication in the fire that broke out at night. The bears were brought for necropsy in the afternoon of the same day. However, the 10th bear, which was affected by the fire but survived, died 5 days later and was brought for necropsy. Himalayan bears (n=3) and brown bears (n=7) were 1 (n=2), 7 (n=2), 9 (n=2), 19 (n=1), and 24 (n=3) years old. Three of the bears were males and 7 of them were females. After the fire, all the bears died and were brought to the Pathology Department for necropsy. After necropsy, tissue samples were fixed in 10% neutral buffered formalin. Then routine process, the samples were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin-eosin (HE).

RESULTS

Macroscopic findings

There were no burns or injuries on the bearskins. The fattening status of the cadavers was normal, and their death stiffnesses occurred. The tracheal mucosa showed markedly hyperemic and petechial to ecchymotic hemorrhages. Additionally, the lumens of the trachea were filled with foamy exudate (Figure 1A).

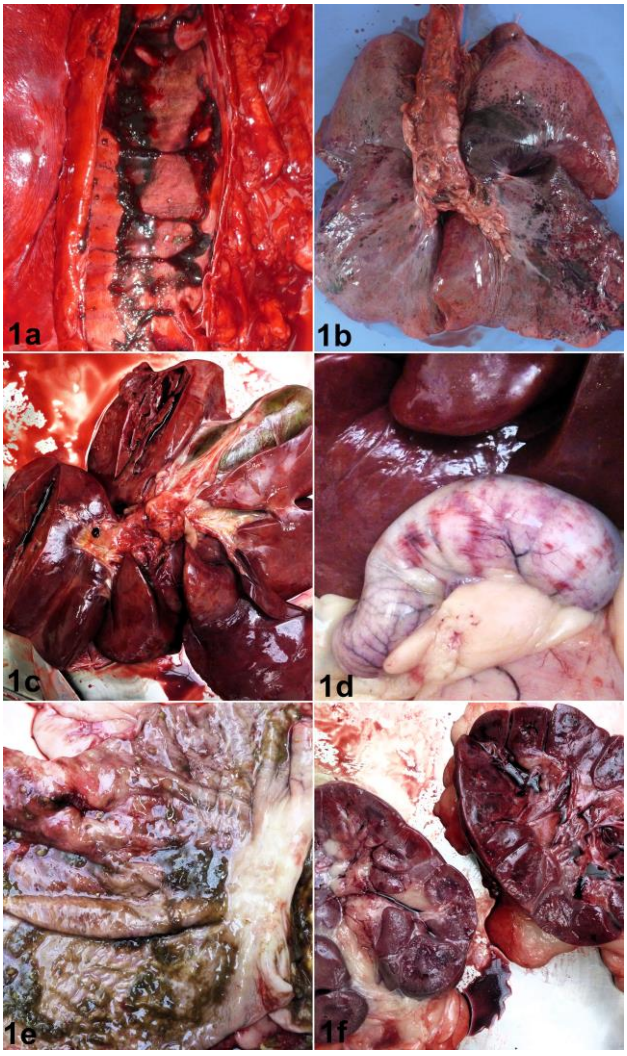

Figure 1. The lumen of the trachea filled with edema and hemorrhage (A), hemorrhage areas ranging from the size of a pinhead to large areas of ecchymosis, spreading diffusely in all lung lobes (B). The blood coming from the cut sections of the liver (C) and kidneys (F), hemorrhage areas at gastric (D) and intestinal mucosa (E).

There were hemorrhage areas ranging from the size of a pinhead to large areas of ecchymosis, spreading diffusely in all lung lobes, including

cyanotic areas in places (Figure 1B). Pulmonary emphysemas were found in varying sizes, especially in the distal lobes. Bullous emphysema was observed in only one of the animals. The livers were swollen, and blood was coming from the cut sections of the livers (Figure 1C). In addition, gastric and intestinal mucosa were hyperemic and had hemorrhage areas (Figure 1D-E). The blood vessels of the brains were swollen and hyperemic. The cut sections of the kidneys were also hyperemic (Figure 1F).

Microscopic findings

In all bronchi, bronchioles, most alveoli, vessels and under the pleura were detected filled areas with erythrocytes (Figure 2A) (n=10).

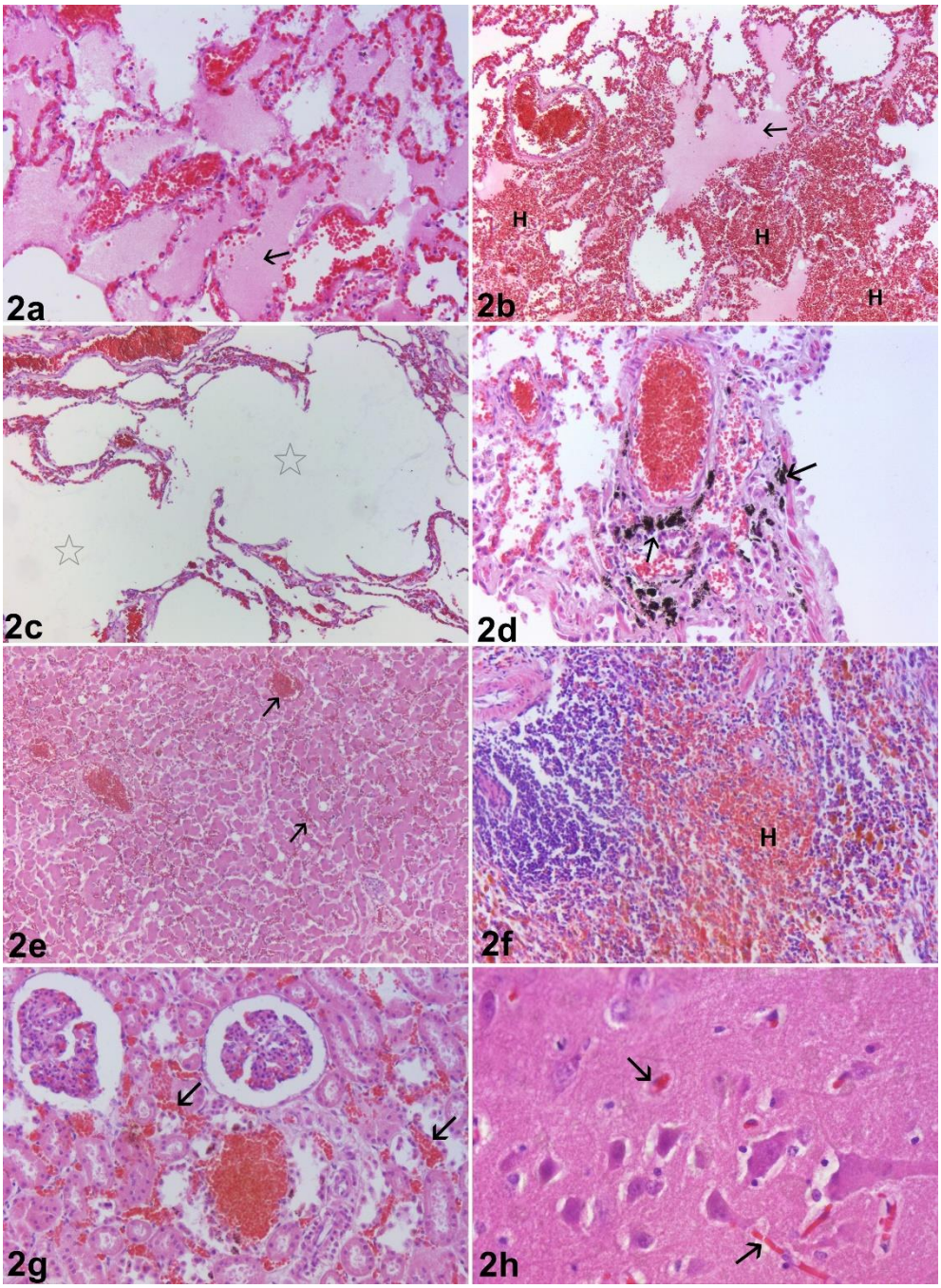

Figure 2. Edema (black arrows) and erythrocyte-filled vessels, bronchioles, alveoli, and hemorrhage (H) areas (A-B), emphysema (stars) in alveoli (C) and carbon pigments (anthracosis) (black arrows) as free and/or in alveolar macrophages (D). Vessels and sinusoids in the liver filled with erythrocytes (black arrows) (E), hyperemic vessels (black arrows) and hemorrhages (H) areas in the spleen (F) and kidneys (G), hyperemic vessels (black arrows) in the brain (H).

The interalveolar septal regions were noted to be thickened by erythrocyte/inflammatory cell infiltration (n=10). The lamina epithelialis layers in some areas were shed to lumens of the bronchi and bronchiole (n=5). Edema was observed in the lumens of some alveoli (n=8) (Figure 2B). The alveoli had an emphysematous appearance in many areas (Figure 2C). In addition, the presence of carbon pigment in alveolar macrophages and/or free (Figure 2D) was determined around the vessels and in interalveolar regions in some areas (n=6).

The vessels and sinusoids in the livers of all animals were filled with erythrocytes. Remak cords were disorganized, and hepatocytes were quite swollen (n=10) (Figure 2E).

In addition, the vessels of the intestine, stomach, spleen (Figure 2F), kidneys (Figure 2G), and heart were hyperemic, and hemorrhages were observed in some areas of these organs (n=10).

The vessels of all brains were severely hyperemic (Figure 2H). While hemorrhage areas were encountered in some areas, perivascular edema was noted in many areas. Vacuolar degeneration was frequently observed in neurons (n=10).

DISCUSSION

Gas inhalation and smoke, followed by respiratory failure, are the important causes of death in living things due to fire (Reczyńska et al., 2018). The degree of inhalation injury depends on the burning material and the components of the inhaled gas. Lower airway damage from smoke inhalation is attributed to toxic gases causing asphyxiation, systemic toxicity, or direct damage to respiratory tissue (Savolainen et al., 1997; Walker et al., 2015). Two known toxic gases are very important in fires, carbon monoxide (CO) and cyanide. Of these, CO is released in slowly burning fires and is lethal to all animals as well as humans. The main cause of death in house and room fires is CO poisoning. CO has approximately 250 times greater affinity for hemoglobin than oxygen, leading to anoxia or hypoxia resulting from failure to oxygenate the blood (Rodkey et al., 1974; Wohlsein et al., 2016). In the study, a fire broke out in the room where the bears were kept, and they were found dead. Therefore, first, CO poisoning was come to mind. Because bearskins were not affected by the fire. There were no wounds or burn marks. Histopathological examinations showed that the bears likely succumbed to smoke inhalation during the fire, as carbon particles were present in their lungs and respiratory tract. The limitation of the study is that since the animals were brought to necropsy dead, unfortunately, their blood could not be examined for carbon monoxide poisoning.

Sheep is the most common animal used in smoke inhalation studies. Hubbard et al. (1991), in a study on 57 sheep, evaluated them for periods ranging from 15 minutes to 4 weeks after smoke inhalation. They stated that necrosis and shedding of the respiratory tract epithelium are the most important lesions caused by smoke inhalation. These findings were seen even in sheep exposed for 15 minutes. Mongrel dogs (Brizio-Molteni et al., 1984) and the New Zealand white rabbits (Thorning et al., 1982) exposed after inhalation of pinewood smoke. Lungs of dogs were examined after 30 min and rabbits up to 72 h after smoke exposure. Extensive hemorrhage and oedema of canine lungs and focal necrosis of rabbit lungs were detected. But, after such a short time, no significant changes to tracheal and/or airway epithelium were found. in dogs, the alveolar spaces were filled with erythrocytes and edema. Several neutrophilic granulocytes were present at some areas. However, no necrosis was encountered in the lungs in this study. But the presence of large amounts of erythrocytes and edema in the lumens of the majority of bronchi, bronchioles and alveoli in the lungs was noted. This was similar to the findings of the mentioned studies. On the other hand, in this study, not only the lung findings in the animals, but all organs

(liver, brain, kidney etc.) were examined in detail and hemorrhage areas and hyperemia were found. In this case, it adds difference to the study.

In the other experimental study (Zhu et al., 2012), for experiment group (9 min time of smoke inhalation) used 48 rats. After smoke inhalation, six rats were killed each time in the at 2 h, 4 h, 6 h, 24 h, 48 h, 96 h, 7 days, and 28 days. When lung histopathologically examined for the effects of smoke inhalation, rat lungs had extensive hemorrhage and accumulation of black particles. 24 hours after smoke inhalation, inflammatory exudates and diffuse hemorrhage were noted, with marked edema in the lungs. At 96 hours, these findings disappeared, and alveolar collapse occurred with partial thickening of the alveolar septum (Zhu et al., 2012). The findings encountered in this study are consistent with those observed in the first 24 hours. Anthracosis pigments, edema, emphysema, and hemorrhage were the most prominent findings. But there was no alveolar septum thickening. It is thought that this situation was not seen because there were no living bears after the fire. Maybe if they had lived for 96 hours, similar findings would have been seen. However, the findings seen in bears that died quickly from smoke inhalation at the fire scene are quite similar to the findings seen in rats in the first 24 hours of the experimental study.

CONCLUSION

Many studies on smoke inhalation are experimental. In this respect, the death findings noted in this study are thought to be very valuable since the bears died naturally. Additionally, symptoms caused by acute smoke inhalation in bears have been described. To the authors' knowledge, this is the first fire-related study in bears, and it is considered that bears have died from carbon monoxide inhalation.

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Conflict of interest: The authors declared that there is no conflict of interest.

Ethical statement: This study was approved by the Ankara University Animal Experiments Local Ethics Committee, Ankara, Türkiye (Approval no: 2023-18-161).

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Microbiological analysis of commercial calf milk replacer and antibiotic resistance in isolated *Enterococcus* spp.

ABSTRACT

One of the reasons why calf milk replacer is preferred over unpasteurized bulk tank milk or waste milk with antibiotics on farms is that it prevents epidemic diseases and antibiotic resistance that may occur on the farm. In this study analyzed commercial calf milk replacer products (n = 12) obtained from dairy farms around Türkiye by microbiological culture and polymerase chain reaction (PCR). In order to evaluate the microbiological quality of calf milk replacer, total bacteria count, coliform *E. coli* and *E. coli* O157-H7, *Salmonella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp. analyses were performed according to microbiological analysis methods determined according to ISO standards. *Enterococcus* spp. was isolated from all 12 calf milk replacer samples analyzed and molecularly confirmed by PCR with the presence of the gross-Es gene. *Salmonella* spp., *E. coli*, *Staphylococcus* spp. and *Streptococcus* spp. were not isolated from the samples. Additionally, in the bacterial counts, an average of $5.3x10^7$ Enterococci were counted from all samples in 1 g of calf milk replacer. Antimicrobial analysis of the isolated bacteria was completed according to CLSI 2022 data, and 11 isolates were defined as multi drug resistance, and one isolate was defined as extensive drug resistance. It was also determined that the isolate defined as extensive drug resistance was resistant to Vancomycin and carried the Van A resistance gene. Many proteins used in the preparation of calf milk replacers are of animal origin and may contain pathogenic bacteria. It is known that milk replacers affect microbiota. It was shown in this study that if calf milk replacers are not prepared under the regulations, they may cause harm rather than benefit to on-farm biosecurity factors. It is concerning that calves are given calf milk replacers containing antibiotic-resistant *Enterococcus* spp. to sustain their lives when they are most vulnerable to disease during the window of susceptibility. When using calf milk replacer in calf feeding, veterinarians should be informed about the microbiological certification of the product and provide information about pasteurization and presentation for consumption.

Keywords: Antibiotic resistance, calf milk replacer, vancomycin resistance Enterococci

INTRODUCTION

Calves can only digest milk or protein in liquid form. A study conducted by USDA 2014 determined that 63.9% of medium-sized dairy cattle farms fed calves with milk replacers (USDA, 2016). Calf milk replacers are usually prepared from whole milk powder, skim milk powder, industrial residues such as casein, whey, and whey protein, plant oil, animal plasma, soybean protein, essential amino acids Lecithin, L-Lysine, DL-Methionine, vitamin and mineral supplements (USDA, 2018). Dairy calves are fed milk replacer until weaning time, especially for economic reasons (when milk is more expensive than milk replacer) (Wood, 2022). Also, farms can use milk replacers in calf feeding to protect against pathogens such as *Salmonella* spp., *Mycoplasma* spp., *Brucella abortus*, *Mycobacterium avium* ssp. *Paratuberculosis* and bovine viral diarrhea virus (BVDV), which are shed with milk (Maunsell

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Research Article

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and Donovan, 2008). *E. coli* and *Enterococcus* spp. found in the flora of the gastrointestinal tract of cattle are opportunistic pathogens (Silva et al., 2012). Especially in bulk-tank milk with high fecal contamination, these agents are more likely to cause infection in calves (Aust et al., 2013). Enterococci are considered non-infectious, however can cause nosocomial infections in humans due to the development of antibiotic resistance mechanisms (Ben Braiek and Smaoui 2019).

Before being placed on the market, milk replacer products must document the results of the microbiological analysis in terms of pathogenic bacteria, and total mesophilic bacteria count, and coliforms (Scheid, 2012). However, the animal-derived products contained in milk replacers also bring a bacterial load (Cooper and Watson, 2013). Soy-derived products, whey, and animal feed containing animal-derived proteins contaminated with *Salmonella* spp. may cause disease in animals that consume the feed (McGuirk, 2008). Calf milk replacers not prepared under the regulations may pose a biosafety threat. Calves are sensitive to pathogens close to the weaning period because of the immunological perspective called the window of susceptibility (Chase et al., 2008). For this reason, some products use antibiotic calf milk replacer in specific proportions, but this solution comes with antibiotic resistance (Langford et al., 2003).

The study aims to investigate microbiological analysis and the antimicrobial resistance of bacteria isolated from commercially available milk replacers in Türkiye.

MATERIALS AND METHODS

Microbiological analysis

Twelve different calf milk replacers available in the market in Türkiye were collected, and the products were numbered 1-12 due to commercial concerns. Calf milk replacer samples were collected according to the sample collection procedures described in ISO 6579-1:2017 (ISO, 2017). Twenty-five g of calf milk replacer was enriched in 225 mL peptone water (CM1049, Lab M) at 37°C for 18 h. As a result of pre-enrichment, 1 mL of enriched culture was passaged into 9 mL of Rappaport Vassiliadis Soy Broth (HP007, LabM). It was incubated at 42°C for 24 h. On the third day, a loopful of this culture was passaged onto XLT-4 agar (CM1061, Thermo Scientific), incubated at 37°C for 24 h and black colonies were tried to be identified (Hadimli et al., 2017). The enriched culture was also passaged onto blood agar for general mesophilic pathogenic bacteria analysis and incubated for 48 hours at 5% CO_2 (Jinneman, 1998). The number of coliform bacteria was detected according to ISO 4832:2013 guidelines (ISO 4832, 2013). MacConkey agar (70143, Sigma Aldrich), Eosin-Methylene Blue agar (70186, Sigma Aldrich) were used for the detection of coliform bacteria and sorbitol MacConkey agar for the detection of *E. coli* O157-H7. Tryptic Soy Agar %5 sheep blood (22091, Sigma Aldrich) was used determine the bacterial load in the gram calves milk replacer, which was determined using the 10-fold dilution method according to ISO 4833-1:2013 guideline (ISO, 2013). Modified Edwards medium (CM0027B, Thermo Scientific) was used to detect *Enterococcus* spp. The isolates were differentiated from *Streptococcus* spp. by the bile-esculin test (Aun et al., 2021).

Antimicrobial susceptibility test

The antibiotic susceptibility of the isolates was determined by the Kirby-Bauer disk diffusion method (Bauer et al., 1966). For the antimicrobial susceptibility test, antimicrobial agents were selected for gastrointestinal system infections of calves. Antibiotic disc used in this study list with abbreviation: VA: Vancomycin, S: Streptomycin, P: Penicillin, AMC: Amoxacillin, RA Rifampicin, L: Lincomycin, TE: Tetracycline, IPM: Imipenem, SXT: Trimethoprim / sulfamethoxazole, CN: Gentamicin, ENR: Enrofloxacin, E:

Erythromycin. Zone diameters were recorded in mm, and antibiotic resistance was determined according to the clinical breaking point reference values prepared for veterinary isolates by the Clinical and Laboratory Standards Institute (CLSI, 2022).

Identification of Enterococcus and AMR with molecular methods

DNA was isolated from the *Enterococcus* spp., according to the Wizard® Genomic DNA Purification Kit Gram-Positive bacteria protocol. The concentrations of the isolated DNA were determined by spectrophotometer (Nanodrop 2000, Thermo Scientific). All *Enterococcus* isolates were PCR confirmed with the forward primer ACAGTTGTTGCAGTCGGTGA and the reverse primer ACATAACTTGGTCGCCTGCT, which was prepared to specifically give an 85 bp PCR product according to the NCBI accession number AY328542 1 for the groES gene. In addition, isolates were screened by PCR for the presence of the vancomycin resistance gene van A (667 Bp), CP036247 1, and NCBI accession number forward primer GTTGCAATACTGTTTGGGGGT reverse primer CAACTAACGCGGCACTGTTT. The primer sets were designed by NCBI primer blast for this study. For the PCR mixture, five µL master mix (5x) (Solis Biodyne, Estonia), one µL forward primer from 10 µM working stock, one µL reverse primer from 10 µM working stock, four µL DNA (25 ng/ µL) 15.9 µL sterile nuclease-free water were added for a total volume of 25 µL. The thermal cycle (T100, Bio-Rad) was repeated 34 times with a pre-denaturation step at 94°C for 10 min, followed by 94°C denaturation for 1 min, 60°C binding for 1 min, 72°C extension for 1 min, and final extension at 72°C for 10 min. A 1% agarose gel was prepared for electrophoresis (maxicell-minicell, EC Apparatus Corporation) of PCR products. Ethidium bromide was added to the gel to a final concentration of 0.5 µg/ mL. Gel wells were loaded with five µL each of PCR products and 100 bp DNA ladder (Solis Biodyne, Estonia). The results were visualized by a gel imaging device (212 Pro, Gel-Logic).

RESULTS

The isolates were negative for *Salmonella* spp., coliform *E. coli*, and *E. coli* O157-H7. However, as a result of selective media analysis and identification, *Enterococcus* spp. was isolated from all milk replacer samples. In the total bacterial count analysis, a calf average of 5.3 x 10^7 CFU *Enterococcus* was counted in 1 gram of calf milk replacer (Table 1).

Table 1. Bacteria isolated from calf milk replacers, total number of bacteria per gram

Calf Milk Replacer Product Number	Isolated bacteria	gr/CFU
1	*Enterococcus* spp.	$7x10^7$
2	*Enterococcus* spp.	$4.2x10^7$
3	*Enterococcus* spp.	$3.6x10^7$
4	*Enterococcus* spp.	$8.2\ x10^7$
5	*Enterococcus* spp.	$4.2\ x10^7$
6	*Enterococcus* spp.	$5x10^7$
7	*Enterococcus* spp.	$3x10^7$
8	*Enterococcus* spp.	$7.6x10^7$
9	*Enterococcus* spp.	$6x10^7$
10	*Enterococcus* spp.	$4.7x10^7$
11	*Enterococcus* spp.	$5x10^7$
12	*Enterococcus* spp.	$5.5x10^7$

According to the antimicrobial susceptibility test result, vancomycin resistance was detected in one isolate. One of the isolates (sample 12) was detected as extensive drug (XDR) resistance, and 11 were seen as multidrug resistance (MDR). Although the isolates were found to be sensitive to penicillin and enrofloxacin, imipenem, rifampicin, and vancomycin (only isolate number 12), which can be used in emergencies for humans, were resistant (Table 2). The isolates were confirmed to be *Enterococcus* spp. molecularly by PCR in terms of the presence of the Gros-ES gene Figure 1.

Table 2. Antimicrobial susceptibility test results of *Enterococcus* spp. isolated from calf milk replacers

Calf Milk Replacer Product Number	VA-30	S-10	CN-30	AMC-30	P-10	E-15	TE-30	RA-5	IPM-10	L-2	SXT-25	ENR-10
1	S	R	R	R	S	R	R	R	R	R	R	S
2	S	R	R	R	S	I	R	R	R	R	R	S
3	S	R	R	R	S	I	R	R	R	R	R	S
4	S	R	R	R	S	I	R	R	R	R	R	S
5	S	R	R	R	S	I	R	R	R	R	R	S
6	S	R	R	R	S	I	R	R	R	R	R	S
7	S	R	R	R	S	S	R	R	R	R	R	S
8	S	R	R	R	S	R	R	R	R	R	R	S
9	S	R	R	R	S	R	R	R	R	R	R	S
10	S	R	R	R	S	R	R	R	R	R	R	S
11	S	R	R	R	S	I	R	R	R	R	R	S
12	R	R	R	R	S	R	R	R	R	R	R	S

VA: Vancomycin, S: Streptomycin, CN: Gentamicin, AMC: Amoxacillin, P: Penicillin, E: Erythromycin, TE: Tetracycline, RA: Rifampicin, IPM: Imipenem, L: Lincomycin, SXT: Trimethoprim/sulfamethoxazole, ENR: Enrofloxacin.

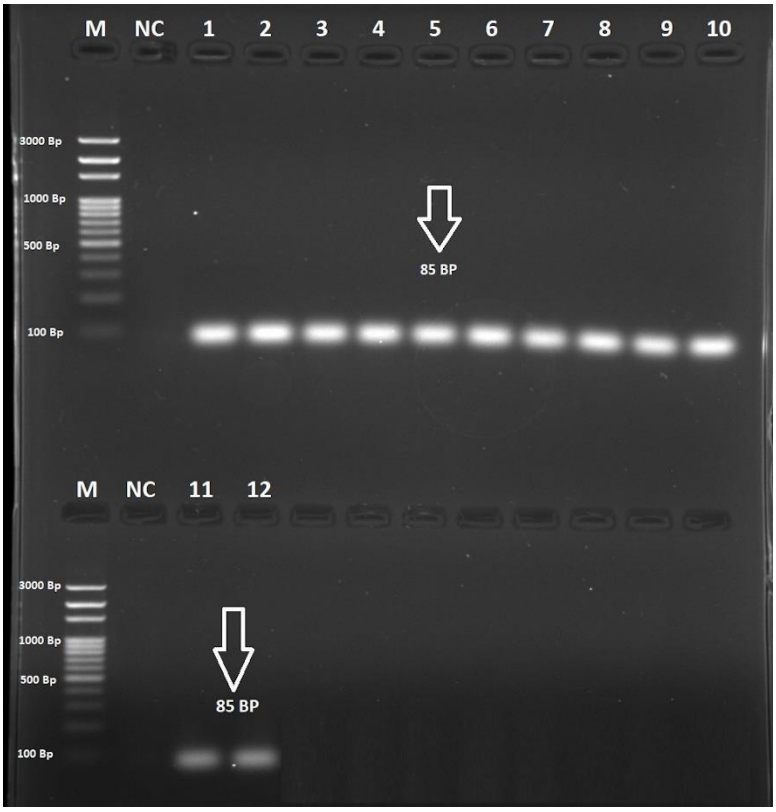

Figure 1. PCR gel image of Enterococcus spp. isolated from calf milk replacers in terms of Gros-ES gene. M: Marker (100 bp), NC: Negative control, Line 1-12: Samples.

The isolates were also screened by PCR for the Vancomycin resistance gene Van A, which is one of the most critical parameters for Enterococcus, and the presence of this resistance gene was detected only in isolate number 12 (Figure 2).

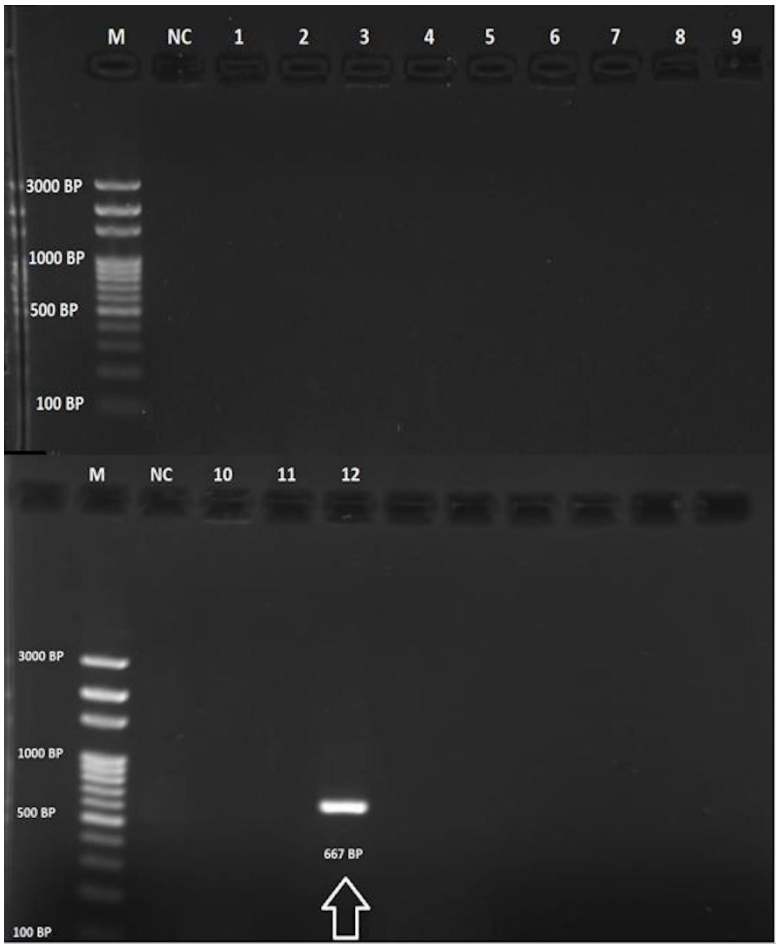

Figure 2. PCR gel image for the Van A gene. M: Marker (100 bp), NC: Negative control, Line 1-12: Samples.

Enterococcus spp. obtained from calf milk replacer number 12 was found to be resistant to vancomycin both with its antimicrobial susceptibility and molecular Van A gene.

DISCUSSION

According to USDA regulations, calf milk replacer must be negative for *Salmonella* (Scheid, 2012). In the study hypothesis, the main isolated was thought to be *Salmonella* because it is known to be abundant in soy-based products, whey. However, in the study, no *Salmonella* was isolated from the calf milk replacers found in the market. In another study conducted in the USA in 2014, *Salmonella* was detected in 5.5% of 55 milk powders and dried whey facilities (Hayman et al., 2020). In another research conducted due to *Salmonella* cases in salmonella-free pig farms in Sweden, 28 different plant-derived *Salmonella* were isolated in samples taken from feed made from imported soybeans in pig feed products (Wierup and Häggblom, 2010).

Enterococcus spp. was isolated from all samples in the study. Although it does not act as the primary pathogen and can be found in the flora, it is thought that it has the power to change the intestinal microbiota and may cause diarrhea and productivity losses. A study investigating how the fecal microbiota of postpartum calves changes within the first week reported that *Enterococcus* spp. was isolated in

the flora starting from the first 6 hours of the birth of a healthy calf (Schwaiger et al., 2020). In microbiota studies conducted on newborn calves, it has been reported that calf milk replacer allows more microflora development compared to the cow milk diet group due to its prebiotic effect and nutritional diversity (Badman et al., 2019; Kumar et al., 2021). Although it does not act as the primary pathogen and can be found in the flora, it is thought that it has the power to change the intestinal microbiota and may cause diarrhea and productivity losses. The slightest change in the GUT microbiota affects the entire flora. In the other study, an increase in the number of *E. coli, Enterococcus spp.*, and a decrease in the number of other beneficial bacteria were detected, depending on the type of antibiotic used (Amin and Seifert, 2021).

Although it is not known how *Enterococcus* spp. contaminates the calf milk replacer, it is thought that it may originate from residues of dairy products such as whey. In their studies, other researchers isolated *Enterococcus* spp. from dairy products and whey (de Sousa et al., 2020; Muguerza et al., 2006). Another study reported that it is included in the starter culture for many cheeses, such as Mozzarella, and can be found in large amounts in whey because it is a thermo-resistant structure (Giraffa, 2003). It was thought that the reason why the number of *Enterococcus* spp. isolated in the study was so high that it was related to the method of obtaining this cheese and whey.

It is a known fact that the multitude of antibiotics used in farm animals indirectly threatens antibiotic resistance in humans. For economic reasons, some farms offer waste antibiotic milk to calves. However, this situation brings with it antibiotic resistance. In a study conducted on 114 calves, it was determined that antibiotic resistance increased in the *E. coli* shed by the feces of calves fed with antibiotic waste milk for 52 days (Aust et al., 2013). To prevent this, farms are recommended to use calf milk replacer (Firth et al., 2021). However, the antibiotic resistance of Enterococcus bacteria isolated from calf milk replacer in the study is thought-provoking. To date, there are nine different types of vancomycin resistance in enterococci. Among these, the three most common variants are types of van A, B and C. Vancomycin is an antimicrobial effective against most Gram-positive bacteria (Cetinkaya et al., 2000). It is considered a 'drug of last resort' and is classified as critically important to human medicine. Therefore, the presence of this resistance gene in enterococcus is considered alarming, and there are many studies on it in farm animals (Nilsson, 2012). Although Enterococci seems to be a commensal agent, its presence in calf milk replacer should be reconsidered by the authorities when considered together with antibiotic resistance.

CONCLUSION

Although *Enterococcus* spp. can be found in the flora of newborn calves, it is thought that it may have a pathogenic effect by changing the microflora structure when given to calves in high numbers under inappropriate conditions. Also, as a result of the study conducted, it was observed that calf milk replacers may pose a biosecurity and antimicrobial resistance threat. It is recommended to use this type of calf milk replacer by pasteurizing it before feeding the calf. In addition to such products, additional probiotic supplements can be recommended to encourage bacterial competition and exclusion for proper rumen and microbiota development.

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