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Research Article

Investigation of plasma pepsinogen level in calves with abomasal distention

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ABSTRACT

The aims of the present study was to determine whether or not abomasal damages occurs in calves with abdominal distention using by plasma pepsinogen levels and the positivity of the fecal occult blood test (FOBT). In the study, 30 calves with abdominal distention (experimental group) and 15 clinically healthy calves (control group), aged between 1-90 days, were used. Plasma samples were used to determine plasma pepsinogen levels, using bovine specific ELISA. Fresh stool samples used to determine occult blood in the samples. Melena and occult blood were detected in 11 and 19 of the stool samples, respectively. The plasma pepsinogen levels of calves with abdominal distention (18.06±7.78 ng/ml) were significatly high compared to those of control group (6.70±2.08 ng/ml), ($p<0.001$). In addition, plasma pepsinogen levels were higher in melena (+) animals than both FOBT (+) animals and control animals ($p<0.001$). It is suggestive that plasma pepsinogen levels could be used to determine abomasal damage in calves, especially with abdominal distention. It can be also useful biomarker to determine the severity of abomasal damage in calves.

INTRODUCTION

Abdominal distention is a general symptom of gastrointestinal disorders in cattle (Peek and Divers, 2018). The main cause of abdominal distention in ruminants is excessive gas production due to abnormal fermentation in the rumen, resulting in ruminal tympany. Certain disorders cause abdominal pain including abomasal ulcers, displacement of the abomasum, intestinal volvulus, and torsion also cause abdominal distention in cattle (Gouda et al., 2020; Radostits et al., 2007).

Abomasal ulcer is one of the causes of abomasal distension in cattle of all the ages. Signs of abomasal epithelial damage can range from the absence of clinical signs to hemorrhage followed by melena and peritonitis if erosive course spread all stratums of the abomasum. In recent years, the frequency of abomasal mucosal diseases in cattle is becoming more endemic with the transition to contemporary compact manufacture (Cable et al., 1998; Radostits et al., 2007).

Detection of abomasal ulcers is often confused with other causes of alimentary disorders with symptoms of indigestion. The clinical symptoms of abomasal ulcers are nonspecific and so variable, which are take place on a very wide scale (Cable, 1998; Radostits et al., 2007). Detection of abomasal ulcers is based on clinical symptoms and fecal occult blood test (FOBT) (Hajimohammadi et al., 2017). However, clinical symptoms are not suffucient for its definitive diagnosis and some abomasum specific biomarkers such as pepsinogen and gastrin need to be analysed.

Pepsinogen is an inactive form of pepsin, which is produced by parietal cells of the abomasal mucosa. Elevated serum pepsinogen activity indicate the presence of abomasal ulcer and also, in some cases, to the presence of other damage to the abomasal mucous membrane (Mesaric, 2005). The increase in plasma pepsinogen level occurs via leakage of pepsinogen from the damaged abomasal mucosa into the blood vessels (Harvey et al., 1983). It is well-known that, increased acidity of stomach contents increase activation of pepsinogen to pepsin, results in ulcers formation in humans and animals (Kataria, 2008; Mesaric, 2005). Biomarkers such as plasma gastrin and pepsinogen (Mesaric et al., 2000, Ok et al., 2001; Zadnik and Mesaric, 1999) have been studied in some gastrointestinal disorders such as abomasum displacement and abomasum ulcer in cattle. However, plasma pepsinogen levels have not been analysed in calves with abdominal distention, associated with abomasal ulcers or other gastrointestinal disorders. On the other hand, plasma pepsinogen levels has been suggested to be a suitable markers for evaluating the degree of gastroentestinal damages (Mesaric, 2005; Kataria, 2008).

The aims of the present study was to define whether or not abomasal damages occurs in calves with abdominal distention using by plasma pepsinogen levels and the positivity FOBT.

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MATERIALS AND METHODS

Animals

In the study, 30 calves with abdominal distension (experimental group) and 15 clinically healty calves (control group), aged between 1-90 days, were used. Routine clinical examinations including body temperature, hearth rate, respiratory rate, skin elasticity test, and control of capillary filling time, mucous membranes, defecation and teeth grinding were performed in all the calves. Presence of rumen atony, abdominal tension, groaning sound on deep palpation over the abomasum from the right side were also examined.

According to the abdominal examination, right or both sided abdominal distention were diagnosed in 30 calves without enteritis and then they were used as experimental group. Furthermore, according to the macroscopic stool inspection and FOBT, 30 animals with abdominal distention were divided into two groups as melena positive (n=11) and FOBT (n=19).

Laboratory analysis

Hematology

Blood samples were collected into blood tubes with K_3EDTA (Greiner Bio-one, Austria). These blood samples were used to establish total white blood cell, granulocyte, lymphocyte, monocyte and red blood cell counts and percentage of hematocrit (Abacus Junior, Diatron MI, Hungary)

Pepsinogen analysis

Blood samples were withdrawn from vena jugularis into heparinized blood tubes (Greiner Bio-one, Austria) and centrifuged to collect plasma samples. Collected plasma samples were kept at -80 ^{o}C until used. The pepsinogen levels were determined by using bovine specific ELISA kits (MyBiosource, San DiegoUSA) according to the producer directives.

The optical density (OD) of each well for pepsinogen was defined with a micro-ELISA plate reader (MR-96A, Mindray-China) at a test wavelength of 450 nm. The concentrations of plasma pepsinogen was calculated regression analysis on the basis of standard curve derived from two-fold dilutions of pepsinogen standard stock solution. The sensitivities of the ELISA kit for pepsinogen was 0.5 ng/ml.

Fecal occult blood test (FOBT)

Rectal fresh stool samples were collected from each animal and used to defined blood in stool samples, using FOBT (Gikan tets, Türklab, İzmir, Turkey) according to the manufacturer instructions.

Statistical analysis

Kolmogorov Smirnov test was used to define normality of distribution of the data. The significance of the differences in values between experimental and control groups were defined by Student’s t test. One way Anova (posthoc Duncan) test was used to compare the differences in values between control, melena (+) and FOBT (+) calves. All the values were

Table 1. Hematologic values in control and experimental group (Mean±SD).

Parameter	Control Group (n:15)	Experimental Group (n:30)	p value
WBC ($x10^9$/l)	9,63±2,39	10,06±5,56	0,722
LYM ($x10^9$/l)	5,61±1,30	4,88±1,82	0,132
MID ($x10^9$/l)	0,17±0,17	0,24±0,31	0,290
GRA ($x10^9$/l)	6,17±8,67	4,94±4,44	0,612
RBC ($x10^{12}$/l)	8,37±0,93	7,77±1,19	0,075
HGB (g/dl)	8,94±1,49	8,59±1,46	0,466
HCT (%)	31,03±5,39	29,89±4,72	0,491
MCV (fl)	36,93±4,62	38,60±4,23	0,252
MCH (pg)	10,70±1,02	11,15±0,97	0,175
MCHC (g/dl)	29,05±1,26	29,00±1,28	0,902
RDWc (%)	29,66±3,80	27,49±3,34	0,072
PLT (x^9/l)	638,00±215,33	585,56±199,92	0,438
PCT (%)	0,43±0,14	0,40±0,14	0,604
MPV (fl)	6,74±0,47	6,91±0,53	0,267
PDWc (%)	33,12±2,09	33,08±2,06	0,944

WBC: total white blood cell count, LYM: lymphocytes count, MID: monocytes and eosinophils, GRA: granulocytes, RBC: red blood cell count, HGB: haemoglobin, HCT: haematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, RDWc: red cell distribution width, PLT: platelet count, PCT: platelet percentage, MPV: mean platelet volume, PDWc: platelet distribution width
Significant level was accepted as p<0.05.

indicated as mean and standard deviations of the mean (mean ± SD). The level of significance was acknowledged as $p<0.05$. SPSS software computer programme (version 14.01 for Windows, SPSS Inc. Chicago) was used to perform all the statistical analyses.

The cut-off value for pepsinogen was determined by adding twice the standard deviation of the control group to the mean of the control group. Concentrations greater than the cut off point (10.87ng/ml) were considered increases for each animal. Thus, the cut off value for pepsinogen was then used to define the number of animals with increased plasma pepsinogen levels (Rastawicki et al. 2011, Sharma and Jain 2013).

RESULTS

Clinical findings

The clinical symptoms were partial or complete anorexia, depression, eye recession into the orbit, increase or decrease

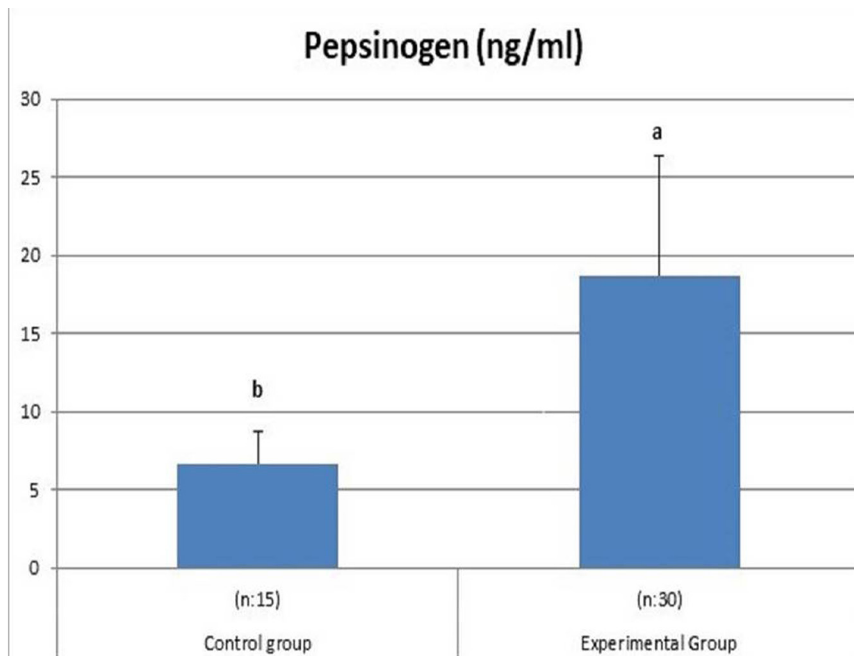

Figure 1. Plasma pespinogen concantrations of control and experimental groups (Mean±SD).

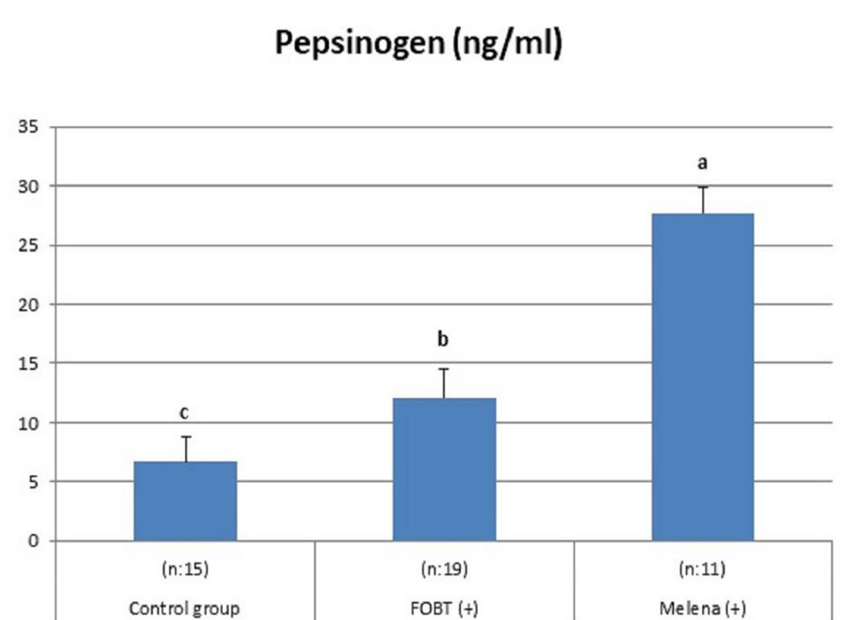

Figure 2. Plasma levels of pepsinogen in control, FOBT (+) and melena (+) calves (Mean±SD).

in appetite and variable heart rate, increase in respiratory rate, decrease in skin elasticity, prolongation of capillary filling time, pale mucous membranes, cold extremities, decreased defecation, and teeth grinding in calves with abdominal distention. In some of these animals, melena, raising their feet towards the abdomen, tachypnea, rumen atony, abdominal tension, groaning sound on deep palpation over the abomasum from the right side and tremors in the muscles were detected.

Laboratory findings

No statistically significant difference was found in the hematological parameters of the experimental and control groups (Table 1). Red blood cell count and hematocrit percentage were tented to be decrease in calves with abdominal distention compared to those of control group. However, red blood cell count and hematocrit percentages of calves with abdominal distention were not statistically different compared to that of control group (Table 1).

In the study, plasma pepsinogen levels were 6.70±2.08 ng/ml and 18.48±7.86 ng/ml in control calves and calves with abdominal distention, respectivelly. The pepsinogen values of calves with abdominal distention were defined to be significantly higher than control group ($p<0.001$) (Figure 1). In addition, 19 calves with abdominal distention were found to be positive for FOBT while, other 11 calves were melana positive. The plasma pepsinogen values in melena (+) calves were significantly high compared to those of FOBT (+) calves and control calves ($p<0.001$) (Figure 2). According to the cut-off value, plasma pepsinogen levels increased in 24 calves with abdominal distention.

DISCUSSION

In the present study, plasma concentrations of pepsinogen were significantly higher in calves with abdominal distention than control group ($p<0,001$) (Figure 1). In addition, 24 calves with abdominal distention had high plasma pepsinojen concentration compared to the cut-off value. Furthermore, plasma pepsinogen values were significantly high in melena pozitif calves compared to those of both FOBT positive and control calves $p<0.001$ (Figure 2). High plasma pepsinogen concentrations are thought to be associated with vascular permeability in the abomasum, such as the melena group in this study, and any damage to the gastric mucosa allows the diffusion of hydrogen ions from the lumen to the mucosal tissues similarly to previous studies. The studies report that the diffusion of pepsin and pepsinogen to the mucosa causes injury to the gastric mucosa (Hajimohammadi et al., 2010; Voros et al., 1984; Zadnik and Mesaric, 1999). These reports also support our results (Figure 2).

Abomasal damage was not confirmed with necropsy in the present study but based on the previous studies in cattle (Hajimohammadi et al., 2010; Voros et al., 1984; Zadnik and Mesaric, 1999), the increase in plasma pepsinogen concentrations detected in this study may indicate the occurrence of abomasal damages in calves.

Abdominal distention is a common manifestation of many gastrointestinal disorders in cattle (Peek and Divers, 2018). Abdominal distention occurs due to abnormal accumulations of food, gas or fluid in the abdominal cavity. This may occur due to accumulation of gases resulting in abnormal fermentation or food retention in case of impaction (Radostits et al., 2007). Furthermore, displacement of the abomasum, volvulus and torsion may also be effective in inducing abdominal distention in cattle (Radostits et al., 2007). Abomasal dilatation can result from mechanical obstruction of the exit from the abomasum at the level of the pylorus (eg, ulcer, foreign body) as well as from the accumulation of feeding, fluid, or gas, or from disruption of the muscular activity of the abomasum by damage to the part of the vagus nerve. Increases in abomasal lumen pressure reduce blood flow to the abomasal mucosa and submucosa. Thus, this pressure predisposes the gastric mucosa to damage and ulceration resulting from the back diffusion of hydrogen ions. (Constable, 1992; Marshall, 2009; Panciera, 2007; Songer, 2005).

In calves, abdominal distention is most frequently seen in the first 1-2 weeks of their life (Jonathan et al., 1987). Common clinical signs in these calves include refusing to drink milk, distending abdomen, grinding their teeth, kicking at the belly, depression, letargy, dropping ears and colic. If these animals are not treated they may die in less than four hours. Abdominal distention may cause various factors and it may be a common indicator of many gastrointestinal disorders in cattle (Peek and Divers, 2018). However, its differential and diagnosis is very diffucult.

Abomasal ulcers in calves are one of the comman complication of abdominal distention, resulting in death in a short period if not treated. Clinical manifestations of abomasal ulcers may be absent or range from haemorrhages and subsequent melena to peritonitis (Cable, 1998; Radostits et al., 2007). The diagnosis of abomasum ulcer is based on clinical symptoms and FOBT. However, clinical symptoms are often quite and non-specific (Mesaric, 2005). Thus, these signs are not sufficient for differential diagnosis of abdominal distention and abamosal ulcers.

Plasma gastrin (Ok et al., 2001) and pepsinogen (Mesaric et al., 2000; Zadnik and Mesaric, 1999) have been studied in some gastrointestinal disorders such as abomasum displacement and abomasum ulcer in cattle. In studies, increases in pepsinogen levels have been detected in cattle and sheep with abomasal ulcers (Hajimohammadi et al., 2010; Mesaric, 2005; Zadnik and Mesaric, 1999). Furthermore, plasma pepsinogen increases have also been shown in cattle with abomasal displacement (Voros et al., 1984) and ostertagia infection (Pitt, 1988). Mesaric (2005) reported that pepsinogen measurement can be a simple serum test for the diagnosis of subclinical abomasal ulcer in cows. Some studies have reported that high pepsinogen concentration may be related to vascular permeability in the displaced abomasum. It has been stated that any damage to the gastric mucosa undergoes diffusion of hydrogen ions from the lumen into the mucosal tissues. In addition, diffusion of pepsin and pepsinogen to the rest of the mucosa has been reported to cause more damage to the mucosa (Hajimohammadi et al., 2010; Hajimohammadi et al., 2017). The increase in abomasum pH is an important stimulant for gastrin secretion

and causes an increase in pepsinogen secretion (Kataria et al., 2008). It is known that increased plasma pepsinogen and gastrin levels indicate abomasal damages in cattle. Thus, these biomarkers have been suggested to be a useful tool for detection of abomasal damages in cattle (Mesaric et al., 2002). However, the role of pepsinogen in the detection of ulcers or damages has not been demonstrated in calves with abdominal distention related to abomasal or other gastrointestinal disorders.

CONCLUSION

In conclusion, high plasma pepsinogen concentrations, melena and a positive FOBT can be used to confirm the suspicion of abomasal damage in calves with abomasal distension. It was found that plasma pepsinogen level could be a useful biomarker to diagnose abomasal damage in calves with abdominal distension.

DECLARATIONS

Ethics Approval

This study was approved by the Local Ethics Committee for Animal Care of the Burdur Mehmet Akif Ersoy University (15/08/2018- decision number: 396). Consent forms were signed by the animal owners.

Conflict of Interest

The authors declare that they have no compet of interests.

Author Contribution

Idea, concept, and design: NM, RY, HİG, TA

Data collection and analysis: NM, RY, HİG, TA

Drafting of the manuscript: NM, RY, HİG, TA

Critical review: NM, RY, HİG, TA

Data Availability

The data used to prepare this manuscript are available from the corresponding author when requested.

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Research Article

Determination of antibiotic resistance and biofilm formation in *Klebsiella* strains isolated from bovine mastitis cases

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ABSTRACT

Mastitis is diseases of dairy cows with a high economic impact. Bovine mastitis is caused by a wide range of bacterial pathogens. As one of the major environmental pathogens Klebsiella spp. was investigated in this study by some phenotypic characteristics like antibiotic resistance patterns and biofilm formation properties. A number of 483 cows by dairy farms around the Konya were examined by California Mastitis Test (CMT) producing 36 positives in terms of subclinical mastitis. A further 19 samples from clinical mastitic udders were also collected. Samples were inoculated onto Trypticase Soy Agar medium enriched with sheep blood and incubated aerobicaly for 24-48 h at 37 °C. By morphological, biochemical and cultural charcateristics 14 isolates out of 37 coliforms were identified as Klebsiella spp. The double disc synergy method and Congo Red Agar test were used to perform antibiotic susceptibility and in vitro biofilm forming properties, respectively. Resistances to the Ampicillin, Carbenicillin, Cephotaxime, Chloramphenicol, Erythromycin, Gentamicin, Neomycin, Oxytetracycline, Sulphamethoxazole/Trimpethoprim, Amoxicillin-Clavulanate and Imipenem antibiotics were 78.5%, 78.5%, 35.7%, 42.8%, 100%, 7%, 7%, 50%, 14%, 21% and 7%, respectively. Three of the total isolets produced biofilm. This appears to the first report on ESBL producing Klebsiella spp. from subclinical cases of bovine mastitis in Konya, Türkiye. Presently, two numbers of antimicrobial combinations to treat bovine cases are recommended by this work. In conclusion, because of costly challange nature of Klebsiella caused bovine mastitis implemantation of an effective mastitis control program should be used in local farms from Konya.

INTRODUCTION

Mastitis, the infection of the mammary gland and one of the most significant diseases of the cattle. It has been leading to the highest economic losses in many national dairy industries (Erdoğdu, 2019). In Türkiye, annual economic loss of 41.5 million TL caused by mastitis was roughly estimated. This amount is mainly due to the decrease in milk production, veterinarian and treatment costs, labor expenses and removal costs of diseased animals (Sabuncuoğlu & Çoban, 2006). Mastitis is caused by a variety of microorganisms, mostly bacteriae. Of these, *Staphylococcus aureus*, *Streptococcus* spp. and *Enterobacteriaceae* are the dominant aetiological factors of bovine cases in many countries of the World including Türkiye (Ajose et al., 2022; Arslan et al., 2009; Kırkan et al., 2005; Tel et al., 2009; Uçan et al., 2005). Of the *Enterobacteriaceae* members, *Klebsiella* spp. is classified as both a major pathogen and an environmental agent. Additionally, in terms of milk loss, *Klebsiella* spp. is the agent that causes the most evident losses both in the first lactations and in the followings (Nielsen, 2009). On the other hand it is the fact that emergence of multi-drug resistance bacteria has been growing problem due to indiscriminate uses of chemotherapeutics in livestock, recently (Feiyang et al., 2021). Although *Klebsiella pneumoniae* (*K. pneumoniae*) which was rather known to cause clinical form of bovine mastitis also causes subclinical form and is presently considered as an emerging issue related to contaminated environments. Although primary species is the *K. pneumoniae* occasionally *K. oxytoca* aslo causes intramammary infections (IMI) in cows (Kleinhenz et al., 2019). Pathogenecity of the *Klebsiella* mastitis in the bovine udder is not fully understood, yet (Cheng et al., 2020). In the virulence of *K.pneumonia* infection in human origin, the factors associated with the infection were reported as those of varieties or presence of capsular serotypes, iron scavenging systems, and fimbriae (Holt et al., 2015). Alternatively,, some factors involved in virulence of *K. pneumoniae* in bovine mastitis were reported as being capsular polysaccharites especially K1 (Capsule) and K2 (Hypermucoviscisity), vmpA, kfu, uge, magA and aerobactin (Osman et al., 2014). Intruguingly, absence of *Klebsiella* spp. isolates from subclinical mastitis was considered that the role of *Klebsiella* spp. in subclinical mastitis was insignificant (Katsande et al., 2013; Swartz & Novello, 1984). However, bedding products (especially materials made by wood) can be a source of *Klebisella* since healthy cows shed *Klebsiella* in their excrements and *Klebsiella* mastitis in both forms originating from the environment may be such a big problem that can only be effectively treated by specific protocols (Fuenzalida et al., 2021; Osman et al., 2014). A rapidly growing veterinary public issue is antibiotic resistance problem. Antibiotic use in farm animals can contribute to the emergence of resistant bacteria that can be transferred from the farm animal species to human somehow. However, the hypothesis, *Klebsiella* might be

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transmitted to human via consumption of contaminated milk or meat is still to be verified (Davis & Price, 2016). Therefore, more researches are needed to reveal various aspects of bovine *Klebsiella* mastitis worldwide. In terms of Türkiye, numerous studies on aetiological agents and prevalances of bovine mastitis have been done in the country, so far (Arda & İstanbulluoğlu, 1979; Bozkır, 1985; Öztürk et al., 2019). However, data on prevalance of *Klebsiella* caused bovine mastitis in Konya needs to be cleared. Occurrence of the agent in the same environment along with the infected cows is also worth of examined. This study aimed to preliminarly present biofilm formation and antibiotic resistance profiles of the *Klebsiella* isolates from local dairy farms at different lactation periods.

MATERIALS and METHODS

A total of 483 cows were included in the study for causative isolation and identification. The samples were transferred to the laboratory maintainig the cold chain and inoculated on trypticase soy agar containing 5% sheep blood then incubated for 24-48 h at 37 °C under aerobic conditions. Based on colony morphology and gram staining characteristics, the isolates suspected of coliform were passaged into Mc Conkey Agar and incubated at 37 °C overnight. Biochemical tests were made by using Lassen triple tube method, MR-VP and citrat utilization test (Hogan et al., 1999; Lassen, 1975). Sensitivities of the isolates to various antimicrobials were detected by Kirby-Bauer Disk Diffusion Method (Bauer, 1966) and performed as per the recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines (Wayne, 2012). Each of the bacteria suspensions was adjusted to the turbidity of 0.5 McFarland Standart and a Mueller Hinton Agar plate was inoculated with the test organism by means of streaking a swab. Then, antimicrobial-impregnated commercial disks Ampicillin (AM10) (10 mg), Carbenicillin (PY100) (100 mg), Cephotaxime (CTX30) (30 mg), Chloramphenicol (C30) (30 mg), Erythromycin (E15) (15 mg), Gentamicin (CN10) (10 mg), Neomycin (N30) (30 mg), Oxytetracycline (T30) (30 mg), Sulphamethoxazole/Trimpethoprim (SXT25) (1.25 mg/23.75 mg), Amoxcillin–Clavulanate (AMC30) (30 mg), Imipenem (IPM10) (10 mg), Flumequin (FLM30) (30 mg) (Bioanalyse, Türkiye) were placed on the surface of the agar. Results were read after 18 h incubation as aerobik condition at 37 °C. Inhibition zones around the disks were measured using a ruler according to Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2012). Colistin sensitivities of the test strains were measured using colimycin (Sigma Aldrich, St. Louis, MO, ABD) by broth dilution test based on the standards of European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC values for Colistin that were ≤2 µg/ml were accepted as sensitive while those >2 µg/ml were noted resistant. The double disc synergy method was used for the antibiotic susceptibility tests and the Extended Spectrum β-Lactamase (ESBL) production in concordance with the procedures stated by the (CLSI).

In order to determine the *in vitro* biofilm forming properties of the isolates, cultivation was performed in Congo Red Agar (KKA) (BHI agar containing 37 g/L, 5% sucrose and 0.8 g/L Congo Red dye) to obtain single colonies (Arslan et al., 2005). After the incubation period, black colonies were accepted as biofilm positive.

RESULTS

All udder lobes of 483 cows from 2 dairy farms located around the Konya were individually sampled and thirty-six milk samples positive by California Mastitis Test (CMT) were included study. In addition, 19 udders with clinical mastitis were also sampled from the same farms. Based on results from biochemical characters, 14 out of 37 coliform suspected isolates were identified as *Klebsiella* spp. (Table 1). The prevalance of *K. pneumoniae* in the both farms were 2.9 % (data not shown). Biofilm formation was observed only in 3 of the strains. ESBL production by double disc synergy method showed an inhibition zone and ESBL production between Amoxicillin–Clavulanate and Cephotaxime in 4 samples (Table 3).

DISCUSSION

Mastitis is likely the costliest disease in dairy breeding. Its prevalance varies between countries. Frequency of mastitis in the dairy cow population, Türkiye seems not to be low, either (Arda & İstanbulluoğlu, 1979; Erdoğdu, 2019; Klaas & Zadoks, 2018; Öztürk et al., 2019)

Klebsiella mastitis has become a major concern in USA and in some parts of the Europe, recently (Fuenzalida et al., 2021; Nielsen, 2009; Osman et al., 2014). In Türkiye, 1277 lactating cows in 3 state ruled farms from the provinces Ankara, Eskişehir and Bursa have been reported to be monitored periodically for 3 years and no *Klebsiella* spp. isolation was made (Arda & İstanbulluoğlu, 1979). Of 14 other studies conducted on bovine mastitis all around the Türkiye during 1979-2019, the prevalance of *Klebsiella* spp. was highest (34.3 %; 23 *Klebsiella* spp. isolation from 162 cows' mastitic udders) in Province Aydın (Erdoğdu, 2019), whereas 0.6 % in Marmara and Paşaeli Regions (Batu et al., 1979) or much later in Marmara 0.2 % (Türütoğlu et al., 1995), 6.8 % in Balıkesir (Çokal & Konuş, 2012). 1.49 % in Bursa and 4 other cities (Büyükcangaz et al., 2012), 0 % in Ankara (Ulusoy, 1985), 4.76 % in Afyonkarahisar (Alaçam et al., 1989) 2.1 % (Muz et al., 1992) and 1.37 % (Gülcü & Ertas, 2004) in Elazığ, 3.83 % (Aydın et al., 1995) and 0 % (Şahin & Çolak, 1997) in Kars, 17.88 % in Burdur (Öztürk et al., 2019) , 1.5 % in Şanlıurfa (Tel et al., 2009) and 1.87 % in Diyarbakır (Yeşilmen et al., 2012). Briefly, different frequencies of *Klebsiella* mastitis in dairy cattle occurred in various regions, provinces or dates in Türkiye.

To the best of our knowledge, the first report on isolation of aerobic bacterial agents from mastitic cow milk stated that no *Klebsiella* was isolated from 150 mastitic milk samples of 691 cows sampled (Bozkır, 1985). Another study form Konya at that time has also noted that *Klebsiella* spp. was not found by examining 39 dairy cattles at the end of the lactation period (Tekeli et al., 1985). First report on *Klebsiella* spp. as a causative agent in mastitis has apparently raised from ovine cases in Konya (Erer et al., 1990) In that study, 1198 sheep were screened for the presence of clinical or subcinical mastitis at a state slaughter house. Roughly six percent of (n=119) milk samples were positive for *K. pneumoniae* growth. Later, no *K. pneumoniae* isolation from bovine mastitis cases was noted

Table 1. Number of samples and isolates based on their animal origins

Cows	Number of sample	Gram(-) bacteria	K.pneumonia	%
With subclinical mastitis	36	21	6	16.66
With clinical mastitis	19	16	8	42.10
Total	55	37	14	25.46

Table 2. Antimicrobial susceptibility of Klebsiella isolates

Antimicrobial agents	Susceptibility		Intermediate		Resistance	
	n	%	n	%	n	%
AM10	0	0	3	21.42	11	78.57
PY100	0	0	3	21.42	11	78.57
CTX30	6	42.85	3	21.42	5	35.71
C30	8	57.14	0	0	6	42.85
E15	0	0	0	0	14	100
CN10	8	57.14	5	35.71	1	7.14
N30	6	42.85	7	50	1	7.14
T30	7	50	0	0	7	50
SXT25	11	78.57	1	7.14	2	14.28
AMC30	11	78.57	3	21.4	0	0
IPM10	13	92.85	1	7.14	0	0
FLM30	14	100	0	0	0	0
Kolistin	14	100	0	0	0	0

(Spanning header above all columns: Susceptibility)

AM10: Ampicillin (10mg), PY100: Carbenicillin (100mg), CTX30: Cephataxime (30mg), C30: Chloramphenicol (30 mg), E15: Erythomycin (15mg), CN10: Gentamicin (10mg), N30 Neomycin (30mg), T30 : Oxytetracycline (30mg), SXT25: Sulphamethoxazole/ Tripethoprim (1.25/23.75mg), AMC30: Amoxcillin- Clavulanate (30mg), IPM10: Imipenem (10mg), FLM: Flumequin (30mg)

Table 3. In vitro slime forming properties and Extended Spectrum β-Lactamase production (ESBL)

Mastitis caused by *K.pneumonia*	Number of samples examined	ESBL	Slime forming (In vitro)	%ESBL	%Slime
Subclinical	6	3	0	50	0
Clinical	8	1	3	12.5	37.5
Total	14	4	3	28.57	21.4

from the same region (Ateş et al., 1991; Dinç et al., 1991) have examined 82 lactating cows from a state farm in Konya and found 43 subclinically mastitic udders, giving 2.32 % *K. pneumoniae*, 4.65 % *Klebsiella* spp and 2.32 % *K. pneumonia* mixed with yeast. In order to early diagnose mastitis in subclinical bovine cases, milk samples from healthy and mastitic udders from 40 number of cows (aged between 4-5) have been sampled and examined bacteriologically in Konya (Nizamlıoğlu et al., 1992). The authors noted that *K. pneumoniae* isolated from a healthy milk sample only (6.25 % of the healthy udders). Later on, using limited samples, an isolation of *Klebsiella* spp has also been reported from cow mastitic udders in Konya (Semacan et al., 2012). In our study, *K. pneumoniae* were isolated 16.7 % and 42.1 % from subclinical and clinical cases, respectively. By comparison with the *Klebsiella* mastitis occurrence in Konya for years, *Klebsiella* mastitis have increased dramatically in the past 3 decades although low numbers of mastitic samples studied mostly.

To update information on antimicrobial resistance pattern of *Klebsiella* isolates from bovine mastitis cases is important since decision on effective treatments almost solely depend on.

The antibiotic resistance of *Klebsiella* from milk with either clinical or subclinical forms mastitic udders was also tested against twelve antimicrobials commonly used by present study. Chloramphenicol showed complete resistance to *K. pneumoniae*. Both flumequin and colistin showed sensitivity (Table 2). Restricted data exists on the issue of antimicrobial resistance patterns of *Klebsiella* from mastitic cows in Konya. (Dinç et al., 1991) have reported that all the isolates they had were sensitive to enroflaxacin at different levels. At that time, this can be an expected outcome since enroflaxacin had been in use for a few years for udder health. At present study, this antimicrobial was not examined since it has not been a first preference for treatment of mastitis for some recent years. Some other researchers from Konya have found that sensitivity of *Klebsiella* spp. isolates to chloramphenicol was 100 %. More than 3 decades later in a neighbouring province sensitivity of the local isolates to the same antimicrobial was nil, likely pointing out a growing resistance in this geography (Alaçam et al., 1989).

A study carried out more recently has highlighted 162 cattle with 23 cases of *Klebsiella* mastitis in Aydın (Erdoğdu, 2019). They stated that resistance to ampicillin by 23 isolates was full (100 %). At present study the isolates were found resistant at 78.57 % against ampicillin. By gaining more data on this issue would clear figures of ampicillin resistance in the Country.

Members of *Enterobacteriaceae* generally contain extanded spectrum beta lactamase (ESBL) enzymes. This class of enzymes confers resistance to penicillins, one to third generation cephalosporins such as cephotaxime. Present study also evaluates the occurrence of ESBL-producing *Klebsiella* spp. in some dairy farms. We found a degree of ESBL (28.57 %), not to a low extent (Table 3). Fifty percent of *Klebsiella* strains isolated from subclinical cases showed ESBL activity is of importance since occurrence of ESBL producing *Klebsiella* spp. from subclinical cases of bovine mastitis in Konya is first reported and supports the observation that ESBL producing bacteria are increasingly being appeared from the livestock and environments (Sivaraman et al., 2021).

Understanding recurrent infections in bovine mastitis is still under investigation. One of the underlying mechanisms is attributed to biofilms formed by different causatives (Hillerton and Kliem 2002, Çökülgen and Uçan 2022). In case of *Klebsiella* spp. mastitis cases, biofilm formation has also been reported by several studies (Schönborn et al., 2017, Rudenko et al., 2021). *Klebsiella* spp. biofilms has been noted to possess a high degree of adhesiveness and strenghtness (Lenchenko et al.,2020). Our study evidences that the occurrence of *Klebsiella* spp. isolates positive for biofim from Konya are not quite high (37,5 %) whereas reports from World that state figures from 60 % to 84 % (Schönborn et al., 2017, Rudenko et al., 2021)

A list of pathogens published by WHO in the context of a global action plan on combating antimicrobial resistance considers various mastitis pathogens such as *E.coli, Klebsiella* and *Staphylococcus aureus* with high priority (Becker et al., 2016; Prigitano et al., 2018; Tacconelli, 2017).

In conclusion, to treat *Klebsiella* mastitis with effective antimicrobials in bovine dairy farms located in Konya appears to be mostly limited by Amoxicilin/Clavulunat or Sulfametaxazole/Trimetoprim combination and to a lesser extent by Gentamicin or Cephotaxim, at present. Moreover imipenem, flumequin and colistin showed complete sensitivity to *Klebsiella* of bovine origin. More data from a bigger sample size needs to be undertaken.

DECLARATIONS

Ethics Approval

For this study, it was unanimously decided that the principles of Selcuk University Veterinary Faculty Experimental Animal Production and Research Center Ethics Committee (SÜVDAMEK) Directive were complied with and that it was "appropriate" in terms of research ethics (18.09.2020- 2020/85).

Conflict of Interest

The authors declare that they have no conflict of interests.

Author contribution

Idea, concept and design: MA, USU

Data collection and analysis: MA

Drafting of the manuscript: USU

Critical review: MA, USU

Data Availability

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

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Research Article

Effect of selenium, vitamin E, and β-carotene administration on fertility of synchronized Awassi ewes during non-breeding season

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ABSTRACT

The aim of this study was to evaluate the effect of selenium (Se), β-carotene, and vitamin E administration on fertility of Awassi ewes synchronized in non-breeding season. The study included 80 multiparous Awassi ewes ranging in age from 2 to 6 years. Intravaginal sponges containing flugestone acetate were inserted and left in for 9 days to allow estrus synchronization. Ewes were divided into two groups at random (group I: study group; group II: control group). In group I (n=40), while the drug containing Se and vitamin E was only administered on the day of sponge insertion, another drug containing β-carotene and Vitamin E was administered three times (sponge insertion, withdrawal, and on day 18 after mating) during estrus synchronization. The control group (group II, n=40) received no supplementary injections. When the sponges were removed, 500 IU PMSG and 0.075 mg d-cloprostenol were administered intramuscularly. The rams joined the herd for an hour twice a day 24 hours after the removal of sponges. Mating occurred after the detection of estrous in ewes. Estrus rates and estrus onset timings of group I and group II were 81.08% and 45.30±1.71 h, and 80.55 % and 43.94±1.72 h, respectively. The conception rates, pregnancy rates, kidding rates, and litter size in group I and group II were 66.66% - 72.41%, 54.05% - 58.33%, 100% - 100%, and 135% - 138%, respectively. There were no statistical differences ($p>0.05$) between the groups in terms of fertility traits. In conclusion, administering Se, β-carotene, and vitamin E at estrus synchronization protocols during the non-breeding season has no positive effect on Awassi sheep fertility traits. However, measuring blood levels of Se, β-carotene, and vitamin E before beginning the treatment may be beneficial in identifying the optimal effect of this treatment.

INTRODUCTION

In ovine breeds that normally deliver once a year, inducing reproductive activity during the non-breeding season allows lambing twice a year or lambing three times in two years. Furthermore, by increasing milk and meat production, these products can be marketed in non-breeding season. In non-breeding season, the most effective methods for stimulating ovarian functions in sheep are progesterone, GnRH, melatonin, and, in addition to these, PMSG or LH effective hormone applications (Gordon, 1997; Wildeus, 2000). A meta-analysis study conducted in Turkey discovered that pregnancy rates were lower in progesterone + PMSG based estrus synchronization protocols (59.36 %) used in non-breeding season compared to the reproductive period (90.37%). The deep anestrus in animals, diminished hormonal effects, and low ovarian activity are the causes of the low pregnancy rates during the non-breeding season (Arikan et al., 2021). The ovarian follicle population in sheep is known to be highly sensitive to dietary intake, and it has been reported that dietary manipulations can increase both folliculogenesis and ovulation (Scaramuzzi et al., 2006). Farm animal reproductive performance, on the other hand, is determined by four major factors: genetics, environment, nutrition, and management. Because of their direct effects on reproduction and potential to mitigate the effects of other factors, nutritional factors are cited as the most important factors. Additionally, nutritional factors more than others are open to adjustment for successful outcomes (Smith and Akinbamijo, 2000). Many minerals and vitamins are required for optimum reproductive performance of animals. Plasma concentrations of these chemicals and reproductive performance are significantly correlated. Some trace element deficiencies, such as those of copper, cobalt, and selenium (Se), can prevent ovulation, cause embryo loss, and potentially lead to fetal mortality (Hostetler et al., 2003; Liu et al., 2014; Zonturlu et al., 2017). The use of intravaginal estrus synchronization devices like sponge and CIDR may also lead to an increase in oxidative stress (Kuru et al., 2016; Farahavar et al., 2020; Eşki et al., 2021). Oxidative stress impairs ovarian function and follicle growth. Vitamin E and beta-carotene are known essential nutrients in the management of oxidative stress (Hostetler et al., 2003; Liu et al., 2014; Zonturlu et al., 2017).

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Ewes must obtain all of their β-carotene needs from the feeds since they are unable to synthesize it. As a result, the season and the type of feed may affect the serum β-carotene levels of ewes (Weiss, 1998). Additionally, β-carotene is also an antioxidant. Similar to vitamin E, which is effective at higher oxygen concentrations, β-carotene scavenges superoxide radicals and neutralizes free peroxide radicals in tissues thus synergizing the antioxidant activity (Arechiga et al., 1998). A lack of β-carotene results in sub-estrous, delayed ovulation, decreased pregnancy rates, an underdeveloped and small-diameter corpus luteum (CL), and decreased progesterone synthesis throughout the cycle and the first trimester of pregnancy, which increases the risk of embryonic death (Ayaşan and Karakozak, 2010). It has been reported that β-carotene has a positive effect on fertility when incorporated into feed or administered parenterally. β-carotene affects fertility either indirectly via vitamin A conversion (Ayaşan and Karakozak, 2010), or directly (Trojacanec et al., 2012).

Vitamin E, also known as the anti-sterility vitamin, is essential for all animal species including humans. It saturates the peroxides and hydroperoxides that disrupt the structure of intracellular membranes thus preventing the peroxide radical formation (Putnam and Comben, 1987; Kott et al., 1998). Vitamin E supplementation can improve ovulation rates and the number of offspring in ewes by playing an important role in oocyte maturation and quality, fertilization, and early embryonic development (Kott et al., 1998).

Se is an essential component of organisms' antioxidant defense system. It contributes to the building of the endogenous antioxidant defense enzyme glutathione peroxidase (GSH-Px) and participates in the catabolism of peroxidase that takes place during lipid peroxidation. Se has biological activity in growth and fertility along with vitamin E, and it also acts as an antioxidant to prevent and repair cellular damage (Hostetler et al., 2003). A study reported that Se contributes to the growth of granulosa cells, the production of estrogen, and the production of prostaglandins (Wichtel et al., 1996). Se also protects follicles from oxidative stress that occurs during follicle growth, maturation, and dominance (Ceko et al., 2014).

Sheep breeding in Turkey is typically spread in a traditional pasture-based manner. According to reports, both types of grass grown in winter and dried in summer for winter feeding have less β-carotene, vitamin E, and Se, each of which has been linked to a variety of effects on reproductive processes. For this reason, it is recommended to add these nutrients during periods of deficiency, especially in pasture-based agriculture, and it is stated that when combined with estrus synchronization, the yield obtained and the economic gain due to this will increase even more (Beytut et al., 2005; Köse et al., 2013). Numerous studies have used these supplements to treat estrus in sheep, goats, and cows. However, its use under local conditions needs to be proven. The purpose of this study was to determine how Se, β-carotene, and vitamin E supplements affected the fertility of synchronized Awassi sheep in non-breeding season.

MATERIALS and METHODS

In the second half of March 2021, the study was conducted on 80 Awassi ewe from a sheep farm in Hatay aged between 2 and 6 years. The ewes weighed 45-60 kg, had lambed once or more, and were in their second month postpartum at least. Study site was situated in Yayladağı, Hatay in the Eastern Mediterranean region of Türkiye (latitude: 35º 90' N; longitude: 36º 06' E). The average ambient temperature during the study period was 17.5°C during the day and 10.8°C at night, with an average day and night length of 13 and 11 hours, respectively.

Standard management and feeding procedures of the holding were applied to the study animals. The ewes, which were not lactating at the time of the trial, were given daily access to 150 g of a concentrate mix comprising 13% crude protein and 2650 kcal/kg energy per animal while grazing on the pasture between the hours of 08:00–18:00 (Table 1).

Table 1. Content of the concentrated feed mix

Raw Materials	%
Corn	33
Barley	30
Coarse wheat bran	11
Dried pitted olive pulp	8
Sunflower meal	14,15
Marble dust	2,6
Salt	1
Vit-Min. premix	0,25

A treatment using intravaginal cylindrical polyurethane sponges impregnated with 20 mg of cronolone (flugestone acetate) (Chronogest CR®, Intervet, Turkey) was administered to each of the 80 ewes that were a part of the study and were not in breeding season. Then, using the random sample technique, the ewes were divided into two equal groups (group I: study group; group II: control group). In Group I, while the medication containing Se and vitamin E (1 mg/1 ml of sodium selenite and 60 mg/ml of vitamin E, Yelvit®, Teknovet, Turkey) was administered only on the day the sponges were inserted intravaginally, another medication containing β-carotene and vitamin E (15 mg/ml of β-carotene and 20 mg of dl-α-tocopherol acetate equivalent to 18.22 mg/ml of vita-

min E, Dalmavital®, Vetaş, Turkey) was administered at the same dose three times (sponge insertion, withdrawal, and on day 18 following mating) during estrus synchronization (Figure 1). The control group (Group II, n=40) did not receive any supplementary injection (Figure 1). The sponges were left in place for 9 days in both groups. In addition, we injected 0.075 mg of d-cloprostenol (Senkrodin®, Vetaş, Turkey) and 500 IU of PMSG (Chronogest/PMSG, 6000 IU, Intervet, Istanbul, Turkey) intramuscularly when removing the sponges. Twenty-four hours after the sponges were removed, the rams joined the herd for an hour twice a day. The ewes that successfully mated after the estrous detection were separated from the herd and put in a different compartment.

parameters that were tested:

Estrus onset time: The period from sponge withdrawal to acceptance of mating (hours)

Estrus rate = (Number of ewes in estrus ÷ Number of ewes treated for estrus synchronization) × 100

Pregnancy rate = (Number of pregnant ewes ÷ Number of ewes in the group) × 100

Conception rate = (Number of pregnant ewes ÷ Number of naturally mated ewes) × 100

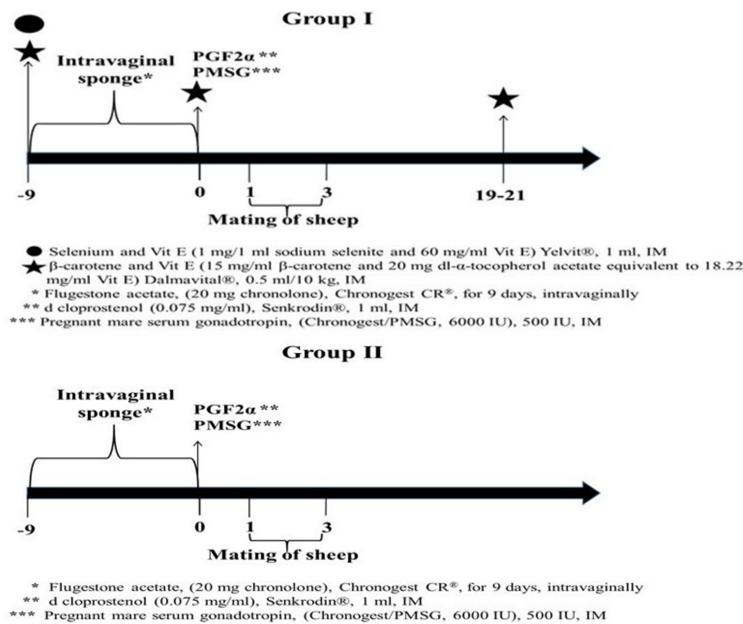

Figure 1. Applications in Group I and Group II

A 6-8 MHz probe real-time ultrasound instrument (Falco, Pie Medical, Netherlands) was used to detect transabdominal pregnancy 50 days after the mating. The existence of fetuses, fetal secretions, placentomes, and a fetal heartbeat were all considered positive evidence of pregnancy.

The SPSS 22.0 software program and the chi-square test were used to statistically examine fertility indicators. A significance level of $p < 0.05$ was used for all statistical analyses.

The following formulas were used to calculate the fertility

Kidding rate = (Number of ewes that have lambed ÷ Number of pregnant ewes) × 100

Litter size = (Number of lambs born ÷ Number of ewes that have lambed) × 100

RESULTS

In the current study, two ewes in group I and three ewes in group II did not retain intravaginal sponges. Additionally,

Table 2. Fertility parameters of Group I and Group II

	Onset of estrus (h)	Estrus rate (%)	Conception rate (%)	Pregnancy rate (%)	Kidding rate (%)	Litter size (%)
Group I	45.30±1.71	81.08 (30/37)	66.66 (20/30)	54.05 (20/37)	100 (20/20)	135 (27/20)
Group II	43.94±1.72	80.55 (29/36)	72.47 (21/29)	58.33 (21/36)	100 (21/21)	138 (29/21)
P	-	-	-	-	-	-

No significant difference between treatment groups (P>0.05).

one ewe from each group was diagnosed with laminitis. These animals were removed from the study. During the sponge treatment, no ewes displayed any symptoms of estrus. Estrus began 24 hours following the removal of the sponges and concluded 60 hours later. After sponge withdrawal in group I, the percentage of ewes in standing estrus was 6.66 % at 24–25 h, 26.66 % at 36–37 h, 53.33 % at 48–49 h, and 13.33 % at 60–61 h. On the other hand, these rates for group II were 6.89% at 24–25 h, 34.48% at 36–37 h, 48.27% at 48–49 h, and 10.34% at 60–61 h. There was no significant difference in fertility traits between the groups (Table 2, p>0.05).

DISCUSSION

The pregnancy rates in groups I and II in the current study were found to be 54.05% and 58.33 %, respectively (Table 2, p>0.05). According to Karagiannidis et al. (2001), progesterone administration to sheep during non-breeding season resulted in fertilization rates of 22% to 70%. According to Gordon (1997), progesterone-treated ewes had pregnancy rates between 70% and 80%. The conception rates for groups I and II in the current study were found to be 66.66 % and 72.41 %, respectively. The fact that the current investigation was carried in non-breeding season may be the cause of the results being comparable to those previously reported by Gordon (1997). The animals used in this study were non-lactating ewes. Moss et al. (1980) suggested that increased serum prolactin concentrations in lactating ewes cause a decrease in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels, lowering the conception rate significantly. The pregnancy and conception rates found in the current study show that ovarian activity was adequately induced during the non-breeding season in non-lactating ewes.

Short-term consumption of β-carotene-supplemented rations increases ovulation rates in goats (Arellano-Rodriguez et al., 2007). Furthermore, prolonged β-carotene supplementation increases pregnancy rates, and long-term supplementation is required to increase the tissue concentration of the β-carotene molecule (Arechiga et al., 1998; De Ondarza et al., 2009). According to Ay et al. (2012), high β-carotene concentrations increase antioxidant activity, creating a favorable uterine environment for implantation and embryo development, and increasing pregnancy rates. Despite a body of research showing that β-carotene supplementation improves fertility (Haliloğlu et al., 2002; Arellano-Rodriguez et al., 2007; De Ondarza et al., 2009), some studies show that it has no effect (Wang et al., 1987; Arechiga et al., 1998; Çelik et al., 2009; Trojacanec et al., 2012). Arechiga et al. (1998) suggested that the failure of β-carotene injections to increase pregnancy rates could be attributed to embryos being resistant to antioxidants during the early developmental stages. These researchers also reported that only long-term dietary supplementation with β-carotene could increase β-carotene levels in the bovine oviduct and uterus. Similar to previous studies (Wang et al., 1987; Arechiga et al., 1998; elik et al., 2009; Trojacanec et al., 2012), the current study found that the pregnancy rate of the β-carotene-supplemented group did not increase (Table2).

Gore and Lehloenya (2020) reported that 50 mg/kg β-carotene added to a 60-day feeding period had no effect on follicle number, follicle diameter, or CL diameter in Saanen goats synchronized with 11-day CIDR during the breeding season. The authors also found that β-carotene increased plasma progesterone concentration and glutathione peroxidase activity without affecting estradiol 17-β concentration. They attributed this finding to the fact that β-carotene did not affect estradiol 17-β and LH concentrations. These two hormones, particularly estradiol 17-β, have a key role in controlling estrous behavior. In this study, we found no positive or negative effects on pregnancy rates with vitamin E, Se, or β-carotene supplementation administered simultaneously with synchronization and continued in the following days. These vitamins and minerals have been reported to positively affect ovarian functions, conception rates, and the sexual cycle in various animal species in some of the studies mentioned above (Haliloğlu et al., 2002; Arellano-Rodriguez et al., 2007; De Ondarza et al., 2009). The presence of an anestrus phase in the ovine sexual cycle, defined by the absence of ovarian functions and the presence of sexual hormones at basal levels, is the main factor that defines treatment success. Furthermore, as Gore and Lehloenya (2020) have suggested, the success of treatment may be related to the fact that estradiol 17-β and LH concentrations are not affected.

Because vitamin E and Se have similar biological effects that are demonstrated together, and their deficiencies are associated with similar symptoms, it is suggested that they should be administered together (Hostetler et al., 2003; Mehdi and Dufrasne, 2016). In a previous study, Awawdeh et al. (2019) used transitional Awassi ewes that were synchronized for estrus with a 12-day treatment with FGA-impregnated intravaginal sponges to administer vitamin E and Se injections at doses of 13.6 mg/kg and 0.045 mg/kg, respectively, at the time of sponge insertion, withdrawal, and 19 days after the withdrawal. The rates of embryonic mortality and pregnancy were 24.3 % and 86.8%, respectively, in the treatment group and 44.8 % and 63.9 %, respectively, in the control group, based on progesterone testing on day 19 post-mating and pregnancy examination on day 40 post-mating. According to Liu et al. (2014), dry grass pastures in the Mediterranean region have insufficient vitamin E content, particularly during the summer and autumn. According to Koyuncu and Yerlikaya (2007), sheep fed grass on dry pastures and stubbles with low vitamin E levels are at a particularly high risk of deficiency. We found no statistically significant differences in fertility parameters between the groups in our study, which may be related to the geographic layout and climatic features of the study area. Because of the region's good pasture conditions, we believe there were no vitamin or mineral deficiencies.

Injections of vitamin E and Se during the breeding season enhanced estrous and pregnancy rates, offspring yields, and improved reproductive metrics in Merino sheep, according to a study by Koyuncu and Yerlikaya (2007). During the mating season, El-Shahat and Abdel Monem (2011) added various amounts of vitamin E and Se to the feed of Baladi sheep and saw a higher pregnancy rate in the experimental groups compared to the controls. There are studies that show supplementation with vitamin E and Se improves sheep fertility (Koyuncu and Yerlikaya, 2007; El-Shahat and Abdel Monem,

2011; Awawdeh vet al., 2019), but there are also reports that claim supplementation has no such beneficial effects (Sanchez et al., 2008; Farahavar et al., 2020). Farahavar et al. (2020) injected Mehraban sheep with 5 ml Ese (0.5 mg/ml sodium selenite and 50 IU DI-α-tocopherol) 2 weeks before CIDR insertion, during CIDR insertion, and during CIDR removal at synchronization during the breeding season. CIDR stayed for 13 days, and the authors discovered no differences in estrous, pregnancy, or twin births between the groups. Se treatment before the breeding season increased embryonic mortality in synchronized sheep, according to Sanchez et al. (2008); this may have a negative impact on fertility. We found no harmful effects of vitamin E or se supplementation on fertility in this study. Supplementing sheep with vitamins and minerals via hormones or injections (as performed here) prior to, during, and after mating shouldn't have any negative consequences on pregnancy or lambing rates.

Köse et al. (2013) investigated sheep in anestrus throughout a 10-day synchronization based on progesterone (20 mg FGA). On the day of sponge removal, pregnancy rates and lamb yields were 59.1% and 45.5% for the β-carotene group (1 mg/kg), 50.0% and 68.2% for the vitamin E + Se (200 mg DL-alpha tocopherol acetate+0.67 mg Se) group, and 64.3% and 57.1% for the control group. According to the authors, the applications had no positive effect on fertility parameters. They also mentioned that the injections were given just before the expected estrous period, so there was no time for vitamin E or Se to exert their biological effects; additionally, only one administration was given. Furthermore, the application occurred shortly before mating, which may have prevented the cellular antioxidant effects of vitamin E and Se on the Graafian follicle and the oocyte within it. The addition of β-carotene, vitamin E, and Se resulted in no increase in estrous or pregnancy rates in the current study. However, unlike Köse et al. (2013), we made the β-carotene and vitamin E injections during both sponge insertion and sponge removal before estrus, suggesting that good pasture conditions during the study period may affect the results.

Kuru et al. (2017) found that injecting barium selenite into anestrus Pırlak ewes on the day of intravaginal sponge insertion had no effect on fertility parameters. Furthermore, Yıldız et al. (2015) discovered that a single injection of vitamin E and Se to dairy cattle prior to Ovsynch protocol treatment increased progesterone levels but did not increase the pregnancy rate. According to these researchers, antioxidants may not always be sufficient in preventing embryonic death or completely counteracting the effects of free oxygen radicals. The present study does not provide any data on embryonic deaths as the study design was based on ultrasonographic examination alone and it was not possible to detect embryonic deaths. According to Sarıbay and Erdem (2007), post-mating embryonic deaths can be detected by ultrasonography if repeated examinations are performed at regular intervals.

Van Niekerk et al. (1996) discovered that Se administration following synchronization/gonadotropin treatment had a negative effect on both pregnancy rate and litter size, with the latter being 19% lower than the litter size of animals in natural estrus. This result was attributed to a significant interaction between Se and the synchronization treatment. According to Sanchez et al. (2008), synchronization/gonadotropin treatments increase Se toxicity and even cause a progressive increase in this toxic effect in ewes. Twin-pregnant ewes were found to be more severely affected by this increased toxicity, according to these authors. According to Scaramuzzi et al. (2006), dietary supplementation with Se and vitamin E raises blood urea levels, which lowers the uterine pH and ovulation rate and results in the release of prostaglandin F2α (PGF2α). This has a negative impact on the pregnancy rate. The ewes' first pregnancy examination was performed on day 50 post-mating in the current study. Due to the lack of pregnancy examinations and plasma progesterone measurements during the embryonic development period, the potential effects (positive or negative) of vitamin E and Se supplementation on embryonic deaths could not be investigated.

While some researchers have reported that vitamin E and/or Se supplementation improves fertility in ewes (Koyuncu and Yerlikaya, 2007; El-Shahat and Abdel Monem, 2011; Awawdeh et al., 2019), others have suggested that supplementation has no effect (Segerson et al., 1986; Köse et al., 2013; Farahavar et al., 2020), and (Scaramuzzi et al., 2006; Sanchez et al., 2008). Awawdeh et al. (2019) attributed varying results in different studies to differences in the administration dose, administration route (dietary supplementation or parenteral injection), and timing of administration (before and/or after mating) of the supplements used, as well as the animals' pre-supplementation initial vitamin E and Se levels. Segerson et al. (1986) suggested that protein, energy, calcium (Ca), magnesium (Mg), and phosphorus (P) levels in the diet could also be effective. According to Van Metre et al. (2001), when investigating the underlying causes of fertility problems in a flock, the effects of all nutrients on fertility should be considered, not just vitamin E and Se. There was no blood analysis performed in this study to determine the pre-supplementation Se, β-carotene, and vitamin E levels in ewes. As a result of the lack of data, the extent to which supplementation had an effect could not be demonstrated.

According to a study on vitamin E and Se supplementation in ewes treated for estrus synchronization, pre-treatment blood vitamin E and Se levels should be measured, and treatment should be adjusted accordingly (Segerson et al., 1986; Van Metre et al., 2001). In the present study, vitamin E and Se supplementation having not caused any alteration in the fertility parameters suggested that the pre-treatment initial vitamin E and Se levels of the ewes should have been determined by blood analyses and supplementary injections should have been performed according to the measured blood levels.

CONCLUSION

As a conclusion, it was found that progesterone-impregnated intravaginal sponges and vitamin E supplementation did not increase reproductive parameters at a synchronized estrus in sheep under range conditions during non-breeding season. More thorough studies examining seasonal nutritional conditions as well as blood analyses for Se, β-carotene, and vitamin E levels could establish the impact of supplementing with Se,

β -carotene, and vitamin E on reproductive parameters in synchronization programs used in non-breeding seasons.

DECLARATIONS

Ethics Approval

This study was conducted pursuant to the 28/01/2021 dated and 2021/01-09 numbered approval of the Local Ethics Board for Animal Experiments of Hatay Mustafa Kemal University.

Conflict of Interest

Any conflict of interest exists not.

Consent for Publication

Not applicable

Author contribution

Idea, concept and design: Sarıbay MK,

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Drafting of the manuscript: Sarıbay MK, Köse AM

Critical review: Sarıbay MK, Köse AM

Data Availability

Data can be accessed from the author when needed.

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Research Article

Analysis and estimation of pathological data and findings with deep learning methods

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ABSTRACT

As in human diseases, rapid diagnosis of animal diseases is of great importance. In order for the disease treatments to be carried out properly, the diagnosis must be of high accuracy, as well as the rapid diagnosis. In this study, the disease types in the data set consisting of the data examined between the years 2000-2020 belonging to the Department of Pathology of the Faculty of Veterinary Medicine of Burdur Mehmet Akif Ersoy University were estimated by using the decision tree classification model and the KNN classification model. Categories such as age, type, city, and gender in the data set were analyzed in graphics. For the estimation and analysis processes to give accurate results, the data set was corrected by going through some pre-processes and the missing data in the data set was completed. It is thought that the results obtained from the estimation and analysis will allow rapid and accurate diagnosis in animal disease diagnoses.

INTRODUCTION

The word pathology is of Latin origin, and it is a definition formed by the combination of the words pathos, which means disease, and logos, which means science (Slauson and Cooper, 1990; Carlton and McGavin, 1995; Cheville, 1999). While Medical Pathology, one of the sub-branches of pathology, deals with human diseases, Veterinary Pathology deals with animal diseases (Slauson and Cooper, 1990; Kahraman, 1996; Cheville, 1999). The pathological data, consists of the data examined between the years 2000-2020 in Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Pathology.

As in human diseases, rapid diagnosis is of great importance in animal diseases. In addition to being rapid, the diagnosis should also have a high accuracy value. Today, computer applications are widely used in the field of health. One of the best examples of this is the use of artificial intelligence applications as a cancer treatment tool (Sütçü & Aytekin, 2018).

When the data in the database are evaluated according to animal species, most of the samples examined belong to ruminants, followed by animals such as dogs and cats. It was observed that digestive and respiratory system diseases were the leading causes of death in adult ruminants, and neonatal septicemia in juvenile ruminants caused significant death. Tumors were most frequently encountered in cats and dogs (Özmen, 2006).

Pandas library was used for data processing and analysis (Pandas-a, 2021). The main purpose of using the Pandas library is to use dataframe structures (Pandas-b, 2021). At the same time, it can import data in a simple way thanks to its data import feature from different formats like excel (Pandas-c, 2021). Since the Pandas library can also work with libraries such as NumPy and Matplolib, it also provides the opportunity to use these libraries. The NumPy library is a library that enables mathematical operations on arrays using matrices and arrays (Numpy-a, 2021; Numpy-b, 2021). Since the Pandas library uses dataframes as objects, it is possible to manipulate these dataframes as arrays with NumPy. Another library, MatPlotLib is a library that provides graphics creation. It can easily create various graphics such as line, bar, or pie plot over the dataframe.

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The method used to complete missing data is the SimpleImputer method in the sklearn.impute library. The SimpleImputer method uses different strategies according to the format of the data (numeric or categorical) (Scikit Learn-a, 2021). Since the data in the database are categorical data, the strategies used in the categorical data were used in the incomplete data completion processes. The methods used in the prediction of disease type are, DecisionTreeClassifier method in the sklearn.tree library and KNeighborsClassifier method in the sklearn.neighbors library (Scikit Learn-b, 2021; Scikit Learn-e, 2021).

Diagnosis has a great role for the treatment and control of diseases in animals. Evaluation of lesions in dead or live animal specimens is important so that the diagnosis can be made rapidly and accurately. Diagnosis is based on understanding general and specific pathology and the application of these categories to diagnosis (Jones & Hunt, 1993; Özmen, 2006). For the correct treatment of diseases, the diagnosis must be made precisely and correctly. Samples for diagnosis can be taken after the death of animals, or pathological samples can be obtained from live animals by appling surgical procedures. In these surgical procedures, reasons such as the inability to obtain tissues using the correct technique or obtaining faulty tissue may lead to incorrect diagnoses and therefore inability to perform treatments properly (Nakhleh, 2015; Özmen, 2021). The correct processing of the taken of pathological samples and the proper evaluation of the pathological findings not only increase the success rate in diagnosis, but also increase the success rate in the treatment process (Özmen, 2021).

As can be seen in Table 1, artificial intelligence and deep learning applications have come a long way from the past to the present, and they can be widely used not only in the field of health but also in many fields. For example, eight of hundred companies that develop artificial intelligence applications, develop applications in the field of health (Sütçü and Aytekin, 2018). As technology develops, the increase in this number and the applications produced can greatly benefit humanity in developing diagnostics that are both rapid and accurate. In this study, it is aimed to analyze animal diseases, to make a quick diagnosis depending on the estimation result, and to have a high accuracy rate in relation to the diagnosis speed. Our aim is to analyze the animal disease types, with animal species and pathological-anatomical diagnoses and their prediction using

Table 1. A timeline of specific AI innovations that resulted in the conquest of cancer (Sütçü & Aytekin, 2018)

Date	Description
1952	Marvin Minsky introduced the Stochastic Neural Analog Reinforcement Calculator (SNARC), the first connectivity neural network learning machine, and possibly the first self-learning machine.
1975	The back propagation algorithm was developed, which solves the difficulties in computer-aided machines, trains multi-layer neural networks, and provides widespread use of neural networks in the 1980s.
Around 2000	The term “deep learning” was used for the first time to describe a machine learning, the creation of networks that can learn from unstructured data in an unsupervised manner.
2011-2012	The convolutional neural network AlexNet has achieved unprecedented accuracy in visual recognition, paving the way for the deep dives into the mainstream.
January 2017	Researchers at Stanford University have developed a deep leaning technology that can visually identify cancerous skin and lesion with the same precision as a human dermatologist.
February 2017	Microsoft founded Healthcare NeXT, a startup design to apply artificial intelligence and machine learning technologies to health problems, including cancer treatment.
March 2017	Google’s GoogleNet deep learning technology detected cancerous tumors with higher accuracy than human clinicans.
October 2017	Intel has announced the Nervana Neural Network Processor (NNP) chip that can accelerate deep learning tasks, including cancer diagnosis.
Around 2021-2026	Microsoft will launch an AI-powered computer inside the human body to detect and reprogram cancerous cells and render them harmless.

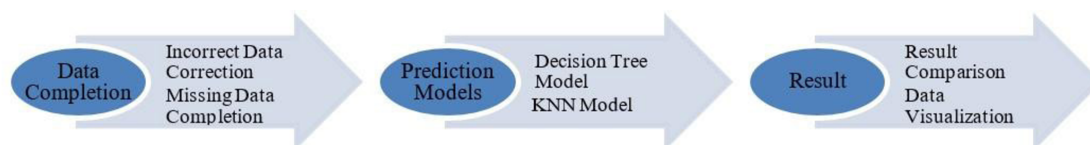

Figure 1. Flow chart followed in the study.

several categories available in the database.

MATERIALS and METHODS

The flow chart that showing of the path to be followed in this study is given in Figure 1. Firstly, data completion processes were carried out, and two different classification models were created using the completed data. The results were compared and then data visualization processes were applied.

Although incomplete and incorrect data are seen in the spelling of letters and similar words in most categories, the Age category may be the one with the most irregularity (Figure 2-c). The reason for this is that data is entered in multiple values (daily, weekly, monthly, etc.), instead of a single standard value. To fix this, each data must be specified over a single value. We can express this in the best way in a daily format.

After correction, the Age category can be seen in Figure 2-d. While there were 647 different categories before the correction, there are 149 different categories after the correction. This shows that it needs to be corrected to achieve a more standard and effective result.

Figure 2. Missing data in the database (a), values for type category (b1, b2), values for age category (c) age category after editing (d), data types (e).

Data Completion

Before proceeding with the analysis and estimation processes, missing or incorrect data in the data set should be corrected. In this way, it is aimed to increase the success rate of the estimation, while providing more accurate analysis. While there are approximately 5500 reviews from 24 different categories in the database, it is seen that some data are incomplete or incorrectly entered. In Figure 2-a, the values of the missing data based on categories are given.

Incorrect Data Correction

The presence of incorrect data in the database, usually due to reasons such as wrong lettering or use of small capital letters, may adversely affect the analysis result. Figure 2-b shows some of the values belonging to the Species category and how many of these values there are. For example, the "Oğlak(goat)" value is written in 3 different ways, and this same value is divided into 3 separate data sets. In another example, it can be seen in the data entered as "Sığr" instead of "Sığır(cattle)". Likewise, the example of "Deve Kuşu" and "devekuşu(ostrich)" can be given as examples of wrong data. In other words, the same species may appear more than once, and some species appear to be expressed with different words (sığır(cattle) vs. inek(cow), etc.). There are 301 clusters in the Species category. After correcting the wrong data, the number of clusters decreases to 69. With this decrease in the number of clusters, a more general and collectively categorized category has emerged, allowing clearer and more precise results in the analysis results.

The "NaN" value that appears in the Age category means "not a number". Some categories are originally referred to as objects, and they must be strings to be edited. When these categories are converted to string values, the null values that were originally "null" do not appear as empty strings. Therefore, "NaN" value is used instead of "null" value (Figure 2-e).

Missing Data

Before completing the missing data, data visualization was made, and it was determined which data were missing. In this way, it is aimed to complete the missing data in a simpler way. The graph shown in Figure 3 gives the distribution of missing data by categories. The protocol number appears to be complete, as it is in the form of an identifier for each entry. In the categories with a large number of missing data, those outside the category of disease type will not be used in examinations and analyzes as they will not affect the result.

Missing Data Completion

After correcting the erroneous data in the data set, data completion operations can be applied. Primarily, the bar graph showing the number of missing data after removing the missing categories and correcting the erroneous data is given in Figure 4. By dividing the number of missing data by the total number of data, we can learn the rate of missing data in the data set. To find out as a percentage value, it will be sufficient

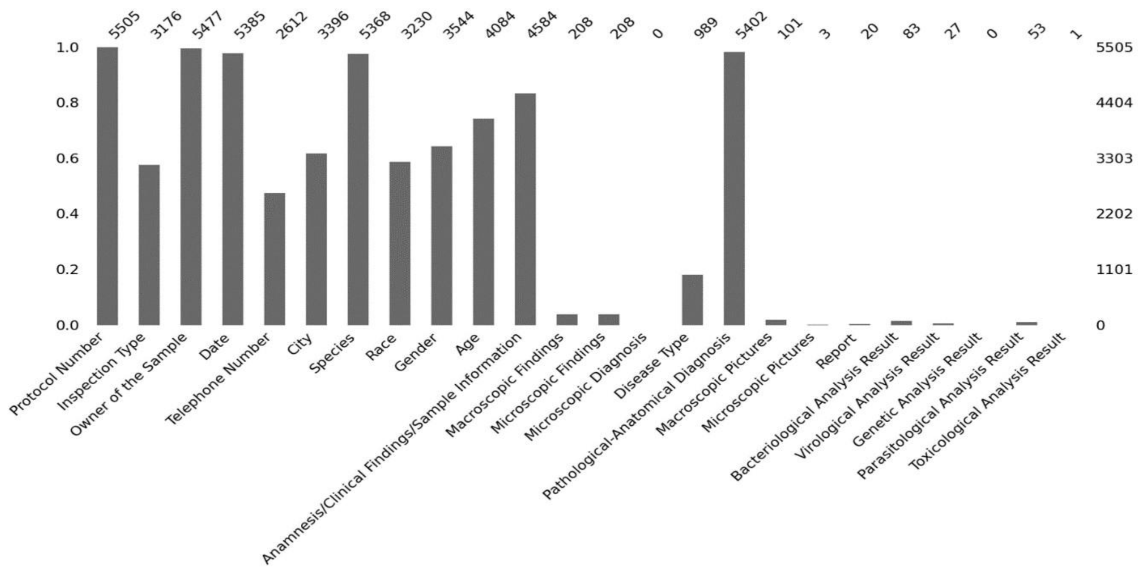

Figure 3. Bar graph representation of missing data.

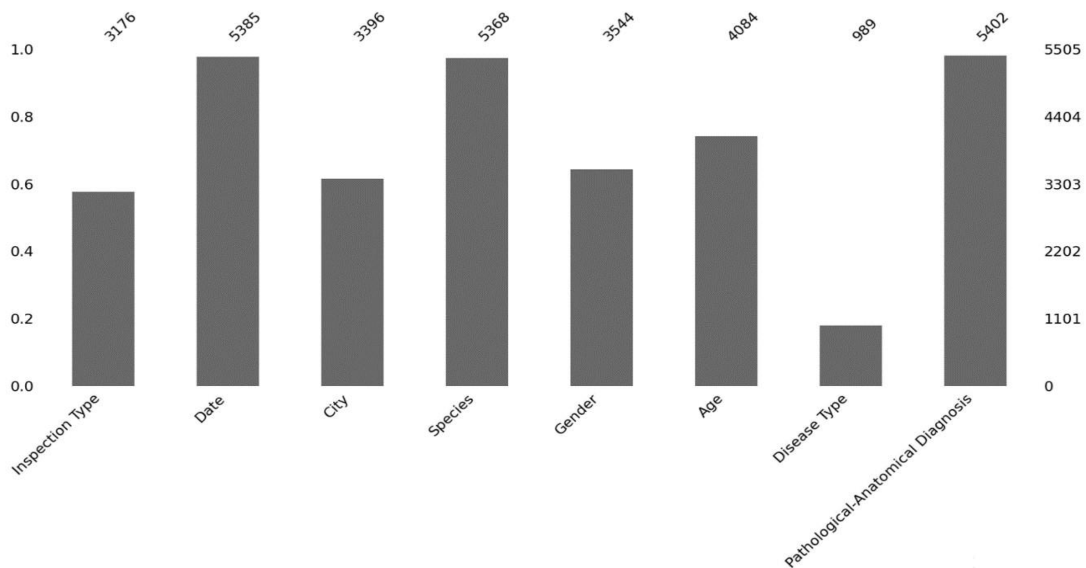

Figure 4. Number of missing data in the data set after missing categories were removed and incorrect data were corrected.

to multiply the result by 100. After performing these operations, it was calculated that there was 59.54% missing data in the data set. The amount of missing data to be used in analysis and estimation processes in the data set after the category removal process is 28.89%. This result shows that almost 1 out of every 4 data is missing.

According to the data graphics after removing the missing categories and correcting the wrong data, it is seen that more than half of the categories other than the Disease Type category are not missing. Among these categories, the Date category is the simplest as incomplete data completion. It is possible to complete the missing data by looking at the date range entered in the data set. This can be done by looking at the date of the data that comes before the missing data. With this method, the Date category is filled in completely.

Another category that can be completed manually is the City category from which the samples come. The missing data were completed by comparing the sample owners with the City categories. The Simple Imputation method of the sklearn.impute library can be used to fill in the remaining categories. This method applies data completion in accordance with both numerical and categorical data. There are two different strategies for categorical data. These are: most frequent, that is, the most frequently found data in the database, and constant, that is, entering a constant value (Scikit Learn-a, 2021). The most frequent method was applied in the remaining missing categories, and the missing data in the categories other than Disease Type were completed.

The reason why the Disease Type category is not completed with the imputation method is that the amount of data in the category is too incomplete and if it is completed with this

method, the result will be the same as the most found data in the category. To complete the Disease Type category, it should be compared with the Pathologic-Anatomical Diagnosis category and the results should be taken according to their common values. As a result of this process, Disease Type category is divided into bacterial, tumoral, parasitic, viral, and other, which can be counted as 5 main categories. Other category includes the conditions in which the remaining four categories are together, as well as anomalies and traumatic lesions.

Forecasting with the Decision Tree Method

After the data completion processes were completed, the model estimation processes were performed on the complete data set. Prediction models can be expressed in two different ways: classification and regression. The prediction obtained by the decision tree method results in classification. Classification is the definition of data into predetermined classes according to their common characteristics. The Disease Type category in the data set was used for classification. Therefore, the category is divided into 5 separate classes: bacteriyal, tumoral, parasitic, viral, and other.

The Decision Tree method used for classification has a structure like the flowchart graph and allows the data to be divided into specified classes (Figure 5). The inductive method is used for classification. In the decision tree method, by performing many tests during classification, the classification result is obtained in the best way. During this process, each test creates one of the branches of the decision tree. For the test process to end, it must reach the leaf node. The order from the tree root to the leaf node containing the classification result is called the if-then rule (Emel and Taşkın, 2005).

The induction method used in the decision tree is called tree induction. This method starts with an empty tree. Tree induction used to fill this tree is an iterative method (recursive) and consists of four steps (Emel and Taşkın, 2005; Zorman et al., 2001):

Step 1: If the result of the training objects in the training set is the same, a leaf node is created using this result and continue from step 4.

Step 2: Using the heuristic evaluation function, starting from the root of the tree, on the way to the current node, the best feature is selected among the previously unused features and a split node is created for this feature. The training set is divided into subsets.

Step 3: Continue from step 1 for each sub-training set created.

Step 4: Recursion is performed by going up one level.

As a result of these four steps, basically two processes take place. These are splitting and pruning. After these processes, the stopping criterion comes to the end of the iteration method (Emel and Taşkın, 2005).

Division is a method that allows the training set to be divided into smaller subsets. In division, which is an iterative method, the first iteration covers the entire training set, including the tree root. The remaining iterations are processed using derivative nodes that include subsets of the training set.

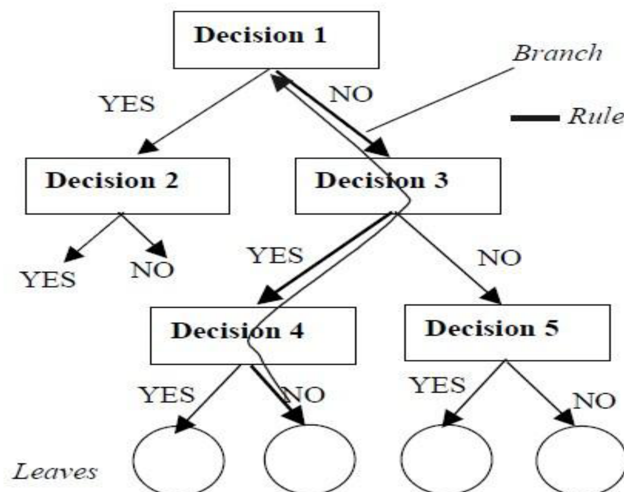

Figure 5. An example decision tree diagram (Bounsaythip and Esa, 2001).

At each division step, the data is analyzed, and the best classification is selected. The most important characteristic of the division process is that it is greedy, therefore, in this method, the algorithm does not look at the steps forward on the tree to find out whether it has achieved the best result (Emel and Taşkın, 2005; Bounsaythip and Esa, 2001).

For the decision tree to be formed, the iterative method must stop. Stop criteria are used for this stopping operation. The stopping criteria usually include a few rules such as the maximum tree depth, the minimum number of items in the node considered for splitting, or the minimum number of items that should be included in the new node. It can change the parameters associated with these rules according to the data type used or the user's request. Generally, applications using this method build trees at maximum depth. While such a tree with maximum depth predicts all the objects in the train-

ing set with a certain probability, they are most likely overfit the data (Bounsaythip and Esa, 2001).

After the decision tree is formed, pruning is used to remove unwanted nodes or subsets due to overfitting. The pruning method removes partitions and their subsets and makes the decision tree more stable. Applications that create trees with maximum depth include an automatic pruning method. After the decision tree training is completed, the estimation process can be applied for the new data by using the path formed from the top of the tree until reaching any result node (Bounsaythip and Esa, 2001).

DecisionTreeClassifier belonging to the Sklearn library is a library used for classification. To make classification, firstly, the categories or columns to be used in the classification and the column containing the classification result should be divided into separate dataframes. To classify the Disease Type, Species and Pathological-Anatomical Diagnosis columns are separated. In some cases, the separated categories need to go through some pre-processing before they can be classified. Here, the one-hot method is used to convert data in categories containing text to numeric values. In the Pandas library, the one-hot method can be easily processed as a single line of code with the pandas.get_dummies method (Pandas-d, 2021). When the categories to be used in classification are ready, model training can be started. In order to determine how successful the model is, the data set should be divided into two sets as training set and test set, before model training. For this separation, the train_test_split method of the Sklearn.model_selection library was used (Scikit Learn-c, 2021). Separation rate can be determined with test_size, which is one of the parameters of this method. This allowed us to separate the data set as the test set as much as the entered parameter size and the rest as the training set. After the data set is divided into two sets as training and test, the model to be used for classification should be defined before starting the model training for classification. This definition can be done by calling the DecisionTreeClassifier function belonging to the Sklearn.tree library. After the defination process, the training is completed using the fit function (Scikit Learn-b, 2021). After the completion of the training, the success rate becomes measurable, and the model is ready to make predictions for the new data to be entered. In order to measure the success rate, the values in the test set are estimated over the trained model by using the predict parameter belonging to the DecisionTreeClassifier function. Then, the accuracy_score method of the Sklearn.metrics library was used to compare these estimates with the actual result values (Scikit Learn-d, 2021).

Forecasting with the KNN Method

The other classification method to be used in this study is KNN (k-nearest neighbors) that classifies according to the mean value of the nearest neighbors in the training set. The k value here indicates the number of neighbors to be selected. In order to find the nearest neighbor, it is necessary to calculate the distance between the points. Different distance metric measurement methods such as Euclid, Manhattan, Minkowski can be used to measure this distance. Generally, Euclidean distance is the most preferred method among these methods (Euclidian Distance, 2021; Scikit Learn-f, 2021). The Euclidean distance used to calculate the linear distance between two points (eg P = (p_1, p_2,…,p_n) and Q =(q_1, q_2,…,q_n) points) is given in formula 1 (Euclidian Distance, 2021).

$$\sqrt{(p_1-q_1)^2+(p_2-q_2)^2+\cdots+(p_n-q_n)^2}=\sqrt{\sum_{i=1}^{n}(p_i-q_i)^2}.$$

Formul 1.

The KNeighborsClassifier method of the Sklearn.neighbors library designed for KNN is a method that includes preliminary steps such as distance measurement (Scikit Learn-e, 2021). As in the decision tree method, before starting the model training, the data set must be pre-processed and divided into two sets as the training set and the test set. For the definition of the model, the KNeighborsClassifier method was used with the k parameter indicating the number of neighbors (Scikit Learn-e, 2021). After this process, the data sets are transferred to the model with the fit method and the training is completed. The predict parameter in the DecisionTreeClassifier library is also available in the KNeighborsClassifier method. Estimation is performed with this parameter. For the success rate, the accuracy_score method of the sklearn.metrics library is used (Scikit Learn-d, 2021).

Calculating Margin of Error with RMSE, MSE and MAE Methods

Among the methods used to calculate the difference between the estimated result and the actual values, there are methods such as MSE (mean squared error), RMSE (root mean squared error) and MAE (mean absolute error). The MSE, or mean absolute margin of error, is the mean value of the absolute difference between both variables in the data set. If the MAE result is close to 0, it indicates that the value gives the best result. The formula for calculating MAE is given in Formula 2 (Mean Absolute Error, 2021).

$$\mathrm{MAE}=\frac{\sum_{i=1}^{n}|y_i-x_i|}{n}=\frac{\sum_{i=1}^{n}|e_i|}{n}.$$

Formul 2.

The MSE method, which is referred to as the mean square error, gives an absolute number indicating how much the predicted results differ from the actual results in the data set. The closer the MSE value is to 0, the better the result is. MSE is given in Formula 3 (Mean Squared Error, 2021).

$$\mathrm{MSE}=\frac{1}{n}\sum_{i-1}^{n}(Y_i-\hat{Y}_i)^2.$$

Formul 3.

RMSE (or RMSD), root mean square error method is the square root of the result of the MSE method and gives smaller

results than the MSE method. For this reason, it is generally preferred over the MSE method. The RMSE is given in Formula 4 (Root Mean Squared Error, 2021).

$$\mathrm{RMSD} = \sqrt{\frac{\sum_{t=1}^{T}(\hat{y}_t - y_t)^2}{T}}.$$

Formul 4.

The mean_squared_error and mean_absolute_error methods of the sklearn.metrics library were used to measure the margin of error between the predictions and the actual result in this study. By changing the squared parameter in the mean_squared_error method to true or false, it is possible to switch between MSE and RMSE methods (Scikit Learn-g, 2021; Scikit Learn-h, 2021).

RESULTS

Graphical Findings

As a result of the analyzes made on the categories belonging to the database, some graphics were produced. While some of these graphics were extracted directly from dataframes using Pandas and MatPlotLib libraries, SPSS 22.00 version program was used for some of them. In order for the graphs to be readable and smooth, the most common values in the category were shown, and the remaining values were arranged in a collective way.

Charts Obtained as a Result of Analysis

There are 24 different values in the City category in the database. These are the 24 provinces from which the samples came. Except for the provinces, it is found in samples from the districts of Burdur province. These are shown in Burdur in the provincial category (Figure 6).

According to this graph, the most samples came from Yeşilova district in Burdur province. On the provincial basis, the most samples came from the province of Burdur. In the evaluation of all animal species in the data set according to Gender, the majority of the samples coming between the years 2000-2020 are female animals (61.14%).

The Inspection Type category applied to the incoming samples is divided into four main areas. These; Biopsy, Necropsy, Cytology, and Organ Sample. The combination of these four fields is shown in the other category (eg Biopsy; Organ Sample). Necropsy was the most common type of examination performed on the samples (75.59%). This is followed by Biopsy (14.28%), Cytology (5.01%), Organ Sample (3.94%) and other categories, respectively.

There are 68 different categories of species appearing in the database in the separation of incoming samples according to species. The first five categories that make up the majority of these are percentaged, while the remaining species are shown as other. According to these values, the most sampled animal species was goat (29.46%), respectively; sheep (17.68%), cattle (15.30%), dogs (14.04%) and cats (5.29%). In the evaluation made according to the age ratio of the incoming samples, it is seen that the most samples come under the age of 1 (79.46%). This is followed by over 5 years (8.37%), 1 year (5.36%), 2 years (4.05%), 4 years (2.52%) and 3 years (0.24%).

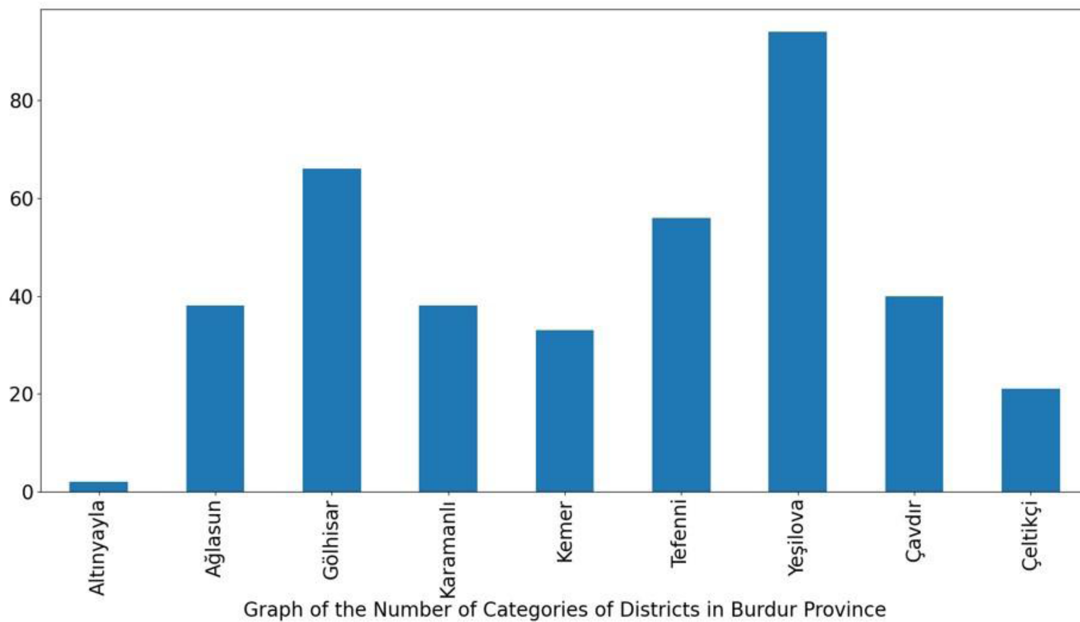

Figure 6. Graph of the number of categories of districts in Burdur province.

The Disease Type category, which is also a classification result category, gives four different results. These are respectively; bacterial (41.34%), tumoral (26.38%), parasitic (10.32%) and viral (6.49%). Results other than these values are given in the other category. The other category, which includes the

combination of these four results, also includes anomaly and traumatic values.

According to the cities, the city with the most samples is Burdur, and the most common disease from this city seems to be bacterial. After the bacterial disease comes the tumoral disease. The most common species from Burdur was goat. Goat type respectively; cattle, dogs, sheep, and cats follow. In the evaluation made according to the examination type, it was observed that necropsy, which is the most common type of examination, was applied more in female animals. Bacterial diseases, which are the most common type of disease among animal species, are most common in goats, while tumoral diseases are most common in dogs. According to the sex ratio of the samples coming between 2000 - 2020, female animals came almost every year more than male animals.

The line chart showing the number of samples received between 2000 and 2020 is given in Figure 7. According to the graph, it is seen that the number of samples increased in 2011, 2012, 2014, 2015, 2018 and 2019. The reason for the decrease in 2020 is due to the fact that the data is until August 2020.

under. The category in which necropsy is the most common type of examination consists of animals aged 1 and younger. In the examination types other than necropsy, it is seen that animals 1 year old and younger are in the majority. Again, it is seen that female animals come more than male animals. According to the distribution of animal species according to age, it comes to the fore that mostly 1 year old and younger animal species come to the fore in all species.

Similar to other results, it is seen that animals under the age of 1 are also in the first place in the category of disease types. In addition, it is seen that bacterial and tumoral disease types are generally seen in other age categories.

Considering the ratio of the sexes of animals to animal species, it is observed that female animals are more common in goat, cattle, sheep, dog, and cat species, while the number of females in the goat category, which is the most common animal species, is even more than twice the number of males. On the other hand, in the category that includes the rest of the animal species, it is seen that the ratio of males is high. In this category, budgerigar, chicken, and fish species are the species with a higher male ratio. Finally, it is observed that the disease types are generally seen on female animals.

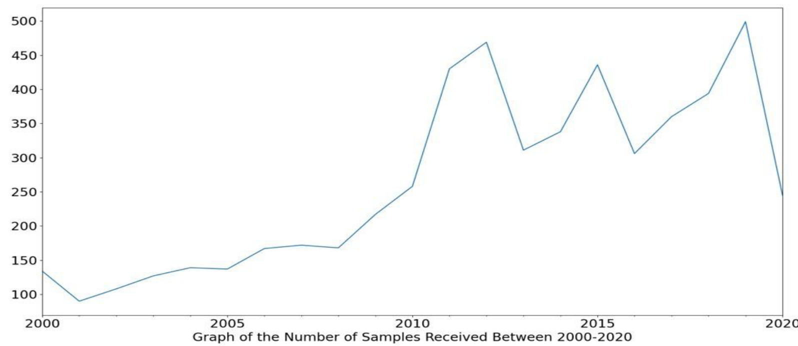

Figure 7. Graph of the number of samples received between 2000-2020.

Graphs Obtained from Statistical Analysis

Statistical analysis of the data of this study was done in SPSS (v.22.00) package program. The same program was used for the graphical representation of the data. When we look at the distribution of animal ages by years, it is seen that the most common cases are in the 1 year and under category, and they came between 2011-2015, while animals aged 10 and over usually come in 2015 and later years. It was observed that the animals coming from Burdur province were mostly 1 year old and

Decision Tree Method Results

During the decision tree classification process, the test_size value entered as a parameter is used to distinguish between training and test data sets. In this study, a test data set of 30 percent was created (3853 training sets, 1652 test sets, a total of 5505 data), and the estimation and error margin results of the models trained with these data sets are given in Table 2. In

Table 2. Table of values obtained as a result of the decision tree model

max_depth	Prediction	MAE	MSE	RMSE
5	0,643	0,752	2,032	1,426
10	0,710	0,615	1,680	1,296
15	0,719	0,568	1,493	1,222
20	0,719	0,568	1,493	1,222
None	0,719	0,568	1,493	1,222

the table, the estimated result values obtained from the models with different depths and the margin of error values of MAE, MSE and RMSE are given. According to the data in the table, it is observed that the result is stable when the depth is defined as 15 and above. The default value of the max_depth parameter to "None" indicates that the depth of the tree has reached the maximum depth it can reach. These value results are added to the last row in the table.

The max_depth value is the parameter that specifies the depth number of the decision tree, and when the default value is set to "None", it goes to the deepest point of the tree. The graph consisting of the results from the table is given in Figure 8. As can be seen from the graph, when the depth number is defined as 15 or more, a fixed value appears in the results and margins of error. For this model, 15 depth values are seen as the best value, and the graph related to this depth is given in Figure 9.

KNN Method Results

For the KNN classification process, the training and test data sets prepared for the decision tree model are used in the same size. The estimation and margin of error results of the KNN model are given in Table 3. In the table, the estimated result values obtained from the models with different near-neighbor numbers and MAE, MSE and RMSE margin of error methods are given. According to the data in the table, the higher the number of neighbors, the better the prediction value in general, and the margin of error values seem lower than the previous value. In the KNN model, the default number of neighbors is 5, and it is seen that the values above the default number of neighbors yield better results.

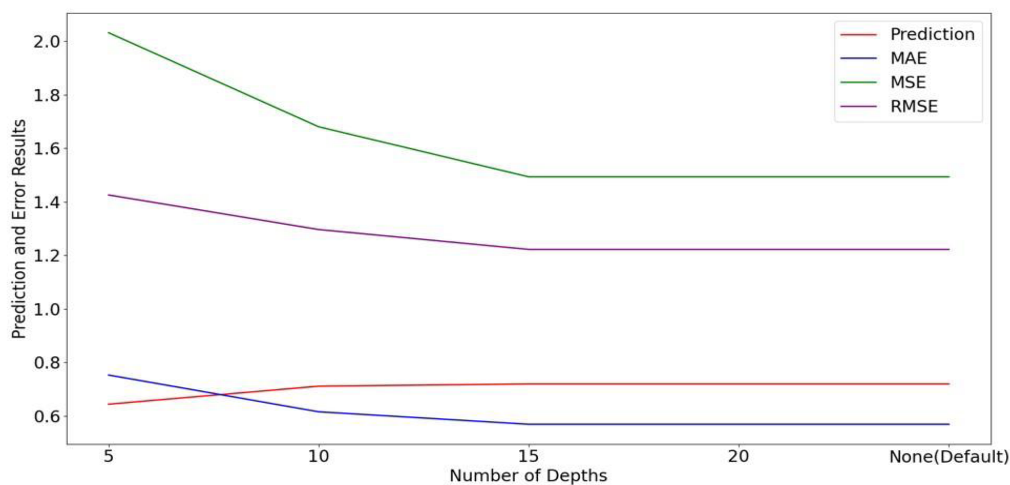

Figure 8. Decision tree model prediction and error margin graph.

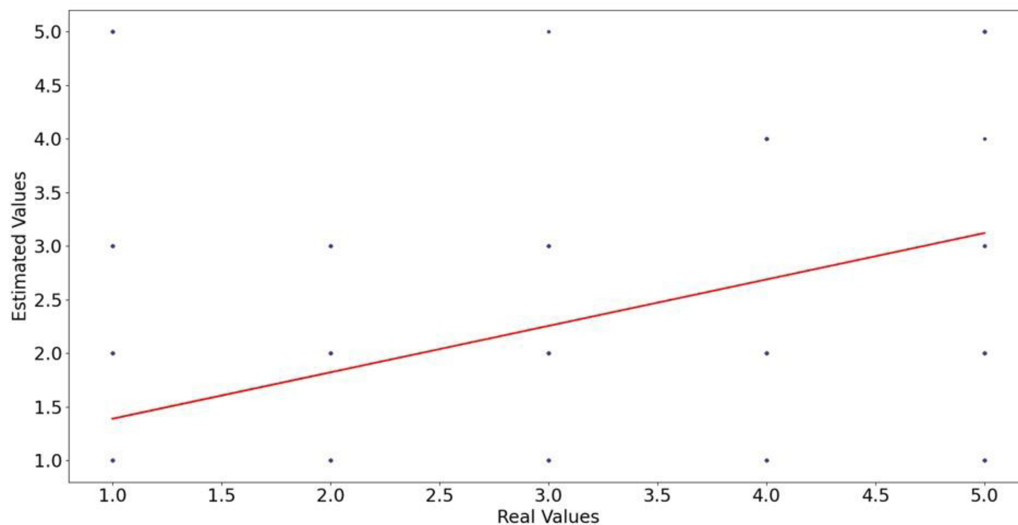

Figure 9. Pattern graphic with depth number 15.

The graph consisting of the results from the table is given in Figure 10. As can be seen from the graph, the higher the number of neighbors, the better the results emerge. The estimation result of the value with 8 nearest neighbors for the KNN model was the highest among the results of other

Table 3. Table of values resulting from the KNN model

n_neighbors(k)	Prediction	MAE	MSE	RMSE
3	0,700	0,665	1,844	1,358
4	0,699	0,661	1,804	1,343
5 (default)	0,699	0,661	1,804	1,343
6	0,709	0,582	1,508	1,228
7	0,704	0,601	1,591	1,262
8	0,718	0,594	1,607	1,268
9	0,710	0,579	1,492	1,221

The n_neighbors(k) value indicates the number of near neighbors, and the default value is 5.

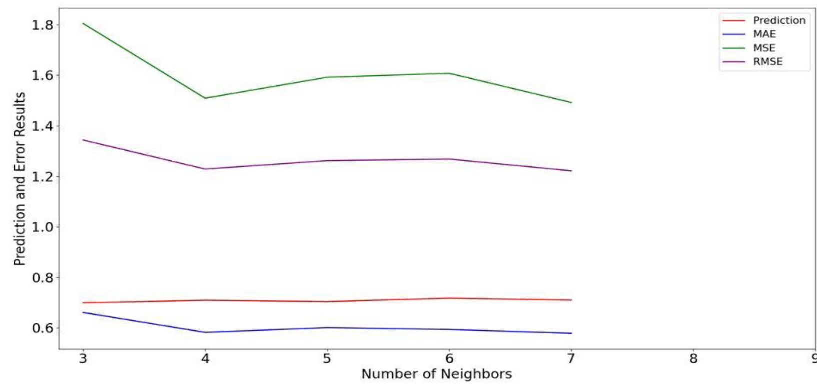

Figure 10. KNN model prediction and margin of error graph.

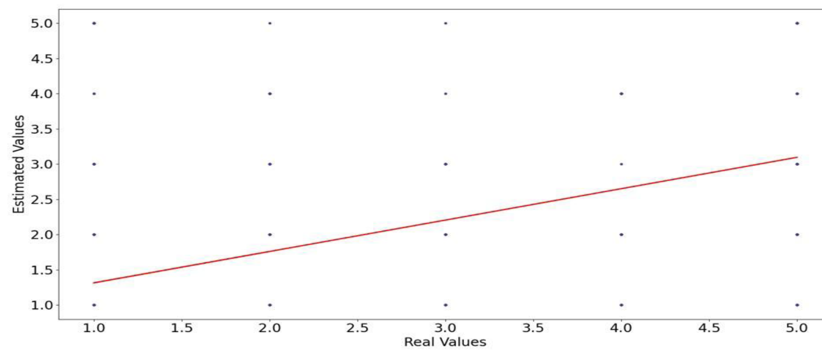

Figure 11. Model graph with 8 neighbors.

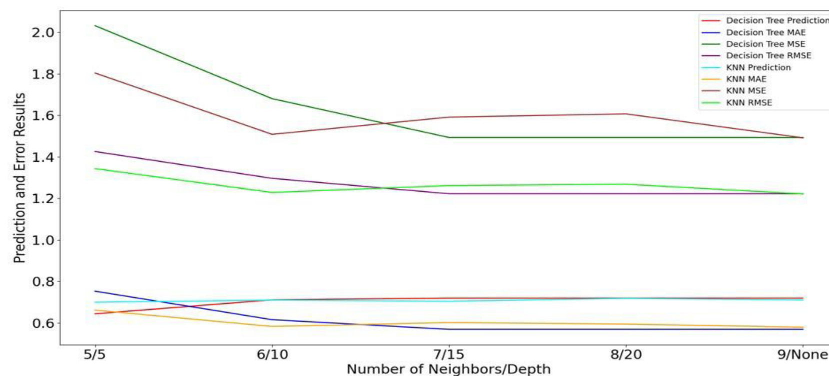

Figure 12. Model result comparison Chart.

values. The graph related to the value results with 8 neighbors is given in Figure 11.

The comparison chart of the results of the two models is given in Figure 12. Since the parameter values of the models are not equal to each other, the values with the number of neighbors of the KNN model less than 5 are not included in the graph. Although the prediction results of the models are almost the same, it is observed that the decision tree model gives a better result with a very small difference.

DISCUSSION

Depending on the developing technologies, there are continuous developments in the field of pathology today. Apart from the development and change of devices and tools used in the field of pathology, the use of computer applications is also increasing and developing. Pathological procedures usually require long-term procedures. Shortening this period may save extra time for the treatment methods to be applied with the diagnosis to be made. Today, a field called digital pathology has emerged that allows the analysis of pathological samples to be transferred to the computer environment, the results can be visualized using various applications, these results can be analyzed, and the results can be stored (Barisoni et al., 2017; Bera et al., 2019; Özmen, 2021).

Advances in the field of pathology enable rapid and accurate diagnosis. For this reason, the technological tools and devices used in the field of pathology and the techniques applied contribute to the rapid and accurate diagnosis. With the advanced computer applications that can be used, it can make a positive contribution to the studies to be done in the field of pathology and provides an opportunity to work more easily. In addition, it is possible to share these studies in the network environment and it is possible to get information from other experts in diagnosis and treatment processes (Niazi et al., 2010; Abels et al., 2019; Chang et al., 2019; McCarty et al., 2006; Özmen, 2021).

Thanks to this study, the data of Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Pathology was analyzed using the data examined between 2000 and 2020, and after the analysis, the classification process was carried out. The results of the analysis are given and explained in graphics. Two different methods were used for the classification process and the results obtained from these methods were compared. During the classification process, the data set was divided into two as 30% test set and 70% training set. While obtaining the results of the models, different values were obtained in terms of obtaining the best result by changing the parameters. While 5 different results were obtained by changing the depth parameter in decision tree classification, 9 different results were obtained by changing the number of neighbors parameter in KNN classification. In the decision tree method, better results were obtained as the depth increased, while a constant value was obtained for the number of depths of 15 and above. In the KNN method, when the number of neighbors is taken less than the default value of 5, it is seen that the values are estimated at a lower rate, while the results of the model trained from the number of neighbors of 5 and above are seen to have better values. In the comparison, it was determined that the two models gave approximately 70% results. It was seen that the decision tree method gave better results with a very small difference. As a result of the high value definition of model parameters, the margin of error values gradually decreased and as a result of this decrease, an increase in the estimation results was observed.

CONCLUSION

The results of the analysis carried out in this study are shown in graphics. According to the results of the graph, the ratios of the data such as type, age, city, and gender of the samples to each other are seen. Thanks to these rates, the probability of making a more effective and faster diagnosis increases. The aim of this study is to make a more effective and rapid diagnosis in animal disease diagnoses. The results of the analysis are expected to show what kind of diseases the incoming samples or animals may encounter under certain conditions.

DECLARATIONS

Ethic Approval

Not applicable

Conflict of Interest

The authors declare that they have no competing interests

Author Contribution

Idea, concept, and design: AAŞ, ÖÖ, Vİ

Data collection and analysis: AAŞ, ÖÖ, Vİ

Drafting of the manuscript: AAŞ, AHI, ÖÖ

Critical review: AAŞ, AHI, ÖÖ, Vİ

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Research Article

An optimized protocol for the electroporation of NCI H929 multiple myeloma cells

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ABSTRACT

Multiple myeloma cell lines are difficult to transfect with non-viral nucleic acid delivery methods. While electroporation is the most efficient tool for the transfection of most hard-to-transfect cells, human multiple myeloma cells differ in their permissiveness and each cell type require different electroporation conditions for an efficient transgene delivery. In this study, various parameters for NCI H929 human multiple myeloma cells are tested to generate an optimized electroporation protocol. Findings from this paper showed that besides the voltage and capacitance settings, cell count, the cell cycle status of cells, the amount of nucleic acid and removal of death cells all impacted the electroporation efficiency and viable cell count. These results are expected to serve as a starting point and a guide for researchers.

INTRODUCTION

In vitro culture and genetic manipulation of animal and human cells are indispensable tools for understanding the function and behavior of cells under normal and pathological conditions. For this purpose, exogenous genetic materials are introduced to cells by various methods to induce or suppress the expression of a target gene. Successful delivery of the nucleic acids into the recipient cells is the most critical and limiting step of the genetic interventions. Various viral and non-viral delivery methods have been developed and employed by the researchers (Chong et al., 2021; Fus-Kujawa et al., 2021; Mizrahy et al., 2017). Despite the diversity of available methods, while some cell types are very permissive for the entry of foreign nucleic acids, others are very resistant and requires special optimization (Canoy et al., 2020; Shih et al., 2019).

Multiple myeloma (MM) cell lines with different genetic composition such as RPMI 8226, U266 and NCI H929 cells are widely used to study the molecular mechanism of the plasma cell malignancy (Hattori et al., 1995). These cells are often modified to examine the role of certain genes or pathways in the transformation of cells and their response to drug candidates (Brito et al., 2010; Sun et al., 2017). While viruses could be successfully used to derive stable changes in these cells, non-viral methods, such as coating nucleic acids with cationic lipid mediators or electroporation, are more convenient when transient modifications, such as small interfering RNA (siRNA)-mediated knockdown, are desired and permanent presence of foreign sequences, such as CRISPR plasmids expressing a DNA-cleaving endonuclease Cas9, are not intended (Brito et al., 2010). However, MM cell lines are not susceptible to most transient transfection methods and electroporation, the common means of gene delivery for these cells, also presents challenges (Steinbrunn et al., 2014). Studies in the literature and databases generated by electroporator manufacturers provide certain parameters such as voltage and capacitance, however crucial details including cell density, nucleic acid amount, the final transfection efficiency and viable cell counts, are oftentimes unreported, which hinders the reproduction of reported findings by other groups (Steinbrunn et al., 2014). The aim of this study is to develop an optimized electroporation protocol for NCI H929 MM cell line. Various parameters are sequentially optimized and incorporated to the protocol. Our findings will guide the researchers step by step in their transient transfection studies for achieving an improved efficiency.

MATERIAL and METHODS

Cell culture

NCI H929 cells are obtained from American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) under 37 ºC temperature and 5% CO_2 conditions. Unless otherwise stated, cells were passaged at a 1:2 ratio and supplemented with fresh complete medium the day before the transfection to promote log-phase in the culture.

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Transfection

Cells were counted and centrifuged at 200xg for 5 min. Pelleted cells were then resuspended in 200 µl serum-free, RPMI 1640 medium. Next, green fluorescent protein (GFP)-expressing pGIPZ empty plasmid (a gift from Dr. Onur Tokgun from Pamukkale University, Turkiye) were mixed with the cells in the tube by gentle stirring with the pipette tip and the DNA-cell mixture was immediately transferred into a 4 mm cuvette (Biorad). The cells in the cuvette were subjected to a single pulse of exponential decay wave using Gene Pulser Xcell (Biorad, California, USA) and 500 µl of pre-warmed media containing only 20% FBS was added to the cuvette and cells were immediately harvested by a transfer pipet. Cells were then seeded and cultured in a 6-well plate with 2 ml prewarmed, 20% FBS-containing media per well. The next day, live-dead cell count was assessed by Trypan blue staining, and cells were spun at 100xg for 5 min to remove dead cells. For complete elimination of dead cells, density centrifugation by Ficoll-Paque Premium (1.084, Cytiva) was performed. For this, at 24 h post-electroporation, 2 ml of cell suspension was laid on 3 ml Ficoll and spun at 400xg for 30 min using a swinging bucket rotor, with brake off. Following centrifugation, live cells were collected with a transfer pipet from the layer between the culture medium and Ficoll solution. The harvested cells were washed twice with 6 ml phosphate-buffered saline (PBS) by spinning the cells at 400xg for 10 min with brake on. At the end, the pelleted cells were resuspended in complete media (RPMI 1640 with 10% FBS and 1% Pen-Strep) and cultured for another day.

Cell imaging and analysis of transfection efficiency

Cells were examined and images were captured at 48 h post-transfection using an inverted fluorescent microscope (Nikon Eclipse Ts2). Total and GFP positive (GFP+) cell counts were determined from the images taken at 20x and 40x magnification. Transfection efficiency was reported as a percentage of GFP+ cells. Overlay images were generated in Image J/Fiji (Rueden et al., 2017). For the statistical analysis, the mean of GFP+ cell percentages were compared using student's t test and p value < 0.05 was considered significant. All statistical analyses were performed using Graphpad Prism 9.

RESULTS

Voltage and Capacitance

First, based on the manufacturer's recommendations and previous literature regarding MM cell lines (Biorad.; Steinbrunn et al., 2014), 950 µF capacitance with a voltage range of 100 V-300 V was tested. Independent of DNA amount, 100 V voltage had the best viable cell count, but the transfection efficiency was below 1% (data not shown). On the other hand, no viable cells remained at 300 V. Compared to previous settings, pulsing cells at 200 V yielded an improved transfection efficiency. The live cell count at 200 V was lower than at 100 V but better than at 300 V. Therefore, 200 V voltage and 950 µF capacitance settings were preferred for the rest of the experiments.

Logarithmic Phase of Cell Culture

Passaging or supplementing cells with fresh media is known to induce cell division (Oyeleye et al., 2016). Moreover, the plasmid vector is taken up by the mitotically active cells and is transmitted to the progenies, which ultimately yields a higher number of GFP + cells (Brunner et al., 2000; Hsu & Uludag, 2012). For NCI H929 cells, feeding cells with fresh media 24 h prior to the transfection, as well as passaging cells at 1:2 ratio were examined. When 2.5 µg and 5 µg of plasmid DNA with the same electroporation settings (950 µF, 200 V, 10^6 cells/200 µl media) were applied to cells, a very low transfection (<%1) efficiency was observed. However, both passaging cells at 1:2 ratio and feeding them with fresh media, 24 h-pre-transfection, drastically improved the transfection efficiency (Figure 1) and viable cell count. Hereafter, this became a part of the optimized protocol for preparing cells for transfection.

The Amount of Nucleic Acid

After an improved efficiency with logarithmic phase cells was observed, increased DNA input was tested. A range of 2.5, 5, and 10 µg pGIPZ-GFP plasmid was used to transfect 10^6 cells in 200 µl serum-free medium. Cells pulsed with predetermined instrument settings (200 V, 950 µF, a single exponential decay pulse) had the highest yield (GFP+ cell count) with 10 µg plasmid (Figure 2). Scaling up or down the cell count, transfection volume and DNA amount also generated equivalent efficiency (data not shown).

Cell Density

The ratio of media volume to cell number is another determinant of transfection efficiency and cell survival. Here, the same number of cells were resuspended in either 100 µl or 200 µl medium volume and were mixed with 5 µg plasmid DNA. As result of electroporation, 10^6 cells in 200 µl volume generated a better live cell count (Figure 3).

By scaling up the DNA accordingly, cell density up to $4x10^6$ cells in 200 µl volume was used for electroporating NCI H929 cells. No compromise in transfection efficiency was observed (Figure 4).

Dead Cell Removal

Electroporation transiently permeates the cell membrane for the exogenous nucleic acid entry, then cells recovers and continue to their normal growth and division cycle (Batista Napotnik et al., 2021). However, depending on the amplitude and duration of the electric pulse, a portion of cells is always lost and remnants of dead cells affect the quality of live cells in culture (Rols, 2017). Here, we first tried low-speed centrifugation (100xg for 10 min) for coarse removal of dead cells. However, dead cells still remained in culture after centrifugation. Alternatively, we performed Ficoll gradient centrifugation and successfully recovered a pure live cell suspension (confirmed by tripan blue staining). Live cells isolated by Ficoll gradient seemed relatively healthier and morphologically normal (Figure 5). GFP+ cell percentage was also significantly higher in cell population isolated by Ficoll gradient.

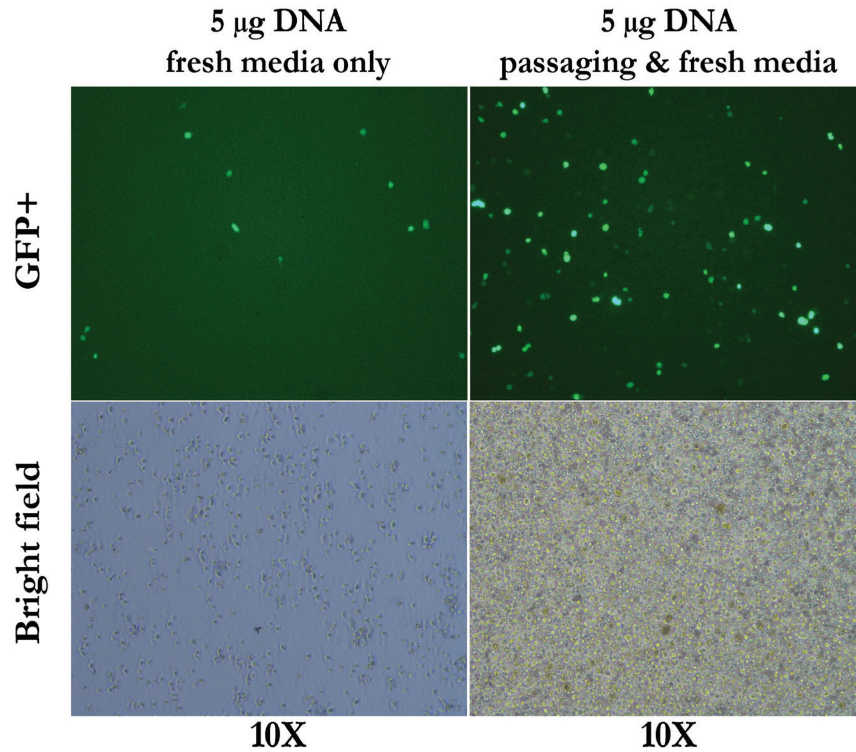

Figure 1. The effect of passaging and fresh media addition on transfection efficiency. Representative images from separate experiments show that 24 h prior to transfection, cells split at 1:2 ratio and fed with fresh media (right) contained higher population of GFP+ cells compared to only fresh media supplemented cells (left). Bright field and fluorescent images are captured 48 h post-transfection.

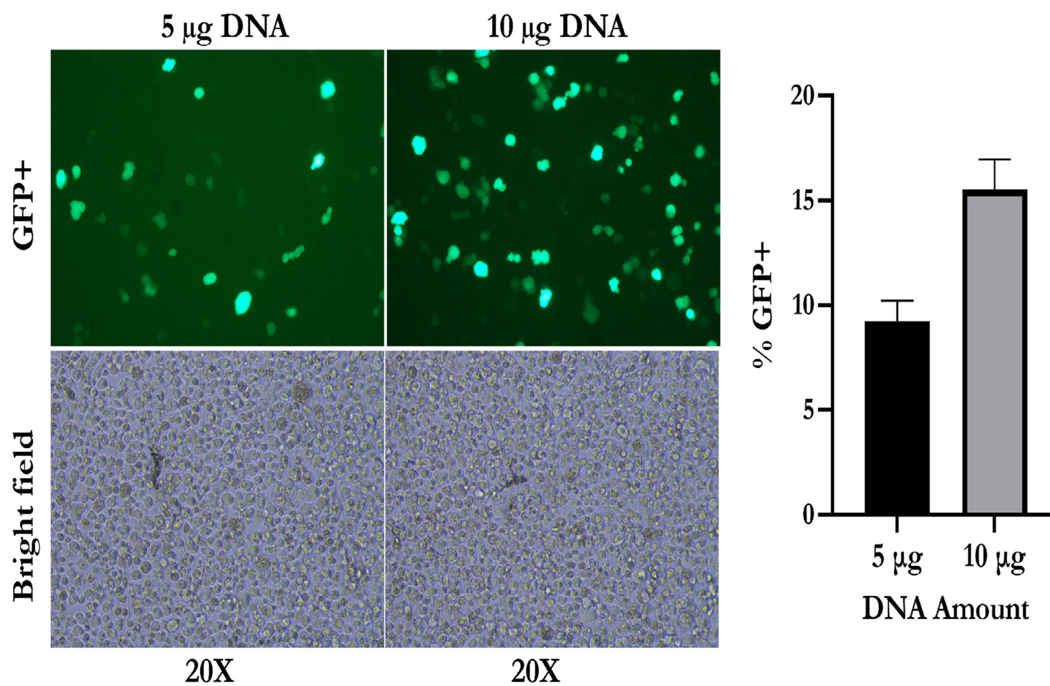

Figure 2. DNA input affects the transfection yield. 106 NCI H929 cells were taken from the culture that was split and fed with fresh media 24 h prior. Cells were pelleted and resuspended in 200 µl serum-free RPMI 1640 medium. Then, cells were mixed with DNA and electroporated (200 V, 950 µF, single exponential decay pulse). Each image is from separate experiments in which all the parameters except DNA amount were kept the same. Images (20x magnification) from at least two different fields were evaluated. The mean of GFP+ cell percentages was calculated and plotted with standard error of means.

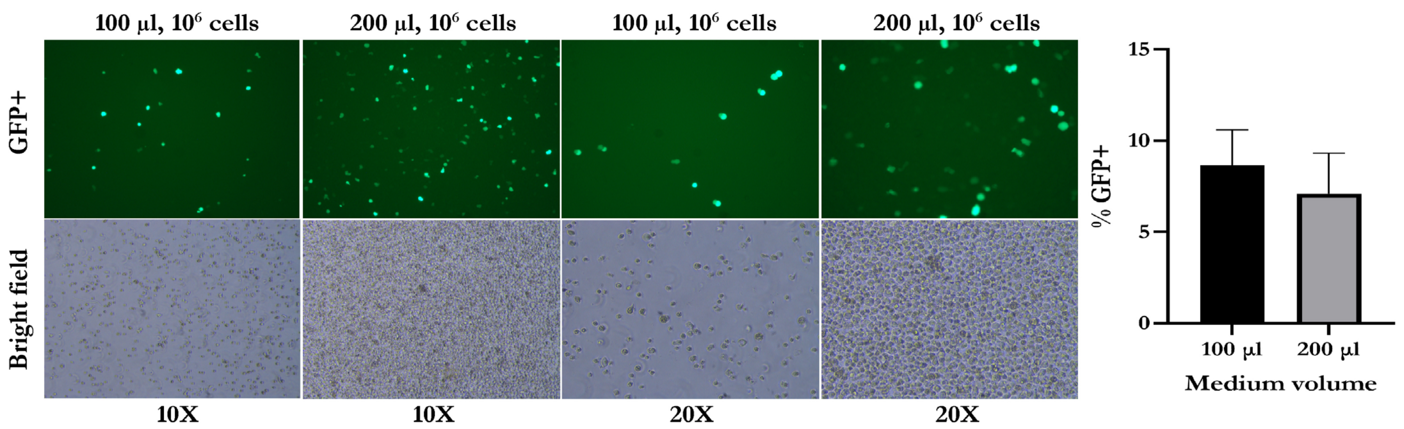

Figure 3. The effect of transfection solution volume on transfection efficiency. 10^6 NCI H929 cells were washed with serum-free RPMI 1640 medium once and resuspended either in 100 µl or 200 µl of the same medium. Cells were mixed with 5 µg pGIPZ plasmid DNA, electroporated (200 V, 950 µF) and seeded on a 6-well plate with a 2 ml prewarmed-media (RPMI 1640 with 20% FBS). At 24 h post-electroporation, cells were spun at 200xg for 5 min to eliminate cell debris. At 48 h post-electroporation, plates were monitored under the inverted fluorescent microscope and images were taken. Images represent the findings from the same experiments. GFP+ cell percentages were determined from at least two different fields. The mean of GFP+ cell percentages was calculated and plotted with standard error of means.

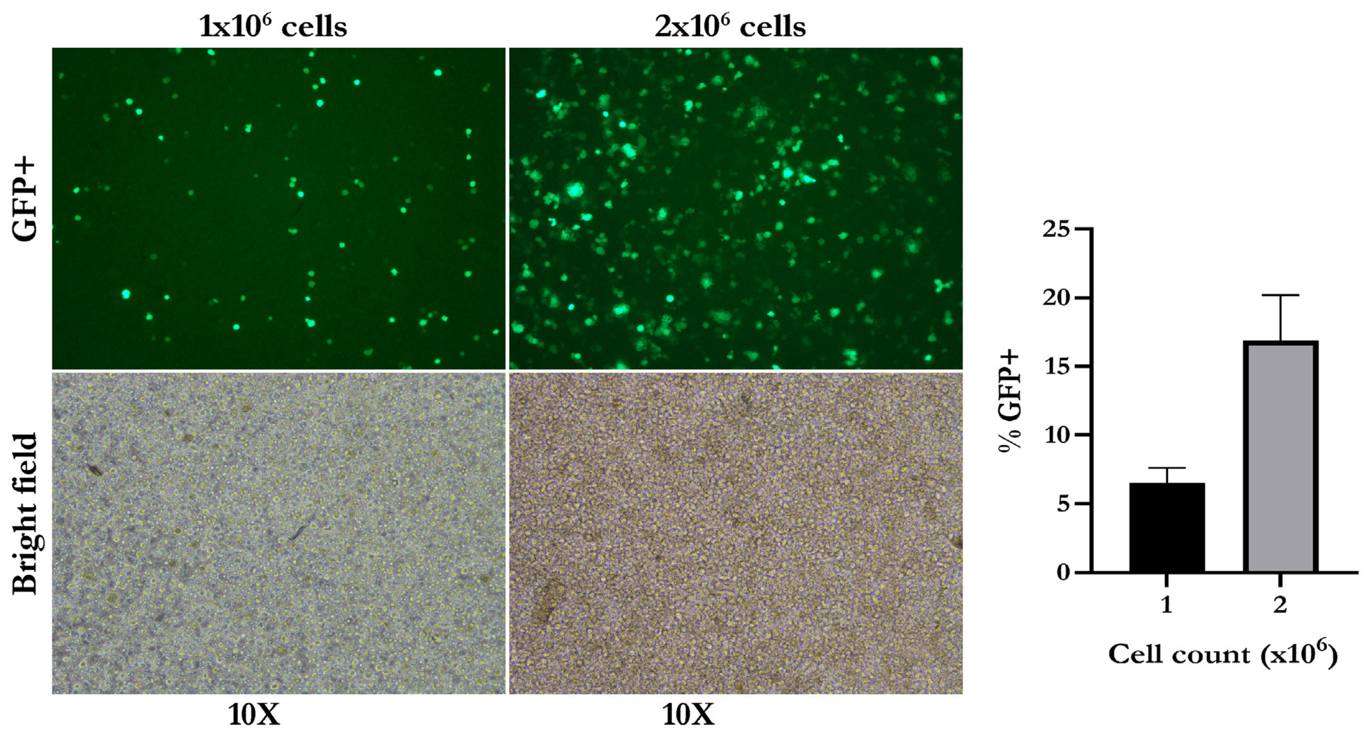

Figure 4. The effect of starting cell number on transfection efficiency. 1, 2 and 4 million NCI H929 cells were mixed in a tube with 10 µg of plasmid DNA and pulsed at 200 V, 950 µF with a single exponential decay wave. Cell images were taken 48 h post-transfection under fluorescent microscope. Compiled cell images in this figure panel reflect the transfection efficiency results from separate electroporation trials. GFP+ cell percentages were determined from at least two different fields. The mean of GFP+ cell percentages was calculated and plotted with standard error of means. unpaired student's t test analysis, p=0.09.

Overall Transfection Efficiency

Total 651 cells from four separate fields in the well were counted. 163 out of 651 cells were counted as GFP+ and the transfection efficiency was determined as 25%.

Final Protocol

- To promote logarithmic phase, cells are passaged and fed with fresh media 24 h prior to electroporation.

- The day of electroporation, one to four million cells per condition are resuspended in 200 µl serum-free RPMI 1640.

- 10 µg plasmid DNA is mixed with cells in a tube by gentle stirring with a pipette tip.

- Cell-DNA mixture is transferred to a 4 mm cuvette and

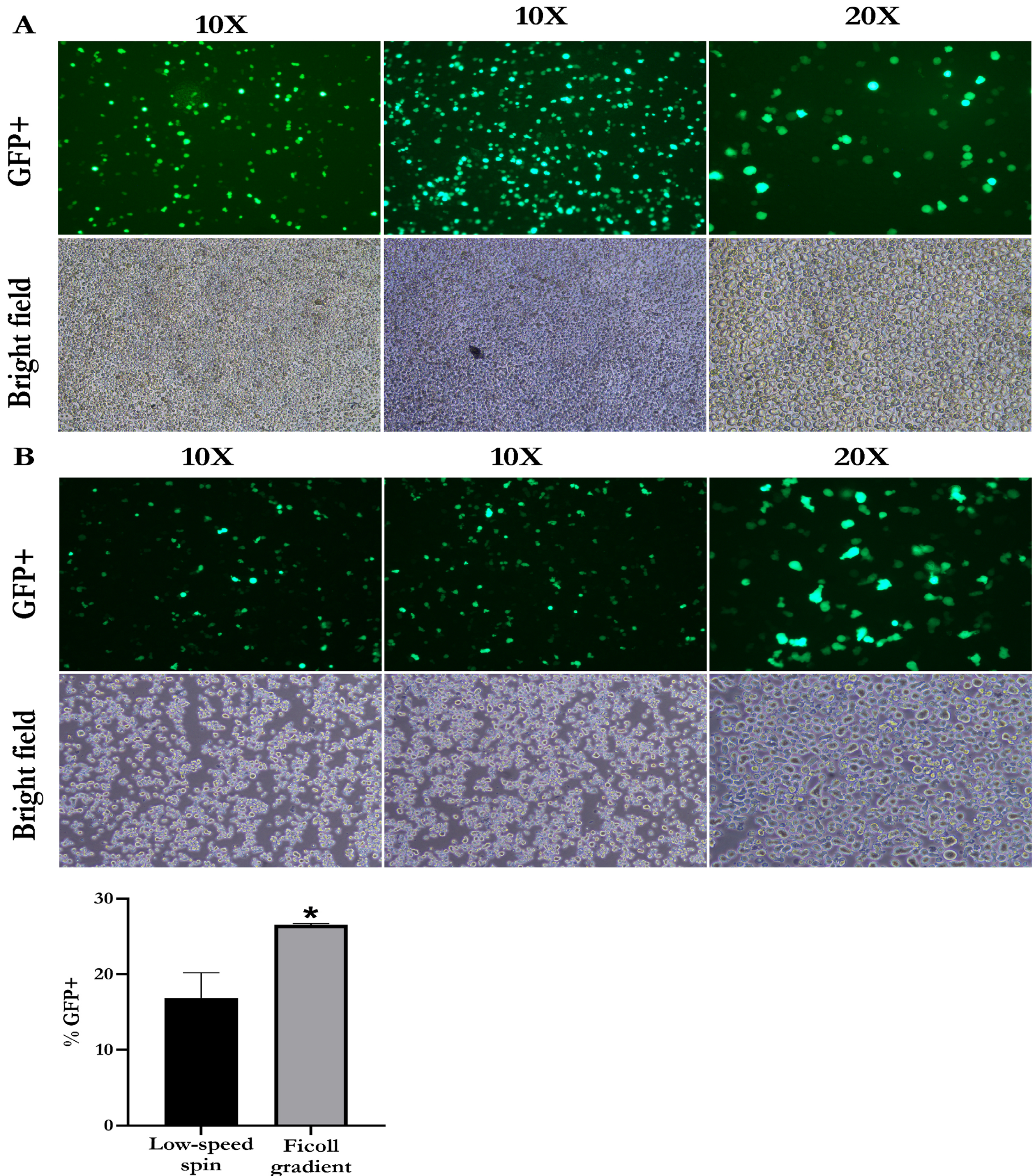

Figure 5. Cells transfected with optimized protocol and subjected either to low-speed centrifugation or Ficoll gradient centrifugation for dead cell removal. 1-4 x10^6 cells (split at 1:2 ratio and fed with fresh media the day before) are washed with and resuspended in 200 µl of serum-free RPMI 1640. 10 µg of pGIPZ plasmid was mixed with cell in a tube and transferred to a 4 mm cuvette. DNA-cell mixture was pulsed with a single exponential decay wave at 200 V, 950 µF. Electroporated cells were harvested after adding 500 µl of warm media (RPMI 1640 with 20% FBS, no antibiotics) into the cuvette and seeded in a 6-well plate containing 2 ml warm media. 24 h post-transfection, cells were either spun at low speed (100xg for 10 min) (A) or subjected to Ficoll-gradient centrifugation (B) to eliminate dead cells and seeded again in a new 6-well plate. Either two days (A) or four days (B) after electroporation, GFP-expressing cells were monitored under a fluorescent microscope and images were obtained. GFP+ cell percentages were determined from at least two different fields. The mean of GFP+ cell percentages was calculated and plotted with standard error of means. unpaired student's t test analysis, p=0.02.

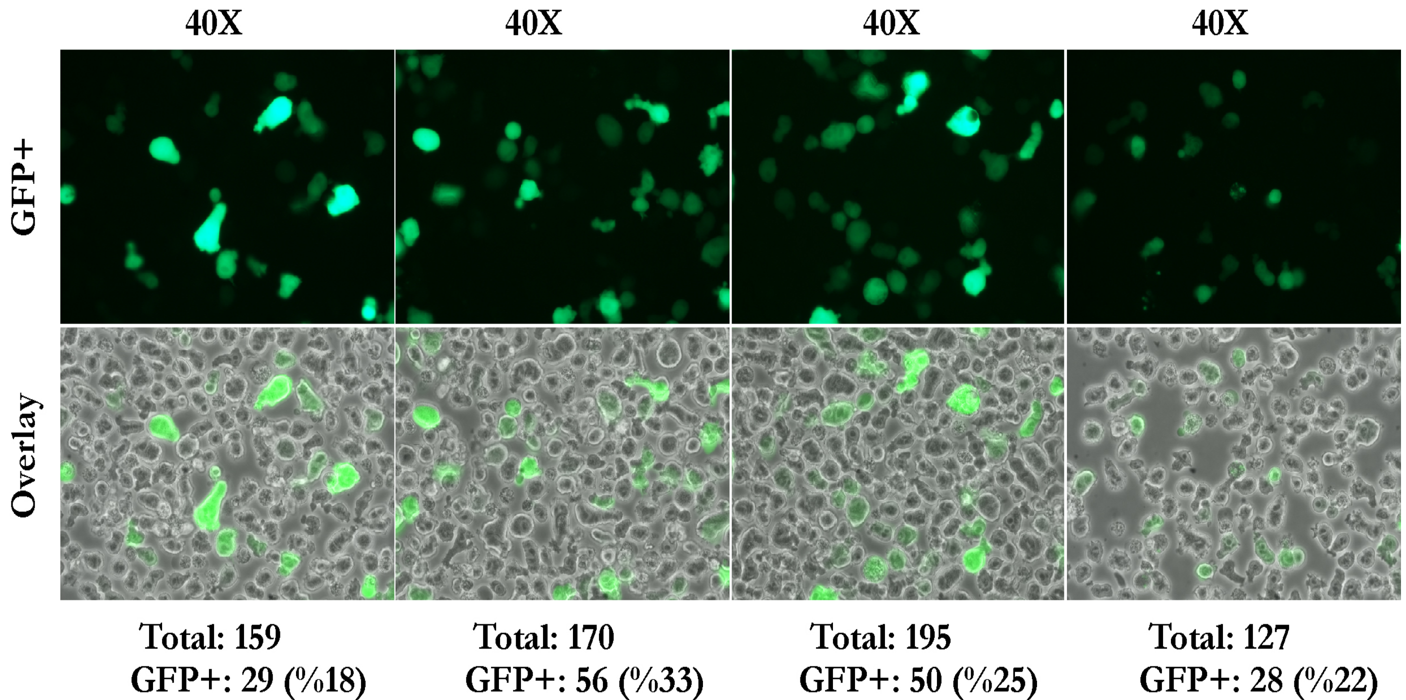

Figure 6. The efficiency of the optimized electroporation protocol. 4 x10^6 NCI H929 cells were mixed with 10 µg of pGIPZ plasmid in 200 µl serum-free medium. After 24 h, dead cells are eliminated by Ficoll gradient centrifugation and cell suspension was cultured in a 6-well plate up to 4 days after electroporation. Cell images were captured under fluorescent microscope. Images at 40x magnification were used to count the GFP+ cells in Image J software.

electroporated at 200 V, 950 µF with a single exponential decay wave.

• 500 µl of prewarmed media was added into the cuvette and cells are harvested by a P1000 pipette tip or a transfer pipet.

• Harvested cells are seeded into a 6-well plate containing 2 ml prewarmed 20% serum-containing, antibiotic free, RPMI 1640 media and cultured for additional 24 h.

• At 24 h post-electroporation, cells are examined and Ficoll gradient centrifugation is performed to eliminate dead cells.

• Isolated live cells are further cultured to expand or immediately used for experiments.

DISCUSSION

Multiple myeloma cell lines are common tools for experimental studies and electroporation of MM cells is a widely used method for allowing the entry of RNA mimics or antisense oligos to drive transient changes in gene expression. Despite the frequent use of electroporation method for these cells, the transfection efficiency is unreported and enrichment of transfectant cells by cell sorting methods are needed to acquire the necessary cell count to conduct an experiment. An ideal transfection protocol is expected to yield a good efficiency as well as cell survival rate. Both high efficiency and less cytotoxicity after transfection depends not only on cell density, transfection solution and electroporator settings but also on the type, size and content of the nucleic acid as well as culture conditions before and after electroporation. Initial settings for this study were based on a good comprehensive work from Steinbrunn et. al (2014). This group aimed to develop a standard procedure for the most common MM cells and suggested further optimization to achieve better outcome for a specific cell type. In contrast to the suggested voltages by Steinbrunn et. al (2014), any voltages above 200 V was not tolerable not only for NCI H929 but also RPMI 8226 and U266 cells in our hands. Moreover, voltages below 200 V (with 950 µF capacitance) increased the viable cell numbers but severely compromised the transfection efficiency. We examined various parameters for optimization and achieved an approximately 25 % electroporation efficiency for NCI H929 cells but could not improve the transfection efficiency for RPMI 8226 cells beyond one per cent (data not shown). The GFP-expressing pGIPZ construct used in this study is a relatively large (11.8 kilobase) lentiviral construct. Using either this construct or a CRISPR vector (~9.1 kb, plasmid #62988, Addgene), the number of recovered cells after 24 h post-transfection was about 1-2 x10^5 cells out of 10^6 cells (10-20%) under optimized conditions. This optimized protocol (950 µF, 200 V, 10 µg DNA, 4x10^6 cells in 200 µl serum-free culture medium) settings might generate different survival rate and transfection efficiency if the input DNA is a smaller plasmid construct or are short oligos such as siRNAs, which was not investigated in this study. Compared to current literature, this protocol provides an improved efficiency and could suffice the need of certain studies where the transformant cells are later enriched by additional methods such as flow cytometry-mediated cell sorting (FACS), magnetic cell sorting or antibiotic-mediated

selection.

DECLARATIONS

Conflict of Interest

The author declares that there is no conflict of interest.

Consent for Publication

I give my consent for this manuscript to be published in the Veterinary Journal of Mehmet Akif Ersoy University.

Author contribution

Idea, concept and design: AK

Data collection and analysis: AK

Drafting of the manuscript: AK

Critical review: AK

Data Availability

The data presented in this study contained within the article.

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Research Article

Classification of digital dermatitis with image processing and machine learning methods

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ABSTRACT

In this study, it was aimed to perform the detection and grading of Digital Dermatitis disease, which is common in dairy cattle and causes serious economic losses, using artificial intelligence techniques in a computer environment with high accuracy without the need for any expert intervention.

Within the scope of the study, pictures of lesions caused by Digital Dermatitis were taken, and four groups were formed according to the degree of size. These examinations were performed on 168 cows of the Holstein breed, aged 4-7 years, whose lameness was detected on dairy farms located in the centre and districts of the Burdur region. The photographs obtained were first labelled according to the degree of disease by a faculty member specialised in podiatry. Afterwards, the tagged photographs were reproduced using artificial intelligence image augmentation techniques, and a sample of 1,000 datasets was carried out for each disease degree. The photographs that make up the dataset were processed using the Inception v3 deep learning algorithm, and 2,148 numerical features were extracted. Then, machine learning models were developed using six different machine learning algorithms to classify these features. The results obtained were examined in detail with the help of tables and graphics, and they showed that the developed artificial intelligence models could be used in the classification of Digital Dermatitis case photos with a cumulative accuracy value above 0.87.

INTRODUCTION

Bovine Digital Dermatitis (DD), known by different names such as papillomatous digital dermatitis, Mortellaro's disease, or hairy heel warts, is a very common infectious foot disease (Biemans et al., 2017; Bruijnis et al., 2012). Treponema spp. plays a role in the occurrence of this disease, which is frequently seen in the hind feet (Clegg et al., 2015; Sogstad et al., 2005). This disease, which was first described in Italy in 1974, was observed to cause serious lameness in the cows in the herd (Biemans et al., 2017). The prevalence of DD is reported to be significantly higher in dairy cows (32.2%) than in beef cows (10.8%) (Hesseling et al., 2019). Digital Dermatitis causes a decrease in milk production, reproductive performance and animal welfare in dairy cows, as well as an increase in treatment costs, and the average cost per case of digital dermatitis is US$ 132.96 (Cha et al., 2010).

Digital Dermatitis is a multifactorial disease, even if Treponema spp. is accepted as the primary pathogen in the formation of the disease. The wet and dirty walking path contributes to the development of this disease (Holzhauer et al., 2008; Trott et al., 2003). In addition, pathogens such as Bacteroidetes, Fusobacteria, Tenericutes, Firmicutes, Proteobacteria, and Actinobacteria play a secondary role in digital dermatitis (Hesseling et al., 2019).

Lesions that are painful and prone to bleeding can be observed above the interdigital space and near the heels, along the coronary band. Filiform papillae may develop in the lesioned area, and the lesions can be surrounded by hyperkeratotic skin with longer than normal hair (Biemans et al.2018). For the classification of these lesions, the scoring system developed by Döpfer et al. is used, and the lesions are scored at four different levels (Döpfer et al., 1997). Döpfer et al. named the cases with 0.5-4 cm diameter and less than 2 mm depth from the epithelial tissue as granulomatous lesions as 1st degree. When this lesion is seen as a classic ulceration area that is deeper than 2 mm from the epithelial level and can reach up to 7 cm in diameter, it is considered grade 2. The appearance of

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the lesion covered with crust in the healing period was classified as grade 3, and when skin lesions were hyperkeratotic and proliferative, they were classified as grade 4.

Today, with the development of computer hardware technology and artificial intelligence techniques, it has become possible to create information systems that can learn and process images without human intervention. In particular, recent developments in Convolutional Neural Networks and deep learning algorithms have been ground-breaking for digitising and classifying images. In our study, DD disease is considered as a classification problem, and the development of models that can classify with high accuracy by using 6 different machine learning algorithms and the performance comparison of these models are discussed in detail.

In the scope of the study, the problem was not only reduced to the binary classification problem of separating diseased individuals from healthy individuals, but it also aimed to determine the degree of the disease by utilising techniques from artificial intelligence. This was accomplished by reducing the problem to its simplest form. In machine learning studies, binary classification problems are generally problems where high performance can be achieved easily. Nevertheless, in most cases, the classification performance of the model deteriorates noticeably as the number of classes grows. With the help of photographs and various techniques that utilise artificial intelligence, the purpose of this study is not only to identify the disease but also to ascertain the severity of the disease that has been identified using as much precision as possible.

MATERIALS and METHODS

To collect data for the study, 206 photographs of the lesions observed on the hind legs of 168 cows of DD disease, Holstein breed, aged 4-7 years, as determined by examinations conducted in the Burdur region, were taken, graded based on the severity of the lesions, and assigned to the appropriate class. Degree 1 represents the mildest form of the disease, while degree 4 represents the most severe case. After the photographs were grouped, there were 60 photographs in the 1st Degree, 71 in the 2nd Degree, 56 in the 3rd Degree, and 19 in the 4th Degree, respectively. Photographic examples of each classification are depicted in Figure 1.

In classification-based machine learning problems, the equal distribution of the amount of data in the classes that make up the data set among the classes is extremely critical to obtaining successful results. For this reason, to create a balanced data set and increase the number of examples, the data set consisting of original photographs was reproduced with 1,000 examples in each class using artificial intelligence techniques. 2,148 numerical features were obtained by using the Inception V3 algorithm, which is a widely used deep learning algorithm in image processing of the reproduced dataset. The numerical data set obtained after this transformation process was classified by running with AdaBoost (AB), Naive Bayes(NB), Stochastic Gradient Descent (SGD), Support Vector Machine (SVM), Random Forest (RF), and Logistic Regression (LR) algorithms, which are widely used in machine learning problems.

The Naive Bayes method is one of the oldest approaches to machine learning. It is founded on the calculation of probabilities and uses simple models. The SVM method involves drawing the border lines between the clusters that represent the classes. It is important to remember that the distance between the cluster centres and the elements should be kept to a minimum, but the distance along the border line between the clusters should be kept to a maximum. The Random Forest algorithm is an example of a method that combines the operations of multiple independent tree algorithms, which allows for higher levels of accuracy to be achieved. The Logistic Regression algorithm is a type of algorithm that was developed before the artificial neural network approach. It makes use of the easily derived sigmoid function, decreases the amount of error produced with each iteration, works quickly, and is simple to train.

A method known as stochastic gradient descent is an example of an iterative approach to optimising an objective function that must have suitable smoothness properties. Since it replaces the actual gradient that was calculated from the entire data set with an estimate of the gradient that was calculated from a randomly selected subset of the data, it can be regarded as a stochastic approximation of gradient descent optimization. This is due to the fact that it calculates the estimate of the gradient from the data. This reduces the extremely high computational burden, which allows for faster iterations in ex-

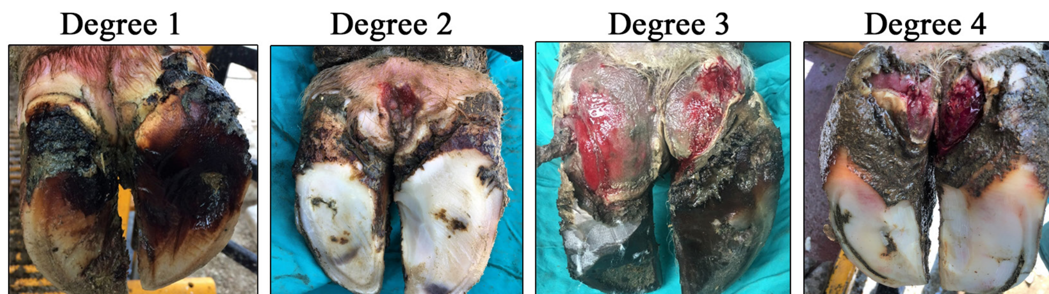

Figure 1. Photo examples representing the 4 different degrees of Digital Dermatitis.
Degree 1. Granulomatous lesion 0.5-4 cm in diameter and less than 2 mm deep from epithelial tissue
Degree 2. A classic ulceration area that is deeper than 2 mm and can reach 7 cm in diameter
Degree 3. lesion covered with crust and healing
Degree 4. skin lesions are hyperkeratotic and proliferative

change for a lower convergence rate. This benefit is especially noticeable when dealing with high-dimensional optimization problems.

AdaBoost, short for Adaptive Boosting, is a statistical classification meta-algorithm and can be used in conjunction with many other types of learning algorithms to improve performance. The final output of the boosted classifier is determined by combining the output of various weaker learning algorithms into a weighted sum. This sum represents the final output of the boosted classifier. AdaBoost is a classification meta-algorithm that, in order to improve performance, can be combined with a wide variety of other kinds of learning algorithms. Specifically, it can be used to improve neural network performance. The output of the other learning algorithms of weak learners is combined into a weighted sum that represents the final output of the boosted classifier. Usually, AdaBoost is presented for binary classification, although it can be generalised to multiple classes or bounded intervals on the real line.

Within the confines of the research project, the supervised learning method was implemented, and test and training data sets were generated by arbitrarily dividing the dataset into 80 percent and 20 percent. In the beginning, each model was initially trained with the help of the training dataset. The validation process utilised 10% of the training data set, and the success of the training was evaluated for each model independently. Figure 2 shows the stages of the classification process. Accordingly, the original data set was first reproduced by performing rotate, zoom in, and zoom out operations in the Python programming language to work with an equal number of samples. After augmentation, a data set was created by randomly selecting 1,000 samples for each class. The dataset created from augmented photographs has been subjected to visual feature extraction so that it can work with numerical data and can be run with widely used machine learning algorithms in the literature. Visual feature extraction is roughly the digitised version of the layer outputs obtained before the fully connected layer in the last layer of a deep learning algorithm.

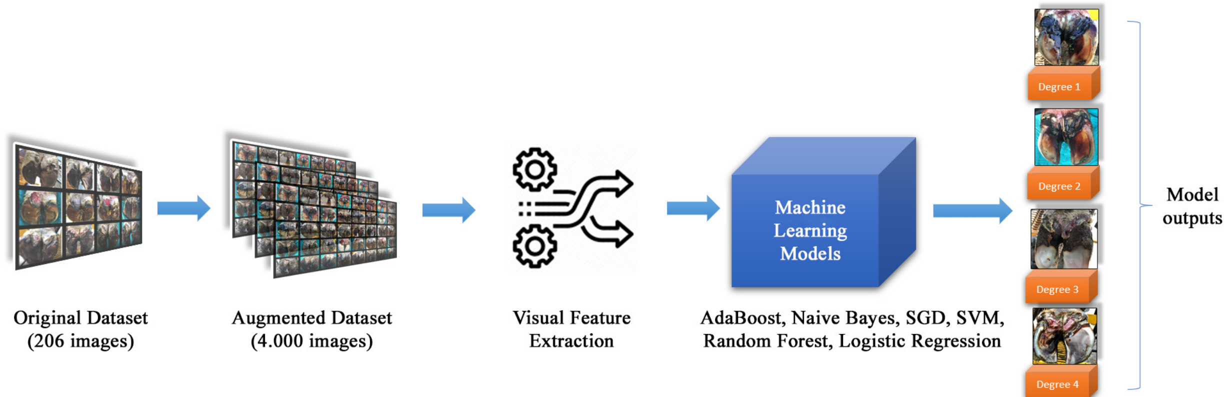

Figure 2. Stages of the artificial intelligence assisted medical photo classification process.

Table 1. Model performance metrics and equations for the classification metrics.

Performance Metric	Equation
Precision	$\frac{TP}{TP+FP}$
Recall	$\frac{TP}{TP+FN}$
Specificity	$\frac{TN}{TN+FP}$
Accuracy	$\frac{TP + TN}{TP + FN + TN + FP}$
F1 Score	$\frac{2. (precision.recall)}{precision + recall}$

TP: True positive, FP: False Positive, TN: True Negative, FN: False Negative

Figure 2 shows the steps of augmentation of the original data set, visual feature extraction, and then classification using machine learning algorithms.

There are many metrics commonly used in the literature to measure model performance. The top among these are Area Under Curve (AUC), Cumulative Accuracy (CA), F1-Score, Precision, Recall, and Specificity measurements. The performance metrics and formulas used in the study are given in Table 1.

RESULTS

The performance data of 6 machine learning models developed and trained within the scope of the study were compared using 6 classification metrics, and the obtained values are given in Table 2 and Figure 3 respectively.

Table 2. Numeric model performance results for all machine learning models.

Model	AUC	Cumulative Accuracy	F1	Precision	Recall	Specificity
AdaBoost	0.6358	0.4538	0.4537	0.4537	0.4538	0.8179
Naive Bayes	0.8167	0.5768	0.5780	0.5831	0.5768	0.8589
SGD	0.9073	0.8610	0.8599	0.8595	0.8610	0.9537
Random Forest	0.8009	0.5795	0.5799	0.5806	0.5795	0.8598
Logistic Regression	0.9778	0.8735	0.8731	0.8729	0.8735	0.9578
SVM	0.9806	0.8730	0.8724	0.8746	0.8730	0.9577

SGD: Stochastic Gradient Descent, SVM: Support Vector Machine, AUC: Area Under Curve.

Figure 3. Graphical model performance comparison for all machine learning models.
AUC: Area Under Curve, CA: Cumulative Accuracy.

Table 3. Numeric train and test time comparison for all machine learning models.

Model	Train time(s)	Test time (s)
AdaBoost	40.8760	2.4560
Naive Bayes	11.6720	2.7450
SGD	20.3750	4.2810
Random Forest	10.1840	1.8400
Logistic Regression	128.6910	2.2080
SVM	151.7910	21.0330

SGD: Stochastic Gradient Descent, SVM: Support Vector Machine

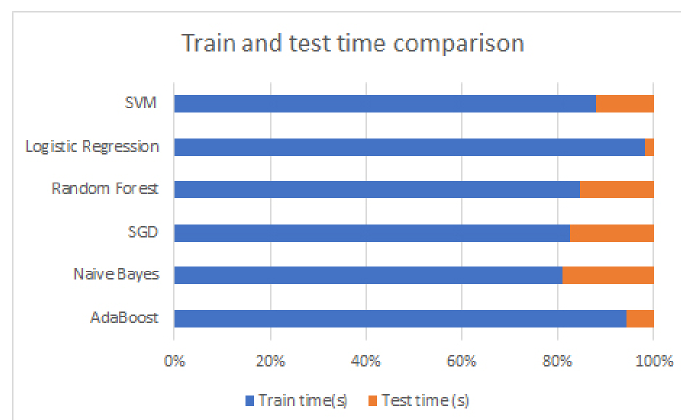

Figure 4. Graphical training and test time comparison for all machine learning models.

Accordingly, when the Cumulative Accuracy performance metric is taken as a basis, LR, SVM, and SGD models have achieved values above 0.86. These results show that all three models can produce a successful classification for this problem. When the models used in the study were evaluated on the precision metric, the LR, SVM, and SGD models showed significantly higher performance than the others. The same is valid for F1-score and recall metrics. Finally, when the specificity metric is considered, it is seen that the LR, SVM, and SGD models show the best performances, although there is no major difference between the models.

In addition to the performance of the model developed using machine learning techniques, the time spent for training and testing is also considered an important metric. In addition to the high classification performance of the developed model, it is desirable that the training and test run times be as short as possible. In Table 3, the training and testing times of the models covered in the study are given in seconds.

The numerical values given in Table 3 are visualized as a bar graph in Figure 4.

When the train and test time values are examined, the longest training time has emerged in the SVM and Logistic Regression models, respectively. The fastest trained model was the Naive Bayes model, with 11.67 seconds. While the longest test time was measured as 21 seconds in the SVM model, the model with the fastest test time was the Random Forest at 1.84 seconds.

DISCUSSION

It is stated that Digital Dermatitis disease, which is common in dairy cattle, is seen especially in Holstein cows (Demirkan et al., 2000). Even though there are cattle breeds like Simental and Montofon in the Burdur region, the fact that the cows diagnosed with DD are all of the Holstein breed lends credence to the study. Although there are cattle breeds such as Simental and Montofon in the Burdur region, the fact that the cows diagnosed with DD are Holstein breeds supports the study.

According to Hernandez et al.'s (2001) research, the aetiology of DD disease can be traced back to conditions such as inadequate hygienic conditions, improper care of the nails, and wet barn floors. The literature data are supported by the fact that comparable images were seen in the various places of business that were investigated as part of the scope of the study.

It is emphasised that DD disease is seen especially in the hind legs and the lesions are in the plantar region (Bassett et al., 2017). Similarly, the fact that DD disease was found in the hind legs and between the heels of cows in the study is consistent with the data that has been found in the previous research.

The review of the relevant literature revealed that there are no artificial intelligence medical image classification studies that have been developed for DD disease. This particular research stands out as an original piece of work due to the aforementioned consideration.

CONCLUSION

Within the scope of the study, the detection and evaluation of digital dermatitis disease, which is common and causes serious economic losses, through photographs was carried out with high accuracy by computer using artificial intelligence techniques.

In our application for the classification of medical images, 6 different machine learning models (AdaBoost, Naive Bayes, SGD, SVM, LR, and Random Forest) were developed and compared using 6 different classification metrics (AUC, CA, F1-score, precision, recall, and specificity). When the results are examined, it is seen that a very high classification success rate of 0.87 has been achieved. Accordingly, the most successful classification models stand out as LR, SVM, and SGD.

All three algorithms are classification algorithms with relatively short working and training times. In terms of the outcomes of the performance tests that were carried out, the differences between them are negligible at best. Although the success of the algorithms varies according to the problem and the data set, when the SVM algorithm is used for problems with high dimension input size, model training takes longer and computational resources are consumed more.

In situations where there is overlap between classes, the classification accuracy of the SVM algorithm is typically lower. The learning phase of the LR algorithm can typically be completed rapidly and with a reduced demand on the available resources of the computer. SGD was determined to be the model that required the least amount of time to train out of these three different models.

DECLARATIONS

EthicsApproval

The ethical approval decision is dated 16.03.2022 and numbered 869 taken from Burdur Mehmet Akif Ersoy University Local Ethics Committee of Animal Experiments.

Conflict of Interest

The authors declare that there have no conflict of interests.

Author ontribution

Idea, concept and design: KY, IK

Data collection and analysis: KY, IK

Drafting of the manuscript: KY, IK

Critical review: KY, IK

Data Availability

The data collected within the scope of the study has not been shared.

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Not applicable

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Research Article

Nutrient profile and digestibility of common vetch *(Vicia sativa)* alone or intercropping with different forages

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ABSTRACT

The present study was conducted to assess the nutrient profile, digestibility and feeding values of common vetch alone or intercropping combinations with different cereal and legume forages in central district of Burdur, Türkiye. Approximately 2 kg fresh matter of common vetch alone or 9 different intercropping combinations was harvested from different locations. Nutrient composition and *in vitro* true dry matter digestibility were analyzed whereas feeding values were calculated. The study showed that intercropping largely decreased the crude protein of common vetch while an increase in fiber and carbohydrate fractions was noted except acid detergent fiber and acid detergent lignin that decreased in intercropping mixtures compared to common vetch alone. In addition, there was an increase in digestibility and feeding values in intercropping mixtures in comparison with common vetch alone. In conclusion, intercropping of common vetch with cereal forages improves the nutrient profile, *in vitro* digestibility, and feeding values despite a reduction in the crude protein content of intercropping mixtures compared to common vetch.

INTRODUCTION

Türkiye has been facing the problem of increasing shortage of forage and roughages due to the feeding practices of farmers that give more importance to the cereal straws in comparison with forages (Arslan & Erdurmuş, 2012). Therefore, the total area for growing cereal crops is greater than that of forage crops especially legumes (TÜİK, 2022). Consequently, the production of leguminous forages is insufficient to sustain the protein needs of animal production. This deficit is largely closed by the import of protein concentrates and protein sources that increase the import bill because of the higher prices of protein sources and increased shipping costs since most of the protein sources (soybean, canola, sunflower, and others) are imported from Latin America and North American. In addition to insufficient forage production, the quality of forages grown in dryland regions of Türkiye is low as well due to climatic conditions. Legume forages, being good sources of protein, are capable of closing the gap between protein requirements and protein produced to sustain the animal production of Türkiye (Alatürk et al., 2018). Common vetch *(Vicia sativa)* is one of the best legume forages after alfalfa due to lesser requirement for irrigation that makes it suitable for drylands as well (Parissi et al., 2022). However, lodging of vetch in the crop fields is very common owing to its weaker stems (Bakoğlu & Memiş, 2002). Besides this, legume forages including common vetch, despite having greater crude protein (CP) levels than cereal forages, are less tasteful due to low carbohydrates in these crops (Ansar et al., 2010).

All these problems can be solved by intercropping of common vetch with other crops especially with winter cereal crops (Zhang & Li, 2003; Hauggaard-Nielsen et al., 2006) that increases the dry matter intake (DMI) in ruminants (Ansar et al., 2010). Intercropping of vetch and winter cereal crops either as main or second crop not only prevents the lodging of vetch but also quality losses (Bakoğlu & Memiş, 2002). In addition, intercropping system ensures the sustenance of nutrient supply in animal production and presents a better control of pests, weeds, and diseases in the crops and fields (Szumigalski & Rene, 2005). Although intercropping of common vetch and cereal crops has increased over the last decade, the nutrient composition and digestibility has remained inconclusive due to the availability of various mixtures, mixing ratios, and locations. Therefore, the present study was conducted to evaluate the nutrient composition and *in vitro* digestibility of different common vetch-cereal crop mixtures grown in Burdur, Türkiye.

MATERIALS and METHODS

The study was conducted in Burdur, Türkiye in 2021. The land is located in the central district of Burdur. Forage crops were harvested in early summer (second week of June 2021). Approximately 2 kg fresh matter was harvested from different sites in the same field. Table 1 shows the common vetch and its various mixtures harvested from central district of Burdur province.

Chemical composition of forage crops

Harvested common vetch and various mixtures were sub-

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Table 1. Abbreviations of common vetch and intercropping mixtures

Abbreviation	Forages[1]
1	*Vicia sativa*
2	*Vicia sativa* + *Pisum sativum*
3	*Vicia sativa* + *Avena sativa* L.
4	*Vicia sativa* + × *Triticosecale* Wittmack L.
5	*Vicia sativa* + *Secale cereale*
6	*Vicia sativa* + *Secale cereale* + *Triticum aestivum*
7	*Vicia sativa* + × *Triticosecale* Wittmack L. + *Hordeum vulgare* L.
8	*Vicia sativa* + *Pisum sativum* + *Avena sativa* L.
9	*Vicia sativa* + × *Triticosecale* Wittmack L. + *Triticum aestivum* + *Secale cereale*
10	*Vicia sativa* + *Pisum sativum* + *Triticum aestivum* + *Secale cereale* + *Avena sativa* L.

[1] *Vicia sativa* = vetch, *Pisum sativum* = pea, *Avena sativa* L. = oat, × *Triticosecale* Wittmack L = triticale, *Secale cereale* = rye, *Triticum aestivum* = wheat, *Hordeum vulgare* L = barley

jected to forced air drying for 48 – 72 h in an oven (Memmert BE 500, Memmert GmbH + Co. KG, Schwabach, Germeny) at 65ºC to measure the dry matter (DM) content in triplicates according to the AOAC method (AOAC, 2000; method 934.01).

The dried samples were further subjected to CP, (method 984.13), crude ash (method 942.05), and ether extract (EE; method 920.39) analyses following AOAC methods (AOAC, 2000). Crude fiber (CF), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were analyzed by fiber analyzer (ANKOM A2000 Fiber Analyzer, ANKOM Technology, NY, United States). The nitrogen-free extract (NFE), non-fiber carbohydrates (NFC), hemicellulose (HEC), DMI, digestible dry matter (DDM), relative feed value (RFV), and net energy for lactation (NE_L) and total digestible nutrients (TDN) of common vetch and mixtures were computed according to the formulas below:

$$NFE = 100 - (CP + \text{Crude ash} + EE + CF)$$

$$NFC = 100 - (CP + \text{Crude ash} + EE + NDF)$$

$$HEC = NDF - ADF$$

$$DMI = 120 \div NDF$$

$$DDM = 88.9 - (ADF \times 0.779)$$

$$RFV = DMI \times DDM \times 0.775$$

$$NE_L = [1.044 - (0.0119 \times ADF)] \times 2.205$$

$$TDN = (-1.291 \times ADF) + 101.35$$

DMI, DDM, RFV, TDN, and NE_L were calculated according to Horrocks and Vallentine (1999). The chemical analyses were conducted in triplicates, averages were taken, and presented as % DM basis.

In vitro true dry matter digestibility

Common vetch and all the mixtures were incubated in Daisy^{II} incubator (Daisy^{II} Incubator, ANKOM Technology, NY, United States) to evaluate the *in vitro* DM digestibility. The samples in triplicates were packed in ANKOM F57 filter bags, placed in Daisy^{II} incubator bottles, ruminal fluid as inoculum was added, and incubated for 48 hours. The *in vitro* DM digestibility (IVTDMD) was calculated followed by the calculation of organic matter (OM) digestibility.

$$IVTDMD = 100 - [(W_3 - (W_1 \times C_1) \times 100] / W_2$$

W_1 = F57 filter bag weight (g)

W_2 = sample weight (dry matter, g)

W_3 = NDF weight after incubation (dry matter, g)

C_1 = blind weight (g)

Statistical analysis

Due to the individual differences among the mixtures, the data were only subjected to descriptive statistical analysis in computer-aided statistical software package (SPSS; version 22.0; Armonk, NY, USA). The data were presented as mean ± standard deviation.

RESULTS

Table 2 presents the proximate analysis of common vetch alone or in combination with different cereal and legume forages. The DM content of common vetch was 21.96% that increased in mixtures containing triticale, wheat, and rye to 36.23%. Common vetch alone had 86.39% OM content almost similar to that intercropped with oat (86.33%) or rye and wheat combined (86.08%). Other intercropping combinations had greater OM content than common vetch alone although the OM content of intercropping combinations was not relatively very high. Common vetch alone had 15.94% CP. Most intercropping combinations had relatively lower CP content than common vetch except the combination with rye only. The CP content was notably lower in combinations cereal forages except rye and pea. In additions, combination involving multiple cereal and legume forages also had very low CP compared to common vetch or in combination with rye or pea.

The EE content of common vetch was 1.94%. Intercropping of common vetch with rye had very high EE content (3.76%) in comparison with all other forages including common vetch alone. Crude ash content was relatively high in common vetch intercropped with rye and wheat (10.12%) compared to all combinations as well as common vetch alone (9.86%). Intercropping combinations of common vetch with pea, triticale, rye, triticale and barley, and multiple forage combinations had considerably lower crude ash concentrations (8.19, 6.77,8.81, 8.03, 6.73, and 8.71%) than common vetch alone and other intercropping combinations. common vetch alone had 30.11% CF content which was lower in comparison with few intercropping combinations with triticale (31.11%), rye and triticale (31.58%), triticale and barley (36.18%), and pea and oat (32.93%). All the remaining combinations had lower CF concentration than common vetch alone or in combination with the afore-mentioned intercropping combinations. The NFE content of common vetch alone (38.40%) and intercropped with rye and triticale (38.09%), or pea and oat (37.76%) was lower in comparison with other intercropping combinations.

Table 2. Proximate analyses of common vetch alone or intercropping with cereal and legume forages (%, dry matter basis)

Forages	Nutrients[1]						
	DM	OM	CP	EE	Crude ash	CF	NFE
1	21.96 ± 2.96	86.39 ± 0.45	15.94 ± 0.29	1.94 ± 0.29	9.86 ± 0.16	30.11 ± 1.30	38.40 ± 2.25
2	24.26 ± 0.38	88.08 ± 0.17	15.68 ± 0.34	1.17 ± 0.31	8.19 ± 0.30	25.90 ± 2.63	45.34 ± 1.93
3	18.97 ± 0.44	86.33 ± 0.38	13.19 ± 0.92	1.87 ± 0.40	9.36 ± 0.45	28.54 ± 1.36	42.73 ± 3.77
4	32.64 ± 0.01	89.87 ± 0.23	11.41 ± 0.06	1.39 ± 0.02	6.77 ± 0.28	31.11 ± 0.43	45.96 ± 0.12
5	27.27 ± 0.01	87.77 ± 0.08	16.39 ± 0.92	3.76 ± 0.23	8.81 ± 0.09	27.54 ± 0.17	40.09 ± 0.23
6	21.71 ± 0.01	86.08 ± 0.07	15.40 ± 0.19	1.03 ± 0.14	10.12 ± 0.06	31.58 ± 0.29	38.09 ± 0.54
7	25.48 ± 0.01	87.90 ± 0.14	10.92 ± 0.32	1.47 ± 0.10	8.03 ± 0.08	36.18 ± 0.83	39.34 ± 0.57
8	24.27 ± 0.01	87.10 ± 0.13	15.21 ± 0.38	1.21 ± 0.16	9.66 ± 0.02	32.93 ± 0.63	37.76 ± 0.28
9	36.23 ± 0.01	90.46 ± 0.16	9.78 ± 0.25	1.43 ± 0.05	6.73 ± 0.16	27.96 ± 0.16	51.31 ± 0.51
10	30.95 ± 0.01	87.12 ± 0.66	11.81 ± 0.14	2.18 ± 0.27	8.71 ± 0.06	26.50 ± 0.38	46.63 ± 0.42

[1]DM = dry matter, OM = organic matter, CP = crude protein, EE = ether extract, CF = crude fiber, NFE = nitrogen free extract

The fiber and carbohydrate fractions of common vetch alone or intercropped with cereal and legume forages have been presented in Table 3. Common vetch had 44.64% NDF, 35.45% ADF, 5.60% ADL, 9.19% HEC, 23.88% NFC, and 68.51% total carbohydrates. Most intercropping mixtures exhibited greater levels of NDF, HEC, NFC, and total carbohydrates whereas lower ADF, ADL were seen.

The digestibility and feeding values of common vetch alone or intercropped with cereal and legume forages has been depicted in Table 4. The IVTDMD, IVOMD, DMI, DDM, RFV, TDN, NE_L of common vetch were 51.56%, 48.18%, 2.69%, 61.29%, 127.89%, 55.59, and 1.37 Mcal/kg, respectively. Intercropping of common vetch increased the IVTDMD and IVTOMD except oat that decreased the IVTDMD and IVTOMD compared to common vetch alone. Common vetch alone had lower DDM, TDN, and NE_L than intercropping mixtures. The RFV of intercropping mixtures was relatively greater than common vetch alone except the intercropping with triticale, rye and triticale, and triticale and barley.

DISCUSSION

Karslı et al. (2005) reported that the DM content ranges between 15.25% and 24.46% in different varieties of common vetch. Intercropping of common vetch with oat had lower DM (18.97%) than that of common vetch alone. This might be attributed to the late maturity of oat due to severe cold climatic conditions of Burdur that prevent the early maturity of oat. Similar CP content of common vetch was reported in previous studies (Lithourgidis et al., 2006; 2007). In contrast, most studies have reported greater CP content of common vetch as opposed to the CP in this study (Karslı et al., 2006; Budakli Carpici & Celik, 2014; Georgieva et al., 2016; Pereira et al., 2020). Karslı et al. (2005) reported that the CP, OM, and crude ash of common vetch varies between 17.75 and 20.30%, 87.36 and 89.6%, and 10.2 and 12.64%, respectively. CP content of common vetch was 21% (Budakli Carpici & Celik, 2014) whereas, CP and crude ash was reported as 24.5% and 8.54%, respectively (Pereira et al., 2020). Similarly, CP and CF ranged from 18.24 to 19.04%, and 25.40 to 26.48%, respectively (Georgieva et al., 2016). Consistent with this study, intercropping of common vetch with oat, barley, triticale, wheat, rye gradually reduced the CP content compared to common vetch alone (Lithourgidis et al., 2006; 2007; Balabanlı et al., 2010; Budakli Carpici & Celik, 2014; Najera et a., 2016). In general, cereal forages are a poor source of CP as opposed to common vetch since common vetch is a legume forage. Therefore, intercropping of common vetch with cereal crops also lowers the CP content of the intercropped mixtures that supports the idea of this study.

Previous studies reported similar fiber and carbohydrate fractions of common vetch (Karslı et al., 2005; Lithourgidis et al., 2006; 2007; Budakli Carpici & Celik, 2014; Georgieva et al., 2016). The NDF and ADF contents of common vetch were 40.76-49.36% and 28.14-32.91% (Karslı et al., 2005). Similarly, common vetch was reported to have 44.31% NDF, 36.58%

Table 3. Fiber and carbohydrate fractions of common vetch alone or intercropping with cereal and legume forages (%, dry matter basis)

Forages	Item[1]					
	NDF	ADF	ADL	HEC	NFC	Total CHO
1	44.64 ± 1.71	35.45 ± 1.29	5.60 ± 0.11	9.19 ± 0.48	23.88 ± 0.85	68.51 ± 1.03
2	43.91 ± 1.96	28.64 ± 0.39	4.02 ± 0.34	15.28 ± 0.64	27.32 ± 0.32	71.23 ± 0.72
3	44.83 ± 0.44	29.40 ± 0.99	3.59 ± 0.86	15.44 ± 0.35	26.44 ± 1.00	71.27 ± 0.68
4	48.07 ± 0.33	30.32 ± 0.27	4.69 ± 0.76	17.75 ± 0.06	29.01 ± 0.02	77.07 ± 0.31
5	38.64 ± 0.09	26.31 ± 0.10	4.04 ± 0.21	12.33 ± 0.18	28.99 ± 0.16	67.63 ± 0.06
6	51.65 ± 0.64	32.63 ± 0.25	4.02 ± 0.32	19.02 ± 0.38	18.01 ± 0.89	69.66 ± 0.25
7	59.00 ± 0.14	35.42 ± 0.01	3.37 ± 0.27	23.58 ± 0.13	16.52 ± 0.13	75.51 ± 0.27
8	44.45 ± 0.45	32.53 ± 0.30	5.07 ± 0.44	11.92 ± 0.75	26.24 ± 0.80	70.69 ± 0.35
9	48.38 ± 0.35	27.02 ± 0.55	2.64 ± 0.14	21.36 ± 0.21	30.89 ± 0.01	79.27 ± 0.35
10	46.48 ± 0.16	28.00 ± 0.32	4.27 ± 0.04	18.39 ± 0.49	26.65 ± 0.63	73.13 ± 0.79

[1]NDF = neutral detergent fiber, ADF = acid detergent fiber, ADL = acid detergent lignin, HEC = hemicellulose, NFC = non-fiber carbohydrates, Total CHO = total carbohydrates

Table 4. *In vitro* rumen digestibility and feeding values of common vetch alone or intercropping with cereal and legume forages (%)

Forages	Item[1]						
	IVTDMD	IVTOMD	DMI[2]	DDM	RFV	TDN	NE_L[3]
1	51.56 ± 2.25	48.18 ± 2.26	2.69 ± 0.10	61.29 ± 1.00	127.89 ± 3.49	55.59 ± 0.66	1.37 ± 0.04
2	57.54 ± 3.01	50.45 ± 2.48	2.80 ± 0.05	66.59 ± 0.30	144.66 ± 3.37	64.38 ± 0.51	1.55 ± 0.01
3	47.65 ± 2.05	39.79 ± 1.75	2.68 ± 0.03	66.00 ± 0.33	136.90 ± 1.94	63.40 ± 0.86	1.53 ± 0.08
4	61.14 ± 2.08	55.39 ± 1.56	2.50 ± 0.02	65.29 ± 0.21	126.32 ± 0.91	62.21 ± 0.34	1.51 ± 0.01
5	60.12 ± 1.32	52.71 ± 1.44	3.11 ± 0.01	68.41 ± 0.08	164.66 ± 0.15	67.39 ± 0.13	1.61 ± 0.01
6	54.28 ± 1.65	45.58 ± 1.36	2.32 ± 0.03	63.48 ± 0.20	114.32 ± 1.25	59.23 ± 0.32	1.45 ± 0.01
7	53.17 ± 1.60	46.42 ± 1.21	2.04 ± 0.01	61.31 ± 0.01	96.65 ± 0.17	55.63 ± 0.01	1.37 ± 0.01
8	52.16 ± 0.97	44.04 ± 0.72	2.70 ± 0.03	63.56 ± 0.32	133.00 ± 0.62	59.36 ± 0.39	1.45 ± 0.01
9	65.30 ± 2.28	61.01 ± 0.21	2.48 ± 0.01	67.85 ± 0.42	130.45 ± 1.24	66.47 ± 0.71	1.59 ± 0.01
10	59.24 ± 1.51	52.18 ± 1.44	2.62 ± 0.01	67.10 ± 0.25	134.26 ± 0.02	65.22 ± 0.42	1.57 ± 0.01

[1]NDF = neutral detergent fiber, ADF = acid detergent fiber, ADL = acid detergent lignin, HEC = hemicellulose, NFC = non-fiber carbohydrates, Total CHO = total carbohydrates

ADF, and 6.85% ADL (Lithourgidis et al., 2006). These researchers, in 2007, reported that common vetch had 43% NDF, 30.4% ADF, 6.74% ADL, and 11.6% HEC (Lithourgidis et al., 2007). Budakli Carpici & Celik (2014) reported 55% NDF and 32% ADF in common vetch. Likewise, NDF, ADF, ADL, and HEC contents of common vetch were 38.03-44.67%, 31.62-39.69%, 5.17-9.33%, and 2.96-8.20%, respectively (Georgieva et al., 2016). Inconsistent results have been reported regarding the fiber levels in intercropped mixtures of common vetch with cereal crops. Partly in line with the findings of this study, intercropping of common vetch with cereal forages increased the NDF and HEC while decreased the ADF and ADL contents (Lithourgidis et al., 2006; 2007; Budakli Carpici & Celik, 2014; Georgieva et al., 2016; Najera et al., 2016). In contrast, Pereira et al. (2020) reported the intercropping of common vetch had no effect on the NDF and ADF contents of common vetch.

Georgieva et al. (2016) reported that the IVTDMD and IVTOMD of different common vetch varieties ranges between 57.7 to 66.2%, and 57.5 to 66.9%. In contrast, the IVTDMD of common vetch varieties differed between 60.96 and 64.67% (Karslı et al., 2005). Lithourgidis et al. (2006) reported 2.71% DMI, 60.4% DDM, 44.15% TDN, 126.85% RFV, and 1.34 Mcal/kg NE_L of common vetch. Similarly, the RFV, TDN, and NE_L of common vetch were 110.4%, 61.9%, and 1.36 Mcal/kg, respectively (Najera et al., 2016).

This study showed that while concentrations of most nutrients, digestibility, and feeding values of common vetch alone or intercropping with cereal forages were consistent with the existing body of literature, there were also differences. It is liable to suppose that these differences might be attributed to the differences in study location, climatic conditions, annual rainfall, soil structure, soil fertilization, agronomic practices, weed control, varieties of forages, seed rate, and the ratios of seeds

in intercropping mixtures. The limitation of this preliminary study was to collect the common vetch and intercropping mixtures from the already sown fields that rendered the researcher devoid of the valuable sowing information, agronomic practices, soil structure, and ratios of seeds in intercropping of common vetch with other forages.

CONCLUSION

In conclusion, the present study showed that common vetch alone or intercropped with certain cereal forages yields forages for animals rich in nutrients with varying degrees of digestibility and feeding values. Although intercropping reduces the crude protein, it may improve the fiber and carbohydrate fractions and digestibility. Experimental studies are necessary to evaluate the ratios of seeds in intercropping mixtures.

DECLARATIONS

Ethics Approval

The present study does not require ethics committee approval.

Conflict of Interest

The author state that no commercial funding was acquired for this study that may be construed as potential conflict of interest.

Consent for Publication

Not applicable.

Author contribution

Idea, concept and design: EK

Data collection and analysis: EK

Drafting of the manuscript: EK

Critical review: EK

Data Availability

The data are available from the corresponding author on reasonable request.

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Research Article

The effects of safranal against bisphenol AF on some reproductive parameters in male new zealand rabbits

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ABSTRACT

Bisphenol AF (BPAF) is used as an analog of the endocrine disruptor Bisphenol A (BPA), whereas safranal is a powerful antioxidant obtained from the saffron plant. In the current study, the possible effects of BPAF and Safranal on some spermatological parameters, reproductive hormones, oxidant/antioxidant enzymes, and histopathological parameters were investigated. A total of 24 male New Zealand rabbits were divided into 4 groups (n= 6 for each group). The groups and the treatments they received by oral gavage for 9 weeks are as follows: The control group received by oral gavage 1 ml/day of corn oil, the BPAF group received by oral gavage 20 mg/kg/day of bisphenol AF, the Safranal group received by oral gavage 100 mg/kg/day safranal, and the treatment group received by oral gavage 20 mg/kg/day bisphenol AF and 100 mg/kg/day safranal. Although the spermatological parameters (sperm concentration, ejaculate volume, progressive motility, ejaculate weight, seminal plasma total protein, and pH) prior to the experiment revealed no differences among the groups, BPAF treatment reduced sperm quantity and motility at the end of the study. BPAF treatments also had a negative impact on testicular MDA and GSH levels. It also caused seminiferous tubule degeneration in testicular tissue. On the other hand, the administration of safranal with BPAF decreased estrogen levels while increasing sperm concentration and motility to control group levels. Thus, the results suggested that safranal could have a beneficial effect in reducing BPAF-induced tissue damage. In conclusion, BPAF may have potentially harmful to the male reproductive system and safranal may exhibit a protective effect against BPAF exposure.

INTRODUCTION

Bisphenol AF (2,2-bis(4-hydroxyphenyl)-hexafluoropropane; BPAF) is a fluorinated BPA derivative with two methyl groups substituted by trifluoromethyl groups. It is used as a monomer in the production of specialized polymers, such as fluoropolymers and fluoroelastomers, polyesters, polyamides, and polyimides. It can also be used found in electronic components (Song et al., 2014). BPA and its derivatives can also be found in the liquid and solid parts of canned foods and beverages, as well as cosmetics and personal care items (Jagne et al., 2016). BPA manufacture has been restricted in recent years due to rising BPA exposure rates and growing public concern about harmful health impacts (Ma et al., 2019). BPA is currently listed 5th on the Environmental Protection Agency's Toxicological Priority Index (EPA, 2010). The development and production of alternative chemicals to replace BPA has been encouraged in recent years by strict regulations and restrictions on BPA. As a result, a number of chemicals known as "BPA analogs" have been utilized as a substitute for BPA in the production of polycarbonate plastics and epoxy resins (Riaz et al., 2021). Parallel to this, the United States (USA) National Institute of Health (NIH) has approved the use of Bisphenol AF (BPAF), an organofluoride chemical created by replacing the methyl hydrogens of BPA with fluorine and a structural analog of BPA (NIH, 2022).

Despite its increasing use, the number of studies on BPAF is still limited. BPAF exposure has been proven to have negative effects on steroidogenesis in studies (Siracusa et al., 2018). BPAF at a concentration of 1 mg/L reduced testosterone levels and affected testicular morphology in male zebrafish (Yang et al., 2016; Shi et al., 2015). BPAF disrupted the blood-testicular barrier and has a negative impact on sperm quality in mice (Wu et al., 2019). It is known that offsprings exposed to BPAF before and after birth have lower testicular testosterone levels and have significantly more alterations in genes involved in cell differentiation and meiosis (Li et al., 2016). These findings suggest that BPAF may be a more potent endocrine disruptor than BPA (Siracusa et al., 2018).

Safranal is a potent antioxidant produced by the saffron (Crocus sativus) plant's secondary metabolism (Hosseinzadeh and Sadeghnia, 2005; Hosseinzadeh et al. 2003). Safranal has also been shown to have anti-inflammatory (Alayunt et al., 2019), anticancer (Zhang et al., 2018), antimicrobial (Khayyat and Elgendy, 2018), antihyperglycemic (Kianbakht and Hajiaghaee, 2011), and neuroprotective (Sadeghnia et al., 2017) ef-

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fects. Saffron, as an antioxidant, improves sperm morphology and motility in infertile men (Heidary et al., 2008). Saffron supplementation reduces the damage to sperm DNA caused by exercise stress (Maleki et al. 2016). It can also eliminate chromatin abnormalities in sperm (Mardani et al., 2014). Saffron helps protect the integrity of the sperm membrane (Vaez et al., 2014) and improved epididymal sperm parameters in rats exposed to cadmium (Asadi et al., 2014). In the testis tissue of diabetic rats, safranal has an antioxidative effect, indicating that it may protect against oxidative stress (Ataei and Rahbarian, 2020).

Therefore, the current study aimed to investigate the potential therapeutic effects of safranal against the possible negative effects of BPAF on some oxidant-antioxidant, histopathological, hormones and male reproductive parameters in male New Zealand White rabbits.

MATERIALS AND METHODS

Experimental Animal Material and Experimental Protocol

Hatay Mustafa Kemal University Rectorate Animal Experiments Local Ethics Board approved the study protocol (2020/07-1;15/12/2020). In the present study, a total of 24 male New Zealand rabbits (Oryctolagus cuniculus) aged 8-10 months were used (n-6). The body weight of the rabbits was between 2400 and 2800 grams at the start of the experiment. They were fed standard pellet feed containing 9% raw ash, 20% crude protein, 14% crude fiber, 0.5% calcium, 0.5% phosphorus, and 0.2% sodium (Mirisan Yem ve Yag Sanayi, Hatay, Turkiye). The rabbits were acclimated for two weeks at a 50-55% humidity, a temperature of 22±2 °C, and a 14:10 hour light: dark cycle prior to the experiment. The experimental period was 9 weeks long [49 days (one spermatogenesis time) + 14 days (sperm storage and transport time in the epididymis)]. Weekly measurements of live weight and feed intake were recorded. Oral gavage applications were performed every day between 17:00 and 18:00.

After a two-week adaptation period, the rabbits were randomly divided into four study groups, each with six rabbits: Control group (C; 1 ml corn oil/day, orally), BPAF group (BF; 20 mg/kg/day Bisphenol AF [Alpha-Aesar A18370.14, Haverhill, Massachusetts, USA] in 1 ml corn oil, orally), Safranal group (SF; 100 mg/kg/day safranal [Sigma-Aldrich W338907, St. Louis, Missouri, USA] in 1 ml corn oil, orally), BPAF+Safranal group (B+S; 20 mg/kg/day Bisphenol AF + 100 mg/kg/day safranal in 1 ml corn oil, orally). There was no statistical difference in the live weights of the rabbits in the groups at the start of the study.

A total of 25 ml of blood was obtained from rabbits via the ear artery one hour after the last oral gavage administration. Blood was collected into EDTA coated (for whole blood and plasma) and non-anticoagulant tubes (for serum). The blood samples were kept at +5-6 °C until they were centrifuged (2000 rpm for 20 minutes) within 30 minutes to obtain serum and plasma samples.

After 24 hours of the last oral gavages, rabbits were euthanized under general anesthesia with isoflurane. The left testicles were washed with chilled saline at +4 °C before being stored in a deep freezer at -80 °C for the measurement of ELISA and oxidative stress parameters. Right testicles were sent for histopathological examination in formaldehyde.

Hormone Assays

Hormone analysis kits were assembled in accordance with the manufacturer's instructions (Bioassay Technology Laboratory, China). Serum, testicular, and seminal plasma testosterone (BT-LAB E0039Rb Rabbit Testosterone ELISA Kit, Standart Curve Range: 0.2-90ng/ml, Intra-Assay: CV<8%, Inter-Assay: CV<10%) and estradiol hormone (BT-LAB E0274Rb Rabbit Estrogen ELISA Kit, Standart Curve Range: 0.5-100ng/L, Intra-Assay: CV<8%, Inter-Assay: CV<10%) levels were assessed using rabbit-specific ELISA kits.

Semen Collection

Rabbits were accustomed to the artificial vagina for 14 days before the study began. Ejaculates were collected once a week through the artificial vagina and directly into graded and warmed glass tubes. After the gel portion of the ejaculate was removed, the volume and weight were measured. Ejaculate samples were stored in a +32°C water bath until spermatological analyses (Ata A., 2018).

Semen Analysis

Ejaculate volume, weight, sperm pH, concentration, and motility were assessed in ejaculates collected at the start and end of the study from rabbits (Ata A.,2018). Hydrogen ion concentrations were measured immediately after the collections using a pH meter (Orion Ross Ultra pH/ATC Triode, Orion 3 Star pH benchtop, Thermo Scientific, USA). The phosphate saline buffer was used to dilute sperm samples ten times. The percentage of sperm motility was determined using a phase-contrast microscope (400X magnification, Nikon E 200) with a heating plate (37.8 °C). Three distinct areas were scanned, and the averages were calculated and expressed as a percentage (%) under the microscope.

Following gel removal, the volume of ejaculate was measured using a graduated tube. A precision balance was used to weigh the ejaculate samples. Sperm count was performed on a Thoma slide with 400 times magnification under the same microscope in 0.1 ml of formalin saline solution. The sperm concentration and ejaculate volume values were used to calculate the number of sperm in the total ejaculate.

Following the measurements, the remaining ejaculates were centrifuged at 2000 rpm for 20 minutes to separate the seminal plasma, and 10μL of the resulting seminal plasma was then added to a refractometer (Atago, SPR-N, Japan) to quantify the total seminal plasma protein levels (SPTP).

Oxidant and Antioxidant Parameters

Testicular tissue samples were homogenized at a 1:10 ratio with 1.15 % KCl and half of this homogenate was used for malondialdehyde (MDA) analysis. The other half was centrifuged for 1 hour at 5000 g (+4 °C), the supernatants were separated, and glutathione (GSH), glutathione peroxidase (GPx),

and catalase (CAT) analyses were carried out from these supernatants. The level of MDA in the study was measured by the method of Ohkawa et al. (1979). The method developed by Beutler et al. (1963) was used to determine the level of reduced GSH. The activity of the CAT and GPx enzyme was assessed using the methods of Aebi (1984) and Beutler (1975), respectively. Protein analyses were carried out from the supernatant generated from the homogenate using the technique of Lowry et al. (1951).

Histopathological Examination

The testicles were dehydrated by passing through graded alcohol after being fixed in 10% formaldehyde for 48 hours. After embedding in paraffin, the tissues were washed with xylene, cut at a thickness of 4-5 µm, stained with Hematoxylin Eosin (Luna, 1968), and inspected for histological alterations under a microscope (Olympus BX50-F4, Tokyo, Japan) at 10-40-100X magnification.

Statistical Analysis

All values are expressed as mean ±SD (Standard Deviation). For statistical analysis, the SAS statistical program's PROC ANOVA was used. The Tukey test was used to compare results with statistical differences. When the difference between groups was $P < 0.05$, the difference was regarded as significant in all statistical applications.

RESULTS

The rabbits had no clinical problems during the course of the experiment. No differences among the groups from the initial sperm samples collected were apparent: sperm concentration, ejaculate volume, progressive motility, ejaculate weight, seminal plasma total protein, and pH were similar in both groups (Table 1). On the other hand, the SF and C groups had the highest sperm concentrations from samples collected at the end of the study (Table 2). Compared to the B+S group, sperm concentrations in the BF group decreased substantially. The total sperm count calculated from the ejaculate volume and sperm concentration data did not differ across the groups. Similarly, the ejaculate volume, testis weights, and epididymis weights did not differ significantly among the groups (Table 2).

There was no difference among the groups for initial serum estrogen and testosterone levels (Table 3). However, there were significant differences in the estrogen levels apparent from the samples taken at the end of the study (Table 4). Estrogen levels were lowest in SF and B+S groups, and there was a significant difference in serum estrogen levels between BF and B+S groups. Moreover, the amount of estrogen in testicular tissue also differed significantly across groups. The lowest levels of estrogen in testicular tissues were in the SF and B+S groups. When compared to the BF group, the B+S group also had significantly lower testicular estrogen levels. However, serum, seminal plasma, and testicular testosterone levels did not statistically differ among the groups.

It is seen that this hormone has the lowest values in SF and B+S groups in serum. Although the serum estrogen amount was at the highest values in the BF group, it was not significant compared to the C group. However, it was found at significantly higher levels compared to the SF and B+S groups. In the analyzes performed on testicular tissue, the amount of estrogen hormone varied significantly between the groups. Estrogen levels in the testicles of the SF group were the lowest compared to the C and BF groups. In the B+S group, statistically significant lower values were observed compared to the BF group. There was no statistical difference between the groups in other reproductive parameters.

Table 5 presents the oxidant-antioxidant enzymes tested in the current study. None of the treatments altered GPx and CAT levels. On the other hand, serum MDA levels were significantly elevated in the BF group. The addition of safranal was able to lower the MDA in the B+S group. Rabbits in the BF group had also significantly lower serum GSH levels when compared to the rabbits in SF and C groups.

Histopathological examination of the testis tissue showed that the basement membrane, germinative epithelium of the seminiferous tubules, Sertoli cells, and Leydig cells in the interstitial area had normal histological features for the rabbits in the C group (Figure 1,2,3). The morphology of the majority of the tubules in the testicles of the rabbits in the BF group was damaged, and some of them were atrophied (Figure 4,5,6,7). Although the tubules in the B+S group were mostly morpho-

Table 1. Initial spermatological parameters (mean±SD) of New Zealand White rabbits prior to the experimental procedure.

	C	BF	SF	B+S	P=
Sperm Concentration(x10⁶/mm3)	272.83±81.25	227.66±77.1	257.83±52.82	244.66±55.19	0.698
Sperm Motility(%)	62.50±5.24	54.16±12.81	60.83±9.7	61.66±6.83	0.393
Ejaculate Volume(ml)	0.63±0.26	0.76±0.24	0.73±0.28	0.85±0.18	0.519
Ejaculate Weight(mg)	0.828±0.296	0.755±0.211	0.841±0.252	0.848±0.207	0.905
Sperm pH	6.80±0.04	6.80±0.13	6.82±0.10	6.80±0.06	0.987
SPTP(mg/dl)	2.35±0.32	2.41±0.51	2.43±0.46	2.38±0.49	0.989
Total sperm (ejaculate volume X concentration, x10⁶/ml)	175.03±80.3	171.88±78.1	179.13±50.0	210.78±72.4	0.768

C= Control group, BF= Bisphenol AF group, SF= Safranal group, B+S= Bisphenol AF+Safranal group. SPTP = Seminal Plasma Total Protein.

Table 2. Effects of BPAF and/or safranal on some reproductive parameters (mean±SD) of New Zealand White Rabbits.

	C	BF	SF	B+S	P=
Sperm Concentration(x10⁶/mm3)	284.2[a]± 67.41	194.0[b]± 65.72	305.83[a] ± 35.27	246.66[ab]± 43.55	0.012
Sperm Motility(%)	64.16[a]± 5.84	45.0[b] ±8.94	67.5[a] ± 8.21	63.33[a] ± 6.05	0.000
Ejaculate Volume(ml)	1.01[a] ± 0.27	0.85[ab]± 0.10	1.0[a] ± 0.23	0.91[ab] ± 0.24	0.557
Ejaculate Weight(mg)	0.96 ± 0.37	0.77 ± 0.19	0.98 ± 0.33	0.88 ± 0.33	0.654
Sperm pH	6.83 ± 0.05	6.75 ± 0.11	6.86 ± 0.1	6.8 ± 0.04	0.183
SPTP (mg/dl)	2.43 ± 0.46	2.43 ± 0.5	2.28 ± 0.41	2.48 ± 0.48	0.892
Testicular Weight(gr)	3.33 ± 0.20	3.14 ± 0.19	3.15± 0.19	3.16± 0.23	0.368
Epididymis Weight(gr)	0.77 ± 0.09	0.78 ± 0.07	0.80 ± 0.08	0.79 ± 0.08	0.888
Total sperm (ejaculate volume X concentration, $x10^6$/ml)	304.0 ±155.4	167.9±68.15	312.1 ±105.0	227.8 ±80.3	0.096

C= Control group, BF= Bisphenol AF group, SF= Safranal group, B+S= Bisphenol AF+Safranal group. SPTP = Seminal Plasma Total Protein.

Table 3. Initial serum estrogen and testosterone concentrations (mean±SD) of New Zealand White rabbits prior to the experimental procedure.

	C	BF	SF	B+S	P=
Estrogen(ng/L)	21.52±2.56	19.05±2.14	19.38±2.21	21.51±3.98	0.293
Testosterone(ng/ml)	12.13±0.90	11.57±0.87	11.79±0.84	11.29±1.31	0.540

C= Control group, BF= Bisphenol AF group, SF= Safranal group, B+S= Bisphenol AF+Safranal group.

Table 4. Effects of BPAF and/or safranal on serum, seminal plasma and testicular testosterone and estrogen levels (mean±SD) of New Zealand White Rabbits.

	C	BF	SF	B+S	P=
Serum Estrogen (ng/L)	20.36[ab] ± 5.02	21.80[a] ± 4.82	15.31[bc] ± 4.25	14.88[c] ± 2.87	0.023
Seminal Plasma Estrogen (ng/L)	29.19 ± 8.94	33.7 ± 5.68	36.8 ± 7.51	33.51 ± 12.35	0.546
Testicular Estrogen (ng/L)	20.92[ab] ± 3.96	29.84[a] ± 7.49	8.07[c] ± 9.15	11.28[bc] ±12.18	0.001
Serum Testosteron (ng/ml)	12.01 ±1.99	11.61 ±1.57	12.17 ± 0.84	11.17 ± 2.05	0.741
Seminal Plazma Testosteron (ng/ml)	42.69 ± 4.63	37.65 ± 4.09	44.70 ± 5.67	44.92 ± 12.73	0.343
Testicular Testosteron (ng/ml)	75.5 ± 14.7	66.5 ± 16.5	75.9 ± 22.5	68.7 ± 16.3	0.731

C= Control group, BF= Bisphenol AF group, SF= Safranal group, B+S= Bisphenol AF+Safranal group. Different superscript letters (a, b, c) in the same line show statistically significant differences between groups as "±".

Table 5. Effects of BPAF and/or safranal on some oksidant-antioxidant parameters (mean±SD) in testis tissues of New Zealand White Rabbits.

	C	BF	SF	B+S	P=
MDA(nmol/gr protein)	3.67[c]±0.47	16.30[a]±6.16	4.64[c]±1.00	8.77[b]±0.55	0.0001
GSH(nmol/gr protein)	1.74[ab]±0.20	1.43[b]±0.17	1.84[a]±0.21	1.97[a]±0.36	0.0094
GPx(IU/gr protein)	26.95±3.52	25.93±2.49	26.69±2.67	25.95±2.11	0.8846
CAT(IU/gr protein)	91.35±5.88	90.13±4.84	90.99±6.02	88.93±5.37	0.8770

C= Control group, BF= Bisphenol AF group, SF= Safranal group, B+S= Bisphenol AF+Safranal group. MDA=Malondialdehyde, GSH=Reduced Glutathione, GPx= Glutathione Peroxidase, CAT= Catalase. Different superscript letters (a, b, c) in the same line show statistically significant differences between groups as "±".

logically normal, morphological changes in some tubules and the interstitial area, including Leydig cells were present (Figure 8,9,10). There was also a slight deterioration in the spermatogenic cell line in the degenerated tubules, as well as a few vacuoles in some germinative cells. However, these changes were milder compared to the changes in the BF group. The basal

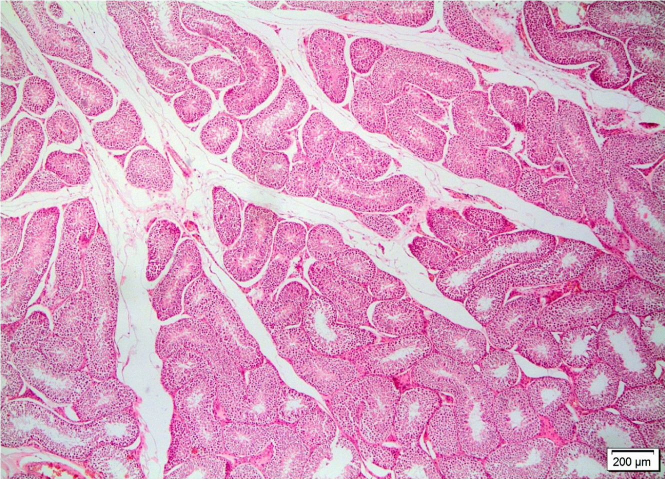

Figure 1. Microscopic view of the normal histological structure of testicular tissue of the control group. H.E. x40

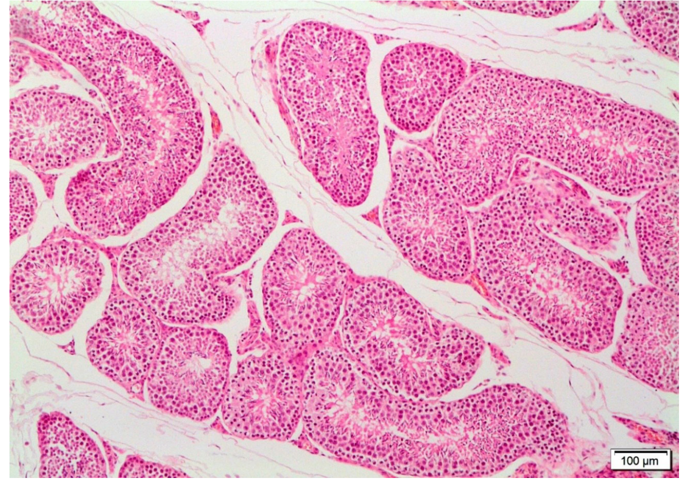

Figure 2. Close microscopic view of the normal histological structure of the testicular tissue of the control group. H.E. x100

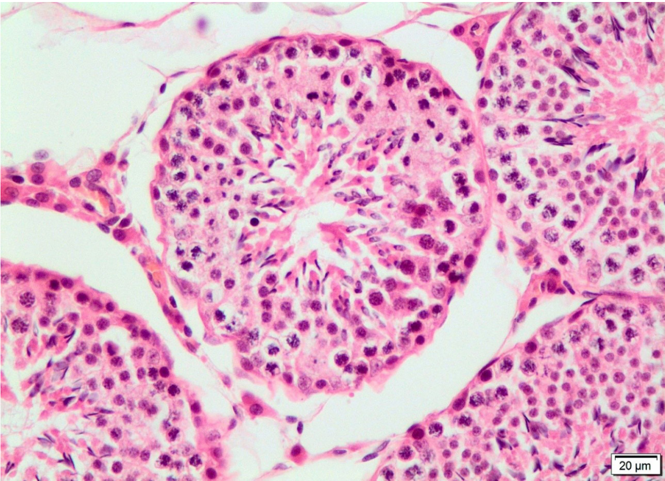

Figure 3. A closer microscopic view of the normal histological structure of testicular tissue belonging to the control group. H.E. x400

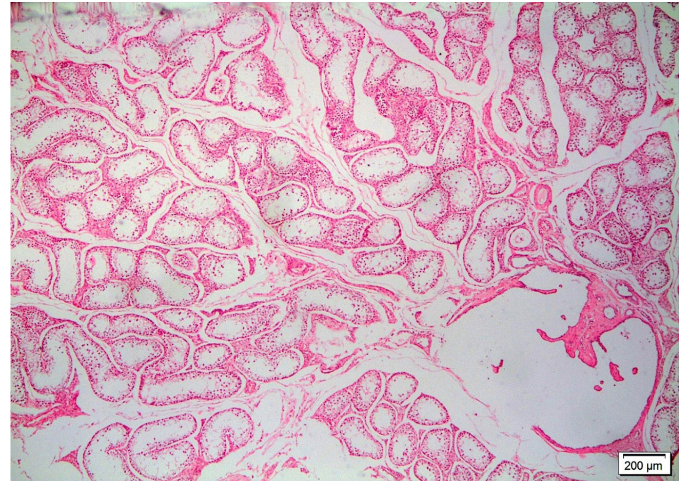

Figure 4. Microscopic view of testicular tissue belonging to the BPAF group. H.E. x40

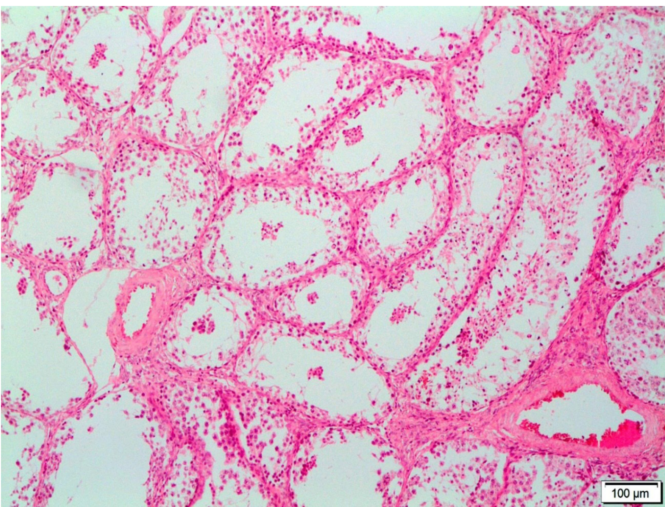

Figure 5. Close microscopic view of seminiferous tubules whose structures are completely destroyed in the testicular tissue of the BPAF group. H.E. x100

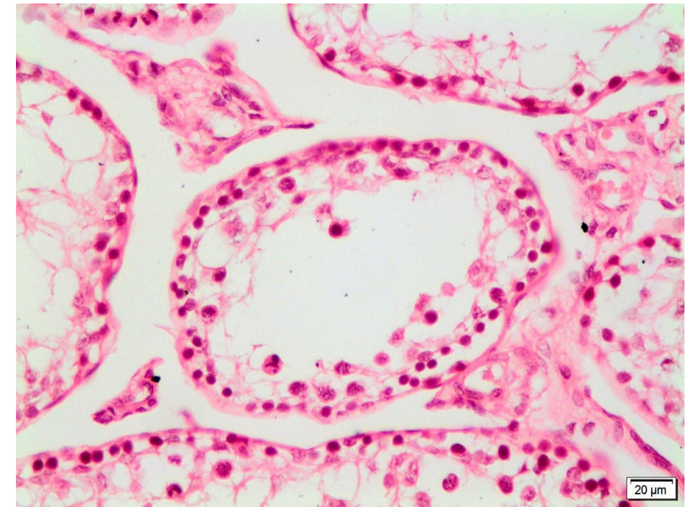

Figure 6. Closer microscopic view of degenerative and necrotic changes in the seminiferous tubule, whose structure is completely destroyed in the testicular tissue of the BPAF group. H.E. x400

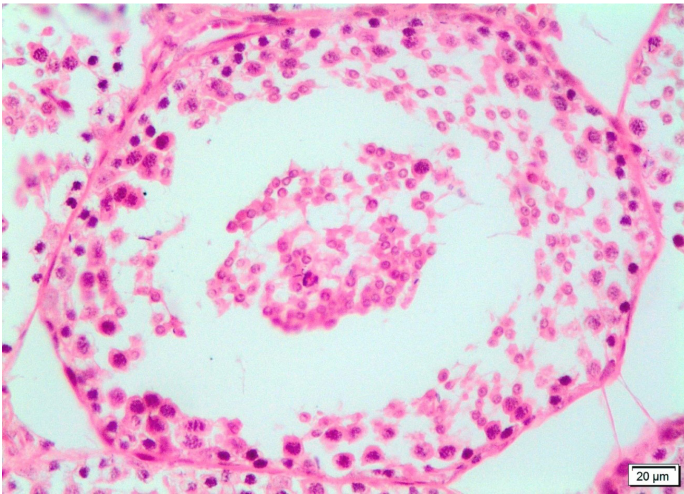

Figure 7. Microscopic view of germinative epithelial cells spilling into the lumen in a degenerated tubule of testicular tissue belonging to the BPAF group. H.E. x400

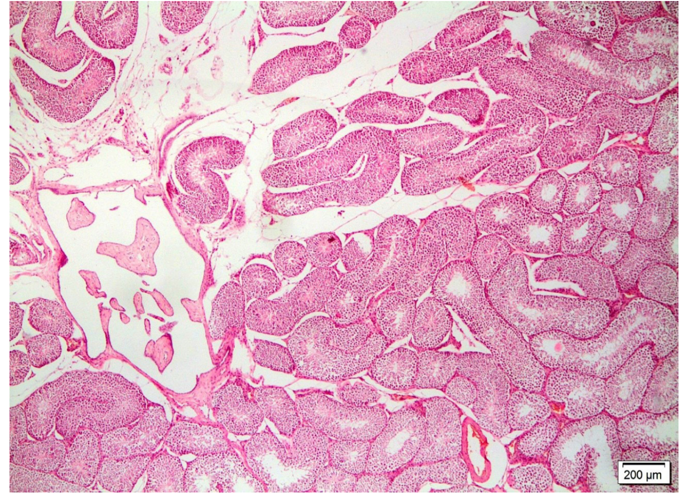

Figure 8. Microscopic view of the nearly normal histological structure of testicular tissue belonging to the BPAF+Safranal group. H.E. x40

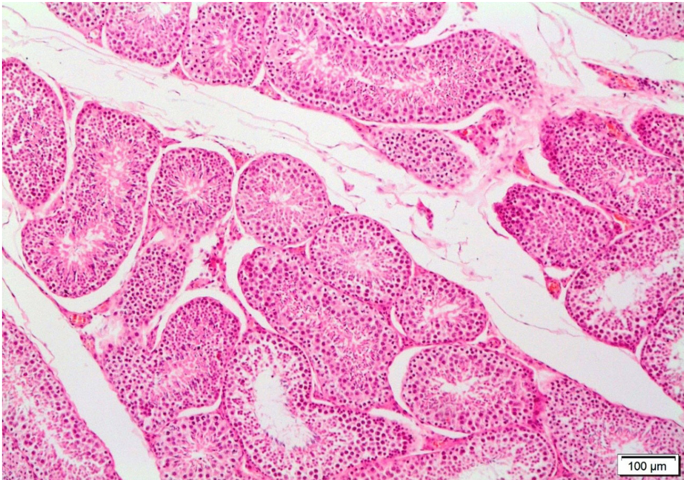

Figure 9. Close microscopic view of the nearly normal histological structure of the testicular tissue belonging to the BPAF+Safranal group. H.E. x100

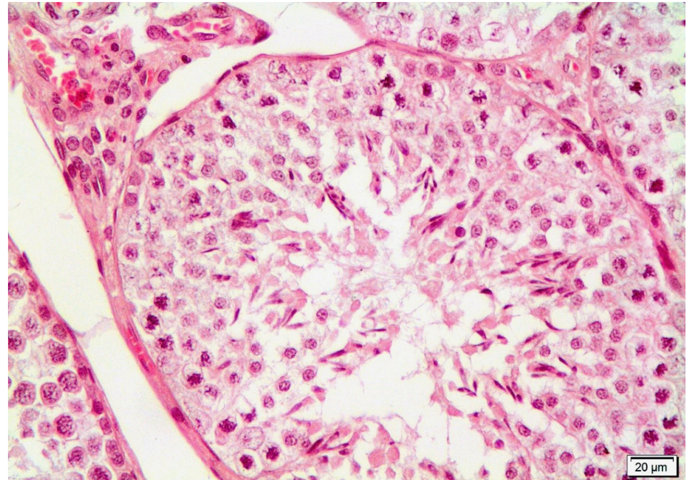

Figure 10. Near-normal microscopic appearance of germinative epithelial cell lines and Leydig cells in the interstitium and vascular structures in the testis tissue of the BPAF+Safranal group. H.E. x400

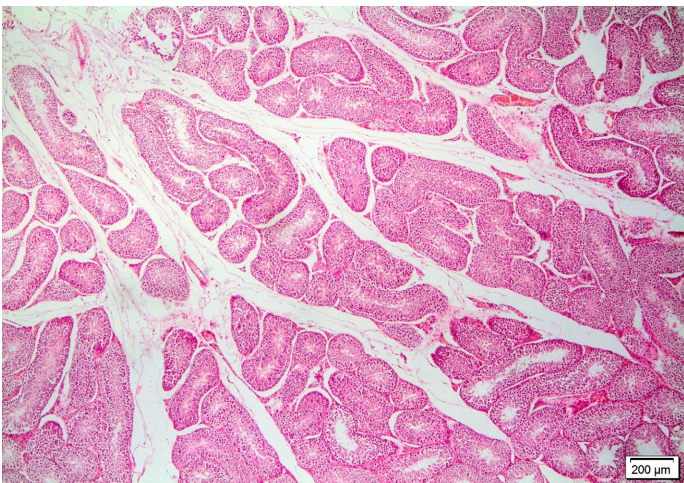

Figure 11. Microscopic view of the normal histological structure of testis tissue belonging to the Safranal group. H.E. x40

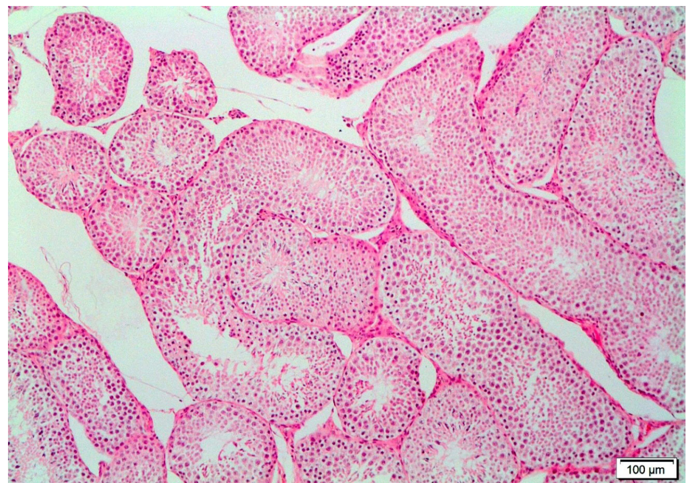

Figure 12. Close microscopic view of the normal histological structure of the testis tissue belonging to the Safranal group. H.E. x100

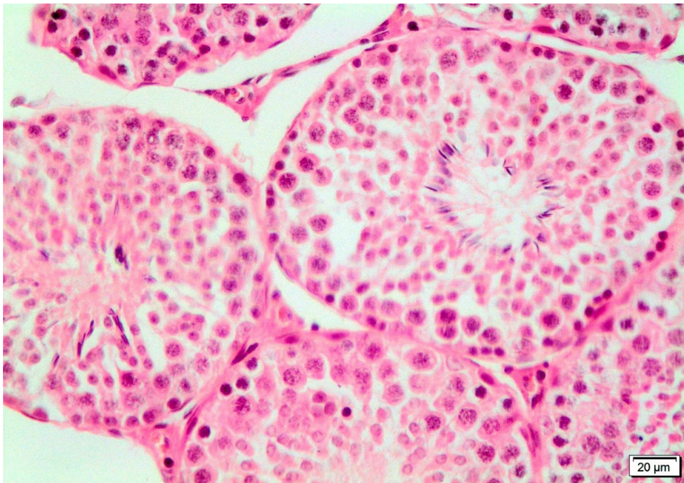

Figure 13. A closer microscopic view of the normal histological structure of the testis tissue belonging to the Safranal group. H.E. x400

membrane, spermatogonia forming the spermatogenic cell lines in the tubular wall, spermatogonia, spermatocytes and spermatid cells, and the interstitial area including the supporting cells Sertoli cells and Leydig cells of the SF group had a histological structure similar to the C group, and spermatogenesis continued normally (Figure 11,12,13).

DISCUSSION

The current study indicated that BPAF has some negative effects on the male rabbits' reproductive system. The results suggested that an oral 20 mg/kg of BPAF every day may lower fertility rates, particularly by lowering sperm concentration and motility. Additionally, when compared to other groups, BPAF tended to increase serum and testicular estrogen levels. As seen in zebrafish, BPAF can strongly bind to estrogenic receptors and might cause an elevation in estrogen secretion (Moreman et al., 2017). Another study also supports our findings that BPAF exposure resulted in increased estrogen levels in both male and female zebrafish (Yang et al., 2016). BPAF decreased serum testosterone levels and testicular weights in rats at a dose of 10 mg/kg, but no change occurred in estradiol levels at the same dose. In the same study, an increase in MDA levels was observed at a 200 mg/kg dose (Yu et al., 2022). In a different study, although serum and testicular estrogen levels were not affected, serum testosterone levels were reduced. It also caused morphological alterations in mice sperm, and reduced sperm mobility (Gao et al., 2022). Feng et al. (2012) reported that BPAF decreased testosterone levels by directly altering testicular function in male mice. In contrast to this study, BPAF exposure elevated the testicular testosterone levels in 23-day-old male rats (Li et al., 2016). The current study confirms earlier findings that BPAF reduces sperm quality and has a detrimental effect on male reproductive processes due to its estrogenic activity. Thus, BPAF should be taken into account as a possible cause of infertility (Wu et al., 2019; Rehfeld et al., 2020; Shi et al., 2015).

BPAF may have caused oxidative damage in testicular tissues by increasing the level of MDA and depleting antioxidant levels such as GSH. In a rat study, BPAF increased testicular MDA levels while decreased antioxidant levels, which confirms our findings (Tian et al., 2022). According to previous reports, administering BPAF to mice did not change the level of MDA in the liver tissue (Meng et al., 2019). MDA is one of the most significant by-products of membrane lipid peroxidation, and the production of might, therefore, aggravate membrane damage. In the current study, the testicular tissue MDA levels increased only in the BPAF treated group compared to the controls. However, safranal addition to BPAF was able to reduce testicular MDA levels in the B+S group. Thus, similar to BPA, BPAF may also result in oxidative stress and tissue deterioration in male rabbits (Karabulut and Gulay, 2022). Furthermore, safranal can be helpful in lowering oxidative stress since antioxidants are chemicals that scavenge free radicals formed as a result of oxidative stress.

The testosterone hormone is produced by Leydig cells from cholesterol. It is essential for the development of the male reproductive system and spermatogenesis (Ye et al., 2011). Testosterone is required for the completion of meiosis during spermatocyte development. Testosterone is also important in preventing the premature release of spermatid. Moreover, testosterone facilitates the discharge of mature sperms into the lumen of the seminiferous tubule (Xiao et al., 2014). In rats, experimental oral administration of BPAF resulted in a substantial decrease in the expression levels of genes and proteins involved in cholesterol production, transport, and steroid biosynthesis activities (Feng et al., 2012). It has been established that BPAF exposure affects testosterone levels by altering genes and proteins in the testosterone biosynthesis pathway (Fic et al., 2015). BPAF has also been shown to reduce serum (Feng et al., 2012; Yu et al., 2022), plasma, and testicular testosterone levels in male laboratory animals (Huang et al., 2020) and in male zebrafish (Yang et al., 2016). However, the current study failed to show a significant change in testicular and serum testosterone levels due to BPAF. However, BPAF dose and species differences may be responsible for the discrepancy between the current study and the current literature.

BPA and its analogs have been shown to generate oxidative stress in plasma, testis, and sperm, resulting in impairment of spermatogenesis (Gules et al., 2019, Ullah et al., 2019). Increased oxidative stress in testicular tissue causes sperm quality to decline and testicular abnormalities due to Leydig, Sertoli, and germ cell dysfunction (Yusoff et al., 2017). BPAF has been demonstrated to inhibit the regeneration of Leydig cells by reducing testosterone synthesis, downregulating the expression of critical steroidogenesis-related genes, and inducing ROS and apoptosis/autophagic cell death (Yu et al., 2022). It has also been found to promote cytoskeleton dysregulation in Sertoli cells and to impair the cellular homeostasis that Sertoli cells execute in the seminiferous epithelium to maintain spermatogenesis. It has been stated that BPAF exposure in mice has a dose-dependent negative effect on blood-testicular barrier integrity, sperm quantity, and sperm quality (Wu et al., 2019). BPAF was found to significantly impair blood-testicular barrier integrity and sperm count, as well as promote human ovarian granulosa cell-like cell death (Huang et al., 2020, Wu et al., 2019). In pubertal rats, BPA lowered daily sperm out-

put (Herath et al., 2004). BPA exposure has been linked to reproductive disorders, such as decreased testicular weight and sperm count, hormonal abnormalities, and poor spermatogenesis (Wang et al., 2016). Similarly, BPAF exposure at various levels reduced testis, epididymis, and body weights in rats (Yu et al., 2022). In the current study, BPAF exposure reduced sperm concentration ratio and sperm motility in male rabbits. The present study suggests that oxidative damage caused by BPAF in the testicular tissue might be the cause of the decrease in these parameters. Safranal treatment significantly reduced the negative effect of BPAF on sperm motility. Thus, safranal could protect testicular tissue from the harmful effects of BPAF and improve spermatological parameters.

Histopathological findings in our study support other spermatological findings. BPAF can cause the disruption of the seminiferous tubules in testicular tissue (Sutherland et al., 2019). Studies in many animal species, including mice, zebrafish, Xenopus laevis (an African clawed frog), and chicken embryos have shown that the histological structure of testicles was compromised due to BPAF exposure (Wu et al., 2019; Yang et al., 2016; Mentor et al., 2020; Cai et al., 2020). BPA exposure has been linked to histological abnormalities in testicles and epididymis, including degeneration, blockage, atrophy, and the loss of germinal cells in rats (Aydoğan et al., 2010). In the current study, the basement membrane, germinative epithelium of the seminiferous tubules, Sertoli cells, and Leydig cells in the interstitial area were normal in C and SF groups. However, the majority of the tubules in testicular tissues were distorted and some were atrophied in rabbits that had only been exposed to BPAF. Although mild morphological abnormalities were seen in some tubules and the interstitial area, most of the tubules in the B+S group were morphologically close to normal.

The majority of exogenous antioxidants are phytochemicals derived from plants (Sarangarajan et al., 2017). It is emphasized that safranal has significant antioxidant activity and may be useful in treating disorders induced by oxidative stress (Rahaiee et al., 2015). Safranal helps to stabilize cell membranes by scavenging ROS and lowering the peroxidation of unsaturated membrane lipids. As a result, safranal may have therapeutic value in conditions where radical scavenging action is important, such as neurodegenerative disorders (Samarghandian et al., 2015). Safranal possesses antioxidant properties and has been shown to lower lipid peroxidation and MDA levels in rats in vivo experiments (Hosseinzadeh et al., 2009). The MDA levels in testicular tissue were considerably lower in the B+S group. This also suggests the probability that longer-term safranal use might help to maintain desired MDA levels in BPAF-exposed rabbits. In diabetic rats, Ataei and Rahbarian (2020) support this positive effect of safranal.

GSH is thought to be the most essential intracellular hydrophilic antioxidant, protecting cells from free radical damage (Pandey and Rizvi 2010). BPAF exposure reduces GSH levels in testicular tissue and induces oxidative damage in rats (Tian et al., 2022). In our study, BPAF exposure significantly decreased the GSH levels in testicular tissue. The treatment with safranal stabilized the GSH levels in testicular tissues of rabbits in the B+S group. The antioxidant enzyme glutathione peroxidase functions as a structural protein in the spermatozoa's mitochondrial capsules and is necessary for the early stage of spermatogenesis (Schneider et al.,2009). Both BPA and BPAF have been shown to cause oxidative stress by downregulating antioxidant defense system expression, including a decrease in superoxide dismutase (SOD) and catalase (CAT) activity in zebrafish and human vascular endothelial cells (Gu et al., 2020). However, it has been reported that BPAF exposure in mice does not result in a significant difference in SOD, CAT, and GSH-Px enzyme activity in liver tissue (Meng et al., 2019). Thus, it should be noted that different criteria such as animal species, BPAF dose, and duration of the study may affect the outcome.

CONCLUSION

The results of this study revealed that exposure to BPAF resulted in oxidative stress and altered estrogen hormone levels in the testicular tissue of male rabbits. These negative alterations possibly had a negative impact on the sperm parameters as well as the histological structure of testicular tissue. It is likely that the effects of BPAF at larger doses would be more evident. The study suggests that safranal exhibited antioxidant activity in testicular tissue and might mitigate the harmful effects of BPAF on testicular tissue. Additional studies at various doses and time intervals could be useful to fully characterize the effects of BPAF in rabbit testicular tissue and the protective effects of safranal.

DECLARATIONS

Ethics Approval

This study was ethically approved by Hatay Mustafa Kemal University Rectorate Animal Experiments Local Ethics Committee with the number 2020/07-1 (15/12/2020).

Conflict of Interest

The authors declare that they have no competitive interests.

Consent for Publication

Not applicable.

Author contribution

Idea, concept and design: M.E, M.Ş.G

Data collection and analysis: M.E, M.Ş.G

Drafting of the manuscript: M.E, M.Ş.G

Critical review: M.E, M.Ş.G

Data Availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Research Article

The effect of organic matter based decontamination technique on *E. coli* inhibition in shrimp

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ABSTRACT

As a result of the increasing world population, people are looking for new sources of nutrition. Alternative seafood, such as crabs, mussels, and shrimps, have gained interest recently as a source of nutrients in addition to traditional seafoods. This study aimed to develop new strategies for reducing *Escherichia coli* count in shrimp. In our study, the effects of nisin, lactic acid, acetic acid and their combinations were investigated in shrimp contaminated with *E coli*. At the end of the study, a statistical difference was found between the effects of single and combined use of all substances ($p<0.05$). In addition, it was observed that lactic acid was the most effective with a decrease of 1.92 CFU (Colony Forming Units)/mL in single use, while acetic acid and nisin had a good synergistic effect with a decrease of 2.2 CFU/mL in combined use.

INTRODUCTION

The aquaculture industry is thought to be a globally expanding field that is effective in supplying human food demands (Macusi et al., 2022). Considering the information in the TUIK 2021 data, it is shown that 5,204 tons of shrimp are caught in our country (Anonymous, 2021). In working societies, consumers are increasingly buying frozen, ready-to-cook goods like fish fillets and shrimp (Arpa et al., 2020). As in the production of all foodstuffs, it is of great importance to produce and consume seafood in a safe and hygienic manner (Yazıcı and Mazlum, 2019). Shrimp meat is easily digested and easily spoiled food with high economic value. The risk of contamination for organisms developing in contaminated habitats rises when water resources and oceans are polluted. Additionally, bacteria may become resistant to antibiotics when they are used for illness prevention and accelerating aquaculture development (Yazıcı and Mazlum, 2019).

One of the most important issues that the food industry focuses on is food quality and safety. Microbial contamination is a major issue, especially about public health. Microbial contamination in seafoods can occur from primary sources as well as secondary sources such as processing and storage conditions (Öztürk and Altınok, 2014). *E. coli* is a bacterium that can be found in environments where shrimp are reared and can cause diseases (Okonko et al., 2008). According to Codex Microbiological Criteria Regulation, 25 g sample should not contain *E. coli* O:157 H:7. (Annex-3) (TGK, 2011).

To preserve freshness and lengthen shelf life of shrimp, various antibacterial substances have been used (Nirmal and Benjakul, 2012; Alparslan et al., 2019). Due to consumer concerns, it is evident that research focuses on organic materials and natural resources. A natural antibiotic called nisin is used to stop bacterial contamination of food (Yehia et al., 2022). Nisin is a widely used food additive in the food industry, as it is generally considered to be safe (GRAS) (FDA, 1988). In general, nisin, which affects Gram (+) bacteria more, has little effect on Gram negative bacteria (Pabon et al., 2021). In addition, it is emphasized that when combined with substances such as lactate, citrate and EDTA, it is also effective against Gram (-) bacteria (Khalafalla et al., 2016). Nisin has been used for bacterial inhibition in dairy products, meat products and canned foods (Li et al., 2022). It has been emphasized that the application of nisin (500 IU/mL) and lactic acid (1.5%) in red meat reduces the number of E. coli (De Martinez et al., 2002).

The use of lactic acid at a rate of 1-3% against pathogenic bacteria in meat, vegetables and fruits is GRAS (Wang et al., 2021). However, it is emphasized that long-term (>30 minutes) use of high concentrations of lactic acid (>2%) in seafood should be avoided so that sensory properties are not adversely affected (Wang et al., 2013). However, studies using 3% lactic acid experimentally were conducted (Shirazinejad et al., 2010; Terzi and Güçlükoğlu, 2010). Although there are studies and recommendations for the use of lactic acid in red meat and poultry for its protective effect, there are very few studies on seafood, especially shrimp (Shirazinejad et al., 2010).

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Acetic acid is produced at the level of 15 million tons/year in the world and it is used commercially in various sectors such as chemistry, textile, food and beverage. It has been used as food additive, food preservative, antimicrobial agent, flavor and flavoring, acidifier, edible packaging material and artificial ripening. Acetic acid, an organic acid, is approved for use in the GRAS category for inhibition of pathogen such as *Salmonella, Escherichia coli* in poultry, pork and red meat (Wali and Abed, 2019). It is stated to be effective against both Gram (-) and Gram (+) bacteria (Deshmukh and Manyar, 2020). Acetic acid has been used in different doses for decontamination in many foodstuffs (Yao et al., 2021; Wang et al., 2021; Sallam et al., 2020).

The study was aimed to reduce the microbial load of shrimps using nisin, lactic acid, acetic acid and their combinations and provide the food safety. Thus, the risk of *E. coli* that may occur in both frozen and ready-to-eat packaged shrimp was tried to be minimized and it was aimed to contribute to the researches in this field.

MATERIALS and METHODS

Materials

E. coli ATCC 25922 strain used in the experimental contamination of shrimp was obtained from the Department of Food Hygiene and Technology, Burdur Mehmet Akif Ersoy University. Shrimps (Balık Dünyası, Turkey) were purchased from local markets. Nisin (Sigma-Aldrich, Germany), Lactic Acid (Merck, Germany), Acetic Acid (Merck, Germany), Sorbitol MacConkey Agar (SMAC-Merck, Germany), Tryptic Soy Broth (TSB-Merck, Germany), Buffered Peptone Water (TPW- Biokar, France) was used.

Experimental Design

Research consisted in the evaluation of nine working groups as follows: K1 (negative control group), K2 (positive control group 10^8 CFU/mL), D1- was immersed in 0.016 mg/mL nisin solution for 20 minutes (Rodpan et al. 2022); D2- was immersed in 3% (v/v) lactic acid solution for 10 minutes (Khalafalla et al., 2016); D3- was immersed in 1% (v/v) acetic acid solution for 10 minutes (Rodpan et al. 2022); D4- was immersed in 0.016 mg/mL nisin solution for 20 minutes and then in lactic acid 3% (v/v) solution for 10 minutes; D5- was placed in a 0.016 mg/mL nisin solution for 20 minutes, followed by a 1% (v/v) acetic acid solution for 10 minutes; D6- was immersed in 3% (v/v) lactic acid solution for 10 minutes followed by 1% (v/v) acetic acid solution for 10 minutes; and D7- was immersed in 0.016 mg/mL nisin solution for 20 minutes, then in lactic acid 3% (v/v) solution for 10 minutes and finally in acetic acid 1% (v/v) solution for 10 minutes.

Preparation of the Inoculum

30 µl of *E. coli* ATCC 25922 strain was added to 10 mL Tryptic soy broth and incubated for 18 hours at 37 °C. At the end of the incubation period, the tubes were centrifuged at 5000 rpm for 5 min (Eppendorf Centrifuge 5810 R, Merck, USA) and the pellet and supernatant were separated. Pellets were dissolved in 1 mL of sterile 0.1% Peptone Water (PW) and then centrifuged. After the second centrifugation, the supernatants were removed and the pellets were dissolved in 1 mL sterile PW again and the inoculum was prepared (Dikici et al., 2013). The bacterial concentration in the contamination liquid was adjusted to 0.5 mcFarland (1.2×10^8 CFU/mL) in 200 mL of TSB. Sterile glass jars were used for all contamination and decontamination solutions.

Contamination Process

The shrimps used in the research were left to completly thaw at +4 °C. Three shrimps, each 3.3 ± 0.3 g, were taken and contaminated by immersion in inoculum solution. At this stage, the contamination process was completed by mixing the shrimps with sterile drumsticks at 30 rpm for 15 min. The shrimps, whose contamination time was completed, were taken into sterile containers and kept at room temperature for 15 min to allow the bacteria to adhere.

Decontamination Solution Control

In order to determine whether there is contamination in the solutions prepared for decontamination, serial dilutions of the liquids were made after all the procedures and inoculated on SMAC agar, and typical colonies were counted as a result of incubation.

Microbiological Analysis

In the negative control group (K1), microbiological analyzes of untreated shrimps were performed to investigate the presence of *E. coli*. In the positive control group (K2), only contamination was performed and microbiological analyzes were performed to determine the bacterial load in the inoculum. All groups were immediately evaluated for microbiological analysis after decontamination. The prawns were taken into a sterile stomacher bag, 90 mL of BPW was added and homogenized in the stomacher (Nr 140/420 IUL) for 2 min. Serial dilutions were prepared from the homogenized samples and the respective dilutions were parallelly plated onto SMAC agar. The petri dishes were incubated at 37 °C for 24 hours, and at the end of the period, typical colonies were counted. All procedures were performed in 3 replications and the results were calculated as $\log_{10}$ CFU/mL.

Statistical Analysis

The study was carried out in 3 parallels and Minitab® 19.1.1 (64-bit) (USA) package program was used to evaluate the data. ANOVA test was applied to the data first and Duncan multiple comparison test was applied to the parameters found statistically significant ($P<0.05$) in the ANOVA test. Data are given in the study as mean ± standard deviation.

RESULTS

Microbiological Analysis and Statistical Results

The decontamination effect of organic acids and their combinations on the inhibition of *E. coli* is shown in Table 1. As a result of the present study, the effect of all organic acids and their combinations on *E. coli* was found to be statistically significant ($P<0.05$). D1 group was found to be the group

with the lowest decrease in *E. coli* counts with approximately 0.85 log CFU/mL compared to the control group. The highest decrease in *E. coli* counts was observed in the D5 group with 2.2 log CFU/mL ($P<0.05$). Considering the inhibition effect of all organic acids alone (D1, D2, D3), it was found that the D7 group containing all of them was more effective ($P<0.05$). While no contamination was observed in lactic acid and acetic acid solutions in plating made from decontamination solutions, 4.03 log CFU/mL *E. coli* was detected in nisin solution.

Table 1. *E. coli* Decontamination Results

GROUPS	K2	D1	D2	D3	D4	D5	D6	D7
Count Results (log_{10} CFU/mL)	5.53± 0.04^{a}	4.68± 0.09^{b}	3.61± 0.06^{ef}	4.14± 0.08^{c}	3.98± 0.07^{d}	3.33 ± 0.07^{g}	3.70± 0.04^{e}	3.55± 0.10^{f}
Count Differences (log_{10} CFU/mL)	-	0.85	1.92	1.39	1.55	2.2	1.83	1.98

^{abcdefg}: Means in the same row with different superscripts are statistically different ($P<0.05$).

Table 2. Bacterial Load in Post-Treatment Decontamination Solutions

Decontamination solutions	Count Results (log_{10} CFU/mL)
Nisin	4.03
Lactic acid	Not detected
Asetic asic	Not detected

DISCUSSION

In the present study, the microbiological quality of frozen shrimp contaminated with *E. coli* was investigated to examine the antimicrobial activity of lactic acid and acetic acid and nisin as organic decontamination agents. Sultana et al. (2021) investigated the total coliform amount in shrimp farms in winter and summer seasons in Khulna district of Bangladesh and determined enterovirulent groups in their study. As a result of the research, coliform was detected in all farms, while *E. coli* was found in 55% of the farms. Monte et al. (2019) recently reported the emergence of *E. coli* carrying clinically relevant resistance genes in seabirds, wild fish and bivalves, highlighting an urgent need for monitoring of marine environments. In another study, *E. coli* was also isolated from shrimp farm wastewater in Ramanathapuram, India (Chinnadurai et al., 2018). These investigations, with similar results, confirm that *E. coli* is a potential public health pathogen in shrimp production.

Khalafalla et al. (2016) investigated the effect of nisin, lactic acid and their combinations on the shelf life and microbiological quality of chicken breasts. In their results, similar to the results of our study, a significant difference was found between the control and treated groups, except for the group treated with nisin on 0. day of storage in coliforms. In addition, the groups treated with lactic acid (1 and 2%) were found to have a higher reduction in coliform counts than the group treated with nisin. On the other hand, the results showed that nisin alone was less effective on coliforms as their cell walls were less permeable to nisin. Similar to the results of our study, it was revealed that the groups treated with nisin and lactic acid were more effective in reducing *E. coli* than the other treated groups, and therefore lactic acid increased the effect of nisin against *E. coli*.

Yehia et al. (2022), it was reported that nisin alone had a bacteriostatic effect on methicillin-resistant *S. aureus*, but did not show the same effect on *S. aureus* ATCC 25923. It has been found that the combination of nisin and reuterin has a bactericidal effect on both microorganisms, and combination of nisin and reuterin can produce a more active effect against both microorganisms. Rodpan et al. (2022) evaluated the inhibitory effects of acetic and propionic acids in combination with nisin in preventing meat and potato spoilage caused by many pathogens. It was found that the synergism of nisin, acetic acid and propionic acids showed a synergistic effect in bacteria such as *E. faecalis, P. aeruginosa, S. Typhimirium* and *E. coli*, which were tested using fractional inhibitory concentration indices, as in our study.

De Martinez et al. (2002) stated that the mixture of nisin and lactic acid provided the highest decrease in total coliform and *E. coli* in beef carcasses in their study on red meats. It has also been reported that this mixture can reduce the total bacterial load by 2 log CFU/g. Mustapha et al. (2002) investigated the antimicrobial effect of 2% low molecular weight polylactic acid, 2% lactic acid, 200 IU nisin and their combinations on raw meat contaminated with *E. coli* O157:H7. Contrary to our study, it was observed that nisin did not increase its antimicrobial effect in combination with acids.

Shirazinejad et al. (2010) evaluated the antimicrobial activity of lactic acid against *V. cholerae, V. parahaemolyticus, S. Enteritidis* and *E. coli* O157:H7 for different durations in a study on fresh raw shrimp. After treatment, 10 min of 3% lactic acid treatment was found to be appropriate in reducing pathogenic bacteria and being organoleptic acceptable. A 10-minute 3% lactic acid treatment has been shown to provide a 2.30 log CFU/

mL reduction in *E. coli*. It is seen that the results obtained are clearly similar and in direct proportion with the decrease of 1.92 log CFU/mL after 10 min of 2% lactic acid treatment as in our study.

Sallam et al. (2020) investigated the effect of lactic acid, acetic acid and trisodium phosphate spray on the microbiological population of cattle carcass surfaces slaughtered in a conventional slaughterhouse in Egypt. It provided complete inhibition of enterococcal growth with lactic acid and acetic acid sprays, and trisodium phosphate was found to be more effective. Hashemi et al. (2021), the effects of relative humidity and temperature on the effectiveness of acetic acid and two different essential oils against pathogens in the vapor phase were investigated. It was determined that the initial population of *B. cereus* (8.1 log CFU/g) was 4.3, 3.9 and it was determined that it decreased to 3.3 log CFU/g.

CONCLUSION

In recent years, we have seen that the consumption of alternative seafood as well as basic seafood has increased and become popular. However, in recent studies, the contamination of marine microflora and consequently the contamination of almost all seafood reveals a frequently encountered situation. In addition, it poses a threat to public health with inadequate sanitation procedures. Considering synthetic preservatives and their negative effects in order to eliminate these problems, natural organic substances have started to be preferred more as preservatives. In cases where the antimicrobial activity of organic agents such as lactic acid and acetic acid is used without impairing the orgonaleptic properties of the foodstuff, and in cases where the effectiveness of nisin alone is not sufficient, new combinations have been tried. It is shown as promising new decontaminant agents that such combinations provide a synergetic effect by eliminating the negative or inadequate conditions in the use of products one by one.

DECLARATIONS

Ethics Approval

Ethics committee approval is not required since humans/animals were not used in our study.

Conflict of Interest

Authors do not have any conflict of interests to disclose nor do they endorse the use of any product/technology/service over the other.

Consent for Publication

Not applicable.

Competing Interest

The authors declare that they have no competing interests

Author contribution

Idea, concept and design: HY, ZP

Data collection and analysis: HY, ZP

Drafting of the manuscript: HY, ZP

Critical review: HY, ZP

Data Availability

Not applicable.

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Research Article

Investigation of serum leptin, ghrelin, irisin, insulin levels and their correlations in cattle with subclinical ketosis

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ABSTRACT

In this study, it was aimed to investigate the correlations with leptin, ghrelin, irisin and insulin levels in the blood serum of cattle with subclinical ketosis. For this purpose, 10 healthy and 10 Holstein cattle with subclinical ketosis obtained from farms in Burdur region were used. A diagnosis of subclinical ketosis was made according to the Rothera test performed in milk, by performing a general clinical examination of the animals. Blood samples were taken from Vena jugularis into tubes without anticoagulant. Serum leptin, ghrelin, irisin and insulin levels were measured in the obtained sera using commercial ELISA kits. In cattle with subclinical ketosis, serum ghrelin, leptin, irisin and insulin values were increased compared to the control group ($p<0.05$). In the correlation findings, a highly and quite significant positive correlation was found between serum ghrelin and irisin values ($r= 0.802$; $p<0.001$). A moderately and quite significant positive correlation was found between serum ghrelin value and insulin value ($r=0.673$; $p=0.001$). A moderately and significant positive correlation was determined between serum ghrelin value and leptin value, between serum irisin value and leptin value, and between irisin value and insulin value ($r=0.623$; $p=0.003$, $r=0.474$; $p= 0.035$, $r=0.558$; $p=0.011$). In conclusion, in this study, correlations were observed between serum levels of leptin, irisin, ghrelin and insulin hormones in animals with subclinical ketosis. However, it is thought that leptin, ghrelin, insulin and irisin hormones, which are associated with lipid and carbohydrate metabolism, can be used as important biomarkers in the diagnosis of subclinical ketosis and in the follow-up of its prognosis.

INTRODUCTION

Ketosis is caused in ruminants when there is a disruption in the metabolism of carbohydrates as well as volatile fatty acids. It is defined as a metabolic disorder that is characterized by a decrease in the amount of blood glucose, the depletion of the amount of glycogen and glucose stored in the liver, and the presence of ketone bodies in the blood, urine, and milk as a result of fatty degeneration in the liver (Gül, 2012). It is possible for either the clinical or the subclinical form of ketosis to develop, but the subclinical form is more prevalent (Detilleux et al., 1994).

Subclinical ketosis is characterized by a persistent deficit in energy balance, and as a result, there are high levels of ketone bodies in the blood, urine, and milk, but there are no clinical signs (Herdt, 2000). It not only reduces the amount of milk that animals produce and their reproductive performance, but it also raises the risk of other metabolic and immunological illnesses that can be noticed in the postpartum period, which results in significant financial losses (Duffield, 2000). It can be occured in cases of nutritional deficits or in cows who have been fed meals low in energy (Youssef and El-Ashker, 2017). According to several studies, the predisposing factors of subclinical ketosis are the number of births, body condition score and season. (Duffield, 2000). It is reported that cattle whose body condition scores are higher than 4.0 have a greater risk of developing subclinical ketosis in the postpartum period compared to cattle whose body condition scores are lower (Duffield et al., 2003). There are numbered studies stating that hormonal control also plays an effective role in the etiology of ketosis and subclinical ketosis (Roh et al., 2016). It is common knowledge that hormones involved in the lipid and carbohydrate metabolism play an important part in the pathophysiology of metabolic illnesses such as ketosis (Roh et al., 2016).

Leptin is a hormone that plays a crucial role in maintaining metabolic balance. It can reduce the amount of food consumed while simultaneously increasing the amount of energy expended. The regulation of energy expenditure is just one of the many functions performed by the hormone leptin, which is released by adipocytes. In addition to the function it serves, it also possesses pleotropic effects, which manifest themselves in a variety of physiological pathways (He et al., 2018). Circulating leptin concentrations in dairy cattle have been the subject of a significant number of investigations (Vargová et al., 2015; Danicke et al., 2018; He et al., 2018).

Since the discovery of the leptin hormone that is secreted by adipocytes till the present day, numerous peptides and cytokines that are responsible for both favorably and negatively regulating the metabolic process have also been found. There are a variety of adipokines, hepatokines, and myokines that all perform the function of cytokines, and the roles that these proteins play in both people and animals are still being researched (Roh et al., 2016).

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The irisin hormone, which is formed as a result of the breakdown of the transmembrane protein containing fibronectin type III domain 5 (FNDC5), has recently been identified as a myokine. The irisin hormone provides the formation of brown adipose tissue from white adipose tissue, contributes to the loss of body weight and improves glucose metabolism. The irisin hormone is usually regulated along with exercise (Boström et al., 2012).

A study was conducted to evaluate the level of bovine FNDC5 protein. The FNDC5 protein from bovines, mice, and humans were all analyzed and compared in this particular study (Komolka et al., 2014). As a consequence of this study, it has been found that the transcript level at the protein locus in cattle is characterized by a greater degree of variability. Additionally, it has been proven that mice and cattle have fundamentally different approaches to the hormonal regulation of the FNDC5 protein and irisin (Komolka et al., 2014). It has been discovered that cold, physical activity, and leptin are among the different factors that modify the quantity of circulating irisin, while circulating irisin is positively connected with muscle mass and body mass index (Huh et al., 2012; Crujeiras et al., 2014; Arhire et al., 2019). On the other hand, contrary to what has been stated, irisin hormone has been shown to have a negative connection with both age and insulin levels (Park et al., 2013; Wen et al., 2013).

Insulin is a polypeptide hormone that is released from β cells in the pancreas, which are located in the centre of the islets of Langerhans (Greenspan and Gardner, 2004). The insulin hormone's primary role is to facilitate the transport of glucose into the cell, which is its primary function. Cows that have hepatic lipidosis and ketosis acquire insulin resistance in the peripheral tissues of their bodies over time (Senoh et al., 2019).

In ruminants, the ghrelin hormone is a peptide that is composed of 27 different amino acids. It is also referred to as the hunger hormone. In ruminants, the abomasum, the small intestine, and the pancreas are responsible for the production of the hormone ghrelin. Acylation only occurs in a minute portion of the hormone ghrelin. This causes both acyl and non-acyl ghrelin hormones to take place in the circulation. Both acylated and non-acylated versions of ghrelin contribute to the regulation of energy homeostasis in the body (Thidar Myint et al., 2006).

In this study, it was aimed to investigate the relationship between insulin, leptin, ghrelin and irisin hormones, which are involved in energy balance, and each other in subclinical ketosis, which occurs as a result of the deterioration of negative energy balance.

MATERIALS and METHODS

This research was conducted with the approval of the Burdur Mehmet Akif Ersoy University Local Ethics Committee for Animal Experiments. (Ethics committee approval number: 992/108-2022)

Animals

In this study, 10 healthy and 10 Holstein cattle with subclinical ketosis obtained from Burdur region farms were used as animal material. It was learned that the animals included in the study were fed with fabricated feed as concentrate feed and with straw, corn silage and fresh beet pulp as roughage. A diagnosis of subclinical ketosis was made according to the Rothera test performed in milk, by performing a general clinical examination of the animals. Blood samples were taken from *Vena jugularis* into tubes without anticoagulant. Sera obtained from blood samples were stored at -20 ºC until analysis.

Analysis of serum hormone levels

Serum leptin, ghrelin, irisin and insulin hormone analyzes were performed using commercial bovine ELISA hormone kits (BT-Lab, China. Catalog numbers of the kits: EA0007Bo, E0262Bo, E2318Bo, E0015Bo), in accordance with kit procedures. ELISA reader device (Biotek EPOCH, USA) was used for measurement.

Statistical analysis

The data from the study were analyzed with the help of the IBM SPSS 22.0 for Windows package program. The Shapiro-Wilk test was utilized in order to determine whether or not the groups in the analyses followed a normal distribution. For the purpose of making comparisons between measurements, a paired t-test was utilized because the data followed a normal distribution. Using the Pearson Correlation analysis, we were able to ascertain the nature of the link between the variables.

RESULTS

The levels of ghrelin, irisin, leptin, and insulin in the serum of cattle with subclinical ketosis were found to be higher than those found in the control group, and a statistically significant difference was found between the two groups ($p < 0.001$), (Table 1).

In correlation findings, a high and very significant positive correlation was found between serum ghrelin value and serum irisin value ($r = 0.802$; $p<0.001$). A moderate and highly significant positive correlation was found between serum ghrelin value and serum insulin value ($r=0.673$; $p=0.001$). A moderate and significant positive correlation was determined between serum ghrelin value and serum leptin value, serum irisin value and serum leptin value, and serum irisin value and serum insulin value respectively ($r=0.623$; $p=0.003$; $r=0.474$; $p=0.035$; $r=0.558$; $p=0.011$), (Table 2).

DISCUSSION

In dairy cattle, the term 'transition phase' refers to the time span that spans the first three weeks before birth and the first three weeks after birth (Grummer, 1995). Changes in hormone levels and a reduction in feed consumption have an impact on metabolism throughout the later stages of pregnancy, resulting in a predominantly negative energy balance (Drackley et al., 1999). Ketosis can occur in animals if their energy needs increase and they develop a negative energy balance (NED), which is especially common in high-yielding dairy cattle in the postpartum period (Guliński, 2021).

In addition to the effects that the insulin hormone has on

Table 1. Serum hormone levels in healthy cattle and cattle with subclinical ketosis

Parameter	Subclinical ketosis (n=10) x̄±ss	Healthy (n=10) x̄±ss	p
Ghrelin (ng/L)	346,26±130,75	117,20±72,07	<0,001
Irisin (ng/mL)	4,63±,1,25	2,00±0,62	<0,001
Insulin (mIU/L)	5,38±2,62	3,41±1,02	0,048
Leptin (ng/mL)	2,42±,46	1,73±,26	0,001

Table 2. Correlation findings of hormone values

		Ghrelin	Irisin	Leptin	Insulin
Ghrelin	Pearson Correlation	1	,802**	,623**	,673**
	Sig. (2-tailed)		,000	,003	,001
Irisin	Pearson Correlation	,802**	1	,474*	,558*
	Sig. (2-tailed)	,000		,035	,011
Leptin	Pearson Correlation	,623**	,474*	1	,319
	Sig. (2-tailed)	,003	,035		,170
Insulin	Pearson Correlation	,673**	,558*	,319	1
	Sig. (2-tailed)	,001	,011	,170	

**The correlation is significant at the 0.01 level (2-tailed).
*The correlation is significant at the 0.05 level (2-tailed).

the way glucose is metabolized, there is evidence to suggest that it also plays a part in ensuring that the body is able to adapt physiologically, particularly at the time of calving. Compared to humans, ruminants have a glucose metabolism that is distinct, characterized by low levels of glucose in the periphery and a diminished insulin response from the periphery's tissues (Zachut et al., 2013). Cows go through a brief period of insulin resistance after giving birth to their young in order to acquire insulin-independent glucose uptake by the mammary gland, which is necessary to maintain milk supply after the calving process. As a result, maintaining blood glucose levels within acceptable physiological ranges during the transition phase is of the utmost importance (De Koster and Opsomer, 2013). In the current study, higher serum insulin levels were found in cows with subclinical ketosis compared to the control group ($p<0.05$). Contrary to the results obtained in this study, some authors reported hypoinsulinemia and hypoglycemia in cows with subclinical ketosis (Tehrani-Sharif et al., 2012) and cows with clinical ketosis (Kerestes et al., 2009; Xu et al., 2014). However, in a study, statistically significantly higher serum insulin levels were reported in cows with subclinical ketosis compared to controls (Mohamed Youssef et al., 2017). It has been determined that the periparturient period in dairy cows may be associated with changes in insulin action, especially in peripheral tissues, and the extent of prepartum insulin secretion is associated with higher insulin action (Zachut et al., 2013).

Insulin levels and leptin hormone levels in ruminants have been shown to have a favorable association. In most cases, elevated levels of insulin are accompanied by elevated levels of leptin. The amounts of circulating leptin have been shown to have a positive correlation with body fat, the amount of food consumed, certain nutrients and hormones (particularly insulin), and the luteinizing hormone pulse. In a nutshell, this hormone is responsible for the connection that exists between a person's nutritional state and their reproductive function (Kadokawa et al., 2006). Yang et al. (2010) reported in their study that blood plasma leptin levels of dairy cows in the subclinical ketosis group showed a lower frequency change within 8 weeks after birth. In the same study, it was reported that the interval of change in leptin level of dairy cows in the before birth term subclinical ketosis group was higher than in the control group. According to the findings of another study, having low serum leptin levels before birth is a significant risk factor for developing subclinical ketosis. On the other hand, it was observed that plasma leptin levels peak after birth (especially at the 7th week) in animals that have subclinical ketosis (He et al., 2018). In the current study, it was discovered that the serum leptin levels of cattle with subclinical ketosis were higher in comparison to the group that served as the control, and that there was a positive correlation between the serum leptin levels and the insulin levels.

As the time for parturition draws nearer, dairy cows have a decrease in their dry matter intake, which is one of the most significant physiological changes that take place throughout the transition phase (Drackley et al., 2001). As a direct consequence of this, the cow will frequently suffer from a wide

variety of metabolic illnesses such as ketosis, fatty liver, hypocalcemia during the period in which it is transitioning (Goff et al., 1997). Intake of food is controlled by a variety of elements, including hormones and metabolites, and is a mechanism that is quite complex (Ingvartsen et al., 2000). Ghrelin is a peptide hormone that is produced in cattle by the abomasal and ruminal tissues (Hayashida et al., 2001; Gentry et al., 2003). According to the findings of a study, the amount of the hormone ghrelin found in the plasma of dairy cows during the time when they were making the transition from pregnancy to lactation increased (Melendez et al,, 2006). Although increased ghrelin secretion is consistent with experimental models of energy deficiency, 36 hours of fasting has been reported to increase plasma ghrelin concentration more than fivefold (Wertz-Lutz et al,, 2006). An rise in serum ghrelin levels was discovered in cattle with subclinical ketosis in the current study ($p<0.05$), which is consistent with the findings of the studies that were discussed before. However, although many studies have reported a negative relationship between serum leptin and ghrelin levels (Nowroozi-Asi et al., 2016; Vargova et al., 2015), a positive relationship was found between serum leptin and ghrelin concentrations in the current study. This relationship is thought to be a part of the acute phase response seen in subclinical ketosis (El-Deeb et al., 2017) rather than hormonal regulation. It has been reported that both serum leptin and serum ghrelin levels increase simultaneously as part of the acute phase response, especially in inflammatory conditions such as sepsis (Das et al., 2011).

Irisin is a recently reported new myokine and adipokine (Boström et al., 2012) produced by cleavage of the precursor protein fibronectin type III domain-containing protein 5 (FNDC5). Various studies in humans and rodents (Boström et al., 2012, Perakakis et al., 2017) show that irisin plays a role in energy homeostasis and irisin is a regulator of glucose metabolism.

Irisin is a hormone that plays a role in the metabolic pathways of the body. This hormone increases the uptake of glucose and fatty acids by the muscles, decreases gluconeogenesis and stimulates glycogenesis in the liver, and converts white adipose tissue (WAT) into brown adipose tissue. Irisin has also been demonstrated to lessen the intensity of inflammation and to have an effect on the function of the kidneys, neurons, bones, endothelial cells, and beta cells of the pancreas (Perakakis et al., 2017; Momenzadeh et al., 2022). A significant number of research organizations have established a connection between irisin level and coronary heart disease, hypertension, and some malignancies (hepatocellular, prostate). It has been suggested that the irisin has a protective effect against these pathologies (Ma and Chen, 2021). Irisin has been linked to having beneficial effects on metabolic disorders, including obesity, type 2 diabetes, dyslipidemia, and nonalcoholic fatty liver disease (NAFLD) (Polyzos et al., 2018). Irisin and NAFLD have a contentious association, and studies that were just recently published have revealed that the concentration of this myokine is elevated in patients who have fatty liver disease (Kosmalski et al., 2022).

Although there has not been a study that measures serum or serum irisin levels in cases of fatty liver or ketosis in ruminants, irisin hormone levels are expected to increase in subclinical ketosis, just as they do in non-alcoholic fatty liver syndrome in humans. In the current study, it was determined that serum irisin hormone levels increase in subclinical ketosis.

Some studies have reported that serum irisin and leptin hormones are positively correlated and these two hormones increase in metabolic diseases such as fatty liver syndrome, obesity and insulin resistance (Sahin-Efe et al., 2018; Li et al., 2019). As a matter of fact, in the current study, both serum leptin levels and serum irisin levels were increased in cows with subclinical ketosis and they had a positive correlation. In addition, other studies examining metabolic disorders (Stengel et al., 2013; De Meneck et al., 2018) reported a positive correlation between insulin hormone and irisin hormone, which is consistent with our findings in the current study.

CONCLUSION

In this study, correlation between serum levels of leptin, irisin, ghrelin and insulin hormones are observed in animals with subclinical ketosis. However, it is thought that leptin, ghrelin, insulin and irisin hormones, which are associated with lipid and carbohydrate metabolism, can be used as important biomarkers in the diagnosis of subclinical ketosis and in the follow-up of its prognosis.

DECLARATIONS

Ethics Approval

This study was approved by Burdur Mehmet Akif Ersoy University Rectorate, Animal Experiments Local Ethics Committee.

Conflict of Interest

There is no conflict of interest.

Consent for Publication

Not applicable

Author contribution

Idea, concept and design: HEE, OM, KB, KV, TA

Data collection and analysis: HEE, OM, KB, KV, TA

Drafting of the manuscript: HEE, OM, KB, KV, TA

Critical review: HEE, OM, KB, KV, TA

Data Availability:

The data of this study are available from the corresponding author upon reasonable request.

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