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RESEARCH ARTICLE

The Comparison of Polymorphisms in the Heat Shock Transcription Factor 1 Gene of Turkish Grey Cattle and Holstein Cattle

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ABSTRACT

Turkish Grey cattle (*B. taurus*) are the only native cattle breed of Türkiye's Thrace and Western Anatolia regions. They can spend the whole year as free herds, including the hottest and coldest months, and can survive, feed, and reproduce without any human intervention. Therefore, Turkish Grey cattle are of interest for genetic marker studies on heat stress tolerance. This study was aimed to identify genetic polymorphism in the DNA binding domain (DBD) encoding region of the heat shock transcription factor 1 (HSF1) gene in Holstein (n: 50) and Turkish Grey (n: 50) cattle. It was determined that the 121 amino acid long sequence encoded by the first three exons of the HSF1 gene was the same in Turkish Grey and Holstein cattle. Two intronic and one synonymous SNPs were identified in Turkish Grey (rs719296338, rs522980029, rs17880386) and one inronic SNP (rs17870386) in Holstein cattle by DNA sequencing. These SNPs were searched in the Bovine Genome Variation Database (BGVD). Interestingly, according to BGVD, rs522980029 was conserved in *B. indicus* but absent in *B. taurus*. Moreover, the rs719296338 locus is monomorphic in western European and Eurasian taurine but is polymorphic in East Asian taurines and *B. indicus*. The results support the view that *B. indicus* is genetic introgression into *B. taurus* cattle in Anatolia. This hybridization may have contributed to Turkish Grey cattle ability to adapt to harsh environmental conditions and heat stress.

Keywords: Genetic introgression, Grey cattle, Heat stress

INTRODUCTION

Global warming is a major concern for the livestock industry worldwide [1]. High ambient temperatures and relative humidity cause heat stress by limiting the capacity of livestock to dissipate heat into their surrounding environment [2]. Decreased feed intake due to heat stress in all livestock species leads to a decrease in egg, milk and meat production [3,4]. Heat stress related economic loss is high in the dairy industry. Moreover, heat stress reduces the immune response against diseases in cattle [5]. Intensive selection has been applied for many years to increase the productivity of livestock. However, these high yielding breeds may be more susceptible to heat stress as they produce higher metabolic heat [6]. The development of a high-yielding breed of cattle that can tolerate heat stress has been an important goal of the researchers, as extreme temperatures are predicted to occur more frequently [7]. Heat stress also reduces of reproductive success, animal welfare and product quality. Heat stress causes a decrease in the content of milk fat and protein and changes in the meat colour and water-holding capacity [8-10].

Cattle breeds show genetic variation in thermotolerance, some being more tolerant than others [11]. This diversity may be due to many different biological mechanisms such as cellular, morphological (coat structure), behavioral and neuroendocrine systems. However, the molecular mechanism of environmental adaptation has not been fully elucidated today [12]. The genes involved in mammalian coping with heat stress have been investigated for decades and many candidate genes have been reported [13-15]. The main biomarker of heat stress in cattle is heat shock proteins (HSP). HSPs are highly conserved protein superfamily that are activated in many different stress situations such as oxidative stress, heat stress, injury, etc. HSPs are important for the maintenance of protein homeostasis. HSPs increase the ability of the cell to survive by reducing the accumulation of damaged or abnormal polypeptides within cells [6]. They restore misfolded proteins, direct irreparable proteins to the degradation pathway, and prevent protein aggregation and apoptosis [16].

HSF1, the major coordinator in the regulation of the heat stress response (HSR), plays a role in physiological

processes such as metabolism, development and aging in non-stress situations [17]. HSR is also triggered by different stress factors such as infectious diseases, heavy metals, oxidative stress, in addition to elevated temperature [11,18,19]. HSF1 induces the transcription of heat shock proteins (HSP), also known as stress proteins, by binding to heat shock elements (HSE). HSE is specific DNA sequences (nGAAn) found in the promoter regions of HSPs [20]. HSF1 basically consists of four functional domains: trimerization domain, DNA binding domain (DBD), regulatory domain, and transcriptional activation domain [21]. HSF1, which is a monomeric conformation (inactive form) in the cytoplasm in the absence of stress, trimerizes under stress and migrates from the cytoplasm to the nucleus. Trimeric HSF1 has the ability to bind to DNA. However, it can upregulate HSP transcription after undergoing phosphorylation [22]. DBD plays a key role in HSF1 recognizing and binding to HSEs [23]. It has been reported that amino acid substitutions in DBD may cause physicochemical changes that alter the ability of HSF1 to bind to HSEs [24].

The HSF1 gene is located on chromosome 14 in cattle. It contains 13 exons and encodes the HSF1 protein contains 515 amino acids. The DBD is the best conserved domain of the HSF1 protein in cattle. The first three exons of the HSF1 gene encode the 106 amino acid long DBD between amino acids 15 to 120 of the HSF1 protein [24].

Native cattle breeds are an important target for genetic marker research. Unlike cultivated breeds that go through rigorous breeding programs, they can carry more genetic variations associated with survival and adaptation to different environmental conditions. Turkish Grey cattle are the only native cattle breed of Türkiye's Thrace and Western Anatolia regions. This breed can spend the whole year as free herds, including the hottest and coldest months, and can survive, feed, and reproduce without any human intervention [25]. Turkish Grey cattle are highly resistant to heat, cold and parasites and can be fed low quality feeds. It is preferred for meat and milk production in rural areas due to its very low breeding costs [25]. Holstein cattle are very vulnerable to heat stress and parasites [26]. Identification of genetic variations that confer superiority in stress tolerance in Turkish Grey cattle may provide the opportunity to apply them in breeding strategies such as marker assisted selection for cattle production systems. This study was designed to identify variations in the DNA loci encoding the DBD of the HSF1 protein in Turkish Grey and Holstein cattle.

Material and Methods

Sample Collection

Tissue samples were collected from 100 animals in two different breeds of *B. taurus*; Holstein cattle (n = 50) and Turkish Grey cattle (n=50). Holstein and Turkish Grey cattle tissue samples were collected from randomly selected carcasses after slaughter in the Thrace region of Türkiye. The muscle tissue samples were obtained from the neck of each carcass. Because the samples were collected on different dates, cattle samples from different farms were included in the study. The samples were stored at -20°C until molecular genetic studies.

Molecular Genetics Analysis

DNA extraction from muscle and blood tissue samples was performed using the Hybrigen (MG-DHDNA-01) DNA extraction kit. The two primer pairs were designed

Table 1. *Primer sequences, amplification regions and PCR fragment lengths*

Primers Name	Primer Sequences	Amplification Region	PCR Product (bp)
Primer E1	F: GTCGAACAACGCCCTCCAA	633178 - 633196	458
	R: CTTTGCGGTCGCTACCTCCT	633635 - 633616	
Primer E23	F: ACAGGCACCTGGTAGAAAGC	618336 - 618355	718
	R: TGAAAACTGTCACCCAGCCT	619053 - 619034	

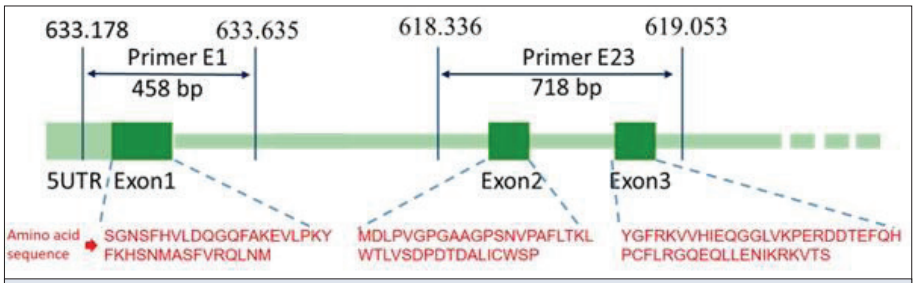

Fig 1. The loci amplified (458 bp and 718 bp) by the primer pairs on the cattle chromosome 14. The encoded amino acid sequences first three exons of the cattle HFS1 gene are written in red letters

using NCBI primer blast *(Table 1)*. The primer pair E1 amplifies exon 1 and partially the 5' UTR and intron 1 regions. The primer pair E23 amplifies exon 2, intron 2, exon 3 and partially intron 1 and intron 3 regions. The HSF1 gene regions amplified by the primers are shown in *Fig. 1*.

PCR amplifications were performed by Proflex thermal cycler (Applied Biosystem). The PCR analyses were performed in a total volume mixture of 25 µL consisting of 12.5 µL of PCR mastermix (K0171, Thermo Scientific), 5 µL of gDNA, 1 µL (10 pmol) of each primer and 5.5 µL of nuclease free water. Sequencing reactions were carried out using a DTCS Quick Start sequencing kit and GenomeLab GeXP Genetic Analysis System (Beckman Coulter, USA). The DNA sequences were analyzed by BioEdit v7.2.5 (Hall, 1999) and Chromas v2.6.6 (Technelysium Pty Ltd, ASTL).

Statistical Analysis

The frequencies of SNPs in cattle breeds worldwide were examined using the Bovine Genome Variation Database (BGVD) [27]. The BDGV includes genomic variation data (SNP, indel, CNV) for 432 samples from 54 modern cattle breeds worldwide. Furthermore, the 54 cattle breeds can be grouped into six early differentiated ancestral populations (Indian indicine, Chinise indicine, East Asian taurine, European taurine, Eurasian taurine, Africa taurine). Distribution patterns of these variations can be obtained using dbSNP ID. Hardy Weinberg equilibrium (HWE) was tested using the HardyWeinberg v1.6.3 package [28] in the R platform.

Results

The exon 1, 2 and 3 loci were amplified in 100 cattle samples using the designed primer pairs *(Table 1)*. The DNA sequencing results were aligned and assembled with v7.2.5 (Hall, 1999). It was determined that the 121 amino acid long sequence encoded by the first three exons of the HSF1 gene was the same in Turkish Grey and Holstein cattle *(Fig. 1)*. However, three SNPs were detected in intron 1 (rs719296338), exon 2 (rs522980029) and intron 3 (rs17880386) *(Fig. 2)*. While rs7192966338 A>G and rs522980029 C>T were detected in Turkish Grey cattle, they were not observed in Holstein cattle. The rs17880386 A>G was identified in both breeds *(Table 2)*. All SNP frequencies were in Hardy Weinberg equilibrium (HWE) in Turkish Grey and Holstein cattle *(Table 2)*.

The three SNPs (rs719296338, rs522980029, rs17880386) were screened in BGVD and map plots were generated for each SNP *(Fig. 3)*. Allele frequencies were obtained for each SNP according to the ancestral populations. The rs719296338 A>G was highly conserved in *B. indicus* but absent in European taurine, Eurasian taurine and African taurine. The frequency of the G allele was 0.014 in East Asian taurine *(Fig. 3-A)*. rs522980029 C>T was absent in *B. taurus* but conserved in *B. indicus*. The T allele frequency was 0.091 in the Indian indicine and 0.684 in the Chinese indicine *(Fig. 3-B)*. The rs17880386 A>G locus was polymorphic in both *B. taurus* and *B. indicus* according to the BGVD database. The G allele frequency was higher in African taurine, Chinese indicine and Indian indicine, and the A allele frequency was higher in other *B. taurus* *(Fig. 3-C)*.

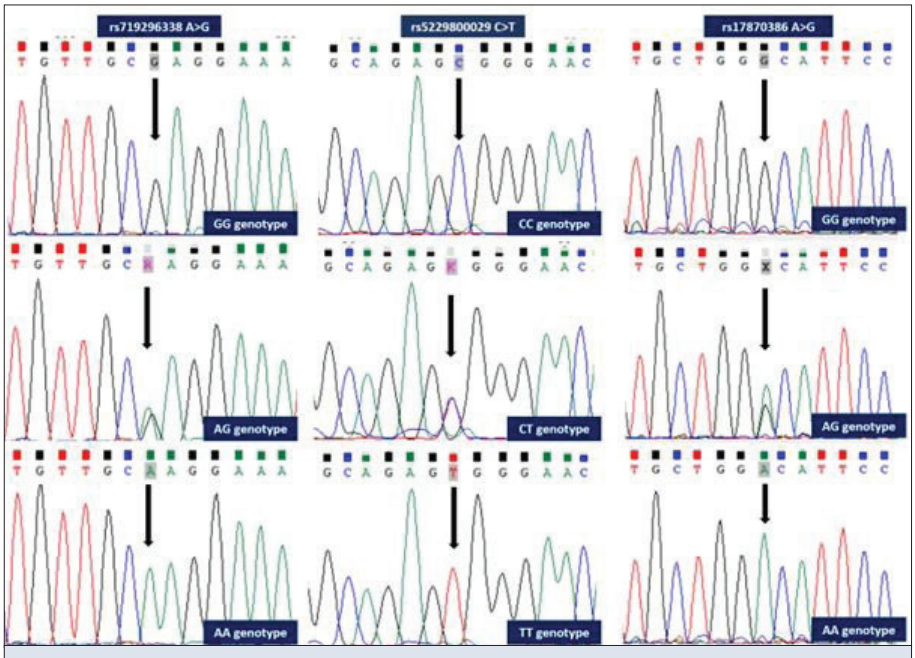

Fig 2. Electropherogram of DNA sequencing results of three SNPs identified in cattle HSF1 gene

Table 2. *Allele and genotype frequencies of three SNPs identified in HSF1 gene in Turkish Grey and Holstein cattle*

SNP/Breed	Genotype Frequencies			Alleles Frequencies		HWE P value
rs719296338	AA	AG	GG	A	G	
Turkish Grey	0.580	0.320	0.010	0.740	0.260	0.234
Holstein	1.000	0.000	0.000	1.000	0.000	-
rs522980029	CC	CT	TT	C	T	
Turkish Grey	0.620	0.340	0.040	0.790	0.210	0.861
Holstein	1.000	0.000	0.000	1.000	0.000	-
rs17870386	AA	AG	GG	A	G	
Turkish Grey	0.240	0.580	0.180	0.530	0.470	0.246
Holstein	0.780	0.220	0.000	0.890	0.110	0.382

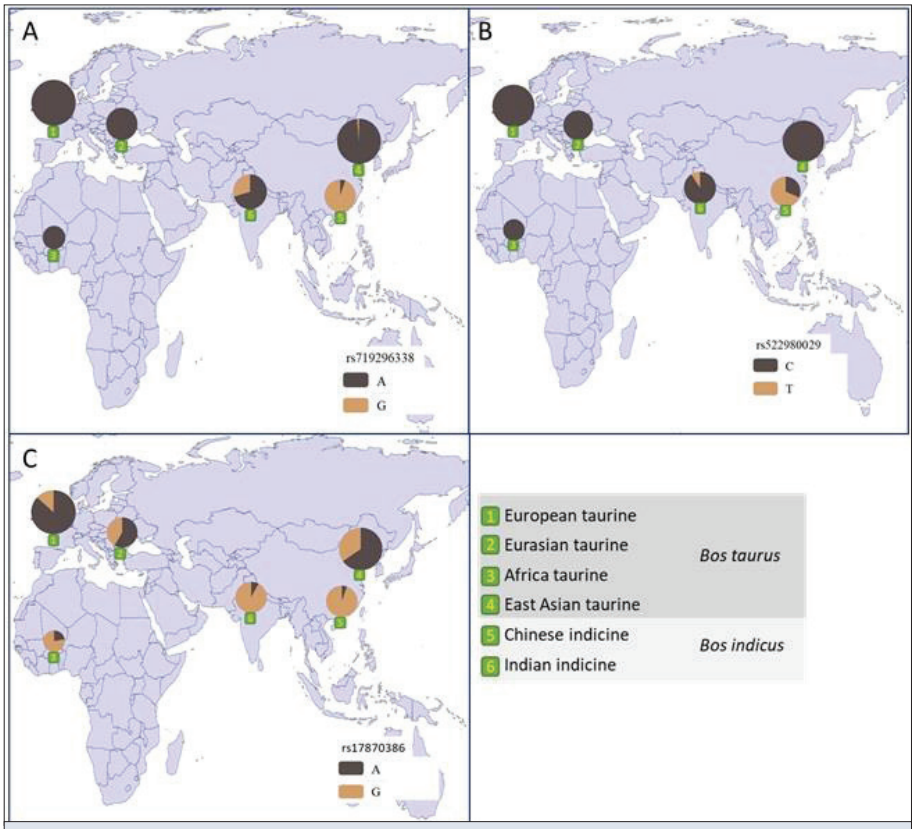

Fig 3. Allele frequency distribution of rs719296338 (A), rs522980029 (B) and rs17870386 (C) in six ancestral cattle groups according to Bovine Genome Variation Database and Selective Signatures (BGVD)

Discussion

Anatolian native breeds, relatives of the first domesticated cattle, are considered the ancestors of many European breeds [29]. One of them, Turkish Grey cattle, also known as Plevne breed in Türkiye, originated from the *B. taurus primigenius* [30]. Although Turkish Grey cattle was a preferred breed by farmers in Thrace and Western Anatolia in the last century, unfortunately, it is under threat of extinction recently. Turkish Grey breeds show high tolerance to cold and heat and high resistance to ecto- and endoparasites [29,31]. They are preferred by poor farmers due to their low breeding costs in the Marmara region in Türkiye [32]. Due to their very high adaptive ability to survive under harsh environmental conditions [33], they are an interesting target for the identification of genetic variants associated with thermotolerance.

Thermotolerance in cattle is a quantitative trait under the influence of multiple genes [34]. Susceptibility to heat stress varies according to genetic potential, species, breed, nutritional status, life stage and body size. Dairy

cows are more vulnerable to heat stress than beef cattle. *B. indicus* breeds and their crosses are better adapted to high environmental temperatures than *B. taurus* [1,35,36]. Traditional breeding methods to improve thermotolerance in cattle have not achieved the expected success. Therefore, genetic marker assisted selection methods can be used to increase the efficiency of traditional selection methods [37].

Maintaining protein homeostasis is essential for the survival of eukaryotic cells. Under stress conditions, proteostasis is dependent on HSR, a cytoprotective mechanism. HSR is triggered by HSF1 upregulating the transcription of a group of chaperones. These chaperones called HSPs, support cell survival by preventing the agglutination of misfolded or defective proteins. HSF1 upregulates the transcription of HSPs by binding to specific sequences called HSEs in their promoter region. The fact that HSF1 expression is higher in cattle in summer than in winter and autumn [38] indicates its relationship with heat stress. The SNPs in the HSF1gene have been reported to be related to heat tolerance in Chinese indigenous cattle [13], Chinese Holstein cattle [39,40] and Angus cattle [41].

In this study, the DBDs of HSF1 protein of two cattle breeds with different thermotolerance capacities were determined to have the same amino acid sequence *(Fig. 1)*. However, two intronic and one synonymous SNPs were detected at the analyzed loci. Three SNPs were observed in Turkish Grey cattle, which is known to have a higher adaptive ability, and one SNP was observed in Holstein cattle. Genetic diversity provides important information about both the evolutionary past and future of a species [42]. Homozygosity increased (decreased genetic diversity) in the Holstein population as a result of intensive selection and inbreeding. Homozygosity is generally detrimental to populations [13,43] and may have increased vulnerability to changing environmental conditions in Holstein cattle.

The synonymous SNP rs522980029 C>T in exon 2 of the HFS1 gene is located at position 618.972 on chromosome 14. It causes an AGC-AGT substitution at the codon 40 encoding the serine residue of the HSF1 protein. All three genotypes were identified in Turkish Grey cattle and the genotypic frequencies of CC, CT, and TT were 0.620, 0.340, and 0.040, respectively. The C>T locus was monomorphic for Holstein cattle, only the CC genotype was observed *(Table 2)*. Interestingly, based on the BGDB distribution models, the variation rs522980029 C>T was absent in *B. taurus* but conserved in *B. indicus*. T allele frequency was 0.091 in the Indian indicine and 0.684 in the Chinese indicine *(Fig. 3-B)*.

The rs719296338 A>G in the intron 1 in the HSF1 gene is located at position 633,222 on chromosome 14. Two alleles and three genotypes (AA, AG, GG) were observed for the rs719296338 in Turkish Grey cattle. Homozygote AA (0.580) had the greatest frequency in these genotypes, and homozygote GG (0.010) had the lowest frequency. The A allele frequency was determined as 0.740 and the G allele frequency as 0.260. The rs719296338 A>G locus was monomorphic for Holstein cattle, only the AA genotype was observed *(Table 2)*. The rs719296338 A>G was screened in the BGVD database. It was highly conserved in *B. indicus* but absent in European taurine, Eurasian taurine and African taurine. According to BGDB, the frequency of the G allele was 0.295 in Indian indicine, 0.947 in Chinese indicine, and 0.014 in East Asian taurine *(Fig. 3-A)*.

The rs17870386 A>G in the intron 2 in the HSF1 gene is located at position 618.721 on chromosome 14. This variation was observed in Turkish Grey and Holstein cattle. While AA (0.240), AG (0.580), GG (0.180) genotypes were determined in Turkish Grey cattle, GG genotype was not determined in Holsteins cattle. The genotypic frequencies of AA and AG in Holstein cattle were 0780 and 0.220, respectively *(Table 2)*. This locus was polymorphic in both *B. taurus* and *B. indicus* according to the BGVD database. The G allele frequency was higher in African taurine, Chinese indicine and Indian indicine, and the A allele frequency was higher in other *B. taurus (Fig. 3-C)*.

These results suggest that there may be a relationship between Turkish Grey cattle and *B. indicus*. Similar results were found in previous studies examining casein gene polymorphisms, and it was reported that genetic introgression of *B. indicus* into Turkish Grey cattle [31,44]. Anatolia, one of the centers where cattle were first domesticated, is accepted as a center of significant introgression with *B. indicus* [45,46]. It has been suggested that Mesopotamia suffered from prolonged drought as a result of significant climatic changes around 4000-3000 years before present [46,47]. It is thought that ancient herders may have brought arid-adapted *B. indicus* populations into the Near East at this time [48].

In order to provide food for the growing world population, it is necessary to have cattle breeds that can cope with the negative effects of global warming. In terms of genetic and physiological adaptability, native cattle breeds are superior to cultivated cattle breeds [26,49]. The study of cattle breeds that respond differently to heat stress can provide very important information to elucidate the molecular mechanisms of adaptation in highly adaptable cattle breeds [50]. SNPs can alter the gene expression transcriptionally and posttranscriptionally and missense SNPs may affect the functionality and stability of proteins [24]. SNPs may cause disruption in the program of biochemical adaptive responses [13,41].

Identification of SNPs in genes involved in thermoregulation may provide important data for marker assisted

selection. The genetic variations found in the Turkish Grey cattle, which has high adaptability to harsh environmental conditions, can have the potential to be used as molecular marker candidates in future studies.

Availability of Data and Materials

Datasets analyzed during the current study are available in the corresponding author (S. Atalay) on reasonable request.

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Conflict of Interest

The authors declare that have no conflict of interest.

Ethica Approval

Ethical approval is not required for this study

Author Contributions

SA and SK designed the study. SA performed the laboratory analysis and wrote the paper. SK reviewed and revised the paper. All authors have read and agreed to the published version of the paper.

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RESEARCH ARTICLE

Comparison of Laying Performance, Egg Quality and Bone Characteristics of Commercial and Türk Laying Hen Genotypes Kept in a Free-Range System [1]

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ABSTRACT

This study was designed to compare of laying performance, egg quality and bone characteristics of commercial and Türk laying hen genotypes kept in free-range system. A total of 720 laying hens (Atabey, Lohmann White, Atak-S, Lohmann Brown; n=180 hens/genotype) were used in the experiment. Production performance was determined as the mean of egg production, egg weight, and FCR value between 54 and 66 weeks of age. Egg quality parameters and bone characteristics of tibia and femur were measured at 66 weeks of age. The mean value of egg production was found to be higher in Lohmann Brown and Lohmann White genotypes compared to Atak-S and Atabey genotypes between 54 and 66 weeks of age ($P<0.01$). The brown eggs obtained from (Lohmann Brown, Atak-S) genotypes tended to be heavier than the white hen genotypes (Lohmann White, Atabey). The lowest mean value of FCR was observed in Lohmann White hens ranged from during the experimental period. The Lohmann Brown and Atak-S eggs obtained from (3.350 g/cm^2 and 3.300 g/cm^2) had a stronger shell strength compared to the Lohmann White and Atabey (2.847 g/cm^2 and 2.910 g/cm^2, $P<0.01$). The breaking strength of tibia was found to be higher in brown hens (366.0 N and 381.2 N) than white hens (267.0 N and 322.2 N) ($P<0.01$). These findings related to different genotypes could be instructive for arranging new management rules and nutritional advice for stronger eggshell and bone strength of hens in free range system.

Keywords: Eggshell breaking strength, Egg production, Laying hen, Tibia strength, Turkish genotypes

INTRODUCTION

Egg production is one of the most significant component of animal production in Türkiye, approximately 19.7 billion eggs produced in a year from over 121 million laying hens in 2021 [1]. Egg production has been achieved mainly by three production systems including cage systems (conventional and enriched cage systems), free-range system and organic production system. Egg production comprises of 78.3% of all eggs produced from cage systems, 19.8% of all eggs from free-range systems and 1.9% of all eggs produced from organic systems during 2018 year in Türkiye [2].

In recent years, consumers have increasingly sensitivity for food safety, animal welfare, sustainability and environmental protection in both European countries and Türkiye. These facts have caused new trends in animal production as like as other agricultural sectors. Therefore, alternative egg production system (free-range and organic systems) have increasingly gained importance due to regarding animal rights and welfare issues [3]. Both of these alternative systems ensure free accessing to pasture that stimulates physical activities, benefit from natural light and sunshine and make possible to exhibit natural behaviors, for example perching, nesting, preening, foraging, dust bathing, and pecking [4].

It is known that egg production and both exterior and interior egg quality are affected by many factors, such as breed, age, husbandry practices, feed composition, and nutritional content [5,7]. The most important quality criterias for the consumers are shell strength, albumen consistency and yolk color [8]. Furthermore, shell quality is largely depending on many factors including genetic factors, egg laying rate, age, health status, housing conditions, and nutrition [9,10]. The differences in nutritional factors among production system has a crucial role in the modulation of

bone mineral homeostasis, influencing the mineralization and mechanical strength of the bones and subsequently shell strength [11,12].

The most important targets in egg production are to produce maximum eggs with saleble quality criterias and FCR, maintain a long-term laying persistence, and preventing skeletal disorders [13]. However, there are some statements that high-yielding hybrids kept in free-range or organic production systems with outdoor access could have some difficulties in adapting to the less controlled environmental conditions in outdoor areas and less equilibrated rations in free-range systems [14]. This could be resulted from these genotypes' suitability for production systems with environmental controlled conditions [15]. Therefore, it could be seen some losses in productivity, egg quality, health and welfare status of commercial layer hybrids kept in free-range system. To minimize these losses, it has been suggested preferring of native genotypes in free range systems, because of their robustness and rusticity [16] and higher adaptive capacity for varying geographical regions and local climate conditions in EU regulation 1804/99 [17] and the final recommendation of the Network for Animal Health and Welfare in Organic Agriculture [18].

The aim of this study is to evaluate egg performance, egg quality and bone characteristics of commercial laying hen genotypes (Lohmann Brown and Lohmann White) and Türk laying hen genotypes (Atak-S and Atabey) in free range system. Atak-S (brown laying hens) and Atabey (white laying hens) are Türk local laying hens that have been developed by Ankara Poultry Research Institute in 2004 [19]. In this study, we focused on the significant differences for productivity, egg quality and bone characteristics of different genotypes during late laying period (54-66 weeks of age) in the free-range system.

Material and Methods

Ethical Approval

The care and use of animals were approved by the ethics committee of Bursa Uludag University and were in accordance with the laws and regulations of Türkiye (License Number 2019-05/09).

Animals and Management Conditions

A total of 720 laying hens of two commercial laying hen genotypes (Lohmann Brown, Lohmann White) and two Türk laying hen genotypes (Atabey, white genotype; Atak-S, brown genotype) were used between 54 and 66 weeks of age. The experimental design included with three subgroups as pens which were considered as replicate for each genotype (n=3 pens/genotype, 60 hens/pen). At 54 weeks of age, the birds were individually weighed on a digital scale with precision ±1 g, and then were randomly allocated to the pens with 3 m × 7 m dimensions.

All birds were reared in a free-range system in accordance with the basic requirements of EU Directive 1999/74/EC [20]. According to these regulations, the free-range system had an indoor and outdoor pasture areas. Wood shavings material was used as litter material to cover pens' floor. Indoor area, all birds were provided as 6.5 cm feeder area per hen with circular plastic feeders and 5 cm drinker area per hen with plastic bell drinkers. Each pen was equipped with perches providing 18 cm perch length per hen, and nesting boxes (3.5 hens per nesting box). The outdoor area with a size of 350 m^2 per pen was covered by wire fences to keep out predators and a shelter. The stocking density was ensured as 2.86 hens per m^2 in indoor area and 5.83 hens per m^2 in outdoor area for each pen. The lighting regime in the pens was applied as 16 h lighting per 24 h period during experimental period.

A standard layer diet for free-range systems was used and the feed ingredients and nutrient composition of diet was analyzed according to [21] *(Table 1)*. Feed and water were offered ad libitum throughout the experiment. The pasture area was comprised of 60% perennial ryegrass *(Lolium perenne)*, 30% alfalfa *(Medicago sativa)*, and 10% white clover *(Trifolium repens)*. The birds could supplement their diets with pasturing and the living small creatures (insects, arthropods, etc.) in the foraging area.

Table 1. *Composition and nutrient content of laying hen diet (54-66 weeks of age)*

Feed Ingredients	**%**
Corn, grain	28.0
Wheat	32.9
Soybean meal, 48%	14.3
Sunflower meal, 32%	3.2
Milled alfalfa	8.6
Soybean oil	2.8
Sodium chloride	0.2
Limestone	8.2
Dicalcium phosphate	1.3
Premix*	0.5
Nutrient Composition	%
ME (kcal/kg)	2803
CP	16.6
Calcium	3.46
Phosphorus	0.48

** 1 kg of premix includes the following compoments: Vit. A, 8.000 IU; Vit. D3, 2.000 IU; Vit. B2, 4 mg; Vit. B12, 10 mg; Vit. E, 15 mg; Vit. K3, 2 mg; Vit. B1, 3 mg; Niacin, 30 mg; Cal-D-pantothenic acid, 10 mg; Vit. B6, 5 mg; Folic acid, 1 mg; D-biotin, 0.05 mg; Vit. C, 50 mg; Choline chloride, 300 mg; Mn, 60 mg; Zn, 50 mg; Fe, 60 mg; Cu, 5 mg; Co, 0.5 mg; I, 2 mg; Se, 0.15 mg*

Data Collection

Laying Performance

The pens were monitored daily basis for egg production (EP) until the end of the experiment. Egg production was calculated by dividing the number of eggs daily collected by the number of hens on the same day. Daily feed intake (DFI) and egg weight (EW) were recorded on a weekly basis. FCR was calculated on a weekly basis as the ratio between DFI and EP multiplied by EW. Egg mass (EM) was calculated as EM = (EP*EW)/100. The FCR was calculated as FCR = DFI/EM. The mean values for EP, EW, EM, DFI and FCR were given as 3 weeks interval (between 54-57, 58-61, and 62-66 weeks of age).

Egg Characteristics

A total of 15 eggs from each genotype were randomly sampled to define external and internal egg quality parameters at 66 weeks of age. The measurements were performed 24 h after the eggs were laid. The eggs were weighed with ±0.01 g precision (Model XB 4200C, Precisa Corp, Zurich, Switzerland), and then the length and width of the eggs were determined using a digital caliper with ±0.01 mm precision (Mitutoyo, 300 mm, Neuss, Germany). The egg shape index was calculated with a formula of (egg width/egg length) × 100 [22]. Eggshell breaking strength (kg/cm^2) was measured by using an eggshell force reader machine (Egg Force Reader, Orka Food Technology, Israel). The eggs were broken to separate the albumen and yolk, and the yolk weight was determined with ±0.01 g precision.

The eggshells were carefully cleaned by washing process and then put in an oven at 105°C (Nüve FN-500, Ankara, Türkiye) during 24 h for drying process. Then, the eggshell weight was determined with ±0.01 g precision. Albumen weight was calculated by subtracting yolk and shell weight from total egg weight. The ratio of yolk, albumen, and eggshell were given as a percentage of EW. Eggshell thickness was measured at three different points of the eggshell as specifically blunt, sharp end, and equator region, using a digital caliper with ±0.01 mm precision. The eggshell thickness was given as the mean of measured three values.

The yolk diameter (YD), albumen length (AL), and albumen width (AW) were determined by using a digital caliper with ±0.01 mm precision (Mitutoyo, 300 mm, Neuss, Germany) to calculate the yolk index (YI), albumen index (AI), and Haugh unit (HU). Albumen height (AH) and yolk height (YH) were measured by using a tripod micrometer. Egg yolk index, albumen index, and Haugh unit were calculated using the formulas given by Funk, Heiman and Carver, and Haugh [23,24], respectively:

$$YI = (YH/YD) \times 100$$

$$AI = (AH/(AL + AW)/2)) \times 100$$

$$HU = 100 \times \log (AH + 7.57 - 1.7 \times EW^{0.37})$$

Yolk color was determined with a Roche yolk color fan with a 15-point scale (Roche Ltd., Basel, Switzerland), according to the pigmentation degree from the lightest (score 1) to the darkest color (score 15).

Bone Characteristics

To evaluate the leg bone characteristics at 66 weeks of age, tibia and femur of both legs (including cartilage) were sampled from randomly selected (n = 15 bone/genotypes) and euthanized by cervical dislocation. After dissection of tibia and femur, the samples were frozen at -20°C until measurements.

After thawing process of bone samples, each bone was checked for any residue of soft tissues and then treated to drying at 22°C for 7 d. Then, the bone weight was measured with ±0.01 g precision, and and bone lenght and width (at 50% of the bone length) were measured by using a digital caliper. The relative weight of the tibia and femur was calculated as the ratio between bone weight and birds' weight. Then, relative asymmetry for bone length was calculated with the formula given by Møller et al.[25]:

$$RA = \{|R - L| / |(R + L)/2\} \times 100$$

in which, RA means relative asymmetry of the left and right bone (%), R means length of the right bone (mm), L means length of the left bone (mm), and |R - L| means the absolute difference between R and L.

Breaking strength (N) for each tibia and femur samples was determined by a 3-point bending test using a fully computerized UTEST tensile and compression testing machine (Model 7014, UTEST Corp, Ankara, Türkiye) that was fitted with a 250 kN load cell. The crosshead movement was at 10 mm/min. The right tibia and femur were ashed, using AOAC method 932.16 [21].

The bone samples were subjected to a temperature of 105°C for 6 h and then defatted with hexane in a Soxhlet apparatus (Model SER148, Şimşek Laborteknik, Ankara, Türkiye) for 4 h. After the extraction of fat, the bones were dried in a forced-ventilated oven at 105°C for 16 h to determine the dry and defatted weights of tibias. Then, the bone samples were crushed and calcined in a muffle furnace at 600°C for 2 h to determine the ash content.

Statistical Analysis

The data on performance parameters (BW, EP, EM, DFI and FCR) for each genotype were analyzed by a one-way analysis of variance procedure (ANOVA) in the statistical analysis software SAS (version 9.4, 2012, Cary, NC, USA) [26]. A completely randomized design was used in the study. For laying performance, egg quality and bone

characteristics, the pens, eggs and, tibia and femur bones were respectively considered as the experimental unit. Significant differences between means were compared using the Tukey test. Analyses of percentage data were conducted after arcsine square root transformation of the data. The data are presented as LSmeans ± SEM for each parameter. Differences were considered statistically significant at P<0.05.

Results

The laying performance of commercial and Türk local laying hen genotypes in free-range system is given in *Table 2*. The mean of egg production showed a similar change between 54-57, 58-61 and 62-66 weeks of age. Lohmann Brown and Lohmann White had a higher egg production percentage during experimental period and varied between 85.6% and 86.5% in Lohmann Brown and 86.5% to 87.6% in Lohmann White during three weeks periods from 54 to 66 weeks of age (P<0.001). During the experimental period, the eggs obtained from brown laying hen genotypes (Lohmann-Brown, Atak-S) tended to be heavier compared to the white genotypes (Lohmann-White, Atabey) (P<0.001). Between 54-57 weeks of age, the mean of egg weight varied from 61.3 g to 65.0 g, whereas the higher mean value of egg weight was observed in Lohmann Brown (65.5 g and 66.1 g) and Atak-S (65.4 g and 65.9 g) genotypes between 58-61 and 62-66 weeks of age respectively. A lower mean of egg mass was observed in Atak-S and Atabey genotypes ranged from 48.6 and 50.1 g, respectively, between 54-66 weeks of age (P<0.001). Similar changes were observed for egg mass during experimental period. White hen genotypes (Lohmann-White, Atabey) had a lower daily feed intake compared to both of brown hen genotypes (Lohmann-Brown, Atak-S). Lohmann Brown laying hens had the

Table 2. *Laying performance of commercial and Türk local laying hen genotypes in free-range system (n= 3 pens/genotypes)*

Parameters	Genotypes	Age (weeks of age)			
		54-57	58-61	62-66	54-66
Egg production (%)	Lohmann Brown	86.5±0.62 [a]	86.2 ±0.80 [a]	85.6±0.65 [a]	86.1±0.65 [a]
	Lohmann White	87.6±0.88 [a]	87.1±0.72 [a]	86.5±0.80 [a]	87.1±0.80 [a]
	Atak-S	75.3±0.83 [c]	74.8±0.80 [c]	74.4±0.72 [c]	74.8±0.78 [c]
	Atabey	79.4±0.58 [b]	78.8±0.60 [b]	78.2±0.52 [b]	78.8±0.57 [b]
	P value	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Egg weight (g)	Lohmann Brown	65.0±0.25 [a]	65.5±0.33 [a]	66.1±0.12 [a]	65.5±0.13 [a]
	Lohmann White	61.3±0.09 [c]	61.7±0.17 [c]	62.3±0.13 [c]	61.8±0.05 [c]
	Atak-S	64.5±0.25 [a]	65.4±0.14 [a]	65.9±0.23 [a]	65.3±0.15 [a]
	Atabey	62.9±0.15 [b]	63.6±0.27 [b]	64.0±0.27 [b]	63.5±0.16 [b]
	P value	*0.001*	*<0.001*	*0.001*	*<0.001*
Egg mass (g)	Lohmann Brown	56.2±0.31 [a]	56.5±0.25 [a]	56.6±0.47 [a]	56.4±0.34 [a]
	Lohmann White	53.7±0.48 [b]	53.7±0.37 [b]	53.9±0.56 [b]	53.8±0.46 [b]
	Atak-S	48.6 ±0.62 [c]	49.0±0.61 [c]	49.0±0.62 [c]	48.8±0.60 [c]
	Atabey	49.9±0.48 [c]	50.1±0.29 [c]	50.1±0.47 [c]	50.0±0.41 [c]
	P value	*0.001*	*<0.001*	*<0.001*	*0.001*
Daily feed intake (g)	Lohmann Brown	130.1±1.39 [a]	130.7±1.54 [a]	131.9±1.18 [a]	130.9±1.37 [a]
	Lohmann White	114.3±0.97 [c]	116.0±1.20 [c]	117.3±1.30 [c]	115.8±1.13 [c]
	Atak-S	121.2±1.02 [b]	122.8±1.16 [b]	124.4±1.10 [b]	122.8±1.06 [b]
	Atabey	115.0±1.59 [c]	115.7±1.45 [c]	116.4±1.39 [c]	115.7±1.30 [c]
	P value	*0.001*	*<0.001*	*<0.001*	*<0.001*
FCR (g feed/g product)	Lohmann Brown	2.31±0.04 [b]	2.32±0.03 [b]	2.33±0.04 [b]	2.32±0.04 [b]
	Lohmann White	2.13±0.03 [c]	2.16±0.04 [c]	2.18±0.04 [c]	2.15±0.03 [c]
	Atak-S	2.50±0.04 [a]	2.51±0.03 [a]	2.54±0.02 [a]	2.52±0.03 [a]
	Atabey	2.30±0.04 [b]	2.31±0.04 [b]	2.33±0.05 [b]	2.31±0.04 [b]
	P value	*0.0007*	*0.0009*	*0.0016*	*0.0009*

[a–c] *values within columns with different superscripts are significantly different (P<0.05)*

Table 3. *Egg characteristics of commercial and Türk local laying hen genotypes (n= 15 eggs/genotypes)*

Parameters	Characteristics	Genotypes				
		Lohmann Brown	Lohmann White	Atak-S	Atabey	*P value*
Egg content	Egg weight (g)	65.3±0.05 [a]	61.5±0.07 [c]	65.1±0.05 [a]	63.9±0.09 [b]	*<0.001*
	Yolk (%)	20.1±0.05	19.0±0.68	17.6±0.64	20.6±0.09	*0.235*
	Albumen (%)	69.4±0.05	70.9±0.72	71.9±0.68	69.0±0.09	*0.284*
	Eggshell (%)	10.5±0.05	10.1±0.06	10.5±0.04	10.4±0.05	*0.138*
Exterior egg quality parameters	Egg shape index (%)	76.7±0.15	75.7±0.30	77.3±0.15	77.0 ±0.26	*0.182*
	Eggshell breaking strength (g/cm^2)	3.350±0.02 [a]	2.847±0.03 [d]	3.300±0.03 [b]	2.910±0.02 [c]	*<0.001*
	Eggshell thickness (mm)	0.370±0.002	0.340±0.007	0.375±0.003	0.357±0.005	*0.180*
Interior egg quality parameters	Yolk index (%)	46.8 ±0.22	47.2±0.49	46.7±0.47	47.4±0.45	*0.937*
	Yolk color	12.3 ±0.15	11.7±0.15	12.3±0.15	12.3±0.15	*0.441*
	Albumen index (%)	11.6 ±0.28	11.9±0.15	10.7±0.24	11.7±0.25	*0.440*
	Haugh unit	89.2±0.70	89.3±0.96	88.6±1.35	89.9±1.04	*0.983*

[a–d] *values within rows with different superscripts are significantly different (P<0.05)*

Table 4. *Morphological and mechanical traits of leg bones in commercial and Türk local laying hen genotypes at 66 weeks age (n=15 bones/genotypes)*

Bone	Characteristics	Genotypes				*P value*
		Lohmann Brown	Lohmann White	Atak-S	Atabey	
Femur	Length (mm)	81.1±0.40 [b]	72.1±0.34 [d]	84.7±0.50 [a]	75.4±0.49 [c]	*<0.001*
	Width (mm)	16.3±0.21 [a]	11.5±0.18 [c]	13.9±0.11 [b]	12.4±0.15 [c]	*0.001*
	Weight (g)	11.4±0.15 [a]	7.4±0.18 [b]	12.1±0.10 [a]	8.2±0.12 [b]	*0.001*
	Relative weight (%)	0.58±0.008 [a]	0.44±0.01 [b]	0.55±0.005 [a]	0.52±0.007 [a]	*<0.001*
	Relative assymmetry (%)	0.278±0.03	0.219±0.04	0.290±0.03	0.358±0.05	*0.559*
	Breaking strength (N)	339.6±6.8 [a]	230.6±4.8 [c]	369.6±5.8 [a]	283.2±7.0 [b]	*<0.001*
	Ash (%)	57.9±0.76 [ab]	48.7±0.46 [c]	60.8±0.70 [a]	55.9±0.61 [b]	*<0.001*
Tibia	Length (mm)	109.2±0.57 [b]	110.2±0.59 [b]	118.7±0.98 [a]	109.9±0.69 [b]	*0.001*
	Width (mm)	15.4±0.19 [a]	11.4±0.20 [b]	14.7±0.20 [a]	12.6±0.15 [b]	*<0.001*
	Weight (g)	13.6±0.12 [b]	8.7±0.16 [d]	15.3±0.22 [a]	9.9±0.13 [c]	*<0.001*
	Relative weight (%)	0.68±0.005 [b]	0.52±0.01 [c]	0.69±0.01 [a]	0.63±0.008 [b]	*<0.001*
	Relative assymmetry (%)	0.169±0.02	0.216±0.03	0.131±0.01	0.264±0.05	*0.318*
	Breaking strength (N)	366.0±9.5 [a]	267.0±7.8 [c]	381.2±10.1 [a]	322.2±6.8 [b]	*<0.001*
	Ash (%)	55.5±0.61 [a]	48.2±0.53 [c]	53.2±0.51 [a]	51.8±0.46 [b]	*<0.001*

[a–d] *values within rows with different superscripts are significantly different (P<0.05)*

highest daily feed intake with values of 130.1g between 54-57 weeks of age, 130.7 g between 58-61 weeks of age, 131.9 g between 62-66 weeks of age (P<0.001). On the other hand, Lohmann White hens had a better FCR than the other white and brown genotypes (2.13, 2.16, 2.18, and 2.15 between 54-57, 58-61, 62-66 and 54-66 weeks of age respectively, P<0.001) *(Table 2)*.

The egg characteristics of commercial and Türk local laying hen genotypes in free-range system is given in *Table 3*. The egg weight was found to be heavier in brown layer hen genotypes (Lohmann-Brown, Atak-S) (P<0.001), whereas any significant differences was observed for the percentage of yolk, albumen and eggshell among the brown and white genotypes (Lohmann-White, Atabey) (P>0.05). A higher mean value of eggshell breaking strength was found in brown eggs (3.350 g/cm^2 and 3.300 g/cm^2) than white eggs (2.847 g/cm^2 and 2.910 g/cm^2, P<0.001). However, egg shape index, eggshell thickness and interior egg quality parameters was found to be similar among the brown and white hen genotypes (P>0.05) *(Table 3)*.

The morphological and mechanical traits of femur and tibia bones of commercial and Türk local laying hen

genotypes in free-range system is given in *Table 4*. The femur length was found to be the highest in Atak-S laying hen genotypes, whereas the femur width was the highest in Lohmann Brown hen genotype (P<0.001). A higher weight of femur was observed in brown hen genotypes (Lohmann-Brown, Atak-S), while the lowest value of femur relative weight with a value of 0.44% was found to be in Lohmann White laying hens (P<0.001). On the other hand, the breaking strength and ash content of femur was the lowest in Lohmann White laying hens than the other hen genotypes (230.6 N and 48.7%, P<0.001). The tibia was observed as the longest in Atak-S laying hens (118.7 mm, P<0.01). The width of tibia was found to be higher in brown laying hen genotypes (Lohmann-Brown, Atak-S) compared to the white laying hen genotypes (Lohmann-White, Atabey) (P<0.001). On the other hand, the weight and relative weight of tibia were the highest in Atak-S laying hens (15.3 g and 0.69%, P<0.001). A higher mean value of breaking strength and ash content was observed in brown laying hen genotypes (Lohmann-Brown, Atak-S) than the white laying hen genotypes (Lohmann-White, Atabey) (P<0.001) *(Table 4)*.

Discussion

The current study clearly indicated significant differences for productivity between commercial (Lohmann-Brown, Lohmann-White) and Türk laying hen genotypes (Atak-S, Atabey) kept in the free-range system. Küçükyılmaz et al.[27] reported a higher production rate for white layer hen genotype than the brown one in both organic production (87.23% vs. 82.50%) and conventional (89.82% vs. 80.43%) system between 23 and 70 weeks of age. On the other hand, brown eggs tended to be heavier compared to the white eggs, whereas eggs obtained from Atak-S and Lohmann White genotypes had a lower egg mass. This could be originated from observed differences for egg production rate and also egg weight.

Sozcu et al.[28] study showed that Atabey hens (75.9%) had a higher egg production level than Atak-S hens in a free range system (70.3%). Otherside, Atak-S hens tended to consume more feed than Atabey hens. Daily feed intake was found to be higher in Atak-S (117.2 g) than in Atabey (109.8 g). Higher FCR between 19 and 72 weeks of age was observed in Atabey than in Atak-S (2.48 vs. 2.54).

There are other contradictory results for differences between layer hen genotypes in free-range system. Küçükyılmaz et al.[27] and Rizzi and Chiericato [29] demonstrated that commercial hybrids had a higher egg production rate and egg mass than native hybrids. On the other hand, Şekeroğlu and Sarıca [30] found a higher egg production rate in native hybrids in free-range system.

The feed intake and FCR were significantly differed among the genotypes in the study. The brown layer hens consumed more feed than white hens and had a worse FCR value. Observed higher feed intake could be attributed to the a higher egg weight in brown eggs, which resulted in increment of feed intake due to a higher energy requirement. On the other hand, the hens had free access to pasture area, therefore possible to more physical activity, such as walking and foraging behaviors, which also increased the energy requirement of laying hens. Küçükyılmaz et al.[27] emphasized that brown layers were more active with scratching and foraging behaviors than white hens in the organic production system and found similar feed intake for brown and white layer hens (Atak-S and Lohmann White) (127.74 g and 127.69 g). These findings are consistent with previous reports by Lampkin [31] and Castellini et al.[32].

Egg exterior and interior characteristics, have importance during commercial handling and transport processes and also consumer preference [33]. In the present study, only eggshell breaking strength showed difference between hen genotypes. Brown eggs had a stronger shell structure with a higher breaking strength value compared to the white eggs. Otherwise, Atak-S Brown hen genotype had higher shape index (77.9%), eggshell breaking strength (3.429 g/cm^2), shell thickness (0.371 mm) than Atabey white hen genotype (76.0%, 2.982 g/cm^2, 0.361 mm) respectively in another study [28].

The observed differences could be attributed to both the genetical differences and more motor activity in pasture. Thus, brown hens could have more time on pasture which provide more ingestion of tiny stone and longer exposure duration to sunlight which promote mineral metabolism. This could lead more accumulation of minerals in the shell, and subsequently more stronger shell structure in brown eggs [34].

The skeleton of birds take parts in eggshell formation (up to 40%) by providing calcium [35,36]. High calcium requirements for shell formation could cause an increment bone mineral resorption from medullary bone. This is the main cause of osteoporosis, and this could mainly develop from age and low calcium content of diet [37,38]. According to these facts, it could cause a negative relationship between egg production and skeletal integrity in layer hens [39,40]. In previous studies, significant negative correlation was found between eggshell quality (shell weight) and bone mineralization (bone ash content). It could be explained that laying hens are more prone to osteoporosis due to mobilizing more calcium for better quality of shell. Furthermore, some studies reported any significant relationship between bone quality, egg production and shell quality [41,42].

Interestingly, current findings demonstrated that femur

and tibia characteristics namely length, width, weight, breaking strength and ash content had higher mean values in brown layer hens compared to the white layer hens. A previous study reported a negative correlation between shell breaking strength and ash content of bones [43]. It could be attributed to more physical activity at outdoor area and directly exposure to sun light of brown layer hens. In this study, both of brown genotypes had more time in pasture area. It is well known that range usage in free range system provides daylight to hens and it stimulates hormone and vitamin D production which could improve bone mineralisation [44].

In a recent study performed by Alfonso-Carrillo et al.[45], it was reported that hens with higher egg production rate and good shell quality had higher body weight and slightly larger uterus (shell gland) compared to the other groups with lower production and poorer shell quality. Therefore, hens with higher production with good quality of shell had a greater capacity to mobilize calcium for shell formation and retained lower amount of medullary bone. In current study, brown laying hens from Lohmann Brown and Atak-S genotypes (1982.1 g and 2209.6 g respectively) had higher body weights than Lohmann White and Atabey white genotypes (1678.5 g and 1575.7 g respectively) at 66 weeks of age (P=0.001).

In conclusion, current data demonstrated the differences between commercial hybrids and Türk genotypes kept in a free-range system between 54-66 weeks of age. These findings clearly showed that white layer hens had a higher productivity level with better FCR, whereas the brown layer hens had a superiority for egg weight, shell strength and bone integrity. To decide the production target between productivity versus welfare, it is important to remember that it is aimed to improve health and welfare status in the free-range systems. As well as priority preference of native genotypes in the free-range system, Atak-S could be preferred to produce heavier eggs with stronger shell structure and bone traits, whereas Atabey could be chosen for a higher egg production and better feed utilization.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (A. Sözcü). The data are not publicly available due to privacy or ethical restrictions.

Ethical Approval

The care and use of animals has been approved by the ethics committee of Bursa Uludag University and comply with Türkiye laws and regulations (License Number 2019-05/09).

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Conflict of Interest Statement

The authors declared that there is no conflict of interest.

Author Contributions

Methodology and collection of data, A.I, A.S., investigation and data analysis, A.S., M.G., writing - review and editing A.S., A.I., M. G. All authors have read and agreed to the published version of the manuscript.

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Research Article

Migration Of Phthalates From Plastic Packages Into Dairy Products

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ABSTRACT

In this study, it was aimed to determine the possible migration from the packaging material by determining the phthalates during the storage period in dairy products. For this purpose, selected phthalate esters were determined by LC-MS Q-TOF from the dairy product samples taken on different times of storage, following the raw milk stage and packaged with plastic. Accordingly, only DEHP was detected from phthalate esters in all samples. In white cheeses, DEHP values was 226 µg/kg in glass packaging and 244 µg/kg in plastic packaging on the 1st day, it increased to 259 µg/kg in glass packaging and 420 µg/kg in plastic packaging in the 9th month. Likewise, the DEHP values of Kashar cheese, which were 236 µg/kg and 262 µg/kg in glass and plastic packaging samples on the 1st day, reached 241 µg/kg and 346 µg/kg, respectively, in the 9th month. In yogurt samples, on the 1st day, there was 253 µg/kg DEHP in the glass package and 255 µg/kg in the plastic package, while on the 30th day, 262 µg/kg in the glass package and 288 µg/kg in the plastic package. Although the determined values are below the legal limits, in case of high phthalate in the packaging material, the potential risk that may occur in foods has been revealed.

Keywords: Cheese, Dairy Products, LC-MS Q-TOF, Phthalates, Yogurt

Introduction

Phthalates are organic compounds defined as plasticizers, especially used to make plastic materials more elastic, lightweight, durable and soft [1,2]. Phthalates, which are not chemically bonded to plastic, have a lipophilic character and tend to evaporate easily. All these properties play an important role in the contamination of these substances to the environment and food [3-5]. Due to they are heavily used in food contact materials to be present in the environment and especially Dimethyl phthalate (DMP), Diethyl phthalate (DEP), Dipropyl phthalate (DPP), Di-isobutyl phthalate (DIBP), Di-n-butyl phthalate (DBP), Butyl benzyl phthalate (BBP), Dicyclohexyl phthalate (DCHP), Di-n-hexyl phthalate (DHP), Di-2-ethylhexyl phthalate (DEHP), Di-n-octyl phthalate (DOP), Di-isononyl phthalate (DINP) and Di-isodecyl phthalate (DIDP) are (DIDP) are common phthalate residues detected in foods [6,7]. The higher molecular weight PAEs, such as DEHP, DINP, and DIDP are used as plasticizing agents to soften poly (vinyl chloride) products. DBP, DEP and BBP as the lower molecular weight PAEs, usually act as thickeners and flavor enhancers in a variety of personal care products [8,9]. Packaging materials, which are also often used in the packaging of milk and dairy products, are the main source of contamination, especially for phthalates such as DEHP, DBP and DIBP. Phthalate contamination of food could occur not only from packaging material, but also from soil, water, air, as well as during transport, production, storage, or even cooking in houses [10-12]. Owing to the lipophilic property of PAEs, milk products with high protein and lipid content are prone to accelerated the migration of PAEs from contacted plastic materials during processing, transport and storage [8,13]. Therefore, the amount of phthalate in packed food depends on both the phthalate concentration in the packaging material, the storage time and temperature, the fat content of the food and the contact surface [14-17]. For this reason, milk and dairy products can be easily exposed to phthalate migration due to their fat content and the use of plastic for their packaging. As

with all raw foods, the amount of phthalates in raw milk is low. It is reported that the amount of phthalates in raw and unprocessed milk does not exceed 120-180 µg/kg. In most cases, phthalates pass into the final product at the processing and packaging stages in milk products [10,15,18]. For example, applications during production in cream and cheese increase the amount of DEHP in the final product by 5-100 times [5]. In one of the studies, total phthalate and DEHP levels in raw milk and dairy products taken during the collection, transportation and packaging were found to average 20 and 280 µg/kg, <10-70 µg/kg in low-fat milk and 1930 µg/kg in cream. In another study, they determined the amount of DEHP in retail dairy products as <10-50 µg/kg and the total phthalate level in dairy products as 3000 µg/kg, the highest in creams [19,20]. Also, Yang et al.[8] investigated ten well known dairy brands of China, and found that the BBP, DEHP and DOP in plastic packages were higher than those in other type of packages.

Phthalates used as softening agent in packaging material and particularly DEHP are specified as "environmental hormones" which have effect of disrupting the endocrine system of animals and humans. The phthalates pose a serious threat to food safety and public health [21]. People can be exposed to phthalates through skin, respiration and digestion. Digestion, in particular, is an important pathway in phthalate exposure. Phthalates are taken into the body by food, supplements and drugs, even young children are exposed to phthalates as a result of swallowing toys or taking them to the mouth. Phthalate contamination of food occurs not in primary parts of production, such as farm and field, but mostly in final stages, such as processing and packaging. Phthalate migration occurs especially from plastic packaging materials to foods. Lipophilic foods such as milk, butter and meat are reported to be the main source of exposure for some phthalates such as DEHP, DBP and DIBP [13,22,23].

Animal experiments conducted in recent years showed that some PAEs, their main metabolites and degradation substances, have toxic effects on various organs, including the liver, reproductive system, kidneys, lungs and heart. DEHP and DBP, the most commonly used phthalate esters during the production, processing and preservation of foods, are evaluated in the 'endocrine disrupting chemical' group since they are estrogen and testosterone antagonists. Among phthalate esters, DBP and DEHP are reported to have negative effects on germ cell development, BBP on epididymal spermatozoa concentration, DINP and DIDP on liver cells. It has been found to cause low sperm count in men, decreased fertility and testicular changes, and low birth weight and malformation in females, especially during pregnancy [24-26]. The effects of phthalates in the organism can vary depending on age, duration of exposure and amount of exposure. In this respect, the most sensitive periods are pregnancy, infancy and puberty [25,27,28]. Col´on et al.[29] suggested a possible association between PAEs with known estrogenic and antiandrogenic activity and the cause of premature breast development in young Puerto Rican girls. In addition, the carcinogenic, teratogenic and mutagenic effect of phthalates revealed by experimental studies is also an important threat to human health [30-32]. The presence of phthalates in foods is legally regulated by some authorities. Because of the potential risks to human health DMP, DEP, BBP, DBP and DEHP phthalate esters, are listed as "Priority toxic pollutants" by United States Environmental Protection Agency (USEPA) in 1976 and reference values for DEHP, BBP and DBP were determined 20, 200, and 100 g/kg body weight (bw)/day, respectively [33,34]. The tolerable daily dose for DBP, BBP, DEHP, DINP and DIDP was determined by EFSA (European Food Safety Authority) as 0.01, 0.5, 0.05, 0.15 and 0.015 mg/kg/day respectively [35-37]. European Union directives set the legal limits for BBP, DEHP, DBP, DINP and DIDP that may generate migration when in contact with food 30, 1.5, 0.3, 9, and 9 mg/kg respectively while total phthalate levels from plastic into food has been limited to 60 mg/kg [38]. Similar practices and limits have also been adapted to Turkey by legal legislation [39].

Yogurt and cheese, which are offered for sale in plastic packaging in Turkey, are dairy products with high nutritional value that are widely consumed by people of all ages. The purpose of this study was, determination of possible potential food safety/public health risks arising from phthalate migration depending on storage period. Yogurt, white cheese and kashar samples were analyzed for six phthalates esters during the shelf life, all samples were produced in an integrated dairy plant using machinery milking.

Material and Methods

Material

In the study, samples were taken before production (raw material), at the beginning of production (pasteurization) and at the end of production (from the final product). Raw milk, pasteurized milk, and white cheese, kashar cheese and yogurt samples produced from this milk, including feed, from an integrated dairy farm with machine milking were used as material in order to determine the presence and amount of phthalates during the storage of the final products. For this purpose, before production 6 samples were taken from each ration of feed and finger milking to evaluate possible phthalate contamination. 18 samples, 6 of each, from raw milk used in the production of yogurt, white cheese and kashar cheese simultaneously after milking with the machine, 18 samples, 6 of each, from milk used in the production of yogurt, white cheese and kashar cheese after pasteurization, and the last stage of

production, a total of 84 samples were taken, 6 of each from yogurt, white cheese and kashar cheese packaged with plastic materials, and 6 of each from yogurt, white cheese and kashar cheese placed in glass jars. All dairy products taken in glass jars were used as the control group. Therefore, to prevent possible phthalate contamination in the glass jars used, the jars, cleaned with ultrapure water, methanol, hexane and acetonitrile respectively and dried. The samples were brought to the laboratory under the cold chain and stored in the refrigerator (4°C) throughout their shelf life. Additionally, a total of nine packaging material samples, three of each used in the packaging of yogurt, white cheese and kashar cheese, were subjected to migration testing and analyzed for phthalate esters.

The presence and amount of DBP, DEHP, BBP, DINP, DNOP, DIDP phthalate esters in all samples were determined by LC/MS Q-TOF. All samples taken from the factory were immediately analyzed in terms of phthalate, and the initial phthalate values of the production date (1st day) were determined. Phthalate analysis was performed on the 1st, 3rd, 6th and 9th months of the shelf life of white cheese and kashar cheese samples while it was performed on the 15th and 30th days of shelf life of yogurt samples in order to determine the effect of storage time on phthalate migration.

Chemicals

Formic acid (98-100%), Acetic acid (100%), Acetonitrile, and Methanol (Hypergrade for LC-MS) from Merck (Darmstad-Almanya). Ultra pure water (18.2MΩ) for mobile phase was from Millipore Simplicity 185 (Millipore SAS, Molsheim, Fransa) water purification system. Analytes DBP, DEHP, BBP, DINP, DNOP, and DIDP referance standards from Dr. Ehrenstorfer (Augsburg -Almanya). Stock solutions were prepared in methanol with concentration of 1 mg/mL, Each of them were weighted 10.0±0.1 and dissolved in 10 mL methanol. Stock solutions were stored in -18°C. Working solutions of 6 compounds were mixed and diluted to 10 µg/ml with methanol. Calibration solutions were prepared diluting 10 µg/mL solution.

Instrumentation

Agilent 1260 series LC coupled with Agilent 6550 LC-MS Q-TOF (Agilent, Santa Clara, USA) was used to determinate DBP, DEHP, BBP, DINP, DNOP, and DIDP. Agilent Zorbax SB-C18 (2.1 mm x 50 mm x 1.8 µm) (Agilent, Santa Clara, ABD) column was used for chromatographic seperation. Precursor ions (M+H) for DBP, DEHP, BBP, DINP, DNOP, and DIDP used or measurement were 279.1595, 391.2847, 313.1436, 419.3156, 391.2847 ve 447.3469 respectively. Referance ions of 121.0508 and 922.0098 were used to correct if mass shift occurs during the run. First 2 min of run was diverted to waste to deliver unretained and matrix components without entering the ion source. All analysis were run in MS and MSMS mode. Injection volume was 1 µL. Parameters for the LC-MS system are shown in *Table 1*.

Glassware

Sample contamination can occur in any steps of sample prep and instrumental part [40,41]. For this reason, only glass materials were used. All the glassware were rinsed with ultra pure water, methanol, hexan and asetonitrile respectively. They left in an oven at 400°C for 12 h. Before use they were rinsed with methanol.

Sample Preperation

Samples were prepared according to FDA [42] method with a slight modification. 5.0±0.1 g of sample weighted and put in a 100 mL beaker and 45 mL methanol was added. It was mixed 30 min in an ultrasonic bath at room temperature. After cooling the volume was filled up 50 mL with methanol. Ten mL of this solution was taken and evaporated under nitrogen at 40°C. The process

Table 1. *LC-MS parameters*

Parameter	Value	Parameter	Value
Mobil phase A	0.1% Formic acid	Column	Agilent Zorbax SB-C18 2.1 mm x 50 mm x 1.8 µm
Mobil phase B	Methanol	Column oven	35°C
Flow rate	0.3 mL/min	Ionisation mode	Positive electrospray
Gradient	0 min - 20% B	Drying gas temp	250°C
	0.5 min - 20% B	Drying gas flow	14 L/min
	3 min - 95% B	Nebuliser	35 psi
	6 min - 95% B	Sheath gas temp	350°C
	6.1 min - 20% B	Sheath gas flow	11 L/min
Analysis time	12 min	Capillary voltage	3000 V
Injection	1 µL	Nozzle voltage	0 V

was stopped when solution volume was left around 1ml. Remaining part dissolved and diluted to 2 mL with methanol. The solution was centrifuged 3500 rpm for 10 min, and 1 mL of supernatant part was transferred to vial and injected to LC-MS.

Migration Test

Migration test was performed acoording to European Directive (EU 10/2011). In this study, 3% acetic acid solution were used for extraction media. 100 mL 3% acetic acid solution put in container and waited in an oven for 10 days at 40°C. Final solution was diluted with methanol (1:1 ratio) in a vial and injected to LC-MS system [36].

Validation

To validate the method, parameters such as selectivity, lineerity, trueness, precision, limit of detection (LOD) and limit of quantitation (LOQ) were determined using the spiked samples according to Eurochem Guide [43]. Standart addition and matrix matched calibration approaches were also used for quantification purposes. Cheese samples were prepared at 50, 100 and 400 µg/kg spiked levels. 20-1000 ng/mL lineer range was used for calibration and first level was checked for signal to ratio to avaoid any contamination. Calibration solutions were prepared in pure solvent and matrix extract. Slope ratio of each compounds calibration were compared to to check matrix effects on each phthalate. LOD and LOQ values were calculated using spiked samples. S/N ratio was taken 3 as LOD and ratio 10 was taken as LOQ. Precision was evaluated at three levels as repeatability and intermediate precision. Trueness was evaluated at three concentration levels (50, 100 and 400 µg/kg) using blank samples spiked with a standard solution. To validate the method used for migration test, blank samples (ultra pure water) were spiked with 50-100-400 µg/L and extracted as the same with migration procedure.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) version 20.0 was used for analysis of the data obtained. Normality assumption was evaluated using the repeated measure variance analysis was used to examine the milk and dairy products change over time. And independent sample t test was used to compare the mean of samples in glass jar, samples in plastic packaging and the finger milking, machine milking groups, mean and standard deviation data were presented. $P<0.05$ was considered to indicate a statistically significant difference among groups.

Results

Within the scope of the study, 93 samples were taken in total, including 84 samples from raw milk, pasteurized milk and the final products (yogurt, kashar cheese, white cheese) and 9 packaging materials of packaged products, and 6 phthalate esters (DBP, DEHP, BBP, DINP, DNOP, DIDP) were analyzed by LC-MS Q-TOF instrument. Chromatographic separations of DEHP and DNOP were achieved by appyling the gradient program due to the fact that DEHP and DNOP of phthalate esters having the same m/z (391.2847) value. DEHP fragmentation products were obtained within certain ion ratios as the major ion 391.2847, and were verified. The fragmentation ions were selected as 279.1591 and 167.0339 ions. *Fig. 1* shows the ion ratios.

Quantification of the positive results, which were obtained, was calculated from the injected 20-1000 ppb series (8 levels). Blank vials were considered before starting the batch run. Blank reading was done at least 3 times before analysis, and the contamination, which may come from the system and solvents, was checked. Due to the fact that the matrix separation cannot be removed efficiently during extraction, recovery value was determined between 65-86% while the RSD value in repeatability studies was obtained in the range of 3-20%. It is a disadvantage that the recovery and repeatability values are at LOQ levels, and it was resulted from the possible matrix components. Particularly, the fat content of the samples and the insufficient removal of it during the extraction could lead the low recovery values [18].

Among the phthalate esters, only DEHP was detected within the performed test for the plastic packages used in the samples *(Fig. 2)*. Other PAEs (DBP, BBP, DINP, DNOP, DIDP) remained below the detection limit ≤50 µg/L).

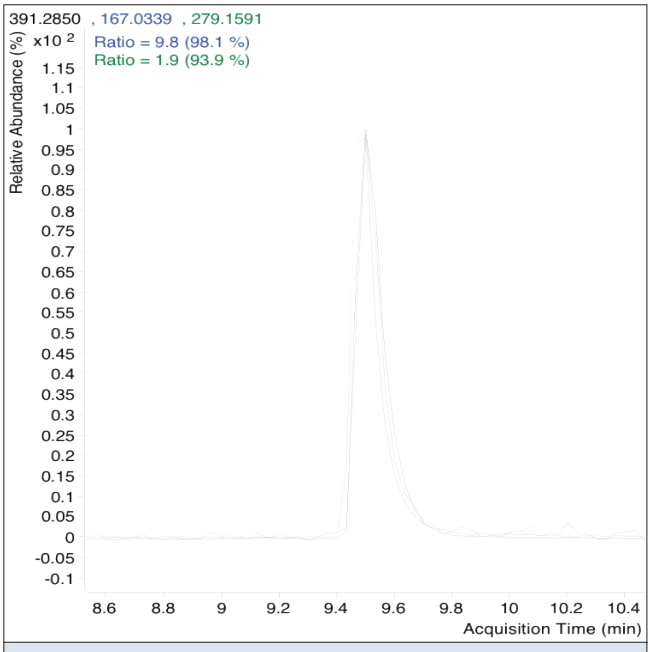

Fig 1. Chromatogram showing the ratios of major ion (391.2847) and fragmentation ions (167.0339 and 279.1591) for DEHP (Ion ratios 9.8% for 167 and 1.9% for 279 ions)

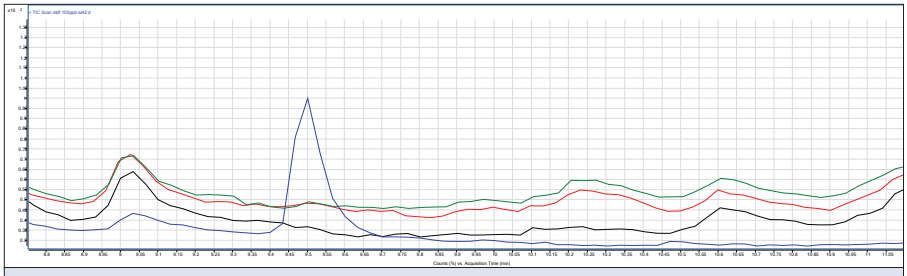

Fig 2. For DEHP, signal at LOQ level (Blue), sample below LOQ level (Green/Red), and solvent injection (Black)

Table 2. *DEHP values of packaging material according to migration test results*

Sample (n=3)	**DEHP Amount* (µg/lt; mean±SD)**
Yogurt Packaging	92±6
White Cheese Packaging	272±7
Kashar Cheese Packaging	118±8

Table 3. *DEHP values of the samples taken from the factory (µg/kg)*

Sample (n=6)		**DEHP (µg/kg; mean±SD)**					
		1st Day	**15th Day**	**1st Month**	**3rd Month**	**6th Month**	**9th Month**
Feed	**Feed**	**<**LOD**	-	-	-	-	-
Milk	Finger milking	161±7	-	-	-	-	-
	Raw milk tank[a]	239±9	-	-	-	-	-
	Raw milk tank[b]	234±9	-	-	-	-	-
	Raw milk tank[c]	226±9	-	-	-	-	-
Yogurt	Pasteurized milk	246±8	-	-	-	-	-
	Glass	253±8	259±9	262±10			
	Plastic	255±9	286±10	288±10			
White Cheese	Pasteurized milk	251±7					
	Glass	246±8		260±8	251±10	253±10	259±8
	Plastic	244±8		268±7	299±10	339±9*	420±8*
Kashar Cheese	Pasteurized milk	256±7	-	-	-	-	-
	Glass	240±10	-	236±10	232±10	244±11	241±11
	Plastic	262±10	-	318±10*	330±10	328±11	346±11

[a]*Value for yogurt milk, Statiscally significant * P=0,017 ** LOD: Limit Of Detection (<50 µg/kg);* [b]*Value for white cheese;* [c]*Value for kashar cheese*

Analyzed amount of DEHP in yogurt, white cheese and kashar cheese packaging samples were determined as 92 µg/L, 272 µg/L and 118 µg/L, respectively *(Table 2)*.

Among the 6 phthalate esters (DBP, DEHP, BBP, DINP, DNOP, DIDP), only DEHP was detected in the samples analyzed for milk and dairy products in the factory, and the obtained values were determined in the range of 161 µg/kg - 420 µg/kg. Other PAEs were detected below detection limit (≤50 µg/kg). The highest DEHP value (239 µg/kg) of the raw milk samples (n=24) taken before production was found in the machine milking to be used for yogurt production, while the lowest value (161 µg/kg) was found in the finger milking sample. The DEHP values in pasteurized milk samples (n=18) were 246 µg/kg, 251 µg/kg and 256 µg/kg for milk used in the production of yogurt, white cheese and kashar cheese, respectively.

The 1st day DEHP values for white cheese samples were determined as (n=6) 246 µg/kg for the white cheese samples in glass jar, and (n=6) 244 µg/kg for the ones in plastic-packaged, respectively. These values at the end of the 9th month were detected as 259 µg/kg for white cheese in glass jar and 420 µg/kg for plastic-packaged cheese. The 1st day DEHP value for kashar cheese were determined as (n=6) 240 µg/kg for the kashar cheese samples in glass jar

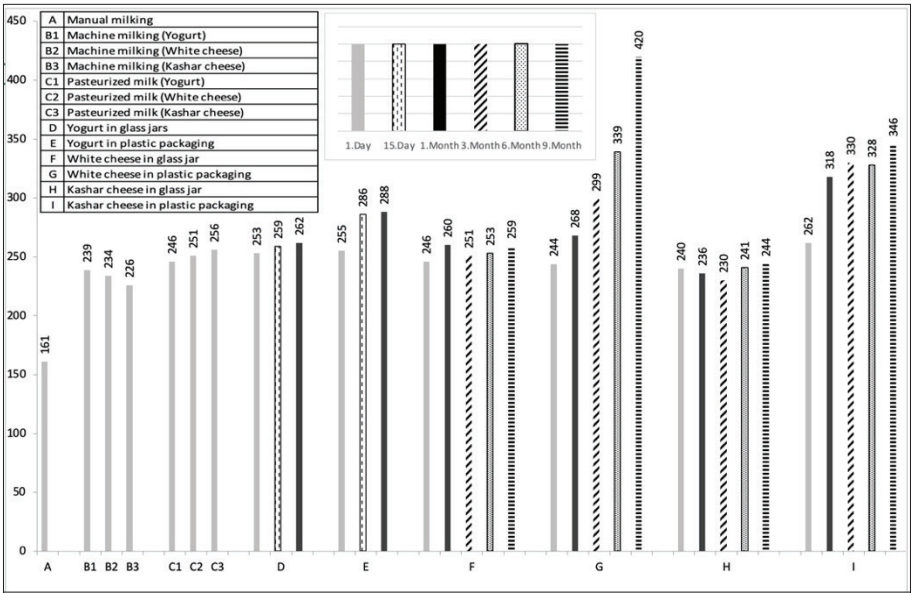

Fig 3. Initial DEHP values of the samples taken from the factory and DEHP change during storage in dairy products (µg/kg)

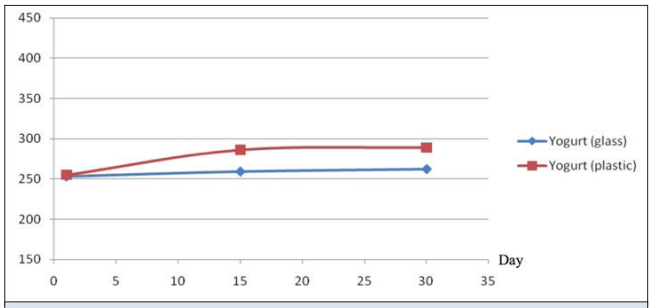

Fig 4. DEHP change of yogurt samples in glass jar and plastic packaged during storage (1st, 15th, 30th day) (µg/kg)

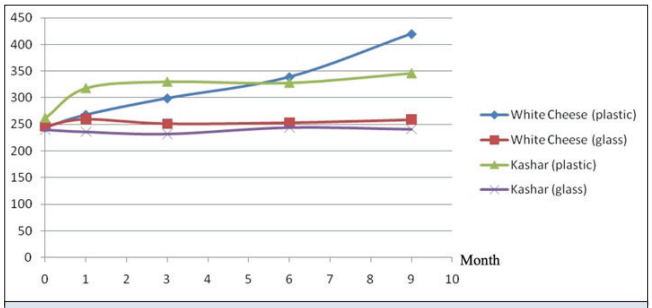

Fig 5. DEHP change in white cheese and kashar cheese samples in glass jar and plastic packaged during storage (1st, 3th, 6th, 9th month) (µg/kg)

and (n=6) 262 µg/kg for the ones in plastic-packaged. In the course of the storage period, the DEHP values of the samples in the glass jar at the 1st, 3rd, 6th and 9th months were found to be as 236, 232, 244, 241, respectively while the ones in plastic-packaged samples were determined as 318, 330, 328, 346 µg/kg, respectively. According to statistical analysis, differences between groups were found to be significant (P<0.05).

The 1st day DEHP value for yogurt samples was determined as (n=6) 253 µg/kg in glass jar yogurt and (n=6) 255 µg/kg in plastic-packaged yogurt samples, respectively. In the analyzes done during storage period, the values for glass jar yogurt samples were found to be as 259 µg/kg at the end of the 15th day and 262 µg/kg at the end of the 30th day, while the values for plastic-packaged yogurt samples were determined as 286 µg/kg at the end of the 15th day and 288 µg/kg at the end of the 30th day. Initial DEHP values of the samples taken from the factory and DEHP change in dairy products during storage period are shown in *Table 3* and *Fig. 3*. The 1st, 15th and 30th day DEHP changes of yogurt samples are demonstrated in *Fig. 4*, and the DEHP changes (1st, 3rd, 6th, and 9th months) of white cheese and kashar cheese samples during the storage period are shown in *Fig. 5*. According to statistical analysis, differences between groups were found to be significant (P<0.05).

Discussion

To know the levels of phthalate contamination before packaging (feed, raw milk and pasteurized milk) are important to clearly demonstrate packaging-induced phthalate migration in dairy products. In this context, analyzes were made in feedi raw milk and pasteurized milk samples in order to understand whether there is phthalate contamination in the pre-packaging stages. Accordingly, DEHP in feed was found below the LOD value. In the study, DEHP values in raw milk samples obtained by machine milking used in the production of yogurt, white cheese and kashar cheese were determined as 239 µg/kg, 234 µg/kg and 226 µg/kg, respectively, and these values were significantly higher than the DEHP value (161 µg/kg) in finger-milked raw milk samples (P=0.05). This difference has been associated with the potential migration of DEHP which will possibly result from the pvc milking pipes in

the milking machine and the milk tank [8,44].

When the 1st day DEHP values of the pasteurized milk used in production and the final products are compared in general, it is seen that there was no remarkable phthalate contamination during the production. Feng et al.[45] examined the PAEs in raw milk samples obtained by finger milking and machine milking from six cows in a factory. In the study, DEHP values of raw milk obtained by machine milking (111.67-283.90 ng/g) were 10-20 times higher than DEHP values of raw milk done by finger milking (8.40-23.72 ng/g), and it was concluded that this difference resulted from the migration of DEHP in the pipes of the milking machines into the milk. In another study which phthalate esters were investigated by GC/TOF-MS in 30 raw milk samples, DEHP values were detected in the range of 0-154 µg/kg in 15 samples, while DEP, BBP and DNOP were not found in any sample [18]. The values of the samples whose milking techniques, not specified, are similar to the values of the samples obtained by finger milking in our study, and these are considerably lower than the values of the machine milking samples.

In white cheese production, the DEHP values of glass jar and plastic packaged white cheese samples on 1st day are 246 and 244 µg/kg, respectively. While no noticeable increase in DEHP was observed in the white cheese samples in glass jars during the storage period (P>0.05), there was a significant increase especially in the 6th and 9th months in packaged samples (P=0.017). It was concluded that the increase possibly resulted from the white cheese packaging material, having the highest DEHP value of 272 µg/kg in the packaging material migration test. In addition, a significant increase in DEHP level towards the end of the storage period, in other words, acceleration of migration, was associated with increased acidity in white cheeses as a result of increased starter culture activity due to ripening [46]. The 1st day DEHP values of the kashar cheese samples in glass jars and plastic packaging for the production of kashar cheese were determined as 240 µg/kg and 262 µg/kg, respectively. Detecting these values similar to the amount of DEHP (251 µg/kg for white cheese, 256 µg/kg for kashar cheese) in pasteurized milk samples (n=18) used in production shows that any significant phthalate contamination is not formed at any stage of production after pasteurization. Any increase in the amount of DEHP was observed during the storage period (9 months) in kashar cheese samples conservation in glass jars. However, the amount of DEHP was 262 µg/kg on the 1st day in packaged samples (P>0.05) while it increased significantly in parallel due to the increasing acidity and became 318 µg/kg (P=0.017) at the end of 30th day. It was determined that this situation is caused by phthalate migration from packaging, as indicated in white cheese.

In yogurt production, regarding the 1st day of sampling DEHP values of the yogurt samples in glass jars and plastic packaging were 253 and 255 µg/kg, respectively. No significant change was observed in DEHP in the yogurt samples in both type packages during storage period (P>0.05). However, DEHP amount in the samples with plastic packaged were detected as 286 µg/kg and 288 µg/kg on 30th day. This increase is quite remarkable when short time of storage (30 days) is considered in terms of migration.

The results of the study could not be compared in this aspect due to the fact that there was no similar study on migration from pack to food. However, there are studies on the presence of phthalates in raw milk and dairy products (milk, yogurt and cheese). Within this scope, a study in which only DBP, DEHP and BBP detected from 6 PAEs by LC-MS/MS in plain yogurt and ayran samples DEHP values were stated as 24-122 µg/kg [47]. In a study carried by Ren et al.[48], 17 PAEs in yogurt and drinking milk by GC/MS were examined, the values of DEHP were determined as ND-144.5 µg/kg. In another study analyzed 16 PAEs in yogurt and drinking milk samples by GC/MS, the average DEHP values were reported as 52.4 µg/kg [49]. In Denmark, a study in which PAEs in raw milk, pasteurized milk, fruit yogurt and baby foods were examined, DEHP determined in all samples was found in the range of 7-138 ng/g [19]. All of these results are quite lower than the findings in our study. Raw milk, milking machine/technique, packaging material, etc. are thought to be the reasons of this situation. In a study the levels of DMP, DEP, DBP, BBP, DEHP, DNOP in fruit yogurt sold in the supermarkets and the migration levels of packaging materials analyzed, and it was stated that DBP, DEHP, DMP, DEP, DNOP and BBP were 76%, 70%, 70%, 54%, 20% and 8%, respectively, and that they were at levels from <LOQ to 1640 µg/kg. In general, it is seen that the values are quite higher than those detected in this study, and it is thought that this may result from fruits that come into contact with various sources of contamination (such as plastic irrigation hoses, plastic boots, plastic transport containers) during production [41]. DEP and DEHP were reported as PAEs determined in all samples from the packaging materials. As known, migration is affected by many factors such as raw materials, production equipment, packaging material, storage conditions. Therefore, it is an expected result that different PAEs are detected at various levels in the studies done on the subject.

The study, in which 6 PAEs in 11 different food groups obtained from Norway market were analysed by Sakhi et al.[50] using gas and liquid chromatography coupled with mass spectrometry, the highest DEHP value in cheese samples found was 173 µg/kg, while this in milk samples was 19 µg/kg. It was observed that the values were

considerably lower than the results of the study. In another study cheese, butter and fatty foods samples in England were also examined. It was observed that the highest DEHP value belonged to cheese sample with 17 mg/kg, and the total phthalate value was 114 mg/kg. In addition, the average DEHP value of the samples was 0.6-3.0 mg/kg. It was stated that the amount of phthalate, detected at a very high level in the samples, resulted from an effective source of contamination such as milking, production, packaging, etc. other than milk-raw material [20].

In conclusion, in this study, the legal limit value of the European Union that can cause migration when in contact with food was taken into account and DEHP values in packaged yogurt, white and kashar cheese samples at the end of the storage period were found below the legal limit (1.5 mg/kg) [19]. Although the phthalate values of the migration test results of the packages are low, the amount of DEHP that migrates to the packaged dairy products is at a level that cannot be ignored, and the risk, which may occur if the amount in the package is high, were showed. In other words, when the migration and the amount of DEHP in the packaged samples and the amount of DEHP detected in plastic packages were evaluated together, how important the material selection used in the food packaging is emphasized. In this respect, the study reveals that the migration test of the packaging material to be used in production and the determination of whether the packaging material is safe have great importance for the protection of public health. In this direction, it is thought that it is necessary to make legal regulations on the subject by taking into account the packaging safety in dairy industry.

Availability of Data and Materials

Data that support the findings of this study are available on reasonable request from the corresponding author (S. D. Korkmaz).

Ethical Approval

The study does not require any ethical approval.

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Competing Interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication

Author Contributions

ÖK and SDK planned and designed the study. SDK and MEŞ performed the experiments; SDK, GİA, and ÖK contributed to the analysis and interpretation of data. SDK, ÖK and GİA drafted the manuscript. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Effects of miRNA-155 on Inflammatory Response and Autophagy Upon Pulpitis Through the NLRP3 Signal

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ABSTRACT

Pulpitis refers to the inflammation of dental pulp tissues caused by infection with dental caries. We aimed to evaluate the effects of micro ribonucleic acid (miR)-155 on inflammatory response and autophagy upon pulpitis *via* the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) signal. Forty rats were randomly assigned to negative control (NC), pulpitis model (PM), anti-miR-155, and anti-miR-155+diethyldithiocarbamate (DDC) groups (n=10). Primary human dental pulp cells were divided into NC, lipopolysaccharide (LPS), anti-miR-155, and DDC groups. Compared with the PM group, the IL-1β, TNF-α, and MDA levels and pulp necrosis rate decreased, while the SOD activity was enhanced in the anti-miR-155 group (P<0.05). Compared to the NC group, the positive expressions of LC3B and Beclin1 and the protein expressions of NLRP3 and Caspase-1 significantly rose in the PM group (P<0.05). Compared with the PM group, the protein expressions of NLRP3 and Caspase-1 significantly decreased, and the positive expressions of LC3B and Beclin1 increased in the anti-miR-155 group (P<0.05). Compared with the anti-miR-155 group, the DDC group had significantly enhanced activity of dental pulp cells, up-regulated mRNA levels of IL-1β, TNF-α, NLRP3, and Caspase-1, and decreased mRNA levels of LC3B and Beclin1 (P<0.05). Suppressing miR-155 expression can relieve inflammatory response and promote autophagy.

Keywords: Autophagy, Inflammatory response, MicroRNAs, Pulpitis

INTRODUCTION

Pulpitis refers to the inflammation of dental pulp tissues mainly caused by infection with dental caries [1]. Pulpitis can cause serious damage to the blood circulation of the teeth, resulting in pulp necrosis, dentition defect, and even tooth loss due to nutritional imbalance. The dental pulp tissue is surrounded by non-tenacious peripheral hard tissues, so the circulation of blood and lymph tissues is poor, leading to uncontrollable pulp inflammation. Besides, long-term severe pain poses a threat to the quality of life of patients [2]. At present, the diagnosis and treatment of pulpitis are still challenging. Pro-inflammatory cytokines can relieve the inflammation of dental pulp tissues, exerting apparent therapeutic effects on pulpitis [3]. The nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome is composed of different endogenous proteins that recognize danger signals and mediate various inflammatory responses [4]. The expression of NLRP3 in the dental pulp tissues of rats with pulpitis obviously increases [5]. Thus, NLRP3 may play a crucial role in the occurrence and development of pulpitis, but whether it is involved in the therapeutic mechanism of pulpitis has rarely been reported. Autophagy is a main protein degradation pathway in eukaryotic cells. As a key defender and metabolic function regulator, autophagy plays an important role in inflammation and infectious diseases [6]. Autophagy can promote the injury repair process [7], but the influence of autophagy on tissue repair after pulpitis-induced injuries remains largely unknown. Micro ribonucleic acids (miRNAs), as endogenous single-stranded non-coding RNAs, can bind messenger RNAs (mRNAs) to inhibit translation, thereby regulating numerous biological activities [8]. MiRNAs play an indispensable role in immune responses [9]. Therefore, they are involved in various infectious oral diseases, including dental caries and periodontitis. In addition, miR-155 shows a high expression in the periodontal ligament of

patients with chronic periodontitis [10], but the expression and mechanism of action in pulpitis are still unclear.

Therefore, this study aimed to evaluate the effects of miR-155 on the inflammatory response and autophagy of rats with pulpitis through the NLRP3 signal.

Material and Methods

Laboratory Animals

The animal experiments have been approved by the animal ethical committee of our hospital (ethical approval number: GYSKLL-KY-20220407-02), and great efforts have been made to minimize their suffering. A total of 40 SPF-grade male Sprague-Dawley rats, weighing 180-200 g, were purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd. [China; Production License No. SCXK (Zhejiang) 2019-0001]. The rats were raised in an animal room at a temperature of 22-25°C and a relative humidity of 50-55%, with a 12/12 h light/dark cycle. They were allowed to eat and drink freely and adapted to the new environment for one week.

Reagents and Apparatus

The reagents used in this study included miR-155 inhibitor (anti-miR-155) (Shanghai GenePharma Co., Ltd., China), hematoxylin and eosin (HE) staining kit, bicinchoninic acid (BCA) protein kit, and enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Sangon Biotech Co., Ltd., China), antibodies against NLRP3 and Caspase-1 (Abcam, USA), and NLRP3 activator diethyldithiocarbamate (DDC). The apparatus applied in this study included a freezing microtome and an inverted microscope (Leica, Germany), electrophoresis apparatus (Beijing Liuyi Instrument Factory, China), and membrane transfer system and a gel imaging system (Bio-Rad, USA).

Grouping and Establishment of a Rat Model of Pulpitis

The 40 rats were randomly divided into negative control (NC) group, pulpitis model (PM) group, anti-miR-155 group, and anti-miR-155+DDC group (n=10/group). Except for NC group, rat pulpitis models were prepared in the other three groups according to a previous reference [11]: The rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium and fixed on the operating table in the supine position. The upper jaw of the rats was opened with forceps to fully expose the maxillary molars, the first and second maxillary molars on the left side were disinfected with alcohol, and then a small hole was drilled in the maxillofacial region with a high-speed turbine until the red part of the dentin near the pulp was seen. Next, the pulp cavity was opened with a reamer, washed, and wiped dry. Then the cotton swab was soaked with complete Freund's adjuvant and placed in the pulp cavity for 30 min. Afterwards, the dental pulp cavity was observed after it was cleaned and dried. The appearance of congestion and abscess in the pulp cavity represented a successfully established pulpitis model.

The anti-miR-155 group was injected with miR-155 inhibitor (80 mg/kg) *via* the tail vein, and the anti-miR-155+DDC group was given NLRP3 activator DDC (300 mg/kg) by gavage 1 h after treatment. Meanwhile, the NC and PM groups were intraperitoneally injected with the same amount of normal saline. The intervention for each group was performed once a day for five consecutive days. Subsequent experiments were carried out 21 d after the intervention.

Detection of Levels of Interleukin-1 beta (IL-1β) and Tumor Necrosis Factor-alpha (TNF-α) in Serum and Levels of SOD and MDA in Dental Pulp Tissues by ELISA

After 21 d, the rats were anesthetized by ether inhalation. Then 2 mL of abdominal aortic blood was taken and centrifuged at 4°C and 3000 rpm for 15 min, after which the supernatant was aspirated. Then ELISA kit was utilized to measure the levels of pro-inflammatory cytokines IL-1β and TNF-α in serum according to the kit's instructions. In addition, the activity of superoxide dismutase (SOD) and the content of malondialdehyde (MDA) in dental pulp tissues were detected by ELISA.

Detection of Pathological Changes of Dental Pulp by HE Staining

After the above detection, the rats in each group were sacrificed by cervical dislocation. The left and right maxillary bones were immediately separated, and the maxilla and maxillary molars were taken out, fixed in paraformaldehyde, decalcified, and embedded in paraffin. The pathological sections with a thickness of 4 μm were made by a freezing microtome. Next, the tissue sections were stained with hematoxylin and eosin and mounted with neutral resin. Later, the pathological changes of the dental pulp in each group were observed under a microscope. Finally, the pulp necrosis rate was calculated based on the formula: pulp necrosis rate (%) = length of necrotic tissue/total length of root canal × 100%.

Detection of Autophagy in Dental Pulp Tissues by Immunohistochemical Staining

The sections prepared in 1.5 were blocked with antigen retrieval and serum at 37°C for 1 h, respectively, and incubated with droplets of working solutions of primary antibodies against LC3B and Beclin1 overnight at 4°C. The tissue sections were rewarmed, washed with phosphate-buffered saline (PBS) three times, and dripped with goat anti-rabbit IgG-labeled secondary antibody. After washing with PBS again for 5 min (3 times in total), the tissue sections were observed under the microscope, and

the distribution of positive cells was analyzed by ImageJ software.

Determination of Expressions of NLPR3 and Caspase-1 in Dental Pulp Tissues by Western Blotting

The molars of rats in each group were cut into pieces and ground into powder in liquid nitrogen. The powder was collected in an EP tube and lysed with RIPA lysate to extract total proteins from dental pulp tissues according to the protein kit instructions. After loading, electrophoresis, membrane transfer, and blocking, the tissue sections were incubated with primary antibodies (1:100 diluted) overnight, with β-actin as the internal control. Next, the HRP-labeled secondary antibody was added for the incubation of the tissue sections for 1 h at room temperature. In the end, ImageJ software was utilized to analyze the gray-scale values of proteins.

Primary Culture of Human Dental Pulp Cells

Fresh extracted third molars or orthodontic teeth were collected from patients aged 12-20 years old in our hospital. The human experiments have been approved by the medical ethical committee of our hospital, and written informed consent has been obtained from all patients. The teeth were washed with PBS containing 10% penicillin-streptomycin. The crowns were split with an osteotome. Then the dental pulp was taken out on the sterile operating table and washed twice with a medium containing the double-antibody solution. Later, the tissues were cut into pieces, spread in a 25 cm^2 culture flask, and cultured with a mixed culture medium (10% fetal bovine serum and 1% double-antibody solution) in a conventional incubator (37°C, 5% CO_2). After passage, the 3rd-5th-generation cells were taken for subsequent experiments.

Detection of Cell Viability by CCK-8 Assay

The cells were inoculated in a 96-well plate, and different cultures were added after the cells adhered to the wall. The cells were divided into NC group, lipopolysaccharide (LPS) group (cultured with 1 mg/L LPS), anti-miR-155 group (cultured with miR-155 inhibitor), and DDC group (cultured with DDC). The cells in each group were incubated for 1 d, 3 d, 5 d, and 7 d, respectively. During the detection, the cells in each well were incubated with 10 μL of CCK-8 solution for 2 h, and the absorbance at 450 nm was measured using a microplate reader.

Detection of mRNA Levels of IL-1β, TNF-α, NLRP3, Caspase-1, LC3B, and Beclin1 in Dental Pulp Cells by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The dental pulp cells were extracted from each group (grouping in 1.9), and lysed with TRIzol to extract total RNA according to the kit's instructions. Total RNA was reversely transcribed into cDNA according to the kit's instructions. The primers were listed below: IL-1β: forward: 5''-AAGACAAGCCTGTGTTGCTGAAGG-3' and reverse: 5'-TCCCAGAAGAAAATGAGGTCGGT C-3', TNF-α: forward: 5'-CTTCTCATTCCTGCTCGTGG and reverse: 5'-GCTACGGGCTTGTCACTCG-3', NLRP3: forward: 5'-CAGCGATGAAGACGCGAGAG-3' and reverse: 5'-AGAGATATGGCACGAAAGCTATCCA-3', Caspase-1: forward: 5'-ACTGCTACACCTGTTGCGCC TCA-3' and reverse: 5'-CTGCCGACGCAGGAAATTC-3', LCB3: forward: 5'-CAGGTTGCCTAGCAGAGGTC-3' and reverse: 5'-GGCATGGACCAGAGAAGTCC-3', Beclin1: forward: 5'-TATAGCAAAGCCCTGCCG-3', and reverse: 5'-AACTGTGCCACAAGCATC-3', GAPDH: forward: 5'-CAACTCCCTCAAGATTGTCAGCAA-3', and reverse: GGCATGGACTGTGGTCATGA. The primers were transiently centrifuged and mixed well with deionized water to prepare a 100 μmol/L stock solution. The forward and reverse primers were diluted to a final concentration of 10 μmol/L. PCR reaction conditions were set as follows: pre-denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 s, and annealing at 66°C for 30 s. At last, the expression level was analyzed by $2^{-\Delta\Delta CT}$.

Statistical Analysis

GraphPad Prism 8.0 software was employed for the analysis of experimental data. All measurement data were expressed as mean ± standard deviation (X±Sx). Repeated measures analysis of variance (ANOVA) and one-way ANOVA were conducted to compare the data among different groups, and the LSD-*t* test was performed for the pairwise comparison of data among groups. $P<0.05$ was considered statistically significant.

Results

Compared with the NC group, the levels of IL-1β, TNF-α, and MDA significantly rose, while the activity of SOD was significantly decreased in the PM group ($P<0.05$). Compared with the PM group, the levels of IL-1β, TNF-α, and MDA decreased, while the SOD activity was enhanced in the anti-miR-155 group ($P<0.05$). In comparison with the anti-miR-155 group, the anti-miR-155+DDC group had significantly increased levels of IL-1β, TNF-α, and MDA and decreased SOD activity ($P<0.05$) *(Fig. 1)*.

In the NC group, the dental pulp morphology was normal. In the PM group, dental pulp tissues were seriously injured, the dentin and fibroblasts were arranged in disorder, most of the cells were significantly changed or even necrosed, and a large number of inflammatory cells were infiltrated. The pulp necrosis rate was (81.36±2.47%) in the PM group. In the anti-miR-155 group, only small quantities of inflammatory cells were observed in the blood vessels

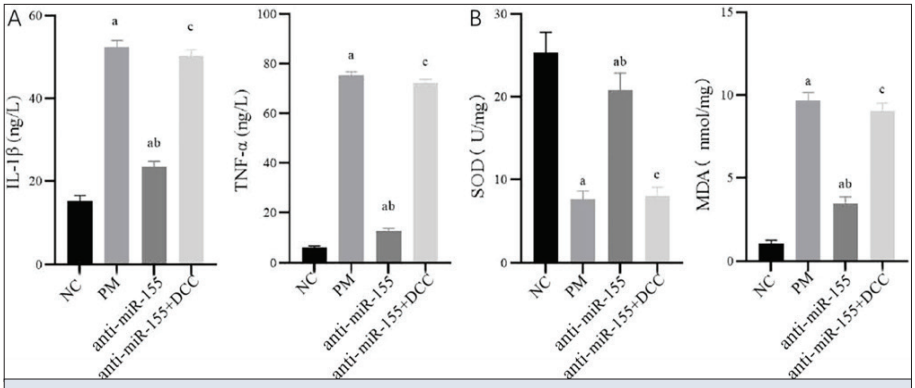

Fig 1. Levels of IL-1β and TNF-α in serum and levels of SOD and MDA in dental pulp tissues. A: Serum IL-1β and TNF-α levels. B: Levels of SOD and MDA in dental pulp tissues. [a]P<0.05 *vs.* NC group, [b]P<0.05 *vs.* PM group, and [c]P<0.05 *vs.* anti-miR-155 group

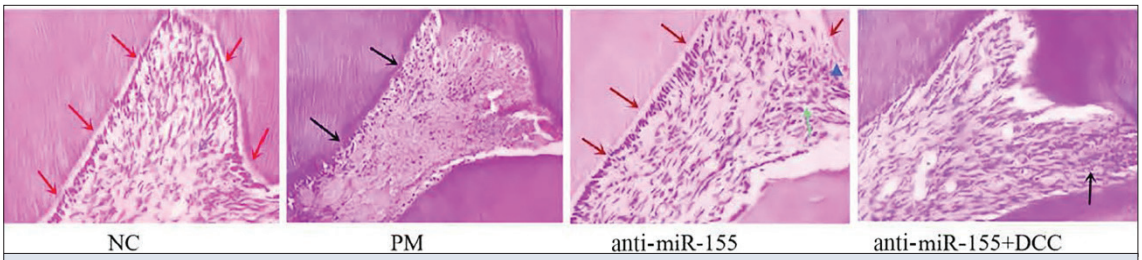

Fig 2. HE staining results of dental pulp tissues (×400). *Red arrows* indicate odontoblasts, *purple arrows* indicate dental pulp cells, *green arrows* indicate the round inflammatory cell aggregation, *blue triangles* indicate vasodilatation and hyperemia, and *black arrows* indicate nucleus fragmentation, cell necrosis, and residual nuclei

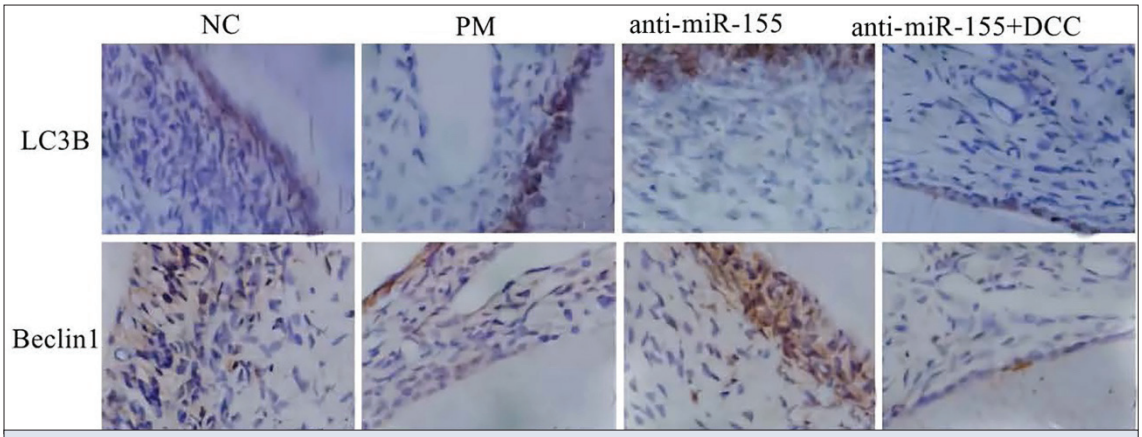

Fig 3. Immunohistochemical staining results of LC3B and Beclin1 expressions in dental pulp tissues (×400)

around the perforating point, which significantly reduced compared with those in the PM group. The pulp necrosis rate was (4.31±1.02%). In the anti-miR-155+DDC group, the cells in the pulp perforation were disordered, and the inflammatory cells gathered around the hyperemic pulp vessels. In addition, the infiltration of inflammatory cells was significantly aggravated in the anti-miR-155+DDC group compared with that in the anti-miR-155 group. The pulp necrosis rate was (75.42±2.26%) in the anti-miR-155+DDC group *(Fig. 2)*.

In the NC group, LC3B and Beclin1 were lowly expressed in dental pulp tissues. In the PM group, the expressions of LC3B and Beclin1 in dental pulp tissues increased, which were observed in the odontoblast layer near the pulp foramen. The anti-miR-155 group had significantly higher positive expressions of LC3B and Beclin1 in dental pulp tissues than those of the PM group, and the positive expressions were widely observed in the odontoblast layer and fibroblasts in the cell layer of all dental pulp tissues. Moreover, the expression levels of LC3B and Beclin1 in the anti-miR-155+DDC group were significantly lower than those in the anti-miR-155 group *(Fig. 3)*.

Compared to the NC group, the protein expressions of NLRP3 and Caspase-1 significantly rose in the PM group

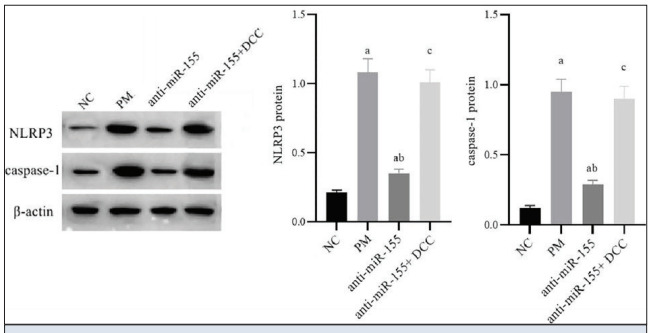

Fig 4. Protein expressions of NLPR-3 and Caspase-1 in rat dental pulp tissues. [a]P<0.05 *vs.* NC group, [b]P<0.05 *vs.* PM group, and [c]P<0.05 *vs.* anti-miR-155 group

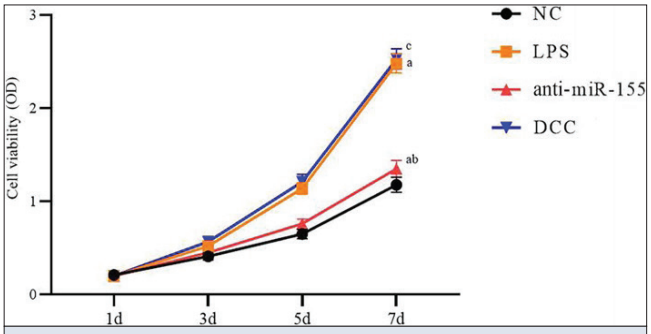

Fig 5. Dental pulp cell viability. [a]P<0.05 *vs.* NC group, [b]P<0.05 *vs.* LPS group, and [c]P<0.05 *vs.* anti-miR-155 group

(P<0.05). Compared with the PM group, the protein expressions of NLRP3 and Caspase-1 in dental pulp tissues significantly decreased (P<0.05). Additionally, the protein expressions of NLRP3 and Caspase-1 in the anti-miR-155+DDC group were significantly higher than those in the anti-miR-155 group (P<0.05) *(Fig. 4)*.

In comparison with the NC group, the activity of dental pulp cells was significantly enhanced in the LPS group (P<0.05). In comparison with the LPS group, the activity of dental pulp cells was significantly reduced in the anti-miR-155 group (P<0.05). Compared with the anti-miR-155 group, the DDC group showed significantly enhanced activity of dental pulp cells (P<0.05) *(Fig. 5)*.

In comparison with the NC group, the mRNA levels of IL-1β, TNF-α, NLRP3, Caspase-1, LC3B, and Beclin1 were significantly raised in the LPS group (P<0.05). Compared to the LPS group, the mRNA expressions of IL-1β, TNF-α, NLRP3, and Caspase-1 significantly declined, and the mRNA expression levels of LC3B and Beclin1 increased in the anti-miR-155 group (P<0.05). Compared with the anti-miR-155 group, the DDC group had significantly up-regulated mRNA levels of IL-1β, TNF-α, NLRP3, and Caspase-1, and decreased mRNA levels of LC3B and Beclin1 (P<0.05) *(Fig. 6)*.

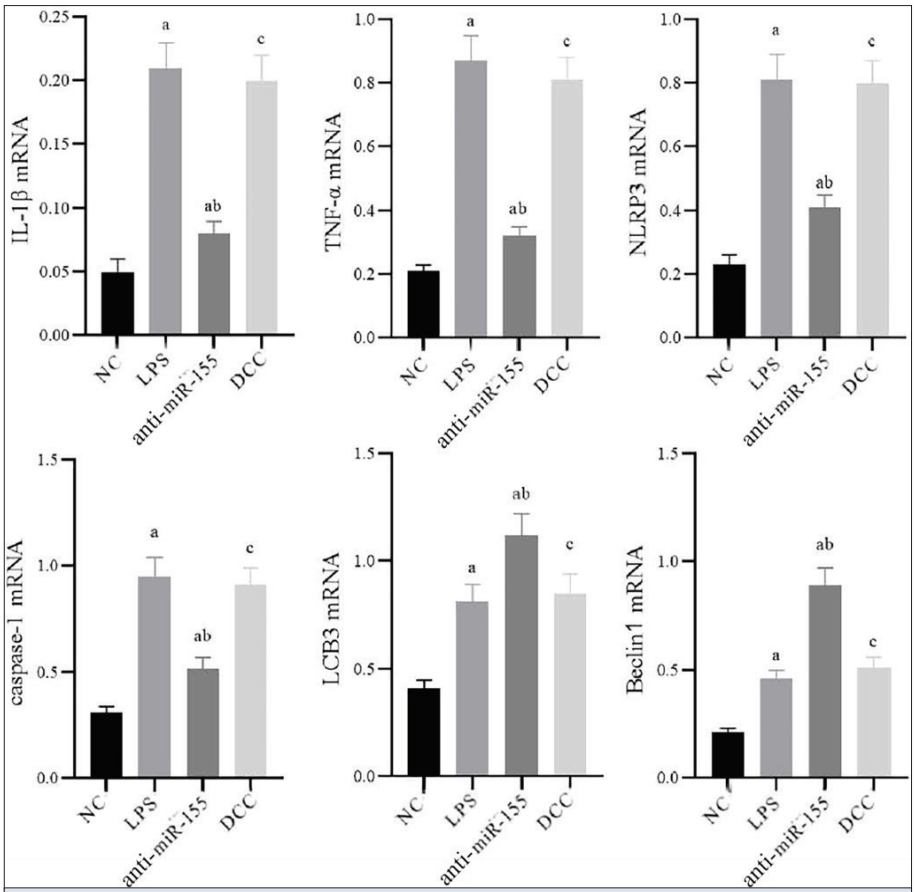

Fig 6. mRNA levels of IL-1β, TNF-α, NLRP-3, Caspase-1, LC-3B, and Beclin-1 in dental pulp cells. [a]P<0.05 *vs.* NC group, [b]P<0.05 *vs.* LPS group, and [c]P<0.05 *vs.* anti-miR-155 group

Discussion

Pulpitis is a common inflammatory disease in dental pulp tissues. External trauma-induced pulp exposure and bacterial infection facilitate inflammatory responses, thereby aggravating the pulp injury [12], so inhibiting inflammatory responses may be a measure for treating pulpitis. Dental pulp tissues are composed of a variety of cells, including odontoblasts and fibroblasts. Odontoblasts are located in the outermost layer of dental pulp tissues. They can express various pro-inflammatory cytokines such as IL-1β and TNF-α through injured dental caries, secrete large quantities of chemokines, accumulate a large number of dendritic cells, and participate in the regulation of immune responses [13,14]. In the case of pulpitis, the expressions of pro-inflammatory cytokines IL-1β and TNF-α are up-regulated, which are involved in the immune response of pulpitis [15]. In this study, in the rats with pulpitis, the dental pulp tissues were significantly injured, and numerous inflammatory cells were infiltrated. The activity of SOD in dental pulp tissues decreased, the level of MDA increased, and the pulp necrosis rate and the levels of IL-1β and TNF-α in serum rose significantly. Taken together, severe inflammation and oxidative stress occurred in dental pulp tissues in the case of pulpitis.

MiR-155 can regulate biological processes such as development, differentiation, activation, proliferation, and homeostasis of T cells, B cells, and macrophages, and exert crucial effects in inflammation, immunodeficiency diseases, and autoimmune diseases [16]. Besides, miR-155 can facilitate the occurrence and development of periodontitis, and its expression in periodontal tissues can be elevated by inflammation [17]. In this study, the levels of IL-1β, TNF-α, and MDA reduced, the SOD activity was enhanced, and the pathological injury of dental pulp tissues was significantly mitigated in the rats treated with miR-155 inhibitor, which indicated that suppressing miR-155 expression inhibited the inflammation and oxidative stress in rats with pulpitis. Liu et al.[18] confirmed that the miR-155 level was up-regulated in periodontal ligament stem cells under an inflammatory environment, and the knockdown of miR-155 facilitated osteogenic differentiation. Additionally, Li et al.[19] reported that the expression of miR-155 was up-regulated in the tissues of mice with pulpitis, and treatment with miR-155 inhibitor significantly alleviated the symptoms.

Autophagy is a key mechanism for maintaining cellular homeostasis. Autophagy is weak under normal conditions, but increases under the stimulation by starvation, oxidative damage, invasion of pathogenic microorganisms, *etc.* Autophagy activity is enhanced in mature dental pulp cells and can mediate odontoblast development. LC3, located on the surface of autophagic vesicles and the autophagic vesicle membrane, can participate in the formation of autophagosomes. The LC3 family has five members, including LC3B and LC3B2. Among them, LC3B is involved in the whole process of autophagy, and it is always located on the autophagosome membrane, which is positively correlated with the number of autophagic vacuoles. Currently, LC3B expression is commonly used to observe autophagy in clinical practice. The results of this study demonstrated that the expressions of LC3B and Beclin1 in dental pulp tissues increased in the rats with pulpitis, and their expressions were observed in the odontoblast layer near the pulp foramen. Additionally, the expressions of LC3B and Beclin1 in the rat dental pulp tissues significantly rose in the rats treated with miR-155 inhibitor, and they were distributed in the odontoblast layer and fibroblasts in the cell layer of all dental pulp tissues, which suggested that suppressing miR-155 expression facilitated autophagy in the dental pulp tissues of rats with pulpitis and thus participated in tissue repairing.

The NLRP3 inflammasome is essential in the innate immune defense. Injured or stimulated by exogenous stimuli, the pulp-dentin complex can trigger the defense activities and responses to external stimuli. The activated immunomodulatory pathway involving NLRP3 can activate Caspases-1, and the activated Caspases-1 participates in the immune inflammatory responses by regulating the synthesis and release of IL-1β, which can mediate the occurrence and development of pulpitis [20]. In this study, the protein expressions of NLRP3 and Caspase-1 in dental pulp tissues were significantly elevated in the rats with pulpitis, which were down-regulated through inhibiting miR-155 expression. Likewise, the expressions of NLRP3 and Caspase-1 in dental pulp cells significantly declined in the rats treated with miR-155 inhibitor, so the immune signaling pathway involving NLRP3 may be the mechanism of pulpitis-induced inflammation. To confirm this postulation, NLRP3 activator DDC was used to inhibit miR-155 expression. It was found that DDC reversed the inhibitory effect exerted by the inhibition of miR-155 on the inflammatory responses of dental pulp tissues and cells in rats with pulpitis, thereby aggravating pulpitis-induced inflammatory responses. Nevertheless, this study is limited. The number of animals was small, and only rats were tested. Further *in vitro* and *in vivo* experiments are still in need to verify our findings.

In conclusion, suppressing the miR-155 expression can inhibit the inflammatory response in rats with pulpitis, promote autophagy, and alleviate dental pulp tissue injury and clinical symptoms, probably by inhibiting the NLRP3 signal.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (S. Guo) on reasonable request.

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Competing Interests

There is no conflict of interest.

Authors' Contributions

YJ and SG designed this study and significantly revised the manuscript; XZ performed this study and wrote the manuscript.

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RESEARCH ARTICLE

Molecular Identification of Goat's Udder Microbes and Nutritional Value of Milk Using Dielectric Constants

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ABSTRACT

This study aims to determine which bacteria are present in goat's udder by using gram dye, nuclear nucleic acid technology, direct 16S rRNA gene sequencing, and dielectric constants. The 16S rRNA gene is frequently sequenced in modern approaches to identifying microorganisms and molecules. Bacteria like *Cohnella suwonensis, Cohnella yongneupensis, and Cohnella faecalis* were isolated and *Staphylococcus* species like *succinus, lentus, xylosus, equorum, stepanovicii, fleurettii, sciuri, kocurii,* and *faecalis* were recorded. The constants such as dielectric constant *(ε′),* spectra *(ε″)* and dielectric loss factor *(ε′+ε″)* spectra, and Total Bacterial Count (TBC) from raw goat milk samples were determined employing network analyzer and plate count methods, respectively. A poor relationship was found between TBC in logarithm and permittivities at single and multiple frequencies. Linear models like multiple linear regression, ridge regression, and absolute shrinking were used to determine TBC based on effective dielectric constant and spectra and their combination; and an excellent TBC performance was determined. In conclusion, various microbes found and isolated were, *Staphylococcus succinus, S. lentus, S. xylosus, S. equorum, S. stepanovicii, S. fleurettii, S. sciuri, S. kocurii, Cohnella suwonensis, C. yongneupensis,* and *C. faecalis***.** This study emphasizes the importance of improved hygienic procedures in ensuring food safety by emphasizing the importance of avoiding contamination and using the appropriate types of processing equipment.

Keywords: Dairy goat, DNA, Milk hygiene, Polymerase chain reaction, 16s rRNA gene

INTRODUCTION

The goat population of the country is very high and serves as an indispensable source of meat and milk producing 82.900 tons of meat and 96.900 tons of milk [1,2].

Mastitis, an infection of the udder, harms both the quantity and quality of milk produced in dairy farms [3,4]. Conventional treatment methods are factors that contribute to the disease's economic impact and therefore management becomes statistically non-significant [5,6]. Mastitis, cannot be completely cured and farmers and other dairy-related business owners are always in search of some alternate disease methods as it is critical to compare the costs of disease control with the costs and losses incurred directly by the disease [7,8]. The risk assessment's goal is to provide a fair and objective evaluation of various pieces of scientific research on the potential dangers of consuming unpasteurized goat's milk.

Molecular markers are used to determine whether phenotypic differences are hereditary or adapted. Many more variants in DNA sequences have been discovered using new DNA techniques, and one of the most important tools is molecular markers which are simply indicators of DNA changes in species. PCR has become extremely important in the field of molecular DNA research, as evidenced by genome mapping, genotyping of bonding strips with desired properties, and detection of polymorphisms in DNA (DNA fingerprint). The sequencing of the 16S rRNA gene is a necessary step in molecular diagnostics and other microbiological determinations [9-11]. The applications of genomic research rely heavily on the development of DNA markers and various molecular biology techniques. Furthermore, it opened up new avenues of possibility for genetic markers, genetic advancement, and animal selection markers.

In contrast to molecular markers, biosensors have also shown promising results in the estimation of various

microbial populations in milk [12]. Therefore, due to high specificity and sensitivity, biosensors could detect a broad spectrum of analytes in complex sample matrices and give real-time results without pre-enrichment. The methods such as fluorescent microscopy [13], quantitative PCR [14], and flow cytometry [15] provide much information about the composition of milk and microbial count. Dielectric spectroscopy is one of the inexpensive methods employed for milk composition analysis [16,17]. Similar techniques have been used by several other workers for the determination of fat and protein from raw cow milk [17]. However, due to the high scale of farming goats in Saudi Arabia, raw goat milk quality determination is a must and a rapid method for detecting TBC of raw goat milk is very important for the local goat milk industry. The dielectric properties such as constant *(ε′)* and dielectric loss factor *(ε″)* are used to describe interactions of materials with electric fields. With the development of microbial populations and nutrient metabolization, the breakdown of macro-molecular occurs bringing change in energy and conductivity as well as resistance as recorded by electrodes. This energy change impacts the response of a material to the magnetic field [18].

It is critical to safeguard human health against illnesses caused by tainted milk or diseases that harm farm animals. Further, it is also very important to safeguard animal health against illnesses caused by contaminated milk. The FDA and the FDA have been implicated in milk contamination [19-21]. Traditional detection methods are time-consuming, costly, and involve several different steps. Molecular identification techniques, such as the 16S gene sequencing method, and dielectric spectroscopy are faster and more accurate [22]. Modern genomic techniques have enabled the discovery of the genetic underpinnings of a wide range of bacterial phenotypes by predicting the effects of variable mutations that occur during the progression of pathogenic populations in animal products. The Phylogenetic analysis was used to gain a better understanding of the diversity found in the expanded areas [23,24].

In the present study, cutting-edge methodologies such as molecular, and microbial identification, as well as 16S rRNA gene sequencing, and die-electric spectroscopy are used to identify and classify bacteria found in goat udder microbiomes.

Materials and Methods

Ethical Statement

This study was approved by the King Abdulaziz University Local Ethics Committee, and animal welfare is ensured through minimal handling and stress during the sampling step (Approval no: 9-15, 2021).

Bacterial Purification

The samples were collected from several goat farms, and all samples were serially diluted by pouring aliquots of 100 mL sterile distilled water (up to 104) with Nutritional Agar (NA) plates and incubating them at 30°C for 72 h. The scope of the investigation was expanded to include the maintenance of morphologically distinct bacterial colonies and bacterial isolates in a 20% glycerol solution. For the determination of bacterial counts and analysis of composition in raw milk, each milk sample was kept in a sterilized plastic bottle and delivered to the laboratory biological biology Dept., in a natural state within 10 min after milking. Samples were stirred for about 1 min using an electric to guarantee the uniform distribution of components. All samples taken were divided into 3 groups to perform chemical analyses, TBC determination, and dielectric spectra acquisition. Gram staining of samples was done exactly as per the procedure given by the Cerny method [25,26].

The Oxidase Test

As a testing reagent, 1% tetramethyl-phenylenediamine solution in water was used according to the method of Whatman [27]. In a glass petri dish, a piece of white paper was placed, followed by two drops of a freshly prepared 1% tetramethyl-phenylenediamine solution. We transferred a loop of bacteria from a culture that had been grown in NA media for 24 h to the impregnated portion of the strip using a sterile toothpick. If the color changed to purple within the first minute and a half, the results were considered positive.

Catalase is Used for Analysis

Bacterial cultures were grown for a full day in NA media to determine catalase activity [27]. A glass loop was mixed with a drop of hydrogen peroxide to see if gas bubbles formed, indicating a positive response to catalase. This process was repeated for each bacterial culture.

Isolation and Purification of DNA from Bacterial Genomes

Following the instructions given in QIAamp genomic DNA Purification Kit, the extraction process was followed and DNA was obtained using the method QIAGEN. Five milliliters of overnight-cultured Nutrient Broth (NB) were removed and centrifuged at 13.000 revolutions per minute to obtain 1.75 mL of each bacterial strain. After 24 h of incubation, the supernatant was removed from a test tube, 180 mL of enzyme lysis buffer was added, and the tube was shaken for the next 10-20 sec at 37°C (AL). After stirring, the liquid was heated to 56°C for 30 min. The centrifuged mixture was diluted with 200 microns of 100% ethanol. The filter was discarded and the column was again centrifuged for 1 min at 13.000 revolutions per minute after being washed with 500 L of AW1 wash buffer solution. The process was completed after incorporating

the mixture with 500 L of AW2 washing buffer and spinning it for 3 min at 13.000 revolutions per minute. In order to preserve the integrity of the DNA sample, it was chilled to -20°C before being frozen.

Polymerase Chain Reaction Amplification of the 16S Ribosomal RNA Gene

Primers with the sequences 5'-CAGCGGTACCAGTTT GCTGCTCAG-3'and5'-CTCTCTGCAGGCTACCTTGT ACGACTTT-3' were used for 16S rDNA amplification. A denaturation polymerase chain reaction (PCR) was performed after 30 cycles of amplification at 94°C for 1 min, 58°C for 30 sec, 72°C for 1.5 min, 10°C for final extension, and 4°C for incubation. After being subjected to agarose gel electrophoresis, the amplified DNA was sequenced after being exposed to ultraviolet light (UV-radiations). A 1% agarose gel with a UV transilluminator was used to visualize the amplified PCR product. This was accomplished by shining a light through the gel.

Genealogical Tree Investigation

The 16S rDNA sequences were then BLAST searched against the GenBank database, which is kept up to date by the National Center for Biotechnology Information. MEGA 7.0.26 was used to create a phylogenetic tree and evolutionary distances were calculated using the Kimura 2-parameter model [28].

Milk Composition Determination

The selected milk samples from goat farms were taken and the nutrient composition including protein, fat, lactose, and SNF content, was determined using a Lactoscan milk analyzer. The moisture and PH were also determined following the method given by AOAC International [29] and PHSJ-3F China, respectively. Biochemical analysis was performed at room temperature (24±1°C).

Determination of Total Bacterial Count (TBC)- Palate Count Method

The palate count method was used to measure TBC following the Chinese National Standard GB4789.2-2010, as described by Zhu et al.[17]. TBC was logarithmically transformed ($\log_{10}$) and the unit of the TBC in this study was $\log_{10}$ colony-forming units per milliliter.

Dielectric Spectra Acquisition

The dielectric measurement system is comprised of an Agilent E5071C vector network analyzer manufactured by Agilent Technologies, Penang, Malaysia. Other essential components include an Agilent 85070E open-ended coaxial line probe, and a few more accessories forming the bases of dielectric spectra acquisition. Frequency is important and the system network ranges from 1 to 10 MHz kept with sampling points in the logarithmic scale of 201. The coefficients or permittivities namely ε' and ε'' were calculated based on the reflection coefficient and material-probe interface, and the detailed information about the setup and calibration procedure of the system was followed as given by Zhu et al.[17]. The system was heated for 0.75-1 h to keep dielectric data steady. The warm-up of the system was immediately followed by calibrating the network analyzer and coaxial line probe. Once the temperature increases to 25°C within 2-5 min, 2 original dielectric spectra (i.e., ε' and ε'' spectra) were inserted and discrete frequencies were measured in periods of about 30-the 40s. Measurements were repeated thrice for each sample from each beaker, and a total mean value of 9 measurements for each milk sample was calculated. The data obtained were used for subsequent analyses.

Quantitative Determination of TBC Using Different Models

Various models used for the quantitative establishment of TBC are as:

a) Multiple Linear Regression (MLR)

b) Ridge Regression (RR)

c) Least Absolute Shrinkage and Selection Operator (LASSO)

MLR Model

This model is based on an output variable Y and an input variable X. The equation is given by:

$Y = X\beta,$

where β represents the regression coefficient matrix. It is determined by the Least Square Method to minimize the Residual Sum of Squares (RSS) as the points of data were represented as (x_1, y_1), (x_2, y_2), (x_3, y_3), …, (x_n, y_n). The x is an independent variable, while is the dependent one.

$RSS_{MLR} = \| \hat{Y}\text{-}X\beta \|^2$

In the above formula, the $y^{\wedge}$ is the predicted values vector of the output variables.

The estimated Regression Coefficient Matrix is expressed as:

$\beta_{MLR} = \| (X^TX)^{-1} \| X^Ty$

The value for the model limits are as $Model_1 <- lm (y_1 \sim x_1, data=df_1)$

In the above equation, df represents a degree of freedom (n-1), y and x as dependent and independent variables, and T represents transposition.

RR Model

In order to limit overfitting and underfitting, the RR

model was used. It was done by introducing a penalizing term, $\alpha \| \beta \|^2$ in the RSS of the MLR model.

The regression Coefficient Matrix is as:

$$\beta_{RR} = (X^T X + \alpha I)^{-1} X^T y$$

In the above equation, α is the ridge parameter. I represent the identity matrix, while α improves the regression condition and minimizes the variance of estimates. The α defines the mixing parameter between ridge ($\alpha = 0$) and LASSO ($\alpha = 1$), the choice of α is often conducted using a *k*-fold cross-validation approach.

LASSO Model

This LASSO model is a regression-based least squares algorithm (TS-LS-LR). The L_i constraint on the regression parameters ranges from 0 to 1. In the RSS model penalizing term, $\lambda \|\beta\| 1$ is added and the regression of all variables is obtained by the formula:

$$\beta_j^{LASSO} = \beta (0, 1\text{-}n\, \lambda / \| \beta^{MLR} \|)$$

Whereas, J = index variable; λ = parameter of LASSO; n = number of samples.

Using the k-fold cross-validation approach, optimal value λ is obtained. Contrary, to the RR model, the LASSO model is advantageous in estimating dependent and independent parameters and the simultaneous selection of discrete variables.

Based on LASSO regression the optimal value λ is as:

$\lambda = 0$, the same coefficient as in simple linear regression

$\lambda = \infty$, all coefficients are zero

$0 < \lambda < \infty$: The coefficient is between 0 and simple linear regression

Model Performance Evaluation

We performed the comparisons of simplified (usually linearized) versions of numerical models. The coefficients especially the calibration set (r_c), prediction set (r_p), RMSE (Root Mean Squared Error), RMSEP (Root Mean Square Error for prediction set), and RDO (Residual Predictive Deviation) were calculated to test model performances. We draw a fit Logistic Regression model with response variables as shown in *Fig. 4*.

Software Used

Matlab version 7.1 (The Mathworks Inc., Natick, MA) was used. Further, Spyder 3.2.6 *(https://www.spyder-ide.org)* was also used to establish the MLR, RR, and LSAAO models.

Results

The 345 samples of goat udder milk collected from ninety different locations in Saudi Arabia were streaked and cultured for 24 h at 30°C before being tested for total bacterial growth (TBG) *(Fig. 1)* and molecular determination of microbiota *(Fig. 2)*. After being placed in the Eppendorf tube, the pure culture was inoculated with blood chocolate and MacConkey media. The phenotypic, morphological, and molecular characteristics of genetically isolated populations were investigated, and a wide variety of useful microbes were found in samples that contributed to the generation of phenotypes *(Fig. 3)*. The microbes found were then used to identify pathogenic and nonpathogenic bacteria. Various morphological characteristics, such as gram-positive and gram-negative staining, were used to select the isolates. As shown in *Fig. 1*, each of the selected isolates has a unique set of morphological characteristics, one of which is the ability to be stained with both gram-positive and gram-negative pigments *(Fig. 1)*.

An Examination of 16S rRNA Gene Sequences-A Tool to Differentiate Bacterial Isolates

During the experiment, high-quality genomic DNA was extracted to investigate and identify bacterial isolates at

Table 1. *Representation of milk composition, total bacterial count, pH, and correlation analysis with total bacterial count*

Parameters	Observations	Power of H^+ Ion Concentration	Moisture Content %	SNF %	Sugar %	Protein %	Fatty Glycerides %	Log_{10} of Total Bacterial Count (TBC)
Static	Minimum	1.31	75	2.2	3.10	3.21	1.56	2.98
	Maximum	5.00	87	5.78	4.78	4.01	5.67	14.93
	Mean	3.21	81	3.99	3.94	3.23	3.61	8.955
	SD	0.54	1.23	0.25	0.15	0.31	0.85	4.43
Coefficient of Correlation	Pxy=Con (rx, ry)/σx σy Pearson's cofficent	-0.31	+0.003	+0.029	-0.01	-0.03	+0.011	-
	P-value	<0.001	0.76	0.29	0.451	0.101	0.09	-

Pxy=Con (rx, ry)/σx σy; *Where as;* ***ρxy*** *= Correlation between two variables;* ***Cov(rx, ry)*** *= Covariance of return X and Covariance of return of Y;* ***σx*** *= Standard deviation of X;* ***σy*** *= Standard deviation of Y*

Table 2. *Dimensional Constatnts from different models used for the determination of total bacterial count (TBC) based on dielectric spectra and dielectric constants and the log_{10} variations of dielectric spectra*

Model Type	Dielectric Constant (ε′)	Dielectric Spectra (ε″)	r_e	Log_{10} cfu /mL (RMSEC)	r_p	Log_{10} cfu /mL (RMSEP)	Residual Productive Derivations (RPD)
LASSO	ε′	ε″	0.87	0.81	0.85	0.80	2.5
	ε′	ε″	0.78	0.70	0.79	0.71	2.4
Dielectric Loss Factor	$ε'_+ ε'$	ε″+ ε″	**1.76**	**1.51**	**1.64**	**1.51**	**4.9**
MLR	ε′	ε″	0.70	0.69	0.90	0.84	2.9
	ε′	ε″	0.88	0.80	0.98	0.90	2.8
Dielectric Loss Factor	$ε'_+ ε'$	ε″+ ε″	**1.58**	**1.49**	**1.88**	**1.74**	**5.7**
RR	ε′	ε″	0.87	0.84	0.97	0.77	1.8
	ε′	ε″	0.60	0.63	0.80	0.88	1.9
Dielectric Loss Factor	$ε'_+ ε'$	ε″+ ε″	**1.47**	**1.47**	**1.77**	**1.87**	**3.7**
P-value	-	-	-	<0.001	-	<0.001	-

ε′=dielectric constant; ε″= dielectric spectra; ε″+ ε′= dielectric loss factor; r_e= correlation cofficent of caliberation set, r_p= correlation cofficent of prediction set

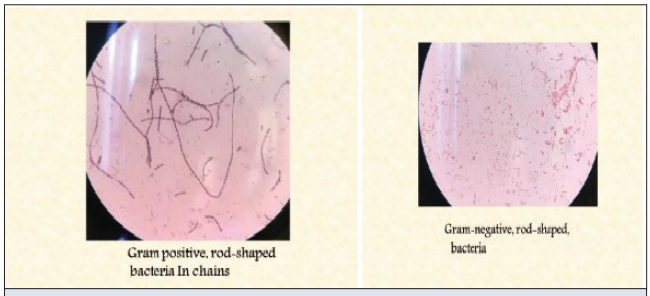

Fig 1. Showed goats udder colony gram staining. **A**- gram-positive *(spiral chain shaped)*, and **B**- Gram-negative *(rod shaped)*

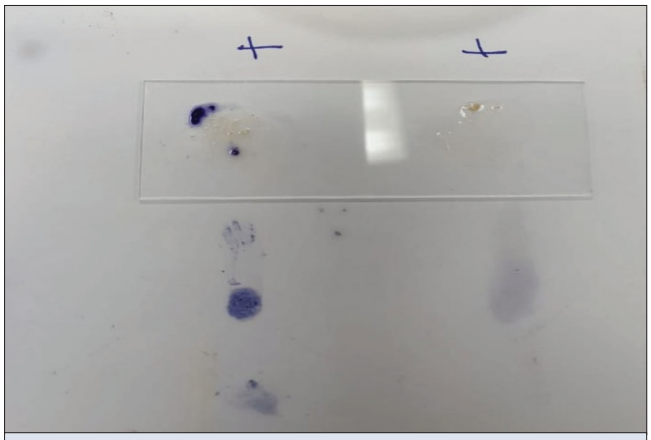

Fig 2. H200729-02 0A07 M A04 1492R *S. lentus* strain F4- 2-25 16S ribosomal RNA gene, partial sequence oxidase variable (catalase-positive). H200729-020 C07 M B04 1492R *S. lentus* strain DQ56 16S ribosomal RNA gene, catalase-positive and oxidase-positive

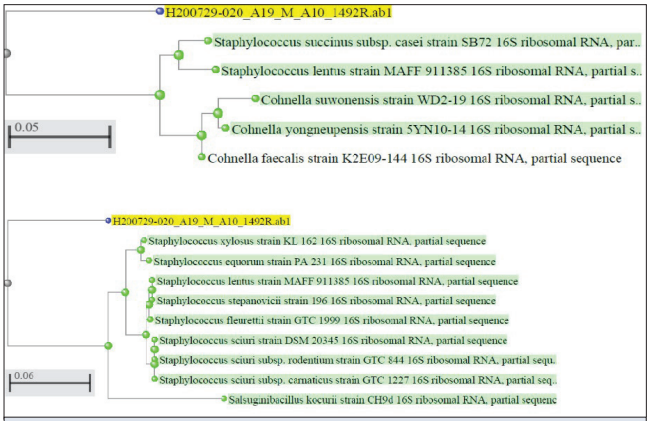

Fig 3. Phylogenetic analysis of bacteria isolates of *Staphylococcus succinus, S.lentus, S. xylosus, S. equorum, stepanovicii, S. fleurettii, S. sciuri, S. kocurii, Cohnella suwonensis, C. yongneupensis, C. faecalis* from goats udder samples based on partial nucleotide sequences of 16S rRNA gene. The tree was constructed using the neighbor-joining

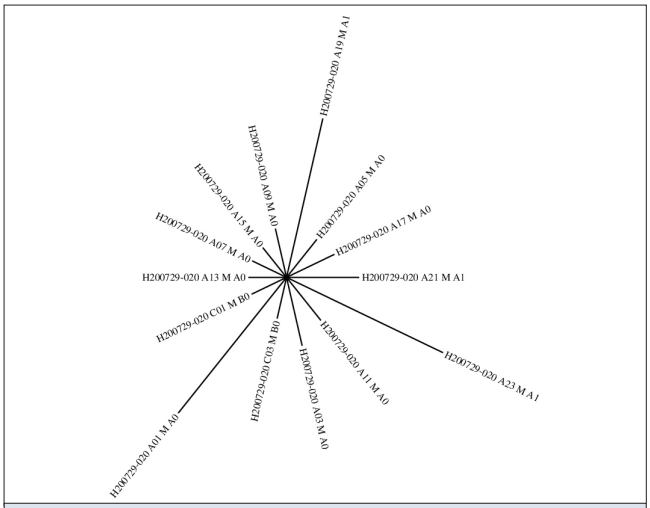

Fig 4. Based on partial nucleotide sequences of the 16S rRNA gene, a phylogenetic analysis of all bacterial isolates from goat udder samples was conducted. The LIAMM method was used to build the tree

the molecular level *(Fig. 4)*. The yield of genomic DNA was in the 60-140 ng/L range. Polymerase chain reaction (PCR) was used to amplify ribosomal DNA, with universal forward and reverse primers for 16S rDNA serving as the starting point *(Fig. 5)*. The dielectric constant (ε′), spectra (ε″), dielectric loss factor (ε′+ε″), and total Bacterial

Fig 5. Gblocks Results 0.91b, input. fasta was the processed file. Alignment is presumptively: DNA new total: 563 places (selected positions are underlined in blue)

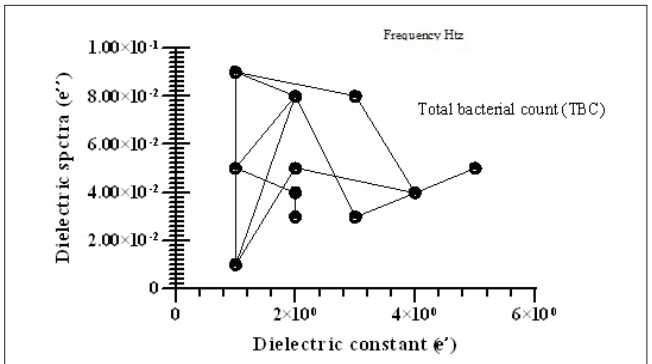

Fig 6. Representation of nonlinear relationship describing the variation of frequency (MHz) with Dielectric constant (ε′), Dielectric spectra (ε″), and Total Bacterial Count (TBC)

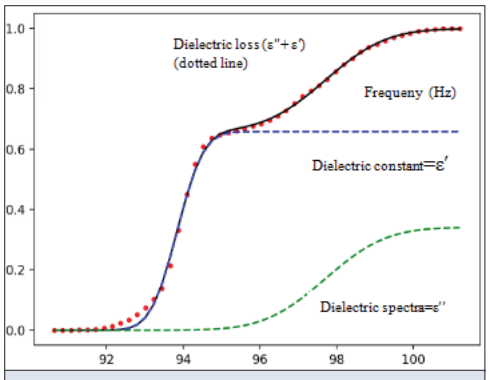

Fig 7. Dielectric constant (ε′) and dielectric spectra (ε″) and frequency (Mhz) of variation of 345 raw milk samples of goat milk taken from goat forms

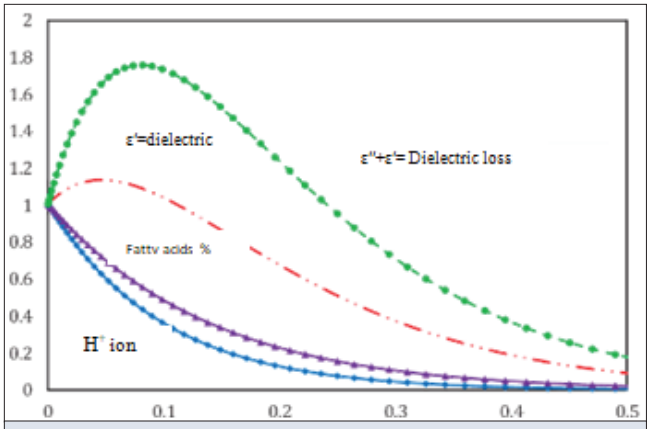

Fig 8. Linear relationship of correlation coefficient (r) between dielectric constant (ε′), dielectric loss (ε″+ε′) and Fatty glycerides and pH of 345 raw goat samples and frequency (Mhz) of variation

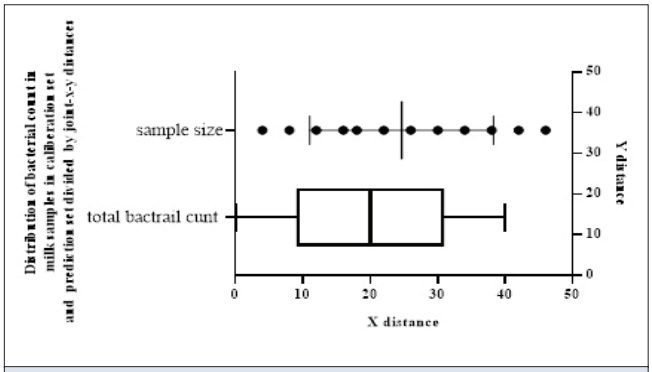

Fig 9. Graphical representation of the distribution of bacterial count in milk samples in calibration set and prediction set in -x-y distances

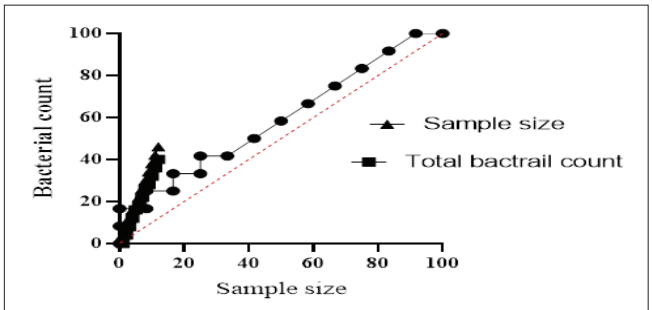

Fig 10. A Fit Logistic Regression Model with response variables Milk sample size and Total Bacterial Count (N=100). Note: in the Regression Fit Line we took only 100 milk samples

Count (TBC) were measured in raw goat milk samples using network analyzer and plate count procedures *(Fig. 6, Fig. 7)*. The association between logarithmic TBC and permittivities at single and multiple frequencies was unsatisfactory *(Fig. 7)*. Correlation studies *(Fig. 8)* and multiple linear regression and ridge regression *(Fig. 9, Fig. 10)*, and absolute shrinkage were utilized to predict TBC based on effective dielectric constant, spectra, and their combination *(Table 1, Table 2)*, resulting in outstanding TBC performance.

Discussion

According to current monitoring in Saudi Arabia, Jeddah. Mastitis disease in goats is significantly prevalent, and its severity can be reduced by improving hygienic practises. Early detection of the disease is critical. Various bacteria such as *Staphylococcus sucinus, S. Lentius, S. xylosus, S. equorum, stepanovicii, St. Flouretii, S. sciuri, S. kocurii, Cohnella suwonensis,* and *C. yongneupensis* were found in this study. Similarly, Alnefaie et al.[30] reported multiple bacteria in milk samples, such as *Klebsiella pneumonia, Serratia marcescens, Micrococcus, Coagulase-negative Staphylococcus, Diphtheroid,* and *Anthracoid.* Recent research has discovered a large number of harmful microorganisms which lead to disease especially Mastitis as it is becoming more common in animals, and this trend is expected to continue as animals become more susceptible at the early milking stage. This pattern has been observed in goats therefore routine monitoring is important. Mastitis was not previously recognized as a community-wide problem in goats (Arabic countries).

In the current study, the bacteria such as *Escherichia coli, Staphylococcus aureus, Citrobacter diversus,* and *Klebsiella* sp. were isolated from goat udder. Similar results were earlier found in Nigeria by Salihu et al.[31]. In Saudi Arabia, goat farms suffer huge economic losses to staphylococcal intramammary infections, *Staphylococcus aureus* is the major cause of clinical mastitis in goats and other ruminants [32].

To save the economic losses the Intramammary infections caused by *S. aureus* needs attention because of its huge prevalence and widespread forms of presentation of the disease. The *Coliform* bacteria cause feces infection contaminating the environment and causing poor hygiene; further, the unsanitary conditions in goat farms were caused by the presence of *E. coli, Klebsiella* spp., and *Citrobacter* species [16].

It has been reported that *coliform* bacteria were isolated from milk of unhygienic farms due to feces contamination and a lack of cleanliness. *Klebsiella* sp. is a gram-negative bacteria that is commonly found in poultry farms [25]. Because *Klebsiella* spp. are only found in unsanitary environments and it has significant public health implications as there is an increased risk of illness, particularly for young children and those with compromised immune systems. Further, when the pH of the urine changes, *Proteus mirabilis* can thrive, resulting in kidney stones [5]. *Citrobacter* can infect many different body systems, including the gastrointestinal tract, lungs, bones, peritoneum, endocardium, meninges, and circulation. The urinary system was the most frequently infected site, followed by the abdomen, skin, and soft tissue (including surgical infection), and caused pneumonia and other diseases in animals. The presence of *Klebsiella* and *Citrobacter* species increases the risk of eggs spoiling in poultry farms [30]. A variety of factors, including vertical and horizontal pollution, contribute to the spread of microbes on the outside and inside of goat farms in Saudi Arabia [10-14].

The goal of this study was to see the molecular identification of microbes and the composition of bacterial contamination in goat udders. There was a slight increase in the total number of microorganisms present in the goat udder in the afternoon samples taken from all farms ($P>0.05$). Because mesophile microorganisms thrive in temperatures ranging from 25 to 40°C, microbial loads are highest in the afternoon as opposed to the morning and evening. These results are consistent with those discovered by Theron et al.[21]. After being exposed to the high temperature, the number of different bacteria species was noticeably higher in the contents of the goats' udders [15,28].

Despite its high nutritional value, goat milk can cause serious health issues if contaminated with harmful germs, and generally goat farming appears to be poorly maintained, based on the high number of bacterial isolates discovered in goat udder and milk content and the unsanitary conditions under which goats were kept. Despite this, it is still advised that strict health regulations be followed in goat farms.

The current study will spend the majority of its time investigating the highly variable 16S rRNA region on chromosome 4. According to Zhang et al.[23]. Research, V4 is an excellent location for gathering information about the target's bacterial population. Furthermore, the ASV inference provides a clear definition of the bacterial diversity found in goat milk, allowing for the identification of variations found within the same species. Firmicutes and Actinobacteria are the two phyla with the highest frequency at the phylum level, according to our findings. Milk samples from various locations, as well as from the market, were found to share common characteristics. The similarities and differences between the core microbiota of goats were also investigated in this study and *Staphylococci, streptococcus*, and other bacteria were found in the core microbiota of all samples; the goats are the only exceptions to have *Corynebacterium* in milk samples. Lactobacillus appears to be the only organism that can be shared with the core microbiota when it comes to obtaining safe samples of cow's milk [14]. A previous study [13] found the same count of *Escherichia* and *Shaegen* in milk which is similar to the current study. Even though *Escherichia* and *Shigella* are not present in the core microbiota of humans, sheep, buffalo, and cow milk. In addition to a descriptive analysis of the microbiota found in goat milk, we presented the findings of a pilot study

designed to assess the potential relationship between SCS, a symptom of subclinical mastitis, and the milk microbiota. Even though the incidence of subclinical mastitis was frequently lower than 5%, it was estimated that the annual prevalence of clinical mastitis in small ruminants was anywhere from 5% to 30% higher than the incidence of subclinical mastitis. The small ruminants had significantly lower abundances of the bacteria species such as *Corynebacterium, Jeotgalicoccus,* and *Escherichia coli/Shigella* compared to the bigger ruminant (Cow) community. Six additional bacterial genera live in the SM community, each of which has the potential to spread illness and unsanitary settings

The most common pathogen that causes mastitis in goats is *Clostridium perfringens* which is consistent with what other researchers discovered in cattle. Further, several different studies have demonstrated this; however, we recognize that the validity of our findings is hampered by the small number of samples analyzed compared to the total population of goats and the conditions in which they are kept. The *Lactobacillus* levels were found to be higher and percentages increased from 12.2% to 19.3%. According to the findings of our study, no *Lactobacillus ASVs* were associated with the SCS trait, and the milk contains less *Lactobacillus* genus that may suppress some important mastitis pathogens. More research is needed to fully understand the role of the genus in subclinical cases of goat mastitis. Additional research on the composition of the microbiota in the goat's udder should be conducted with the assistance of a larger animal population to determine whether or not it can improve SCC quality. Based on previous knowledge [28], this is the completive study to characterize the microbiota found in goat udders using a culturally independent metagenomic method based on 16S V4 hypervariable region sequencing and dielectric properties and models.

If the TBC of the milk was greater than 8.955 log10 cfu/mL, the milk was regarded as hygienic. In the current studies, many of the milk samples analyzed were found to have good hygiene. As shown in *Table 1*, the Pearson correlation coefficients were -0.31, +0.003, +0.029, -0.01, -0.03, and +0.011 respectively for pH, Moisture, SNF, sugar, protein, fatty acids, and total bacterial count. Statistically, the P-value was high so significance was recorded *(Table1). Table 1* shows the Pearson correlation coefficient and the P-value of each milk component and pH with TBC. Among the investigated compositions and pH, only the pH was highly correlated ($P<0.01$) with TBC, but The Pearson correlation coefficient was only -0.31. The poor correlation coefficients of the milk components and pH with TBC indicate that the milk composition and pH had difficulty in predicting the TBC of milk. So, any general conclusion from pH was difficult. Dimensional constants obtained are as shown in *Table 2* which showed the original dielectric constant (ε), dielectric spectra (ε″), and dielectric loss (ε″+ ε′) of 345 raw goat milk samples: dielectric constant (ε) decreased with the increase of frequency over the whole frequency range as shown in *Fig. 2*. While the dielectric spectra (ε″) decreased with an increasing frequency and as shown in *Fig 2*. While the dielectric loss (ε″+ ε′) showed an increase *(Fig 2)*. The correlation of calibration and prediction obtained are given in *Table 2*. Similar effects of frequency on ε′ and ε″ of raw milk have been found in several studies [17]. Determination of milk quality and microbiota in milk is very important. In current studies, various microbes are found and isolated. The bacteria present in goats' udder were determined using gram dye, nuclear nucleic acid technology, and direct 16S rRNA gene sequencing. For quantitative estimation of bacteria count, dielectric spectra were used.

In conclusion, various microbes found in goat's udder and isolated microbes were *Staphylococcus succinus, S. lentus, S. xylosus, S. equorum, S. stepanovicii, S. fleurettii, S. sciuri, S. kocurii, Cohnella suwonensis, C. yongneupensis,* and *C. faecalis*. This study emphasizes the importance of improved hygienic procedures in ensuring food safety by emphasizing the importance of avoiding contamination and using the appropriate types of processing equipment.

Availability of Data and Materials

The authors declare that the data and materials are available on request from the corresponding author (M.M.M. Ahmed).

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Ethical Statement

This study was approved by the King Abdulaziz University Local Ethics Committee (Approval no: 9-15, 2021).

Competing Interest

The authors declared that there is no competing interest.

Author Contributions

F.A.A.: Desing of experiment, Mothdolgy, Lab. Collect samples; M.M.M.A.: Written MS and review; A.M.A.: Collect samples, Lab. and review; S.K.: Reviewer MS

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Research Article

Determination of the Mycotoxin Activity of Filamentous Fungi Isolated from the Intestinal Region of Adult Honey Bees by the PCR and UHPLC-Orbitrap-HRMS Methods

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ABSTRACT

Honey bees are threatened by many fungal, parasitic and bacterial diseases. This study was aimed at identifying filamentous fungi colonizing the intestinal region of dead adult honey bees and determining resultant mycotoxin activity and its potential adverse implications for bee and human health by the PCR and UHPLC-Orbitrap-HRMS methods. For this purpose, dead bees were collected from the ground in front of 95 hives displaying mass mortality in the Hatay, Iğdır and Bingöl provinces, and 22 filamentous and 6 yeast-like fungi were isolated and identified from the intestinal region of these bees. Of the filamentous fungal isolates, 8 were identified as *Aspergillus* spp., 3 as *Fusarium* spp., 2 as *Alternaria* spp. 5 as *Penicillium* spp., 4 as *Mucor* spp., and 2 as *Rhizopus* spp.. The PCR analysis of the filamentous fungi using primers targeting the aflatoxin and ochratoxin A genes revealed the presence of aflatoxin in only 1 out of the 22 samples. Aflatoxin and ochratoxin were not detected in any of the other samples. The UHPLC-Orbitrap-HRMS method revealed the presence of aflatoxin B1 in 17, both B1 and B2 in 6, B1, B2 and G1 in 5, and aflatoxin G2 in 1 of the filamentous fungal samples. The results obtained in the present study suggest that filamentous fungi may produce mycotoxins in the intestinal region of honey bees, and thereby, honey bees may distribute mycotoxins into the environment and cause indirect adverse effects on human and animal health.

Keywords: Filamentous fungus, Honey bee, Mycotoxin

Introduction

Honey bee diseases were first recognized by beekeepers and recently, have gained increased importance in the veterinary field [1]. Many parasites and pathogens threaten honey bees [2], and microbes associated with honey bees are either pathogenic or nonpathogenic. The common pathogenic bacteria of honey bees are *Escherichia coli* and species of the genera *Klebsiella, Proteus* and *Pseudomonas*. Fungal agents isolated from honey bees are mostly identified as *Penicillium* spp., *Aspergillus* and occasionally as *Torulopsis* spp. While fungal agents can colonize the intestinal system of honey bees without causing any harm, sometimes colonization may result in mass bee deaths [3].

Although fungal agents are generally known as plant and insect pathogens, they also cause diseases in vertebrates [4].

Fungal pathogens can be life threatening for both plants and animals, and their distribution has increased with climate change and rising temperatures [5].

Toxic substances known as mycotoxins are produced by mold fungi and can cause food poisoning and damage to organs such as the liver and kidney. Mycotoxins are produced primarily by species belonging to the genera *Aspergillus, Penicillium* and *Fusarium*, but other fungal species can also produce these toxic compounds [6].

Mycotoxins are defined as secondary metabolites of mold fungi that adversely affect human, animal and plant health. The contamination of food products with mycotoxins is a serious problem worldwide. Approximately 500 different mycotoxins have been identified to date, and new mycotoxins continue to be discovered [7].

Mold fungi are reproduced by spores, which are resistant to almost all kinds of environmental conditions, and their growth in crops may adversely affect plant products. Mycotoxins associated with filamentous fungi may harm animal and human health through the intake of contaminated feed and food products [8].

The toxic effects of mycotoxins are well-known in veterinary practice and are encountered in both developed and developing countries consequential to the growth of filamentous fungi under favourable social, economic and meteorological conditions (such as temperature and humidity) [9].

Filamentous fungi are widely distributed in nature and can survive adverse environmental conditions in the spore form, which is resistant to even very high temperatures. The growth of filamentous fungi is followed by the production of mycotoxins, which are toxic to animals and humans even at small concentrations [10].

Farm animals are frequently exposed to mycotoxins, and pigs are especially very sensitive to their adverse effects [11]. In the livestock industry, mycotoxins are common feed pollutants. Studies have shown that mycotoxins cause immunotoxicity and adversely affect the reproduction of animals [12].

Fungi, along with bacteria, are commonly observed in plants and animals, and although known as plant pathogens, can also colonize the intestinal region of honey bees, making these insects an important vector of these agents [13].

Honeybees and their hives host many saprophytic fungi and mycotoxins. When below a certain concentration, most mycotoxins are able to be neutralized by honey bees [14], yet even a dose of 5 ppm of some mycotoxins such as aflatoxin can cause the death of bee larvae [15]. The intestinal fungi of honey bees not only use the food stores in the hive, but also cause toxicity in bees by producing mycotoxins [16].

Due to the very low concentrations of mycotoxins, sensitive analytical procedures are required for their detection in biological samples. Most of the analytical methods used for mycotoxins are performed with liquid chromatographic (LC) quantification systems combined with other detection techniques such as spectrophotometry, fluorescence, mass spectrometry (MS) or MS/MS. Recently, liquid chromatography (LC) Orbitrap high resolution mass spectrometry (HRMS) has been effectively used for the identification and screening of non-target compounds in metabolomic strategies for the study of the bioaccumulation, toxicokinetics and excretion of mycotoxins and their metabolites as well as the target analysis of mycotoxins [17].

Maintaining the health of honey bee colonies is of great importance for the global agricultural sector, given the critical role of honey bees in crop pollination. Several factors including adverse environmental conditions, stress, pesticides and pathogens cause bee mortality [18,19].

In the present study, it was aimed to identify the fungal agents colonizing the intestinal region of dead honey bees and investigate whether they were related to mycotoxin activity, honey bee mortality, and indirectly human health.

Material and Methods

Ethical Statement

This study was performed with the permission of the Local Ethics Committee of Kafkas University (KAÜ-HADYEK, Decision number: 2022-213, Date: 27. 12. 2022).

Handling and Culture of Specimens

Dead bees were collected into ziplock bags from the ground in front of a total of 95 randomly-selected hives displaying mass mortality in three provinces, including Hatay (H) (17 hives), Bingöl (B) (54 hives), and Iğdır (I) (24 hives). The pile of dead bees in front of each hive was sampled from the top, middle and bottom layers to represent the whole population. The samples were delivered to the laboratory after each bag was numbered.

In the laboratory, the samples were sterilized first with 70% ethanol for 60 sec, then with 5% NaOCl for 60 sec to prevent external surface contamination, and washed in distilled water [20,21]. Next, the samples were transferred to 10 mL-cryotubes and chopped in a tissue slicer (Qiagen Tissue Lyser). After being inoculated onto Saburoud dextrose agar, the samples were incubated at 25°C under aerobic conditions. Fungal colonies that grew in the culture medium were conventionally identified under the light microscope as described by Navi et al.[22].

Table 1. Primers targeting the aflatoxin and ochratoxin A genes

Gene	Sequences	Length Base Pair (bp)	Reference
*Afl*D (Nor1)	F: 5'-GTCCAAGCAACAGGCCAAGT-3' R: 5'-TCGTGCATGTTGGTGATGGT-3'	66bp	[23]
Ochratoxin-A	F: 5'-AGCATCTATGCTGGCCAATC-3' R: 5'-AATGTACTCTCGCGGGCTAA-3'	187bp	[24]

Molecular Methods

DNA Isolation

For the molecular identification of mycotoxins, one piece of each of the identified filamentous fungi was transferred to a cryotube containing 500 µL of physiological saline and mechanically disrupted in a cell disintegrator.

DNA isolation from the disrupted fungi was performed with the chloroform isoamyl alcohol method. Primers targeting the aflatoxin and ochratoxin A genes were used for PCR *(Table 1)*.

PCR and PCR Conditions

For each sample, a PCR reaction mixture of 25 µL, which consisted of 2.5 µL DNA, 16.5 µL DNA-free water, 1 µL of each primer (total 2 µL), 0.5 µL dNTP mix, 0.5 µL Taq polymerase enzyme and 3 µL buffer, was prepared. The reaction products were incubated at 98°C for 30 sec, annealed at 94°C for 10 sec and amplified in a thermal cycler at 52°C for 15 sec, at 72°C for 15 sec and at 72°C for 1 min. The amplified products were run on 1.5% agarose gel. The bands that formed during electrophoresis were viewed under ultraviolet light. Bands of 66 bp corresponded to aflatoxin, and those of 186 bp corresponded to ochratoxin A [22-24].

Reagents and Chemicals for Mycotoxin Detection and Quantitation

Methanol (99.95%) of LC-MS grade and formic acid (99%) of LC-MS grade were purchased from CARLO ERBA (Val-de-Reuil Cedex, France), and ammonium formate of LC-MS grade was purchased from Honeywell™ Fluka™ (Seelze, Germany).

One-mL of an analytical standard mixture in acetonitrile (purity 99.9%) containing Aflatoxin B1 (1.2 µg/mL), Aflatoxin B2 (0.32 µg/mL), Aflatoxin G1 (0.88 µg/mL), and Aflatoxin G2 (0.28 µg/mL) was purchased from n'Tox (Bordeaux, France) (Certificate Number: 1622614458). Furthermore, 1.1 mL of Ochratoxin A in 100 µg/mL acetonitrile (purity 99.9%) was purchased from n'Tox (Bordeaux, France) (Certificate Number: 1604676695).

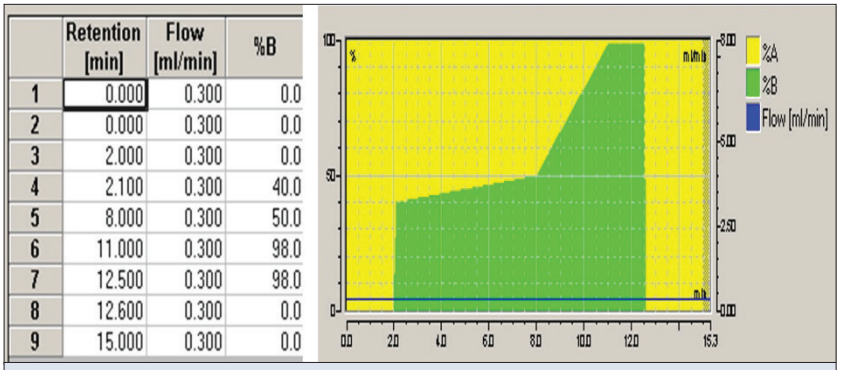
Ultra-High Performance Liquid Chromatography Coupled to Orbitrap High-Resolution Mass Spectrometry (UHPLC-Orbitrap-HRMS)

UHPLC-Orbitrap-HRMS analyses were conducted using an UHPLC system equipped with a DIONEX UltiMate 3000 RS pump, DIONEX UltiMate 3000 RS autosampler and DIONEX UltiMate 3000 RS column oven, and a high-resolution Orbitrap mass spectrometer (Orbitrap-HRMS, Exactive PlusTM, Thermo Fisher Scientific, Bremen, Germany) with a heated electrospray ionization interface (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA). The Orbitrap-HRMS instrument was calibrated with positive (Pierce™ LTQ Velos ESI Positive Ion Calibration Solution) and negative calibration solutions (Pierce™ Negative Ion Calibration Solution) using an automatic syringe injector (Thermo Fisher Scientific, USA). In the UHPLC-Orbitrap-HRMS analyses, UHPLC and part of MS were run simultaneously with the TraceFinder 3.2 program (Thermo Scientific) installed on the system computer, and the data were collected and recorded with the Xcalibur software version 2.1.0.1140 (Thermo Fisher Scientific).

Chromatography and High-Resolution MS Conditions

A Thermo SCIENTIFIC part no 17326-102130 (Dim. 100 mm × 2.1 mm, particle size: 2.6 µm) column was used for chromatographic analyses. The column oven was operated at a temperature of 30°C. The elution gradient was set as 1 mM ammonium formate (Fluka) prepared in ultrapure water obtained by the Ultrapure water system (GFL 2004/ Human power 1) for use as the mobile phase A, and methanol (Sigma) of 99.9% purity and LC-MS grade and 1mM ammonium formate (Fluka) for use as the mobile phase B. Separation was carried out applying the conditions presented in *Fig. 1*, with a sample injection volume of 20.0 µL and gradient elution conditions at a flow rate of 0.3 mL min-1. The analysis time was set to a total of 15 min.

The heated electrospray interface (HESI II, Thermo Fisher Scientific, San Jose, CA, USA) was set in only positive (ESI) mode using the following operating parameters: spray



	Retention [min]	Flow [ml/min]	%B
1	0.000	0.300	0.0
2	0.000	0.300	0.0
3	2.000	0.300	0.0
4	2.100	0.300	40.0
5	8.000	0.300	50.0
6	11.000	0.300	98.0
7	12.500	0.300	98.0
8	12.600	0.300	0.0
9	15.000	0.300	0.0

Fig 1. Subsequent gradient conditions in elution

voltage 3.5 kV; sheath gas (N2>99%) 35 (adimensional); auxiliary gas (N2>99%), 7 (adimensional); aux gas heater temperature 350°C; and capillary temperature 350 °C; S-lens RF level 50.

Mass spectra were obtained using two alternative acquisition functions: (1) Full MS, ESI+ (high collision dissociation (HCD) collision cell was closed without fragmentation), (2) All Ion Fragmentation (AIF), MS/MS, ESI+ operated fragmentally (HCD on, collision energy = 25 eV). The MS scan range was 200-450 m/z for the Full MS and AIF mode; the resolution was 17500; automatic gain control (AGC target) was $5x10^6$; and maximum IT was set to 2 ms. Mass accuracy was checked daily with multiple compound standards and calibrated weekly with mass accuracy standards.

Method Validation

UHPLC-Orbitrap-HRMS analysis was performed to determine the mycotoxin profiles of the samples. The stock standards of Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2 and Ochratoxin A were diluted to different concentrations as shown in *Table 2* with a 1:1 methanol-water solution. The external calibration curves given in *Fig. 2* were constructed and all measurement data were calculated using these calibration graphs.

Quan peak (Parent Ion) and confirming ions (Fragment Ions), retention time (RT), concentration ranges, ion mode (polarity), and the HCD collision energy of the mycotoxins are given in *Table 3*.

Table 2. *Calibration solutions of the Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2 and Ochratoxin A standards at different concentrations*

Mycotoxins	Level 1 (ng/mL)	Level 2 (ng/mL)	Level 3 (ng/mL)	Level 4 (ng/mL)	Level 5 (ng/mL)	Level 6 (ng/mL)
Aflatoxin B1	0.043	0.214	0.429	1.071	2.143	4.286
Aflatoxin B2	0.011	0.057	0.114	0.286	0.571	1.143
Aflatoxin G1	0.031	0.157	0.314	0.786	1.571	3.143
Aflatoxin G2	0.010	0.050	0.100	0.250	0.500	1.000
Ochratoxin A	-	0.100	0.250	0.500	1.000	2.500

The validation parameters of the UHPLC-Orbitrap-HRMS mycotoxin analysis method were used as performance criteria for method validation. The quan peak (parent ion), fragment ions (m/z) (confirming ion-1 and confirming ion-2), chromatograms and calibration curves used in UHPLC-Orbitrap-HRMS are shown in *Fig. 2*. The determination coefficient (R2), limit of detection (LOD) (ng/mL), limit of quantification (LOQ) (ng/mL), and recovery (%) (In the sample without analyte, 0.250 ng/mL for Ochratoxin, Level 2 concentrations in *Table 2* for Aflatoxin B1, Aflatoxin B2, Aflatoxin G1 and Aflatoxin G2 were spiked[17].) for the analysis of mycotoxins using the UHPLC-Orbitrap-HRMS method's validation parameters are provided in *Table 4*.

Table 3. *Optimized Orbitrap-HRMS parameters for the mycotoxins*

Mycotoxins	RT (n=9)	Parent Ion *(m/z)*[b]	Fragment Ions *(m/z)*		Concentration Range (ng/mL)	Adduct	Ion Mode	HCD CE
		Quan Peak (m/z)	Confirming Ion-1 (m/z)	Confirming Ion-2 (m/z)				(Fragmentation) (eV)
Aflatoxin B1	7.67±0.005	313.07066	285.07486	270.05148	0.043-4.286	M+H	Positive	25
Aflatoxin B2	7.14±0.004	315.08631	287.09082	259.05988	0.011-1.143	M+H	Positive	25
Aflatoxin G1	6.48±0.005	329.06558	311.05423	243.06474	0.031-3.143	M+H	Positive	25
Aflatoxin G2	6.07±0.005	331.08123	313.08123	303.08578	0.0100-1.000	M+H	Positive	25
Ochratoxin A	11.00±0.005	404.08954	257.02072	239.00957	0.100-2.500	M+H	Positive	25

RT: Retention time, HCD CE: Higher collisional dissociation collision energies.

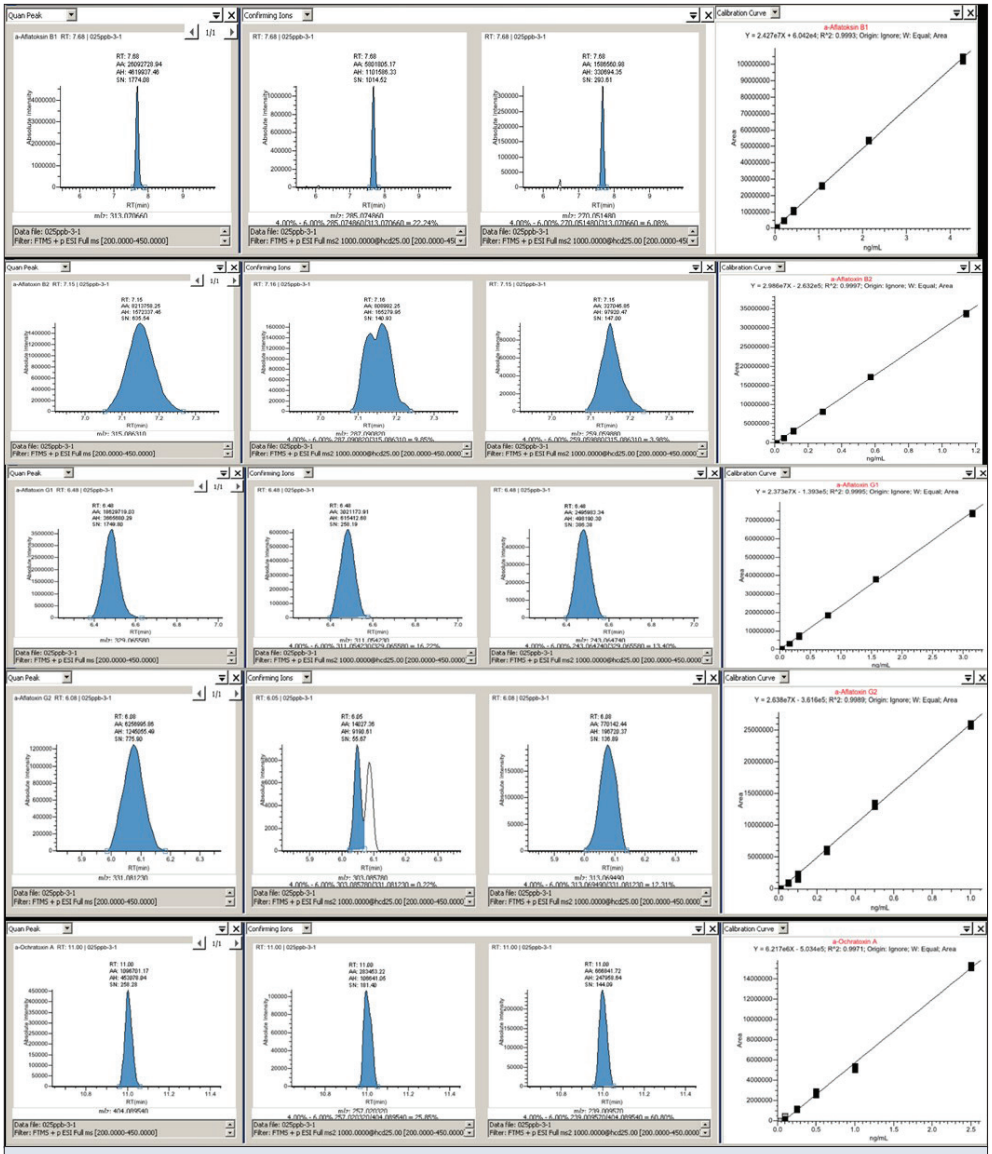


Fig 2. UHPLC-Orbitrap-HRMS Quan Peak (Parent Ion), Fragment Ions (m/z) (Confirming Ion-1 and Confirming Ion-2) chromatograms of mycotoxins, calibration curves

Table 4. Validation parameters of the UHPLC-Orbitrap-HRMS method used for mycotoxin analysis

Mycotoxins	% Recovery (n=7)	% RSD (n=7)	LOD (µg/kg) (n=7)	LOQ (µg/kg) (n=7)	R^2
Aflatoxin B1	96.19	0.88	0.005	0.018	0.9993
Aflatoxin B2	94.24	1.64	0.003	0.009	0.9997
Aflatoxin G1	93.99	1.81	0.008	0.027	0.9995
Aflatoxin G2	99.91	4.01	0.006	0.020	0.9989
Ochratoxin A	106.80	4.63	0.037	0.124	0.9971

% RSD: Relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantification, R^2: Correlation coefficient

Results

Microscopy

Fungi that grew in the culture medium were examined under a light microscope, and 22 out of the 95 samples were identified as filamentous fungi whilst 6 were identified as yeast-like fungi. Of the filamentous fungi, 8 were identified as *Aspergillus* spp., 3 as *Fusarium* spp., 2 as *Alternaria,* 5 as *Penicillium* spp., 4 as *Mucor* spp. and 2 as *Rhizopus* spp. *(Fig. 3) (Table 5).*

Table 5. *Fungi isolated from the intestinal tract of dead bees*

Provinces	Number of Hives	Culture-negative	*Aspergillus* spp.	*Alternaria* spp.	*Mucor* spp.	*Fussarium spp*	*Penicillium* Spp.	*Rhizopus* spp.	Yeast-like
Hatay	17	9	3	0	0	0	1	2	1
Iğdır	24	15	2	1	0	1	2	0	2
Bingöl	54	34	3	1	4	2	2	0	3
Total	95	57	8	2	4	3	5	2	6

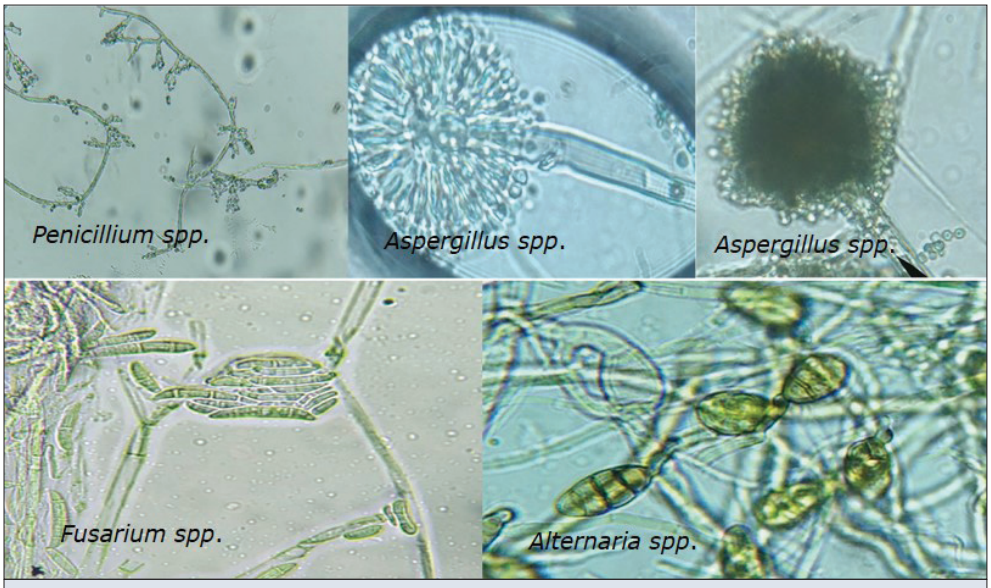


Fig 3. Micrographs obtained under the light microscope

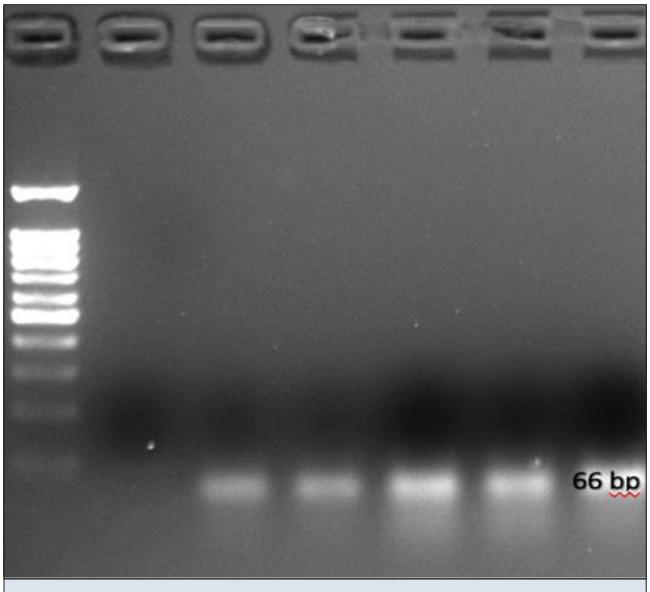


Fig 4. Electrophoresis image of the PCR products

According to PCR analyses performed with primers targeting the aflatoxin and ochratoxin A genes in filamentous fungi, aflatoxin was detected in only 1 out of the 22 samples, and neither aflatoxin nor ochratoxin were detected in the other samples.

Results of Mycotoxin Analysis by UHPLC-Orbitrap-HRMS

The qualitative and quantitative analyses of the mycotoxin content of the samples were determined using an UHPLC-HR-Orbitrap-MS device and a validated method *(Table 6)*.

Table 6. *Results of mycotoxins analysis by UHPLC-HR-Orbitrap-MS*

No	Sample Name	Aflatoxin B1 (µg/kg)	Aflatoxin B2 (µg/kg)	Aflatoxin G1 (µg/kg)	Aflatoxin G2 (µg/kg)	Ochratoxin A (µg/kg)
1	B8	nd	nd	nd	nd	nd
2	B8-1	nd	0.038	nd	nd	nd
3	B13	nd	nd	nd	nd	nd
4	B19	nd	nd	nd	nd	nd
5	B19-1	nd	0.034	nd	nd	nd
6	B22	nd	0.045	nd	nd	nd
7	B26	nd	0.074	nd	nd	nd
8	B31	nd	nd	nd	nd	nd
9	B33	nd	0.036	nd	nd	nd
10	B38	nd	nd	nd	nd	nd
11	B54	nd	0.037	nd	nd	nd
12	H1-6	0.076	0.037	0.035	nd	nd
13	H2-6	0.026	0.043	nd	nd	nd
14	H10-1	0.027	0.049	5.167	nd	nd
15	H10-2	0.081	0.038	6.284	nd	nd
16	H11	0.255	0.047	0.041	nd	nd
17	H11-2	0.078	0.058	0.170	nd	nd
18	H12	nd	0.460	nd	nd	nd
19	H20	nd	0.212	nd	nd	nd
20	H22	nd	0.133	nd	nd	nd
21	I10	nd	0.037	nd	nd	nd
22	I16	nd	0.036	nd	0.033	nd

nd: not detected

Ochratoxin A was not detected in any of the samples. Aflatoxin G2 was determined at a concentration of 0.033 µg/kg in only sample I16. Aflatoxin B2, which was the most common mycotoxin in the samples, was detected within a concentration range of 0.034-0.460 µg/kg. Aflatoxin B8, B13, B19, B31 and B38 were not detected in the samples. Aflatoxin B1 was detected in only 6 samples: H1-6 (0.076 µg/kg), H2-6 (0.026 µg/kg), H10-1 (0.027 µg/kg), H10-2 (0.081 µg/kg), H11 (0.255 µg) /kg), H11-2 (0.078 µg/kg)), and aflatoxin G1 was detected in only 5 samples: H1-6 (0.035 µg/kg), H10-1 (5.167 µg/kg), H10-2 (6.284 µg/kg), H11 (0.041 µg/kg), H11-2 (0.170 µg) /kg).

Discussion

Honey bees are exposed to the threat of multiple pathogenic microorganisms throughout their lives. Fungi can affect bee larvae without showing any symptoms, especially with an increase in air temperature. *Aspergillus flavus, A. niger* and *A. fumigatus* facilitate the exposure of bees to other disease-causing agents by suppressing their immune system [25].

Nearly a hundred species of mycotoxins produced as secondary metabolites by filamentous fungi have been identified, and most of them cause poisoning, organ damage, and carcinogenic effects in humans and animals [26]. In the present study, aflatoxin B2 was detected in 17 out of 22 filamentous fungi samples, aflatoxin B1 and B2 were detected in 6 out of 17 samples, and aflatoxin B1, B2 and G1 were detected in 5 samples. These results show that bees can be exposed to mycotoxins due to unhygienic conditions and environmental effects, spread them to plants, and infect bee larvae and other bee products. Thus, mycotoxins are likely to show adverse effects on the health of humans and animals directly or indirectly.

Aspergillus spp. and *Ascosphaera apis* are pathogenic to bees. *Aspergillus* spp. cause chalkbrood disease and *A. apis* causes stonebrood disease in bee larvae [27]. In the present study, *A. apis* was not isolated and identified from any of the samples, but *Aspergillus* spp. was identified in 8 out of 22 samples. *Aspergillus* species cause stonebrood disease in bee larvae and affect adult bees by suppressing their immune system [28]. In this study, the identification

of *Aspergillus* spp. suggests that these fungal agents could have caused the death of adult bees, and that they could have been transmitted from adult bees to larvae during feeding.

Isayeva et al.[29] examined the mycobiota of bees and isolated fungi from 130 out of 250 samples. These 130 fungal isolates were identified as belonging to 52 species, including among others *Alternaria alternata, Aspergillus flavus, Candida albicans,* and *Cladosporum herbarum*. These fungal species are reported to have toxigenic and allergenic effects on human and animal health. The identification of *Alternaria* spp., *Aspergillus* spp., and other filamentous fungi in honey bees in the present study shows similarity to the results reported by Isayeva et al.[29] and some differences are attributed to climatic and environmental conditions.

In the present study, while the aflatoxin gene was detected in only 1 sample by the PCR method, ochratoxin A was not detected in any of the samples. UHPLC-Orbitrap-HRMS results revealed the presence of aflatoxin B2 in 17 samples and aflatoxin B1 and aflatoxin B2 in 6 samples. In previous studies aimed at the investigation of the presence of mycotoxins by PCR, this method was proven to be useful in determining the presence of some mycotoxin genes [22,30]. Levin [31] reported that, unlike bacterial toxins encoded by a single gene, mycotoxins are encoded by multiple genes and cannot be detected using the PCR method. Furthermore, it has been reported that the presence of mycotoxin genes does not mean that the particular fungus species synthesize mycotoxins. Levin's findings explain the difference between the PCR and UHPLC-Orbitrap-HRMS results obtained in the present study.

Mycotoxin production by filamentous fungi has been demonstrated in several studies and aflatoxin B1 has been reported to be produced by *Aspergillus* spp. Honey bees are highly sensitive to aflatoxin B1, but an understanding of their sensitivity to other mycotoxins requires further research. It has been shown that 5 μg g^{-1} of dietary aflatoxin can cause high mortality in honey bees [32]. Aflatoxins reduce the resistance of honey bees to mycotic infections by directly affecting the central nervous and endocrine systems [33]. In the present study, aflatoxin B2 was detected in 17 of the filamentous fungi identified. Both B1 and B2 were detected in 6 samples, B1, B2 and G1 were detected in 5 samples, and aflatoxin G2 was detected in 1 sample. Aflatoxin was detected in 18 samples, corresponding to 82% of the 22 samples, and suggested that the cause of death of the collected adult bee samples could be mycotoxins produced by filamentous fungi.

In a study conducted by Decker et al.[13] on the intestines of 45 bees, it was reported that the fungal species identified could differ with the bacterial flora of the sampling site, and that bees could be the vector of fungi and transmit plant diseases. The same study pointed out to the need for further research to identify the species and potential roles of fungi in honey bees.

In a study conducted by Kis et al.[34] on 30 honey samples collected in Croatia between 2012 and 2017, *Penicillium, Alternaria* and *Cladosporium* were isolated and identified from 47.3% of the samples. It was also reported that *Mucor, Aureobasidium, Acremonium, Botrytis, Stachybotrys* and *Paecylomyces* were isolated and identified from 5.26% of the samples.

The present study suggests that fungal agents isolated and identified from adult honey bees can be transmitted to honey by adult bees, and that these fungal agents may threaten both human and animal health due to mycotoxin production.

The relationship between plant pathogens and honey bees has been demonstrated in many studies. Honey bees, which are important pollinators, also act as a vector of fungal, parasitic and viral plant pathogens. Honey bees can resist these agents up to certain limits [35]. In the present study, it was determined that the fungal agents isolated from the intestinal region of dead honey bees showed mycotoxin activity. AFB1, AFB2, AFG1 and AFG2 are toxic for both humans and animals. These aflatoxins are reported to have hepatotoxic, nephrotoxic and immunotoxic effects. Also, AFB1 is carcinogenic for humans. These mycotoxins may accumulate over time and cause mortality in honey bees. The destruction or treatment of plants infected with fungal diseases may reduce the spread of these mycotoxin-producing fungi into the environment by honey bees.

In conclusion, filamentous fungi can produce mycotoxins in the intestinal tract of honey bees. These mycotoxins can be spread to the environment by bees and adversely affect animal and human health. The mycotoxin activities of filamentous fungal agents, which are plant pathogens and for which pollinator honey bees may act as a vector, increase over time and cause bee mortality. Although reports are available on the isolation of filamentous fungi from the intestinal region of honey bees, further research is needed to determine the mycotoxin activity of these fungal agents in bees. This study is expected to contribute to future research in this field.

Availability of Data and Materials

Datasets analyzed during the current study are available in the author (S. Tarhane) on reasonable request.

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Conflict of Interest

The authors declare that there is no conflict of interest and they do not have any financial interests.

Author Contributions

All authors contributed to the understanding and design of the study. Sample collection was done by ST, İD, AKT, ŞK. The samples were prepared and analyzed by ST, İD, AKT, AG. The first draft of the article was written by ST and all authors commented on previous manuscripts of the article. All authors have read and approved the final article.

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Research Article

Immunohistochemical Assessment of MDA and 8-OHdG Expression in the Skin, Lungs and Kidneys of Lambs Naturally Infected with Sheeppox Virus Confirmed with PCR

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ABSTRACT

This study aimed to assess the role of free radicals in the pathogenesis and progression of sheeppox with the immunohistochemical investigation of MDA and 8-OhdG expressions. The study material comprised of 24 lamb carcasses (suspected of being infected with sheeppox), which were referred to the Pathology Department for routine diagnosis, and 6 healthy lambs used for control purposes. Commercial MDA and 8-OHdG were used for labeling with the avidin-biotin-Peroxidase complex technique by the manufacturer's instructions. By using PCR the correct-sized amplicon was obtained from eight of the samples. Characteristic pock nodules were detected in the skin, lung, and liver tissues. In histopathological examinations, sheeppox cells and Guarneri bodies, which are quite typical for the diagnosis of the disease, were found in the internal organs. All of the sheeppox-infected cases yielded positive results for MDA and 8-OHdG immunostainings. When compared to the control group, the sheeppox-infected group displayed statistically higher levels of MDA and 8-OHdG expressions. In conclusion, increased MDA and 8-OHdG expressions in the visceral organs of lambs naturally infected with the sheeppox virus demonstrated that sheeppox is associated with the disruption of the antioxidant/oxidant balance and the occurrence of significant oxidative stress-induced damage to macromolecules such as lipids and the DNA. The positive correlation detected between the severity of the disease and the expression of these biomarkers showed that free radicals are actively involved in the development of pox lesions.

Keywords: Free radicals, Immunohistochemistry, Lipid peroxidation, Oxidative stress, Sheeppox virus

Introduction

Sheeppox is a highly contagious viral disease of sheep [1,2]. The causative agent of the disease, the sheeppox virus (SPPV), together with the goatpox virus (GTPV) and lumpy skin disease virus (LSDV), belongs to the genus Capripoxvirus, which is classified under the family Poxviridae [3]. The disease is spread primarily by inhalation. Nonetheless, the causative viral agent may also be transmitted mechanically by infected animals and insect bites, and through contact with contaminated material. Experimental infection can be induced by intradermal and subcutaneous injections [4]. The clinical manifestation of the disease may range from moderate to severe, depending on viral factors and the host status [5]. Sheeppox is characterized by fever, conjunctivitis, rhinitis, dyspnea, generalized and internal pox lesions, and lymphadenopathy [6]. The typical lesions of the disease are localized mainly to the skin [7,8]. However, in cases of systemic infection, lesions also occur in the visceral organs, including among others, the lungs, kidneys, liver, heart, adrenal glands, thyroid, and pancreas [9]. Skin lesions are of either papulovesicular or nodular form and are localized particularly to the inguinal region, axillar region, underneath the tail, perineum, face, ears, lips and periocular region [10]. While sheeppox occurs in animals of all ages, the mortality rate of the disease may rise to 80-100% mostly in young animals [11].

Although having been eradicated in Europe, sheeppox still exists in several African (except South Africa) and Asian countries, including Turkey [12,13]. Being the cause of mortality, stillbirth, abortion, decreased milk production, wool and leather quality, sheeppox results in major economic losses in the international trade of animals and animal products [14,15]. Thus, sheeppox is classified by the World Organization for Animal Health (OIE) as a notifiable disease, which has a potential for very serious and rapid spread and requires primary and immediate control [2,6,16].

In a healthy organism, there is a balance between oxidants and antioxidants. The disruption of this balance in favor of oxidants results in the development of oxidative stress [4]. Oxidative stress leads to the generation of free radicals [5]. Free radicals attack macromolecules such as lipids, carbohydrates, proteins, and nucleic acids, and thereby, cause oxidative damage [4,17]. Malondialdehyde (MDA), the end-product of the oxygenation of poly-unsaturated fatty acids, is a highly reliable biomarker used for the detection of lipid peroxidation [18,19]. MDA is one of the major molecules involved in free radical-induced cell degeneration [5,6]. On the other hand, 8-hydroxy-2'-deoxyguanosine (8-OhdG) is one of the most common parameters used to assess reactive oxygen species (ROS)-induced DNA oxidation [20].

This study was aimed at the immunohistochemical investigation of MDA and 8-OhdG expression to assess the role of free radicals in the pathogenesis and progression of sheeppox.

Material and Methods

Ethics Board Approval

This study was conducted under the approvals of the General Directorate for Food and Control of the Ministry of Agriculture and Forestry of the Republic of Türkiye (E-71037622-325.01-7907977) and the Local Ethics Board for Animal Experiments of Kafkas University (KAÜ-HADYEK-2021/065).

Animals

The study material comprised 24 lamb carcassess (suspected of being infected with sheeppox), which were referred to the Pathology Department of Kafkas University Faculty of Veterinary Medicine for routine diagnosis, and 6 healthy lambs used for control purposes.

Molecular Methods

Paraffin-embedded tissue blocks were used as the material for nucleic acid extraction. Ten-micrometer-thick sections were cut from the blocks and placed into 1.5-mL polystyrene tubes. Extraction was performed as described by Pikor et al.[21]. The polymerase chain reaction (PCR) technique was employed for the investigation of the presence of Capripox virus nucleic acids. Primer pairs targeting the conservative A4L gene homologue were chosen. Forward primer and reverse designs are 5'-GGCCATGGCGATGGACTTCATGAAAAAATAT AC-3' and 5'-GGAAGCTTTTTGCTGTTATTATCATC TAG-3' respectively. PCR was performed with PCR Master Mix (Hibrigen, Türkiye), 10 picomols of each primer and 3 µL of template DNA under optimized cycling parameters: 95°C for 5 min, 35 cycles of denaturation (95°C for 45 sec), annealing (53°C for 45 sec) and extension (72°C for 1 min), followed by a final extension step at 72°C for 10 min [22]. The expected amplicon size was 486 bp. PCR products were visualized on an UV transilluminator after running electrophoresis in 1% agarose gel containing the Safe-Red DNA stain (Safe ViewTM Cat No: G108-R, Canada).

Histopathological Examination

Tissue samples taken at the systemic necropsy of the animals were fixed in a 10% formaldehyde solution for 24-48 h. After routine tissue processing, 5-micrometer-thick serial sections were cut from the paraffin blocks on a rotary microtome. For the examination of the histopathological findings in the tissue samples, the sections were stained with hematoxylin and eosin (H&E). The sections were examined under a light microscope and pox-associated findings were imaged with a digital camera attached to the microscope.

Immunohistochemical Examination

Four-micrometer-thick serial sections, cut from the paraffin-embedded tissue blocks, were stained with the avidin-biotin-peroxidase complex (ABC) technique using commercial anti-MDA and anti-8-OHdG antibodies in accordance with the manufacturer's instructions. The clone numbers, incubation periods, and dilution rates of the primary antibodies used in this study are presented in *Table 1*. All immune labeling were performed using the Thermo Scientific Histostain IHC Kit (HRP, broad-spectrum, REF: TP-125-HL). Amino-ethyl carbazole (AEC, Thermo Scientific, REF: TA-125-HA) was used as a chromogenic substrate and was incubated for 15 min. The

Table 1. *Information on the primary antibodies used in the immunohistochemical stainings*

Primary Antibodies	Pretreatment	Company and Catalogue Numbers	Dilution	Incubation Conditions
MDA	Microwave open	Abcam, ab6463, Polyclonal	1/1500	Overnight, 4°C
8-OHdG	Microwave open	Bioss Antibodies, bs-1278R, Polyclonal	1/1500	Overnight, 4°C

slides were washed with distilled water for 5 min before being stained with Mayer's hematoxylin. Next, the slides were coverslipped with an AEC-mounting solution.

The preparations were examined under a light microscope (Olympus Bx53) and were imaged with the Cell ^P software (Olympus Soft Imaging Solutions GmbH, 3,4). Detailed analyses of the images were made with the Image J software (1.51j8).

The results of the immunohistochemical staining were analyzed with a scoring system. System is based on the number of positive cells in the regions displaying the most intense staining as identified by the assessment of immunopositive reactions. For each tissue section, three different areas were examined at 50x magnification. The number of cells displaying positive staining was recorded for each area. Than the mean value of the five areas was noted as the mean number of positive cells in that particular case. The scoring was performed as follows: (-) no immunoreactivity; (+) weak, 1-10% positivity; (++) moderate immunoreactivity, 11-59% positivity; (+++) strong immunoreactivity, ≥60% positivity [23].

Statistical Analysis

The statistical analyses of the results were performed with the SPSS® software (SPSS 26.0, Chicago, IL, USA). The comparison of the control group and the sheeppox-infected group was made with the Mann-Whitney U-test. The results are given as mean ± standard error (SE) of the mean. When assessing the results, statistical significance was set at $P<0.05$.

Results

Molecular Results

Eight of the samples demonstrated correct sized (approx. 486 bp) amplicon *(Fig. 1)*.

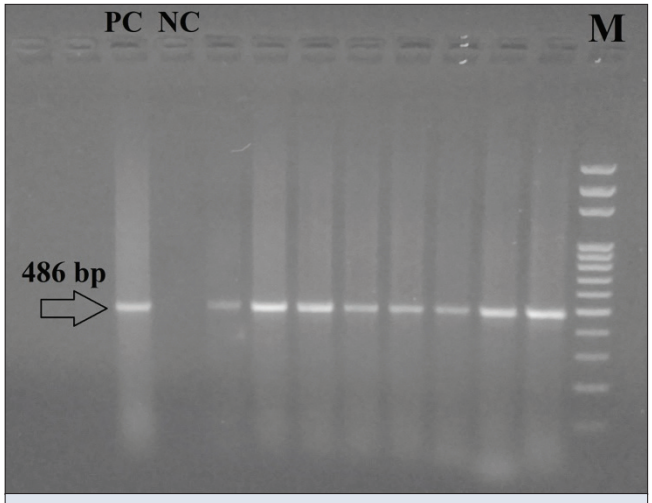

Fig 1. Agarose gel image of the positive samples. Lane marked with M contains the DNA ladder (100 bp plus Thermo-Scientific), PC: Positive Control, NC: Negative Control. Samples 1-8 are placed between the DNA ladder and the negative control

Macroscopic Findings

Papulovesicular skin lesions were localized mostly to regions either lack of fleece or covered with very little fleece. These regions include the ventral surface of the tail, the inner surface of the legs, the preputium, mammary glands, vulva, scrotum, eyelids, bucca and nostrils *(Fig. 2-a)*.

Multiple whitish round nodules were observed in the lungs, and particularly in the dorsocaudal lobes. The diameter of the nodules ranged from a few millimeters to 2-3 cm. These nodules were very hard and demarcated from the surrounding normal pulmonary tissue by a hyperemic ring-shaped region *(Fig. 2-b)*.

Whitish pale-colored miliary foci of varying size were observed throughout the renal cortex *(Fig. 2-c)*.

Microscopic Findings

The epidermis layer of the skin displayed acanthosis, as well as hyperkeratotic and parakeratotic alterations. Furthermore, cells lining the stratum spinosum presented balloon-like hydropic degeneration. Fluid-filled micro-vesicles, formed by the merge of these degenerated cells after undergoing necrosis, were observed. Degenerated epithelial cells contained intracytoplasmic eosinophilic inclusion bodies, also known as Guarneri bodies. Severe mononuclear cell infiltration was observed in the dermis. The histiocyte-like sheeppox cells (cellules claveleuses),

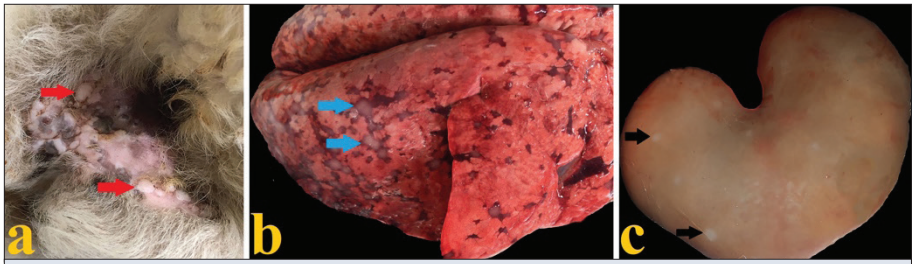

Fig 2. a: Papular lesions on the skin and the ventral surface of the tail *(red arrows)*, **b:** Lung, multiple pox nodules, each surrounded by a hyperemic ring and some of which were merged, in the dorsocaudal lobes *(blue arrows)*, **c:** Kidney, pale whitish foci distributed throughout the cortex *(black arrows)*

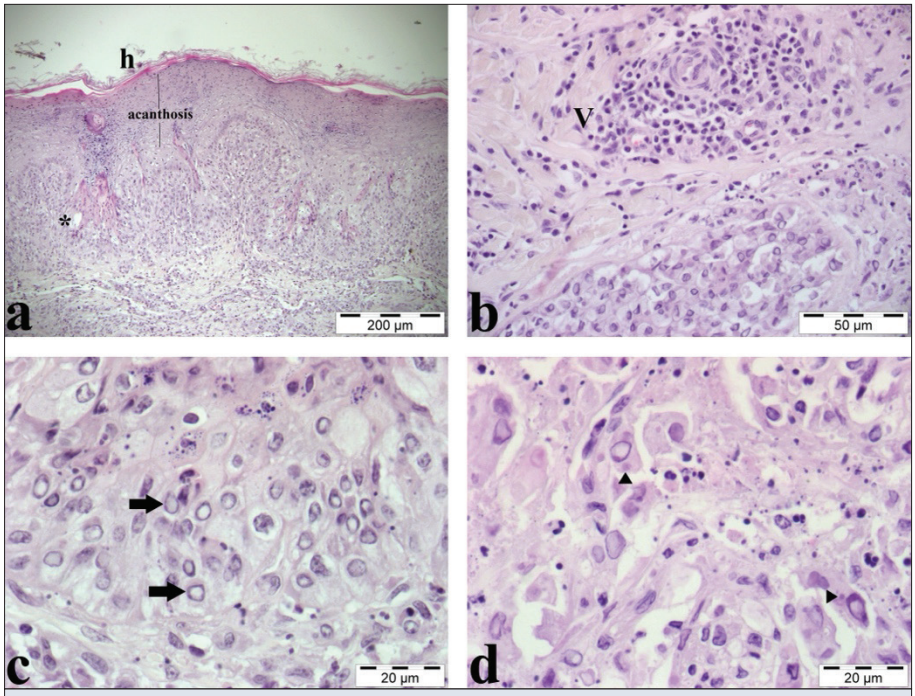

Fig 3. a: Skin, hyperkeratosis and acanthosis in the epidermis, microvesicle *(star),* Bar=200 microns, **b:** Severe vasculitis in the dermis layer (V), Bar = 50 µm, **c:** Sheeppox cells displaying marginal hyperchromasia *(arrows),* Bar=20 µm, **d:** Intracytoplasmic inclusion bodies, Bar=20 µm

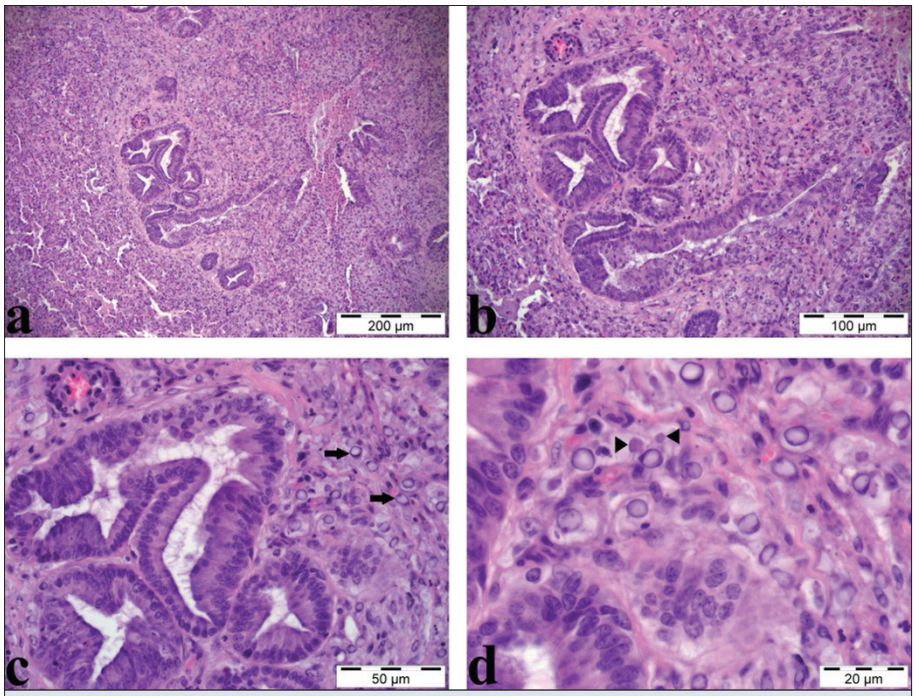

Fig 4. a-b: Lung, hyperplasia of the bronchiolar epithelium, **c:** Sheeppox cells *(arrows),* Bar=50 µm, **d:** Guarneri bodies *(arrowheads),* Bar=20 µm

which are characteristic of the disease and aid in differential diagnosis, presented with marginal hyperchromasia in their nuclei. Eosinophilic inclusion bodies were also detected in the cytoplasm of these cells. Inflammatory cell infiltration, comprised mainly of mononuclear cells, but also of edema, was present. Few neutrophil leukocytes were detected. Moreover, the dermis also presented with vasculitis (*Fig. 3*).

The most prominent finding in the lungs was the epithelial hyperplasia of the alveoli, bronchi, and bronchioles. A single layer of cuboidal type-2 pneumocytes lined the alveolar wall, largely. Intracytoplasmic inclusion bodies were

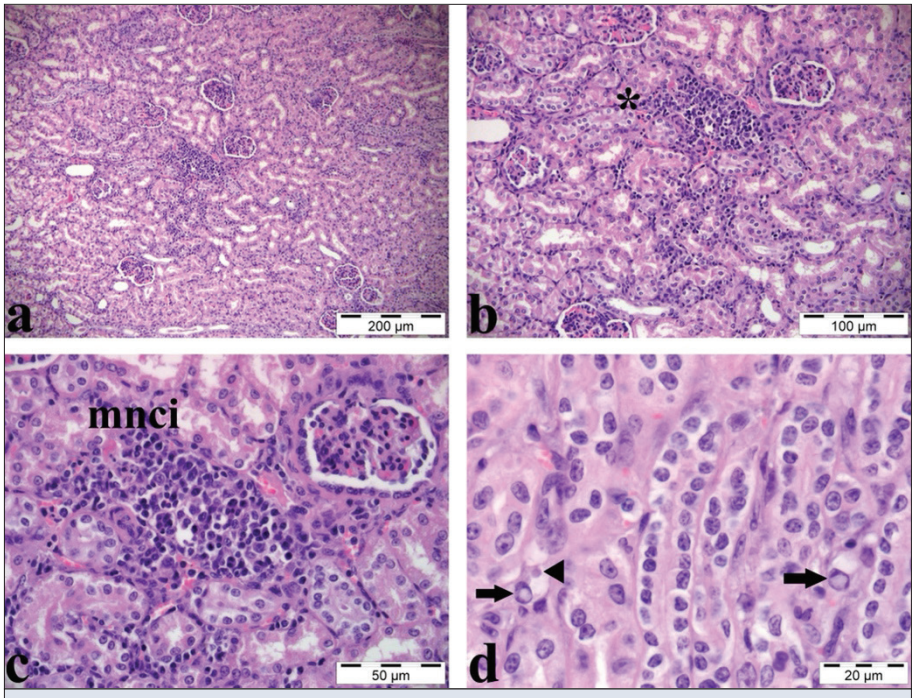

Fig 5. a: Kidney, **b:** Higher magnification, inflammatory focus peripheral to the glomerulus, Bar=100 µm, **c:** Mononuclear cell infiltration, Bar=50 µm, **d:** Sporadic sheeppox cells in the periphery of the renal tubules *(arrows)* and eosinophilic inclusion body in the cytoplasm *(arrowhead),* Bar= 20 µm

detected in the proliferated epithelial cells of the alveoli and bronchioles. Thickening of the alveolar wall was observed due to the infiltration of mononuclear cells, sheeppox cells, and a few neutrophil leukocytes. The sheeppox cells displaying marginal hyperchromasia had a slightly basophilic cytoplasm. In the proliferated epithelium of the bronchioles, cells with hydropic degeneration presented with both marginal hyperchromasia and inclusion bodies. Perivascular, peribronchial, and peribronchiolar lymphoid cell infiltrations were observed in the areas, to which the lesions were localized. Some cases displayed slight fibrotic changes and alveolar edema *(Fig. 4)*.

The kidneys presented focal nonpurulent interstitial nephritis. Mononuclear cell infiltration was observed in the periphery of the glomeruli and blood vessels. Different from the skin and lungs, the kidneys contained only very few sheeppox cells *(Fig. 5)*.

Immunohistochemical Findings

Although at low levels, MDA and 8-OHdG expressions were detected in the healthy pulmonary tissues of the control animals. All of the sheeppox-infected cases yielded positive results for MDA and 8-OHdG immunostainings. When compared to the control group, the sheeppox-infected group displayed statistically higher levels of MDA and 8-OHdG expressions *(Table 2)*. Stainings for these two oxidative stress markers were much more intense in cases with more severe disease-related pathological findings. MDA-positive stainings were detected mostly in the cytoplasm of the sheeppox cells in the dermis layer. Apart from these cells, mononuclear cells that had infiltrated into the same layer were also positive for MDA immunoreactivity. MDA expression was also detected in the degenerated keratinocytes of the epidermis. In the lungs, inflammatory cells in the interalveolar septa, as well as alveolar macrophages and the sheeppox cells displayed cytoplasmic MDA-positive staining. Moreover, proliferated epithelial cells of the bronchi and bronchioles were also identified as being immunopositive for MDA expression. In the kidneys, mostly the interstitial cells displayed MDA expression. Positive stainings were observed in very few mononuclear cells in the inflammatory foci. Weak reactions were present in the epithelium of the degenerated and necrosed renal tubules *(Fig. 6)*. 8-OHdG-positive stainings were most intense in the cytoplasm of the sheeppox cells found in the skin, and particularly in the epidermis. Apart from these cells, degenerated cells of the stratum spinosum and mononuclear cells also displayed intracytoplasmic reactions. In the lungs, positive stainings were localized particularly to the cytoplasm of the proliferated epithelial

Table 2. *Mean ± standard error values of the sheeppox-infected and control groups*

Groups	8-OHdG	MDA
Control	0.33±0.21	0.50±0.22
Sheeppox-infected	1.96±0.14	2.63±0.10
P value	0.0003	0.0001

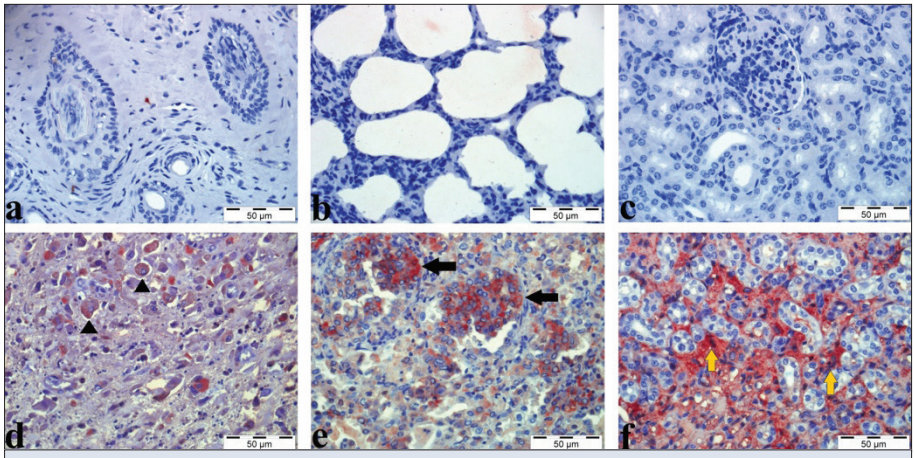

Fig 6. a: Control group, skin, Bar=50 µm, **b:** Control group, lung, Bar=50 µm, **c:** Control group, kidney, Bar=50 µm **d:** Sheeppox-infected group, skin, intracytoplasmic MDA-positive staining in the sheeppox cells *(arrowheads),* Bar=50 µm, **e:** Sheeppox-infected group, lung, strong MDA expression in the cytoplasm of sheeppox cells in the proliferated bronchioles *(black arrows),* Bar=50 µm, **f:** Sheeppox-infected group, kidney, intracytoplasmic MDA-immunoreactivity in cells localized to the interstitium *(yellow arrows),* Bar=50 µm

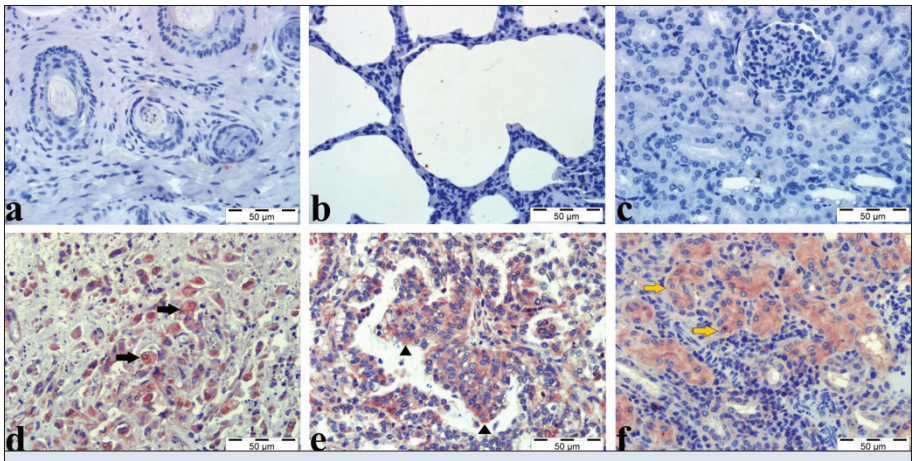

Fig 7. a: Control group, skin, Bar=50 µm, **b:** Control group, lung, Bar=50 µm, **c:** Control group, kidney, Bar= 50 µm, **d:** Sheeppox-infected group, skin, 8-OHdG-immunoreactivity in the cytoplasm of sheeppox cells displaying marginal hyperchromasia in the dermis layer *(black arrows),* Bar=50 µm, **e:** Sheeppox-infected group, lung, intracytoplasmic 8-OHdG-positive staining in the proliferated epithelial cells of the bronchioles, Bar=50 µm, **f:** Sheeppox-infected group, kidney, 8-OHdG expression in degenerated tubular epithelium *(yellow arrows),* Bar=50 µm

cells lining the bronchi and bronchioles. Moreover, the inflammatory cells and sheeppox cells in the alveolar wall also reacted positively for 8-OHdG expression. In the kidneys, 8-OHdG immunoreactivity was predominant in the cytoplasm of the epithelial cells lining the degenerated tubules. Furthermore, mononuclear cells presented with weak reactions *(Fig. 7)*.

Discussion

Free radicals play an important role in the pathogenesis of many diseases, and several viruses, including poxviruses, are known to increase the generation of free radicals [5]. Previous research has demonstrated that while poxviruses cause damage to visceral organs, oxygen-based reactants play an active role in this damage [24]. Literature review showed that there are reports on the blood MDA levels of sheep naturally infected with the sheeppox virus [4-6,18]. In the present study, it was determined that, compared to the control animals with no pathological lesions, the animals naturally infected with sheeppox displayed significantly higher levels of MDA expression in the dermal, pulmonary, and renal tissues. Blood levels reported in previous research [4-6,18] confirm the results of the present study. Moreover, MDA expression was ascertained to increase in parallel with the severity of lesions and inflammatory infiltration in the visceral organs in lambs. These findings suggest that free radical-induced lipid peroxidation is involved in the progression of organ damage associated with sheeppox.

By interacting with DNA, ROS cause the generation of

more than 20 products of oxidative base damage. Among these products, 8-OHdG is one of the most common biomarkers used to detect oxidative stress-induced DNA damage [25-27]. It is considered that the increased expression of 8-OHdG may be associated with poor prognosis in several types of cancer [28]. In multiple studies carried out in human medicine, the hepatitis B and C viruses, herpes simplex virus (HSV), and human immunodeficiency virus (HIV) have been reported to trigger oxidative stress, and it has also been indicated that individuals infected with these viruses display significantly increased levels of 8-OHdG [29-33]. The present study is valuable in that it provides data on the detection of oxidative stress-induced DNA damage in cases of sheeppox infection. As was the case with lipid peroxidation, 8-OHdG-positive stainings were observed to be more intense in cases with severe lesions, localized particularly to the proliferated epithelia of the bronchi and bronchioles. Based on these findings, it is evident that in cases of infection with the sheeppox virus, ROS-induced DNA damage occurs in the skin and several visceral organs including the lungs and kidneys.

In conclusion, increased MDA and 8-OHdG expressions in the visceral organs of lambs naturally infected with the sheeppox virus demonstrated that sheeppox is associated with the disruption of the antioxidant/oxidant balance and the occurrence of significant oxidative stress-induced damage to macromolecules such as lipids and the DNA. The positive correlation detected between the severity of the disease and the expression of these biomarkers showed that free radicals are actively involved in the development of pox lesions.

Availability of Data and Materials

The authors declare that the data that support the findings of this study are available from the corresponding author (E. Karakurt), upon reasonable request.

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Competing Interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication

Authorship Contribution

Histopathological and immunohistochemical stainings: HN, AY, EKu, Histopathological and immunohistochemical analysis: EB, SD, EKa, Molecular analysis: NC, VY, Idea, concept, and writing the article: EKa, NC

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RESEARCH ARTICLE

Evaluation of Peppermint (*Mentha piperita* L.) Essential Oil as a Digestive Tract Regulator in Broilers

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ABSTRACT

The aim of this study was to investigate the effects of peppermint *(Mentha piperita L.)* essential oil on duodenal and colonic contractions of broilers *in vitro*. For this purpose, 10 broiler intestines (42 days old) obtained from a slaughterhouse were brought to the laboratories by immersed in Krebs solution (NaCl, 118 mmol/L; KCl, 4.7 mmol/L; $CaCl_2$, 2.5 mmol/L; $MgSO_4$, 1 mmol/L; KH_2PO_4, 1 mmol/L; glucose, 11 mmol/L; $NaHCO_3$, 25 mmol/L) at +4°C. Isolated duodenal and colon tissue strips were placed in a four-channel, isolated organ bath system, where they were exposed to Krebs solution aerated with a gas mixture of 95% O_2 - 5% CO_2, at 39°C. The effect of peppermint essential oil (PEO) obtained by hydrodistillation method on spontaneous duodenum and colon contractions was evaluated in the concentration range of 0.1-1000 µg/mL. Subsequently, the effects of 300 and 1000 µg/mL PEO were reevaluated on acetylcholine-induced contractions after incubation with Nω-Nitro-L-arginine (L-NNA, NOS inhibitor) and Methylene Blue (MB, cGC inhibitor). It was determined that the PEO dose-dependently decreased the amplitude of spontaneous and acetylcholine-induced contractions in both isolated tissues. This relaxant effect persisted after L-NNA and MB incubations. In conclusion, PEO induced relaxation in isolated duodenum and colon intestinal smooth muscles not through the nitric oxide-sGMP pathway.

Keywords: Broiler, Colon, Contraction, Duodenum, Peppermint essential oil

INTRODUCTION

Antibiotics are prohibited from being used as feed additives in poultry nutrition in the European Union because they cause a decrease in beneficial microorganisms in the intestinal microflora of animals, create residual risk in animal products, and adversely affect the health of people fed with these products [1]. Accordingly, studies on the development of alternative feed additives to antibiotics have been accelerated. In this context, the use of natural and reliable herbal products (aromatic plants and essential oils extracted from these plants) for poultry nutrition has gained importance [2,3]. Essential oils obtained by steam distillation or squeezing from the leaves, flowers, bark, seeds, and roots of plants have volatile and fragrant properties at room temperature [4]. Many studies have reported that these products protect the endocrine and immune systems [3,5], increase the digestibility and absorption of nutrients [6-8] and improve performance [9-11], as well as antioxidant, anti-inflammatory, and anti-microbial effects [12].

Mentha plants are one of the most popular plants used since ancient times for their medicinal and aromatherapy properties [13]. The essential oil of peppermint, which is the most important Mentha specie, has been reported to have antioxidant, antidiabetic, antibacterial, anti-mutagenic, antifungal, and anticarcinogenic effects [14]. Studies conducted on the use of Mentha plants in poultry nutrition have evaluated the effects on performance [13,15], carcass traits [16,17], immunity, and some serum biochemistry parameters [18] during the growth period in broilers and on performance, egg quality and serum parameters in laying hens [19,20]. It is also stated that peppermint has a beneficial effect on the absorption surface area by improving the small intestine length and villi structure in quails [18]. These plants are used for the treatment of gastrointestinal (GI) system diseases [21-23].

The regulation of intestinal motility is significant as its adequate digestion of food and minimizes the risk of colonization by intestinal pathogens in the lower GI tract [24,25]. Prior studies have provided mixed results regarding the effect of essential oils and extracts derived from aromatic plants on intestinal contractions, as noticed in studies conducted on rats [22,23] and broilers [26]. This study was designed to evaluate the effect of various levels of POE on contractions occurring in the duodenum and colon of poultry. Assessing the potential impact of POE on contractions in the chicken GI tract will contribute to the development of potential uses for POE, which serves as an alternative to synthetic agents, such as antibiotics, in the field of poultry nutrition.

Material and Methods

Ethical Statement

This study was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (Approval no: AKÜ-HADYEK-272-13).

Extraction of PEO

Fresh mint (*Mentha piperita L.*) was obtained from a local herbal market in Afyonkarahisar. Turkey. After being cleared of weeds and parts, it was dried at room temperature without sunlight and then ground into powder using a mechanical grinder. 50 g of ground mint were transferred to a glass flask filled with 500 mL of distilled water. Hydrodistillation was carried out in a Clevenger apparatus for 180 min [26]. Before starting the distillation process, 1 mL of n-hexane was added to the water surface of the side arm of the Clevenger apparatus, and the condensed essential oil droplets were collected, reducing their distribution with water, and increasing efficiency. The resulting essential oil was dried over anhydrous Na_2SO_4 (Sigma-Aldrich, 238597) and stored in a closed dark glass bottle in a refrigerator at 4°C until use [25].

Animal and Tissue Preparation

This study was carried out on 10 broilers (Ross 308; 42 days old). They were obtained from the local private poultry slaughterhouse with standard regulations; the birds were sacrificed via a neck cut and bled for 120 sec. The birds were eviscerated manually [27]. The tissue strips were processed approximately 10 min after the animal was sacrificed, 2 cm-long strips of the mid-duodenum and colon were obtained. Then, these were immersed in a cold, freshly prepared Krebs solution (NaCl, 118 mmol/L; KCl, 4.7 mmol/L; $CaCl_2$, 2.5 mmol/L; $MgSO_4$, 1 mmol/L; KH_2PO_4, 1 mmol/L; glucose, 11 mmol/L; $NaHCO_3$, 25 mmol/L) and transferred to the laboratory. After removing fat and connective tissue from the intestinal segments, longitudinal strips (0.6 cm long and approximately 3-4 mm wide) were placed in a four-chamber organ bath (IOBS 99 Isolated Tissue Bath Stand Set; Commat) with 20 mL Krebs solution (pH 7.4) in 95% O_2 and 5% CO_2 at 39°C. Longitudinal smooth muscle strips were attached to platinum ring electrodes along one edge using 2:0 silk ligatures. The opposite edge of the tissue was connected to a force-displacement transducer (model 10-A; MAY; Commat, Ankara, Türkiye). The isometric smooth muscle activity of the intestine samples was monitored and recorded by the computer using the force transducer and an acquisition system (model MP30 WSW with Biopac Student Lab, PRO Software, Biopac Systems; Commat). To maintain the contractile activity of the tissue, optimal tension relationships for the strips were achieved with resting tensions of 1 g. It was allowed to equilibrate for an hour and the Krebs solution was changed every fifteen minutes. The viability of the strips was verified by adding Acetylcholine (Ach) at a concentration of 10^{-5} M at the beginning and/or end of the experiment.

Monitoring the GI Tract Muscle Activity

Subsequently, the effectiveness of the doses (300 and 1000 µg/mL) of PEO that cause inhibition in duodenal and colonic contractions was reevaluated after incubation with the NOS inhibitor L-NNA (10^{-5} M) and the cGC blocker MB (10^{-2} M). In addition, the effect of 8-bromo-cyclic GMP (8-Br-cGMP; 10^{-8}, 10^{-7} and 10^{-6} M), a cGMP analog, on the amplitude of isolated duodenal and colon spontaneous contractions was evaluated.

Statistical Analysis

Statistical analysis was done using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The Shapiro–Wilk test was used to test the data for normality. All values are presented as mean ± S.E.M. One-way ANOVA was used for the statistical evaluation of the data. The Tukey test was performed to compare individual means of treatment groups. Differences were considered significant at $P<0.05$.

Results

Effect of PEO on the Amplitude of Spontaneous Duodenum and Colon Contractions

It was determined that the cumulative application of PEO at concentrations of 0.1, 0.3, 1, 3, 10, 30 100, 300 and 1000 µg/mL caused inappropriate relaxation to the gradual concentration response in the duodenum *(Fig. 1-A)* and colon *(Fig. 1-B)* tissue samples.

The amplitude of spontaneous duodenal and colonic contractions was reduced by PEO at concentrations of 300 µg/mL ($P<0.01$) and 1000 µg/mL ($P<0.001$). However, no statistically significant influence on these contractions was seen at concentrations from 0.1 to 100 µg/mL. It was

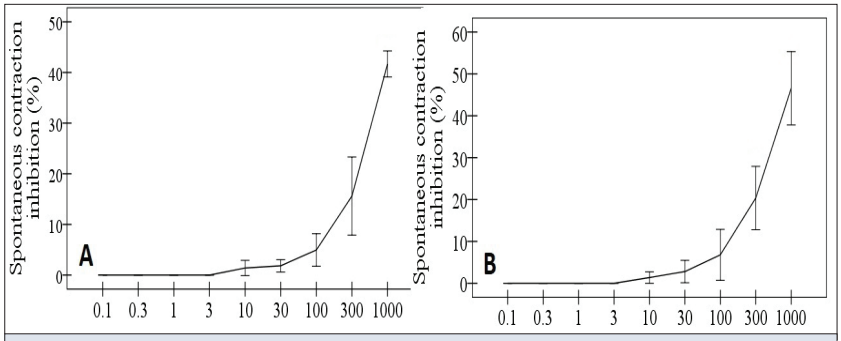

Fig 1. The dose-response curves of PEO in isolated broiler duodenum and colon (A: Duodenum, B: Colon, PEO: Peppermint essential oil)

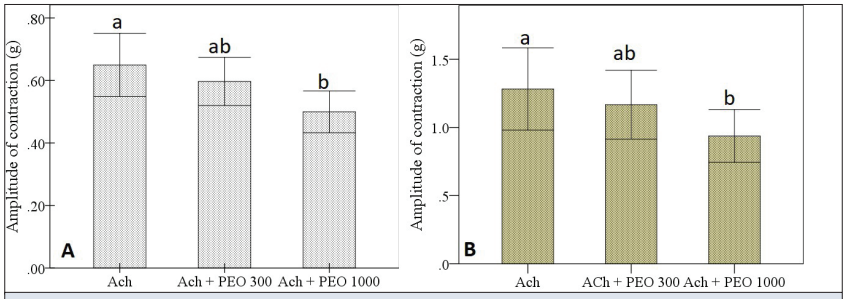

Fig 2. Effect of 300 and 1000 µg/mL PEO on Ach-induced duodenum and colon contractions. (A: Duodenum, B: Colon; PEO: Peppermint essential oil). Amplitude values of contractions in groups induced by EFS in the presence of PEO (300 and 1000 µg/mL. Letters (a and b) indicate significant differences between them in each column (P<0.05)

observed that the contractions returned to normal after washing the tissues with Krebs solution.

Effect of PEO on Ach-Induced Duodenum and Colon Contractions

The effects of 300 and 1000 µg/mL PEO on duodenal and colonic contractions induced by Ach (10^{-4} M), respectively, are shown in *Fig. 2*. It was determined that 1000 µg/mL PEO reduced the contraction effect of Ach in both tissues (P<0.05).

Effect of PEO on Duodenum and Colon Contractions Induced by Ach Following L-NNA and MB Incubation

The effects of 300 and 1000 µg/mL PEO on the amplitude of duodenal and colonic contractions induced by Ach (10^{-4} M) following L-NNA and MB incubations, respectively, are given in *Table 1* and *Table 2*. It was determined that 1000 µg/mL PEO decreased the amplitude of contraction in both tissues (P<0.05), and a similar effect was observed after MB incubation (P<0.05).

Effect of 8-Br-cGMP on Spontaneous Duodenum and Colon Contractions

The effect of 8-Br-cGMP (10^{-8} - 10^{-6} M) on spontaneous duodenal and colonic contractions is shown in *Fig. 3*. It was observed that 10^{-8} M dose of 8-Br-cGMP had no effect on the contraction intensity of both tissues, whereas 10^{-7}

Table 1. *Effect of PEO on the amplitude of Ach-induced contractions following L-NNA incubation (g)*

Tissue	L-NNA + Ach	L-NNA + Ach + PEO 300	LNNA ± Ach + PEO 1000	P-value
Duodenum	0.661±0.052[a]	0.608±0.036[ab]	0.511±0.026[b]	0.048
Colon	1.301±0.151[a]	1.190±0.135[ab]	0.955±0.092[b]	0.032

L-NNA: *Nω-Nitro-L-arginine;* ***Ach:*** *Acetylcholine;* ***PEO:*** *peppermint essential oil*
Letters (a, b) in the same line indicate significant differences between different letters

Table 2. *Effect of PEO on the amplitude of Ach-induced contractions following MB incubation (g)*

Tissue	MB + Ach	MB + Ach + PEO 300	MB ± Ach + PEO 1000	P-value
Duodenum	0.676±0.053[a]	0.608±0.037[a]	0.496±0.021[b]	0.010
Colon	1.313±0.152[a]	1.208±0.146[ab]	0.945±0.093[b]	0.042

MB: *Methylene Blue;* ***Ach:*** *Acetylcholine;* ***PEO:*** *peppermint essential oil*
Letters (a, b) in the same line indicate significant differences between different letters

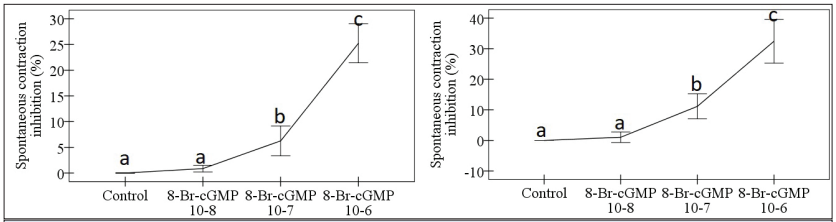

Fig 3. The dose-response curves of 8-Br-cGMP in isolated broiler duodenum and colon (A: Duodenum, B: Colon). Letters (a, b) in the same line indicate significant differences between different letters

M and 10^{-6} M doses depressed the contraction intensity ($P<0.001$).

Discussion

In this study, PEO decreased the amplitude of spontaneous duodenum and colon contractions in broilers in a dose-dependent manner. Similarly, we have previously demonstrated that myrtle, rosemary, and thyme essential oils inhibit contractions of the small intestine [26]. Essential oils have been added to poultry diets for several purposes. Recently, they have also been used to increase the digestibility and absorption of nutrients in poultry [7,28,29]. Essential oils have been demonstrated to increase the secretion of some digestive enzymes [6] and stimulate the appetite center due to their aroma-enhancing properties [30,31]. Since essential oils obtained from different plants regulate the contractions in the GI tract of rodents, the use of these plants is recommended to support the digestive tract [32,33]. Mentha plants have been used in traditional medicine for many years in the treatment of GI disorders [13]. In this study, the spasmolytic effect created by the essential oil of peppermint, known as Mentha spicata, was demonstrated in isolated duodenum and colon smooth muscle.

In the GI tract, Ach is an important neural mediator of parasympathetic innervation in poultry, similar to in other animals. It binds to muscarinic receptors and induces smooth muscle contraction [34,35]. Our previous study showed which Ach concentrations of 10^{-4} M induce maximal contractions in the duodenum of broilers [26]. In this study, duodenum and colon contractions were induced by using Ach (10^{-4} M), and the effectiveness of PEO (300-1000 g/mL) was reevaluated. The spasmolytic activity of PEO has been demonstrated by reducing the amplitude of Ach-induced contractions, similar to spontaneous duodenal and colonic contractions.

In this study, after determining the relaxation effect of PEO, we hypothesized that it may be efficient through the NOS-NO-cGC. The smooth muscle layers are innervated by enteric inhibitory and excitatory motor neurons that directly regulate GI motility [36]. Nitric oxide (NO), which is released in response to nerve stimulation of the myenteric plexus, causes relaxation of smooth muscle in addition to the protection of the digestive tract mucosa as a result of the regulation of gastric mucosal blood flow and fluid secretion. Thus, it plays an important role in regulating stomach and intestinal motility [37,38]. In the myenteric plexus, neuronal NO synthase (nNOS) generates NO from L-arginine [36]. Due to their ability to inhibit NOS, L-arginine analogs, specifically N-nitro-L-arginine (L-NNA) and N-ω-Nitro-L-arginine methyl ester, are frequently used to prevent the production of NO [40,41]. In contraction trials, when the nNOS inhibitor L-NNA is at a concentration of 1 mM, it blocks the NOS enzyme [41,42]. In the present study, it was determined that the relaxing effect of PEO continued in the duodenum and colon, induced by Ach, after inhibition of the NOS enzyme with L-NNA. Therefore, the results of the study show that PEO does not create a relaxant effect by increasing NO formation.

The primary receptor for NO is soluble guanylate cyclase (sGC) [40]. Nitric oxide activation of sGC leads to the formation of cGMP. As a second messenger, cGMP interacts with protein kinases, phosphodiesterases, and ion channels, leading to the cellular effects of NO. The cGMP induces relaxation in smooth muscle tissue by two mechanisms [44]. First, it increases the permeability of K^+ channels by lowering the level of Ca^{++} in the cell. Thus, it hyperpolarizes the plasma membrane [45,46]. Second, cGMP blocks the myosin/actin interaction by activating cGMP-dependent protein kinase (PFG), which causes the dephosphorization of myosin light chains [47]. Moreover, some of the NO-induced smooth muscle relaxations were also reported to be independent of cGMP [36,48]. However, there are no previous studies in which the effects and dose were demonstrated in the anatomical formations of the small and large intestines, although the effect of cGMP was shown in different smooth muscle cells in poultry. In this study, it was determined that the amplitude of duodenum and colon contractions decreased. In this context, a cGMP-dependent relaxation was observed in the poultry intestine.

Methylene blue prevents cGMP-dependent relaxation by inhibiting cGC inhibitor and cGMP formation [49]. It is known that the dose of 10^{-5} M blocks cGC in *in vitro*

experiments [42,50]. The present study provided evidence that the intensity of Ach-induced duodenal and colonic contractions was reduced by PEO after inhibition of MB and cGC, whereas MB did not reduce PEO activity. In conclusion, it is considered that the effectiveness of PEO is not a cGMP dependent.

As a result, it was determined that a cGMP-dependent relaxation was observed in the duodenum and colon of broilers, whereas PEO reduced the amplitude of contractions in the same tissues without using the NOS-cGC-cGMP pathway. It is suggested that >10 µg/mL of PEO be added to broiler diets due to its spasmolytic effect, but it is also recommended to conduct trials in terms of *in vivo* activity.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (T. Bülbül) on reasonable request.

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Ethical Statement

This study was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (Approval no: AKÜ-HADYEK-272-13).

Conflict of Interest

There is no conflict of interest.

Author Contributions

TB designed this study, prepared this manuscript, and analyzed experimental data; VÖ analyzed experimental data; AB analyzed experimental data.

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Research Article

Radiographic Pelvimetry in Budgerigars with and without Egg Retention

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ABSTRACT

Egg retention, particularly in budgerigars, is an important reproductive issue. This study aimed to obtain osteometric measurement values, determine the length/width ratios between measurement values, and reveal the biometric differences in X-ray images of the pelvis of normal and egg-retention budgerigars. Pelvimetric measurements (linear distance, angle, length/width) were obtained from ventrodorsal images of the pelvis of 15 normal and 15 egg-retention budgerigars. The measured values were subjected to statistical analysis. Examining the pelvimetric data showed that the average measurement values of L1 (cranial ilium width), L4 (middle pubis width), L5 (caudal pubis width), L6 (caudal ischium width), A1 (iliac arch), LA2 (left ischiopubic angle), and RA2 (left ischiopubic angle) were significantly higher in the normal group than those of the egg retention group ($P<0.05$). Additionally, all length/width ratio measurements were higher in egg retention budgerigars. However, while the mean length/width ratio measurement values of L7 (left iliopubic length)/L2 (preacetabular tubercle width), L8 (right iliopubic length)/L2, L9 (synsacrum length)/L2, L7/L3 (acetabula width), L8/L3, and L9/L3 were higher in egg retention budgerigars compared to the normal group, this difference was not statistically significant ($P>0.05$). A1, LA2, and RA2 measurement values in normal and egg retention budgerigars were respectively found at 113.93±3.39 (°) and 95.73±2.22 (°); 115.93±2.25 (°) and 104.67±2.32 (°); 115.13±2.10 (°) and 105.07±1.98 (°). Consequently, the osteometric measurement parameters of the pelvis in the normal and egg retention budgerigars were determined using X-ray images. The morphometric data acquired through this study is believed to hold potential value for veterinarians involved in clinically assessing egg retention in budgerigars.

Keywords: Bird, Budgerigars, Egg retention, Pelvis, Radiographic Pelvimetry

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Introduction

Budgerigars, among the parrot species, are domesticated bird species spread worldwide from Australia. These birds can be small and long-tailed, with patterned wings and different colors. Owing to their attractive appearance, budgerigars are intensively bred as pets worldwide. In addition, budgerigars are among the most popular pets because they are easy to live in, train, buy, and inexpensive to meet their needs. Budgerigars average 18-20 cm in length and 30-40 g in weight, and their lifespan is between 6-8 years. It is noteworthy that these birds possess speech skills [1,2].

Many reproductive problems can occur in birds. One of these problems is egg retention. In avian practice, egg retention is defined as the inability of a bird to successfully pass an egg without assistance. Among bird species, it is more common in budgerigars, cockatiels, lovebirds, finches, and canaries. The causes of egg retention include vitamin deficiencies, metabolic calcium syndrome related to calcium exhaustion or lack of absorption or availability, systemic diseases, malformed eggs, obesity, malnourishment, heredity, and pelvic factors. In addition, the evaluation of avian reproductive anatomy is of vital importance for understanding this situation [3,4].

Imaging methods are widely used in many areas of veterinary medicine [5]. Among these imaging methods, radiography is critical [6]. Radiography is used in poultry and other animal species [3-6].

This study aimed to present useful information to the veterinary field as a result of the data obtained by

comparing pelvic measurements in budgerigars with and without egg retention.

Material and Methods

Ethical Statement

All procedures were performed with the approval of the Van YYU Animal Experiments Local Ethics Committee (VAN YUHADYEK), approval number 2023/03-06.

Animals

This study was conducted between the years of 2018-2022. In this study, 30 budgerigars with 15 egg retention, and 15 without egg retention, between the ages of 1-2 were used. The mean age of normal budgerigars was 17.80±3.47 months, while the mean age of egg retention was 17.73±3.77 months. In addition, the mean body weights of normal and with egg retention budgerigars were calculated as 27.67±1.45 g and 32.40±2.06 g, respectively. The body weight of the egg retention budgerigars was measured together with the egg weight in the reproductive tract. Animal materials were obtained from private veterinary clinics in Bursa Province of Türkiye and the required permissions were obtained (permission approval number: BETA/RI-I). Radiographic images of the pelvis of birds were used. Budgerigars that were healthy and could lay eggs regularly were formed without the egg retention group *(Fig. 1-A)* while budgerigars that could not hatch their eggs naturally and had egg retention as a result of X-ray examination, and underwent the necessary intervention formed the egg retention group *(Fig. 1-B)*. Consequently, the study encompassed budgerigars that exhibited no anatomical disorders, neoplastic growths, infectious conditions, congenital irregularities, or traumatic skeletal system abnormalities upon examination. The images obtained in this study were retrospectively evaluated based on routine clinical examination procedures.

Radiological Imaging

The Has Vet 838R X-ray device was used for X-ray examination of normal and egg retention budgerigars. The birds were placed in the ventrodorsal position on a disposable drape laid on the X-ray table. During the shooting, the film-focus distance was determined as 10-20 cm for the stationary system in the parameters of the X-ray device. The exposure times were determined as 50-90 KV, 30 mA, 0.1-6.3 seconds. Thus, ventrodorsal images of the normal and egg-retention budgerigar pelvis were obtained, in which the anatomical structures were clearly visible. All image analyses were performed using open-source image analysis software (Horos v3.3.6, *https://horosproject.org/,* MacPro Quad Core, Apple, Inc. Cupertino, CA). Images were measured with electronic calipers using a software program.

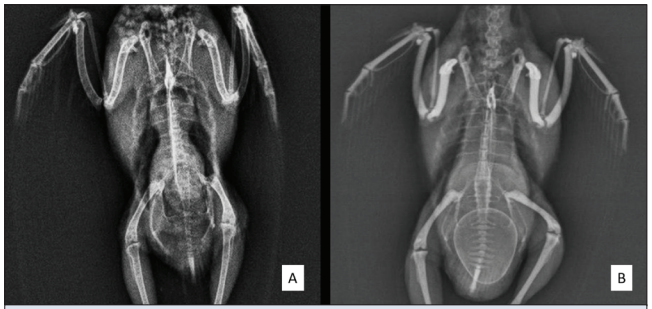

Fig 1. Ventrodorsal projection of the pelvis in normal (A) and egg retention (B) budgerigars

Taking Measurements

Nomina Anatomica Avium was used to name the anatomical reference points used in the measurements [7]. The morphometric measurements were based on the measurement points specified in the literature [8]. Linear measurements taken from the ventrodorsal images of the pelvis of budgerigars were determined as L1 (Cranial ilium width), L2 (Preacetabular tubercle width), L3 (Acetabula width), L4 (Middle pubis width), L5 (Caudal pubis width), L6 (Caudal ischium width), L7 (Left iliopubic length), L8 (Right iliopubic length), and L9 (Synsacrum length). In addition, 3 angle measurement values were obtained: A1 (Iliac arch), LA2 (Left ischiopubic angle), and RA2 (Right ischiopubic angle). Finally, the length/width ratios between the morphometric measurements obtained from the ventrodorsal images of the budgerigar pelvis were evaluated. In this measurement value obtained from the pelvis, centimeters (cm) were used for linear measurements, and degrees (°) were used for angle measurements. The measurement points of the pelvis, abbreviations, and definitions of these measurements in the normal and egg retention budgerigars are presented in *Table 1*.

All the budgerigars were carefully positioned using a dorsal recumbency method within the positioning device, ensuring consistency across all ventrodorsal radiological images. To maintain an upright posture, the birds' necks were supported by a guillotine-like apparatus. A slight, gentle sway of the wings was observed in all specimens. The pelvic limbs were cautiously extended caudally and secured, thus standardizing the ventrodorsal alignment for all budgerigars. By adhering to these meticulous parameters, the objective was to achieve homogeneity, thereby safeguarding the precision of the measurements across all stances. Furthermore, to assess the reliability of the measurement outcomes, each pelvic dimension of the budgerigars underwent triple measurements by the same anatomist. The resulting average of these measurements was subsequently utilized for analysis.

Table 1. *Measurement points, abbreviations, and explanation of the pelvis in budgerigars with normal and egg retention*

Abbreviation	Measurements	Definition
Linear distances (cm)		
L1	Cranial ilium width	Horizontal distance greatest width between the cranial end of the os ilium
L2	Preacetabular tubercle width	Horizontal distance greatest width between the preacetabular tubercle
L3	Acetabula width	Horizontal distance between greatest width between the both acetabula (pelvis width)
L4	Middle pubis width	Horizontal distance between greatest width between the middle pubis
L5	Caudal pubis width	Horizontal distance between greatest width between the caudal end of the os pubis
L6	Caudal ischium width	Hhorizontal distance between smallest width between the caudal end of the os ischii
L7	Left iliopubic length	Distance between the cranial end of the left ilium and caudal end of the left pubis.
L8	Right iliopubic length	Distance between the cranial end of the right ilium and caudal end of the right pubis.
L9	Synsacrum length	Distance between the cranial and caudal end of the synsacrum.
Angle measurements (°)		
A1	Iliac arch	The angle between the cranial ilium arch
LA2	Left ischiopubic angle	The angle between the caudal point of the left ischium with the left pubis in the ventral image
RA2	Right ischiopubic angle	The angle between the caudal point of the right ischium with the right pubis in the ventral image
Ratios (length/width)		
L7/L1: Left iliopubic length/Cranial ilium width L8/L1: Right iliopubic length/Cranial ilium width L9/L1: Synsacrum length/Cranial ilium width L7/L2: Left iliopubic length/Preacetabular tubercle width L8/L2: Right iliopubic length/Preacetabular tubercle width L9/L2: Synsacrum length/Preacetabular tubercle width L7/L3: Left iliopubic length/Acetabula width L8/L3: Right iliopubic length/Acetabula width L9/L3: Synsacrum length/Acetabula width		L7/L4: Left iliopubic length/Middle pubis width L8/L4: Right iliopubic length/Middle pubis width L9/L4: Synsacrum length Middle pubis width L7/L5: Left iliopubic length/Caudal pubis width L8/L5: Right iliopubic length/Caudal pubis width L9/L5: Synsacrum length/Caudal pubis width L7/L6: Left iliopubic length/Caudal ischium width L8/L6: Right iliopubic length/Caudal ischium width L9/L6: Synsacrum length/Caudal ischium width

Statistical Analysis

In the calculation of the sample size of this study, which was carried out to determine the morphometric measurements of the pelvis radiologically in normal and egg-retention budgerigars, Power of test was determined by taking at least 80% and Type-1 error 5% for each variable. Shapiro-Wilk (n<50) and Skewness-Kurtosis tests were used to determine whether the continuous measurements in the study were normally distributed. Parametric tests were performed because measurements were normally distributed. Descriptive statistics for the study variables are expressed as mean, standard deviation, median, minimum, maximum, number (n), and percentage (%). "Independent T-test" was performed to compare the measurements according to the groups. The statistical significance level (α) was taken as 5% in the calculations and the SPSS (IBM SPSS for Windows, ver. 26) statistical package program was used for analysis.

Results

In this study, linear morphometric measurements were obtained from 9 parameters using radiological images of the ventrodorsal projection of the pelvis in normal and egg-retention budgerigars. Three measurement values were determined iliac arch, right ischiopubic angle, and left ischiopubic angle. The osteometric reference points used for the pelvic measurements of budgerigars are shown in *Fig. 2-A,B*. Finally, a total of 18 parameter ratio measurement values of the pelvis of budgerigars were calculated using the ratios of length and width measurements in morphometric measurements. The comparative statistical differences between these linear

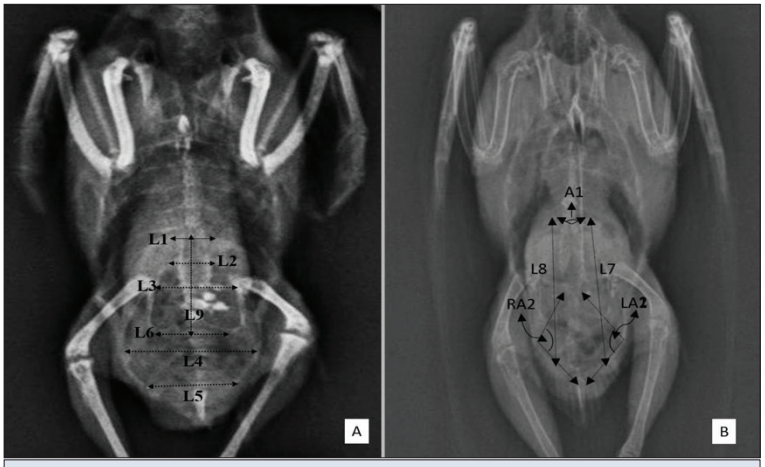

Fig 2. Ventrodorsal radiograph, pelvis measurements of egg retention (A) and normal (B) budgerigars, measurements: L1 (cranial ilium width), L2 (preacetabular tubercle width), L3 (acetabula width), L4 (middle pubis width), L5 (caudal pubis width), L6 (caudal ischium width), L7 (left iliopubic length), L8 (right iliopubic length), L9 (synsacrum length), A1 (iliac arch), LA2 (left ischiopubic angle), RA2 (right ischiopubic angle)

Table 2. *Descriptive statistics and comparison of pelvimetric measurement values in budgerigars with normal and egg retention*

Measurement Parameters	Normal (n=15)					Egg Retention (n=15)					P
	Mean	Std. Dev.	Median	Min.	Max.	Mean	Std. Dev.	Median	Min.	Max.	
L1	.851	.022	.850	.810	.890	.732	.037	.730	.660	.790	.001
L2	.991	.100	.990	.790	1.140	.981	.079	.970	.870	1.200	.779
L3	1.200	.028	1.190	1.160	1.250	1.194	.024	1.190	1.160	1.230	.526
L4	1.341	.072	1.360	1.150	1.430	1.197	.065	1.190	1.110	1.320	.001
L5	1.130	.111	1.170	.930	1.270	.895	.081	.880	.780	1.100	.001
L6	1.170	.035	1.180	1.100	1.230	.859	.062	.870	.760	.970	.001
L7	2.835	.077	2.850	2.690	2.970	2.846	.064	2.860	2.720	2.930	.682
L8	2.862	.048	2.860	2.780	2.950	2.851	.054	2.860	2.770	2.970	.573
L9	2.134	.041	2.140	2.050	2.200	2.144	.050	2.150	2.070	2.210	.553
A1	113.933	3.390	115.000	108.000	119.000	95.733	2.219	95.000	93.000	99.000	.001
LA2	115.933	2.251	116.000	112.000	119.000	104.667	2.320	105.000	101.000	109.000	.001
RA2	115.133	2.100	115.000	111.000	118.000	105.067	1.981	105.000	101.000	108.000	.001

**P<0.05; Independent sample T-test*

measurements, angle, and ratio measurements between the groups are presented in *Table 2* and *Table 3*. Statistically significant differences (P<0.05) between the measurement values in the tables were noted.

Comparative descriptive statistics of pelvimetric measurement values in normal and egg retention budgerigars are presented in *Table 2*. According to the table data, it was determined that other measurements, except for the L7 and L9 measurement values, were higher in normal laying budgerigars than in egg retention budgerigars. In addition, the mean measurement values of L1, L4, L5, L6, A1, LA2, and RA2 were significantly higher in the normal group than in the egg retention group (P<0.05). However, there was no statistically significant difference between the mean linear measurement values of L2, L3, L7, L8, and L9 of the pelvis of normal and egg retention budgerigars (P>0.05). Angle measurement values in normal and egg retention budgerigars were 113.93±3.39 (°) and 95.73±2.22 (°) respectively for iliac arch, 115.93±2.25 (°) and 104.67±2.32 (°) respectively for left ischiopubic angle; and 115.13±2.10 (°) and 105.07±1.98 (°) respectively for right ischiopubic angle.

The descriptive statistics and comparison of the ratios between the pelvimetric measurement values in normal and egg retention budgerigars are presented in *Table 3*. When we look at the table data in general, it was observed

Table 3. Descriptive statistics and comparison of the ratios between pelvimetric measurement values in in budgerigars with normal and egg retention

Ratios (length/ width)	Normal (n:15)					Egg Retention (n:15)					P
	Mean	Std. Dev.	Median	Min.	Max.	Mean	Std. Dev.	Median	Min.	Max.	
L7/L1	3.332	.113	3.326	3.112	3.561	3.897	.202	3.887	3.654	4.364	.001
L8/L1	3.364	.119	3.356	3.135	3.605	3.904	.207	3.917	3.564	4.348	.001
L9/L1	2.508	.088	2.506	2.303	2.651	2.936	.166	2.945	2.620	3.221	.001
L7/L2	2.891	.325	2.869	2.482	3.696	2.917	.233	2.960	2.358	3.225	.804
L8/L2	2.918	.317	2.859	2.527	3.570	2.924	.246	3.010	2.350	3.345	.959
L9/L2	2.175	.230	2.155	1.877	2.734	2.196	.163	2.216	1.792	2.483	.772
L7/L3	2.365	.105	2.367	2.187	2.560	2.385	.080	2.403	2.244	2.504	.563
L8/L3	2.386	.058	2.376	2.306	2.521	2.389	.063	2.372	2.301	2.560	.896
L9/L3	1.779	.053	1.782	1.691	1.856	1.796	.049	1.802	1.724	1.889	.370
L7/L4	2.120	.142	2.082	1.986	2.487	2.385	.150	2.420	2.142	2.622	.001
L8/L4	2.140	.121	2.142	2.000	2.417	2.390	.153	2.356	2.181	2.628	.001
L9/L4	1.595	.100	1.560	1.470	1.860	1.797	.092	1.840	1.580	1.940	.001
L7/L5	2.533	.272	2.475	2.118	3.118	3.205	.304	3.172	2.609	3.744	.001
L8/L5	2.558	.285	2.424	2.282	3.108	3.210	.287	3.241	2.600	3.667	.001
L9/L5	1.909	.213	1.830	1.640	2.300	2.412	.192	2.390	2.010	2.720	.001
L7/L6	2.425	.089	2.433	2.261	2.544	3.329	.266	3.247	2.938	3.789	.001
L8/L6	2.448	.086	2.441	2.309	2.588	3.334	.241	3.291	3.033	3.803	.001
L9/L6	1.825	.057	1.817	1.740	1.920	2.507	.189	2.471	2.155	2.797	.001

**P<0.05; Independent sample T-test*

that all the length/width ratio measurement values of the budgerigars were higher in the egg retention budgerigars than in the normal group. However, L7/L1, L8/L1, L9/L1, L7/L4, L8/L4, L9/L4, L7/L5, L8/L5, L9/L5, L7/L6, L8/L6, and L9/L6 the length/width ratio measurement values were found to be statistically significantly higher in egg retention budgerigars compared to the normal group. No statistically significant differences were found between the other length/width ratio measurements (L7/L2, L8/L2, L9/L2, L7/L3, L8/L3, and L9/L3) (P>0.05).

Discussion

Osteometric analyses in animals provide morphometric data for important scientific fields such as developmental, evolutionary, and forensic sciences. In addition, these morphometric data are frequently used for research on different animal species, determining morphological variations within species, taxonomic classification of animals, and determination of sexual dimorphism [9,10]. In addition, knowing the shape, morphologic, and morphometric features of the pelvis in birds, revealing allometric and phylogenic features, determining the ecological diversity, diversifying the locomotor modes, and evaluating the pathological conditions related to the pelvis are of great importance [2-4,11,12]. Exotic pet animals (such as birds, small mammals, and reptiles) medicine, and surgery have made great progress depending on developments in computer technologies, especially in the field of medical imaging. Diagnostic imaging modalities such as digital radiography and computed tomography are routinely used to examine any anatomical structure and to evaluate the efficacy of diagnosis, and treatment of various diseases in these animals [13]. In recent years, radiographic anatomical descriptions of other bones in the body, including the pelvis, have been made, especially for bird species such as parrots, partridges, ducks, and some raptors. In this way, the morphological features of osteological structures in radiological images have been determined [14-17]. This study was performed to obtain pelvimetric measurement values (linear osteometric measurements and angle), to determine the length/width ratios between pelvimetric measurements, and to determine the correlations between these measurements values using ventrodorsal X-ray images of the pelvis of normal and egg retention budgerigars.

Regarding the determination of the morphological and morphometric features of the pelvis of birds; common hawk cukoo *(Hierococcyx varius)* and yellow billed babbler *(Argya affinis)* [18], ostrich *(Struthio camelus),* emu *(Dromaius novaehollandiae),* domestic fowl *(Gallus gallus*

domesticus) and duck *(Anas platyrhynchos domesticus)* [19], blue and yellow macaw *(Ara ararauna)* [20], Guinea fowl and pigeon [21], Indian eagle owl *(Bubo bengalensis)* [22], Chinese goose *(Anser cygnoides)* [23], peahen *(Pavo cristatus)* [24], crested serpent eagle *(Spilornis cheela)* and brown wood owl (*Strix leptogrammica)* [25], emu *(Dromaius novaehollandiae)* [26], ostrich *(Struthio camellus)* [27], domestic duck *(Anas platyrhynchos domesticus)* [28], peacock and peahen [29], Japanese quail *(Coturnix coturnix japonica)* [30], scientific studies have been carried out on many bird species. In the present study, comparative descriptive statistics of pelvimetric measurement values in normal and egg retention budgerigars were examined. Accordingly, the mean measurement values of L1, L4, L5, L6, A1, LA2, and RA2 were significantly higher in the normal group than in the egg retention group ($P<0.05$). Based on these findings, we concluded that the pelvis of normally laying budgerigars was larger than that of the egg retention group. This may cause the egg to protrude more easily from the pelvic canal. In addition, there was no statistically significant difference between the mean linear measurement values of L2, L3, L7, L8, and L9 taken from the pelvis of normal and egg retention budgerigars ($P>0.05$). In this case, it is thought that this cause egg retention by causing further elongation of the pelvic canal.

According to some studies, on the egg retention problem, one or more eggs through cloaca, the oversized or malformed eggs, and deformed egg maternal abnormalities include a misshapen pelvis, reproductive disorders such as an oviducal stricture, dysfunction of oviductal muscle, oviduct and cloaca damage, or a nonreproductive mass such as an abscess or cystic calculi, or a complication during oviposition such as an egg fractures within the or two eggs try to pass through the pelvis [31-33]. Additionally, vitamin deficiency, systemic disease, disorders of calcium metabolism, improper nesting site, improper temperature, malnutrition, dehydration, and poor physical condition of the female may all lead to egg retention [33,34]. Delving into the multifaceted causes of egg retention, the study directed its attention towards discerning pelvic dimensions and uncovering morphometric insights within both typical budgerigars and those experiencing egg retention. The premise underlying this focus was the potential influence of pelvic bone structure in precipitating such conditions. The findings from this analysis are anticipated to furnish crucial supplementary parameters, valuable for the comprehensive clinical assessment of egg retention, and even extend their utility to the selection of breeding candidates.

Egg dimensions exhibit variability corresponding to the age of the hens, while feeding and management practices distinctly influence egg size [33]. Concurrently, researchers have indicated a positive correlation between pelvic length and body mass, with an observed decrease in relative egg size as pelvic length extend [34-36]. Furthermore, findings suggest a negative relationship between egg mass and female body mass [37]. In summary, a prevailing trend among birds indicates that the proportional dimensions of eggs tend to rise as pelvis size decreases overall [36]. Given the study's primary emphasis on pelvis morphometrics, metrics like egg size or egg mass remained unattainable. Consequently, an exploration of the correlation between pelvis size and egg dimensions could not be undertaken. However, drawing from analogous measurements in existing research, there exists potential for a comprehensive clinical investigation into the intricate interplay between pelvis size and egg dimensions.

In the process of reviewing the literature, no specific measurement parameters were identified that encompassed the length/width index measurements of avian pelvises. Nevertheless, a study by Anten-Houston et al.[35] addressed this gap by examining pelvis dimensions across 146 bird species. The study underscored the significance of discerning morphometric linear measurements, including pelvis length and width, as crucial for unveiling insights into matters such as locomotor behaviors, ecological diversity, and phylogenetic attributes within avian populations. In this study, when the ratios between the pelvimetric measurement values in normal and egg retention budgerigars were examined, it was observed that all the length/width ratio measurement values of the pelvis of budgerigars were higher in egg retention budgerigars than in the normal group. In addition, although the mean length/width ratio measurement values of L7/L2, L8/L2, L9/L2, L7/L3, L8/L3, and L9/L3 were higher in egg retention budgerigars compared to the normal group, this difference was not statistically significant ($p>0.05$). These findings lead to the conclusion that a higher length/width ratio may result in a probability of egg retention in budgerigars.

There were some limitations in our study. Firstly, in the presented study, we only took morphometric measurements from the ventrodorsal images of normal and egg-retention budgerigars aged 1-2 years. It could be evaluated by taking measurements comparatively in different age groups or by laterolateral and dorsoventral projection. However, we were only able to access ventrodorsal radiological images of normal and egg retetion budgerigars with this age group. Another limitation is that in this study, measurements were obtained on X-ray images since it was affordable. Advanced medical imaging methods such as Computed Tomography (CT), Magnetic resonance imaging (MRI), or measurements could have been taken by making 3D models on these images, but these imaging methods could not be used due to the expense of these devices and the need for specialist personnel. Finally, besides pelvic

measurements of budgerigars, detailed assessments of relationships could have been conducted by including measurements of body length, egg size, and egg mass. However, in this study, we focused on the morphometric pelvic measurements of both normal and egg-retained budgerigars.

In this study, radiographic pelvimetry of normal and egg-retention budgerigars was first evaluated in a comparative and comprehensive manner. With regard to the pelvis of female budgerigars, important descriptive morphometric data that could assist clinician veterinarians in the evaluation of radiographic images in various clinical application areas and could serve as a basis for future studies were obtained.

Availability of Data and Materials

The data sets generated for this study are available from the corresponding author (D. Koca) on reasonable request.

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Ethical Statement

All procedures were performed with the approval of the Van YYU Animal Experiments Local Ethics Committee (VAN YUHADYEK), approval number 2023/03-06.

Competing Interests

The authors declared that there is no conflicts of interest.

Author Contributions

D.K., O.Y. conceived, designed, and supervised the research procedure. M.E.S., T.A. collected data. D.K., O.Y., M.E.S., T.A. performed the anatomical analysis and measurements. D.K., O.Y. performed the statistical analysis, the imaging stage, and the language editing of the final manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version

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RESEARCH ARTICLE

Melatonin Protects Bovine Embryos from Heat Stress and Oxygen Tension and Improves Embryo Production *In vitro* [1]

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ABSTRACT

The objective of this study was to determine melatonin's ameliorating effects against heat stress and oxygen tension in developing bovine embryos *in vitro*. The oocytes were collected from ovaries obtained from a local abattoir, followed by *in vitro* maturation, fertilization, and embryo culture. During *in vitro* culture, embryos were exposed to 5% (Group I) and 20% (Group II) oxygen tension with 10^{-3}, 10^{-6}, and 10^{-9} molar (M) melatonin, along with the control group without melatonin (Group III). Compared to the control group, melatonin at 10^{-6} and 10^{-9} concentrations increased *in vitro* development rates and decreased caspase 3/7 activity at 5% and 20% oxygen tension ($P<0.01$). One-half of the zygotes were cultured under normal temperature (38.5°C) during the culture period, and the other half of the zygotes were heat stressed at 41°C for six hours. Then they transferred into the normal culture conditions for the rest of the period using 0, 10^{-6}, and 10^{-9} M of melatonin (Group IV). Under normal temperature (38.5°C), melatonin at 10^{-9} M was beneficial for *in vitro* development and DNA integrity. Under heat stress at 41°C, melatonin at 10^{-6} and 10^{-9} M was useful for *in vitro* development and DNA integrity ($P<0.05$). Supplementation of melatonin to embryo culture medium did not alter the caspase 3 and 7 activities ($P>0.05$). In conclusion, melatonin prevents the adverse effects of heat stress and O_2 tension on preimplantation bovine embryos *in vitro*.

Keywords: DNA integrity embryo, Heat stress, Melatonin, Oxygen concentration

INTRODUCTION

Bovine embryos produced *in vitro* are vital in accelerating cattle genetics, and *in vitro* culture conditions are far from optimum. The continuing efforts to develop optimum culture conditions for *in vitro* embryo production and better protocols for improving *in vivo* survival could help advance cattle breeding and genetics for precision farming of the animals.

One of the main issues with *in vitro* embryo culture system is the oxygen tension (O_2). Tubal and uterine O_2 tension is lower than atmospheric O_2 tension routinely used in mammalian embryo culture [1]. Culturing embryos in an environment with high O_2 content (20%) *in vitro* can result in the production of reactive oxygen species (ROS) containing more free radicals [2]. Excessive physiological levels of ROS have harmful effects on embryos and can cause significant damage to cell structures [3,4]. Therefore, there is a need to pursue research on oxidative stress and its prevention using different oxygen concentrations and antioxidants to improve embryo culture systems.

Sensitivity of the embryo to temperature depends on the cleavage stages [5,6]. Embryos at the zygote to the eight-cell stage are more sensitive to high temperatures than blastocysts or morula [7]. Temperatures ranging between 40.0°C and 42.0°C reduce the *in vitro* development of embryos at the zygotic to eight-cell stage but do not significantly affect *in vitro* development to morula and

blastocyst stages. Exposure of early embryos to high temperatures can cause damage to microfilaments and microtubules and swelling of mitochondria [8]. With increasing temperature, apoptotic cell numbers increase in 2-cell embryos [9].

Melatonin (N-acetyl-5-methoxy tryptamine), an endogenously produced indole, is found throughout mammalian species, vertebrates, invertebrates, algae, bacteria, and a variety of plants. The metabolites produced when melatonin scavenges free radicals are also highly effective scavengers [10]. For example, melatonin and its metabolites can directly scavenge hydroxyl free radicals, organic oxygen free radicals, peroxy free radicals, peroxynitrite anions, nitric oxide, and singlet oxygen [11]. In addition, melatonin can repair some oxidized molecules [12].

This study was designed to test the hypothesis that melatonin improves bovine embryo production by protecting them against heat stress and oxygen tension *in vitro*.

Materials and Methods

In vitro Maturation (IVM) and Fertilization (IVF)

The ovaries were obtained from a local abattoir within lactated ringer solution including 1000 IU Penicillin/mL + 1000 µg/mL streptomycin at 37°C were transferred to Parrish Lab (Department of Animal and Dairy Science, University of Wisconsin, Madison, WI, USA). Cumulus-oocyte complexes were aspirated from antral follicles sized 1-5mm using an 18-gauge needle. The standard IVM and IVF protocols were used according to the established methods as previously described in the Parrish lab [13]. For IVM, in all groups, oocytes were matured in TCM-199 with the following supplementations: 10% heat-treated FBS, 1 µg/mL of estradiol 17-ß, 0.2 mM Na-pyruvate, 5 µg/mL of LH (NIH-oLH-26), 0.5 µg/mL of FSH (NIH-FSH-S-17), and 50 µg/mL gentamycin. For the purpose of IVF, *in vitro* matured oocytes were fertilized with frozen-thawed semen after Percoll gradient separation.

In Vitro Embryo Culture (IVC)

Presumptive zygotes were cultured in KSOM medium with different melatonin concentrations (0, 10^{-3}, 10^{-6}, and 10^{-9} M) under 20% *(Group I)* and 5% *(Group II)* oxygen conditions in an adjustable three gas incubator with no FCS supplementation during the culture period. To compare the differences between 20 and 5% oxygen, zygotes from the same batch were cultured using the same melatonin concentrations (0, 10^{-3}, 10^{-6}, and 10^{-9} M) in *Group III*. While half of the zygotes were cultured under normal temperature (38.5°C) during the culture period, the other half of the zygotes were heat stressed with an adjustable incubator for 6 h at 41°C. Then they were transferred into the normal culture conditions for the rest of the period using 0, 10^{-6} and 10^{-9} M melatonin *(Group IV)*.

Nick End Labeling (TUNEL)

The percentage of DNA fragmented blastomeres and average cell numbers were determined using the TUNEL assay followed by the Fluorescein Apoptosis Detection System. Day 8 embryos with intact zona pellucida were fixed and permeabilized, according to Brison and Schultz [14]. For this purpose, embryos were kept in 2% TritonX-100 (Bio-Rad) solution for 180 minutes to ensure permeabilization, washed twice in PBS-polyvinylpyrrolidone (PVA) solution, and incubated in fluorescent-conjugated dUTP and TdT solution (Roche Diagnostics, Tokyo) in the dark at 37°C for 1 h. Next, they were kept in DNAse (1000 IU mL-1) solution as a positive control at 37°C for 20 min. Embryos in the negative control groups were incubated in a fluorescent-dUTP solution without TdT. Following the TUNEL, the embryos were washed three times, and propidium iodide (PI) (50 µg-1) was added to stain all nuclei after adding RNAse (50 µg RNAse for 60 min at room temperature). Day 8 blastocysts were collected and labeled with a TUNEL assay Fluorescein Apoptosis Detection kit. The total number of cells and TUNEL-positive blastomeres were counted for 0, 10^{-6}, and 10^{-9} M melatonin in both normal and heat-stressed culture conditions.

Caspase 3/7 Activity

Eight-cell embryos (10 embryos per assay) were incubated at room temperature for eight more hours. Then, the resulting luminescent signal representing the amount of caspase activity in the sample was measured using a luminometer. Briefly, embryos were subjected to Caspase-Glo® 3/7 Assay and Caspase-Glo® 8 Assay (Promega Corporation, Madison, WI, USA) kits. Again, 30 µL of the reagent included in the kit was used. After 30 minutes of incubation in the dark, values were measured with the GloMax®-96 Microplate Luminometer using the Caspase-Glo® Program.

Statistical Analysis

The percentages of blastocysts were calculated from the number of presumptive zygotes. The arcsine square-root remodeled information was analyzed with a randomized complete block style exploitation SAS mixed procedure to see the numerous variations among the treatments. Significant differences in blastocyst cell numbers and the TUNEL-positive cells per blastocyst were determined by employing a unidirectional analysis of variance. Differences at $P<0.05$ were considered significant. In the study, three replicates were run for caspase 3/7 activity (10 eight-cell embryos/replicate), and five replicates

Table 1. Development of embryos cultured under 20% oxygen tension in the presence of different melatonin concentrations

Melatonin (M)*	Zygotes (n)	Oocytes Cleaved		Embryos Developed >		Blastocyst on Day 8 (%)
		at 32 h (%)	at 48 h (%)	8-cell (%)	16-cell (%)	
0	380	39.7	73.9[b]	39.9	22.0[a]	10.7[b]
10^{-3}	387	35.7	76.6[b]	25.1	7.5[b]	0.0[c]
10^{-6}	380	38.9	85.7[a]	38.2	22.5[a]	17.5[a]
10^{-9}	374	42.6	85.8[a]	47.3	26.1[a]	16.9[a]

**Presumptive zygotes were cultured in KSOM medium with different melatonin concentrations (0, 10^{-3} 10^{-6} and 10^{-9} M: molar) with no FCS supplementation during the culture period. Data represent the mean from six replicates. a-c: Values with different superscripts in the same column were significantly different (P<0.05)*

Table 2. Development of embryos cultured under 5% oxygen tension in the presence of different melatonin concentrations

Melatonin (M)*	Zygotes (n)	Oocytes to Blastocyst		Hatched Blastocyst Day 9 (%)
		Day 8 (%)	Day 9 (%)	
0	394	14.9 [b]	17.1 [b]	4.7 [b]
10^{-3}	408	1.6 [c]	2.3 [c]	0.0 [c]
10^{-6}	400	21.4 [a]	24.9 [a]	6.6 [a]
10^{-9}	414	21.8 [a]	23.3 [a]	5.1 [a]

** Presumptive zygotes were cultured in KSOM medium with different melatonin concentrations (0, 10^{-3}, 10^{-6} and 10^{-9} M: molar) with no FCS supplementation during the culture period. Data represent the mean from six replicates. a-c: Values with different superscripts in the same column were significantly different (P<0.05)*

were performed (50-75 zygotes for group/replicate) for blastocyst development, DNA fragmentation, and blastocyst cell number.

Results

Melatonin improved embryo development in 20% oxygen tension in vitro

We found that melatonin supplementation at 10^{-6} and 10^{-9} M concentrations increased embryo cleavage rate in 20% oxygen tension compared to the control group at 48 h, in 16-cell embryo rates, and in blastocyst rate on day 8 (P<0.05). But, melatonin supplementation at 10^{-3} M concentration decreased embryo cleavage according to the control group in 16-cell embryo rates and blastocyst rate on day 8 (P<0.05) *(Table 1)*.

Melatonin improved cleavage rates in 5% oxygen tension in vitro

We found in Experiment II that melatonin supplementation at 10^{-6} and 10^{-9} M concentrations increased embryo cleavage rate as compared to the control group on day 8 and day 9 embryos cultured under 5% oxygen tension (P<0.05). Conversely, melatonin supplementation at 10^{-3} M concentration decreased embryo cleavage rate according to the control group on day 8 and day 9 embryos cultured under 5% oxygen tension (P<0.05) *(Table 2)*.

High doses of melatonin increased caspase 3/7 activity in 20% tension

We showed that 10^{-3} M concentrations of melatonin

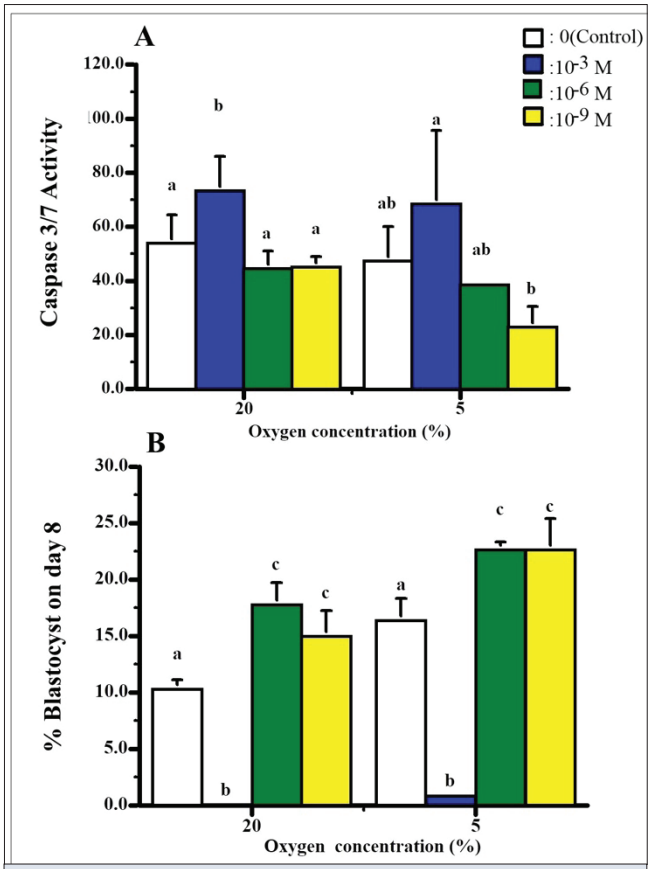

Fig 1. Effect of melatonin on caspase 3/7 activity (A) and blastocyst development (B) of preimplantation bovine embryo cultured under different oxygen concentrations (20% vs. 5%). a-b: Values with different superscripts indicate significant difference within the same culture temperature (P<0.05)

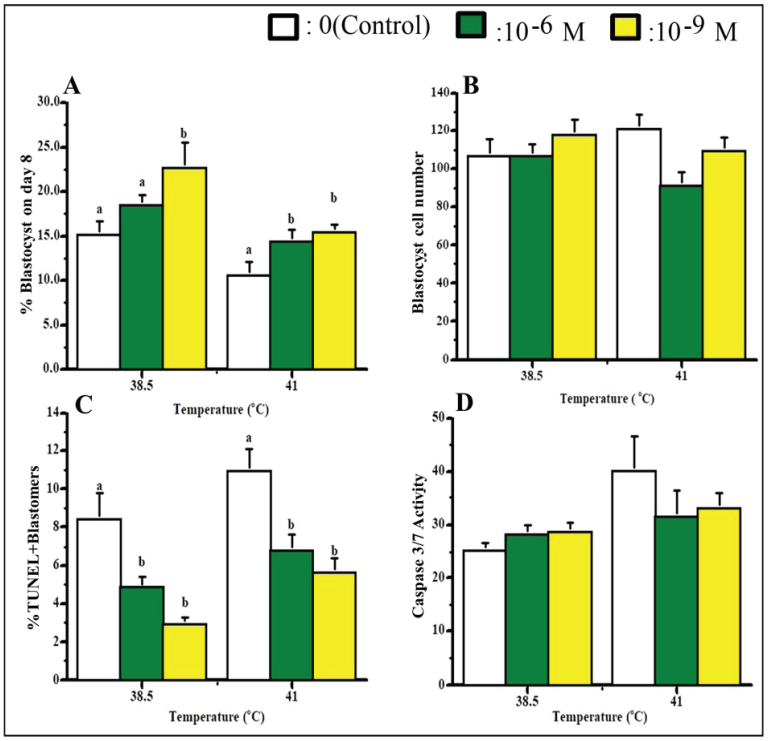

Fig 2. The effects of melatonin on blastocyst development (A), total cell number (B), DNA fragmentation (C) and caspase 3/7 activity (D) of bovine embryos cultured under normal (38.5°C) and heat stressed (41°C) conditions. a-b: Values with different superscripts indicate significant difference within the same culture temperature (P<0.05)

increased caspase 3/7 activity at 20% oxygen concentration in *Fig. 1-A* (P<0.05) but did not change at 5% oxygen tension (P>0.05). In *Fig. 1-B*, 10^{-6} and 10^{-9} M melatonin concentrations increased blastocyst rates at 20% and 5% oxygen tension (P<0.05).

Melatonin decreased DNA fragmentation and increased blastocyst rates in vitro in high temperatures

We found that supplementation of 10^{-9} doses of melatonin increased the blastocyst rates at both 38.5°C and 41°C compared to the control *Fig. 2-A*, while blastocyst rates were also increased with 10^{-6} at 41°C *Fig. 2-A* (P<0.05). In addition, both doses of melatonin decreased DNA fragmentation at the temperature of 38.5°C and 41°C *Fig. 2-B* (P<0.05). However, it was determined that blastocyst cell numbers and caspase 3/7 activity rates did not change *Fig. 2-B* (P>0.05).

Discussion

As an effective free radical scavenger and antioxidant, melatonin and its metabolites (Cyclic 3-hydroxy melatonin, N^1-acetyl-5-methoxykynuramine, and N^1-acetyl-N^2-formyl-5-methoxykynuramine) are widely used to protect embryos cultured *in vitro*. Adding melatonin to the culture medium can promote early embryonic development in mice [15]. The beneficial effects of melatonin on the development of *in vitro* production (IVP) embryos include increased blastocyst formation rate, average cell number, and hatching rate [16]. Melatonin improves the quality and survival rate of sheep embryos *in vitro* and *in vivo* [17,18] and increases the division rate of embryos and the total cell number of blastocysts [19]. In particular, its hydrophilic and hydrophobic structure allows melatonin to pass through membranes and disperse into tissues quickly [20]. Although it is known that melatonin has antioxidant properties, plays an antiapoptotic role and protects DNA integrity; it is unknown how it affects *in vitro* developmental rates, DNA integrity, apoptotic effect, and cell numbers in blastocysts exposed to high oxygen tension or temperature. The goal of this research was to pursue the intriguing idea that melatonin can protect embryos from damage that may occur at high oxygen tension and temperatures by taking advantage of the mentioned effects.

It was shown in this study that *in vitro* culture with a low dose of melatonin (10^{-6} M, 10^{-9} M) on heat stress and oxygen tension may have significant effects on the final development rate and cell number of bovine embryos. Incubation with 10^{-6} and 10^{-9} M melatonin concentrations improved bovine embryo development cultured in the KSOM media at both oxygen levels regarding blastocyst development and cleaved embryos at 48 h compared to

no melatonin supplementation. However, when 10^{-3} M melatonin was added to the KSOM media with no serum during embryo culture, melatonin decreased embryo development beyond 16-cell stages regardless of oxygen concentration. Moreover, the higher doses of melatonin (10^{-3} M) increased the caspase 3/7 activity, a sign of apoptosis at 8-cell stage embryos. Furthermore, the blastocyst development was higher when cultured under 5% oxygen tension compared to 20% with no melatonin supplementation. Additionally, 10^{-6} and 10^{-9} M melatonin concentrations improved blastocyst development when embryos were cultured under 5% oxygen ($P<0.05$). Although the blastocyst cell numbers did not differ, heat stress caused a decrease not only in blastocyst development but also increased DNA damage, both of which were improved by culturing embryos with melatonin.

Low concentration (5%) of oxygen tension has been shown to have a positive effect on *in vitro* growth rates in buffalo [21] and cattle [22]. In addition, low-concentration oxygen plays a stimulatory role but does not change blastocyst rates. However, other researchers [24,25] claim thatneither high nor low oxygen concentrations affects *in vitro* growth rates. On the other hand, adding melatonin to the culture medium has been shown to increase the *in vitro* growth rate [26]. In fact, supplementing melatonin under high oxygen concentration promoted the development of *in vitro* bovine embryos [27]. Similarly, in this study, we demonstrated that adding 10^{-6} and 10^{-9} M melatonin increased *in vitro* growth rates in both low (5%) and high oxygen concentrations (20%) compared to the control group. However, addition of 10^{-3} M melatonin decreased the blastocyst rates. It is evident that H_2O_2, which may result from a high concentration of O_2 ratio, can be removed by melatonin.

Several studies have demonstrated that melatonin can protect embryos and oocytes exposed to heat stress in different animal species [28-30]. In particular, embryonic deaths have been reported to occur with increased summer temperatures, particularly in the northern hemisphere [31]. The primary issue caused by heat stress is the production of ROS [32]. In this study, adding 10^{-6} M and 10^{-9} M melatonin to the culture medium increased the blastocyst rates. Heat stress has been reported to increase ROS production in embryos, increase the number of apoptotic cells, and decrease *in vitro* development [33]. Moreover, ROS generated by the increase in temperature can cause DNA damage in cells [34]. In cattle, heat shock protein 70 begins to be produced during early embryonic development, and its expression continues to increase until the morula stage [35]. Thus, embryos are highly sensitive to temperature changes during the early stages of embryonic development in cattle [36]. As shown in *Fig. 2*, blastocyst rates decreased with an increase in the temperature (41°C); however, adding 10^{-6} and 10^{-9} M melatonin preserved the *in vitro* growth rates compared to the control group. The cleavage data might include cytoplasmic divisions stemming cytoplasmic fragmentation as well.

During preimplantation embryo development, apoptosis plays an essential role in removing defective cells and proceeds in a coordinated manner. This process continues to develop in a certain harmony with the expression of antiapoptotic (BCL-W, BCL-2, BCL-XL) and proapoptotic (BAK, BAX, BAD) members of the BCL-2 family proteins expressed at different levels [37,38]. However, there are reports that this coordination progresses abnormally under the culture conditions [39,40]. Phosphorylated BECLIN1 can bind to BCL2 in the process of apoptosis, increase free BAX in cells, and cause mitochondrial-dependent apoptosis [41]. Melatonin ameliorates reactive oxygen species by promoting the expression of MnSOD and SIRT1.

Melatonin's function is dependent on melatonin receptors 1 and 2 (MT1 and MT2), G protein-coupled membrane receptors that can heterodimerize with the G protein receptor, affecting cellular functions [42]. Caspase 3/7 activity is also a method used to determine apoptosis [43]. In our study, caspase 3/7 activity was evaluated under 5% and 20% oxygen tensions and heat stress. The group that used 10^{-9} M melatonin at 5% oxygen tension showed the lowest caspase 3/7 activity ($P<0.05$), while in control, 10^{-6} M, and 10^{-9} M melatonin groups at 20% oxygen tension, the lowest caspase 3/7 activity was observed ($P<0.05$). The results showed that the use of 10^{-3} M melatonin increased caspase 3/7 activity under both oxygen tensions. Researchers state that 10^{-3} and higher doses of melatonin are insoluble, hydrophobic, and cannot penetrate cells and that high-dose melatonin has an apoptotic effect [44,45]. In our study, we demonostrated that high-dose melatonin at both 5% and 20% oxygen tensions increased caspase 3/7 *(Fig. 1),* consistent with other studies. It was determined that the activity of caspase 3/7 did not change at 38.5°C and 41°C in both the control and the groups containing melatonin. Although it does not alter caspase 3/7 activity, melatonin can modulate the up- or down-regulation of different genes related to temperature and apoptosis.

Researchers have stated that melatonin protects DNA integrity by preventing ROS in embryos and oocytes [46,47] in many animal species [48]. In this study, when DNA damage was examined, it was revealed that melatonin protects DNA at high temperatures compared to the control group ($P<0.05$). The resulting DNA damage is thought to be due to the failure of HSP70 proteins to be produced at the beginning of the embryonic development period, resulting in the formation of ROS and damage to the cell DNA.

Studies show that melatonin positively affects both oocyte maturation [49] and *in vitro* embryo development rates. Similarly, in this study, as in other studies [26,50], melatonin allaviated oxygen tension and heat stress, improving blastocyst development and decreasing the number of TUNEL-positive nuclei by protecting embryos from damage caused by oxidative and heat stress. The result of this study suggest that the adding melatonin in culture media at concentrations of 10^{-6} M or 10^{-9} M can positively impact embryo development.

Data Availability

All data in this article can be obtained through the corresponding authors.

Ethical Approval

Ethics committee approval is not required for this study.

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Conflict of Interest

The authors declared no interest.

Authors' Contributions

AK and EM conceived the research idea, and AK conducted the research. MH, AK, and EM searched literatures. AK, JJP and JS performed quality assessment. AK and JJP completed data analysis and MH, AK, EM, MB, and EM drafted the manuscript. EM, AK, JJP, JS, and MH revised the article. All authors discussed and contributed to the final manuscript.

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RESEARCH ARTICLE

Evaluation of Some Systemic Inflammatory Biomarkers in Canine Malignant Mammary Tumors [1]

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ABSTRACT

The aim of this study is to investigate whether neutrophil-to-lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR), platelet-to-lymphocyte ratio (PLR), systemic immune-inflammation index (SII), albumin-to-globulin ratio (AGR), and prognostic nutritional index (PNI) parameters could be used as biomarkers for canine malignant mammary tumors (MMTs), and the changes in these parameters according to different tumor (T), lymph node (N), and metastasis (M) stages (TNM I-II-III, TNM IV, TNM V) and the number of affected mammary glands (single, multiple). Thirty-seven with MMT and 20 healthy dogs were used in this study. Complete blood count and biochemistry analysis were performed in all dogs. Tumor material is removed by tru-cut and sent to the pathology laboratory for diagnosis. NLR, PLR, and SII values increased, and LMR and PNI values decreased in dogs with MMT. Median NLR values increased and median LMR and PNI values decreased as the TNM stage progressed. In dogs with a single MMT, median NLR, and PLR values were found to be lower than in dogs with multiple MMTs, and median LMR, SII, AGR, and PNI values were higher. The present results indicated that NLR, LMR, PLR, SII, and PNI parameters could be used as biomarkers for canine MMT. Also, NLR, LMR, PLR, SII, PNI, and AGR parameters may be valuable biomarkers that reveal the degree of systemic immune response according to different TNM stages and the number of affected mammary glands.

Keywords: Canine, Malignant mammary tumor, Inflammation, Biomarkers, Oncology

INTRODUCTION

Cancer begins when the cell becomes unable to respond to the mechanisms that control its division due to a number of structural defects in the cell [1]. Initially, cells that have undergone neoplastic transformation are detected by the host immune system and eliminated by various mechanisms. However, this effect of the immune system against tumors can be weak and insufficient, and even some components contribute to tumor development at the same time [2,3]. In fact, the tumor-associated immune response is more likely to contribute to tumor growth, progression, and immunosuppression than it is to form an effective host antitumor response [4]. Revealing the links between cancer and inflammation has implications for the prevention and treatment of cancer [3,5]. It has been stated that cancer-related inflammation is associated with changes in circulating white blood cells and some biochemical parameters both in humans and dogs [6-8]. Lymphocytes have the most important role in the immune response against cancer [9,10]. Neutrophils and monocytes also have critical roles in antitumor immunity, they exhibit their antitumor functions directly or work with lymphocytes. However, when they gain a cancer-supportive structure, they start to exhibit quite important pro-tumor functions and contribute to tumor progression, promote metastasis, and show immunosuppressive activity [11,12]. Also, neutrophils can prevent the anticancer functions of lymphocytes [13]. Platelets become active by interacting with cancer cells and show many functions that participate

in cancer progression, metastasis, and inflammation [14]. Therefore, parameters such as neutrophil-to-lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII) are calculated in many studies conducted to date. High NLR, PLR and SII values and a low LMR value are associated with advanced disease and poor prognosis. Moreover, they have been shown to be useful for the selection of the appropriate treatment method and the management of the disease in several neoplastic conditions both in humans [5,6,15-20] and dogs [21-23]. The albumin-to-globulin ratio (AGR) parameter is a useful biomarker for revealing systemic inflammation associated with malignancies and has prognostic value in various types of cancer including breast cancer in humans [24-26]. The prognostic nutritional index (PNI) is a new systemic immune-nutrition index and represents the immune and nutritional status of the host [27].

It has been demonstrated in many studies that the parameters we mentioned above can be used as biomarkers in the diagnosis, management of the disease, and prediction outcome in many cancer types, including a large population of breast cancer [15,19,24,27]. Only NLR has been reported to have a prognostic value in dogs with mammary tumors [28]. Therefore, this study aimed to investigate, firstly, whether the parameters indicating the systemic inflammatory response associated with cancer could be used as biomarkers for canine MMT, and secondly, the changes of these parameters according to different TNM stages and the number of affected mammary glands.

Material and Methods

Ethical Statement

The study was approved by the Ethics Committee on Animal Research of Bursa Uludag University (Approval No: 2020-03/03).

Animals

The first group of dogs in this study included 37 dogs with malignant mammary tumor (MMT) aged 3 to 16 years. The breeds of the dogs included Terrier (7), Golden Retriever (5), Crossbreed (5), Cocker (4), Doberman (2), Siberian Husky (2), Doberman Pinscher (1), Jack Russel Terrier (1), Dogo Argentino (1), Rottweiler (1), German Shepherd (1), Chihuahua (1), Pekingese (1), Beagle (1), Cane Corso (1), American Staffordshire Terrier (1), Kurzhaar (1) and Alabai (1). The second group of dogs in this study included 20 healthy dogs aged 2 to 10 years from different breeds (Crossbreed (11), Alabai (3), Golden Retriever (2), Labrador Retriever (1), Dogo Argentino (1), Border Collie (1) and Chow Chow (1)) as controls. None of the dogs had concurrent systemic inflammatory or immune-related diseases. Two of the dogs with MMT had cancer-related cachexia. The diet of the dogs in the study was not uniform. Characteristics of dogs with MMT and the control group are summarized in *Table 1*.

Table 1. *Characteristics of the dogs with malignant mammary tumors (MMTs) and the control group*

Characteristics	n		Percentage (%)	
	Dogs with MMTs	Control	Dogs with MMTs	Control
Age				
≤8	17/37	17/20	45.95	85
Neutering Status				
Neutered	6/37	6/20	16.22	30
Intact	30/37	14/20	81.08	70
Remnant Ovary	1/37	0	2.70	0
Pseudopregnancy History	6/37	2/20	16.22	10
Contraception History	4/37	0	10.81	0
TNM Stage				
I-II-III	14/37		37.84	
IV	15/37		40.54	
V	8/37		21.62	
Number of Tumors				
Single	10/37		27.03	
Multiple	27/37		72.97	
Histopathological Evaluation				
Scirrhous Adenocarcinoma	9/37		24.32	
Solid Adenocarcinoma	6/37		16.22	
Tubular Adenocarcinoma	5/37		13.51	
Invasive Ductal Carcinoma	3/37		8.11	
Adenocarcinoma	3/37		8.11	
Papillary Adenocarcinoma	2/37		5.41	
Malignant Mixed Tumor	2/37		5.41	
Tubulopapillary Carcinoma	2/37		5.41	
Tubular Carcinoma	2/37		5.41	
Spindle Cell Carcinoma	1/37		2.70	
Invasive Cribriform Carcinoma	1/37		2.70	
Carcinoma	1/37		2.70	

All dogs had a general examination, complete blood count, and biochemistry analysis, in addition to that thoracic radiography and intra-abdominal ultrasonography were done in dogs with mammary tumors. Ultrasound-guided biopsy samples were obtained from mammary tumors with a 14-gauge tru-cut biopsy needle. Fine-needle biopsy was performed from local lymph nodes with a 21-gauge needle. Lidocaine Hydrochloride (Jetocaine, ADEKA, Samsun, Turkey) was used at 4 mg/kg for local anesthesia before biopsies. All biopsies were sent to the pathology laboratory. 37 dogs that were found to have MMT according to the pathological examination were included in the study. All dogs with MMT were evaluated

according to the modified TNM system [TNM I-II-III ($T_{1-2-3}N_0M_0$), TNM IV ($T_{1-2-3}N_1M_0$), TNM V ($T_{1-2-3}N_{0\_1}M_1$)] as described by Goldschmidt et al.[29]. Those with tumors in one mammary gland were grouped as "single" and those with tumors in more than one mammary gland were grouped as "multiple".

Biochemical, Hematology Analyses and Biomarkers

Blood was taken from the vena cephalica antebrachii into EDTA tubes for complete blood count and into dry tubes for measurement of biochemical values. The blood samples centrifuged at 4000 rpm for 10 min and their serums were separated on the day of the examination. A complete blood count was performed with the "Hasvet VH5R, Automated Hematology Analyzer" (Urit, China) device. Total Protein (TP), Albumin (ALB), Alkaline Phosphatase (ALP), Glucose (GLU), Total Bilirubin (TBIL), Inorganic Phosphorus (IP), Total Cholesterol (TCHO), Gamma-Glutamyl Transferase (GGT), Alanine Aminotransferase (ALT), Calcium (Ca), Creatinine (CRE), Blood Urea Nitrogen (BUN), Globulin (GLOB), values were determined by "FUJI DRI-CHEM NX500V IC Chemistry Analyzer" (FUJIFILM, Japan) device.

The NLR was determined by dividing the neutrophil (10^9/L) by lymphocyte counts (10^9/L) [21]. The LMR was calculated by dividing the lymphocyte (10^9/L) by monocyte counts (10^9/L) [21]. The PLR was detected by dividing the platelet (10^9/L) by lymphocyte counts (10^9/L) [21]. The SII was calculated by multiplying the neutrophil count (10^9/L) by the platelet count (10^9/L) and dividing by the lymphocyte count (10^9/L) (N × P/L) [21]. The AGR was estimated by dividing the albumin (g/dL) by globulin (g/dL) [24]. The PNI parameter was obtained by summing 10 times serum albumin (g/dL) and 0.005 times lymphocyte count (per mm^3) (10 × ALB + 0.005 × L) [27].

Histopathological Evaluation

Biopsy samples of mammary tumors were fixed in 10% buffered formalin and embedded in paraffin. Sections of 4 µm thickness were taken from the tissues passed through alcohol and xylol and stained with hematoxylin&eosin. Tubule and mammary alveolar formation, nuclear polymorphism, pleomorphism, mitosis index, inflammatory infiltration, necrosis, adjacent tissue invasion, and lymph node metastasis were evaluated according to the World Health Organization criteria for canine mammary tumors [30].

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 28 for Windows. Shapiro-Wilk test was used to examine whether the data were normally distributed. Nonparametric tests were used for data that did not show normal distribution. The comparison of the values of the dogs with MMT and the control group was done by using the Mann-Whitney U-test, which is one of the nonparametric tests. The Kruskal-Wallis test was used for the comparison according to the TNM stage and the number of affected mammary glands. Significance values had been adjusted by the Bonferroni correction for multiple tests. Statistical significance was set at P value <0.005.

Results

The mean age of dogs with MMT and the control group was 9.59±3.02 and 6.20±2.31 years, respectively (P<0.001). 86.48% of dogs with MMT and 45% of the control group were pure-breed dogs. The most common breeds of dogs with MMT included Terriers 18.92% (7/37), Golden Retrievers 13.51% (5/37), Crossbreeds 13.51% (5/37), and Cockers 10.81% (4/37). Characteristics of dogs with MMT are summarized in *Table 1*. Hematological and biochemical data and reference intervals of dogs with MMT and control group are given in *Table 2* and *Table 3*. Radiographic images of two dogs with lung metastases and histopathological image samples of dogs with MMTs are given in *Fig. 1* and *Fig. 2*. Differences between the dogs with MMT and the control group were found statistically significant for NLR, LMR, PLR, SII and PNI parameters (P<0.05). The AGR value did not show any differences between the groups (P=0.496). The data are summarized in *Table 4*.

In the comparison made according to TNM staging, the

Table 2. *Hematological parameters of the dogs and the referance ranges*

Variable	Dogs With CMT		Control		Referance
	Median(min:max)	Mean±SEM	Median(min:max)	Mean±SEM	
NEU10^9/L	8.19 (3.47:36.1)	9.36 ± 0.90	6.05 (3.74:9.66)	6.13±0.37	2.7-9.4
MON 10^9/L	0.61 (0.12:4.02)	0.75 ± 0.11	0.32 (0.09:0.64)	0.34±0.03	0.1-1.3
LYM 10^9/L	1.55 (0.78:4.47)	1.79 ± 0.15	2.48 (1.15:4.91)	2.53±0.19	0.9-4.7
PLT 10^9/L	349 (147:693)	377.78 ± 24.51	263 (172:384)	265.95±12.35	186-545

Data expressed as median (min:max) and mean ± SEM. NEU=neutrophils, MON=monocytes, LYM=lymphocytes, PLT=platelets.

Table 3. *Biochemical parameters of the dogs and the referance ranges*

Variable	Dogs With MMTs		Control		Referance Values
	Median(min:max)	Mean±SEM	Median(min:max)	Mean±SEM	
TP g/dL	6.8 (5.6:8.2)	6.71±0.10	6.5 (5.9:7.1)	6.48±0.08	5.5-7.2
ALB g/dL	3.4 (2.3:4.5)	3.39±0.08	3.4 (3:4)	3.39±0.06	3.2-4.1
GLOB g/dL	3.1 (2.5:5.4)	3.29±0.11	3.1 (2.6:3.7)	3.09±0.06	1.9-3.7
ALP U/L	56 (14:287)	80.31±11.35	38 (19:86)	43.63±4.93	7-115
GLU mg/dL	104 (64:134)	104.2±2.38	107.5 (70:123)	101.95±3.50	68-104
TBIL mg/dL	0.3 (0.2:0.6)	0.28±0.02	0,2 (0.2:0.4)	0.24±0.02	0-0.2
IP mg/dL	3.6 (1.5:6.5)	3.61±0.17	3.5 (2.9:4.6)	3.65±0.10	2.7-5.4
TCHO mg/dL	287 (169:437)	302.18±15.49	165 (108:275)	175±12.59	136-392
GGT U/L	<10	<10±0	<10	<10±0	0-8
ALT U/L	41.5 (10:149)	45.16±3.94	36.5 (20:75)	41.25±3.25	17-95
CA mg/dL	10.6 (9.3:12.1)	10.65±0.11	11.1 (10.2:11.9)	11.15±0.11	9.4-11.1
CRE mg/dL	0.75 (0.41:1.6)	0.81±0.05	0.69 (0.43:0.97)	0.66±0.031	0.6-1.4
BUN mg/dL	14.05 (5:29)	14.62±1.09	13 (5:19.9)	12.43±1.01	9-26

Data expressed as median (min:max) and mean ± SEM. ALB=albumin, GLOB=globulin, APL=alkaline phosphatase, GLU=glucose, TBIL=total bilirubin, IP=inorganic phosphorus, TCHO=total cholesterol, GGT=gamma-glutamyl transferase, ALT=alanine aminotransferase, CA=calcium, CRE=creatinine, BUN=blood urea nitrogen

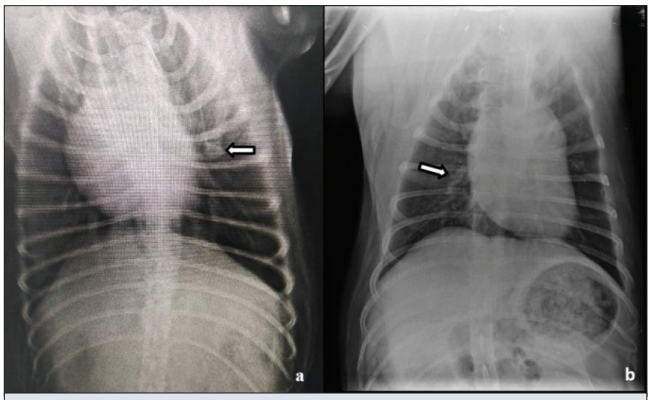

Fig 1. Ventrodorsal radiographic views of the thorax in a 5 years old Terrier (a) and in an 8 years old Cane Corso (b) with MMTs

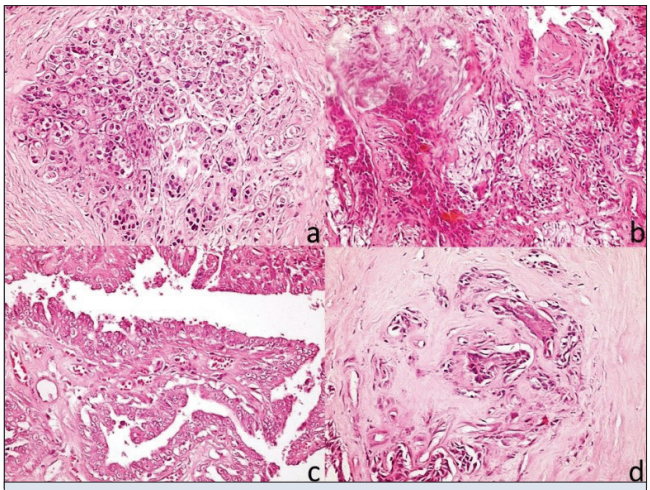

Fig 2. a- Solid Adenocarcinoma. Neoplastic cells exhibit a pronounced pleomorphism and fill the alveolar lümen, **b**- Malignant Mixed Tumor. The neoplasm contains multiple clusters of carcinoma cells as well as regions characterized by the proliferation of myoepithelial cells, **c**- Tubulopapillarycarcinoma. Neoplastic cells have a vesicular appearance and are binucleated. Tubular epithelia have formed multiple layers, **d**- Scirrhous Adenocarcinoma. Excessive increase in fibrous stroma led to deterioration of lobular structure. Alveoli and ducts are not visible

NLR value did not show statistical significance between the control group and TNM I-II-III stage (P=0.069), while the differences between each of TNM IV and TNM V and the control group were significant (P<0.001, P=0.012, respectively). The LMR values were found to be statistically significantly lower in each of the TNM I-II-III, TNM IV, and TNM V stages compared to the control group (P=0.032, P<0.001, P=0.006, respectively). The PLR values were determined significantly higher in TNM I-II-III and TNM IV stages versus the control group (P=0.03, P=0.001, respectively). However, the difference between the PLR values of the TNM V group and the control group was not significant (P=0.076). The SII values were significantly higher in TNM I-II-III, TNM IV, and TNM V stages versus the control group (P=0.018, P<0.001, P=0.038, respectively). The overall test did not show significant differences between the groups for the AGR parameter (P=0.344). The PNI values did not show statistically significant differences between TNM I-II-III and TNM V stages versus the control group (P>0.05), but the difference in the PNI value between TNM IV and control group was significant (P=0.039). There was no significant difference in the biomarkers assessed among TNM I-II-III, TNM IV, and TNM V stages *(Table 5)*.

The NLR, LMR, PLR, and SII values were found to be

Table 4. *Comparison of dogs with malignant mammary tumors (MMT) and control group*

Parameters	Dogs with MMTs		Control		P Value
	Median (min : max)	Mean ± SEM	Median (min : max)	Mean ± SEM	
NLR	5.01 (1.56:29.35)	6.34±0.80	2.77 (1.12:4.30)	2.63±0.20	<0.001
LMR	2.68 (0.31:12.83)	3.89±0.56	6.99 (3.82:27.56)	9.26±1.44	<0.001
PLR	242.11 (47.09:689.66)	262.79±25.92	107.28 (48.73:236.36)	117.80±10.66	<0.001
SII	1791.98 (383.58:12855.12)	2574.62±420.64	724.18 (199.87:1342.55)	713.05±70.23	<0.001
AGR	1.07 (0.52:1.73)	1.08±0.05	1.06 (0.92:1.54)	1.11±0.03	0.496
PNI	41.93 (31.9:56.65)	43.15±6.59	45.38 (39.75:58.55)	46.47±1.08	0.022

Data expressed as median (min:max) and mean ± SEM. NLR=neutrophil-to-lymphocyte ratio, LMR=lymphocyte-to-monocyte ratio, PLR=platelet-to-lymphocyte ratio, SII=systemic immune-inflammation index, AGR=albumin-to-globulin ratio, PNI=prognostic nutritional index.

Table 5. *Comparisons according to TNM stages of the dogs with malignant mammary tumors.*

Parameters	TNM I-II-III		TNM IV		TNM V		Control		P Value
	Median (min : max)	Mean ± SEM	Median (min : max)	Mean ± SEM	Median (min : max)	Mean ± SEM	Median (min : max)	Mean ± SEM	
NLR	4.66^{ab} (1.86:14.55)	4.84 ±3.21	5.83^{a} (3.02:29.35)	7.97±1.70	6.51^{a} (1.56:8.62)	5.9±0.92	2.77^{b} (1.12:4.30)	2.63±0.20	<0.001
LMR	3.07^{a} (0.81:12.83)	5.00±1.08	2.54^{a} (0.31:10.59)	2.99±0.60	2.27^{a} (1.07:12.25)	3.63±1.34	6.99^{b} (3.82:27.56)	9.26±1.44	<0.001
PLR	200.37^{a} (47.76:689.66)	252.21±48.37	255.45^{a} (47.09:617.50)	286.08±40.52	254.74^{ab} (55.03:431.03)	237.64±44.23	107.28^{b} (48.73:236.36)	117.80±10.66	0.001
SII	1478.84^{a} (525:8731.03)	2159.07±601.15	2165.96^{a} (538.24:12855.12)	3272.57±840.97	2082.66^{a} (383.58:3723.86)	1993.18±399.91	724.18^{b} (199.87:1342.55)	713.05±70.23	<0.001
AGR	1.19 (0.76:1.73)	1.74±0.08	0.94 (0.52:1.54)	1.02±0.10	0.94 (0.84:1.25)	1.01±0.07	1.06 (0.92:1.54)	1.11±0.03	0.344
PNI	43.88^{ac} (37:56.65)	45.56±1.61	41.25^{bc} (31.9:54.90)	41.10±1.87	40.80^{ac} (33.95:54.35)	42.10±2.54	45.38^{a} (39.75 8.55)	46.47±1.08	0.029

$^{a-c}$ *Different superscripts indicate values that within the row are significantly different. Data expressed as median (min:max) and mean ± SEM. NLR=neutrophil-to-lymphocyte ratio, LMR=lymphocyte-to-monocyte ratio, PLR=platelet-to-lymphocyte ratio, SII=systemic immune-inflammation index, AGR=albumin-to-globulin ratio, PNI=prognostic nutritional index*

Table 6. *Comparisons according to the number of affected mammary glands.*

Parameters	Single		Multiple		Control		P Value
	Median (min : max)	Mean ± SEM	Median (min : max)	Mean ± SEM	Median (min : max)	Mean ± SEM	
NLR	4.73^{a} (2.17:8.62)	4.88±0.72	5.34^{a} (1.56:29.35)	6.87±1.06	2.77^{b} (1.12:4.30)	2.63±0.20	<0.001
LMR	3.23^{a} (1.07:11.00)	4.70±1.19	2.68^{a} (0.31:12.83)	3.59±0.63	6.99^{b} (3.82:27.56)	9.26±1.44	<0.001
PLR	232.58^{a} (97.40:382.58)	240.23±32.57	242.11^{a} (47.09:689.66)	271.15±33.60	107.28^{b} (48.73:236.36)	117.80±10.66	<0.001
SII	1889.92^{a} (525.00:3723.86)	1898.28±314.00	1734.58^{a} (383.58:12855.12)	2825.12±560.49	724.18^{b} (199.87:1342.55)	713.05±70.23	<0.001
AGR	1.12 (0.52:1.62)	1.09±0.12	1.03 (0.61:1.73)	1.08±0.06	1.06 (0.92:1.54)	1.11±0.03	0.781
PNI	43.10 (33.95:56.30)	43.66±2.26	41.70 (31.90:56.65)	42.96±1.33	45.38 (39.75:58.55)	46.47±1.08	0.066

Different superscripts indicate values that within the row are significantly different. Data expressed as median (min:max) and mean ± SEM. NLR=neutrophil-to-lymphocyte ratio, LMR=lymphocyte-to-monocyte ratio, PLR=platelet-to-lymphocyte ratio, SII=systemic immune-inflammation index, AGR=albumin-to-globulin ratio, PNI=prognostic nutritional index

significantly different between dogs with a single MMT and control group (P=0.037, P=0.024, P=0.009, and P=0.002, respectively). Similarly to that, the same values of the dogs with multiple MMTs and the control group were found significantly different (P<0.001, P<0.001, P=0.001, and P<0.001, respectively). In dogs with a single MMT, the median NLR and PLR values were lower and the median LMR and SII values were higher than in dogs with multiple MMTs, but no statistical significance was found (P>0.05). The overall test did not show significant differences between the groups for the AGR and PNI parameters (P=0.781, P=0.066, respectively) *(Table 6)*.

Discussion

There are strong similarities between human breast cancer (HBC) and canine mammary tumors [31-33]. Also, neoadjuvant chemotherapy can be used in the treatment of canine mammary tumors similar to that in humans [34]. As in HBC [6,11,35], it has been noted that there are some remarkable changes in blood values in the presence of mammary tumors in dogs [7,8]. In veterinary medicine, it has been stated that NLR, LMR, PLR, and AGR, parameters may be potential biomarkers in certain malignancies of dogs [21-23,28]. But until recently, no literature data were evaluating LMR, PLR, SII and PNI parameters in dogs with MMTs. Uribe-Querol et al.[28] reported that a high NLR value (NLR<5) before treatment was associated with a lower survival rate in dogs with mammary tumors. They also stated that NLR could be used as a prognostic marker for disease severity, but AGR value did not show any predictive value on tumor malignancy. In this study, we found that in dogs with MMT, NLR, PLR, and SII values were high, and LMR and PNI values were low as in human breast cancer [15,18,27]. However, in our dogs with MMT, there was no significant difference compared to our control group in the AGR parameter [24-26]. In the study by Lallo et al.[36], it was stated that AGR values were lower in malignant MMTs. Unlike this, the AGR value did not differ in dogs with MMT when compared with healthy ones in the study by Uribe-Querol et al.[28] and in this study. We recommend evaluating the AGR parameter in larger populations.

Median NLR, PLR, LMR, and SII values show differences among TNM stages in HBC. It has been said that NLR, PLR, LMR, and SII parameters can be valuable and guide in the staging of HBC [18]. Low AGR has been found to be associated with advanced-stage of HBC and low PNI parameter has been found to be associated with advanced disease [24,27]. High NLR values have been associated with advanced or aggressive HBC [5,15,20]. It has been shown that NLR values increase as the disease progresses in TNM stages of HBC [17,18]. In our study, although not statistically significant, the median NLR values increased as TNM stages progressed (4.66, 5.83, 6.51, respectively). Low LMR is correlated with advanced disease and TNM stages in HBC [18,20]. Compatible with this, median LMR values decreased as TNM stages progressed (3.07, 2.54, 2.27, respectively) in our study, but this decrease was not statistically significant. It was shown that the PLR parameter was correlated with advanced disease and TNM stages [18,20], and another study reported that it was associated with lymph node metastasis but not with advanced T stages [16]. Unlike them, Elyasinia et al.[17] reported that there was no relationship between PLR values and different TNM stages of HBC. Similarly, PLR values did not show a significant difference among TNM stages in the dogs with MMT. The SII parameter has been found to be associated with the advanced TNM stage in HBC [18,19]. The median SII values in TNM IV and V stages were found to be higher than the median SII value in the TNM I-II-III stages in our study. Low AGR has been found to be associated with the advanced stage of HBC [24]. In our study, the highest median AGR value was observed in the TNM stage I-II-III group, in which no metastases have formed. A low PNI parameter has been found to be associated with advanced disease in HBC [27]. Although there was no statistically significant difference in our study, median PNI values decreased as TNM stages progressed (43.88, 41.25, 40.8, respectively). Similar changes were detected in NLR, LMR, PLR, and PNI parameters at TNM stages in dogs with MMT as in HBC. These biomarkers reveal the balance between the immune system and systemic inflammation. In the early stages of the disease, tumors can be detected and destroyed by the immune system. As the disease progresses, the immunogenic capacity of the tumor decreases and its inflammatory capacity increases. As detectable tumors develop, cancer cells develop different mechanisms that mimic peripheral immune tolerance to avoid tumorocidal attack [2,37]. In our study, although the changes in SII and AGR parameters according to TNM stages showed some differences from HBC, median values in TNM I-II-III stages suggested that less systemic inflammatory response occurred in the early stages of canine MMT *(Table 5)*.

Lymph node involvement seems to be the most important factor in predicting prognosis using systemic inflammatory parameters in HBC [16,19,20,25,27]. In our study, it was found that there was no significant increase in NLR values up to TNM stage IV, where the disease affected the lymph nodes for the first time. The highest NLR, SII, and median PLR, SII values, with the lowest LMR, AGR, and PNI values were obtained in TNM stage IV *(Table 5)*. The most significant P values were obtained for the NLR, LMR, PLR, and SII parameters compared to the control group and TNM stage IV. Interestingly, the PNI parameter showed a significant difference only when TNM stage IV

and the control group were compared *(Table 5)*. However, no statistical difference was found between TNM IV and V groups. According to the current literature, the rate of bilateral HBC development humans is low [38] but, multiple tumors are more common in dogs [39,40]. In dogs with a single MMT, median NLR, and PLR values were found to be lower than in dogs with multiple MMTs, and median LMR, SII, AGR, and PNI values were higher in our study *(Table 6)*. These results suggest that the systemic inflammatory response may be higher when the disease metastasizes to other mammary glands than when it is localized in a single mammary gland. More research should be done about this subject in larger populations.

Advanced clinical staging is known to be associated with hematologic parameters and provide prognostic information for canine mammary tumors [8]. The data of this study showed that some combinations (NLR, LMR, PLR, SII, and PNI) of hematological and biochemical data routinely measured for systemic inflammatory response, varied between healthy dogs and dogs with malignant mammary tumors. It is also suggested that these biomarkers could be used as biomarkers in different TNM stages of MMTs and in cases where the disease metastasizes from the mammary gland of the primary tumor to other mammary glands. However, further studies are needed to determine the value of these biomarkers in determining the prognosis of the disease, choice of treatment modality, or prediction of response to chemotherapy.

Availability of Data and Materials

Data supporting these findings are available upon request from the corresponding author (D. NAK) on reasonable request.

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Statement

The study was approved by the Ethics Committee on Animal Research of Bursa Uludag University (Approval No: 2020-03/03).

Author Contributions

D.N. and Z.M.E. wrote the manuscript, conducted experiments and interpreted the results. M.O.O. and Z.A.K perfor.med the histopathological examination. F.E.K. and T.A. made a significant contribution to and supported the experiments. D.K. and O.G. participated in the experiments. Y.N. and all authors critically reviewed and revised the manuscript draft and approved the final version for publication.

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RESEARCH ARTICLE

Effect of LED Light Color and Stocking Density on Growth Performance, Carcass, and Meat Quality Characteristics of Japanese Quails

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ABSTRACT

This study was conducted to evaluate the effect of LED light color and stocking density on the growth performance, carcass, and breast meat quality characteristics of quails. The experiment comprised 720 1-day-old mixed-sex Japanese quails (*Coturnix coturnix japonica*) randomly divided into 6 experimental groups with 4 replicates in each group. Experimental treatments included three different LED light colors (white, monochromatic blue, and monochromatic green) and two different stocking densities (100 and 200 cm^2/bird) in a 3x2 factorial design. The feed intake and body weight of quails were measured weekly. At 42 days of age, 20 quails per treatment group were randomly selected for carcass and breast meat quality characteristics analysis. Body weight gain was significantly higher in the blue and green LED light treatments ($P<0.05$). Quails reared at 200 cm^2/bird significantly determined the highest body weight gain ($P<0.001$). Quails reared at 200 cm^2/bird had the best ratio of feed conversion ratio ($P<0.01$). Breast meat yield and weight were higher in quails reared at blue LED light. Blue LED light tended to increase the water-holding capacity of breast meat ($P<0.01$). The breast meat L* value decreased significantly as stocking density (200 cm^2/bird) increased ($P<0.05$). However, despite the difference in pH_{15}, pH_u and L* value of breast meat, other meat quality characteristics were not different among the stocking density groups. In conclusion, it is shown that mono-crop monochromatic blue or green LED light and 200 cm^2/bird floor space have a utilization effect on quail performance traits.

Keywords: LED light color, Meat quality, Performance, Quail, Stocking density

INTRODUCTION

The feed conversion ratio, growth rate, and food security of poultry species have continually developed with the advancement of intensive genetic selection, management, and nutrition [1]. Birds have highly improved vision and are sensitive to light as management practices. The primary biological rhythms in poultry, as in other animals, are seasonal and diurnal, both mediated by light. The circadian rhythms in quails are essential for their survival and reproduction. These rhythms are influenced by the detection of light through photoreceptors in their eyes, which signal the suprachiasmatic nucleus in the brain to coordinate various physiological and behavioral processes with the daily and seasonal changes in light. This synchronization helps quails adapt to their environment and optimize their chances of reproductive success [2].

The suitable light environment (light duration, source, wavelength, and intensity) has indirect or direct effects on the economically significant growth performance traits, physiology, metabolism, and welfare of birds [3-5]. The stimulation of the hypothalamus by different wavelengths of light can affect various physiological events in poultry, such as their circadian rhythm and growth. For example, exposure to short wavelengths of light (blue and green light) can suppress melatonin production and stimulate activity, while exposure to long wavelengths of light (red and orange light) can promote melatonin production and induce sleepiness [6-8].

Having used monochromatic lighting on broilers and turkeys in the breeding process using LED light, Mohamed et al.[3] and Oke et al.[9] indicated that blue and green LED light had positive effects on growth performance. Halevy et al.[10] reported that green and blue light accelerated the growth development of chicks. It has been reported that green light accelerates broiler muscle growth during the early period of development, while blue light stimulates growth in the later period [11,12]. It was found that the usage of white or red color light as more body weight gains (BWGs) than blue light in turkey [13]. Yang et al.[14] found

significant differences in broiler body weight, and some meat quality among the quails raised under blue, green, yellow, or red LED lights. Environmental, physiological, and biological factors during the growth period can influence the susceptibility of birds to PSE (pale, soft, and exudative) meat, ultimately impacting meat quality characteristics. Additionally, light, as a significant environmental factor, may influence the quality of breast meat [15]. Ke et al.[16] determined that green-blue light improves carcass weight and quality by increasing pH value and water holding capacity (WHC) in broilers. Also, blue and green (short wavelength light) LED lighting has been shown to reduce measures of fear in broilers [3] and ducks [17].

Stocking density is essential factor for the birds among all the management practices. The quails are typically provided with a certain amount of floor area per bird to ensure they have enough space for movement and to reduce stress. The guideline you mentioned recommends a floor area of 130-150 cm^2 per quail during this period. This allows for a suitable environment for quails to lay eggs and move around without overcrowding. The high stocking density can reduce the growth performance of the bird [18,19]. In addition, a high stocking density can be stressful and can negatively affect quail's immunological and physiological performance [20]. Önel and Aksu [21] found no significant difference in the meat color variables (L*, a*, and b*) among the quails raised stocking density.

Yet, further investigations are needed to understand how light color in combination with different stocking density affects quail production, carcass, and meat quality. Therefore, the present study was designed to investigate the effect of different LED light colors (white, blue, and green) and stocking density (100 and 200 cm^2/bird) on growth performance, carcass, and meat quality characteristics of Japanese quails during their growth period extending from day 1 to day 42.

Material and Methods

Ethical Statement

All the experimental procedures involved in this study were performed after ethical approval was taken from the Animal Care and Use Committee of Aydin Adnan Menderes University (64583101/2022/96).

Experimental Design and Treatments

This study was conducted at the Poultry Research Unit of Aydin Adnan Menderes University, Aydın, Türkiye for six weeks.

In this way, LED light bulbs were arranged inside the cages, and bulbs were installed with LED (CATA CT-4277, Türkiye) of 9W power in each cage. The LED light color treatments were assessed by light wavelength. LED light groups were white (400-770 nm), blue (480 nm), and green (560 nm) in which LED light was applied continuously (24 h light a day). Each one of the LED light color groups is managed under one of two different stocking density (100 and 200 cm^2/bird) groups.

A total of 720 1-day-old mixed-sex Japanese quails *(Coturnix coturnix japonica)* chicks were initially weighted individually so that the cages had similar initial weight distribution and were randomly assigned to six groups in a 3x2 factorial design, involving three LED light color (white, blue, and green) and two stocking density (100 and 200 cm^2/bird). Each group consisted of 4 replicates with 20 quails in each; hence, a total of 80 quails were subjected to the treatment. The quail chicks were kept in chick-rearing cages with a size of 25x44x30 cm and in the same position and number of heaters, feeders, and drinkers throughout the experiment. The quail chicks were subjected to a temperature of 33°C during the first three days of age followed by a gradual reduction of 3°C every week until 23°C was attained. The relative humidity was 50-60% constant throughout the experiment.

General Management

All quails were fed with balanced diets (0-14 d; 2910 kcal ME/kg, 24% crude protein, and 15-42 d; 2900 kcal ME/kg, 22% crude protein). Feed and water were ensured *ad libitum* throughout the study [22].

Performance Parameters

Weekly weights of quails were evaluated as individuals. Quails were weighed at the start of the experiment and at the end of each week to ascertain the body weight and cumulative BWG of the overall experimental duration. The feed intake was also weekly recorded on a per-replicate basis. The feed conversion ratio (FCR) was calculated by dividing the feed intake with BWG. Experimental quails were observed and recorded for mortality rate in each cage daily, and survival analysis was conducted.

Carcass and Meat Quality Measurements

At the end of the experiment, five quails from each replicate cage (20 quails/group; 120 quails in total) were randomly selected, weighed, and slaughtered by decapitation followed by soft scalding, evisceration, exposition of the carcass, cutting and weighting the boneless breast meats, and finally storage at 4°C for 24 h. The dressing percentage was determined by dividing the hot carcass weight by the preslaughter weight and multiplying the result by 100. Additionally, the breast meats were separated, weighed, and expressed as percentages of the slaughter weights.

In the present study, breast meats were used to evaluate meat quality characteristics. The pH of breast meat was

measured at 15 min (pH_{15}) and 24 h (pH_u) after slaughter using a digital pH meter (Testo 205, Lenzkirch, Germany). A pH meter was used to measure the breast pH by inserting the probe 2-2.5 cm into the breast meat. Breast meat color was measured by using the digital color meter (Konica Minolta Sensing, Inc., Osaka, Japan) by the principles of the International Commission on Illumination (CIE). Color values of L* b*, and a* indicate lightness, yellowness, and redness, respectively. Breast meat pH and color were measured followed by the measurement of cooking loss (CL) and WHC. In the CL method described, meat samples were placed in polyethylene bags and cooked in a water bath until they reached an internal temperature of 75°C. After cooking, the samples were cooled for 15 min under running tap water, removed from the bags, dried with filter paper, and weighed [23]. To measure WHC, the meat samples were first placed between two filter papers to remove excess surface moisture. Then, a 2250 g weight was applied on top of the filter papers for 5 minutes, to extract the water from the meat sample. After removing the weight, the samples were weighed again to determine the final weight. The WHC was calculated by subtracting the final weight from the initial weight of the sample and dividing this by the initial weight. The result was then multiplied by 100 to express the value as a percentage [24]. Three different measurements were taken for breast meat color and pH, and then the average of these values was used to minimize the variations.

Statistical Analysis

The data were analyzed using the SPSS 22.0 (Statistical Package for the Social Sciences for Windows, IBM Corp., Armonk, NY, US). Data were tested for normality using Shapiro-Wilk's test. Using Levene's test, the assumption of homogeneity of variances was verified. Analysis of variance was performed with the GLM (Univariate General Linear Model) procedure to reveal the effects of performance, carcass, and meat quality characteristics. Significant differences among group means were determined by Duncan's multiple range tests as post-hoc tests. The chi-square test was used for the mortality rate. Survival curves were plotted using the Kaplan–Meier method, and data were analyzed by the log-rank test, with $P<0.05$ indicating statistical significance.

Table 1. *Influences of LED light color and stocking density on body weight gain of quails*[1]

Treatment Main Effects	**Body Weight Gain (g)**											
	n	**d 0-7**	**n**	**d 0-14**	**n**	**d 0-21**	**n**	**d 0-28**	**n**	**d 0-35**	**n**	**d 0-42**
Expected mean (μ)	720	22.63		59.77		106.03		153.05		193.02		209.42
LED light treatment												
White	240	21.78^{b}	240	58.65	240	106.45	236	150.77^{b}	236	189.57^{b}	235	205.63^{b}
Blue	240	23.22^{a}	240	60.46	240	105.97	239	153.21^{ab}	238	195.34^{a}	239	212.24^{a}
Green	240	22.88^{a}	239	60.21	238	105.67	234	155.17^{a}	233	194.16^{a}	231	210.39^{ab}
Stocking density												
200 cm²/bird	240	24.07	240	61.34	240	109.22	240	157.14	236	195.41	235	214.83
100 cm²/bird	480	21.18	479	58.20	476	102.84	476	148.97	471	190.64	470	204.01
Pooled SEM[2]		0.18		0.36		0.57		0.67		0.92		1.12
LED color x stocking density												
White - 200 cm²/bird	80	23.09		60.20		107.89^{abc}		153.65		192.18		210.36
White - 100 cm²/bird	160	20.47		57.09		105.00^{bcd}		147.89		186.97		200.90
Blue - 200 cm²/bird	80	24.93		62.08		110.90^{a}		157.20		196.90		217.76
Blue - 100 cm²/bird	160	21.52		58.86		101.03^{d}		149.22		193.78		206.71
Green - 200 cm²/bird	80	24.20		61.78		108.86^{ab}		160.56		197.16		216.36
Green - 100 cm²/bird	160	21.56		58.64		102.47^{cd}		149.79		191.16		204.43
Pooled SEM[3]		0.44		0.88		1.38		1.61		2.21		2.70
Treatment interaction effects	**P Value**											
LED light color		0.003		0.090		0.853		0.026		0.025		0.044
Stocking density (SD)		<0.001		<0.001		<0.001		<0.001		0.009		<0.001
LED light color x SD		0.589		0.999		0.044		0.309		0.802		0.901

n: *The total number of quails in the group,* [a, b, c, d] *Means with different superscript letters in the same column differ (P<0.05)*
[1] *Data presented as the least square means,* [2] *Pooled SEM for main effects,* [3] *Pooled SEM for interaction effect*

Table 2. *Effect of LED light color and stocking density on cumulative feed intake consumption, feed conversion ratio, and mortality of quails*

Treatment Main Effects	Cumulative Feed Intake (g/bird)							Cumulative Feed Conversion Ratio (g of feed/g of gain)						Total Mortality, %
	n	d 0-7	d 0-14	d 0-21	d 0-28	d 0-35	d 0-42	d 0-7	d 0-14	d 0-21	d 0-28	d 0-35	d 0-42	
Expected mean (μ)	24	35.42	119.19	204.04	273.85	332.59	325.04	1.56	1.99	1.93	1.79	1.72	1.56	
LED light treatment														
White	8	33.88	114.69	201.23	278.57	330.29^{b}	332.48^{a}	1.55	1.96	1.93	1.85^{a}	1.74^{a}	1.62^{a}	2.5^{ab}
Blue	8	36.08	121.41	205.70	173.53	341.26^{a}	331.45^{a}	1.58	2.02	1.95	1.76^{b}	1.76a	1.58^{a}	0.4^{b}
Green	8	36.31	121.46	201.23	269.46	326.22^{b}	311.21^{b}	1.56	2.01	1.90	1.76^{b}	1.67^{b}	1.47^{b}	3.8^{a}
Stocking density														
200 cm²/bird	12	39.66	126.48	212.27	285.82	344.71	322.85	1.65	2.06	1.95	1.82	1.77	1.51	2.5
100 cm²/bird	12	31.18	111.89	195.81	261.88	320.47	327.24	1.47	1.92	1.91	1.76	1.68	1.61	2.1
Pooled SEM[1]		0.41	1.22	1.30	1.57	1.98	3.23	0.02	0.01	0.01	0.01	0.01	0.02	
Significance of main effects					**P value**						**P value**			**P value**
LED light color		0.051	0.055	0.330	0.087	0.017	0.024	0.790	0.172	0.347	0.005	0.031	0.003	0.044
Stocking density (SD)		<0.001	<0.001	<0.001	<0.001	<0.001	0.505	<0.001	<0.001	0.116	0.008	0.006	0.006	0.721
LED light color x SD		0.892	0.601	0.323	0.634	0.662	0.088	0.808	0.455	0.802	0.773	0.621	0.067	

n: *The number of pens,* [a, b] *Means with different superscript letters in the same column differ (P<0.05),* [1] *Pooled SEM for main effects*

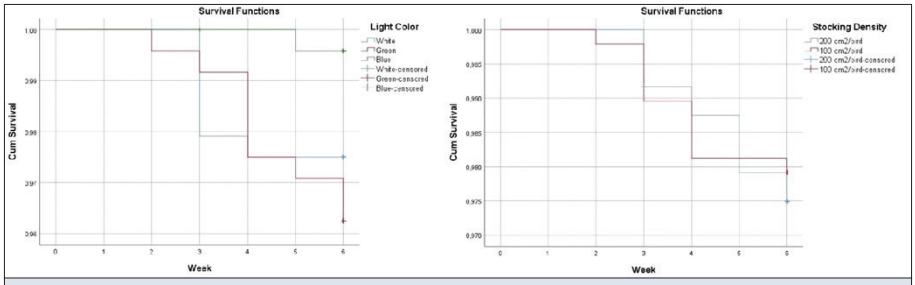

Fig 1. Effect of LED light color and stocking density on survival rates of quails

Results

The BWG was found the highest in the blue LED light (212.24 g) group (P<0.05), and 200 cm²/bird (214.83 g) group (P<0.001). Blue light-200 cm²/bird combined was found highest (217.76 g), but no significant between interactions at 0-42 d *(Table 1)*. Cumulative feed intake (CFI) was suppressed green LED light group compared to white and blue LED light (P<0.05) at 0-42 d. There was no significant between stocking density groups for FCR. LED light color affected CFI (P<0.01) of quails from 0 to 42 days of age. Quails reared under 200 cm²/bird density groups had better FCR than those 200 cm²/bird groups (P<0.01). It's apparent from the obtained results mortality rate was significantly (P<0.05) reduced with blue LED light. However, stocking density has no significant effect on the mortality ratio *(Table 2)*. A survival curve showing the survival probability of quails over time across farms is shown in *Fig. 1*. At the beginning of the study, the white, blue, and green color groups had 240 chicks, per group. In the 6th week, the survival rate in the white, blue, and green groups was 97.5%, 96.3%, and 99.6% respectively (P<0.05). The 200 cm²/bird, and 100 cm²/bird groups had 240 and 480 chicks at the beginning of the study. In the 6th week, the survival rate was 97.5%, and 97.9% in the corresponding treatments (P<0.05). LED light color significantly affected the yield and weight of the breast (P<0.01). The stocking density has a statistically not significant effect on live weight, carcass yield, breast yield, and weight *(Table 3)*. Compared with the white LED group, blue and green LED light increases in pH_{15} pH_u value of breast meat. The differences between 100 and 200 cm²/bird stocking density groups for pH_{15}, pH_u, and L* values were significant *(Table 4)*.

Table 3. The least-square means for live weight, carcass, and breast meat yield in LED light color and stocking density groups[1]				
Treatment Main Effects	**Live Weight (g)**	**Carcass Yield (%)**	**Breast Meat Yield (%)**	**Breast Meat Weight (g)**
Expected mean (μ)	214.21	75.75	19.70	31.86
LED light treatment				
White	214.73	75.75	19.78[a]	32.00[a.b]
Blue	215.88	75.86	20.61[a]	33.65[a]
Green	212.03	75.65	18.72[b]	29.93[b]
Stocking density				
200 cm²/bird	219.12	75.72	19.62	32.48
100 cm²/bird	209.30	75.79	19.79	31.23
Pooled SEM[2]	2.51	0.17	0.21	0.43
Treatment effects				
LED light color	0.814	0.872	0.002	0.003
Stocking density (SD)	0.053	0.822	0.697	0.150
LED light color x SD	0.436	0.271	0.834	0.251

[a, b] Means with different superscript letters in the same column differ (P<0.05), [1] Data presented as the least square means
[2] Pooled SEM for main effects

Table 4. Effects of LED light color and stocking density groups on breast meat quality characteristics of quails[1]							
Treatment Main Effects	**Breast Meat**						
	pH_{15}	**pH_u**	**L***	**a***	**b***	**CL (%)**	**WHC (%)**
Expected mean (μ)	6.43	5.57	43.42	16.18	12.29	20.88	5.05
LED light treatment							
White	6.52[a]	5.63[a]	44.22	16.69	12.48	21.85	4.76[b]
Blue	6.36[b]	5.46[c]	42.62	15.48	12.50	21.03	5.64[a]
Green	6.39[b]	5.55[b]	43.43	16.38	11.87	19.78	4.76[b]
Stocking density							
200 cm²/bird	6.38	5.59	42.74	16.36	12.03	20.77	4.94
100 cm²/bird	6.47	5.51	44.11	16.01	12.54	20.99	5.16
Pooled SEM[2]	0.02	0.01	0.29	0.21	0.23	0.43	0.13
Treatment effects							
LED light color	0.002	<0.001	0.080	0.056	0.459	0.145	0.009
Stocking density (SD)	0.019	0.004	0.019	0.407	0.276	0.798	0.402
LED light color x SD	0.142	0.744	0.341	0.268	0.887	0.149	0.614

[a, b] Means with different superscript letters in the same column differ (P<0.05), [1] Data presented as the least square means, [2] Pooled SEM for main effects

Discussion

BWG, which is the production parameter, was significantly increased in poultry reared under blue and green LED light compared to those reared under white LED light with the best performance achieved. In the study, the green LED light resulted in better FCR of quails at 0-35 d and at 0-42 d. In line with previous a study [4], the broilers reared under white LED added with blue-green LED light had higher BW than broilers reared under white LED light. Mohamed et al.[3] determined higher BWG and better FCR in blue and green LED light groups compared to those in the white LED group. The findings suggest that the color or wavelength of light used in quail housing can impact their growth and feed efficiency. This observation could have practical implications for quail farming, as optimizing lighting conditions may improve production outcomes. However, some studies have reported no effect of light color (light wavelength) on FCR and BW [25,26]. In the study, the highest FI was determined in the group

with white LED lighting (P<0.05). Some studies indicate that when exposed to short wavelength light quails [27], ducks, and turkeys [13] broiler chickens [12] tend to have faster growth rates compared to when they are exposed to long wavelengths. Mohamed et al.[28], reported that due to the calming effect of blue and green LED light makes birds less active and fearful. This could explain the better growth performance of the quails in blue and green LED light groups, as their visible spectrum included blue and green due to a light short wavelength.

The present study showed that high stocking density (HSD) decreased the BWG and FCR but had no significant effect on feed intake. Cicek et al.[29], Aro et al.[30], and Abdel-Azeem [31], Mahrose et al.[32] also reported HSD in quail farming can have various effects on bird performance, including BWG, FCR, and FI. The specific effects of stocking density can vary depending on various factors, including quail genetics, management practices, and environmental conditions. Scientific researchers often conduct studies to determine the optimal stocking density for quail production to balance growth performance, feed efficiency, and animal welfare. The performance parameters including BWG and FCR were not affected by stocking density in Japanese quails [33]. The non-significant alterations in feed intake in the study disagree with decreased feed intake as the field per quail decreases as stated by other researchers [34,35]. Decreasing floor allowance in quail was associated with a marked significant decrease in BW. The BWG for quails reared at the low SD was high due to more availability of fields for drinking and feeding. This statement suggests that the amount of space quails have in their environment can have a significant impact on their body weight and growth. Providing quails with more space for movement and access to food and water seems to result in better body weight gain.

There was no significant effect of LED light color on carcass yield in the study. The LED light color was the variable of interest, and the carcass yield of quails was the outcome measure [25,26,36,37]. The blue LED light group found the highest breast meat yield and weight (P<0.01). Consistent with those results, Cao et al.[12] reported that the breast weights of broilers exposed to blue LED lighting were higher than those of other light groups. The blue or green LED lighting may promote post-hatch muscle growth because of increased satellite cell density in the breast muscle as well as a 1.6-fold increase in expression of the growth hormone receptor during early post-hatch stages [10]. According to these results, it can be said that the blue LED lighting improved breast yield and weight.

Low stocking density has led to an increase in live weight, but this has not reached statistical significance. Carcass yield, breast meat yield, and weight were not significantly influenced by the stocking density. The effect of stocking density on carcass yield and breast meat weight was found to be statistically non-significant in quails [18,32]. On the other hand, Abdel-Azeem [31] and Hassanein [38] observed that increasing stocking density negatively affects carcass weight. According to these results, it can be said that the effects of the stocking density on performance parameters and carcass yield of quail are contentious due to different density rates. The effects of light color and stocking density on quail performance parameters and carcass yield can vary depending on multiple factors, including the specific conditions of the quail operation. Research in this area is ongoing to find optimal combinations of lighting and stocking practices that balance production efficiency with the welfare and well-being of the birds. Quail producers need to consider these factors carefully and potentially conduct their trials to determine the best practices for their circumstances.

In this study, white lighting increased breast meat pH_u indicating better breast meat quality that was characterized by increased meat color (a*) and lower protein damage which confirms previous results [39,40]. It was shown that breast meat WHC was significantly decreased by the white and green LED light group. In the study, no effects of LED light color were found on L*, a*, b* values, and CL of quail breast meat. Zhang et al.[41] showed that the green light may slightly decrease the WHC of breast meat, and this effect was not significant. Overall, white, and green LED lights only affect a few of the general meat quality characteristics, and this effect was significant enough to be beneficial to meat quality.

El-Moniary et al.[42] reported that the pH value is a direct response to muscle acid content, which affects the color and WHC of breast meat. Our results suggest breast meats of quails in 200 cm^2/bird had significantly higher lightness, compared with those in the 200 cm^2/bird no perch group (P<0.05). It can be said that the increased pH_u and decreased L* value found here with 200 cm^2/bird indicates their utility effect on meat quality and results in improved welfare due to the low stocking density.

In overall conclusion, despite that white and blue LED light was effective in reducing mortality, the monochromatic blue and green light was beneficial to the growth performance of quails. This study showed that a lower stocking density provided more action space for quails. The low stocking density suppresses the growth performance traits of quails. The blue LED light resulted in heavier breast meat compared to those of the green LED light group. In contrast, stocking densities did not significantly influence the carcass parameters of quails. During the rearing period, the white and green LED lights resulted in higher pH_u and lower WHC. Therefore, a blue or green LED light is recommended for the growth

performance of quails reared at low stocking density. The suitability of the blue-colored, 200 cm^2 per bird combination treatment for quails will depend on scientific evidence and the well-being of your quail flock. Be sure to conduct thorough research and consider all relevant factors before implementing any changes in scientific studies. It is essential to have scientific data or research that supports the use of this combination treatment. If this treatment has been tested and proven to be effective in improving quail health, well-being, or productivity, it can be a suitable choice.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (E. Dereli Fidan) upon reasonable request.

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Competing Interests

The authors reported no potential conflicts of interest.

Author Contributions

EDF contributed to literature searches and study design. EDF and MK conceived and supervised the study. EDF and MK collected and analyzed data. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

Coagulation Profile Alterations in Dogs Co-Infected with Visceral Leishmaniasis and Monocytic Ehrlichiosis

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ABSTRACT

Canine Visceral Leishmaniasis (CVL) and Canine Monocytic Ehrlichiosis (CME) are zoonotic diseases that cause coagulation disorders, abnormalities in various organs and systems along with vasculitis. Since it was reported that the presence of co-infection may cause more severe abnormalities, this study aimed to investigate the synergistic effects of co-infection on some coagulation analytes (prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (FIB) and D-Dimer). Twenty dogs, all were mixed breed, aged 2-4 years, and determined to be co-infected with CVL and CME were used. As a result of the coagulation profile analyte measurements, the values of APTT, PT, and the concentration of FIB were determined to be higher in the Co-infected Group compared to the Control Group ($p<0.05$). Receiver operating characteristic (ROC) analysis revealed that PT (AUC: 0.980); APTT (AUC: 0.959), FIB (AUC: 1.000), and D-dimer (AUC: 0.929) had outstanding diagnostic discrimination. As a result, it was concluded that the presence of co-infection deteriorates the coagulation profile more severely in co-infection with CVL and CME.

Keywords: Activated partial thromboplastin time, D-Dimer, Dog, Fibrinogen, Prothrombin time

INTRODUCTION

Canine Visceral Leishmaniasis (CVL), which is described as an emerging zoonotic disease by the World Health Organization, has a poor prognosis and causes damage to various organs and systems in dogs [1,2]. It was previously reported that in CVL, not only vasculitis is present [3,4] but also abnormalities in the coagulation profile [5-7]. On the other hand, Canine Monocytic Ehrlichiosis (CME) is another vector-borne disease of dogs. It was previously reported that CME causes significant vascular and coagulation abnormalities as in CVL [8-12].

Hemostatic disorders (epistaxis, hemorrhagic diarrhea and hematuria) are reported to be common findings in both diseases [6,7,9,10]. Studies have shown that hemostatic changes in CVL-infected dogs are associated with the severity of clinical signs and thrombocytopenia [13], however, platelet dysfunction has been reported in CME-infected dogs [14].

Prothrombin time (PT) and activated partial thromboplastin time (APTT) are frequently used important biomarkers in the interpretation of the coagulation profile in dogs with bleeding tendency [15]. Fibrinogen (FIB), which is a glycoprotein, is synthesized by the liver and converted to fibrin by thrombin during homeostasis. Although FIB is one of the most important factors in the coagulation cascade, it was reported that its concentration may increase in several clinical conditions such as acute infections, hemodynamic disorders, heart and lung diseases and malignant conditions [16]. D-dimer, which is an important biomarker used primarily in the diagnosis of disseminated intravascular coagulopathy (DIC), is known as a degradation product of cross-linked fibrin that can increase with clot formation and fibrinolysis. Apart from DIC, it may increase due to infection, metabolic disorders, neoplasia and following an operation [17-19].

A study that evaluated platelet aggregation and haemostatic response in dogs co-infected with CME and CVL detected that there was a synergistic effect on haemostatic disorders in co-infected dogs with CME and CVL [20]. Therefore, this study, it was aimed to determine and validate the possible synergistic alterations in the coagulation profile

by evaluating coagulation profile analytes including PT, APTT, FIB and D-dimer in dogs co-infected with CVL and CME.

Material and Methods

Ethical Statement

This study was approved by the Harran University Animal Experiments Local Ethics Committee (Approval no: 01-11, session number 2021/009).

Animals

The animal material of the present study consisted of 20 dogs, all were mixed breed, aged 2-4 years, and were admitted to animal hospital for diagnosis/treatment or routine check-up/vaccination purposes. All the dogs underwent clinical examination, including body temperature, capillary refill time, lung and heart auscultation, and assessment of palpable lymph nodes. In addition, rapid diagnostic test kits (SNAP Leishmania®, IDEXX, USA and SNAP 4Dx Plus®, IDEXX, USA) were applied to all the dogs in accordance with the manufacturer's instructions. After the clinical examinations and rapid test kit evaluations, 10 dogs (4 male, 6 female, all were intact with a median age of 3.71 years (2-4 years) and a median body weight of 20 (7-28) kg) were diagnosed to be co-infected with CVL and CME, and all were included in the Co-infected Group (n:10). The other 10 dogs (4 females, 6 males, all were intact with a median age of 2.86 (2-4) years and a median body weight of 15 (10-18) kg) were determined to be healthy as a result of the clinical examinations and rapid test kit evaluations, and included to the Control Group (n:10).

Inclusion/Exclusion Criteria

In order to rule out the diseases (*Dirofilaria sp., Anaplasma phagocytophilum, Anaplasma platys* and *Borrelia burgdorferi)* that may cause similar clinical findings similar to CVL and CME infections, rapid diagnostic test kits (SNAP 4Dx Plus®, IDEXX, USA) were applied to the dogs of the Co-infected Group of the study. Dogs with any comorbid disease were excluded from the study. The same examinations and tests were applied to the dogs of the Control Group. All examination and test results were negative in context of differential diagnosis.

Clinical Examination

In accordance with the anamnestic data which were obtained as a result of face-to-face interviews with the animal owners, it was learned that all of the dogs were living at home, were taken to a walk 2-3 times a day and were fed on commercial dry dog food. Heart rate, respiratory rate, body temperature and capillary refill time (CRT) measurements, evaluation of dehydration status and palpable lymph nodes were assessed in all the dogs within the scope of clinical examinations. In addition, to detect pulmonary (pulmonary edema, dyspnea, etc.) clinical pathological changes secondary to cardiac disorders and cardiac clinical pathological changes (arrhythmia, valvular leaks, etc.), auscultation of the lungs and heart (mitral valve, in line with the costochondral junction of the left 5th intercostal space; aortic valve, just above the costochondral junction of the left 4th intercostal space; pulmonary valve, just above the sternum in the left 2nd-4th intercostal space region; tricuspid valve, right 3rd-5th intercostal space near the costochondral junction) was performed as previously reported [21].

Positive cases included in the study were not hospitalized. The routine treatment procedures reported for CVL [22] and CME [23] were followed.

Collecting Blood Samples

Venous blood samples (5-10 mL) were obtained via *vena cephalica* venepuncture with minimal restraint in order to not cause stress to all the dogs. The blood samples were taken into tubes without anticoagulant (BD Vacutainer® SST™ II Advance, BD, United Arab Emirates) and citrated anticoagulant tubes (BD Vacutainer® Citrated Tube, BD, United Arab Emirates), all were centrifuged at 3000 rpm for 10 minutes and their serum and plasma were extracted. Some portions of the serum and plasma samples (0.5-1 mL) were used for rapid diagnostic test kit evaluations (serum D-dimer and plasma PT, APTT, FIB, respectively). Centrifugation and rapid diagnostic test kit evaluations were performed within 10-15 min after the blood sampling.

Rapid Diagnostic Test Kit Applications

Confirmation of CVL and CME infections in the dogs, which were included in the Co-infected Group, was based on the clinical findings such as anorexia, lethargy, exercise intolerance, and arrhythmia, and as a result of rapid ELISA-based diagnostic test kits (SNAP 4Dx Plus®, IDEXX, USA) evaluations from the serum samples of the dogs of the Co-infected Group in accordance with the manufacturer's instructions. CVL and CME infections were confirmed according to the rapid diagnostic test kit results along with the compatible clinical findings as previously reported [24]. The same examinations and diagnostic tests were also applied to the dogs of the Control Group and all were determined to be negative.

Coagulation Profile Analyte Measurements

Plasma APTT, PT, FIB and serum D-dimer concentrations were measured using a commercial analysis system (Precil® C2000-4 four channel semi-automatic coagulometer and Wondfo Finecare® Fluorescent Immunoassay, respectively). The linear range (min-max) of the Finecare®

Table 1. *Temperature (T), Pulsation (P), Respiration (R) findings of all animals (according the groups)*

Parameters	Control Group (n:10)		Co-infected Group (n:10)	
	No	Value	No	Value
T (°C)	1	38.6	1	40.2
	2	38.5	2	40.6
	3	39.1	3	40.7
	4	38.5	4	39.3
	5	38.5	5	39.0
	6	39.0	6	38,9
	7	38.6	7	39.0
	8	39.1	8	38.8
	9	39.0	9	39.2
	10	39.3	10	39.0
P (beat/min)	1	80	1	175
	2	76	2	175
	3	84	3	180
	4	76	4	104
	5	100	5	88
	6	88	6	100
	7	96	7	96
	8	72	8	96
	9	104	9	84
	10	100	10	80
R (breaths/min)	1	16	1	62
	2	16	2	60
	3	24	3	68
	4	28	4	24
	5	18	5	28
	6	16	6	26
	7	15	7	28
	8	30	8	16
	9	16	9	24
	10	20	10	24

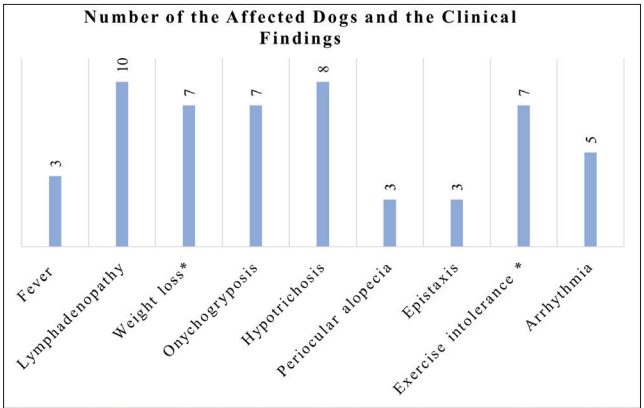

Fig 1. The clinical findings and their distribution of the co-infected group
*Based on body condition score and daily diet intake [25]

Fluorescent Immunoassay was 0.1-10 mg/L for D-dimer. Measurements below the lower detection limit (0.1 mg/L for D-dimer) were used for statistical analysis.

Statistical Analysis

All the data were evaluated using SPSS 25.00 (SPSS for Windows®) statistical software. One sample Kolmogorov-Smirnov test was applied to determine whether all data were parametric or non-parametric. It was found that the data were not normally distributed. Therefore, non-parametric data were evaluated with the Mann Whitney U, Kruskal-Wallis test. In addition, Receiver operating characteristic curve (ROC) analysis was used to distinguish the healthy dogs from the co-infected ones using coagulation profile analytes. Within the scope of ROC analysis, Area Under Curve (AUC), standard error (std. error), cut-off, sensitivity, and specificity parameters were evaluated. Within the scope of ROC analysis, it was accepted that an AUC of 0.5 suggests no discrimination (i.e., ability to diagnose patients with and without the disease or condition based on the test), 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is considered excellent, and more than 0.9 is considered outstanding. Moreover, the Spearman correlation test was performed to determine possible correlations between PT, APTT, FIB, and D-Dimer. Statistical significance was accepted as $P<0.05$ for all the data.

Results

Clinical Examination Results

As a result of clinical examinations, among the dogs of the Co-infected group, three had elevated body temperature (40.2°C, 40.6°C and 40.7°C) and three had prominent tachycardia (175, 175 and 180 beats/min). Temperature (T), Pulsation (P), and Respiration (R) findings of all animals (according to the groups) were given in *Table 1*. In addition, lymphadenopathy, hypotrichosis, exercise intolerance and onychogriposis were prominent clinical findings in the dogs of the Co-infected Group. The distribution of the clinical findings of the Co-infected Group are presented in *Fig. 1*.

Coagulation Profile Analyte Measurement Results

Within the scope of coagulation profile analyte measurement, the values of APTT, PT and the concentration of FIB were determined to be higher in the Co-infected Group compared to the Control Group ($P<0.05$). All coagulation profile analyte measurement results are presented in *Table 2*.

ROC Analysis Results

ROC analyses (CI: 95%) were performed to evaluate the diagnostic performances/efficacies of PT, APTT, FIB and

Table 2. *Coagulation profile analyte measurement results between the co-infected and healthy dogs*

Parameters	Control Group (n:10) median (min-max)	Co-infected Group (n:10) median (min-max)	P-Value
PT (sec)	7.97 (7.66-8.91)	9.84 (8.59-10.9)	0.000
APTT (sec)	11.7 (11-12.1)	18.4 (11.8-38.2)	0.025
FIB (mg/dL)	120 (103.3-189.2)	272 (201-752.1)	0.019
D-Dimer (mg/L)	0.09 (0.09-0.09)	0.7 (0.09-5.1)	0.083

PT: Prothrombin time, APTT: Activated partial thromboplastin time, FIB: Fibrinogen

Table 3. *ROC analyses data*

Parameters	AUC	Std. Error	P-Value	Asymp. 95% CI		Cut-off	Sensitivity	Specificity
				Lower Bound	Upper Bound			
PT (sec)	0.980	0.032	0.003	0.917	1.000	8.51	100%	85.7%
APTT (sec)	0.959	0.050	0.004	0.861	1.000	11.95	85.7%	85.7%
FIB (mg/dL)	1.000	0.000	0.002	1.000	1.000	195.4	100%	100%
D-Dimer (mg/L)	0.929	0.082	0.007	0.767	1.000	0.29	85.7%	100%

PT: Prothrombin time, APTT: Activated partial thromboplastin time, FIB: Fibrinogen, AUC: Area under curve, Std. Error: Standard Error, Asymp: Asymptotic tests, CI: Confidence interval

Table 4. *Spearman correlation test result*

Method	Parameters	Relation	Parameters			
			PT	APTT	FIB	D-Dimer
Spearman's rho	PT	Correlation Coefficient	1	0.647*	0.691**	0.836**
		Sig. (2-tailed)		0.012	0.006	0.000
	APTT	Correlation Coefficient		1	0.827**	0.869**
		Sig. (2-tailed)			0.000	0.000
	FIB	Correlation Coefficient			1	0.860**
		Sig. (2-tailed)				0.000
	D-Dimer	Correlation Coefficient				1
		Sig. (2-tailed)				

** Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed). PT: Prothrombin time, APTT: Activated partial thromboplastin time, FIB: Fibrinogen*

D-dimer in the determination of the coagulation disorders and the distinguishment of the healthy dogs from the diseased ones. As a result of the comparative ROC analysis, it was determined that PT (AUC: 0.980, sensitivity: 100%, specificity: 85.7%); APTT (AUC: 0.959, sensitivity: 85.7%, specificity: 85.7%), FIB (AUC: 1.000, sensitivity: 100%, specificity: 100%) and D-dimer (AUC: 0.929, sensitivity: 85.7%, specificity: 100%) had outstanding diagnostic discrimination. Comparative ROC analysis results are presented in *Table 3*.

Spearman Correlation Test Results

Spearmen correlation tests were performed for the analytes evaluated within the scope of coagulation profile. As a result, a strong positive correlation was found between PT and D-dimer, APTT and D-dimer, APTT and FIB,

FIB and D-dimer and a moderate positive correlation was found between PT and APTT, PT and FIB. The results are presented in *Table 4*.

Discussion

Bleeding disorders caused by CVL and CME are reported to cause significant clinical problems that cannot be ignored [5-12]. As expected, prominent clinical findings such as fever, lymphadenopathy, weight loss, onychogryposis, hypotrichosis, periocular alopecia, epistaxis, arrhythmia, and exercise intolerance which were previously reported in cases of CVL and CME [1,7,8] were also observed in the Co-infected Group of the present study.

There are studies evaluating the abnormalities in the coagulation profile of CVL-infected dogs. A previous study conducted on 28 CVL-infected dogs [7] evaluated the changes in the coagulation profile of dogs which were classified according to the disease stage. As a result of the aforementioned study, a statistically significant difference in PT, APTT, FIB and D-dimer concentrations between the dogs with an advanced stage (stage IV) disease and the healthy ones were determined. In another study conducted on 33 dogs infected with CVL, it was reported that PT, APTT and D-dimer concentrations were statistically significantly different in the dogs with an advanced stage (stage IV) disease compared to the previously reported findings [26]. They were also reported that this change might be related to the altered vitamin D concentrations. In a study investigating the cause of epistaxis, which could develop in dogs naturally infected with CVL, it was reported that no statistical difference was determined in the coagulation profile parameters including PT, APTT, FIB, von Willebrand Factor deficiency. It was reported that epistaxis may be associated with a secondary homeostasis mechanism or the presence of DIC [26]. In the present study, statistically differences in the PT, APTT and FIB concentrations (prolongation of PT and APTT duration, increase in FIB concentration) were determined similar to the dogs with an advanced stage (stage IV) disease in the previous studies [7,26]. On the contrary to the previous report [27], prolonged PT and APTT, and increased FIB concentrations were observed in the present study. The most prominent difference between the studies [7,26] with abnormalities in the coagulation profile and the present study that did not is the use of the CVL staging system as an inclusion criterion that was defined in the previous article [1]. the staging system was not used in the group whose coagulation parameters did not alter, it is noteworthy that the cases included in our study may not be in the initial stages (Stage I, II) and/or in the advanced stage (Stage IV). Also, Juttner et al.[27] reported that there may be a possibility of the presence of DIC, but fibrin degradation products such as D-dimer, which may increase in the presence of DIC, were not included in the study. Although there was no statistically significant difference in the present study, the concentration of the measured D-dimer was above the reported [17] cut-off value (0.3 mg/L). In the present study, the reason for the more severe alteration in the coagulation profile may be related to the presence of co-infection with CME.

There was a statistically significant increase was found in APTT [9,10] and D-dimer [28], while no statistically significant change was found in PT [9,10] value in studies conducted in dogs co-infected with CME compared to healthy dogs. In the present study, a statistically significant difference in APTT value and a higher cut-off value of D-Dimer than the previously reported concentrations were determined. Unlike the previous studies, a statistically significant increase in PT value was observed in the present study. This finding was thought to be related to the synergistic effect of co-infection.

In co-infected dogs with CVL and CME, a statistical increase in APTT values was detected, while no difference in PT value and FIB concentration was reported, which did not support the expected hypothesis. It was reported that the low sensitivity of the commercial PT kit that was used in the study may be a reason for not detecting an increase [20]. In the present study, prolongation in PT and an increase in FIB concentrations were determined in the co-infected dogs. The increase in FIB concentrations was thought to be related to the presence of acute inflammatory changes [29]. The prolongation of PT and APTT can be explained by the synergistic effect of CVL and CME on the coagulation profile.

In the ROC analysis of the present study, the sensitivity and specificity of PT, APTT, FIB and D-dimer (100% and 85.7%; 85.7% and 85.7%; 100% and 100%; 85.7% and 100%, respectively) were determined to have outstanding diagnostic discrimination/performance. These findings may be related to the low number of animals in the present study. The cut-off value of the D-dimer concentration was determined to be 0.29 mg/L, similar to the previously reported cut-off value (0.3 mg/L) [17]. The cut-off values of PT, APTT and FIB were 8.51 sec, 11.95 sec and 195.4 mg/dL, respectively *(Table 3)*. Since there is no study reporting the cut-off values of PT, APTT and FIB in dogs infected with CVL and/or CME, the cut-off values determined in the present study are promising in terms of diagnosis.

In the present study also demonstrated that a strong positive correlation was found between PT and D-dimer, APTT and D-dimer, APTT and FIB, FIB and D-dimer and a moderate positive correlation was found between PT and APTT, PT and FIB. The positive correlations identified in the study were expected due to the disturbances in both the extrinsic and intrinsic coagulation cascade caused by

the CVL [7,13,20,26] and CME [6,9,10,12,20]. We believe that this correlation data makes an unique contribution to the scientific literature.

This study has some limitations. The low number of animals, along with the use of laboratory analyses including hemogram and serum biochemistry profiling for inclusion criteria and to form a differential diagnosis list, and the fact that the laboratory analysis results were not discussed, can be considered as limitations. For this reason, it is recommended to evaluate the coagulation profile along with laboratory analysis results.

In the present study, promising findings were determined. These included prolonged PT, APTT and higher FIB value which was interpreted that the presence of co-infection deteriorates the coagulation profile more severely in dogs co-infected with CVL and CME. In addition, these promising findings validated the previous study.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (C. Balıkçı) at the University of Harran, Şanlıurfa, Türkiye.

Financial Support

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Competing Interest

The authors declared that there is no conflict of interest.

Ethical Statement

This study was approved by the Harran University Animal Experiments Local Ethics Committee (Approval no: 01-11, session number 2021/009).

Author Contributions

C. B. and E. G. designed, planned and drafted the research and converted into the manuscript. C. B., E.G. A.Ş. and İ.G. conducted and collected the data. C. B., E. G. analysed the data. C.B., E.G. and A. Ş. performed interpretation of data, conception and reviewed the manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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RESEARCH ARTICLE

Melatonin Treatment Affects Leptin and Nesfatin-1 Levels but not Orexin-A Levels in REM Sleep Deprived Rats

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ABSTRACT

Sleep contributes to the energy balance of body. This study aims to investigate how rapid eye movement (REM) sleep deprivation (SD) or recovery sleep affects rat weight and the serum levels of Nesfatin-1, Orexin-A, and Leptin; additionally, seeks to determine the impact of melatonin administration on these parameters. Male, Sprague Dawley rats were randomized into two groups (n=9). REMSD was induced using the modified multiple platform method (MMPM). Melatonin was used as a treatment (20 mg/kg). Study Group I was created to investigate the effectiveness of the treatment during REMSD. Study Group II was established to analyze the possible therapeutic role of melatonin and recovery sleep after REMSD-induced damage. The rats' weights were recorded during the experiments. Blood samples were collected from all rats via decapitation after experiments. The levels of serum Nesfatin-1, Orexin-A, and Leptin were analyzed using the ELISA method. REMSD affected weight of the rats and altered the levels of serum Nesfatin-1 and Leptin. Melatonin administration influenced weight gain and affected Nesfatin-1 and Leptin levels. REMSD or melatonin did not affect Orexin-A levels. REMSD and melatonin play significant roles in the body's energy balance. This study will contribute to elucidating the role of SD in metabolic processes and will play a role in assessing the impact of melatonin, a commonly used treatment in human and veterinary medicine.

Keywords: Leptin, Melatonin, Nesfatin-1, Orexin-A, Sleep deprivation

INTRODUCTION

We spend one-third of our lives sleeping. Sleep influences every system in the body and is crucial for physical, emotional, and mental well-being. The American Academy of Sleep Medicine and Sleep Research Society considers 7-8 h of sleep per night important for maintaining people's overall health [1]. It is also well known that sleep contributes to the homeostasis of energy [2]. Many people experience sleep deprivation (SD), especially due to changing living and working conditions [3]. Research indicate that sleep deprivation is associated with elevated risk of cardiovascular diseases, metabolic disorders, cerebrovascular pathologies, and accidents [4]. It is suggested that changes in metabolism resulting from sleep deprivation may cause these health issues. Some of these are hyperphagia, weight loss, high energy expenditure, elevated plasma catecholamines, hypothyroidism, deterioration in physical appearance, low anabolic hormone levels, and weakened immune system [5]. Furthermore, it has been discovered that light exposure can suppress melatonin production and DNA damage repair capability, thereby raising the risk of developing chronic illnesses [6].

Animal studies have shown that the effects of SD on body weight differ from those reported in humans. Sleeping less than 7 hours per night causes weight gain in humans [7]. On the other hand, rapid eye movement (REM) SD causes weight loss despite increased food intake (hyperphagia) in rats [8]. Hyperphagia was thought to be linked to higher oxygen consumption and increased energy metabolism [9]. The occurrence of weight loss despite increased food intake suggests that SD has a negative effect on energy balance [10].

The hypothalamus and brain stem regulate eating behavior [11]. There are also many hormones responsible for the control of body weight and nutrition. One of them, Leptin, known as the satiety hormone. The impact of sleep deprivation on Leptin levels appears to vary, with a study suggesting an increase [12] while another indicate no change [13]. Additionally, it was shown that insufficient sleep leads to higher energy expenditure and reduced Leptin levels [14], also it has been determined that Leptin

levels decrease as a result of sleep deprivation [5,15]. Leptin is thought to affect Orexin neuronal activity to control energy homeostasis [16]. Orexin receptors are found throughout the central nervous system (CNS), particularly in the hypothalamus. Orexin-A can cross the blood-brain barrier and promotes adipocyte glucose uptake [17]. Additionally, melatonin has been shown to induce sleep by inhibiting Orexin neurons [18]. Another hormone, Nesfatin-1 is an anorexigenic hormone that suppresses appetite [19]. Nesfatin-1 is strongly associated with various diseases, including neurogenic diseases, certain psychiatric disorders, diabetes, and obesity [20]. Nesfatin-1 expression has been shown to decline after 72 hours of REM sleep deprivation and it rises again after 3 hours of rebound sleep. Furthermore, intracerebroventricular Nesfatin-1 injection has been shown to reduce REM sleep temporarily while increasing slow-wave sleep [21]. Nesfatin also plays a crucial role in sleep regulation, and Nesfatin neurons are primarily located in the dorsolateral hypothalamus lateral hypothalamic and perifornical regions of the CNS [22]. These areas significantly contribute to the sleep-wake cycle [23]. Nesfatin-1 is believed to reduce appetite independently to the Leptin pathway by activating the melatonin system [24].

Melatonin promotes healthy sleep and plays an important role in regulating human sleep [25], it has also been shown to increase REM sleep percentage [26]. Sleep loss alters both melatonin cycle and eating behavior [27]. It has been demonstrated that REM sleep stabilizes the hypothalamic representation of feeding behavior and influences future food intake. This highlights the significant impact of sleep and SD on food intake [28]. Although various studies have unveiled the role of melatonin in the pathologies arising from SD and the impact of SD or recovery sleep on metabolic processes, no investigation has been found that examining the connection and underlying mechanisms between these factors. The purpose of this study is to investigate the effects of REM SD or recovery sleep on the weight of rats and the serum levels of Nesfatin-1, Orexin-A, and Leptin. In addition, we scoped to determine the impact of melatonin administration on these parameters.

Material and Methods

Ethical Statement

The study has been approved by the Bursa Uludag University Local Ethics Committee on Animal Research under decision number 2022-17/07.

Animals and Groups

Experiments were conducted in accordance with the NRC Guide for the Care and Use of Laboratory Animals. The rats were acclimatized to laboratory conditions for 2 days before the onset of the experiments. The room was temperature-controlled (23-25°C) with a 12 h light-dark cycle. Male, 8-12-week-old Sprague Dawley rats were randomly assigned to groups (n=9). The modified multiple platform method (MMPM) was used to induce REMSD. This was achieved by placing 3 animals on 6 platforms inside a large tank for 6 consecutive days. The tanks were filled with water at 24°C and platforms (6.5 cm diameter) were placed 2 cm above the water surface. When the animals lost muscle tone during REM sleep, they fell into the water, thereby waking up and experiencing REMSD. For the animals in the control group, same-size tanks were used, but grids were placed on the platforms to prevent the animals from falling into the water when they entered REM sleep. This ensured that the control group was exposed to the same environmental conditions without SD. Melatonin was used as a treatment and the dosage of melatonin (20 mg/kg) was determined based on the previous study showing its effectiveness [29]. The melatonin was dissolved in dimethyl sulfoxide (DMSO) and intraperitoneally (i.p.) administered at 08:00 am, adjusted as the end of the night cycle. The animals were divided into the following groups:

Study Group I: These groups were created to investigate the molecular changes that occur during REMSD and the effectiveness of the treatment. The animals in these groups were monitored in their appropriate cages for 6 days with daily i.p. injections, and on the 7th day, they were decapitated *(Fig 1)*. The animals in Control Groups were placed on grids according to MMPM, were provided access to food and water under optimal conditions: C+S: Saline treated control group (n=9); C+DMSO: DMSO treated control group (n=9); C+MEL: Melatonin treated control group at a dose of 20 mg/kg (n=9). The animals in REMSD Groups were placed on small platforms (6.5 cm diameter) according to MMPM, were provided access to food and water under optimal conditions: REMSD+S:

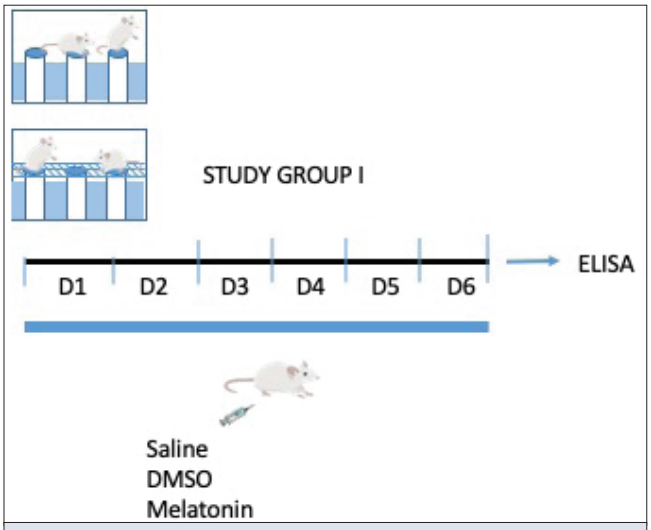

Fig 1. Schematic diagram of Study Group I

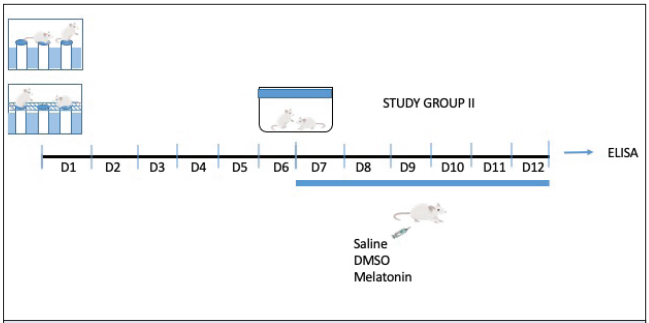

Fig 2. Schematic diagram of Study Group II

Saline treated REMSD group (n=9); REMSD+DMSO: DMSO treated REMSD group (n=9); REMSD+MEL: Melatonin treated REMSD group at a dose of 20 mg/kg (n=9).

Study Group II: These groups were created to analyze the possible therapeutic role of treatment and recovery sleep after damage caused by REM SD. Animals were housed in appropriate cages (according to MMPM) for 6 days, and then followed in standard laboratory cages from days 7-12. Intraperitoneal injections were administered once daily on days 7-12 and animals were decapitated on 13th day *(Fig. 2)*. Animals in Control Groups were placed on grids according to the MMP method, provide optimal access to food and water: RC+Saline: Saline treated recovery control group (n=9); RC+DMSO: DMSO treated recovery control group (n=9); RC+MEL: Melatonin treated recovery control group at a dose of 20 mg/kg (n=9). Animals in REM Sleep Deprivation Groups were placed on small platforms (6.5 cm in diameter) according to the MMPM, provide optimal access to food and water: RREMSD+S: Saline treated recovery REMSD group (n=9); RREMSD+DMSO: DMSO treated recovery REMSD group (n=9); RREMSD+MEL: Melatonin treated recovery REMSD group at a dose of 20 mg/kg (n=9).

Enzyme-Linked Immunosorbent Assay (ELISA) Analyses

Rats were anesthetized using sevoflurane and blood was obtained from all rats after decapitation between 09.00-11.00 am and collected in glass centrifuge tubes. Blood was centrifuged for 15 min at 3000 r.p.m., serum was collected and stored at -80°C until used. Serum Leptin, Orexin-A, and Nesfatin-1 levels were analyzed spectrophotometrically following the commercial kit protocols based on the ELISA principle (BT-LAB, Shanghai Korain Biotech Co., Ltd, People's Republic of China).

Statistical Analyses

Analyses were performed using Sigma Plot version 12.5. All values are reported as the Means ± SEM. Statistical analyses for weight of the animals in Study Group I were performed using One-way ANOVA followed by Holm-Sidak test, for weight of the animals in Study Group II were performed using One-way ANOVA RM. In addition, statistical analyses for ELISA tests were performed using One-way ANOVA followed by Holm-Sidak test. The correlation between OD values scored with ELISA was measured by the Pearson correlation. Statistical significance level was set at P<0.05.

Results

Weights of the Animals

Animal weights were recorded before and after the experiments. The data and statistical analyses for weight-related measurements of rats were presented in *Table 1* and *Table 2*. No significant differences were observed in the weights of animals in the Control Groups. However, a statistically significant decrease in weights on day 7 compared to day 1 was observed in rats subjected to REMSD in Study Group I (at least P<0.01) *(Table 1)*.

The weights of animals in Study Group II were compared at the beginning of the experiment (day 1), the day of transfer to normal laboratory cages (day 7), and the end of the experiment (day 13). A statistically significant increase in weights on day 7 compared to day 1 was observed in Control Groups (at least P<0.01). Rats in RC+S and RC+DMSO continued to gain weight, and the weight

Table 1. *Weights of animals in Study Group I at the beginning (day 1) and end of the experiment (day 7)*

Groups	Day 1	Day 7
C+S	248.9±8.7	242.2±8.0
C+DMSO	271.1±7.5	266.7±8.5
C+MEL	381.7±7.5	363.3±4.2
REMSD+S	292.2±4.0	241.1±4.5***
REMSD+DMSO	296.7±5.5	247.8±8.3***
REMSD+MEL	383.3±8.7	343.3±6.5**

***P<0.01, ***P<0.001 compared to day 1, n=9, mean±standard error*

Table 2. *Weights of animals in Study Group II at the beginning (day 1), transferred to normal laboratory cages (day 7) and end of the experiment (day 13)*

Groups	Day 1	Day 7	Day 13
RC+S	394.4±2.4	408.9±2.0***	413.3±2.4***
RC+DMSO	364.4±1.8	387.8±2.2***	390.0±2.9***
RC+MEL	344.0±2.2	358.0±2.5**	350.0±3.0
RREMSD+S	342.2±5.7	322.2±2.2***	338.9±3.5^{+++}
RREMSD+DMSO	316.7±2.4	302.2±2.8**	313.3±3.3^{++}
RREMSD+MEL	316.7±2.4	297.8±2.8***	304.4±2.4**

***P<0.01, ***P<0.001 compared to day 1, $^{++}$P<0,01, $^{+++}$ P<0,001 compared to day 7, n=9, mean±standard error*

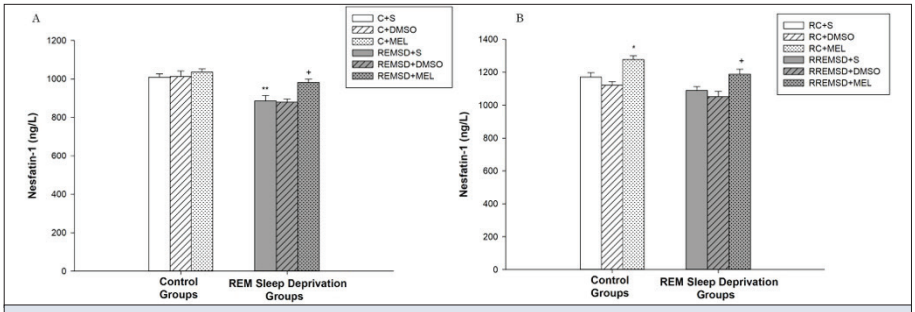

Fig 3. A- Serum Nesfatin-1 levels in Study Group-I, **P<0.01 indicates significant differences compared to the C+S group, +P<0.05 indicates significant differences compared to the REMSD+S group. **B**- Serum Nesfatin-1 levels in Study Group-II, *P<0.05 indicates significant differences compared to the RC+S group, +P<0.05 indicates significant differences compared to the RREMSD+S group. Values are presented as mean ± SEM. n=7

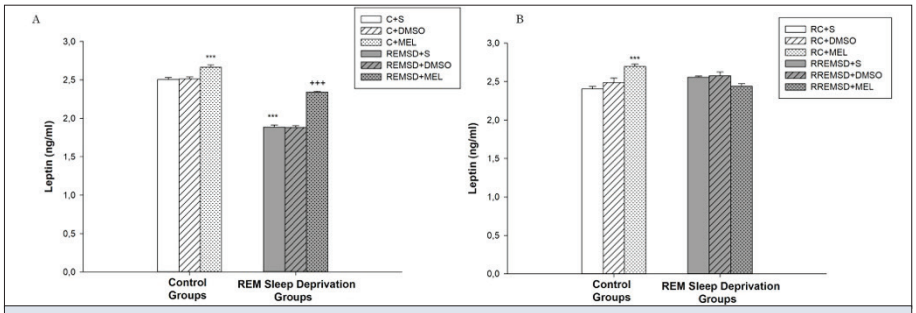

Fig 4. A- Serum Leptin levels in Study Group-I, ***P<0.001 indicates significant differences compared to the C+S group, +++P<0.001 indicates significant differences compared to the REMSD+S group. **B**- Serum Leptin levels in Study Group-II, ***P<0.001 indicates significant differences compared to the RC+S group. Values are presented as mean ± SEM. n=7

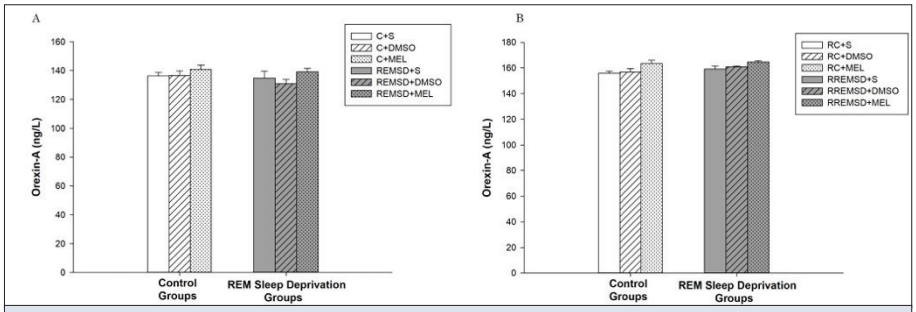

Fig 5. A- Serum Orexin-A levels in Study Group-I. **B**- Serum Orexin-A levels in Study Group-II. Values are presented as mean ± SEM. n=7

on day 13 was significantly higher than that of day 1 (P<0.001). Although not statistically significant, but there was a decreasing trend of weight on day 13 compared to day 7 in rats in RC+MEL group *(Table 2)*. On the other hand, rats subjected to REMSD showed a statistically significant decrease in weights on day 7 compared to day 1 (at least P<0.01). In addition, rats treated with Saline and DMSO in RREMSD groups showed a significant increase in weights on day 13 compared to day 7 (respectively, at least P<0.001 and P<0.01) *(Table 2)*.

ELISA Analyses

1- Serum Nesfatin-1 Levels

Since there was no significant difference between the animals treated with saline and DMSO, all comparisons were made according to the Saline group. In Study Group I, there was a significant decrease in serum Nesfatin-1 levels in REMSD+S group in comparison to C+S group (P<0.01). In addition, serum Nesfatin-1 level was higher in REMSD+MEL group compared to REMSD+S group (P<0.05) *(Fig.3-A)*. Melatonin treatment increased serum Nesfatin-1 levels compared to those received saline (P<0.05) in Study Group II *(Fig. 3-B)*.

2- Serum Leptin Levels

There was a significant increase in serum Leptin levels of C+MEL group (P<0.001), while there was a significant decrease in serum levels of REMSD+S group in comparison to C+S group (P<0.001). In addition, serum Leptin level was higher in REMSD+MEL group compared

to REMSD+S group (P<0.001) *(Fig. 4-A)*. Melatonin treatment also increased serum Leptin levels of RC+MEL group compared to those received saline (P<0.001) in Study Group II *(Fig. 4-B)*.

3- Serum Orexin-A Levels

There was not any significant difference in serum levels of Orexin-A between the groups *(Fig. 5-A,B)*.

4- Correlation of Nesfatin-1, Leptin and Orexin-A Levels

When the results of the Pearson correlation analyses were reviewed, it was determined that there were no significant correlations between Nesfatin-1 and Orexin-A, Nesfatin-1 and Leptin, or Orexin-A and Leptin in any of the groups.

Discussion

The present study provides novel information on the changes of serum Leptin, Orexin-A, and Nesfatin-1 levels following REMSD and recovery sleep and explains the connection between weight changes observed during melatonin treatment.

Sleep plays a crucial role in energy homeostasis [30]. According to the results of our study, a notable decrease was observed in the weight of rats subjected to REMSD. However, after a period of recovery sleep, the rats' body weight significantly increased. In a sleep restriction research, it was shown that while weight loss was observed at the beginning, there was weight gain afterwards [15]. The results of our study, in parallel with the study [31], show that SD causes weight loss and recovery sleep reverses this loss.

When the effects of melatonin on body weight were examined, the C+MEL group experienced a higher percentage weight loss than the C+S group. This result is likely related to the reduced appetite caused by an increase in Leptin levels. Furthermore, the group subjected to REMSD and treated with melatonin (REMSD+MEL) exhibited less weight loss compared to the REMSD group without melatonin treatment (REMSD+S). It is known that animals lose weight during REMSD despite increased food intake due to a negative energy balance. It is thought that nutrition increases due to increased energy needs [31]. The reduced weight loss with melatonin treatment may be attributed to the positive effects of melatonin on negative energy balance. The significant increase in Nesfatin-1 and Leptin levels in animals in the REMSD+MEL group compared to the REMSD+S group also supports these findings.

In the comparison of recovery sleep results between the 7th and 13th days, it was observed that there was weight loss in the RC+MEL group and weight gain in the other groups. This weight loss is thought to be related to the increased levels of Nesfatin-1 and Leptin which have an anorexigenic effect. Similarly, it was concluded that the less weight gain in the RREMSD+MEL group compared to the RREMSD+S group was related to increased levels of Nesfatin-1. Melatonin treatment is thought to regulate energy balance by reducing oxidative stress [32] through its antioxidant properties [33] in animals subjected to SD.

Melatonin administration has been shown to reduce body weight and body mass index in people with metabolic syndrome [34]. It has been observed that the administration of melatonin in rats with fructose-induced metabolic syndrome leads to weight loss [35]. A recent meta-analysis reported that melatonin has a weight-reducing effect [36]. Melatonin increases brown adipose tissue activity and mass [37]. When the effects of melatonin on nutrition and weight were examined, it was shown that animals supplemented with 0.4 µg/mL melatonin in their drinking water and followed for 12 weeks did not show any differences in food intake compared to the control group, but a significant decrease in weight was observed [38]. These findings support previous research [35-38] indicating that melatonin supplementation leads to a decrease in body weight.

Nesfatin-1 is an important anorexigenic peptide produced in the hypothalamus, defined as the satiety hormone, and plays a significant role in nutrition and glucose metabolism [19,39]. Nesfatin-1 has a primary effect on reducing appetite, independent of the leptin pathway, leading to decreased food intake and weight gain, as well as increased glucose uptake into tissues [40]. It has been shown that Nesfatin-1 mRNA and protein expression are decreased in the dorsolateral hypothalamus of rats after 72 hours of REMSD [21] . Consistent with these findings, the results of our study showed that Nesfatin-1 levels decreased in the REMSD group compared to the Control group.

Leptin receptors are present in both the CNS and peripheral tissues [41]. The results regarding changes in Leptin levels following sleep restriction are controversial. A previous study showed no change in Leptin levels [13], while another indicate an increase [12], or a decrease [42]. In animal studies, SD has been found to reduce Leptin levels [14,43]. Similarly, our study revealed a decrease in Leptin levels due to SD. A previous study has demonstrated that melatonin reduces Leptin levels [44], while another shows that it increases Leptin expression [45]. In our study, melatonin increased Leptin levels in both the control and SD groups.

Orexin plays an important role in normal sleep, energy metabolism and food intake. Orexin is a peptide produced from the lateral hypothalamus and basically increases food intake [46]. While Orexin release is low during the daytime wakefulness, it is high at night. Orexin receptor

subtype has a varied role in regulating NREM and REM sleep [47]. In the study in which 7 days of REMSD was created with the multi-platform method, values of Hypocretin-immunoreactive neurons were investigated and no significant difference was observed in Wistar rats compared to the control group, while an increase was observed in Wistar-Kyoto type rats, used as a depression model, compared to the control group [48]. Another study analyzed changes in Orexin-A levels in different brain regions after 96 h of REMSD. While there was no significant difference in the hippocampus and pedunculo-pontine area, Orexin-A levels significantly increased in the locus coeruleus, hypothalamus and cortex, and returned to normal level after recovery [49]. Similarly, in a study with mice, in which both Orexin-A and Orexin-B levels were examined in the whole brain after 96 hours of REM SD and 24 hours of recovery sleep, it was shown that neither SD nor recovery sleep changed Orexin levels [50]. The results of our study also showed that Orexin-A levels did not change with SD or recovery sleep, as reported in the previous studies [48-50]. In addition, no effect of melatonin treatment was observed on these parameters. There were no significant correlations between Nesfatin-1 and Orexin-A, Nesfatin-1 and Leptin, or Orexin-A and Leptin in any of the groups. The absence of a significant correlation was attributed to the limited sample size.

In conclusion, our study provides insights into the effects of REMSD, recovery sleep, and melatonin treatment on weight regulation and hormone levels related to the body weight change and nutrition. The most important limitation of this study is the inability to monitor the feeding of animals in this SD method. This study will shed light on future studies on sleep and nutrition and has added new information to research on the effects of melatonin, which is frequently used in treatment. Furthermore, this study will contribute to elucidating the role of sleep deprivation on eating behavior, evaluating the effects of melatonin metabolic processes, which is a widely used treatment in human and veterinary medicine, in accordance with the one health concept. The results obtained from this study will aid in predicting the effects that may occur on metabolic processes following the treatment.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (A. Cakir).

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Ethical Statement

The study has been approved by the Bursa Uludag University Local Ethics Committee on Animal Research under decision number 2022-17/07.

Competing Interest

The authors declared that there is no conflict of interest.

Author Contributions

A. C.: Conceptualization; Methodology; Investigation; Data curation; Writing original draft, Writing - Review & Editing and Funding acquisition. S. S.: Methodology; Investigation; Data curation; and Writing original draft. C. K.: Methodology; Investigation; Data curation; and Writing original draft, Writing - Review & Editing. N. K.: Conceptualization; Methodology; Writing - original draft; Writing - Review & Editing; Supervision; and Funding acquisition.

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RESEARCH ARTICLE

Effect of Celery Powder as an Alternative Nitrite Source on Some Quality Properties and Nitrosamine Formation in Sucuk

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ABSTRACT

This study aimed to determine the effects of celery powder (nitrate converted to nitrite) and cooking time on the formation of nitrosamine in sucuk. The microbiological and physicochemical properties were also investigated. Four sucuk batters were prepared: T1 - 100 mg/kg synthetic sodium nitrite, T2 - 150 mg/kg synthetic sodium nitrite, T3 - celery powder equivalent to 100 mg/kg nitrite, T4 - celery powder equivalent to 150 mg/kg nitrite. After ripening (initial fermentation temperature: 24±1°C, ripening time: 7 days), the samples were subjected to the analyses. Lactic acid bacteria and *Micrococcus/Staphylococcus* were not affected by the treatment. T4 treatment showed higher pH values than T1 and T2. The celery powder groups (T3 and T4) showed lower a_w values than other groups (T1 and T2). No significant differences were observed between the treatments in terms of thiobarbituric acid reactive substance (TBARS) value, residual nitrite level, N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) contents. However, N-nitrosopiperidine (NPIP) content was found to have higher in T4 treatment. Cooking time, especially 3 min, caused a significant increase in nitrosamine content. The effect of cooking time on nitrosamines was also revealed in principal component analysis.

Keywords: Sucuk, Fermented sausage, Nitrosamine, Celery powder, NDMA, NDEA, NPIP

INTRODUCTION

Fermented sausages have an important place among meat products with their characteristic sensory properties. These products are classified according to such criteria as raw material, type and amount of fat, seasonings, moisture content, water activity (a_w), moisture:protein ratio, weight loss, the mincing size of meat and fat, casing diameter and geographical region. However, the most common classification is based on a_w value or moisture:protein ratio [1]. The maximum a_w value for dry fermented sausages is below 0.90. Heat treatment is generally not applied in this type of products. In semi-dry fermented sausages, the minimum and maximum a_w values are 0.90 and 0.95, respectively, and heat treatment/smoking may also take place in the process of these products [2]. Moreover, dry fermented sausages often have a moisture:protein ratio of less than 2.3:1. This ratio may go up to 3.7:1 in semi-dry fermented sausages [1].

Two different types of fermented sausages are produced in Türkiye: sucuk and heat-treated sucuk. While the history of sucuk production dates back to ancient times, heat-treated sausage started to be produced in the 1980s [2,3]. Sucuk has a final pH of 5.4, moisture:protein ratio lower than 2.5:1 and fat:protein ratio lower than 2.5:1. There are three main stages in the production of this type of fermented sausage: batter preparation, fermentation (fast or slow), and ripening/drying. In the heat-treated sausage, the moisture:protein ratio must be <3.6:1, the fat:protein ratio must be <2.5:1, and the pH value must be ≤5.6 [4].

Depending on the ripening time, nitrate and/or nitrite are used as curing agents in fermented sausages [5]. However, nitrate must be converted to nitrite in products using nitrate. Nitrite is a multifunctional additive and shows antimicrobial and antioxidant activity. It also contributes to the formation of color and the development of flavor. Yet, nitrite has an important effect on the formation of nitrosamines, which have carcinogenic, mutagenic, and teratogenic properties [6]. The level of nitrosamines in fermented meat products varies depending on the product type. Factors such as ingoing nitrite, residual

nitrite, catalysts and inhibitors, cooking method and time, pH and a_w play an important role in the formation of nitrosamines. However, the most important factors are the cooking degree, level of ingoing nitrite and residual nitrite [7].

Since nitrite poses a risk to consumer health, research has been conducted for a long time on the use of nitrate-rich vegetables such as celery, Swiss chard, and beetroot powder as an alternative curing agent, and the effects of these alternative additives on product properties have been investigated [8-12]. The number of studies on nitrosamine formation by vegetable extracts is quite limited, and the effects of Swiss chard and celery powder on nitrosamine formation in heat-treated sausage were investigated in these studies [13,14]. There is no study about the effect of vegetable extract on nitrosamine formation in dry fermented sausages such as sucuk. In the present study, the use of celery powder (nitrate converted to nitrite) as an alternative curing agent and the effect of cooking time on nitrosamine formation in sucuk were investigated. In addition, microbiological and physicochemical properties of the samples were determined at the end of ripening.

Material and Methods

Material

Beef and beef fat were used as raw materials in production. Celery powder (Veg stable® 506) (2.10% nitrite) (nitrate converted to nitrite) was obtained from a commercial company (Florida Food Products, USA). *Lactiplantibacillus plantarum* GM77 (at the level of about 10^7 cfu/g) and *Staphylococcus xylosus* GM92 (at the level of about 10^6 cfu/g) strains isolated and identified from sucuk by Kaban and Kaya [15] were used as starter cultures.

Sucuk Production

Four sucuk batters were prepared: T1 -100 mg/kg synthetic sodium nitrite, T2 - 150 mg/kg synthetic sodium nitrite, T3 - 100 mg/kg nitrite from CP, T4 - 150 mg/kg nitrite from CP. The basic formulation (meat, fat, spices, salt and saccharose) given by Akköse et al.[16] was used in the production. The productions were carried out at three different times using three different raw materials at each production.

Laboratory type cutter (Mado Typ MTK 662, Dornhan, Schwarzwald) was used to prepare the batter, and laboratory type filler (Mado Typ MTK 591, Dornhan, Schwarzwald) was used for the filling. Each sucuk had an approximate weight of 200 g. Ripening was carried out in the climate chamber (Reich, Urbach, Germany) as follows: 1 days at 24±1°C, 1 days at 22±1°C, 2 days at 20±1°C and 3 days at 18±1°C. The relative humidity was gradually decreased from 90±2% to 84±2%.

Cooking Procedure

The samples were sliced at a thickness of 0.5 cm, and the cooking was carried out at three different cooking times (0, 1 and 3 min) at 180°C on a hot plate. The surface temperature of the hot plate was accurately measured with a digital thermocouple (Testo 926, Testo, Titisee-Neustadt, Germany).

Microbiological Analyses

Twenty-five g samples were homogenized with 225 mL of sterile physiological water (0.85% NaCl) in a stomacher (Lab Blender 400-BA 7021, London, UK). For the enumeration of lactic acid bacteria and Enterobacteriaceae, de Man Rogosa Sharpe Agar (MRS, Merck, Darmstadt, Germany) and Violet Red Bile Agar (VRBD, Merck, Darmstadt, Germany) were used, respectively, and incubations were carried out under anaerobic conditions (at 30°C for 48 h); for *Micrococcus/Staphylococcus,* Mannitol Salt Phenol Red Agar (MSA, Merck, Darmstadt, Germany) was used, incubation was carried out for at 30°C 48 h [17].

Physicochemical Analyses

To determine the pH value of samples, 10 g samples were homogenized with 100 mL distilled water using an Ultra Turrax (IKA Werke, Breisgau, Germany), and the pH values were determined using a pH-meter (Mettler Toledo, Greifensee, Switzerland). The a_w value was determined using the a_w device (TH-500 a_w Sprint, Novasina, Pfaffikon, Switzerland) [18]. For the determination of thiobarbituric acid reactive substance (TBARS) value, the method by Lemon [19] was used, and the results were given as µmol malondialdehyde (MDA)/kg. For residual nitrite analysis, the method given by NMKL [20] was used. The residual nitrite was determined using high-performance liquid chromatography coupled with a diode array detector (Agilent 1100, USA). Hamilton PRP-X100 (5 µm × 150 × 4.6 mm, USA) was used as a column. In the system, UV wavelength and flow rate were set 220 nm and 2 mL/min, respectively. The LOD (limit of detection) and LOQ (limit of quantification) values were determined adding standard at different rates (1-20 mg/L). The LOD and LOQ values of nitrite were 1.05 mg/L and 3.00 mg/L, respectively. The coefficient of the regression line (R2) of standard curve was 0.9999. The results were expressed as mg/kg.

Nitrosamine Analysis

The extraction was carried out according to the method given by Wang et al. [21]. To determine the nitrosamine level of the extract, GC/MS (Agilent 6890 N/Agilent 5973, USA) was used. In the system, helium was used as carrier gas and DB-5MS (30 m × 0.25 mm × 0.25 µm) (Agilent, USA) was used as column, and selected ion monitoring mode was used for quantification. The oven temperature programme was gradually increased; first kept at 50°C for

2 min, increased to 100°C at a rate of 3°C/min and kept at this temperature for 5 min, then increased to 250°C at a rate of 20°C/min. Standard nitrosamine mix (EPA 521 Nitrosamine Mix, Sigma-Aldrich, USA) was used for identification and the results were given as µg/kg.

The validation of the analysis was carried out by adding standard at different rates (0.5-20 µg/L). The LOD (limit of detection) and LOQ (limit of quantification) values were determined for N-Nitrosodimethylamine (NDMA, LOD = 0.32, LOQ = 0.97), N-Nitrosodiethylamine (NDEA, LOD = 0.37, LOQ = 1.12), N-Nitrosomethylethylamine (NMEA, LOD = 0.39, LOQ = 1.21), N-Nitrosopyrrolidine (NPYR, LOD = 0.37, LOQ = 1.13), N-Nitrosodipropylamine (NDPA, LOD = 0.45, LOQ = 1.37), N-Nitrosopiperidine (NPIP, LOD = 0.32, LOQ = 0.98) and N-Nitrosodibutylamine (NDBA, LOD = 0.38, LOQ = 1.14). The coefficients of the regression line (R2) for nitrosamine standard curve were all >0.999.

Statistical Analyses

The treatment (T1:100 mg/kg synthetic sodium nitrite, T2: 150 mg/kg synthetic sodium nitrite, T3: celery powder equivalent to 100 mg/kg nitrite, T4: celery powder equivalent to 150 mg/kg nitrite) was evaluated as the main effect, and replications were evaluated as random effects. The experiment was carried out according to a completely randomized block design with three replicates (three batters for each treatment). For the evaluation of nitrosamines, treatment and cooking time were taken as factors. The analysis of variance was applied to the results, and the means of significant sources of variation were compared using Duncan's multiple range tests at the < 0.05 level. The statistical analysis was performed using the SPSS program (IBM SPSS Inc., Chicago, IL, USA). In addition, the relationship between treatment and cooking time factors and nitrosamines was evaluated by principal components analysis (Unscrambler, CAMO vs. 10.1, Oslo, Norway).

Results

The effect of using celery powder as curing agent on lactic acid bacteria (LAB), *Micrococcus/Staphylococcus*, pH, a_w, TBARS and residual nitrite of sucuk is given in *Table 1*. The treatment had no significant effect on LAB and *Micrococcus/Staphylococcus* (P>0.05). In all treatments, the numbers of LAB and *Micrococcus/Staphylococcus* were determined 10^8 cfu/g and 10^6 cfu/g, respectively. The number of Enterobacteriaceae was below the detectable limit (<2 log cfu/g) in all groups (data not shown). pH was affected by treatment, and the highest mean pH value was found in T4. However, the mean pH value of this treatment was not differ T3 group (P>0.05). The a_w value was ranged from 0.870 to 0.890, and the treatments with synthetic sodium nitrite showed higher a_w values compared to the T3 and T4 groups (*Table 1*) (P<0.05).

TBARS value indicating the extent of lipid oxidation was not affected by the treatment (P>0.05). Similarly, residual nitrite level was not also affected by treatment (P>0.05). In all groups, residual nitrite amount was found to have under 20 mg/kg *(Table 1)*.

Only three nitrosamines (N-Nitrosodimethylamine (NDMA), N-Nitrosodiethylamine (NDEA) and N-Nitrosopiperidine (NPIP)) were determined in sucuk samples. N-Nitrosomethylethylamin (NMEA), N- Nitrosopyrolidine (NPYR), N-Nitrosodipropylamine (NDPA) and N-Nitrosodibutylamine (NDBA) were not dedected in the samples. The effects of using celery powder and cooking time on NDMA, NDEA and NPIP of sucuk are shown in *Table 2*. The treatment had no significant effects on NDMA and NDEA (P>0.05). In contrast, NPIP was affected by treatment, and the highest mean NPIP level was found in T4 group (P<0.05). Cooking time had a significant effect on NDMA, NDEA and NPIP (P<0.01). The NDMA level increased as the cooking time progressed, but a statistical difference was observed in only 3 min of cooking. NDEA was found to be below the LOD value in raw samples. After 1 min of cooking, an average NDEA of 0.64±0.16 µg/kg was determined. The 3 min cooking process caused an increase in the amount of NDEA. The NPIP level increased as the cooking time progressed (P<0.05). However, treatment x cooking time interaction did not show any significant effect on nitrosamines (P>0.05).

Table 1. *The effect of using celery powder on lactic acid bacteria, Micrococcus/Staphylococcus, pH, a_w, TBARS and residual nitrite of sucuk*

Treatment	Lactic Acid Bacteria (log cfu/g)	*Micrococcus/ Staphylococcus* (log cfu/g)	pH	a_w	TBARS (µmol MDA/kg)	Residual Nitrite (mg/kg)
T1	8.68±0.19^a	6.38±0.22^a	4.81±0.06^b	0.890±0.008^a	7.02±1.94^a	15.96±4.52^a
T2	8.39±0.31^a	6.49±0.19^a	4.86±0.09^b	0.888±0.004^a	6.45±0.94^a	17.01±4.98^a
T3	8.46±0.40^a	6.64±0.28^a	4.94±0.05^{ab}	0.875±0.005^b	6.83±0.62^a	17.61±2.75^a
T4	8.26±0.25^a	6.50±0.31^a	5.01±0.05^a	0.870±0.008^b	6.78±0.58^a	19.69±2.88^a
P value	> 0.05	> 0.05	<0.05	<0.05	> 0.05	> 0.05

T1: *100 mg/kg synthetic nitrite,* ***T2:*** *150 mg/kg synthetic nitrite,* ***T3:*** *celery powder equivalent to 100 mg/kg nitrite,* ***T4:*** *celery powder equivalent to 150 mg/kg nitrite;* $^{a-b}$ *Means marked with different letters in the same column are statistically different (P<0.05)*

Table 2. *The effects of using celery powder and cooking time on NDMA, NDEA and NPIP levels of sucuk (µg/kg)*

Treatment (T)	NDMA	NDEA	NPIP
T1	0.82±0.36[a]	0.46±0.38[a]	1.39±0.49[b]
T2	0.98±0.20[a]	0.49±0.40[a]	1.34±0.50[b]
T3	0.96±0.25[a]	0.52±0.42[a]	1.50±0.53[b]
T4	1.04±0.26[a]	0.57±0.48[a]	1.74±0.68[a]
P value	> 0.05	> 0.05	<0.05
Cooking time (min) (CT)			
0	0.75±0.26[b]	< LOD	0.95±0.17[c]
1	0.87±0.13[b]	0.64±0.16[b]	1.49±0.41[b]
3	1.23±0.16[a]	0.89±0.17[a]	2.05±0.32[a]
P value	<0.01	<0.01	<0.01
The interaction of T x CT	>0.05	>0.05	>0.05

NDMA: *N-Nitrosodimethylamine,* ***NDEA:*** *N-Nitrosodiethylamine,* ***NPIP:*** *N-Nitrosopiperidine.* ***T1:*** *100 mg/kg synthetic nitrite,* ***T2:*** *150 mg/kg synthetic nitrite,* ***T3:*** *celery powder equivalent to 100 mg/kg nitrite, T4: celery powder equivalent to 150 mg/kg nitrite;* [a-c] *Means marked with different letters in the same column are statistically different (P<0.05). LOD: Limit of detection*

The relationship between the factors and nitrosamines was evaluated by Principal Components Analysis (PCA) and is shown in *Fig. 1*. The first two principal components (PC1: 95% and PC2: 4%) accounted for 99% of the variance. The groups that were not applied cooking (T1/0, T2/0, T3/0 and T4/0) and the groups of T1, T2 and T3, which were cooked for 1 min, were on the negative side of PC1. On the other hand, all groups cooked for 3 min and T4 (T4/1) group cooked for 1 min were on the positive side of PC1. In other words, the raw and 1 min cooked samples of T1, T2 and T3 showed close correlations and, at the same time, differed from the 1-min cooked group of the T4 group. The groups cooked for 3 min and the T4/1 group showed close correlation with nitrosamines.

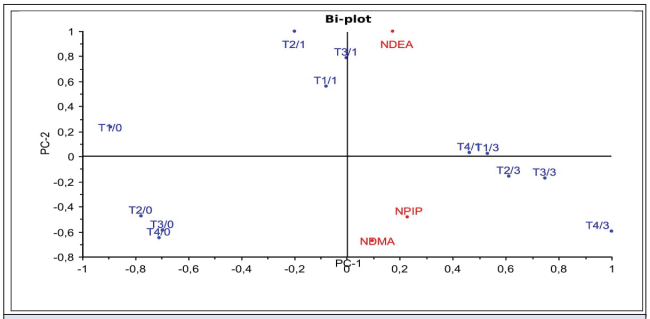

Fig 1. Principal component analysis of the relationship between the factors (treatment and cooking time) and nitrosamines (T/cooking time, NDMA: N-Nitrosodimethylamine, NDEA: N-Nitrosodiethylamine, NPIP: N-Nitrosopiperidine)

Discussion

In this study, autochthonous strains (*Lactiplantibacillus plantarum* GM77 and *Staphylococcus xylosus* GM92) were used as starter cultures. Both strains showed good adaptation to the sucuk environment and remained in high numbers. Similar results were also reported by Akköse et al. [16]. The use of celery powder in sucuk did not have a significant effect on the number of LAB. Similarly, Işıksal [22] reported that the use of celery in sucuk and heat-treated sucuk did not have a significant effect on the LAB count, while Pennisi et al.[23] reported a similar result for in Italian-type dry fermented sausage. Yılmaz Oral [13], on the other hand, reported that the use of celery powder equivalent to 150 mg/kg nitrite in heat-treated sucuk causes a decrease in LAB count, albeit slightly. Micrococci and staphylococci, another group of microorganisms that are technologically important in fermented sausages, show slow growth during fermentation since they are sensitive to acid [16-18]. Therefore, the number of *Micrococcus/Staphylococcus* in the final product increased slightly and did not exceed 10^7 cfu/g. The use of celery powder did not have an effect on these microorganisms *(Table 1)*. The members of the Enterobacteriacea family were found below the detectable limit in the final product due to their low a_w and pH values, which are in line with the results found in other studies [16,18].

pH is an important parameter in terms of both product safety and the development of sensory properties in sucuk and similar fermented sausages. LAB (spontaneous or starter culture) form lactic acid during fermentation, bringing the pH closer to the isoelectric point of muscle proteins, thus reducing the water holding capacity and facilitating drying [2]. In the present study, pH value increased slightly when using celery powder equivalent to 150 mg/kg nitrite. However, this increase was not at a level that would negatively affect product characteristics. On the other hand, Yılmaz Oral [13] reported that the pH value of the heat-treated sucuk increased as the proportion of celery powder use increased. It was also reported in a

study on ham that celery juice concentrate increased the pH value [9]. In terms of a_w, the groups containing synthetic nitrite gave higher a_w values than the groups containing celery powder. It is thought that this was resulting from the water holding capacity of celery powder. A similar result was also reported by Yılmaz Oral [13]. On the other hand, Işıksal [22] reported that the addition of different nitrate/nitrite sources did not affect the basic composition of the sucuk.

The TBARS value, which is a good indicator of the degree of lipid oxidation in meat products, was not affected by the use of celery powder. TBARS value was found below 1 mg MDA/kg in all groups. However, the use of celery powder in heat-treated sucuk slightly increased the TBARS value, but this value did not exceed 1 mg MDA/kg in any samples [13]. Sindelar et al.[8] also reported that the use of celery powder in ham production caused an increase in the TBARS value.

Residual nitrite level is an important factor in terms of nitrosamine formation in cured meat products [7,24]. In this study, residual nitrite levels were found below 20 mg/kg in all treatment groups and were not affected by the use of celery powder. In contrast, Işıksal [22] stated that residual nitrate level was higher in sucuk and heat-treated sucuk samples containing vegetal nitrate/nitrite both during production and storage stages. In this current study, it is thought that the low amount of residual nitrite is due to rapid acidification. The decrease in pH during fermentation accelerates the conversion of nitrite to nitric oxide and thus a significant decrease in the residual nitrite level occurs [25].

N-nitrosodimethylamine (NDMA) is one of the most commonly detected nitrosamines in fermented sausages [26,27]. This nitrosamine, which is formed as a result of the reaction between the nitrosating agent and dimethylamine, which is a secondary amine, is in the group of probably (Group 2A) carcinogenic compounds for humans [28]. Amines formed as a result of proteolysis during the ripening of fermented sausages can cause an increase in the level of this nitrosamine [29]. In this study, an increase in NDMA was not observed due to the use of celery powder. It was also reported in another study that the use of celery powder in heat-treated sucuk did not cause a significant change in NDMA content [13]. In addition, it was reported that the NDMA content of the Swiss chard powder was not affected in this fermented sausage type [14]. In the present study, NDMA content increased after 3 min of cooking. It was also determined in other studies that the NDMA content increased with cooking [24,27,30].

One of the probably (Group 2A) carcinogenic compounds for humans determined by the International Agency for Research on Cancer is NDEA [28]. This nitrosamine was unaffected by the use of celery. While it was below the LOD value in raw samples, it increased with cooking. The highest NDEA level (0.89±0.17) was obtained after 3 min of cooking. Ozel et al.[31] also determined that the NDEA content ranged between 0.10-0.95 µg/kg in sucuk.

In this study, it was determined that celery powder, when added at a level to match the nitrite level of 150 mg/kg, increased the NPIP level in the final product when added to the sucuk batter. A similar result was observed in heat-treated sucuk [13]. However, in a study using Swiss chard powder, no increase in NPIP was observed in heat-treated sucuk [14]. Black pepper, which is included in the formulation in sucuk and many other fermented sausages, plays a role in the formation of NPIP due to the piperine and piperidine it contains [7,32]. Piperidine is also found in celery [33]. In the present study, it is thought that the increase in NPIP level may be related to the piperidine content of celery powder. On the other hand, it was reported that lipid oxidation promotes the formation of NPIP [34]. In addition, as with other nitrosamines, cooking time caused an increase in NPIP, which is possible carcinogenic compounds (Group 2B) [28]. It has also been reported in other studies that the cooking time in sucuk and heat treated sucuk increases the NPIP content [13,24,27,32]. According to the PCA results, the increase in cooking time caused an increase in the level of nitrosamines. However, the effect of cooking on the formation of nitrosamines becomes more evident in the case of using celery powder at a level to meet the nitrite level of 150 mg/kg.

As a result, the use of celery powder as a curing agent in sucuk did not have a significant effect on the microbiological properties of the product. This vegetable product also had no significant effect on residual nitrite and lipid oxidation. The changes in pH and a_w values did not occur at a level that would affect the product properties. However, the addition of celery powder to the sucuk batter equivalent to 150 mg/kg nitrite caused an increase in the NPIP content in the final product. The cooking process also caused an increase in the content of nitrosamines, but especially the NDEA and NPIP content increased as the time progresses. Considering all these aspects, it was concluded that the use of celery powder as an alternative curing agent in sucuk is not effective in preventing the formation of nitrosamines and even slightly increases the NPIP content, and cooking is a more important factor in terms of nitrosamine.

Availability of Data and Materials

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

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Competing Interest

The author declared that there is no conflict of interest.

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RESEARCH ARTICLE

The Histopathological Evaluation of Effects of Application of the Bovine Amniotic Fluid with Graft on Peri-Implant Bone Regeneration

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ABSTRACT

This study aimed to determine the effects of bovine amniotic fluid combined with bone graft in treating peri-implant bone defects with guided bone regeneration. Twenty female Sprague–Dawley rats were divided into two groups. Bone sockets with a diameter of 4 mm in the coronal part and a diameter of 2.5 mm in the apical part of the implant were created into the corticocancellous bone in the metaphyseal parts of the right tibia bones of all subjects. Implants with a length of 4 mm and a diameter of 2.5 mm were placed in the bone sockets. In the sham surgery group (n = 10) was the circumferential bone defect equivalent to half of the 4-mm implant length, which occurred between the implant and the bone, filled with bovine xenograft. Bovine xenografts were filled with amniotic fluid mixture in the experimental group (n = 10). After 8 weeks of recovery, all rats were sacrificed. The implants were extracted from the soft tissues and the surrounding bone. Subsequently, the bones were decalcified and prepared for histological analysis. The percentage of newly regenerated bone (NRB) formation and fibrosis in the bone defect area around the implant was calculated from all sections. NRB was found in 37.4±4.4% of controls and 41.4±2.63% of test animals ($P<0.05$ and $P=0.024$, respectively). Fibrosis formation was found at a rate of 38.6±5.06% in the control group and 33.2±5.38% in the test group ($P<0.05$ and $P=0.033$, respectively). It was considered that combining bovine amniotic fluid with bone transplant could be a useful way of treating bone abnormalities.

Keywords: Bone graft, Bovine amniotic fluid, Guided bone regeneration, Peri-implant bone defect, Tibial bone

INTRODUCTION

Titanium-manufactured implants have recently become one of the most popular options in orthopedics, oral and maxillofacial surgery, and prosthetic dentistry [1]. With their functionality, aesthetics, ease of use, and comfort, titanium-made implants are a trustworthy material in fracture treatment [1,2]. Successful treatments with titanium implants in orthopedic and maxillofacial implant operations depend on the extent of osseointegration, which is described as direct contact of the bone with the titanium implant surface, as well as the properties of the implant material. In this regard, bone formation and regeneration mechanisms are important parameters for effective osseointegration [3].

Guided bone regeneration (GBR) applications have proven to be effective in treating abnormalities and deficiencies in peri-implant bone tissue. In the presence of bone defects and insufficient bone tissue, GBR is necessary for 40% of patients with implants to ensure healthy osseointegration. The survival rates of patients with GBR-treated implants have been comparable with those of non-GBR-treated implants. Titanium implants have a high survival rate after GBR treatment and a good survival rate after functional loading. Studies demonstrating a 95% implant survival rate after horizontal or vertical GBR treatment with

various bone graft materials have been recorded in the literature. In this regard, GBR is a scientifically approved and successful treatment method frequently used with various bone graft materials, including otogen, allogeneic, xenogeneic, and alloplastic, for treating bone tissue defects around the titanium implant [1-4].

Stem cells were obtained from amniotic fluid and these stem cells attracted the attention of researchers. Amniotic mesenchymal stem cells can be isolated from amniotic fluid. Therefore, their use does not fall within the ethical concerns associated with the use of embryonic stem cells. Furthermore, like other fetal-derived stem cells, amniotic mesenchymal stem cells are easy to store and can be obtained at a low cost. Populations of amniotic mesenchymal stem cells can be easily grown, and these cells can be kept for a long time without any adverse effects. Therefore, amniotic fluid can be considered a source of pluripotent and multipotent stem cells for organ regeneration [5-8].

Bovine amniotic fluid contains various proteins, minerals, and cells [8]. Cattle have a well-developed allantoic cavity containing abundant amniotic fluid. Because of their nature, they can be a valuable source for obtaining amniotic fluid to treat various diseases [8]. Unlike embryonic stem cells, amniotic fluid stem cells do not develop teratomas when injected subcutaneously into mice [9-12]. Therefore, amniotic fluid stem cells are considered an intermediate stem cell type as they exhibit embryonic and adult stem cell characteristics. Wound repair and regeneration can be aided by amniotic tissue products [13].

Amniotic fluid contains high concentration of various growth factors that may be involved in bone formation mechanisms [9-14]. This study examined the efficacy of bovine amniotic fluid in combination with bone graft in the regeneration of experimentally created tibia peri-implant bone defects in rats.

Material and Methods

Ethical Approval

The study's experimental and surgical methods were implemented in the experimental research center of Fırat University Elazığ, Turkey. Before the experimental applications were carried out, ethical approval was obtained from the Fırat University Animal Experiments Local Ethics Committee (Protocol Number: 24/02/2020-380123). Rats were cared for and kept in their special cages in accordance with the Declaration of Helsinki.

Animal and Study Design

In the study, 20 female Sprague-Dawley rats were divided into two groups. In the control implant + graft group (n = 10), titanium implants 4 mm long and 2.5 mm in diameter were applied to the tibia bones of the rats. Titanium implants were inserted into implant beds developed in the corticocancellous bone tissue of the right tibia bones of the rats. A bovine bone graft was implanted in the circumferential bone defect surrounding the implants, accounting for 2 mm of implant length. During the 8-week healing period, no additional procedures were performed on the rats in this group. Following decalcification, the bone tissue containing implants was separated from the soft tissues and subjected to histological analysis. The percentages of new bone development and fibrosis in the defect around the implant were calculated [3,4]. In the implant + graft + amniotic fluid group (n = 10), titanium implants with 2.5 mm diameter and 4 mm length were applied to the tibia bones of the rats. The implants were inserted in the corticocancellous bone tissue of the rats' right tibial metaphyseal bones, and amniotic fluid and bovine bone graft were combined and placed in the bone defect created in the neck region, corresponding to 2 mm of the implant length. Bone grafts were obtained by mixing 2 mL bovine amniotic fluid with 1 mL bone graft. During the 8-week study period, no experimental procedures were used. Rats were sacrificed after an 8-week experiment. After removing the soft tissues, the implants and surrounding bone tissues were collected and histologically analyzed. The percentages of new bone formation and fibrosis in the defect around the implant were calculated [3,4].

The area of the peri-implant defect was initially measured before assessing new bone growth and fibrosis. The regions of new bone tissue and fibrosis that had grown within the defect were measured. The percentages of new bone formation and fibrosis were calculated by dividing the new bone tissue and fibrosis values by the defect area [3,4].

Obtaining Bovine Amniotic Fluid

During cesarean delivery at the Fırat University Animal Hospital, bovine amniotic fluid was taken from a healthy pregnant cow using an injector. The sample was brought to the laboratory in an ice box without breaking the cold chain, and it was stored in a deep freezer at −20°C until the day of surgery. It was used after allowing the sample to dissolve at room temperature for 15 min.

Surgical Procedures

Following general anesthesia, all surgical applications were performed under sterile conditions. General anesthetics (10 mg/kg xylazine HCl [Rompun®, Bayer, Germany] and 40 mg/kg ketamin HCl [Ketasol®, Richter Pharma, Germany]) were administered intraperitoneally using a rat-specific injector. During the surgical application preparation phase, the surgical area was shaved and then cleansed with povidone-iodine. An incision was made

over the right tibia bones of the rats with a no. 11 scalpel during the surgical implantation of the implants, and the metaphyseal part of the bone was reached. The surgical region was cleansed with physiological saline to prevent warmth while the implant sockets were opened. A hollow with a width of 4 mm from the coronal part and a diameter of 2.5 mm in the apical half was opened to half the length of the implant while forming the implant sockets. Following implant placement, the circumferential bone defect of 1.5-2 mm developed surrounding the implant was filled with bovine bone graft in the sham surgery group and amniotic fluid mixture with bovine bone graft in the test group [3,4]. After surgical insertion, the soft tissues were sutured with a 4-0 absorbable suture (polyglactin). Antibiotics (50 mg/kg Penicillin [Ampisina®, Gensenta İlaç San. Tic. A.Ş, Turkey]) and analgesics (0.1 mg/kg Tramadol hydrochloride [Ramadex®, Haver Trakya İlaç San. Tic. A.Ş, Turkey]) were administered intramuscularly for 3 days following all surgical operations to avoid infection and pain. Whole rats were slaughtered after 8 weeks of surgical procedures [3,4].

Histological Analysis

After removing the soft tissues, titanium implants and surrounding bone were collected for histomorphometric analysis. All bone samples were preserved in 10% formaldehyde for tissue fixation. After that, the bone tissues were softened in 10% formic acid. The implants were gently removed from the bone after the surrounding bone tissues relaxed. After drying, the softened samples were embedded in paraffin wax. Before microscopic analysis, all samples were stained with hematoxylin, eosin, and Masson trichrome. Histological assessment was performed using a light microscope. A light microscope was used to examine bone tissue sections with a total thickness of 6 µm surrounding the implants. The percentage of bone filling and intradefect fibrosis was evaluated [1-3].

Statistical Analysis

The Student's t-test was used to determine whether there was a significant difference in the experiment data (due to the availability of two more test groups not stated in this article). All values included in the statistical phase of the study were expressed as mean ± standard deviation (SD), and P<0.05 was considered the statistically significant value.

Results

Table 1 shows the distribution of newly regenerated bone (NRB) and fibrosis among groups. The graft with the bovine amniotic fluid group had the highest NRB, whereas the sham control group had the lowest. The mean values of NRB in the BAF graft and sham control groups

Table 1. *Histologic new bone formation (NRB) an fibrosis parameters of the groups after the H&E eosin and masson trichrome staining*

Parameters	Groups	N	Mean (%)	SD	P*
NRB	Amnion	10	41.4	2.63	0.024
	Control	10	37.4	4.4	
Fibrosis	Amnion	10	33.2	5.38	0.033
	Control	10	38.6	5.06	

** Student T Test (p<0.05). Statistically significant difference detected between the groups*
NRB: Newly Regenerated Bone, SD: Standard Deviation

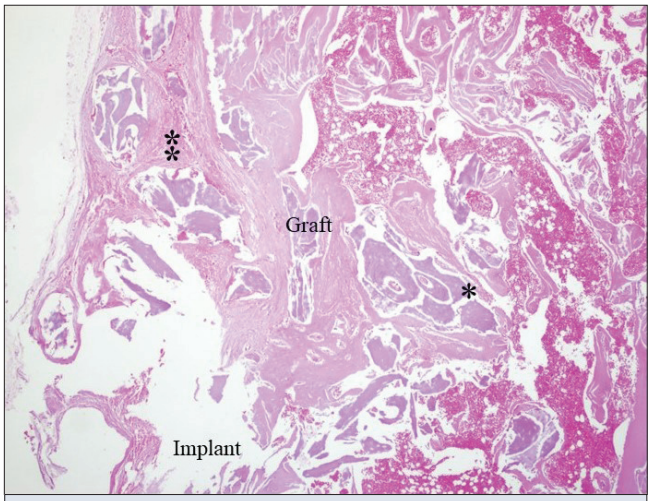

Fig 1. Decalcified histologic images of the sham control group (4 *g* magnification, Hematoxylin-Eosin). Bovine xenografts appeared to ossify at the implant neck defect site and are surrounded by fatty bone marrow. Ossification areas are surrounded by fibrosis. * Newly regenerated bone, ‡ Fibrosis

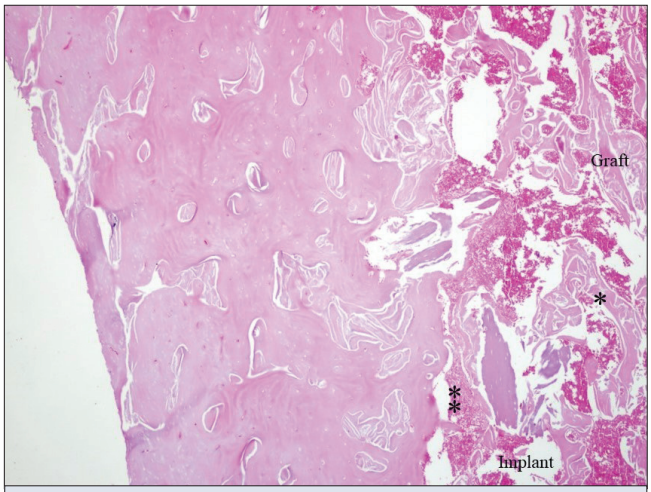

Fig 2. Decalcified histologic images of the bovine amniotic fluid and graft mixation group (4 *g* magnification, Hematoxylin-Eosin). Bovine xenografts appeared to ossify at the implant neck defect site and are surrounded by fatty bone marrow. Ossification areas are surrounded by fibrosis. Bone filling and maturation of the defect in the experimental group were statistically higher than in the sham group. * Newly regenerated bone, ‡ Fibrosis

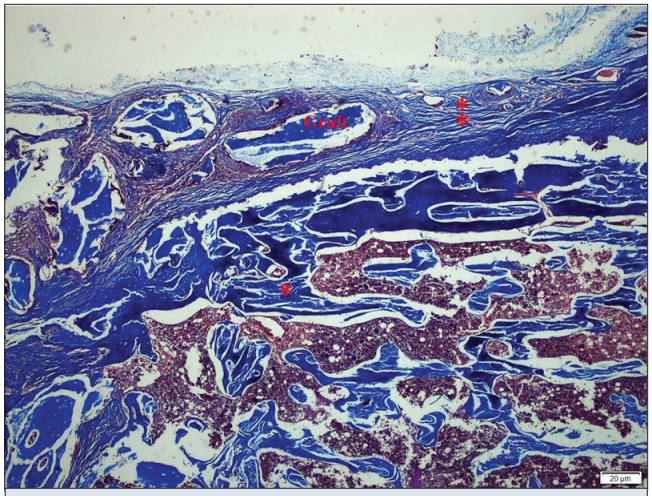

Fig 3. Decalcified histologic images of the sham control group (10 *g* magnification, Masson Trichrome). Bovine xenografts appeared to ossify at the implant neck defect site and are surrounded by fatty bone marrow. Ossification areas are surrounded by fibrosis. * Newly regenerated bone, ‡ Fibrosis

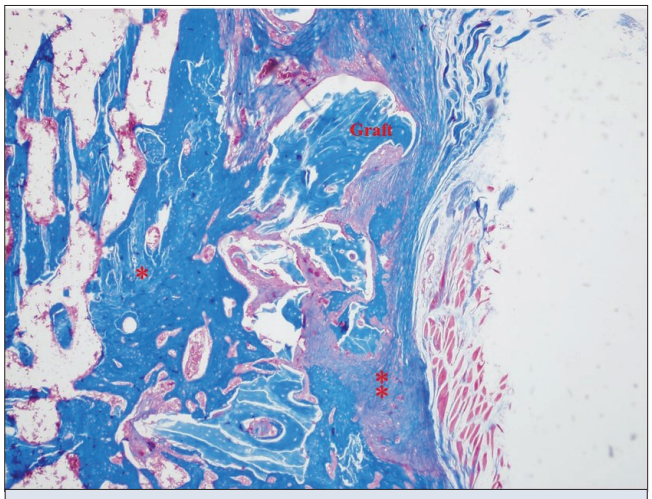

Fig 4. Decalcified histologic images of the bovine amniotic fluid and graft mixation group (10 *g* magnification, Masson Trichrome). Bovine xenografts appeared to ossify at the implant neck defect site and are surrounded by fatty bone marrow. Ossification areas are surrounded by fibrosis. Bone filling and maturation of the defect in the experimental group were statistically higher than in the sham group. * Newly regenerated bone, ‡ Fibrosis

were 41.4±2.63% and 37.4±4.4%, respectively (P<0.05 and P=0.024). The graft with the bovine amniotic fluid group had the highest fibrosis level, whereas the lowest in the sham control group had the lowest. The mean values of fibrosis in the bovine amniotic fluid graft and sham control groups were 33.2±5.38% and 38.6±5.06%, respectively (P<0.05 and P=0.033). There were statistically significant differences between groups in NRB and fibrosis values (*Fig. 1*, *Fig. 2*, *Fig. 3* and *Fig. 4*).

Discussion

Bovine amniotic fluid is a relatively new material, and research on its effects on bone metabolism is ongoing. Positive effects of bovine amniotic fluid on bone tissue, tendon, and nerve regeneration have been reported in the literature [15,16]. Furthermore, some researchers have found that bovine amniotic fluid is less expensive and easier to obtain than human amniotic fluid. Mesenchymal cells, insulin growth factors, other growth factors, and macromolecules, such as hyaluronic acid and hyaluronic acid stimulating factor, are found in bovine amniotic fluid [17,18]. The increased percentage of newly formed bone tissue in the bone defects and lower fibrosis levels reported in the participants transplanted with bovine amniotic fluid are assumed to be to these characteristics of bovine amniotic fluid.

This study aims to generate new perspectives for improving the rate of bone formation and bone quality. Bovine amniotic fluid is a successful method in GBR with bone graft. The components of bovine amniotic fluid have allowed it to be used in various applications [19]. Insulin-like growth factor, for example, can be actively produced by the placenta of ruminant animals [20]. Insulin-like growth factor-1 can directly and indirectly increase osteoblast matrix production [21]. Therapeutic applications, such as growth factors and other regenerative agents, may be required to enhance bone formation around implant surfaces. Furthermore, an osteogenic coating on implant surfaces has been proposed to improve osseointegration and bone tissue regeneration around the implant [22]. Therefore, using bovine amniotic fluid is believed to play a role in increasing GBR around the implant. Previous studies reported that amniotic fluid stem cells activated the bone morphogenic protein gene [23]. The bone morphogenic protein is involved in regeneration by generating the signal that causes osteoblast maturation. Bone morphogenetic proteins are the most critical growth factors for bone formation and repair among bone-related growth factors [24,25]. Osteoblast maturation is also initiated by bone morphogenetic protein [26]. Bovine amniotic fluid can be considered a diverse supply of mesenchymal cells. These mesenchymal cells can develop into various cell types, including adipose, muscle, bone, and neural lineages [24-26].

In this study, GBR was performed, a procedure commonly used to treat bone defects around the implant and has gained popularity in the literature. The data gathered at the end of the trial confirmed that the biological ingredients in bovine amniotic fluid, when used in conjunction with the graft to repair bone defects, could yield more beneficial results. In the presence of insufficient bone tissue, bone grafts, commonly used in oral-maxillofacial surgery, periodontology, oral implantology, and orthopedic surgery, are an essential aspect of the treatment. In the present literature, autogenous bone grafts are still considered the gold standard. However, due to difficulty in

acquiring autogenous grafts, defects in the donor location, neurovascular injuries in obtaining autogenous grafts, and early resorption, clinicians typically use animal-derived xenografts, synthetic alloplastic grafts, and human-derived allogeneic grafts [1-4,5,7]. This study examined bone healing between xenograft mixed with bovine amniotic fluid and the xenograft alone group. This study hypothesized that biological components can accelerate bone healing, such as hyaluronic acid, mesenchymal stem cells, and growth factors in bovine amniotic fluid. The xenograft was combined with enough bovine amniotic fluid to make it plasmatic, and the circumferential bone defect around the implant was filled [1,3,4,15,16,24-26].

The bovine xenografts used in the study were freeze-dried and obtained through various laboratory processes, have reduced antigenic properties, and are commonly used therapeutically clinically in orthopedic and oral maxillofacial surgery. Furthermore, the use of rats in this study was indicated by the study by Ozgenel et al.[16] They used macroscopic and histological methods to compare nerve recovery using human amniotic fluid in sciatic nerve injury with controls and found no immunogenic response in rats in the human amniotic fluid group.

This study evaluated bovine amniotic fluid's effectiveness in guided bone regeneration when used with xenograft using a histological approach. Bovine amniotic fluid, which contains chemicals such as mesenchymal stem cells, hyaluronic acid, and growth factors, has become a material that can positively influence bone tissue healing. The findings of this study suggest that using bovine amniotic fluid in conjunction with bone transplant may be an effective method in GBR, which is used in treating peri-implant bone defects.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (Ozmen Istek) at the University of Mus Alparslan University, Faculty of Health Sciences, Department of Nursing, Mus, Turkiye.

Funding

There is no funding.

Conflict of Interest

There is no conflict of interest.

Ethical Approval

Ethical approval was obtained from the Fırat University Animal Experiments Local Ethics Committee before the experimental applications were carried out (Protocol Number: 24/02/2020-380123).

Author Contributions

O.I., M.T., and S.D. designed the study. O.I., S.D., and M.T. did the experimental procedures. H.E. and B.K. did the histological procedures. B.K., S.D., M.T., and H.E. did the histological analysis. S.D. did the statistical analysis. O.I., R.G., E.C.O., S.D., and M.B.B. wrote the study. R.G., M.B.B., and E.C.O. controlled the manuscript.

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Review

Thiamethoxam Toxicity: A Review in One-Health Perspective

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ABSTRACT

Extensive and frequent use of pesticides has induced numerous abnormalities in target and non-target exposed organisms. Among different various pesticides, neonicotinoids are extensively employed in agro-production sectors. Thiamethoxam (TMX) plays an essential role in keeping the crop safe from insect attack, but on the other hand, it has been reported to induce adverse effects in both humans and animals. Previously, it was thought that neonicotinoids have low toxicity potential in mammals, but widespread use has made it evident that these pesticides have serious toxic effects on both invertebrates and vertebrates. Extensive applications of pesticides also pose serious eco-toxicological threats to aquatic and terrestrial organisms in the ecosystem. This review describes the chemistry, pharmacodynamics, and toxic effects of various TMX on living organisms. Moreover, this review summarizes the excretion/deposition of TMX in different tissues along with potentially adverse effects on production potential, immunity, blood parameters, and male/female reproductive systems. Though the pros of TMX surpass the cons, its reported intrinsic toxicity stresses the need to develop new pesticides that have high potency with little harm to humans and animals. Hence, there is a need for hours to address knowledge gaps related to TMX and design effective rational usage of TMX strategies to keep the ecosystem safe from the potentially harmful effects of TMX.

Keywords: Thiamethoxam, Pesticides, Neonicotinoids, Toxicity, Residues, Human, Animals

Introduction

The massive rise in the human population and livestock farming with food and fodder shortages has ultimately highlighted the need for improved agriculture production. Insecticides/pesticides/herbicides are extensively used in agriculture, public health management, and veterinary practice to control insects/pests, and parasites and to enhance crop yield [1-3]. Globally, there has been a massive rise in the usage of insecticides and pesticides to protect crops and preservation of agricultural products [4,5]. However, irrational and indiscriminate use leads to various harmful effects on the exposed organisms including humans and animals [6-8]. Nutrient swings, climate change, habitat loss, acidification, and biological invasions have seriously threatened public health. Even the labeled use of different synthetic chemicals in agriculture leads to aquatic ecosystem contamination and thus becomes dangerous to aquatic life [9]. These synthetic compounds induce oxidative stress that may lead to decreased reproductive efficiency, oxidative disorder, poor erythropoiesis, and genotoxic effects on their consumers [10-13]. Extensive usage of pesticides increases the chances of serious risk to the environment, animals, human beings, and birds living in the same ecology [7,14]. Pesticides are commonly used in agriculture systems to increase crop yield and eradicate a variety of parasites from livestock populations [15]. Previously, it was thought that neonicotinoids have low toxicity potential in mammals, but widespread use has made it evident that these pesticides have serious toxic effects on both invertebrates and vertebrates [16,17]. The European Union has banned neonicotinoid pesticides, based on the threat they pose to pollinators [18]. Extensive applications of pesticides also pose serious

eco-toxicological threats to aquatic and terrestrial organisms in the ecosystem [10,19,20]. Pesticides continue to bio-accumulate in aquatic organisms, depending on their Physico-chemical properties, and can therefore interact with tissues and cells to induce the process of biotransformation [21-23]. Over the last two decades, the extensive use of these synthetic chemicals has increased dramatically and tends to accumulate these chemicals in groundwater, agricultural products, soils, and surface water [24]. Other parameters are potentially affected, such as growth, reproductive capacity, and even survival of aquatic organisms [25-27]. Among different pollutants that induce carcinogenic and mutagenic effects, pesticides are the most hazardous compounds responsible for numerous tissue changes in target and non-target organisms. The objective of this review is to provide insights about thiamethoxam in the One-Health interface.

Impacts of Pesticides on Terrestrial and Aquatic Life

Terrestrial Life

Pesticides lead to the induction of harmful effects on domesticated animals, plants, insects, and human beings. The severity of the toxic effects of pesticides depends upon the dose and the type of organism exposed [28]. Death of the target and non-target exposed species is the most obvious sign of toxicity that leads to a decline in number. The frequency of the mortalities is directly proportional to the route, and the dose of pesticide exposure. In addition, a fall in the number of members of one species in a community leads to a decrease in the population size of the other species with which the target species interact [29].

The forms of life are more often exposed to more than one pesticide at a time, which has either additive or synergistic toxic effects [30,31]. Pesticide toxicity also leads to weight loss in association to decrease food and water intake [32], memory loss, loss of aggressive behavior, and lack of mobility desire, making the affected animal prone to predation by various predators [33].

Malfunctioning of endocrine glands due to exposure to sublethal levels of pesticides can lead to impaired growth and development, and reproductive failures [34]. The thyroid hormone is crucial for metamorphosis and development. Studies have shown that exposure of birds to DDT, OP, carbamates, and pyrethroids [35,36], goldfinches to linuron, and amphibians and fishes to endosulfan [37] leads to developmental abnormalities as a result of decreased thyroid hormone levels. In many studies, the lower hatching success and impaired reproduction in bald eagles and alligators in Florida have been reported due to heavy contamination of agricultural lands with bifocal and DDT [38]. Moreover, in that area, the testicles of male alligators were found to be poorly organized and the morphological characteristics of female ovaries were abnormal [39].

Aquatic Life

Fishes are one of the important sources of proteins for human beings and occupy a vital place on the food web [40]. owever, contamination of the river and oceanic water due to different environmental pollutants, particularly insecticides, has induced adverse impacts on aquatic life by affecting growth, survival, and reproduction. Unfortunately, the extensive use of pesticides to improve crop production has led to deleterious effects on aquatic life [41,42]. Contamination of water bodies with pesticides is not only a serious threat to the food supply but also has a negative effect on the health of aquatic life [43]. Chronic toxicities with pesticides may lead to the extinction of endangered species due to loss of natural defense, induction of blindness, hyperexcitability, weakness, and sterility [44-46]. In addition, prolonged exposure of organophosphates to aquatic life has resulted to induce the abnormal swimming of fish and peroxidative damage to gills and brains [47,48]. Moreover, chromosomal aberrations and cellular hyperproliferation are the main cellular events that may appear as a result of exposure to pesticides [49-51]. The reduced protein level in muscle [52,53], poor feed utilization, and poor self-defense from predation are the other consequences that may appear due to pesticide exposure in various aquatic organisms [44,54]. According to [53], pesticides have been reported to have negative impacts on the reproductive potential of fish, causing abnormal sexual development, male feminization, abnormal sex ratio, and unusual mating habits. Some studies also indicate that long-term exposure to lufenuron causes DNA damage, increases oxidative stress, decreases the profile of enzymatic antioxidants, and causes histological lesions in the visceral organs of Nile tilapia [43]. A brief overview of the toxic effects of different pesticides on various forms of life is highlighted in *Table 1*, *Table 2*, *Table 3*, *Table 4*.

Synthesis of Neonicotinoids

The discovery of neonicotinoids was a breakthrough in pesticide research. Neonicotinoid pesticides are one of the fastest-growing classes of insecticides [94]. With no cross-resistance to the other conventionally used insecticides, this class started to replace other insecticides like organophosphates, pyrethroids, and carbamates [95].

During the 1980s, neonicotinoid insecticides were synthesized to regulate the pest population dynamics [96]. This class of chemicals is one of the most important classes of acetylcholine receptor inhibitors accounting for up to 25% of the commercial insecticides available in the market [97]. According to the classification by Hussain et al.[98], these insecticides can be grouped into three categories based on their chemical nature and generations. *Fig. 1* shows the representative members of each class along with the year of commercialization into

Table 1. Effects of different pesticides on Micro-Organisms and Soil Metabolism

Community	Pesticide	Adverse Effects	Reference
Microorganisms and Soil Metabolism	Benomyl and Captan	Reduces soil basal respiration	[55,56]
	2,4-D picloram and Glyphosate	Reduces soil basal respiration	[57, 58]
	Atrazine and Metolachlor	Alters the soil community structure Reduces methanotrophic bacteria	[59,60]
	Carbofuran insecticide	Increases nitrogen fixation by Azospirillum in rice paddies	[61]
	Glyphosate	Suppresses soil bacteria; Increases plants' susceptibility to pathogens; Inhibitory effect of phosphatase in the presence of glyphosate	[62,63]

Table 2. Effects of different pesticides on arthropods community

Community	Pesticide	Adverse effects	Reference
Arthropods	Aldrin, Dieldrin, Heptachlor, Chlordane and DDT	Reduced Springtails species *(Collembola)*, mites, and Myriapoda	[64-66]
	DDT, Endosulfan, Aldrin, Chordane and Heptachlor, Carbamate, Aldicarb, Carbofuran	Negative effect on predatory mites' population	[66-68]
	Gaseous pesticides	Destroys mite population; Decreases soil biodiversity	[69,70]
	DDT, Aldrin, Carbamates, Organophosphates	Detrimental to the centipede population	[71,72]
	Lindane, Carbamates and Organophosphates	Destructive to springtail population	[73,74]
	Simazine	Destroys Diptera larvae significantly; accumulation of dead organic matter	[75]
	Ivermectin	Reduction in the emergence of *Liatongus minutes* and flies from cowpats	[31,76]

the market. These chemicals irreversibly bind to nicotinic acetylcholine receptors very tightly [99,100], resulting in blockage of acetylcholine binding to the receptors leading to spastic paralysis (overstimulation of the cells) and death of cells and/or of individuals [101,102].

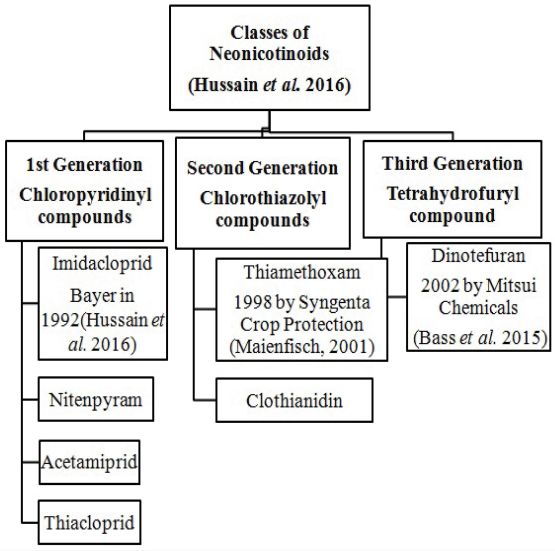

Fig 1. Classification of neonicotinoids (Created by Biorender)

Thiamethoxam

Thiamethoxam [3-(2-chloro-1, 3-thiazol-5-methyl) 25-methyl-4-nitroimino-perhydro-1,3,5-oxadiazine)] is a nitro-substituted second-generation neonicotinoid [103]. The empirical formula of TMX is $C_8H_{10}C_lN_5O_3S$ having a molecular weight of 291.7 g/mol. TMX is a crystalline powder with a slight cream color. The physicochemical properties of TMX are mentioned in *Table 5* [104].

Discovery and Synthesis

Thiamethoxam was first discovered and developed by Ciba Crop Protection in 1991 [105-107]. Since 1998, TMX has been marketed with different trade names like Actara® and Cruiser®. N-methyl-nitroguanidine treated with formaldehyde and formic acid leads to laboratory synthesis of thiamethoxam [108]. Alkylation with 2-chlorothiazol-5-ylmethyl chloride in N, N-dimethyl-formamide, and potassium carbonate as a base yield the active ingredient in good amounts [106].

Table 3. *Effects of different pesticides on other vertebrates community*

Community	Pesticide	Adverse Effects	Reference
Other Vertebrates	Lindane, Carbamate, and Organophosphates	Reduce saprophytic and beneficial nematodes	[59,75]
	Captan	Reduces fungal-feeding nematodes' population	[77]
	Thiobencarb and Simetryne	Increase plant-root parasites	[78]
	Chlormethoxyfen	Decimate predaceous mononchids	[78]
	Copper fungicides and Arsenates	Kill earthworms; increase avoidance behavior	[79,80]
	Imidacloprid	Adversely affect the burrowing activity of earthworm	[81]
	Chlordane, Heptachlor, Phorate, and Carbofuran	Toxic to all worms	[82]
	All fumigants	Kill earthworms	[75]
	Carbendazim	Harmful to earthworm	[83]
	DNOC, Chlorpropham, Atrazine, Simazine, Monuron	Rreduce earthworm populations	[83,84]

Table 4. *Effects of different pesticides on vertebrates community*

Community	Pesticide	Adverse Effects	Reference
Vertebrates	Organochlorines (OC)	Accumulate in tissues of all organisms and progressively released	[85]
	Consumption of invertebrates contaminated with OC insecticides	Causes the death of bats and other insectivorous birds	[86]
	OC insecticides, DDT	Reproductive impairment in birds and fish-eating birds	[87]
	Herbicides and Insecticides	Breeding failure; Chick starvation; Poor survival	[88,89]
	Routinely used pesticides	Adversely affecting the bird population, Pronounced teratogenic and histopathological effects in the liver were also observed in birds at higher dosages	[3]
	Combined effects of insecticides and herbicides	Negatively affect feeding patterns and growth of tadpoles; Sublethal effects due to promotion of trematode infection development	[90,91]
	Continuous use of herbicides in rice paddies	Reduces population of diving ducks	[92]
	Glyphosate sprays	Reduction in insectivorous and granivorous bird population	[93]

Mechanism of Action

TMX shows its action by binding to post-synaptic nicotinic acetylcholine receptors present inside the central nervous system and at neuromuscular junctions [109,110]. As a result of the irreversible binding of TMX to its receptors, nerve impulses are produced initially, followed by the collapse of the neurons to generate any further impulses [111]. Constant activation of these receptors appears due to the failure of acetylcholinesterases to break down TMX [112]. The potential of TMX binding to its receptors in insects is quite stronger as compared to the affinity for mammalian receptors [113,114].

Absorption

TMX is rapidly absorbed through the oral route and almost 90% is excreted in the urine of rats. TMX has low toxicity in experimental studies conducted on rats through oral, inhalation, or cutaneous routes. It is non-irritant to the eyes and skin with no sensitizing abilities [104,106]. TMX has very low dermal absorption both in humans and rats. After 6 h of exposure time to TMX, the systemic

*Table 5. Physicochemical properties of TMX**		
Color	**Slight Cream**	**Reference**
Physical state	Crystalline powder	[104]
Melting point	139.1	
Vapor pressure (at 25°C)	$6.6*10^{-9}$	
Solubility in water	4.1	
Solubility in methanol	10.2	
Dissociation constant pKa (at 20°C)	No dissociation in range pH 2–12	

absorption ranged between 0.4 and 2.7%. After 48 h of initial exposure, the systemic absorption was slightly increased ranging between 0.8 and 2.9% [107].

Metabolism

Studies have shown that 85-95% of TMX is excreted in the urine while only 2.5-6% is excreted in feces as the parent compound after 24 h of administration. Hydrolysis and N-demethylation are the metabolic pathways by which TMX is excreted out of the body. Hydrazine is produced as a result of hydrogenation of the N-nitro group which later on conjugates with acetic acid or 2-oxo-propionic acid resulting in the production of several metabolites. *Fig. 2* highlights the mechanism of action of TMX.

Animal and Human Toxicity

Due to heavy intoxication of TMX, clinical signs like hypersalivation, diarrhea, vomiting, muscular weakness, and ataxia are most seen in mammals [115]. Further, uncoordinated gait, reduced physical activity and tremors have also been reported due to TMX toxicity [116]. Signs of toxicity disappear soon but a very high toxic dose may lead to death within 24 h [117,118].

TMX is capable of producing Phenobarbital-like induction of enzymes in mouse liver. Repeated doses of TMX have led to affecting the liver and kidneys of rats and induced the production of liver tumors [104,119]. A yearlong study on dogs given TMX showed that TMX causes changes in blood biochemical parameters, alterations in testicular weights, and tubular atrophy at a toxic dose of 1500 ppm.

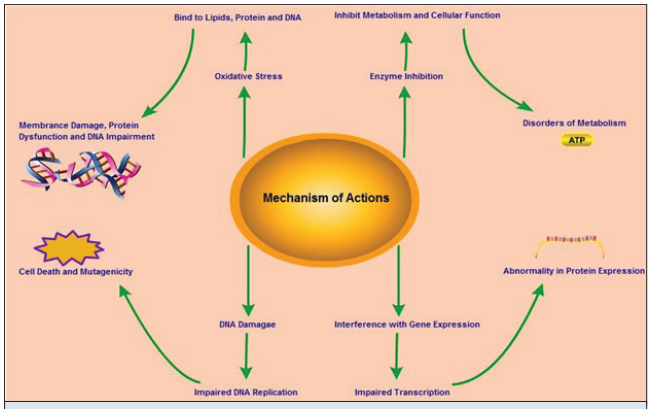

Fig 2. Mechanisms of actions of TMX toxicity (Created by Biorender)

According to previous investigations, under laboratory conditions, TMX degrades at slow rates and gets photolyzed in water very quickly. Moreover, TMX has low toxicity to birds and is non-toxic to fishes and mollusks through ingestion. Earthworms and green algae are insensitive to TMX [104]. TMX has a slight to moderate potential to cause harmful effects in beneficial insects and has proven to be safe for mites [107]. Moreover, TMX has no bioaccumulation abilities and degrades at a moderate to fast pace in the field [106].

The data related to TMX toxicity in humans is scarce. Fever, disorientation, dizziness, and vomiting are the most commonly seen side effects [103]. Tachycardia, hyperpnea, and profuse sweating have also been reported in cases of TMX toxicity [120]. Inhalation toxicity cases may lead to agitation, breathlessness, and disorientation in the affected human being [121]. Various toxicological properties and environmental toxicity profiles are mentioned in *Table 6* and *Table 7*.

Genotoxic Effect of TMX

After exposure to insects and birds, insecticides interact with DNA [122]. There are three possible ways of chemical-DNA interaction [123,124]. There may be electrostatic interactions between the chemical moieties of insecticide and charged phosphate backbone of DNA. Moreover, intercalative binding of chemicals within the stacked base pairs of DNA leads to disruption of conformation. Finally, groove binding interactions cause a significant change in

Table 6. Toxicological properties of TMX	
Study Type	**Comment**
Acute oral (LD50 mg a.i. /kg bw	4366
Acute dermal (24 h) (LD50 mg a.i. /kg bw)	>2000
Acute inhalation (4 h, aerosol) (LC_{50} mg a.i. /m^3 air)	3720
Skin irritation (4 h)	No irritation
Eye irritation	No irritation
Skin sensation	No skin sensation
a.i. active ingredient	

Table 7. Environmental profile of TMX	
Study Type	**Level**
Mallard duck (LC_{50} mg a.i. /kg diet)	>5200
Bobwhite quail (LD_{50} mg a.i. /kg bw)	1552
Rainbow trout (LC_{50} mg a.i. /l)	>125 (96 h)
Bluegill sunfish (LC_{50} mg a.i. /l)	>114 (96 h)
Water flea, *Daphnia magna* (EC_{50} mg a.i. /l)	>100 (48 h)

DNA conformations. As a result of the interaction between chemical exposure and subsequence reactions, genetic changes appear that influence biological parameters like fertility, fecundity, and longevity of the exposed organisms [19,125].

Oxidative Stress

TMX toxicity has been known to induce oxidative stress in different flora and fauna [126]. Chronic exposure to TMX has also been reported to cause oxidative damage in honey bees [127]. It is a fact that oxidative stress is one of the most important reactions to take place within the body after exposure to hazardous chemicals. As a result of oxidative stress, reactive oxygen species (ROS), which are normal products of cell metabolism, are released in very high amounts leading to cellular damage, and are considered to be the biomarkers of toxicity [128]. In order to prevent such destructions, cells activate different defensive mechanisms to counter the effects of ROS by releasing enzymatic and non-enzymatic antioxidants [129]. ROS causes cellular damage and necrosis through protein denaturation, lipid peroxidation, and DNA damage [130]. Superoxide dismutase (SOD), Glutathione-S-Transferase (GST), Peroxidases (POX), Catalases (CAT), and Ascorbate Peroxidases (APX) are the anti-oxidant enzymes while non-enzymatic antioxidants include ascorbic acid, thiols, and α-tocopherol which play their role in the process of detoxification. Oxidative stress leads to lipid peroxidation and the generation of hydroperoxides and various other free radicals which are efficiently removed by Glutathione-S4 Transferase (GST) [131,132]. Malondialdehyde (MDA), the end product of lipid peroxidation, causes injuries both at the cellular and subcellular levels [133,134]. TMX induces its toxic effects via different mechanisms. No exact report is available about the exact mechanisms of its toxicity. However, it is determined that TMX induces its adverse effects due to the induction of oxidative stress ensues leading to cell membrane damage and damage to DNA, and eventually cell death. Moreover, impaired transcriptional process as a consequence of TMX toxicity leads to abnormal protein synthesis and inhibition of various enzymatic reactions paving the way to abnormal metabolic processes.

One-Health Perspective of Thiamethoxam (TMX)

TMX is amongst the most commonly used insecticides throughout the world against insect pests of different crops [135,136] because of its wide-spectrum insecticidal activity [137,138].

TMX is used as prophylactic protection of fruits, vegetables, rice, and cotton against aphids, beetles, and thrips [139]. In addition, to control the pest attack on crops like barley, cotton, maize, sorghum, and wheat, TMX is one of the most frequently used insecticides in poultry farms [140,141]. There are several ways in which TMX can be applied. Of them, foliar sprays, root drenches, and seed dressings are the most frequently used methods of application [142-144]. Once TXM is within the insect/plant, it is readily metabolized by cytochrome P450 enzymes via desulphuration and transformed into its metabolite clothianidin [101,145,146]. This metabolite is highly toxic to the insect and adds a continuing environmental hazard of TMX to poultry birds [147].

TMX is not readily degraded and remains in the soil as well as crops/food products for a long time posing serious risks to humans, animals, and birds [148-151]. Environmental contamination is a major drawback of TMX as its residues have been detected in cocoa farmlands [152], soils of parks, residential areas [153], and arable soils [154], with concentrations ranging from μg kg^{-1} to mg kg^{-1} [138,142]. Humans and animals get the TMX into their bodies either through dermal absorption, inhalation, or ingestion. *Fig. 3* gives a brief overview of the pharmacokinetics of TMX.

Animal and plants product with persistent pesticides is the main source of human exposure to pesticides. According to a study conducted in China, 94.90% of pesticide exposures are through ingestion whereas only 5.1% of exposures occur through inhalation or dermal contact [155]. Poultry meat and eggs are the major portions of the human diet as it meets the protein requirement with the least cost [156]. These pesticides in the human lead to chronic health effects by irritating eyes and skin, damaging the nervous system, causing asthma, affecting hormones leading to reproductive issues, fetal death, and neurodevelopmental issues [157-159]. Previous studies show that the daily intake of these pesticides by the chicken is excreted as its residues in the egg and meat [160].

TMX Setbacks to the Poultry Sector

Dusting with insecticides to control insect attacks on the birds within the premises of poultry farms is the most

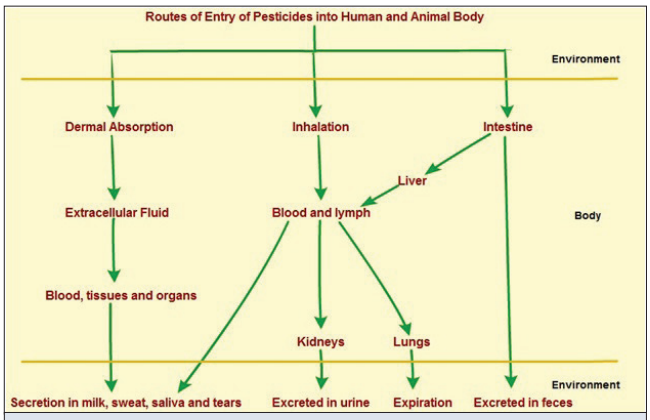

Fig 3. Mechanisms of actions of TMX toxicity (Created by Biorender)

Table 8. *Excretion/deposition of TMX and its metabolites in different animal species^*

Animal	TMX excretion/deposition	Other mMetabolites	
Rats	70-80% as TMX in urine	10% N- (2-chlorothiazol-5-ylmethyl)-N'-methyl-N"-nitroguanidine in urine	*Data collected from FAO/WHO Meeting on Pesticide Residues [161]
Goats	1% in milk	44-45% milk N- (2-chlorothiazol-5-ylmethyl)-N'-methyl-N"-nitroguanidine	
	36% goat fat	Metabolites in liver (up to 10%) N-(2-chlorothiazol-5-ylmethyl)-guanidine) N-(2-chlorothiazol-5-ylmethyl)-N'-methyl-guanidine 3-(2-chloro-thiazol-5-ylmethyl)-5-methyl- [1,3,5] oxadiazinan-4-ylidene]-Hydrazide 3-(2-chlorothiazol-5-ylmethyl)-5-methyl- [1,3,5] oxadiazinan-4-ylidineamine	
	51% muscles		
	22% kidneys		
Laying hens	Most dose excreted in droppings	Egg white 45% (N-(2-chlorothiazol-5-ylmethyl)-N'-nitroguanidine	
	Eggs 0.1%	Yolk 54% (N-(2-chlorothiazol-5-ylmethyl)-N'-nitroguanidine	
	Tissues 1-1.5%	Fat and skin 57%(N-(2-chlorothiazol-5-ylmethyl)-N'-nitroguanidine	
		Liver 39% N-(2-chlorothiazol-5-ylmethyl)-N'-methyl-N"-nitroguanidine	

^ *Food and Agriculture Organization Joint Meeting on Pesticide Residues*

important source of insecticides toxicities in the broilers and layers. According to the estimates, nearly 670 poultry birds acquire direct toxicities from pesticides in the USA throughout the year while the mortality rate is around 10% in severe toxicity cases. These figures can be much higher as mortality rates are complicated to estimate since the death of birds may occur away from the site of contact with pesticides. Moreover, the presence of scavengers in/around the farm also has a role in the underestimation of mortality rate figures [162]. The use of insecticides has a one-health perspective as poultry food products may contain pesticide residues that would be unfit for human use [163].

Various other toxic effects of TMX include cellular damage, genotoxicity, and immunosuppression in birds [164,165]. Studies have shown that TMX affects different facets of bird physiology in a dose-dependent manner. The probable impacts of TMX on birds are increasing as they are at risk to be exposed in multiple ways and have been recognized to show adverse effects even at sublethal concentrations [166]. Behavioral changes and mortality have been observed in pigeons and partridges following TMX toxicity. Accumulation of toxic levels of chemicals in the liver and kidney along with weakened locomotory ability were also found in pigeons and partridges [167,168].

Effects on Production and Immunity

Ingestion of seeds and crops treated with pesticides is the main route of exposure and induces mortality. Acute toxicity in sub-lethal doses can produce various clinical manifestations including lethargy, decreased production, and dropped immunity leading to the emergence of different infections in exposed species [169]. In addition, TMX toxicity has been reported to cause drop in egg production and thinning of the eggshell [170-172]. Neonicotinoids insecticides like TMX adversely affect cell-mediated immunity by lowering type-IV hypersensitivity reactions and lowering the T-lymphocyte stimulation index to phytohemagglutinin [169,173]. Furthermore, TMX toxicity may lead to multiple infection susceptibility in the birds as it lowers the phagocytic activity of macrophages and turn down lymphoproliferative activity leading to failure of the mounting effective immune response [174]. Previously, TMX has been regarded as the safest neo-nicotinoid but the recent studies covering biochemical, hematology, and behavioral aspects of laboratory animals have shown that even the sub-lethal doses can cause toxic effects in the birds [169,175].

Adverse Effects of TMX on Blood Cells and Biomarkers of Liver and Kidneys

In laboratory animals like rats and guinea pigs, the adverse effects of TMX on hematological, biochemical, and behavioral parameters have been reported. The oral administration of TMX causes a decline in erythrocyte and leukocyte count, and low hematocrit and hemoglobin value [140,169,175]. A recent study was performed to check the

Table 9. *Toxicological monograph for risk assessment of TMX toxic effects*

Species	Trial Period	Effect	NOAEL*	LOAEL**	
Mouse	3-month study	Toxicity	100 ppm	1250 ppm	***Data collected from FAO/WHO Meeting on Pesticide Residues**
	18-month study	Toxicity	20 ppm	500 ppm	
		Carcinogenicity	20 ppm	500 ppm	
Rat	Single-dose study	Toxicity	100mg/kg bw	500 mg/kg bw	
	3-month study	Toxicity	250 ppm	1250 ppm	
	24-month study	Toxicity	1000 ppm	3000 ppm	
		Carcinogenicity	3000 ppm	-	
	2 generations study	Reproductive toxicity	2500 ppm	-	
		Parental toxicity	1000 ppm	2500 ppm	
		Offspring toxicity	30 ppm	1000 ppm	
	Developmental toxicity study	Maternal toxicity	30 mg/kg BW	200 mg/kg BW	
		Embryo and fetal toxicity	200 mg/kg BW	750 mg/kg BW	
Rabbit	Developmental toxicity study	Maternal toxicity	15 mg/kg BW	50 mg/kg BW	
		Embryo and fetal toxicity	50 mg/kg BW	150 mg/kg BW	
Dog	3-month study	Toxicity	250 ppm	1000 ppm	

**No Observed Adverse Effect Level; **Lowest Observed Adverse Effect Level; ^ Food and Agriculture Organization Joint Meeting on Pesticide Residues (Page 318-319)*

hematological and biochemical changes in chickens. In birds, a significant decline in red blood cells, white blood cells, packed cell volume, and hemoglobin concentrations has been reported. Albumin, globulin, creatinine, urea, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were also noticed which showed that high dose of TMX leads to a significant decrease in the albumin globulin and creatinine value whereas a rise in the urea, ALT, and AST were seen [140,176]. Such alterations in renal and hepatic functioning can lead to debilitation [177], poor performance, and reduced feed conversion in poultry birds and these findings suggest conducting clinical trials on dairy animals to ascertain the role of TMX on these parameters.

Toxic Effects of TMX on the Reproductive System

TMX has been found to affect male fertility by either causing direct damage to spermatozoa or altering the functioning of Leydig or Sertoli cells. In addition, TMX also has the potential to disrupt the endocrine functions at a few stages of hormonal regulation [178]. Sperm nuclear proteins are changed by TMX toxicity leading to male infertility [179]. Epidemiology-based studies have indicated that the semen quality (sperm morphology, viability, motility, and fertilization capability) of agricultural workers is poor where TMX is frequently used [180].

Optimum female reproductive health determines profit maximization in the dairy sector. Poor reproductive performance has long-lasting effects on livestock farm economies worldwide. In-vitro studies have revealed that TMX has the potential to cause harmful effects on the developing embryo [97]. Sub-lethal doses of TMX have shown negative effects on the female reproductive system the disruption of ovarian structure, delayed sexual maturity, and reduced egg production [181]. This deposition of toxin within the eggs means that developing embryos are prone to be affected more easily by toxic effects being more sensitive than adult birds. Granivorous birds have shown a number of abnormalities after ingestion of seed coated with TMX. Reduced chick survival and lower egg fertilization rates in partridges [182], reduced clutch size, weight loss, and lower chick survival in northern bobwhite quail [166], and shorter embryos, testicular abnormalities, and increased rates of DNA damage in Japanese quail [183] have been reported. There is also evidence that neonicotinoid residues present in the environment can be deposited into eggs. A recent investigation showed that 24 clutches (out of 52) of Patridge collected from a habitat contained 15 different pesticides in trace amounts including thiamethoxam [184]. Such exposure has been reported to lead to changes in organogenesis [185-187] and anatomical abnormalities [188,189]. Damage to cellular structure and genotoxic effects can affect both growth as well as development in unpredictable ways. *Table 9* summarizes the results of TMX toxicity studies conducted with no observed adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL).

Conclusion

The use of pesticides to keep save crops from insect attacks is advantageous as it boosts the economy of the agricultural

sector. Though, irrational, unwise, and extensive use of chemicals like TMX has deleterious effects on both animal and human health. The presence of toxic levels in the environment leads to the cellular level of harmful changes as well as many grossly observed alterations in metabolism, productivity, reproduction potential, and general health status of the exposed animals/humans. This review highlighted many injurious consequences of TMX toxicity including oxidative stress, hematological alterations, hepatic and renal parameter changes, and thrashes to the reproductive health of both males and females. Thus, we recommend that future research on determining acceptable exposure levels should be undertaken in One-Health Interface. Further, policy-making about the cogent use of TMX and awareness programs among farmers on the grass-root level about TMX toxic effects should be designed.

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Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author (Mughees Aizaz Alvi) on reasonable request.

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Competing Interests

The authors declare that they have no conflicts of interest.

Authors' Contributions

Conceptualization, methodology, formal analysis, investigation, resources, data curation, writing-original draft preparation, W.Q., M.US., M.I., M.M.A., A.F. and R.Z.A.; writing-review and editing, W.Q. and M.A.A.; project administration, R.Z.A. and R.H. All authors have read and agreed to the published version of the manuscript.

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Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

• **Peer-review Process**

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• **Publication Process of an Article**

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

NO PUBLICATION FEE

Processing and publication are free of charge with the journal. There is no article processing char-ges, submission fees or any other fees for any submitted or accepted articles.

RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

ARCHIVE POLICY

The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

Kafkas Universitesi Veteriner Fakultesi Dergisi
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INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

The official language of our journal is English. Additionally, all the manuscripts must also have Turkish title, keywords, and abstract (translation will be provided by our journal office for foreign authors).

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and supplementary files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

3- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

4- Authors should know and take into account the issues listed in the "Ethical Principles and Publication Policy" section regarding scientific research and authors.

5- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

6- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

7- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z: Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg*, 23 (5): 767-772, 2017. DOI: 10.9775/kvfd.2017.17726

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: Mcllwraith CW: Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

Follow the link below for EndNote Style of Kafkas Universitesi Veteriner Fakultesi Dergisi;
https://researchsoftware.com/downloads/journal-faculty-veterinary-medicine-kafkas-university

8- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

9- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

10- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with a broad database of academic publications.

11- There is no copyright fee for the authors.

12- The authors are charged a fee on acceptance of the manuscript to cover printing costs and other expenses. This payment information can be found at http://vetdergi.kafkas.edu.tr/

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- Cover letter

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction).

• Other information has been added that should be known by the editorial board (e.g.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere.

- Title page

• Title, running title (should be a brief version of the title of your paper, no exceed 50 characters)

• The author's name, institutional affiliation, Open Researcher and Contributor ID (ORCID)

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• Title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

- Availability of Data and Materials

- Acknowledgements

- Funding Support

- Competing Interests

- Authors' Contributions

Further considerations

• Journal policies detailed in this guide have been reviewed

• The manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided