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Whole genome molecular characterization of Infectious Pancreatic Necrosis Viruses isolated in Turkey

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Abstract: Infectious pancreatic necrosis virus (IPNV; *Birnaviridae, Aquabirnavirus*) causes infectious pancreatic necrosis (IPN) in fish. IPN disease was first found in 2002 in Turkey. In this study, 10 IPNV isolates were isolated between 2005 and 2013 and propagated in RTG-2 cell cultures. RNAs obtained from cell lysates were used as template and VP1, VP2, VP3, VP4 and VP5 genes of the virus were amplified in full length. Sequence analyses of the genes were made. The obtained sequences were compared with international reference strains from GenBank and phylogenetic analyses and genogrouping of the viruses were conducted. Turkish isolates were found to show a genetic similarity of between 93.5% and 99.8% in terms of molecules. When segment A sequences were compared with each other, a similarity between 97.8% and 99.8% was found; when they were compared with international reference strains, they were found to have the highest similarity (99.3%) with France AJ622822 isolate and the lowest (65.7%) similarity with Canada NC001915 isolate. When segment B sequences of the isolates were compared with each other, a similarity between 93.5% and 99.5% was found; when they were compared with international reference strains, they were found to have the highest similarity (98.9%) with Canada M58757 isolate and the lowest (52.7%) similarity with Finland KY548519 strain. As a result of phylogenetic analyses conducted, Turkish isolates were found to be closely related with France, Spain and Iran strains in serotype A2 and genogroup 5 they were placed in.
Keywords: IPNV, molecular characterization, RT-PCR, Turkey, whole genome.

Türkiye'den izole edilen İnfeksiyöz Pankreatik Nekrozis Viruslarının tüm genom moleküler karakterizasyonu

Özet: İnfeksiyöz pankreatik nekrozis virusu (IPNV; *Birnaviridae, Aquabirnavirus*) balıklarda infeksiyöz pankreatik nekrozis (IPN) hastalığını oluşturmaktadır. IPN hastalığı Türkiye'de ilk kez 2002 yılında tespit edilmiştir. Bu çalışmada 2005 ve 2013 yılları arasında izolasyonu yapılan 10 adet IPNV izolatı RTG-2 hücre kültürlerinde üretildi. Hücre lizatlarından elde edilen RNA'lar kalıp olarak kullanılarak virusun VP1, VP2, VP3, VP4 ve VP5 genleri tam uzunlukta amplifiye edildi. Genlerin dizi analizleri gerçekleştirildi. Elde edilen sekanslar GenBank'tan sağlanan uluslararası referans suşlar ile karşılaştırılarak virusların filogenetik analizleri ve genogruplandırılmaları yapıldı. Türkiye izolatlarının moleküler yönden % 93,5 ile % 99,8 oranında genetik benzerlik gösterdiği saptandı. Segment A sekansları kendi aralarında % 97,8 ile % 99,8 oranında, uluslararası referans suşlarla karşılaştırıldığında ise en yüksek oranda (% 99,3) Fransa AJ622822 izolatı ile en düşük oranda (% 65,7) Kanada NC001915 izolatı ile benzerlik bulundu. Segment B sekansları kendi aralarında % 93,5 ile % 99,5 oranında, uluslararası referans suşlarla karşılaştırıldıklarında ise en yüksek oranda (% 98,9) Kanada M58757 izolatı ile en düşük oranda (% 52,7) Finlandiya KY548519 suşu ile benzerlik bulundu. Yapılan filogenetik analizler sonucunda Türkiye izolatlarının serotip A2 ve genogrup 5 içinde yerleştikleri Fransa, İspanya ve İran suşları ile yakın ilişkili oldukları belirlendi.
Anahtar sözcükler: IPNV, moleküler karakterizasyon, RT-PCR, tüm genom, Türkiye.

Introduction

Infectious pancreatic necrosis virus (IPNV) is a small non-enveloped virus of *Aquabirnavirus* genus from *Birnaviridae* family; it has genome 2 segmented (A and B) RNA structure, it has double stranded and linear with a diameter of 60 nanometer and it is surrounded with an icosahedral capsid (8, 25). IPNV causes high levels of mortality in fry and young farm fish (1, 27). The fish which survive the infection remain as life-long asymptomatic carrier (19). In our country, this disease was first diagnosed in 2002 and it was found that the disease was more common in many trout farms (4).

The larger segment, A, is 3,097 nucleotides long and encodes a 107-kDa precursor protein (9, 13, 28) in a single

large open reading frame (ORF), which is cotranslationally cleaved by the viral nonstructural (NS) protease, VP4, generating VP2 and VP3 structural proteins (5, 8-10). Segment A also encodes a 15-kDa arginine-rich protein from a small ORF partly preceding and overlapping the polyprotein ORF (6, 11). VP2 also includes virulence markers (3, 17). In virulent IPNV strains, there are Threonine and Alanine (Thr217/Ala221) at 217 and 221 positions of VP2, respectively, while there are Proline and Ala (Pro217/Ala221) amino acid residues at this position in moderate or low virulence strains. Strains with 221 Threonine position are almost avirulent (21, 24). VP3 is an internal protein. The smaller genomic segment, B, is 2784 nucleotides (nt) long and encodes VP1, the virion-associated RNA-dependent RNA polymerase (10, 15).

Aquabirnaviruses are grouped in 4 (A-B-C-D) serogroups (16, 27). Most of the aquabirnaviruses are in Serogroup A and this group is divided into 9 serotypes (A1-A9). These serotypes are represented by West Buxton (A1), Sp (A2), Ab (A3), He (A4), Te (A5), Can1 (A6), Can2 (A7), Can 3 (A8) and Jasper (A9) reference strains (19). Serogroup B consists of a single serotype B1. Serotype A1 includes America isolates, while serotype A6-A9 includes Canada isolates and serotypes A2-A5 and B1 include Europe and Asia isolates (3, 16, 17). Aquabirnaviruses have been classified according to the phylogenetic analysis results of VP2 gene and 6 genogroups have been reported in 9 serotypes of Serogroup A (1, 3). Genogroup 1 includes serotypes A1 and A9, genogroup 2 includes serotype A3, genogroup 3 includes serotypes A5 and A6, genogroup 4 includes serotypes A7 and A8, genogroup 5 includes serotype A2 and genogroup 6 includes serotype A4 (20, 23, 25). In addition to these 6 genogroups, it has been proposed to classify all aquabirnaviruses as genogroup 7 (3, 13, 19).

The purpose of this study is to conduct the whole genome molecular characterization of native IPNV isolates, to genogroup them and to compare the viruses with known European and American genotypes.

Material and Methods

Cells and virus isolates: 15 number ethical board approval was taken for the study from local ethics committee of Veterinary Control Institute. Rainbow trout gonad cells were obtained from Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Virology. In this study a total of 10 virus isolates were selected from 66 isolates that were used number 2130156 Project of TUBITAK conducted by Ondokuz Mayıs University in 2014 and 2017 years. These viruses are isolated by Bornova Veterinary Control Institute Department of Virology (Muğla07 KY606185, Hatay07 KY606187, Aydın07 KY606192, Antalya07 KY606213, Uşak05 KY606229, Ankara10 KY606221), Samsun Veterinary Control Institute Virology Laboratory (Almus KM972672), Trabzon Central Fisheries Research Institute Fish Health Diseases Laboratory experts (Hah-2 KM972673, Hah-3 KM972674, Hah-4 KM972675) during outbreaks and routine field screening. Three of them from the province of Trabzon, and one from each of the provinces of Muğla, Hatay, Aydın, Antalya, Uşak, Ankara and Tokat (9 rainbow trout and 1 turbot origin), isolated between 2005 and 2013, were used.

Cell culture and propagation of viruses: The local IPNV isolates were propagated in RTG-2 cells at 15°C. The cells were grown at 23°C in L-15 medium (Gibco, 1929865) supplemented with 10% fetal bovine serum (FBS) (Sigma, 094M3335) and 2 mML-glutamine (Gibco, 1788084). For preparation of local IPNV virus stocks, confluent RTG-2 cells grown at 25°C were infected at a multiplicity of infection (MOI) of 0.001 in MEM (Gibco, 12492013) with 5% FBS. After 1 h of adsorption at 15°C, the inoculum was removed, and the cells were incubated at 15°C until extensive cytopathic effect (CPE) was observed. The supernatant was collected 7 days after post-infection, clarified and stored at −80°C for further processing. Second passage of viruses were used further studies.

PCR analysis of genes: RNA extraction was made with commercially obtained kit (Thermo GeneJET, K0732) and RT-PCR studies were conducted with OneStep RT-PCR kit (Qiagen, 163012798), according to the directions of the manufacturer firm. VP2, VP5 (2), VP3, VP4 and VP1 (6) gene areas of the viruses were amplified in vitro by using specific primers and using genomic RNAs stocked at -20 °C as template (Table 1) (18). Mix preparation and PCR conditions of all genes were the same; however, since VP1 gene was long, it was amplified in two pieces as VP1a and VP1b and for VP1 gene, annealing heat was changed as 50°C. Since VP5 gene and VP2 gene overlapped except the first 7 nucleotide, the amplification of these genes was made on single fragment. A reaction of 11 PCR was prepared for the amplification of each gene. For this purpose, reaction tubes which included a 50 µl mixture of 10 µl 5X Buffer (12.5 mM $MgCl_2$), 1 µl (0.2 mM) dNTP mix, 2 µl each primer (10 pmol), 1 µl enzyme (RT, DNA pol), 2.5 µl (5 mM) DTT, 5 µl RNA and 26.5 µl distilled water were put in thermal cycler. The steps of amplification on thermal cycler were set up as follows: The reaction mixture was incubated at 50°C for 30 minutes. The reverse transcriptase enzyme was then inactivated by holding at 95°C for 15 minutes was followed consecutively by 30 seconds at 55°C, 70 seconds at 72°C, and 30 seconds at 94°C, which were repeated 35 times. Amplification was terminated by final extension at 72°C for 10 minutes. The resulting DNA products (amplicon) were analyzed on agarose gel (1.5%) after electrophoresis at 80 V for 30 minutes. The DNA bands were observed under ultraviolet light (Figure 1).

Table 1. DNA sequences and target regions of the primers.

Primers	Aligment	Localisation	References
FVP1A	5'- ATG TCG GAC ATC TTC AAY TCA CC -3'	101-123	Dadar et al. (6)
RVP1A	5'- GAG CCG TCC TCG TTT GTC CA -3'	1379-1398	
FVP1B	5'- CAC ATG CAG GCA ATG ATG TAC TAC -3'	1340-1364	Dadar et al. (6)
RVP1B	5'- CCT AGT TTC TTC TCT GCT TCT C -3'	2614-2636	
FVP3	5'- GCA TCC GGG ATG GAC GAG GA -3'	2207-2226	Dadar et al. (6)
RVP3	5'- TTA CAC CTC AGC GTT GTC TCC -3'	2956-2977	
FVP4	5'- GGA CCA GAG TCT TCA ACG AAA TCA CG -3'	1275-1300	Dadar et al. (6)
RVP4	5'- TAG ATC TCG GCG TCC TGG ACT TC -3'	2377-2400	
SPAF 1	5'- GGA AAG AGA GTT TCA ACT TTA GTC G -3'	1-24	Albayrak (2)
SPAR 1	5'- GAC TCC AGC CTG TTC TTG AGG -3'	1675- 1686	

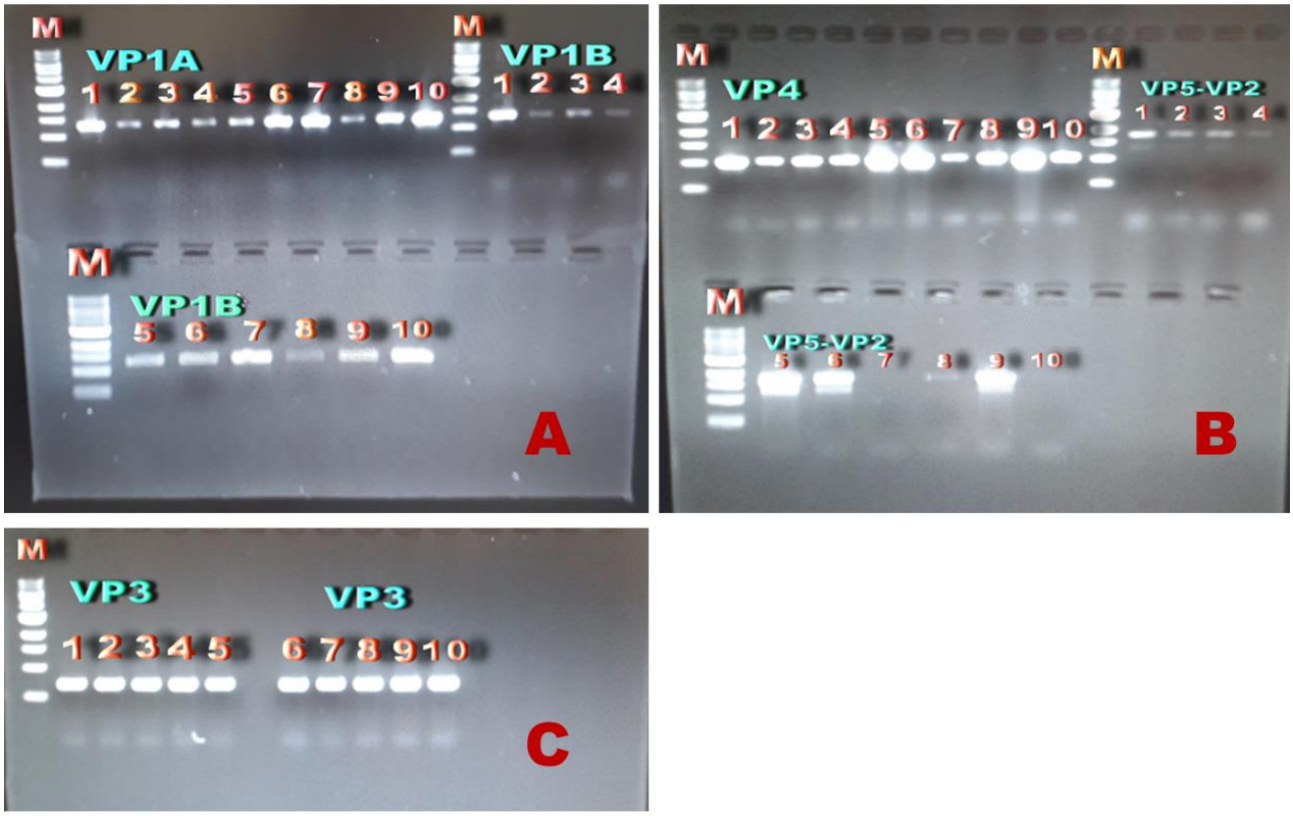

Figure 1. Specific bands of VP1, VP2, VP3, VP4 and VP5 genes. **A.** 1297 bp VP1A and 1296 bp VP1B specific PCR, **B.** 1125 bp VP4 and 1662 bp VP5-VP2 specific PCR, **C.** 770 bp VP3 specific PCR. M: 500 bp molecular weight standard; 1: Almus isolate; 2: Uşak05 isolate; 3: Hah-2 isolate; 4: Hah-3 isolate; 5: Hah-4 isolate; 6: Antalya07 isolate; 7: Muğla07 isolate; 8: Ankara10 isolate; 9: Hatay07 isolate; 10: Aydın07 isolate.

Sequencing of PCR products: A total of 50 PCR product 2 direction (as forward and reverse) 100 sequencing with 5 different primer pairs was performed by a commercial firm by using Sanger method. Sequencing and assessment was performed with BioEdit sequence alignment editor program, while integration was performed with Contig express program. Segment A and Segment B sequences were compared within their own groups and also compared with reference virus sequences. Phylogenetic trees were created and genogroup comparisons were made.

Results

After the first passage of the viruses, CPE formation was observed in 6 isolates, while it was seen in all of the 10 isolates after the second passage. In PCR analyses, VP1 specific bands were observed at 1296 and 1297 bp, VP3 specific bands were observed at 770 bp, VP4 specific bands were observed at 1125 bp and VP5 and VP2 specific bands were observed at 1662 bp. No band was observed at VP5-VP2 gene area for Muğla07 and Aydın07 isolates (Figure 1).

Table 2. Informations of IPNV strains which were made phylogenetic analysis.

Genogroup	Strain	GenBank accession number	Geographical origin	Reference region	Nucleotide sequence
I	Ja	NC001915	Canada	Segment A	1-3097
I	WB	AF078668	Canada	Segment A	1-3097
II	Ab	AF342729	Denmark	Segment A	1-2904
III	C1	AF342732	Canada	Segment A	1-2904
III	TE	AF342731	England	Segment A	1-2904
IV	1146	AJ489222	Spain	Segment A	1-2919
IV	C2	AF342733	Canada	Segment A	1-2904
IV	C3	AF342734	Canada	Segment A	1-2904
V	Hatay07	MH614926	Turkey	Segment A	1-3097
V	Antalya07	MH614927	Turkey	Segment A	1-3097
V	Uşak05	MH614928	Turkey	Segment A	1-3097
V	Almus	MH614929	Turkey	Segment A	1-3097
V	Hah-2	MH614930	Turkey	Segment A	1-3097
V	Hah-3	MH614931	Turkey	Segment A	1-3097
V	Hah-4	MH614932	Turkey	Segment A	1-3097
V	Heh-5	KF991533	Turkey	Segment A	1-1572
V	Almus-1	KF914646	Turkey	Segment A	1-1779
V	SP	KF279643	Iranian	Segment A	1-2916
V	31-75	AJ622822	France	Segment A	1-3096
V	N-137	HQ457181	Norway	Segment A	1-1510
V	I-2	HQ457195	Ireland	Segment A	1-1510
VI	He	AF342730	Germany	Segment A	1-2904

Segment B sequencing was performed in all of the 10 studied isolates; however, all sequences of segment A was not performed in Muğla07, Aydın07 and Ankara10 isolates. Whole Segment A was sequenced in 7 isolates. It was found that VP5 termination codon of Almus, Antalya07, Hah-2, Hah-3, Hah-4 and Uşak05 isolates was terminated at 496 nucleotide base, while Hatay07 isolate was terminated as 511 nucleotide base. Amino acid residues of VP2 gene at 217/221/247 positions related with virulence were identified as Proline/Threonine/ Glutamic acid (Glu) (PTE) in Almus and Hah-4 isolates, while it was identified as PTA in Antalya07, Hah-2, Hah-3, Hatay07 and Uşak05 isolates. It was found that small ORF on segment A started a protected sequence at nucleotide 112 with ATGCAA and encoded 2 different VP5 proteins – one protein cut with early termination codon at 496 nucleotide and a 15 kDa whole protein at 511 base. It was found that large ORF terminated at nucleotide 3037 with TAA termination codon it started at nucleotide 119, 7 nucleotides after small ORF, and encoded VP2, VP3 and VP4 proteins. Amino acid residues of Turkish isolates were found as Pro217, Thr221, Ala247 or Glu247, respectively. All the sequence data analyzed were stored at GenBank Database and 13 reference IPNV representing all genogroups obtained from GenBank was compared with Segment A sequence and phylogenetic assessments were made (Table 2).

While the amino acid similarity of segment A was between 98% and 99.8% in Turkish isolates (Table 3), amino acid similarity of segment B was between 93.5% and 99.1% (Table 4). Segment B amino acids have higher rates of differences. The closest similarity of native isolates in segment A nucleotides was found with France isolate AJ622822 between 97.3% and 98.6%, with Iran isolate KF279643 between 97.8% and 98.5%, with Spain isolate AJ489222 between 98.3% and 98.8%. Nucleotides of these isolates and native isolates were found to have a similarity of more than 97.3%. The highest difference between nucleotides was found as 34.3% between native isolates and Canada isolate NC001915 and as 23.3% between native isolates and Germany isolate AF342730 (Table 3). With phylogenetic studies, IPNV isolated from Turkey were classified within genogroup 5 (Figure 2) and this genogroup includes Europe and Asia isolates (Iran Sp, France 31-75, Norway N-137 and Ireland I-2). 10 Segment B sequences analyzed were stored in GenBank Database and their data were compared with 25 IPNV segment B sequence data and their phylogenetic analysis was made (Figure 3) While the segment B amino acid similarity of native isolates among themselves was found to be between 93.5% and 99.1%, their nucleotide similarity was found to be between 97.4% and 99.5% (Table 4). While more than 97.5% nucleotide similarity was found between native isolates and Canada M58757 isolate, the lowest similarity was found with Finland KY548519 strain at a rate of 64.2%. Between native isolates and segment B amino acids of reference strains, more than 93.3% similarity was found between Canada M58757, France AJ622823, Norway AY379743, AY823633, AY379739, America AY354522, AY354523, AY354524, AY379741 isolates.

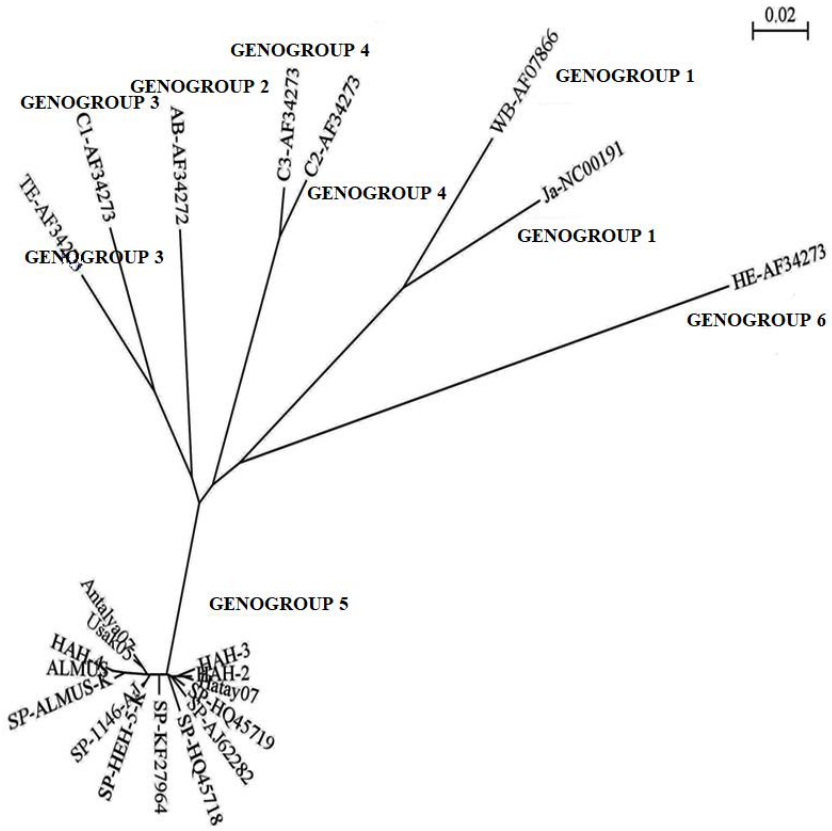

Figure 2. Dendrogram obtained with the neighbor-joining method using 1000 bootstrap in Turkish isolates and reference strains according to Segment A.

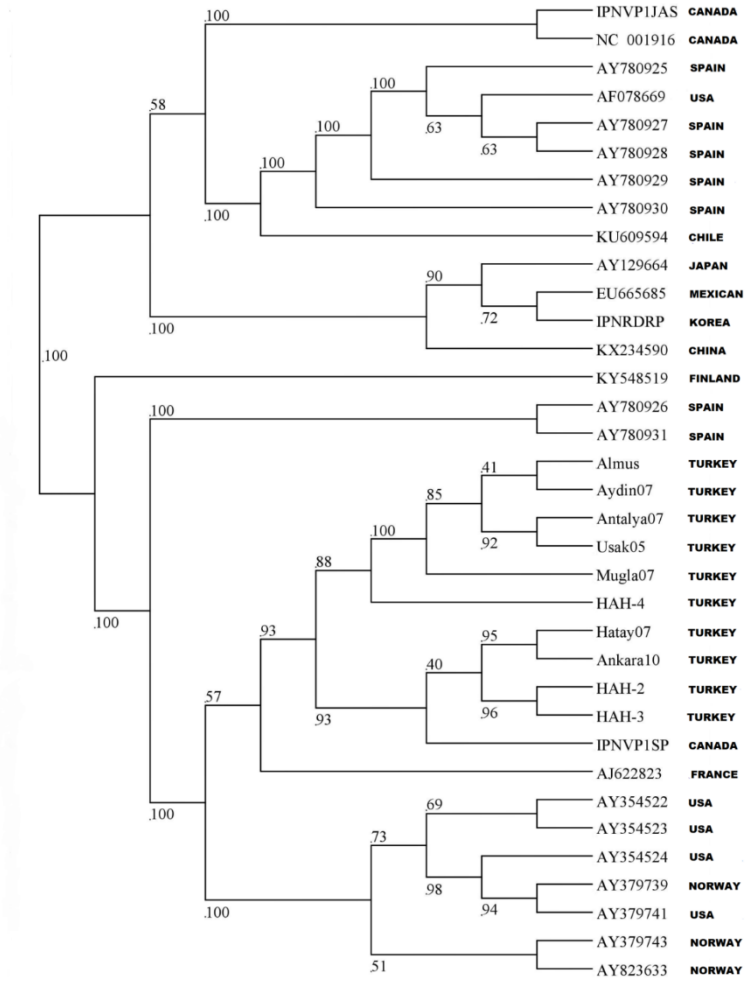

Figure 3. Phylogram obtained with the neighbor-joining method using 1000 bootstrap in Turkish isolates and reference strains according to Segment B.

Table 3. Percentages of Segment A nucleotide and amino acid sequences.

Nük/Aa	Hatay07	HAH-2	HAH-3	Antalya07	Usak05	HAH-4	Almus	KF914646	AJ489222	KF991533	KF279643	AJ622822	AF342729	AF342732	AF342731	AF342733	AF34273	AF342730	NC001915	AF078668	HQ457181	HQ457195
Hatay07	ID	0,998	0,988	0,985	0,984	0,981	0,984	0,979	0,983	0,982	0,981	0,993	0,9	0,905	0,899	0,881	0,879	0,843	0,844	0,85	0,507	0,51
HAH-2	0,996		0,989	0,984	0,985	0,98	0,983	0,978	0,982	0,981	0,98	0,992	0,899	0,904	0,898	0,88	0,878	0,842	0,843	0,849	0,506	0,509
HAH-3	0,991	0,994		0,986	0,987	0,981	0,984	0,979	0,984	0,983	0,986	0,99	0,896	0,902	0,898	0,879	0,878	0,838	0,837	0,842	0,508	0,505
Antalya07	0,982	0,983	0,984		0,996	0,989	0,992	0,987	0,988	0,991	0,985	0,985	0,896	0,905	0,899	0,882	0,881	0,843	0,843	0,848	0,506	0,505
Usak05	0,983	0,984	0,985	0,993		0,987	0,99	0,986	0,987	0,99	0,984	0,984	0,895	0,904	0,898	0,881	0,88	0,841	0,841	0,846	0,505	0,504
HAH-4	0,979	0,978	0,979	0,984	0,987		0,996	0,987	0,982	0,985	0,98	0,981	0,892	0,904	0,897	0,876	0,875	0,838	0,84	0,843	0,501	0,5
Almus	0,98	0,98	0,979	0,985	0,988	0,998		0,99	0,985	0,988	0,983	0,984	0,895	0,906	0,899	0,879	0,878	0,841	0,843	0,846	0,504	0,503
KF914646	0,979	0,98	0,979	0,984	0,986	0,989	0,991		0,994	0,995	0,982	0,981	0,891	0,903	0,896	0,881	0,876	0,84	0,839	0,842	0,503	0,502
AJ489222	0,984	0,984	0,985	0,988	0,988	0,983	0,983	0,992		0,996	0,987	0,985	0,895	0,901	0,897	0,881	0,876	0,842	0,839	0,844	0,507	0,506
KF991533	0,984	0,983	0,984	0,989	0,99	0,984	0,985	0,993	0,997		0,986	0,984	0,894	0,904	0,898	0,883	0,878	0,842	0,84	0,845	0,506	0,505
KF279643	0,981	0,982	0,985	0,983	0,983	0,978	0,978	0,981	0,988	0,987		0,985	0,893	0,9	0,896	0,88	0,877	0,841	0,841	0,846	0,51	0,507
AJ622822	0,985	0,986	0,984	0,979	0,98	0,973	0,975	0,976	0,981	0,98	0,98		0,903	0,908	0,904	0,884	0,882	0,843	0,843	0,849	0,509	0,511
AF342729	0,863	0,863	0,861	0,86	0,861	0,855	0,857	0,859	0,862	0,862	0,86	0,865		0,918	0,915	0,865	0,87	0,822	0,84	0,842	0,472	0,473
AF342732	0,863	0,862	0,86	0,861	0,86	0,856	0,857	0,859	0,86	0,861	0,859	0,864	0,876		0,963	0,87	0,873	0,822	0,835	0,833	0,473	0,474
AF342731	0,866	0,867	0,867	0,865	0,866	0,861	0,862	0,863	0,867	0,867	0,865	0,87	0,871	0,912		0,865	0,868	0,829	0,831	0,835	0,474	0,474
AF342733	0,842	0,843	0,843	0,841	0,841	0,837	0,838	0,842	0,843	0,844	0,842	0,846	0,829	0,836	0,828		0,974	0,827	0,832	0,835	0,473	0,472
AF342734	0,842	0,842	0,843	0,843	0,843	0,838	0,84	0,84	0,842	0,843	0,843	0,846	0,829	0,841	0,831	0,97		0,824	0,829	0,83	0,472	0,47
AF342730	0,768	0,768	0,767	0,768	0,767	0,768	0,77	0,771	0,77	0,771	0,767	0,766	0,756	0,757	0,762	0,758	0,754		0,801	0,804	0,456	0,458
NC001915	0,659	0,658	0,657	0,659	0,658	0,658	0,658	0,658	0,658	0,659	0,66	0,659	0,655	0,649	0,646	0,649	0,648	0,612		0,972	0,459	0,459
AF078668	0,788	0,788	0,785	0,788	0,786	0,784	0,785	0,785	0,787	0,788	0,787	0,787	0,791	0,782	0,777	0,776	0,775	0,736	0,763		0,459	0,46
HQ457181	0,504	0,505	0,504	0,502	0,502	0,497	0,499	0,499	0,503	0,503	0,505	0,505	0,449	0,445	0,452	0,439	0,441	0,4	0,343	0,417		0,991
HQ457195	0,51	0,51	0,507	0,504	0,504	0,5	0,501	0,502	0,507	0,506	0,507	0,508	0,45	0,447	0,452	0,442	0,443	0,404	0,345	0,418	0,981	

Tablo 4. Percentages of Segment B nucleotide and amino acid sequences.

Nük/Aa	Almus	Aydin07	Antalya07	Usak05	Mugla07	HAH-4	Hatay07	Ankara10	HAH-2	HAH-3	M58757	AF078669	AY780925	AY780927	AY780928	AY780929	AY780930	KU609594	M58756	NC001916	AY129664	EU665685	D26527	KX234590	KY548519	AY780926	AY780931	AJ622823	AY379743	AY823633	AY354522	AY354523	AY354524	AY379739	AY379741
Almus	ID	0,983	0,97	0,98	0,972	0,973	0,947	0,952	0,957	0,952	0,949	0,558		0,558	0,558	0,558	0,558	0,556	0,559	0,559	0,588	0,578	0,582	0,582	0,528	0,701	0,723	0,945	0,942	0,944	0,942	0,94	0,945	0,942	0,944
Aydin07	0,994		0,978	0,977	0,973	0,97	0,948	0,947	0,957	0,952	0,952	0,555	0,555	0,555	0,555	0,555	0,555	0,552	0,557	0,557	0,586	0,576	0,581	0,58	0,531	0,699	0,722	0,948	0,945	0,947	0,944	0,944	0,947	0,945	0,946
Antalya07	0,988	0,992		0,982	0,964	0,955	0,935	0,936	0,946	0,945	0,941	0,562	0,562	0,562	0,562	0,562	0,562	0,558	0,552	0,552	0,588	0,576	0,584	0,584	0,531	0,694	0,719	0,935	0,934	0,936	0,933	0,933	0,938	0,935	0,936
Usak05	0,992	0,991	0,993		0,967	0,959	0,941	0,947	0,952	0,952	0,945	0,556	0,556	0,556	0,556	0,556	0,556	0,553	0,556	0,556	0,588	0,576	0,582	0,582	0,527	0,691	0,716	0,94	0,936	0,939	0,935	0,935	0,94	0,938	0,939
Mugla07	0,989	0,99	0,986	0,987		0,963	0,943	0,945	0,952	0,942	0,952	0,555	0,555	0,555	0,555	0,555	0,555	0,553	0,558	0,558	0,586	0,572	0,58	0,58	0,534	0,7	0,725	0,939	0,939	0,944	0,94	0,94	0,945	0,941	0,944
HAH-4	0,985	0,985	0,979	0,98	0,982		0,959	0,959	0,973	0,965	0,966	0,561	0,561	0,561	0,561	0,561	0,561	0,557	0,565	0,565	0,586	0,576	0,58	0,581	0,534	0,7	0,722	0,948	0,954	0,955	0,952	0,949	0,954	0,952	0,95
Hatay07	0,979	0,98	0,975	0,977	0,978	0,983		0,991	0,977	0,967	0,976	0,553	0,553	0,553	0,553	0,553	0,553	0,55	0,562	0,562	0,582	0,569	0,576	0,578	0,531	0,697	0,725	0,946	0,957	0,959	0,958	0,955	0,955	0,951	0,951
Ankara10	0,981	0,979	0,975	0,979	0,978	0,982	0,996		0,983	0,976	0,977	0,552	0,552	0,552	0,552	0,552	0,552	0,549	0,561	0,561	0,582	0,568	0,575	0,577	0,528	0,695	0,724	0,945	0,955	0,96	0,959	0,957	0,957	0,952	0,952
HAH-2	0,979	0,979	0,976	0,978	0,977	0,99	0,989	0,991		0,988	0,984	0,553	0,553	0,553	0,553	0,553	0,553	0,55	0,562	0,562	0,584	0,569	0,577	0,578	0,533	0,699	0,728	0,947	0,961	0,966	0,965	0,96	0,963	0,958	0,958
HAH-3	0,978	0,978	0,975	0,977	0,974	0,986	0,985	0,988	0,995		0,972	0,55	0,55	0,55	0,55	0,55	0,55	0,548	0,557	0,557	0,577	0,562	0,57	0,571	0,527	0,693	0,722	0,938	0,952	0,957	0,955	0,951	0,953	0,948	0,948
M58757	0,977	0,978	0,975	0,975	0,979	0,983	0,989	0,989	0,989	0,985		0,557	0,557	0,557	0,557	0,557	0,557	0,553	0,564	0,564	0,586	0,572	0,578	0,58	0,528	0,694	0,723	0,949	0,959	0,964	0,963	0,958	0,96	0,957	0,955
AF078669	0,667	0,666	0,669	0,667	0,666	0,667	0,665	0,665	0,666	0,663	0,666		1	0,998	0,998	1	1	0,984	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
AY780925	0,667	0,666	0,669	0,667	0,666	0,667	0,665	0,665	0,666	0,663	0,666	1		0,998	0,998	1	1	0,984	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
AY780927	0,666	0,666	0,669	0,666	0,665	0,667	0,665	0,665	0,665	0,662	0,666	0,999	0,999		1	1	0,998	0,983	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
AY780928	0,666	0,666	0,669	0,666	0,665	0,667	0,665	0,665	0,665	0,662	0,666	0,999	0,999	1		0,998	0,998	0,983	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
AY780929	0,667	0,666	0,669	0,667	0,666	0,667	0,665	0,665	0,666	0,663	0,666	1	1	0,999	0,999		1	0,984	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
AY780930	0,667	0,666	0,669	0,667	0,666	0,667	0,665	0,665	0,666	0,663	0,666	1	1	0,999	0,999	1		0,984	0,741	0,741	0,763	0,757	0,762	0,76	0,496	0,551	0,554	0,561	0,568	0,562	0,561	0,562	0,563	0,561	0,564
KU609594	0,666	0,666	0,668	0,666	0,665	0,666	0,664	0,664	0,664	0,662	0,665	0,992	0,992	0,992	0,992	0,992	0,992		0,732	0,732	0,752	0,747	0,751	0,751	0,494	0,552	0,555	0,558	0,565	0,559	0,558	0,558	0,561	0,558	0,562
M58756	0,669	0,669	0,669	0,669	0,668	0,671	0,669	0,669	0,67	0,667	0,67	0,899	0,899	0,899	0,899	0,899	0,899	0,894		1	0,727	0,736	0,741	0,728	0,486	0,575	0,573	0,567	0,572	0,568	0,569	0,567	0,567	0,567	0,571
NC001916	0,669	0,669	0,669	0,669	0,668	0,671	0,669	0,669	0,67	0,667	0,67	0,899	0,899	0,899	0,899	0,899	0,899	0,894	1		0,727	0,736	0,741	0,728	0,486	0,575	0,573	0,567	0,572	0,568	0,569	0,567	0,567	0,567	0,571
AY129664	0,676	0,675	0,675	0,675	0,675	0,676	0,673	0,673	0,674	0,671	0,674	0,907	0,907	0,907	0,907	0,907	0,907	0,902	0,891	0,891		0,926	0,949	0,936	0,475	0,568	0,574	0,591	0,593	0,587	0,591	0,592	0,591	0,59	0,593
EU665685	0,672	0,672	0,671	0,671	0,671	0,672	0,669	0,669	0,671	0,667	0,67	0,906	0,906	0,905	0,905	0,906	0,906	0,901	0,893	0,893	0,97		0,946	0,916	0,477	0,562	0,564	0,577	0,577	0,571	0,577	0,576	0,572	0,575	0,576
D26527	0,674	0,674	0,674	0,673	0,674	0,674	0,672	0,672	0,673	0,67	0,673	0,908	0,908	0,908	0,908	0,908	0,908	0,903	0,896	0,896	0,98	0,977		0,935	0,476	0,564	0,568	0,583	0,586	0,58	0,583	0,584	0,583	0,582	0,586
KX234590	0,676	0,676	0,676	0,675	0,676	0,677	0,674	0,674	0,675	0,672	0,675	0,905	0,905	0,905	0,905	0,905	0,905	0,901	0,891	0,891	0,972	0,965	0,973		0,485	0,573	0,577	0,584	0,584	0,582	0,584	0,582	0,581	0,58	0,584
KY548519	0,643	0,644	0,643	0,642	0,643	0,644	0,643	0,642	0,644	0,64	0,641	0,761	0,761	0,76	0,76	0,761	0,761	0,76	0,762	0,762	0,756	0,755	0,757	0,759		0,52	0,52	0,531	0,54	0,541	0,537	0,54	0,537	0,535	0,536
AY780926	0,734	0,733	0,732	0,731	0,733	0,732	0,732	0,732	0,731	0,728	0,73	0,803	0,803	0,802	0,802	0,803	0,803	0,803	0,813	0,813	0,811	0,808	0,809	0,814	0,778		0,965	0,965	0,965	0,965	0,965	0,965	0,965	0,699	0,703
AY780931	0,741	0,741	0,74	0,739	0,741	0,739	0,741	0,741	0,741	0,737	0,739	0,804	0,804	0,803	0,803	0,804	0,804	0,804	0,813	0,813	0,813	0,809	0,811	0,817	0,779	0,986		0,724	0,735	0,732	0,728	0,725	0,728	0,725	0,73
AJ622823	0,819	0,82	0,816	0,816	0,816	0,817	0,818	0,817	0,816	0,812	0,817	0,801	0,801	0,801	0,801	0,801	0,801	0,801	0,802	0,802	0,812	0,808	0,81	0,812	0,78	0,876	0,886		0,955	0,958	0,957	0,957	0,959	0,957	0,957
AY379743	0,818	0,818	0,815	0,816	0,816	0,82	0,822	0,822	0,822	0,817	0,822	0,805	0,805	0,804	0,804	0,805	0,805	0,804	0,806	0,806	0,814	0,809	0,812	0,814	0,783	0,88	0,891	0,981		0,985	0,983	0,982	0,984	0,979	0,979
AY823633	0,818	0,819	0,816	0,816	0,818	0,82	0,823	0,823	0,823	0,819	0,823	0,803	0,803	0,803	0,803	0,803	0,803	0,802	0,804	0,804	0,812	0,807	0,811	0,814	0,783	0,88	0,891	0,981	0,994		0,985	0,984	0,986	0,982	0,982
AY354522	0,817	0,818	0,814	0,814	0,816	0,819	0,823	0,823	0,823	0,818	0,822	0,803	0,803	0,802	0,802	0,803	0,803	0,802	0,805	0,805	0,814	0,809	0,812	0,814	0,782	0,878	0,889	0,981	0,993	0,994		0,986	0,989	0,984	0,984
AY354523	0,817	0,818	0,814	0,815	0,816	0,819	0,822	0,822	0,822	0,817	0,822	0,803	0,803	0,803	0,803	0,803	0,803	0,802	0,805	0,805	0,814	0,809	0,813	0,814	0,782	0,879	0,889	0,981	0,993	0,994	0,995		0,989	0,984	0,984
AY354524	0,819	0,82	0,816	0,817	0,818	0,82	0,823	0,823	0,823	0,818	0,822	0,804	0,804	0,803	0,803	0,804	0,804	0,803	0,805	0,805	0,814	0,808	0,812	0,813	0,782	0,88	0,89	0,982	0,994	0,994	0,994	0,995		0,995	0,995
AY379739	0,817	0,818	0,815	0,815	0,817	0,819	0,821	0,821	0,821	0,817	0,82	0,803	0,803	0,802	0,802	0,803	0,803	0,802	0,805	0,805	0,814	0,808	0,812	0,813	0,781	0,878	0,888	0,981	0,992	0,993	0,993	0,994	0,997		0,995
AY379741	0,818	0,819	0,816	0,816	0,818	0,819	0,822	0,822	0,821	0,817	0,821	0,804	0,804	0,803	0,803	0,804	0,804	0,803	0,806	0,806	0,814	0,808	0,812	0,814	0,782	0,88	0,89	0,981	0,993	0,993	0,994	0,994	0,997	0,998	

Discussion and Conclusion

Our study is the first to perform whole genome sequence and molecular characterization of rainbow trout and turbot originated IPNV isolates in Turkey. Molecular analyses showed very small genetic differences among isolates. These small genetic differences show that fish and their eggs are transferred among farms from geographically remote areas and that the virus is spread in a wide area.

Since mutation was reported after 8th passage on CHSE-214 cell lines in previously conducted studies with IPNV (25), passages were made on RTG-2 cell lines and low passage numbered strains were studied. Our phylogenetic analysis results show that Turkish isolates are within genogroup 5 and serotype A2 and they are closely related with France, Spain and Iran strains. Almost all of the Thr221 containing isolates in genogroup 5 are avirulent and they are responsible for the occurrence of carrier or persistent infections in salmons (20, 22, 25). RNA viruses are known to adapt quickly to environmental conditions. In non-enveloped viruses, adaptation mutations generally occur in external capsid proteins which function as virus cell binding and receptor defining protein (25). In our study, Almus and Hah-4 isolates were found to undergo a point mutation from Ala to Glu at VP2 gene 247 position, unlike the GenBank accession number KM972672 and KM972675 parent virus. The beginning of VP5 protein start codon may change (7). Start codon of VP5 has been shown to be placed at 68 or 112 position (12). In addition, Weber et al. (26) and Shivappa et al. (23) showed that in Sp strains, second start methionine codon is responsible for starting VP5. According to Segment A sequences, VP5 start codon was found to be placed at 112 nucleotide and be responsible for starting VP5 translation of the second methionine in Turkish strains.

In small ORF encoding VP5 at IPNV genome, a great number of hotspot points are observed. VP5 mutations include amino acid residue 29, 36, 45 and 106. As a result of the changes in these areas, 3 different types of VP5 are occurred. The first stop codon in this ORF normally appears at nucleotide 511, resulting in a polypeptide of 133 amino acids. Some isolates had a premature stop codon at nucleotide 496, encoding a polypeptide shorter by five amino acids. Others could encode a truncated NS protein of 105 amino acids, having a stop codon at nucleotide 427 (14). In all of the 7 isolates sequenced in our study, this gene encodes VP5 protein. Isolates were found to encode both 133 aa full length VP5 protein and 128 aa cut VP5 protein.

As a result of phylogenetic analyses, amino acids of segment A in Almus, Antalya07, Uşak05 and Hah-4 isolates were found to show more than 99% similarity. In Hah-2, Hah-3, Hatay07, Uşak05 and Antalya07 isolates, all nucleotides of virus genome were found to be more than 99% similar. This result brought to mind that the source of these viruses which were isolated in different years and cities were the same and they were circulated among the cities. Hah-2 strain was isolated from turbots and Hah-3 strain was isolated from a salmon in Trabzon in 2010, while Hatay07 strain was isolated from a salmon in Hatay in 2007. The result that more than 99% molecular similarity was found between salmon isolate Hah-2 and turbot isolates Hatay07 and Hah-3 brought to mind that this virus was transferred between fish species.

Segment A sequences of Turkish isolates were found to show a similarity between 97.8% and 99.8%. When compared with international reference strains, while the lowest similarity (65.7%) was found between Canada strain NC001915 and Hah-3 isolate, the highest similarity (99.3%) was found between France isolate J622822 and Hatay07. Segment B sequences of Turkish isolates were found to show a similarity between 93.5% and 99.5%. When compared with international reference strains, while the lowest similarity (52.7%) was found between Finland strain KY548519 and Uşak05 and Hah-3 isolates, the highest similarity (98.9%) was found between Canada isolate M58757, Hatay07, Ankara10 and Hah-2.

The results of this study can help in developing specific sanitation methods in protection from IPNV and epidemiological connections. The presence of IPNV carriers in brood stock fish is an alarming situation especially for spawners. Through larvae and eggs taken from these spawners, the infection is transferred to other farms. In order to protect from vertical infection of IPNV, the source of the disease should be eliminated by routine checks, determination of IPNV carrier spawners and taking out the carrier brood stock from aquaculture units. Producers should buy eggs and fries after having IPNV control tests and certifications should be made compulsory especially for hatcheries. While fishery industry is developing fast in many countries of the world, failure in fighting this infection is resulting with economic loss. Due to the presence of "persistent IPNV strains" in hatcheries, the elimination of this disease is difficult. Developing effective vaccines and presenting these to the market will contribute to fighting the disease.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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3 tesla magnetic resonance imaging and multiplanar reconstruction of the brain and its associated structures in sheep

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Abstract: The purpose of the study was to scan the brain and related structures in sheep with high-resolution magnetic resonance imaging (MRI) and three-dimensional (3D) multiplanar reconstruction for defining the anatomical regions. Six adult sheep, three of six were male and three of six were female used as cadaver, were utilized in this research. Heads were scanned at 3 Tesla Siemens Magnetom Spin-Echo MRI devices using the human head coil. The processes were acquired in both T1 and T2 weighted slices and were reconstructed by using Leonardo workstation. The cranioencephalic structures and anatomical details were defined and labeled in all slice of 3 planes that were obtained 1 mm thickness sequential images in sagittal, frontal, and transversal planes. According to the MRI measurements obtained intracranially from the brain, the average value of length, height, and width of the brain were 87.1 ± 0.3, 46.8 ± 0.7, and 62.2 ± 0.4 mm, respectively. It was observed that in T1 weighted images were more effective to identify deep brain structures and anatomical details. On 3D reconstructed images obtained from the study can be used as a reference in head and brain MR scans in multidisciplinary studies where sheep are used as animal models.
Keywords: Animal model, MRI, nervous system, sheep, 3D reconstruction.

Koyunda beyin ve ilişkili yapıların 3 tesla manyetik rezonans ile görüntülenmesi ve multiplanar rekonstruksiyonu

Özet: Bu çalışmada, koyun beyni ve beyin çevresindeki anatomik yapıların, yüksek çözünürlüklü manyetik rezonans ile görüntülenmesi ve 3 boyutlu (3B) multiplanar rekonstruksiyonu ile bölge anatomisinin özelliklerinin belirlenmesi amaçlandı. Araştırma için 3 erkek, 3 dişi toplam 6 yetişkin koyun kullanıldı. Tüm kafalar 3 Tesla Siemens Magnetom Spin-echo manyetik rezonans görüntüleme cihazı ile insan kafa koili kullanılarak tarandı. Tarama işlemi hem T1, hem de T2-ağırlıklı olarak yapıldı ve Leonardo workstation kullanılarak rekonstrüksiyonu yapıldı. Sagittal, frontal ve transversal düzlemlerde alınan yaklaşık 1 mm kalınlığındaki ardışık kesit görüntülerinin üzerinde kafa yapıları ve anatomik detaylar üç düzlemde ve her kesit üzerinde tanımlanarak işaretlendi. Kesit görüntüleri üzerinden yapılan kafa içi ölçümlerde encephalon'un ortalama uzunluğu, yüksekliği ve genişliği sırasıyla 87.1 ± 0.3, 46.8 ± 0.7 ve 62.2 ± 0.4 mm olarak ölçüldü. T1 ağırlıklı görüntülerin, derin beyin yapılarının ve anatomik detayların tanımlanmasında daha etkili olduğu görülmüştür. Hayvan modeli olarak koyunun kullanıldığı multidisipliner çalışmalarda, 3B rekonstrüksiyon ile elde edilen görüntülerin, baş ve beyin MR taramalarında referans olarak kullanılabilir.
Anahtar sözcükler: Hayvan modeli, koyun, MRG, sinir sistemi, 3B rekonstrüksiyon.

Introduction

The advantages of using magnetic resonance imaging (MRI) are being the noninvasive clinical approach, a keen diagnostic tool (9, 13, 14, 24) and acquiring comprehensive information about neuroscience (4-6). Improvements in MRI techniques can make the determination of pathologies or injuries on the brain and spinal cord easily (2, 9, 11). It also finds a use for diagnosing zoonoses such as hepatica fascioliasis or cerebral coenurosis (10, 17). In addition to these MRI is accepted as a gold standard method for examination of muscle morphometric parameters such as cross-section areas and muscle fatty infiltrates (25).

An ovine model can be used because of having anatomical and physiological similarities to the human body than other experimental species, such as the size of fetal sheep pregnancy and imaging features (6, 18). This animal model has become important for surgical and

anatomical cerebral studies because of its size (6, 14, 27), biomedical imaging and implant testing studies (21), biomechanics of the spine disorders (13, 28), diagnosing of diseases on prematurity (22, 24), monitoring the effectiveness of treatment on diseases such as epilepsy or neurologic decompression sickness (11, 23). The shape of the sheep skull shows similarity like humans, so this can be useful for studies (16). Sheep is also become an alternative animal model due to a physiological examination of genes for reproductive functions (1, 7) and endocrine regulations related to reproductive physiology (5).

The aim of this study was to define imaging features of the normal anatomical structures of the brain and round tissues in the sheep MR images. By this way, it helps the clinicians to compare the head pathologies with normal anatomy and as a reference to the biomedical investigations for researchers.

Material and Methods

This study was carried out with the 2013/18 numbered permission of the Bilkent University Animal Experiments Ethics Committee. In this study, 3 male and 3 female total 6 adult Akkaraman sheep fresh cadavers obtained from the faculty farm were used. The weights of the sheep were 40 ± 3 kg. The heads of the sheep were placed in "prone" position and were scanned using a 3-Tesla Siemens Magnetom Spin-echo magnetic resonance imaging device (Siemens Magnetom Trio, Erlangen, Germany) with an 8-channel human head coil and a 15-channel human knee coil. Whole brain and associated structures were scanned with three-dimensional (3D), T1-weighted (W) gradient echo inversion recovery (IR) multiplanar reconstruction (MPR) sequence in the sagittal plane with isotropic voxels for reformation and 3D reconstructed by Leonardo Workstation software (Siemens Medical Solutions, Erlangen, Germany). Parameters for T1-weighted images during scans; TE: 12 ms; TR: 600 ms; Section Thickness: 1 mm; FOV: 230 × 230 mm; Number of sections: 125; Scanning time; 8 minutes, parameters for T2-weighted images; TE: 404 ms; TR: 3000 ms; Section Thickness: 1 mm; FOV: 230 × 230 mm; Number of sections: 125; Scanning time; 11 minutes. Nomina Anatomica Veterinaria was used for the nomenclature (12). Identification of the anatomical structures were based on the textbooks (3, 15).

Results

All animals successfully underwent MRI progress. In MR images, anatomical structures of the brain did not contain any differences in shape or signal intensities described. T1 and T2 weighted MR images were performed in detail for whole brain and associated structures in three planes that were acquired 1 mm thickness consecutive images in sagittal, frontal and transversal planes. Morphological features of the MR images were illustrated in Figure 1, 2, and 3. According to the measurements of the brain obtained intracranially with MRI, the average values of length, height, and width of the brain were 87.1 ± 0.3 mm, 46.8 ± 0.7 mm and 62.2 ± 0.4 mm, respectively.

After the colored reformation of images in T1 weighted series scanned with 3D-IR-MPR sequence, the anatomic details were clearly defined, and the grey and white matters were also recognized more easily from each other (Figure 1A, 1B, and 1C). It has been observed that anatomical details and deep brain structures can be easily detected on T1 weighted images. The grey matter was more hypointense when compared with the white matter (Figure 1D, 2B, and 3D). The brain tissue could be easily separated from other surrounding tissues (head bones, muscles, fat tissues) because of the high tissue contrast provided by the MR images (Figure 1C, 2C, 3B, and 3C). In the image scans, the bones were monitored isointense (Figure 1C), muscles were hypointense appearance (Figure 1D), and fat tissues were identified hyperintense (Figure 3B).

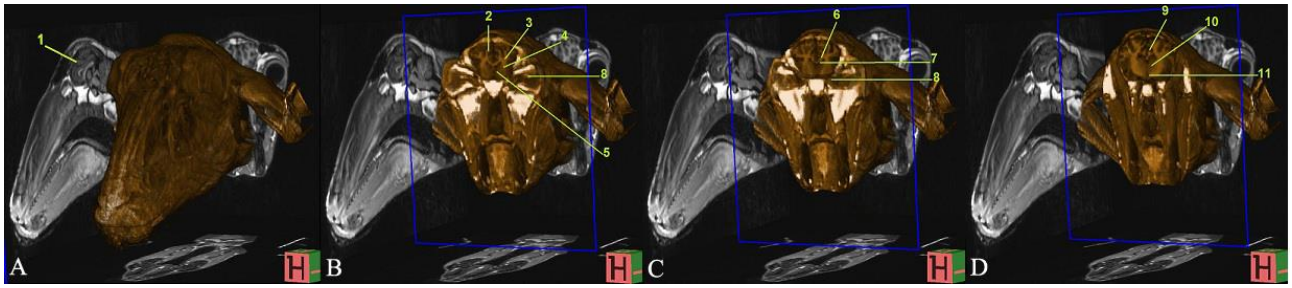

Figure 1. The transversal 3D-IR-MPR images of the head (**A.** Three dimensional view of the sheep head, **B.** At the level of caudate nuclei, **C.** At the level of lateral ventricle and **D.** At the level of middle of thalamus). 1. Sagittal T1 weighted image, 2. Cerebral cortex, 3. Semioval center, 4. Internal capsule, 5. Caudate nucleus, 6. Corpus callosum, 7. Lateral ventricle, 8. Optic chiasma, 9. Cingulum, 10. Thalamus, 11. Third ventricle.

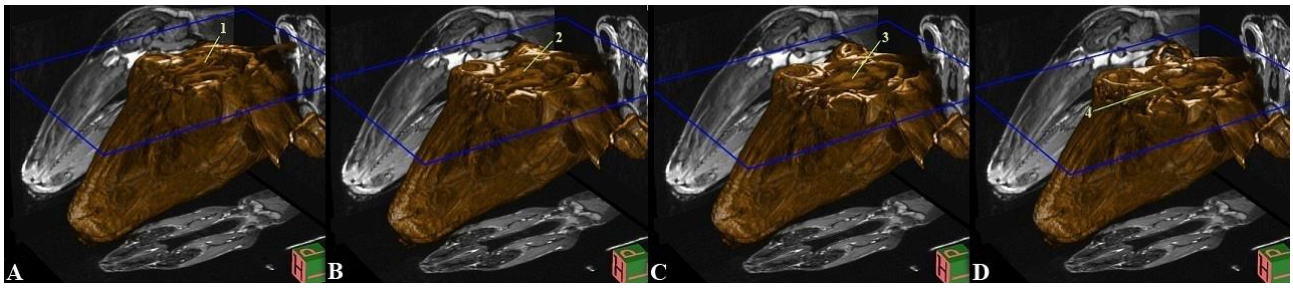

Figure 2. The frontal 3D-IR-MPR images of the head (**A.** At the level of lateral ventricle, **B.** At the level of rostral colliculi, **C.** At the level of middle of thalamus and **D.** At the level of optic chiasm). 1. Lateral ventricle, 2. Thalamus, 3. Cerebral aqueduct, 4. Optic chiasm.

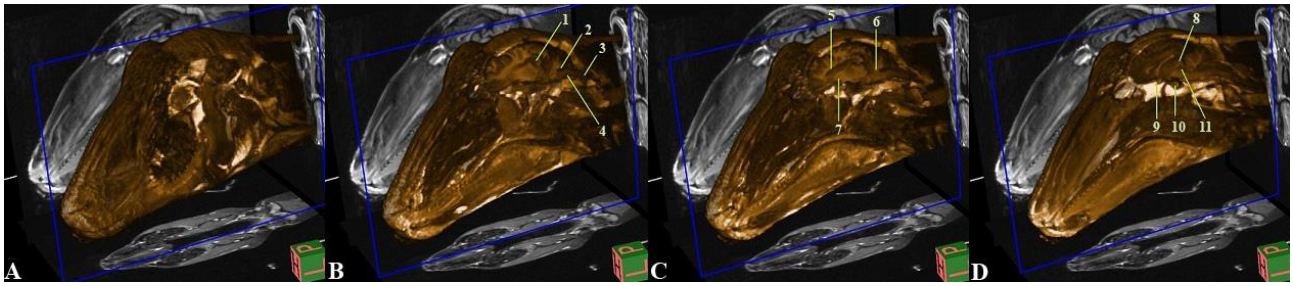

Figure 3. The sagittal 3D-IR-MPR images of the head (**A.** At the level of beginning of left cerebral hemisphere, **B.** At the level of left hippocampus, **C.** At the level of left lateral ventricle and **D.** At the level of middle of brain). 1. Hippocampus, 2. Fourth ventricle, 3. Spinal cord, 4. Medulla oblongata, 5. Lateral ventricle, 6. Middle cerebellar peduncles, 7. Internal capsule, 8. Rostral colliculus, 9. Optic chiasma, 10. Pituitary gland, 11. Cerebral aqueduct.

In addition to these, the anatomical details of deep cerebral structures such as cortex cerebri, capsula interna, nucleus caudatus, corpus callosum, ventriculus lateralis, chiasma opticum, thalamus and ventriculus tertius on transversal sections were identified from 3D images (Figure 1A-D). Ventriculus lateralis and aqueductus mesencephali were also observed in coronal sections (Figure 2A-D). Hipophysis, aqueductus mesencephali, colliculus rostralis, hypocampus, pedunculus cerebellaris medius, medulla oblongata, ventriculus quartus, and medulla spinalis were identified on a sagittal section of 3D images (Figure 3 A-D). Orbita and its surrounding tissues on coronal sections (Figure 2D), mandibula, pharynx, skull bones, meninges and muscles on transversal sections (Figure 1C and 1D) and cavum nasi, lingua, and palatum on sagittal sections (Figure 3D) were also seen as well.

Discussion and Conclusion

The present study demonstrated the applicability of the 3D-IR-MPR imaging for brain and its surrounding tissues in a healthy sheep model. The use of sheep as an anatomical model is developing. Many studies made in recent years have strengthened this consideration due to the sheep brain and its anatomical characteristics (5, 6, 16).

In previous studies, it has been stated that MR images are used in the diagnosis process of diseases (8, 29). It has also been suggested that MR studies on specimens to see the similarities or differences for normal anatomical structures, were important for other researchers such as surgeons, radiologists (4, 16, 19, 20, 26). It was thought that the images obtained from this study can be used as a teaching material in anatomy education, as well as the normal structure, size, position, etc. on these 3D images can be compared with the pathological features that may occur in the brain, brain membranes or cerebrospinal fluid. In addition to the 3D images from this study, this colored 3D reconstructed images to transmit and visualize the interaction of the brain with surrounding tissues better compared with T1 weighted and T2 weighted images.

As a result, MRI has become one of the routine imaging techniques in many veterinary fields. It is considered that reconstructed three-dimensional images obtained from MR images can be used as an important source not only the recognition of pathological tissues and during the preparation phase of operation in the clinic but also in veterinary anatomy training for understanding the morphology of the structures more easily.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Effects of *Urtica dioica*, *Matricaria chamomilla*, and *Vitex agnus-castus* extracts on *in vitro* rumen fermentation under normal and acidosis conditions

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Abstract: The aim of this study was to investigate the effects of dry extracts of *Urtica dioica*, *Matricaria chamomilla*, and *Vitex agnus-castus* with high phenolic contents on rumen microbial fermentation as compared with those of monensin, a common ionophore antibiotic, using Rumen Simulation Technique (RUSITEC) under normal and acidosis conditions. The treatments were as follows: negative control (no additive), positive control (5 mg/d monensin), and extracts of *U. dioica* (500 mg/d), *M. chamomilla* (500 mg/d), and *V. agnus-castus* (500 mg/d). Neither the plant extracts nor monensin altered the ruminal pH under normal or acidosis conditions. All the treatments affected total volatile fatty acid (VFA) production, propionate production, and dry matter digestibility (DMD), regardless of the fermentation conditions. All three extracts increased (P<0.05) total VFA production similar to that observed with monensin (P<0.05). *M. chamomilla* and *V. agnus-castus* increased propionate production and DMD similar to that obtained with monensin (P<0.05). In contrast to the monensin treatment, all three extracts increased acetate production under normal conditions (P<0.05). Under acidosis conditions, acetate production remained unchanged in the *U. dioica* and *V. agnus-castus* treatments, as well as in the monensin treatment. Under both conditions, the acetate-to-propionate (A:P) ratio decreased only in the monensin treatment (P<0.05). *U. dioica* and *M. chamomilla* had antiprotozoal effects (P<0.05) similar to those of monensin, regardless of the condition. The NH_3-N concentration declined only in the *V. agnus-castus* treatment under acidosis conditions (P<0.05). Similar to the monensin treatment, lactate concentrations remained unchanged in the *V. agnus-castus* treatment under both conditions. In conclusion, plant extracts stimulated fermentative activity of rumen microorganisms under normal and acidosis conditions. Although they did not improve ruminal pH, *U. dioica* and *V. agnus-castus* extracts had more favorable effects on some fermentation parameters under acidosis conditions.

Keywords: Acidosis, plant extracts, rumen fermentation, RUSITEC.

Urtica dioica, *Matricaria chamomilla* ve *Vitex agnus-castus* ekstraktlarının normal koşullar ve asidoz koşulları altında rumen fermentasyonuna *in vitro* etkileri

Özet: Bu çalışmada, *Urtica dioica*, *Matricaria chamomilla* ve *Vitex agnus-castus*'un yüksek fenolik içerikli kuru ekstraktlarının normal koşullar ve asidoz koşulları altında rumen mikrobiyal fermentasyonu üzerine monensin ile karşılaştırmalı etkilerinin Rumen Similasyon Tekniği (RUSITEC) kullanılarak araştırılması amaçlanmıştır. Deneme grupları, negatif kontrol (katkı maddesi yok), pozitif kontrol (5 mg/gün monensin) ve *U. dioica* (500 mg/gün), *M. chamomilla* (500 mg/gün) ve *V. agnus-castus* (500 mg/gün) ekstraktlarından oluşmuştur. Bitki ekstraktları ve monensin ruminal pH'yi normal koşullar ve asidoz koşulları altında değiştirmemiştir. Deneme gruplarının toplam uçucu yağ asidi (UYA) ve propiyonat üretimi ile kuru madde sindirilebilirliği (KMS) üzerine etkilerinin koşuldan bağımsız olarak gerçekleştiği gözlenmiştir. Üç ekstrakt da monensin'e benzer şekilde toplam UYA üretimini arttırmıştır (P<0,05). *M. chamomilla* ve *V. agnus-castus*, propiyonat üretimi ve KMS'yi monensin'e benzer şekilde arttırmıştır (P<0,05). Monensin'in aksine, normal koşullar altında her üç ekstrakt da asetat üretimini arttırmıştır (P<0,05). Asidoz koşulları altında ise asetat üretimi monensin'in yanı sıra *U. dioica* ve *V. agnus-castus* gruplarında da değişmeden kalmıştır. Asetatın propiyonata oranı (A:P), her iki koşulda da sadece monensin grubunda azalmıştır (P<0,05). *U. dioica* ve *M. chamomilla* koşuldan bağımsız olarak monensin'e benzer şekilde antiprotozoal etkiler göstermişlerdir (P<0,05). NH_3-N konsantrasyonu, asidoz koşulları altında sadece *V. agnus-castus* grubunda azalmıştır (P<0,05). Laktat konsantrasyonu, *V. agnus-castus* grubunda her iki koşulda da monensin'e benzer şekilde değişmemiştir. Sonuç olarak, bitki ekstraktları normal koşullar ve asidoz koşulları altında rumen mikroorganizmalarının fermentatif aktivitelerini uyarmıştır. Ruminal pH'yi iyileştirmemiş olmalarına rağmen, *U. dioica* ve *V. agnus-castus* ekstraktları bazı fermentasyon parametreleri üzerine asidoz koşulları altında daha olumlu etkiler oluşturmuşlardır.

Anahtar sözcükler: Asidoz, bitki ekstraktları, rumen fermentasyonu, RUSITEC.

Introduction

Sub-therapeutic doses of ionophore antibiotics have been used since the 1970s to avoid ruminal energy and nitrogen losses and to control metabolic disorders, including acidosis, by selectively inhibiting Gram-positive rumen bacteria and protozoa (29). The use of antibiotics as feed additives was banned in the European Union as of 21 January 2006 due to antibiotic residues in animal products and the development of bacterial resistance (27). Following the ban, there has been intense interest in the development of safer antimicrobial agents that can serve as alternatives to antibiotics as feed additives. Most recent studies have focused on plant extracts and secondary bioactive plant metabolites due to their potential to modify ruminal fermentation (4, 18). However, experimental data on the effects of plant extracts on rumen microbial fermentation under acidosis conditions particularly following normal conditions as in the practice are scarce. Such data would reveal the potential of plant extracts to prevent acidosis.

Urtica dioica (stinging nettle), *Matricaria chamomilla* (chamomile), and *Vitex agnus-castus* (chasteberry) extracts have been used for centuries in traditional medicine and industrial applications, as they contain antimicrobial phenolic compounds, mainly flavonoids (i.e., isorhamnetin, kaempferol, quercetin, rutin, apigenin, and luteolin) and phenolic acids (i.e., caffeic acid, formic acid, malic acid, and chlorogenic acid) (17, 26, 28). In previous studies, extracts of *U. dioica*, *M. chamomilla*, and *V. agnus-castus* were more effective against Gram-positive bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus* spp., and *Enterococcus* spp. than Gram-negative bacteria (2, 11, 20), similar to those of ionophore antibiotics, suggesting that these plant extracts may have potential to modify ruminal fermentation. There are some reports on regulatory effects of *U. dioica* on ruminal pH (21) and in the fermentation process of sausage (22) and a few studies on the effects of other dry extracts on various ruminal fermentation parameters under normal rumen conditions (16, 18).

The use of disease models, such as acidosis, that have a negative effect on animal well-being is problematic under *in vivo* conditions due to ethical issues. The standardized semi-continuous Rumen Simulation Technique (RUSITEC) offers an appropriate alternative to such disease models. Therefore, the objective of the present study was to investigate the effects of *U. dioica*, *M. chamomilla*, and *V. agnus-castus* extracts as compared with those of monensin, a commonly used ionophore antibiotic, on *in vitro* rumen microbial fermentation under normal and acidosis conditions.

Material and Methods

Plant extracts: Dry extracts of *U. dioica*, *M. chamomilla*, and *V. agnus-castus* were supplied by Herbal Extracts Plus Co. Ltd. (Croydon, US). The phenolic contents of the plant extracts are summarized in Table 1.

Table 1. Phenolic compounds of plant extracts (μg/g).

Phenolic compounds	Plant extracts		
	U. dioica	***M. chamomilla***	***V. agnus-castus***
Chlorogenic acid	566.6	394	ND
Caffeic acid	36.9	ND	ND
P-Coumaric acid	10.3	47.7	15.6
O-Coumaric acid	ND	5.6	ND
Syringic acid	ND	38.5	ND
Gallic acid	ND	ND	126.9
Caffeic acid	ND	ND	63.3
Rutin	206.9	ND	ND
Quercetin	263.2	542.9	ND
Apigenin	ND	75.9	ND
Luteolin	ND	ND	344.1

ND: not determined.

Incubation technique: RUSITEC was performed as described by Czerkawski and Breckenridge (13). Ten incubation vessels with a nominal volume of 0.75 L were simultaneously used. Inoculum was obtained from a freshly slaughtered 2-y-old healthy Holstein bull (mean body weight: 500 kg) at a commercial slaughter facility. The inoculum transferred in a warm (39°C) insulated flask for use in the *in vitro* system within 30 min. The ruminal fluid was mixed and filtered through three layers of cheesecloth to partition it into liquid and solid (digesta) fractions. Each fermentation vessel was filled with 750 mL of filtered ruminal fluid. Two nylon bags (80 × 120 mm; 150 μm pore size), one containing 80 g of solid digesta and the other containing 16 g of an experimental diet (12.8 g of barley straw cut into 1-cm lengths and 3.2 g of concentrate on a dry matter basis), were placed in the inner perforated containers at the beginning of the experiment. The concentrate was composed of barley, corn, wheat bran, corn gluten meal, sunflower seed meal, dried sugar-beet pulp, molasses, rice bran, vegetable oil, sodium chloride, sodium bicarbonate, calcium carbonate, and a vitamin-mineral premix. According to information obtained from the owner of the Holstein bull, the animal had been fed barley straw ad libitum and 10 kg of a concentrate diet every morning and evening. The same feedstuffs were used in the *in vitro* incubation trial. The chemical composition of the experimental diet is shown in Table 2. After 24 h, the nylon bags containing the solid

digesta from the rumen were replaced with another feed bag containing a fresh experimental diet. Thereafter, only one feed bag was replaced with a new bag daily, and the other bag remained in the system for a further 24 h. Therefore, each feed bag remained in the fermentation vessel for 48 h. The fermentation vessels were maintained at a constant temperature (39°C) and received a continuous infusion of buffers at a rate of 750 mL/d. The chemical composition of the buffer solutions is shown in Table 3. Pure CO_2 was applied to the fermenters when changing the feed bags for continuity of anaerobic conditions.

Table 2. Chemical composition of the experimental diet.

	Barley straw	Concentrate
Dry matter (g/kg)	941.5	927.5
Crude protein (g/kg DM)	37.17	153.10
Crude fat (g/kg DM)	15.93	40.97
Crude fiber (g/kg DM)	445.03	80.86
Ash (g/kg DM)	83.90	78.71
Acid detergent fiber (g/kg DM)	547	-
Metabolizable energy (MJ/kg DM)	6.29	12.10

DM: Dry matter.

Table 3. Chemical composition of the buffer solutions (g/L).

Chemicals	Adaptation period and normal conditions	Acidosis conditions
NaCl	0.470	0.470
KCl	0.570	0.570
$CaCl_2.2H_2O$	0.053	0.053
$MgCl_2.6H_2O$	0.128	0.128
$Na_2HPO_4.12H_2O$	3.720	0.620
$NaHCO_3$	9.800	2.450
pH	8.6	8.6

Experimental procedure: The experiment lasted 21 days (21 d). The first phase of the study (d 1 to d 7) was considered as an adaptation period for the microorganisms to the *in vitro* conditions. In the second phase of the study (d 8 to d 14), 10 RUSITEC fermenters (vessels) were divided into five groups, with two vessels in each group, to investigate the effects of the plant extracts under normal conditions. The five groups were as follows: group 1, no additives (negative control); group 2, 500 mg/d (667 mg/L) of *U. dioica* extract; group 3, 500 mg/d of *M. chamomilla* extract; group 4, 500 mg/d of *V. agnus-castus* extract; and group 5 (positive control), 5 mg/d of monensin (monensin sodium, Fluka). In the third phase of the study (d 15 to d 21), acidosis was established in the RUSITEC fermenters by changing the forage-to-concentrate ratio to 20:80 and reducing the amount of buffering compounds in artificial saliva solution (15). The same amount of each substance was added to the vessels under acidosis conditions.

Sampling and analytical procedures: The phenolic contents (Table 1) of the plant extracts were quantified using a high-performance liquid chromatography (HPLC) (Shimadzu) device equipped with a photodiode array detector. An Agilent Eclipse XDB-C18 (250 × 4.60 mm) 5 µm column at 30ºC and 0.8 mL/min flow speed was used.

The dry matter (920.36), crude protein (984.13), crude fat (920.39), crude fiber (978.10) and ash (942.05) contents of the experimental diet (Table 2) were analyzed according to the procedure of the Association of Official Analytical Chemists (1). The acid detergent fiber was analyzed according to the criteria of Van Soest et al. (32). All samples were ground finely before the chemical analysis.

The pH values were measured daily in each fermentation vessel at the time of feeding using an epoxy body pH electrode (WD-35801-00, Oakton) connected to a pH meter (Ion 6; Acorn series, Oakton). The overflow flasks in the RUSITEC system were placed on ice throughout the experiment to halt microbial activity and to preserve the fermentation products. The liquid effluent was collected daily for VFA, lactate, and NH_3-N determination. Effluents (5 mL) taken for VFA and lactate analysis were stored at -20°C after adding 90 µL of 12 N H_2SO_4. Samples for NH_3-N analysis were frozen directly after collection. The ruminal samples were allowed to thaw completely at 4°C before the analysis. The VFA and lactate concentrations were quantified by HPLC as described previously (14). The NH_3-N concentration was determined with indophenol blue method using the spectrophotometer (UV-150-02; Shimadzu) at 546 nm (9).

The dry matter was determined by drying the feed bags at 65°C for 48 h. The digestibility of the dry matter after 48 h was calculated as the original dry matter sample weight minus the dry matter residue weight divided by the original sample weight (33).

For protozoa counting, rumen fluid samples were removed from the fermenters daily immediately before substrate exchange. The total number of protozoa was counted as described by Demirtas et al. (14).

Statistical analysis: Statistical analysis was performed using the General ANOVA/MANOVA repeated measures factor design, with three fixed effects: two levels of rumen conditions, five levels of treatments, and seven levels of time course. Statistica 5.5 (StatSoft,

Tulsa, OK, USA) was used for the analysis. The effects of time course on microbial fermentation parameters, except for ruminal pH, were not presented in this article. Data of protozoa were transformed by Log10 before variance analysis (25). Significant differences between the means were analyzed using Duncan multiple range test using MstatC software v 1.4 (Michigan State University, 1989). P value of ≤0.05 was considered statistically significant.

Results

Under normal conditions, no significant differences were observed in the ruminal pH for 7 d, and the pH ranged between 6.93 and 6.99. The ruminal pH significantly decreased during the first 3 d when it was switched to acidosis conditions (P<0.05). The pH was 5.65 on d 18 and remained constant thereafter until the end of acidosis. There were no significant differences in the ruminal pH values in the monensin or plant extract groups under the normal and acidosis conditions (Figure 1).

The effects of the plant extracts and monensin treatments on the VFA profile and DMD are shown in Tables 4 and 5, respectively. All the plant extracts increased acetate production under normal conditions (P<0.05), whereas acetate production remained unchanged in the *U. dioica* and *V. agnus-castus* treatments similar to the monensin treatment under acidosis conditions. Similar to monensin, *U. dioica* and *M. chamomilla* had no significant effect on butyrate production under normal conditions. In contrast, under acidosis conditions, the plant extracts increased butyrate production (P<0.05), while monensin decreased butyrate production (P<0.05). All the treatments affected total VFA and propionate production and DMD, regardless of the fermentation conditions. Propionate production and DMD increased in the *M. chamomilla* and *V. agnus-castus* groups (P<0.05), similar to monensin, but remained unchanged in the *U. dioica* group. Total VFA production also increased within all the additive groups (P<0.05). Under both conditions, only monensin reduced the acetate-to-propionate (A:P) ratio (P<0.05), with no significant change observed in any of the plant extract groups. The total protozoa number decreased in the *U. dioica* and *M. chamomilla* groups (P<0.05) similar to that observed in the monensin group, regardless of the fermentation conditions. Under normal conditions, none of the additives had any effect on the NH_3-N concentration, and only *V. agnus-castus* reduced the NH_3-N concentration under acidosis conditions (P<0.05). Lactate concentrations remained unchanged in the *U. dioica* extract treatment under normal rumen conditions and in the *V. agnus-castus* extract treatment under both conditions, similar to monensin treatment.

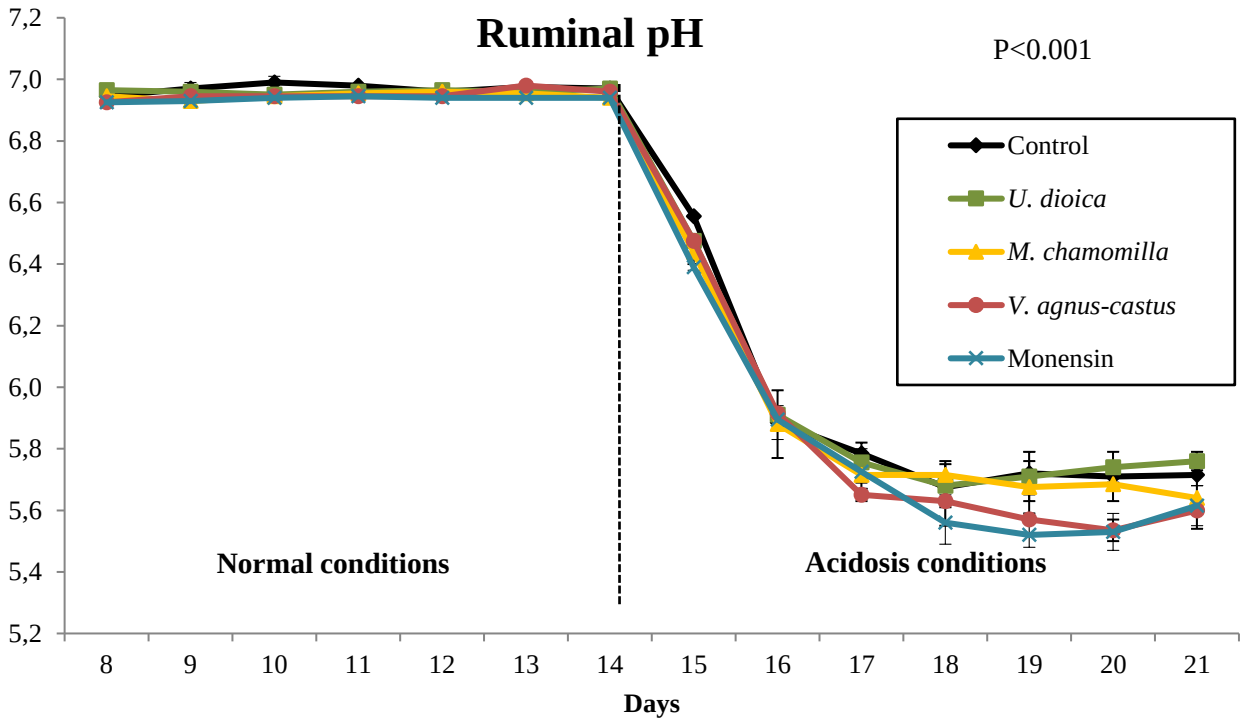

Figure 1. Effects of *U. dioica*, *M. chamomilla* and *V. agnus-castus* extracts as compared with those of monensin on ruminal pH during normal and acidosis conditions. Bars indicate standard error. The P value denotes the interaction between rumen conditions and time.

Table 4. Effects of *U. dioica*, *M. chamomilla* and *V. agnus-castus* extracts as compared with those of monensin on the production of VFA (mmol/d) and the A:P ratio under normal and acidosis conditions.*

Rumen Condition (RC)	Treatment (T)	Acetate	Propionate	Butyrate	Total VFA	A:P
N	Control (0)	11.89±0.38^{c}	4.33±0.13	2.47±0.09^{e}	18.68±0.58	2.75±0.04^{a}
N	*U. dioica*	13.45±0.36^{b}	5.13±0.16	3.22±0.12^{de}	21.79±0.61	2.63±0.05^{a}
N	*M. chamomilla*	14.47±0.73^{ab}	5.40±0.31	3.44±0.28^{de}	23.31±1.26	2.69±0.04^{a}
N	*V. agnus-castus*	15.47±0.44^{a}	5.63±0.20	3.82±0.11^{d}	24.92±0.73	2.76±0.05^{a}
N	Monensin	11.44±0.58^{cd}	6.54±0.21	2.93±0.17^{de}	20.91±0.71	1.79±0.14^{b}
A	Control (0)	10.33±0.86^{d}	7.77±0.33	7.39±0.48^{b}	25.49±0.75	1.38±0.14^{c}
A	*U. dioica*	10.85±0.98^{cd}	8.00±0.38	8.45±0.50^{a}	27.29±0.84	1.38±0.12^{c}
A	*M. chamomilla*	11.91±0.89^{c}	9.46±0.54	8.54±0.61^{a}	29.91±0.95	1.32±0.12^{c}
A	*V. agnus-castus*	11.41±0.61^{cd}	9.30±0.75	8.80±0.74^{a}	29.51±1.17	1.35±0.14^{c}
A	Monensin	10.16±0.64^{d}	12.63±0.99	5.87±0.44^{c}	28.66±1.42	0.86±0.07^{d}
Main effects						
N		13.34±0.29^{a}	5.40±0.13^{b}	3.18±0.09^{b}	21.92±0.44^{b}	2.53±0.05^{a}
A		10.93±0.36^{b}	9.43±0.35^{a}	7.81±0.28^{a}	28.17±0.50^{a}	1.26±0.06^{b}
	Control (0)	11.11±0.49^{c}	6.05±0.37^{c}	4.93±0.53^{b}	22.09±0.80^{c}	2.06±0.15^{a}
	U. dioica	12.15±0.57^{b}	6.56±0.34^{bc}	5.83±0.56^{a}	24.54±0.73^{b}	2.00±0.14^{a}
	M. chamomilla	13.19±0.62^{a}	7.43±0.50^{b}	5.99±0.59^{a}	26.61±1.00^{ab}	2.01±0.15^{a}
	V. agnus-castus	13.44±0.54^{a}	7.46±0.52^{b}	6.31±0.60^{a}	27.21±0.81^{a}	2.06±0.15^{a}
	Monensin	10.80±0.44^{c}	9.58±0.77^{a}	4.40±0.36^{b}	24.78±1.08^{ab}	1.32±0.12^{b}
P values						
RC		<0.001	<0.001	<0.001	<0.001	<0.001
T		<0.001	0.001	<0.001	0.005	<0.001
RC × T		0.041	0.131	0.021	0.626	0.011

*The values for the main effects of the RC and RC × T are the means of 7 d ± SEM, and the values for the main effects of T are the means of 14 d ± SEM. ^{a,b,c,d,e} Means in the same column followed by different superscripts differ significantly (P<0.05). N: Normal rumen conditions, A: Acidosis conditions, RC: Rumen conditions, T: Treatment, RC × T: Interaction between RC and T, VFA: Volatile fatty acids, A:P: Acetate-to-propionate ratio.

Table 5. Effects of *U. dioica*, *M. chamomilla* and *V. agnus-castus* extracts as compared with those of monensin on DMD coefficients, total protozoa (log 10/mL), and NH_3-N and lactate concentrations (mmol/L) under normal and acidosis conditions.*

Rumen Condition (RC)	Treatment (T)	DMD	Protozoa	NH_3-N	Lactate
N	Control (0)	0.16±0.01	3.22±0.05	0.96±0.07^{c}	0.054±0.015^{cd}
N	*U. dioica*	0.16±0.01	2.89±0.23	1.38±0.18^{c}	0.089±0.016^{c}
N	*M. chamomilla*	0.18±0.01	2.72±0.40	1.41±0.18^{c}	0.131±0.016^{b}
N	*V. agnus-castus*	0.20±0.01	3.37±0.09	1.20±0.12^{c}	0.033±0.014^{d}
N	Monensin	0.18±0.02	2.39±0.35	0.80±0.04^{c}	0.089±0.013^{c}
A	Control (0)	0.37±0.03	1.22±0.39	3.23±0.31^{a}	0.140±0.011^{b}
A	*U. dioica*	0.37±0.02	0.42±0.29	2.93±0.38^{ab}	0.193±0.026^{a}
A	*M. chamomilla*	0.38±0.02	0.00±0.00	2.69±0.28^{ab}	0.186±0.016^{a}
A	*V. agnus-castus*	0.38±0.02	0.20±0.20	2.35±0.20^{b}	0.164±0.016^{ab}
A	Monensin	0.38±0.02	0.00±0.00	3.12±0.22^{a}	0.161±0.019^{ab}
Main effects					
N		0.18±0.01^{b}	2.92±0.12^{a}	1.15 0.06^{b}	0.079±0.008^{b}
A		0.38±0.01^{a}	0.37±0.12^{b}	2.86±0.13^{a}	0.169±0.008^{a}
	Control (0)	0.26±0.03^{c}	2.22±0.27^{a}	2.10±0.27	0.097±0.012^{c}
	U. dioica	0.27±0.02^{bc}	1.65±0.30^{bc}	2.15±0.26	0.141±0.018^{ab}
	M. chamomilla	0.28±0.02^{a}	1.36±0.33^{bc}	2.05±0.21	0.158±0.012^{a}
	V. agnus-castus	0.29±0.02^{a}	1.79±0.32^{ab}	1.78±0.16	0.099±0.017^{c}
	Monensin	0.28±0.02^{ab}	1.19±0.29^{c}	1.96±0.25	0.125±0.013^{b}
P values					
RC		<0.001	<0.001	<0.001	<0.001
T		0.023	0.008	0.445	<0.001
RC × T		0.111	0.183	0.049	0.028

*The values for the main effects of the RC and RC × T are means of 7 d ± SEM, and the values for the main effects of T are means of 14 d ± SEM. ^{a,b,c,d} Means in the same column followed by different superscripts differ significantly (P<0.05). N: Normal rumen conditions, A: Acidosis condition, RC: Rumen conditions, T: Treatment, RC × T: Interaction between RC and T, DMD: Dry matter digestibility.

Discussion and Conclusion

In this study, we investigated the effects of plant extracts on *in vitro* ruminal fermentation parameters under two pH conditions; normal and acidosis. Similar to *in vivo* conditions, acidosis followed normal conditions. One of the aims of the study was to evaluate the potential of the plant extracts to prevent acidosis. Based on our results, neither the plant extracts nor monensin had a significant effect on ruminal pH under normal or acidosis conditions. To the best of our knowledge, there are no reports on the effect of *V. agnus-castus* extract on ruminal pH. Some studies reported that *M. chamomilla* had no effect on ruminal pH (16, 18), as found in the present study. There are a few studies on regulatory effects of *U. dioica* on ruminal pH values under normal rumen conditions. Humphries and Reynolds (21) reported a quadratic increase in *in vivo* ruminal pH values in lactating dairy cows fed a diet supplemented with 10% dried *U. dioica*. However, in their study, *U. dioica* was employed as a whole plant, rather than as an extract; therefore, it was a component of animal ration/substrate by 10%, with a high rate, rather than a feed additive. Active components responsible for antimicrobial action in a sample may vary, depending on how the plant material is used.

In the present study, *U. dioica*, *M. chamomilla*, and *V. agnus-castus* extracts at a dose of 500 mg/d (about 667 mg/L) stimulated the fermentative activity of rumen microorganisms and resulted in elevated production of total VFA and increased DMD, regardless of the fermentation conditions. The stimulatory effects of *U. dioica*, *M. chamomilla*, and *V. agnus-castus* extracts on total VFA production and ruminal fermentation at a dose of 500 mg/d (667 mg/L) suggest that these extracts have no toxic effects on ruminal microbes.

The effects of plant extracts used for modifying ruminal fermentation were generally considered positive, when propionate production increased, acetate and butyrate production decreased, and/or the A:P ratio decreased. *M. chamomilla* and *V. agnus-castus* extracts increased propionate production similar to that obtained using monensin, irrespective of the rumen conditions. On the other hand, under normal conditions, all three extracts increased acetate production, whereas monensin did not. Therefore, the A:P ratio decreased only in the monensin treatment. Monensin shows antimicrobial activity against Gram-positive bacteria, which mainly synthesize acetate and butyrate, rather than propionate-producing Gram-negative bacteria (30). In the present study, under normal rumen conditions, all three plant extracts increased acetate production, suggesting that they do not exhibit selective antimicrobial activity against Gram-positive bacteria. Thus, they appear to affect microbial metabolism by a mechanism different from that of monensin.

The *U. dioica*, *M. chamomilla*, and *V. agnus-castus* extracts used in the present study were rich in flavonoids, such as rutin, quercetin, apigenin, and luteolin, and phenolic acids, such as chlorogenic acid, caffeic acid, coumaric acid, and gallic acid (Table 1). Broudiscou et al. (4) reported that flavonoid-containing dry plant extracts *Lavandula officinalis* and *Solidago virgaure*, administered at a dose of 500 mg/d -as in the present study- increased the production of total VFA and strongly promoted fermentation. Therefore, they have the potential to modify ruminal fermentation. The authors ascribed these effects to the high flavonoid contents of these plant extracts. The effects of flavonoid-rich plant extracts on rumen microorganisms have been attributed to one or a combination of the following hypotheses: *(i)* the inhibitory effects of flavonoids, *(ii)* stimulatory effects of degradation products of flavonoids, and *(iii)* direct actions of other secondary metabolites (5). Interestingly, some studies have also provided support for the second hypothesis which is based on the flavonoids and phenolic acids were hydrolyzed by bacterial enzymes and converted to more bioactive forms which stimulated the enzymatic activity of certain groups of bacteria via the synthesis of aromatic amino acids (3, 24). Cellulolytic bacteria protect themselves against the toxic effects of phenolic compounds in this way and that they use hydrolyzation end-products as a carbon source (10). Greathead (19) classified the stimulatory effect of some herbs and spices on some bacterial species as a prebiotic-type effect and suggested that this effect may be used for manipulating ruminal metabolism (i.e., promoting fiber-digesting bacterial populations). Therefore, the phenolic compounds of plant extracts used in the present study may have generated prebiotic-like effects on some bacterial groups in the rumen, mainly cellulolytic bacteria, considering the increase in the production of acetate under normal rumen conditions.

On the other hand, in the present study, the effects of the treatments on some parameters showed differences in acidosis conditions compared to normal conditions. For example, in the *U. dioica* and *V. agnus-castus* treatment groups, acetate production did not change similar to those of monensin under acidosis conditions but increased under normal conditions. Likewise, the *V. agnus-castus* extract decreased the NH_3-N concentration under acidosis conditions but not under normal rumen conditions. Cardozo et al. (8) reported that the effects of plant extracts on ruminal fermentation might differ, depending on the ruminal pH, and that oregano, garlic, capsicum, yucca extracts, and cinnamaldehyde had more favorable effects on fermentation parameters at pH 5.5 than 7.0. The authors attributed these positive effects to the tendency of active molecules to become undissociated in low pH conditions.

Undissociated forms are more hydrophobic and therefore interact more readily with cell membranes of bacteria and exert antimicrobial effects (8). Active phenolic components of *U. dioica* and *V. agnus-castus* extracts may have inhibitory effects on some strains of Gram-positive bacteria with a similar mechanism, when the ruminal pH is low and have more favorable effects under acidosis than normal rumen conditions.

In the present study, *U. dioica* and *M. chamomilla* extracts exhibited antiprotozoal effects similar to those observed in the monensin treatment, irrespective of the rumen conditions. *U. dioica* and *M. chamomilla* extracts contain rutin, quercetin, and apigenin, in addition to chlorogenic acid, all of which have been reported to have antiprotozoal, antiplasmodial, and antitrypanosomal effects (6, 7, 23, 31). Therefore, flavonoids can interact with microorganisms in a negative, as well as in a positive way (4).

Lactate concentrations remained unchanged in the *U. dioica* extract treatment under normal rumen conditions in the present study and in the *V. agnus-castus* extract treatment under both conditions, similar to monensin treatment. Lactate is an intermediate in rumen metabolism and can be converted to other VFAs or long-chain fatty acids. Previous research reported that 60–95% of lactate produced after concentrate-rich feeding was converted to propionate by the acrylate pathway and that 20–30% was converted to butyrate by *Megasphaera elsdenii* (12). In the present study, lactate might be converted to propionate and butyrate in the *V. agnus-castus* extract group, considering that this extract was unique additive, which increased the production of propionate and butyrate but did not change lactate concentrations under both normal and acidosis conditions.

In conclusion, *U. dioica*, *M. chamomilla*, and *V. agnus-castus* extracts positively affected *in vitro* ruminal fermentation by stimulating the fermentative activity of rumen microorganisms under both normal and acidosis conditions. However, the mode of action of these plant extracts appears to differ from that of monensin, particularly under normal rumen conditions. Although none of the plant extracts prevented acidosis, *U. dioica* and *V. agnus-castus* extracts had more favorable effects on some fermentation parameters such as the NH_3-N concentration and acetate production under acidosis conditions. The effects of higher concentrations of *V. agnus-castus* on lactate production should be studied, although it did not exert prominent effects in the present study. Further *in vivo* studies are required to determine the value of these extracts as feed additives in enhancing the efficiency of ruminal fermentation and animal performance.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Economic and efficiency analysis of beekeeping activity in Turkey: Case of Çanakkale Province

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Abstract: This study was carried out to determine the economic structure and efficiencies of the beekeeping enterprises in Çanakkale Province. Beekeepers were divided into three groups/strata according to number of hives: 1-75 hives (first group), 76-150 hives (second group) and 151 hives and above (third group). Survey sample size was determined as 87 based on stratified random sampling method. The portion of fixed costs were 65.25% and variable costs were 34.75% in averages of production costs of all enterprises. The cost of honey production per kg changed between 2.04 US$ to 3.54 US$ among to enterprises. The average production cost of honey per kg was 2.92 US$. Average yield amount per hive was 16.24 kg and production cost per hive was 53.32 US$. Besides, average gross profit, net profit and relative profit were determined as 77.53 US$, 42.74 US$ and 1.80, respectively. According to the average of the enterprises, total technical efficiency (constant return to scale), pure technical efficiency (variable return to scale), scale efficiency, allocative efficiency and economic efficiency were found as 0.64, 0.89, 0.70, 0.74 and 0.66, respectively. It was determined that producer's age and agricultural activity apart from beekeeping affected the economic efficiency negatively and land size, income per hive and honeycomb changing frequency affected the economic efficiency positively. According to the results, it was concluded that honey production was a profitable activity in Çanakkale Province and relative profits and efficiencies of the enterprises in the third group were higher than the other groups.
Keywords: Beekeeping, cost, efficiency, honey, profitability.

Türkiye'de arıcılık faaliyetinin ekonomik ve etkinlik analizi: Çanakkale ili örneği

Özet: Bu çalışma Çanakkale ilinde faaliyet gösteren arıcılık işletmelerinin ekonomik yapısının ve etkinliklerinin belirlenmesi amacıyla yapılmıştır. İşletmeler kovan sayılarına göre üç gruba/tabakaya ayrılmıştır: 1-75 kovan (birinci grup), 76-150 kovan (ikinci grup) ve 151 kovan ve üzeri (üçüncü grup). Tabakalı tesadüfi örnekleme yöntemine göre örnek büyüklüğü 87 olarak belirlenmiştir. İşletmeler ortalamasına göre üretim masraflarının %34,75'i değişken masraflardan, %65,25'i sabit masraflardan oluşmaktadır. İşletmelerde 1 kg balın maliyeti 2,04 US$ ile 3,54 US$ arasında değişmektedir. Ortalama bal maliyeti 2,92 US$'dir. Ortalama kovan başına verim 16,24 kg, kovan başına üretim masrafı 53,32 US$'dır. Ortalama brüt kâr 77,53 US$, net kâr 42,74 US$, nispi kâr ise 1,80 olarak belirlenmiştir. İşletmeler ortalamasına göre toplam teknik etkinlik (ölçeğe sabit getiri) 0,64, saf teknik etkinlik (ölçeğe değişken getiri) 0,89, ölçek etkinliği 0,70, kaynak dağıtım etkinliği 0,74, ekonomik etkinlik 0,66 olarak bulunmuştur. İşletme sahibinin yaşının ve tarımda arıcılık dışı faaliyetle uğraşma durumunun ekonomik etkinliği negatif, arazi büyüklüğünün, kovan başına elde edilen gelirin ve petek değiştirme sıklığının pozitif yönde etkilediği belirlenmiştir. Bu sonuçlardan hareketle, Çanakkale ilinde gerçekleştirilen bal üretiminin kârlı bir üretim faaliyeti olduğu, ancak üçüncü gruptaki işletmelerin nispi kârlarının ve etkinliklerinin diğer gruplara göre daha yüksek olduğunu söylemek mümkündür.
Anahtar sözcükler: Arıcılık, bal, etkinlik, kârlılık, maliyet.

Introduction

Beekeeping is a producing activity of living substances such as queen and drone and products such as honey, beeswax, pollen, royal jelly, bee gum and bee venom by using herbal sources, bee and labor together (24). Beekeeping is dependent on nature due to the lifestyle of the honey bees and the collection of the raw materials of the products from nature (27). Beekeeping has a significant role in rural development in many countries in the world and Turkey (21).

In 2018, there were approximately 91 million beehives in the world, and the produced honey amount was 1.8 million tons (5). The average honey yield per hive in the world was 22 kg and this value was 50-51 kg in

China, 39 kg in Mexico, 26-27 kg in Argentina and 26 kg in the USA. Honey production per hive was 14.6 kg in Turkey and it was ranked at the twelfth in the world. Turkey takes third place in terms of total hive number and takes second place in terms of total production amount (14).

Turkey contains 20% of the bee races in the world (8). Turkey has 75% of world honeyed plant flora. Turkey has a great beekeeping potential in terms of rich flora, suitable ecology, colony existence and genetic variation in bee population (29). According to the data of 2017, total number of the hives, honey production, beeswax production and total number of the enterprises were 7 991 072, 114 471 tons, 4488 tons and 83 210, respectively (31).

As in Turkey, beekeeping activity, which can be done with little capital and expense by not being dependent on an area, has been a significant side income source in Çanakkale Province. Çanakkale is an appropriate nutrition area for the bees in terms of plant species and variety (18). Blossom honey is produced in the province predominantly besides the honeydew honey production. According to the data of 2018, total number of the hives was 79 635 and 1689 tons of honey was produced (14). Biga, Central, Çan and Yenice districts come into prominence in beekeeping activities. Organic beekeeping activities are conducted in Gökçeada and Ezine districts (6).

The aim of this study was to determine the economic structure of beekeeping activity in Çanakkale Province. Honey costs of the beekeeping enterprises were calculated, the incomes and the expenses were examined and the profitability of beekeeping activity was introduced. Besides, efficiency analysis (technical efficiency, allocative efficiency and economic efficiency) at enterprise level was done and some effective socio economic factors of economic efficiency were analyzed.

Material and Methods

The material of the study was the survey data which were collected from the beekeepers who were registered to Çanakkale Beekeepers Union. Besides, it was utilized from the local and foreign studies related to the research subject and the statistics.

The data concerning the number of the hives of the beekeepers were obtained from Çanakkale Beekeepers Union. The beekeepers who have the number of 30 and above hives are affiliated to beekeepers unions. It was determined from the data that there were 368 registered beekeepers to Çanakkale Beekeepers Union in 2018. As the variation coefficient was high, stratified sampling was done. In stratified random sampling method, the following formulas were used (13).

$$= \frac{N \sum[N_h(S_h)^2]}{N^2\, D^2 + \sum N_h(S_h)^2} \text{ and } n_i = \frac{N_h}{\sum N_h} * n$$

$D^2 = (d/Z)^2$, d= deviation from average , Z= degree of freedom, N_h= number of the enterprises in the strata, S_h= Standard deviation of the strata, S_h^2= Variance of the strata, n_i= sample number in the strata n= sample size

The number of the beekeepers were divided into three strata, including 1-75 hives (first group), 76-150 hives (second group) and 151 hives and above (third group). The number of the surveyed beekeepers was determined as 87 with 5% sampling error margin and in 95% confidence interval. The surveyed beekeepers were distributed to the strata proportionately (32). There were 27 surveyed beekeepers in the first group, 38 surveyed beekeepers in the second group and 22 surveyed beekeepers in the third group.

It was utilized from some descriptive analysis such as average, standard deviation, and percentages, statistics, parametric and non-parametric tests for the evaluation of the data. The convenience of the continuous data to normal distribution was determined by Kolmogorov-Smirnov test. Variance analysis was used for normally distributed continuous data, Kruskal Wallis test was used for non-normally distributed continuous data and Chi-square test was used for discrete data in order to determine the differences between the groups.

Man labor unit was used for the determination of the labor potential used in the production. Man labor unit is the labor that an adult male employee (15-49 ages) reveals by working average 10 hours in a day and 300 days in a year (7).

The amortization ratio for the calculation of device-machine amortization was taken as 10% (24). When calculating the device-machine and bee capital interest compensations, Ziraat Bank real interest rate (6%) was applied to half of the device-machine and bee capital value (20). General administration expenses was calculated by taking 3% of the total variable costs. The wage level paid to alien labor was taken in exchange for the family labor fee.

Feed (sugar) costs, drug costs, honeycomb costs, fuel-transport costs, jar-tin costs, accommodation costs, temporary labor costs, repair and maintenance costs and circulating capital interest were taken as variable costs. General administration expenses, family labor fee, bee capital interest, device-machine amortization, device-machine capital interest, subscription and insurance fee were taken as fixed costs.

Circulating capital interest was calculated by applying 6% (the agricultural credit interest rate applied by Ziraat Bank) to half of the variable costs. For the calculation of the production cost of one kg of honey, byproduct (beeswax, pollen, bee gum) income is subtracted from total production costs and this value is divided to total honey amount (3). Gross output value is calculated by the addition of the honey income and byproduct income. Gross profit is found by subtracting the

variable costs from gross output value. Net profit is found by subtracting the production costs from gross output value. Relative profit is found by dividing the gross output value to the production costs.

Data envelopment analysis was used for the efficiency analysis. In data envelopment analysis, the unit which obtains the maximum output with minimum input is determined and an efficient frontier is composed with these units. The efficiencies of the other decision-making units are determined by measuring the radial distances to this frontier.

The achievement of the enterprise on producing the maximum output by using the input combination properly is named as technical efficiency. Allocative efficiency is the achievement of selecting the input combination which will minimize the production cost by considering the input and output prices of the enterprise. Price information is not required in technical efficiency but allocative efficiency is calculated over this information. Economic efficiency is obtained by the combination of technical efficiency and allocative efficiency (30).

Technical efficiency that shows whether enterprises operate effectively or not is divided into two subgroups as pure technical efficiency and scale efficiency (11).

Pure technical efficiency indicates the efficient usage of the inputs according to variable return to scale assumption. If technical efficiency values for constant return to scale and variable return to scale are different for a specific production unit, this indicates that the production unit has scale inefficiency. Accordingly, scale efficiency could be explained in this way (33).

Technical efficiency (CRS) = Pure technical efficiency (VRS) x Scale efficiency

In the efficiency analysis, enterprises with efficiency coefficient between 0.95 and 1 are considered as effective, between 0.90 and 0.95 are considered as less effective and less than 0.90 are classified as ineffective enterprises (10). Since producers have more tendency to control their inputs than their outputs, efficiency measurements of Farrell (15) relating to inputs was used in this study. DEAP 2.1 statistical package program was used for the estimation of the efficiency measurements.

Total income was accepted as output and labor, variable costs, fixed costs and number of frame were accepted as inputs in the model. A model was designed with four inputs and one output. The relations between the efficiency scores and demographic, economic and social characteristics was put forward by Tobit model. Tobit model was developed by James Tobin, and also named as censored or discrete regression model (16). EViews 4 program was used for the estimation of Tobit model.

Results

Economic analysis: The average variable costs, fixed costs and total production costs per hive were found at 18.53 US$, 34.79 US$ and 53.32 US$, respectively. According to Kruskal Wallis test, feed, drug, honeycomb, jar-tin, temporary labor expenses, bee capital interest, device-machine amortization, device-machine capital interest, union subscription, total fixed costs and total production costs changed according to the enterprise size groups (Table 1).

Table 1. Variable, fixed and total costs of beekeeping in the enterprises (US$/hive).

Cost items	Enterprise groups			Average	P
	First group	Second group	Third group		
Variable costs	17.52	18.55	19.74	18.53	0.916
Feed (sugar) costs	2.28	4.66	5.49	4.13	0.038**
Drug costs	2.89	1.76	2.38	2.27	0.010*
Honeycomb costs	3.44	2.06	2.76	2.67	0.035**
Fuel-transport costs	5.46	6.87	5.24	6.02	0.116
Jar-tin costs	1.66	1.15	1.63	1.43	0.047**
Accommodation costs	0.47	0.41	0.36	0.42	0.571
Temporary labor costs	0.07	0.70	0.83	0.54	0.082*
Repair and maintenance costs	0.73	0.40	0.47	0.52	0.868
Circulating capital interest	0.51	0.54	0.57	0.54	0.912
Fixed costs	46.10	31.81	26.07	34.79	0.004***
General administration expenses	0.53	0.56	0.59	0.56	0.910
Family labor fee	33.90	25.68	22.09	27.32	0.274
Bee capital interest	6.98	2.47	1.35	3.59	0.000***
Device-machine amortization	2.64	1.74	1.17	1.87	0.001***
Device-machine capital interest	1.15	0.72	0.52	0.80	0.002***
Subscription	0.42	0.18	0.11	0.24	0.000***
Insurance fee	0.48	0.47	0.24	0.41	0.930
Total production costs	63.62	50.36	45.81	53.32	0.057*

*: Significant at 10% significance level; **: Significant at 5% significance level; ***: Significant at 1% significance level; 1 US$= 4.813 TL in 2018 (average).

According to the average of the enterprises, it was determined that 34.75% of the total production costs were variable costs whereas 65.25% was fixed costs. The ratio of variable and fixed costs in total production costs changed according to the groups. Fuel-transport costs had the highest ratio (11.29%) in the variable costs and this was due to the migratory beekeeping. The ratios of feed (sugar) costs and honeycomb costs were 7.74% and 5.00%, respectively. Family labor fee had a significant ratio with 51.24% in total production costs (Table 2).

According to the average of the enterprises, honey production amount per hive was found as 16.24 kg and this value increased according to the enterprise size groups. The income obtained from honey production was found as 90.23 US$ according to the average of the enterprises (Table 3). In the enterprises, besides the honey production, the production of the byproducts such as royal jelly, beeswax, pollen and bee gum, was carried out. However, as the amount of these products was low, it was determined that the producers preferred to evaluate the bee gum in their enterprises and sell royal jelly, beeswax and pollen to various markets. From this viewpoint, it is expected that the increase of the amounts of byproducts affects the operating profits of the enterprises positively.

Table 2. Ratio of the costs items in total production costs (%).

Cost items	Enterprise groups			Average
	First group	Second group	Third group	
Variable costs	27.54	36.84	43.09	34.75
Feed (sugar) costs	3.58	9.25	11.99	7.74
Drug costs	4.54	3.50	5.20	4.26
Honeycomb costs	5.41	4.10	6.03	5.00
Fuel-transport costs	8.59	13.64	11.45	11.29
Jar-tin costs	2.61	2.28	3.55	2.68
Accommodation costs	0.74	0.81	0.78	0.78
Temporary labor costs	0.10	1.39	1.81	1.01
Repair and maintenance costs	1.15	0.80	1.03	0.98
Circulating capital interest	0.80	1.07	1.25	1.01
Fixed costs	72.46	63.16	56.91	65.25
General administration expenses	0.83	1.11	1.29	1.04
Family labor fee	53.29	50.99	48.22	51.24
Bee capital interest	10.98	4.91	2.96	6.73
Device-machine amortization	4.15	3.45	2.54	3.51
Device-machine capital interest	1.80	1.42	1.13	1.50
Subscription	0.66	0.36	0.24	0.45
Insurance fee	0.75	0.92	0.52	0.77
Total production costs	100.00	100.00	100.00	100.00

Table 3. Economic analysis results.

Profitability indicators	Enterprise groups			Average	P
	First group	Second group	Third group		
Honey production amount (kg/hive)	15.78	14.98	18.97	16.24	0.120
Honey production value	88.26	82.77	105.54	90.23	0.132
Bee products production value (beeswax, pollen, bee gum, royal jelly)	7.81	3.73	7.02	5.83	0.443
Gross output value	96.07	86.50	112.56	96.06	0.074*
Variable costs (US$/hive)	17.52	18.55	19.74	18.53	0.916
Fixed costs (US$/hive)	46.10	31.81	26.07	34.79	0.004***
Production costs (US$/hive)	63.62	50.36	45.81	53.32	0.057*
Production cost of 1 kg of honey	3.54	3.11	2.04	2.92	0.104
Gross profit	78.55	67.95	92.82	77.53	0.068*
Net profit	32.45	36.14	66.75	42.74	0.027**
Relative profit	1.51	1.72	2.46	1.80	0.013**

*: Significant at 10% significance level; **: Significant at 5% significance level; ***: Significant at 1% significance level; 1 US$= 4.813 TL in 2018 (average).

The average production cost of one kg of honey was calculated as 2.92 US$. In terms of the enterprise size groups, the production cost of one kg of honey was found as 3.54 US$ in the first group, 3.11 US$ in the second group and 2.04 US$ in the third group. According to these results, it was determined that the honey cost had the highest value in the first group.

Gross profit is accepted as a significant success criterion on the determination of the competitive power of the production activities (19). According to the average of the enterprises, gross output value and gross profit were found as 96.06 US$/hive and 77.53 US$/hive. The ratio of the gross profit in gross output value was determined as 80.71%. The net profit, indicating the investment and administration income, was found as 42.74 US$/hive and the ratio of the net profit in gross output value was found as 47.37%. When the net profit values were examined in terms of enterprise size groups, it was concluded that the net profit value increased due to the increase of the hive number. It was determined that the enterprises in the third group obtained more net profit according to the enterprises in the first and second groups.

It is accepted that the relative profit is a preferable criterion for the producers on the investments of a production activity. The average relative profit was found as 1.80 in the enterprises. This value indicated that 1.80 US$ profit was obtained for 1 US$ of expense for honey production in the enterprises. In terms of enterprise size groups, the relative profit value was determined as 1.51 in the first group, 1.72 in the second group and 2.46 in the third group. According to these results, it can be said that the enterprises in the third group were more advantageous than the other enterprises.

According to Kruskal Wallis test, it was determined that there were statistically differences ($P<0.05$) between the groups in terms of gross output value, total fixed costs, total production costs, gross profit, net profit and relative profit (Table 3).

Efficiency analysis: According to the average of the enterprises, total technical efficiency (constant return to scale) was found as 0.64 and pure technical efficiency (variable return to scale) was found as 0.89. This value indicated that the inefficient enterprises could reduce the inputs in the ratio of 11% by not decreasing the outputs. Pure technical efficiency values were found as 0.84, 0.89 and 0.94, respectively by the groups (Table 4). Accordingly, it can be said that the enterprises in the third group were more efficient than the enterprises in the first and second groups, technically.

Technical inefficiency is generally based on two main factors; operating with inappropriate input combination and operating in inappropriate scale. According to the data envelopment results, average scale efficiency was found as 0.70. This indicated that technical inefficiency was generally based on operating in inappropriate scale.

The enterprises were classified according to the technical efficiency. Accordingly, it was determined that 48.15% of the enterprises in the first group, 50% of the enterprises in the second group and 63.64% of the enterprises in the third group were technically efficient (Table 5). Chi-square test results indicated that the technical efficiency changed according to the groups ($P=0.017$).

Table 4. Descriptive statistics of technical efficiency scores.

Efficiency level	First group			Second group			Third group			Average		
	CRS	VRS	SE	CRS	VRS	SE	CRS	VRS	SE	CRS	VRS	SE
Minimum	0.17	0.50	0.18	0.20	0.61	0.26	0.24	0.70	0.26	0.17	0.50	0.18
Maximum	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Average	0.53	0.84	0.61	0.64	0.89	0.70	0.78	0.94	0.83	0.64	0.89	0.70
Standard deviation	0.30	0.18	0.27	0.26	0.12	0.24	0.23	0.10	0.22	0.28	0.14	0.26

CRS: Total technical efficiency (constant return to scale); VRS: Pure technical efficiency (variable return to scale); SE: Scale efficiency.

Table 5. Classification of the enterprises according to the technical efficiency scores.

Efficiency status	First group		Second group		Third group		Total	
	Number	%	Number	%	Number	%	Number	%
Efficient ($0.95\leq TE\leq 1$)	13	48.15	19	50.00	14	63.64	46	52.87
Less efficient ($0.90\leq TE\leq 0.949$)	2	7.41	0	0.00	4	18.18	6	6.90
Inefficient ($TE\leq 0.899$)	12	44.44	19	50.00	4	18.18	35	40.23
Total	27	100.00	38	100.00	22	100.00	87	100.00
P=0.017								

According to the average of the enterprises, 77.01% of the enterprises had increasing return to scale, 18.39% of the enterprises had constant return to scale and 4.60% of the enterprises had decreasing return to scale (Table 6). It was concluded that the ratio of constant return to scale index in the third group was higher than the other groups. Chi-square test results indicated that returns to scale did not change according to the groups.

Allocative efficiency values changed between 0.31 and 1 by the groups and it was found as 0.74 on the average (Table 7). This value indicated that a great majority of the beekeepers made production with improper input combination in current technology level when the current input prices were taken into consideration. The enterprises made expenses in the ratio of 26% more than the input combination with minimum costs. Allocative efficiency coefficients differed by enterprise size groups (F=4.166, P=0.019).

The enterprises were classified according to the allocative efficiency. According to the average of the enterprises, it was determined that 20.69% of the producers allocated the sources efficient and this value was found as14.81% in the first group, 13.16% in the second group and 40.91% in the third group (Table 8). Chi-square test results indicated that the allocative efficiency changed according to the groups (P=0.018).

Table 6. Returns to scale by enterprise land sizes.

Return to scale	First group		Second group		Third group		Total	
	Number	%	Number	%	Number	%	Number	%
Increasing returns to scale	21	77.78	31	81.58	15	68.18	67	77.01
Constant returns to scale	5	18.52	5	13.16	6	27.27	16	18.39
Decreasing returns to scale	1	3.70	2	5.26	1	4.55	4	4.60
Total	27	100.00	38	100.00	22	100.00	87	100.00
P=0.728								

Table 7. Descriptive statistics of allocative efficiency scores.

Groups	Average	Standard deviation	Minimum	Maximum
First group	0.66[a]	0.21	0.35	1.00
Second group	0.76[b]	0.16	0.42	1.00
Third group	0.81[b]	0.20	0.31	1.00
Average	0.74	0.20	0.31	1.00

[a, b]: Values within a column with different superscripts differ significantly at P<0.05.

Table 8. Classification of the enterprises according to allocative efficiency scores.

Efficiency status	First group		Second group		Third group		Total	
	Number	%	Number	%	Number	%	Number	%
Efficient ($0.95 \leq TE \leq 1$)	4	14.81	5	13.16	9	40.91	18	20.69
Less efficient ($0.90 \leq TE \leq 0.949$)	2	7.41	1	2.63	3	13.64	6	6.90
Inefficient ($TE \leq 0.899$)	21	77.78	32	84.21	10	45.45	63	72.41
Total	27	100.00	38	100.00	22	100.00	87	100.00
P=0.018								

According to the average of the enterprises, it was determined that the economic efficiency changed between 0.21 and 1 and it was found as 0.66 on average. This value meant that the inefficient enterprises should reduce the operation expenses in the ratio of 34% in order to reach the level of the efficient enterprises. Economic efficiency values were found as 0.56, 0.68 and 0.77, respectively by the groups (Table 9). Accordingly, it can be said that the enterprises in the third group operated economically more efficient than the enterprises in the first and second groups. Economic efficiency coefficients differed by enterprise size groups (F=5.585, P=0.005).

The enterprises were classified according to the economic efficiency. According to the average of the enterprises, it was determined that 14.81% of the enterprises in the first group, 13.16% of the enterprises in the second group and 27.27% of the enterprises in the third group were economically efficient and this value was found as 17.27% according to the average of the enterprises (Table 10). Chi-square test results indicated

that the economic efficiency changed according to the groups (P=0.008).

Effect of some factors on economic efficiency: The effect of some factors on economic efficiency was determined. Average, standard deviation, minimum and maximum values of the variables used in Tobit model are given in Table 11.

The average age, education period, family size, beekeeping experiences of the producers were found as 54.71 years, 9.28 years 3.02 persons and 19.37 years, respectively. The average land size was 13.94 da. The average income per hive was 96.96 US$ whereas the nonagricultural income per hive was 3535.06 US$. Performing an agricultural activity apart from keeping was very low whereas migratory keeping was quite prevalent. Honeycomb changing frequency was found as 2.69 years.

The education periods, family sizes, nonagricultural incomes of the beekeepers and migratory keeping were determined to have negative effects and experience of the beekeepers was determined to have positive effects on economic efficiency. These variables were not statistically significant (P>0.10).

Producer's age affected the economic efficiency negatively (P=0.0490). As the ages of the producers increased, the economic efficiency decreased. This can be explained that as the education levels of the young producers increased, they were more interested in the innovations and by this way, they could obtain more yield and accordingly more income.

Table 9. Descriptive statistics of economic efficiency scores.

Enterprise size groups	Average	Standard deviation	Minimum	Maximum
First group	0.56[a]	0.25	0.21	1.00
Second group	0.68[b]	0.19	0.33	1.00
Third group	0.77[b]	0.22	0.31	1.00
Average	0.66	0.23	0.21	1.00

[a, b]: Values within a column with different superscripts differ significantly at P<0.05.

Table 10. Classification of the enterprises according to economic efficiency scores.

Efficiency status	First group		Second group		Third group		Total	
	Number	%	Number	%	Number	%	Number	%
Efficient (0.95≤TE≤1)	4	14.81	5	13.16	6	27.27	15	17.24
Less efficient (0.90≤TE≤0.949)	2	7.41	0	0.00	5	22.73	7	8.05
Inefficient (TE≤0.899)	21	77.78	33	86.84	11	50.00	65	74.71
Total	27	100.00	38	100.00	22	100.00	87	100.00
P=0.008								

Table 11. Descriptive statistics of the variables used in Tobit model.

Variables	Average*	Standard deviation	Minimum	Maximum
Demographic characteristics				
Producer's age (year)	54.71	1.64	30.00	80.00
Education period (year)	9.28	4.04	5.00	15.00
Family size (person)	3.02	0.99	2.00	5.00
Beekeeping experience (year)	19.37	9.91	3.00	48.00
General characteristics of the enterprise				
Land size (da)	13.94	27.10	0.00	175.00
Income per hive (US$)	96.06	48.19	25.15	237.90
Dealing with an agricultural activity apart from beekeeping	0.21	0.41	0.00	1.00
Nonagricultural income (US$)	3535.06	1977.67	0.00	12466.24
Beekeeping activities				
Migratory beekeeping	0.87	0.33	0.00	1.00
Honeycomb changing frequency (year)	2.69	0.78	1.00	5.00

*: Arithmetic mean was used in distance and ratio data as measure of central tendency.

Table 12. Tobit analysis results: Factors affecting the economic efficiency.

Variables	Coefficient	Standard error	P
Producer's age	-0.005937**	0.003016	0.0490
Education period	-0.009026	0.006517	0.1660
Family size	-0.008608	0.026548	0.7457
Beekeeping experience	0.002494	0.002773	0.3684
Land size	0.001672*	0.000871	0.0549
Income per hive	0.000369***	0.000101	0.0002
Agricultural activity apart from beekeeping [1]	-0.092220*	0.055445	0.0963
Nonagricultural income	-0.00000001	0.000000027	0.4730
Migratory beekeeping [2]	-0.045193	0.068585	0.5099
Honeycomb changing frequency	0.055705**	0.027872	0.0456
Likelihood ratio	13.19***		

*: Significant at 10% significance level; **: Significant at 5% significance level; ***: Significant at 1% significance level; [1]: Dealing with an agricultural activity apart from beekeeping (1: yes; 0: no); [2]: Migratory beekeeping (1: yes; 0: no).

Land size affected the economic efficiency positively (P=0.0549). It was concluded that the beekeepers hired the agricultural areas rather than farming. This can be explained that by hiring the agricultural areas, they could obtain additional income and they could transfer this income to beekeeping activity.

Income per hive affected the economic efficiency positively (P=0.0002). As the income per hive increased, economic efficiency increased. The economic analysis results were in this way.

Dealing with and agricultural activity apart from beeping affected the economic efficiency negatively (P=0.0963). As the producers performed an agricultural activity apart from beekeeping, they could not spare enough time and interest to beekeeping and consequently this case had a negative effect on economic efficiency.

Honeycomb changing frequency affected the economic efficiency positively (P=0.0456). As the honeycomb changing frequency increased, the quality and the amount of the yield increased and accordingly, this case caused the increase of the income and consequently, the economic efficiency (Table 12).

Discussion and Conclusion

In this study, expenses, costs and profitability of beekeepers in honey production period in 2018 in Çanakkale Province were determined. Average honey yield per hive was found as 16.24 kg and this value is over the average yield value (14.6 kg) of Turkey.

Honey yield per hive was found as 12.32 kg in Mediterranean Region (26) and 19.27 kg in İzmir Province (23). As the production amounts of other bee products (royal jelly, beeswax, pollen, and bee gum) are little, it can be said that increasing the production of these products is necessary for the profitability of the enterprise.

The ratio of the fixed costs in the first group was higher than second and third groups. If the ratio of the variable costs is high, this means that the enterprises operate more intensive (4). From this point of view, it can be said that the enterprises in the third group operated more intensive. The ratio of family labor fee is higher than temporary labor expenses and this can be commented that the producers mostly maintain the beekeeping activity based on the family labor.

It was determined that fuel-transport and feed (sugar) costs had the highest share in the variable costs. Similar results were obtained from the study conducted in Adana (24). Low interest loan can be provided for the inputs used in the transport for arriving to the places for honey production and used in the feeding which is done in order to strengthen the colony in certain times of the production period, especially in spring, in migratory beekeeping. It can be applied low prices in sugar purchasing to the beekeepers who are the members of the unions or the cooperatives. Besides, bee accommodation areas and flora intensity should be determined contemporarily and rearranged for increasing the yield. The producers should be encouraged for insurance in order to reduce the effects of negative climate conditions and increase of insurance premium ratio should be provided by the government. The subsidy amount per hive should be increased and support of the inputs such as beeswax, bee cake should be provided. The cooperation of the related organizations in the region should be provided in order to give practical technical training to the producers on the subject of beekeeping and health protection.

As the scale of the enterprise increased, the cost of one kg of honey decreased in this study. Similarly, in previous studies conducted in Adana (24), Mediterranean Region (26) and Gökçeada (25) and Aegean Region (12), it was stated that the cost of one kg of honey decreased as

the number of hive increased. It was obviously seen that the cost of 1 kg honey decreased according to the increase of the number of the hives. It was concluded that the hive number had a significant effect on honey production cost. The necessary supports should be provided to the producers in order to increase the number of the hives.

Technical efficiency, allocative efficiency and economic efficiency were determined as 0.89, 0.74 and 0.66, respectively. The average pure technical efficiency values were found as 0.55 in Nigeria (2), 0.85 in Adana (24), 0.66 in Greece (22), 0.89 in Gana (1), 0.84 in Turkey (9) and 0.57 in Niğde (17). The technical efficiency coefficient of this study was the same as the result of the study conducted in Gana (1). In the study conducted in Turkey (9), the average allocative efficiency and economic efficiency of the beekeeping enterprises in Turkey were found as 0.75 and 0.62.

The number of the completely efficient enterprises; in other words, the technical efficiency values were 1, was determined as 40. In the study carried out in the Rocky Mountain region in the USA, it was determined that 25% of the enterprises were technically efficient (28). The number of the enterprises, which were fully economically efficient, in other words the economic efficiency values of which were 1, was determined as 13.

Technical efficiency scores were found to be higher than the economic efficiency scores. This result indicated that the producers required information on the subject of selecting suitable input combination on data price level rather than the technical information. It was determined that the effect of honeycomb changing frequency on economic efficiency was positive. The awareness of the producers should be raised on the necessity of honeycomb changing and hive control for honey production performance, honeycomb changing and hive control should be provided in appropriate frequency.

Economic and efficiency analysis results indicated that the relative profits and efficiencies of the enterprises in the third group were higher than the other groups.

The most important problems which the beekeepers encountered in beekeeping activity were marketing of the crops, struggling with diseases, accommodation areas, transportation and organization. The beekeeping unions should be more efficient for the solution of the problems in beekeeping sector. The beekeeping unions should be more active for the marketing of honey and other honey products. The determination of the locations of the hives will be useful for the solution of the accommodation area problem. The accommodation areas should be determined according to the floristic variety for the solution of the problems which the migratory beekeepers. It was concluded that illness and wintering losses affected the honey production. The employment of expert veterinarians should be provided in public establishments and unions for the recognition and treatment of the illnesses.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Comparison of the effects of zinc-silver cream and *Nigella sativa* oil on wound healing and oxidative stress in the wound model in rats

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Abstract: The present study was undertaken to evaluate of *Nigella sativa* oil (NSO) and zinc-silver cream in a rat model of wound healing by means of clinical appearance, histopathology, oxidative stress parameters, and biomechanical tests. The rats were divided into three groups, each having twenty animals. Group 1 served as a control having wound. Other two groups received either NS oil or zinc-silver cream in addition to the wound. The study was initiated after the animals rested for 2 weeks for acclimation. Blood samples were collected from 10 randomly selected animals from each group at days 0, 3, 7 and 14 for oxidative stress measurements. Differences among groups for blood lipid peroxidation and antioxidant values were determined with respect to biochemical oxidative stress results ($P<0.01$). Wound sites of 10 animals from each group were allocated for histopathological examinations, and those of the remaining 10 animals from each group were used in biomechanical testing. Histopathological examination revealed that epithelization occurred in all groups. Inflammatory response with an intensive vascularization was present in the samples of the silver group ($P<0.01$). Consequently, zinc-silver group has differed positively in terms of biochemical and biomechanical and histological aspects compared to other groups for wound healing and oxidative stress.

Keywords: *Nigella sativa* oil, oxidative stress, rat, wound healing, zinc-silver.

Çinko-gümüs krem ve çörekotu yağının rat yara modelindeki yara iyileşmesi ve oksidatif stres üzerine etkilerinin karşılaştırılması

Özet: Bu çalışma, rat yara modelinde çörekotu yağı ve çinko gümüş kreminin klinik görünüm, histopatoloji, oksidatif stres parametreleri ve biyomekanik testlerle değerlendirilerek yara iyileşmesi üzerine etkilerinin karşılaştırılması amacıyla yapıldı. Çalışmada kullanılan ratlar, her bir grupta 20 hayvan bulunan üç gruba ayrıldı. Grup 1, kontrol grubu olarak belirlendi. Diğer iki gruba sırasıyla yara oluşturulduktan sonra çörekotu yağı ve çinko-gümüş krem uygulandı. Çalışma, hayvanların adaptasyonu için 2 hafta dinlendirilmesinden sonra başlatıldı. Oksidatif stres ölçümleri için 0, 3, 7 ve 14. günlerde her gruptan rastgele seçilen 10 hayvandan kan örnekleri alındı. Kan lipid peroksidasyonu ve antioksidan değerleri için gruplar arası farklılıklar biyokimyasal oksidatif stres sonuçlarına göre belirlendi ($P<0.01$). Her gruptan 10 hayvanın sırt kısmındaki yara bölgeleri histopatolojik inceleme için tahsis edildi. Kalan 10'ar hayvanın sırt derileri ise biyomekanik testler için kullanıldı. Histopatolojik inceleme tüm gruplarda epitelizasyonun olduğunu ortaya koydu. Çinko-gümüş grubunun örneklerinde yoğun damarlanma ile inflamatuar yanıt tespit edildi ($P<0.01$). Sonuç olarak, çinko-gümüş grubu, yara iyileşmesi ve oksidatif stres için diğer gruplara kıyasla, biyokimyasal, biyomekanik ve histolojik yönleri bakımından pozitif olarak farklılık gösterdi.

Anahtar sözcükler: Çinko-gümüş, çörekotu yağı, oksidatif stress, rat, yara iyileşmesi.

Introduction

Wound healing is a multifactorial process which results in contraction and closure of the wound and restoration of a barrier. Repair of injured tissues occurs in the order in which events occur, including inflammation, proliferation, and migration of various cell types (27). It is a permission that reactive oxygen species (ROS) which are injurious to the wound healing process due to the detrimental effects on cells and tissues (3). Oxidative stress and free radicals have been implicated in impaired wound healing. Topical applications of products with free-radical-purifier properties in animals have shown that the improvement was significantly on wound healing and protecting tissues from oxidative damage (28). Defense

systems that function in the body are called antioxidants. Their task is to prevent the formation of ROS to prevent the damages of these substances and provide detoxification (16, 24).

The uses of natural products, as well as wound dressings with or without antiseptics, are continuously increasing as a consequence of infections related to resistant bacteria in the wound sites (15). There have been numerous studies conducted on wound healing in the recent decade (5, 12, 13). Materials containing silver ions at different concentrations release them into wound sites at various rates. These silver ions bind to the bacterial cell walls and enzymes. Then, they disrupt the bacterial cell wall structure and consequently prevent bacterial colonization at the wound site. Recently, silver has emerged as an alternative treatment option for combating infection in tissue-injured wounds as well as a treatment for chronic ulcers. Eventually, various silver-based preparations have been introduced to the market in the last few years (5, 13).

Experimental studies have shown that unsaturated fatty acids and essential oils, which constitute the most important chemical composition of *Nigella sativa* oil (NSO), have a wide range of pharmacological effects similar to antipyretic, analgesic, anti-inflammatory, and antimicrobial drugs. It is known that NSO has been used for various medical treatment purposes due to beneficial effects (14, 30). There are several studies reporting that NSO has antioxidant properties (10, 31). In addition to the antimicrobial and anti-helminthic effects (1, 4), it has been shown through clinical and experimental studies that NSO also has anti-inflammatory (19), anti-tumoral (29), antidiabetic (20), and antiulcerogenic effects (2).

This study aims to investigate the effects of Zinc-silvercream containing nanosilver and NSO on wound healing in a rat wound model through clinical, biochemical, and histological studies.

Material and Methods

The study was conducted following the approval of the Kırıkkale University Local Ethics Committee for Animal Experiments (13/06).

***Animals*:** Sixty healthy adult male albino Wistar rats weighing between 250-300 g were used in the study. The rats were fed a standard rat diet during the study period. Feed and water were given as *ad libitum*. Rats were divided into 3 groups: 2 treatment groups (NSO group n = 20 and zinc-silver group n = 20) and 1 control (control group n = 20). Each group contained 20 animals. Animals were allowed to acclimate for 10 days before treatment.

At the beginning of the study, blood samples were collected from 10 randomly selected animals for control purposes and used in oxidative stress measurements. These animals were rested for 10 days before any surgical applications. The wound was created in all animals by a surgical application. Prior to surgery, animals were treated with 4 mg/kg xylazine hydrochloride, 45 mg/kg ketamine hydrochloride for anesthesia. A 4 x 4 cm area was shaved on the back of the subjects and a full layer (dermis + epidermis) defect (wound) was created with a 20 mm diameter sterile punch biopsy instrument after the necessary antisepsis was achieved. For post-operative analgesia, subjects were administered flunixin meglumine subcutaneously at a dose of 2.5 mg/kg. Starting at the day of surgery, a cold cream containing NSO was applied to the NSO group, and a Zinc-silver cream was applied to the nano-silver group once a day for 14 days. The rats in the control group received physiological saline in a similar manner. The wound site was not covered with any kind of dressing. During the study, all animals were caged separately.

On the 14th day of the study, the animals were sacrificed under deep anesthesia, and then 10 animals from each group were used for histological examination, and the remaining 10 animals were used for biomechanical measurements. In addition, wound healing site was evaluated clinically and then photographed.

Biochemical analyses: Blood samples were taken from the heart and collected into both anticoagulated and non-anticoagulated tubes from 10 randomly selected animals from each group on days 0, 3, 7, and 14 of the study for biochemical analyses. The lithium heparin-anticoagulant blood samples was centrifuged at 3000 rpm for 10 min at +4 °C to obtain plasma. Blood taken to non-anticoagulated tubes were centrifuged for 10 min at 3000 rpm, and then the serum were separated. Plasma and serum samples were stored at -80 ^{0}C until analysis. Among oxidative stress markers, Malondialdehyde (MDA) levels in plasma samples were determined by a plate reader as reported by Buege and Aust (8). Furthermore, enzyme activities of catalase (CAT) and superoxide dismutase (SOD) were determined by using commercial ELISA test kits by obtaining a double reading. The amount of nitric oxide in serum samples was determined by the "Vanadium-3-chloride-Gries Reaction" method (21).

Histopathological examinations: The skin samples were collected at the wound sites in 1 cm^2 size and kept in 10% formaldehyde. Fixed tissues were passed through a graded alcohol series and blocked in paraffin through a routine histology procedure. Serial sections at a thickness of 5 μm were taken from paraffin blocks at an interval of 300 μm. Sections were stained with Crossman's modified triple staining technique (11) and examined under a light microscope. Histologic sections were assessed semi-quantitatively for changes in the epithelium, inflammatory reaction, and vascularization by using a modified wound scoring from 0 to 4 (Table 1) (17).

Table 1. The modified scoring system for histological evaluation of wound healing.

Degree	Epithelization type	Inflammatory reaction	Vascularization
4	Complete epithelization	Very intense inflammatory cell	Extensive vascularization
3	Basal membrane + middle-level epithelium	Medium intense inflammatory cell	Medium intensive vascularization
2	Basal membrane + partial epithelium	Less intense inflammatory cell	Less intensive vascularization
1	Basal membrane + 1-2 epithelium	1-2 inflammatory cell	Very little vascularization
0	No epithelization	No inflammation cell	No vascularization

*Modified from the scoring system used by Gibson-Corley et al. (17).

Biomechanical tests: For biomechanical tests, skin samples at a size of 2 x 2 cm, which includes the wound site, were excised out and placed in physiological saline. Measurements were made within 2 hours on fresh tissues. The skin samples, of which the panniculus layer was already removed with prerecorded size, were placed in the machine for tension and tear (Figure 1). Tissue specimens for mechanical testing were measured mechanically with the aid of a tissue stretching and tear-off device.

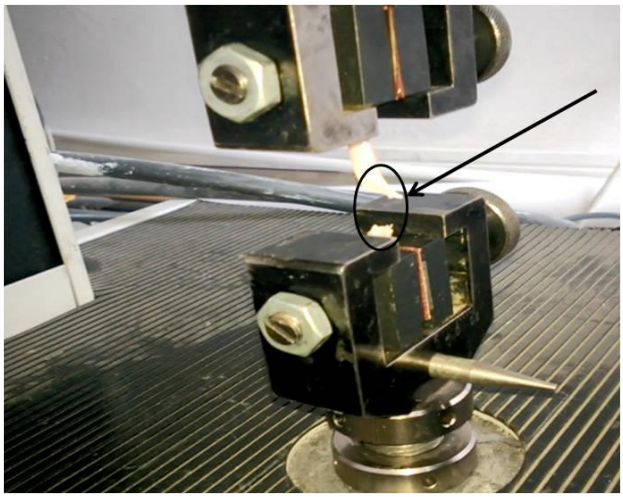

Figure 1. Tissue tension and tear-off test on the 14th day after artificial wounds in rat skin.

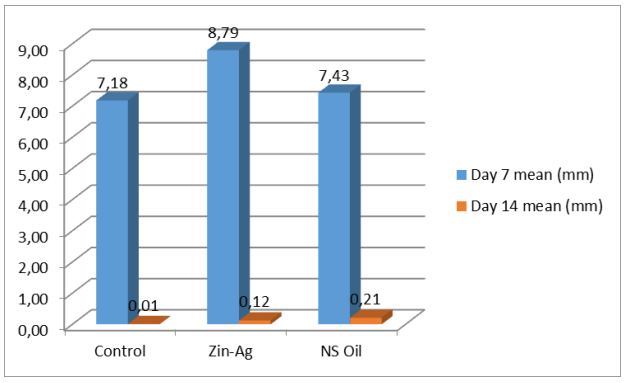

Figure 2. Wound contraction rates of wounded rat skin (mean, mm^2).

Statistical analysis: The statistical software SPSS 15.0 was used to evaluate the data. The significant difference among groups for biochemical analyses was determined by Kruskal-Wallis test and Mann-Whitney U test after a normality test. Statistical analysis of histopathological findings did not reveal a normal distribution in a normality test. For histopathological analysis, the Kruskal-Wallis test, which is a nonparametric test, was used to determine the differences among groups, and the Mann-Whitney U test was performed to evaluate significance levels. The Spearman's test was used to examine the correlation for epithelialization, inflammation, and vascularization. In order to examine the results of the tissue tensile test, One-way ANOVA and the Tukey-Kramer multiple comparison tests were performed to compare and reveal differences for wound durability. A P value of < 0.01 was considered significant for all analyses.

Results

During the study, there were no unexpected conditions that could affect the course or results of the study. And death was not seen. The wound closure period was the fastest in the control group, and secondly in the silver group, and the lowest in the NSO group. The wound closure rate in mm with respect to time is graphed in Figure 2. In the control group, the wound line was completely closed in all animals on the 14th day. In the silver group, on the 14th day, the closure rates were close to the control group, but the wound closure was incomplete, and there were wound incrustation in some animals. In the NSO group, the wound contraction rate was the least, and the wound incrustation was observed in more subjects than the latter groups. However, there were no statistically significant differences among groups for the wound closure rate ($P>0.05$).

Among the oxidative stress parameters, blood lipid peroxidation and antioxidants including MDA, NO, and CAT were measured with the presence of statistically significant differences among groups ($P<0.01$). Compared either to initial blood samples or to the other groups, animals in the control group had the highest serum MDA level while the highest NO level was observed in animals

of the silver group. Some increases were detected in all groups compared to pre-study SOD value. However, the highest increase was observed in the control group. But, these increases were not statistically significant (Table 2).

Histopathological examinations were evaluated in terms of epithelialization, inflammation, and vascularization at the wound site. The best epithelialization rate was observed in the NSO group, secondly in the control group, and the least in the silver group (Figure 3). The severity of inflammation was the highest in the silver group and the lowest in the NSO group. The degree of vascularization in the silver group was higher compared to the other groups, control, and the NSO groups, both of which had a similar degree of vascularization. There was a negative correlation between epithelialization and severity of inflammation in all groups (P<0.01). Unlike the other groups, a positive correlation was found between inflammation and vascularization in the NSO group (Table 3).

Table 2. Oxidative stress parameters measured at various periods following forming artificial wounds in rats.

Day	Group	MDA (nmol/L)	NO (µmol/L)	CAT (nmol/min/ml)	SOD (U/ml)
10 days prior to study		0.86±0.62	25.57±3.81	135.19±59.71	5.57±0.06
Day 0	Control	0.92±0.30	35.80±5.96*	125.85±28.33	5.63±0.04
	Zinc-silver	1.03±0.76	34.66±7.35	120.40±81.66	5.57±0.15
	NSO	1.51±1.57	37.69±6.55*	118.85±69.70	5.64±0.03
Day 3	Control	1.05±0.68	28.27±7.20	127.26±42.24	5.35±0.53
	Zinc-silver	0.92±0.73*	31.70±5.49*	102.2±83.88*	5.32±0.40
	NSO	1.01±0.44	30.43±6.25	109.3±89.72	5.52±0.07
Day 7	Control	2.26±1.84	37.89±6.30*	97.94±57.13	4.66±1.41
	Zinc-silver	0.90±0.63*	32.17±7.84	66.80±23.48*	5.39±0.24
	NSO	1.39±0.91	40.46±6.11*	141.57±60.95	5.51±0.09
Day 14	Control	2.85±2.27	34.10±11.09	178.85±8.,32	5.38±0.19
	Zinc-silver	1.03±0.58*	36.01±6.06*	114.56±19.63*	5.47±0.08
	NSO	1.16±0.75	34.70±7.12	138.35±25.34	5.46±0.13

Data are expressed as mean ± SD (n= 10).
*Indicates significant differences. When compared to the values measured 10 days prior to the study.
P< 0.01.
MDA: Malondialdehyde, NO: Nitric oxide, CAT: Catalase, SOD: Superoxide dismutase.
Day 0: The day right after surgery.

Table 3. The modified scoring system data for histological evaluation of artificial wounds in rats.

	Control group	Zinc-silver group	NSO
Epithelization	3.5 (0-4)	3.4 (1-4)	3.9 (2-4)
Inflammation	1.4 (0-4)	1.5 (0-4)	0.6 (0-4)
Vascularization	3.3 (2-4)	3.9 (2-4)	3.3 (2-4)

Data are given median (min-max). n=10

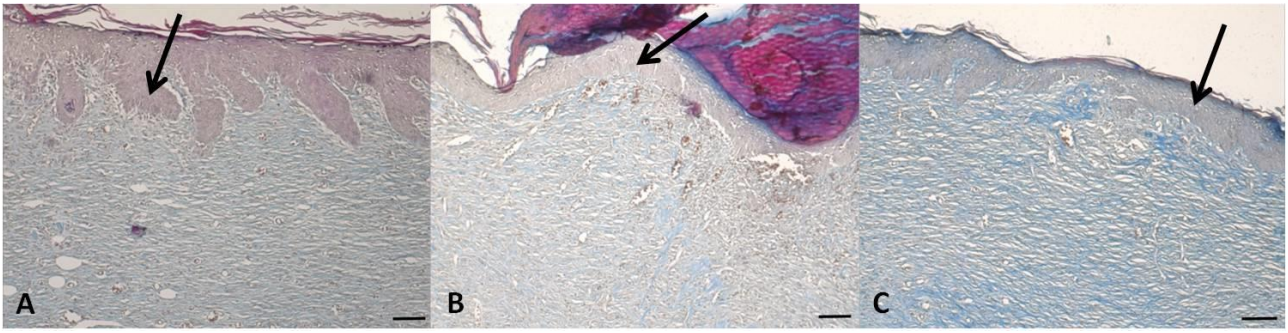

Figure 3. Histopathological images of the 14th day in the rat wound model. A: control group, B: NSO group, C: Zinc-silver group (H&E, 10x, bar: 100µm), Arrows: Epidermis.

In the control group, it was determined that no load could be carried during the biomechanical tests despite the good completion of the epithelialization in the histopathological examination. As seen in Figure 4 representing the control samples, the tissue was Instantaneously ruptured from the wound site during the tension and tear test, and there was a brief decrease in the tissue load carrying capacity. The first breakpoint on the test chart of the control group shows that the holding force of the wound is very low.

There were statistically significant differences among groups for a time, load, tissue tension, and stress based on the tissue tension and tear test (P<0.01). In the tissue tension and tear test, the control group had no durable skin resistance despite the best wound healing appearance (Table 4). The strongest tissue to bear the highest load was those in the silver group (Figure 5). Then, all of the data were evaluated in Table 4, and mean values were found to be relatively high.

Table 4. Evaluation of tissue tension and tear test results.

Test Parameters	Group	Mean±SE	Median	IQR	Mean rank	P	Z
Times	Control	112.20±3.21*	93.67	97.81	1557.05	0.001	-0.730
	Zinc-silver	90.25±1.75*	86.87	85.27	1449.84		
	NSO	87.86±1.68*	85.01	85.67	1442.14		
Load	Control	5.60±0.35*	6.87	14.58	1607.69	0.001	-4.232
	Zinc-silver	10.41±0.28*	8.79	14.14	1491.51		
	NSO	8.75±0.25*	6.25	12.13	1329.66		
Extension	Control	18.25±0.53*	15.29	16.50	1577.92	0.001	-0.736
	Zinc-silver	14.29±0.29*	13.68	14.01	1439.61		
	NSO	13.87±0.28*	13.41	14.15	1411.38		
Strain	Control	0.61±0.02*	0.51	0.55	1510.52	0.001	-4.755
	Zinc-silver	0.48±0.01*	0.46	0.47	1369.19		
	NSO	0.55±0.01*	0.54	0.57	1549.40		
Stress	Control	0.45±0.01*	0.36	0.53	1508.32	0.001	-3.522
	Zinc-silver	0.38±0.01*	0.32	0.52	1392.25		
	NSO	0.47±0.01	0.34	0.67	1528.59		

Data are expressed as mean ± SE (n= 10).
(*) Indicates significant differences among groups P< 0.01.

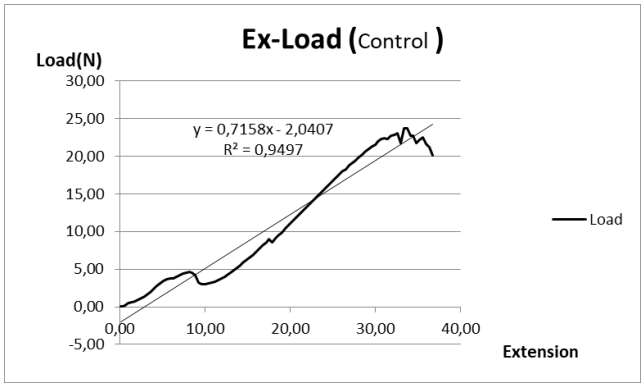

Figure 4. The control group's extension-load graph, obtained through measurement of the wounded rat skin.

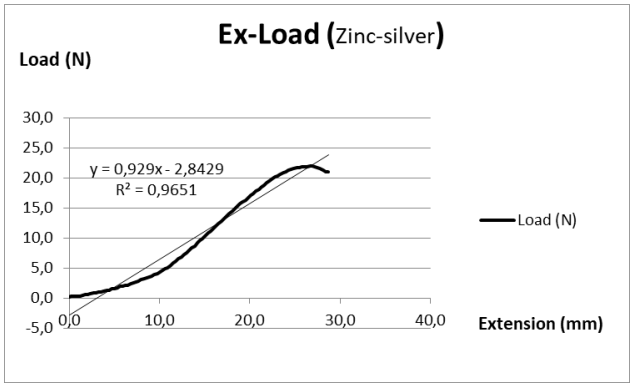

Figure 5. The zinc-silver group's extension-load graph, obtained through measurement of the wounded rat skin.

Discussion and Conclusion

Wound closure involves a series of events and concomitant interaction of numerous molecules including antioxidants (22, 23, 26). Antioxidants are substances that prevent the progression of peroxidation and reacts quite rapidly with radicals. Since free radicals play a definite role in the pathogenesis of wound, antioxidant activity has been investigated in many studies (6). The results of our study showed that topical silver application increases antioxidant activity by inhibiting lipid peroxidation, and also NO level increases in the silver group when compared to baseline values (Table 2). These results confirm the potent wound healing activity of silver. Thus, the wound healing activity may occur because of the potent-radical-scavenging activity. Lipid peroxidation begins as a consequence of free radicals oxidating polyunsaturated fatty acids. MDA is the last product of lipid peroxidation (9, 22, 23, 26). Increase in the serum MDA levels occurs either as a consequence of an increase in free radicals in the body or reduced antioxidant defense mechanisms. In the present study, the highest increase in serum MDA level was observed in the control group while the silver group had the lowest increase. This confirms that nanosilver may have a reducing effect on the lipid peroxidation products, or it may itself have a strong antioxidant effect (9, 22).

The increase of free radicals and lipid peroxidation in injured tissue may thereby predispose to accelerated catalase and catalytic activity for poor wound healing and organ failure because of the disrupted cell membrane structure and permeability (16). In the present study, the lowest plasma serum CAT level was observed in zinc-silver group. This result may be due to possible anti-inflammatory effects (9, 22).

SOD is known as a protective enzyme against oxidative stress, which converts superoxide radical to hydrogen peroxide and to molecular oxygen. During the study, a further increase in the SOD activity was found in the control group as compared to the other groups. Also, in this study, there were some changes seen in SOD levels in all the groups; however, no statistically significant change was observed. Thus, SOD may not be involved in the healing process of the rat wound model we applied.

NO production is an expected outcome during a normal wound healing process. Some studies have reported that nitric oxide synthase inhibitors delay wound healing, while the administration of nitric oxide accelerates wound healing (22, 26, 32). Some authors have reported that inducible nitric oxide synthase (iNOS) reaches peak levels 4 - 6 days after wound formation and accelerates wound healing (9). Studies have shown that nitric oxide is effective in wound healing, and also that it plays an important role in collagen accumulation (7, 18, 25). Increase in NO level may accelerate wound healing by increasing angiogenesis, endothelial and epithelial cell proliferation, and migration (26). In our study, serum NO levels in all study groups were found to increase during the study period according to the control measurements, and they reached their highest level on the 7th day. The increase of NO level to the maximum level in the nano-silver group on the 14th day (after collecting the laboratory samples) suggests that the best contributory effect in the wound healing processes is in this group. This was also confirmed by histopathological examination and mechanical tests.

Silver is widely used in wound healing due to its anti-inflammatory and antiseptic effects, although there are also some data causing controversies as well. In fact, even though nano-silver-containing products for clinical applications are currently in use, it is also possible to encounter many opposing views within these multiple data sets. It is clear that commercial nano-silver-containing products that have been tested for a cytotoxic activity will contribute to further knowledge about the subject, supported by further, planned in vivo studies in terms of antibacterial effects and inflammation. It is thought that some data obtained in the present study are similar to other groups, and there are statistical differences between them, but still, it is thought that the silver group is superior to other groups in terms of wound healing. Histologically, the reason for the increase in inflammation compared to the other groups is thought to be that the nanosilver stimulates the inflammatory response and contributes to the formation of connective tissue. The silver group, which has been found to be the best group for tissue bond development, has also been the group with the highest load carrying capacity (21.95 N) in the tissue stretching and breakout test; thus, making it the best group for skin healing. It has been concluded with the post healing data, zinc-silver group differs positively in terms of clinical and biochemical and histological aspects compared to other treatment groups, and thus it may be a clinically useful product for wound healing.

Studies on experimental burn wounds in rats have reported that epithelialization and granulation are better in subjects treated with NSO than in other groups (30). In the present study, the NSO group's epithelization in the histological section was found to be the best group that contradicts with the previously published articles. The inflammatory response was found to be at the least level in NSO group as well. In the histological evaluation, Epithelization and connective tissue proliferation were better in NSO group compared to those in the control group (Figure 4). But, vascularization and connective tissue formation were found to be lower compared to those in the silver group. This finding is also supported by the tissue tensile tear test results of the study (Figure 6).

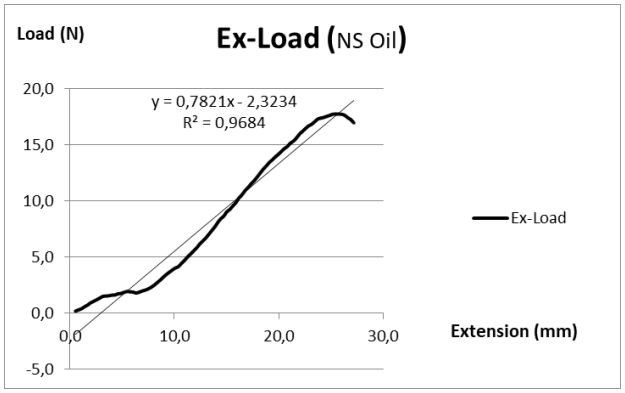

Figure 6. The NSO group's extension-load graph, obtained through measurement of the wounded rat skin.

Mechanical resistance is a characteristic that determines the quality of healing in skin wounds. The mechanical properties of the skin are used to examine changes in the skin due to aging or in vivo or ex vivo evaluation of wound healing studies in experimental animals. In the biomechanical tests, the control group showed the lowest load carrying capacity (Mean Load: 4.60 N). The lowest load carrying capacity is also reflected in the histomorphological study in which epithelialization and connective tissue formation were incomplete in this group.

In conclusion, although lower clinical healing appearance, zinc-silver seems to have a better healing capacity in a rat wound healing model due to best histological healing with an increase anti-oxidant induction and a higher load carrying capacity. Healing capacity resulting from NSO lower compared to that of zinc-silver. Further studies are needed to reveal the molecular background of zinc-silver and NSO effects on wound healing, such a complex multifactorial process.

Acknowledgments

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Toxic effects of cutaneous and oral exposure to aluminum and magnesium nanoparticles on brain tissue in rats

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Abstract: In this study, it was aimed to research the effects of cutaneous and oral exposure to aluminum nanoparticles (Al-NPs) and magnesium nanoparticles (Mg-NPs) on the brain tissue, which is vitally important in terms of its structure and functions. The study was performed on Wister-Albino rats, which were divided into 10 groups, such as control groups (groups 1 and 2), groups, to which Al and Mg NPs were applied as 500 mg/kg and 1500 mg/kg orally (groups 3-6) and 1000 mg/kg and 2000 mg/kg cutaneously (groups 7-10)**.** The dosages were administered as a single dose. While brain tissue and serum MDA levels as well as brain tissue TNF-α and IL-6 levels have significantly increased in the group, to which 1500 mg/kg Mg-NPs was applied orally, significant decreases have also been observed in brain tissue GPX and SOD levels of the same group. Additionally, meaningful decreases in brain tissue SOD levels and significant increases in TNF-α and IL-6 levels have been observed in the group, to which 1500 mg/kg Al-NP was applied orally. On the other hand, it was found that brain tissue GPX and SOD levels of the group, to which 2000 mg/kg Mg-NP was applied cutaneous, have been decreased significantly. Histopathological examinations have also supported these findings. At the end of the study, it was observed that the toxic effect of Al and Mg NPs has varied, depending on the application method, dosage and duration.
Keywords: Aluminum nanoparticle, brain toxicity, cutaneous, magnesium nanoparticle, rat.

Ratlarda deri ve ağız yoluyla alüminyum ve magnezyum nanopartiküllere maruziyetin beyin dokusu üzerindeki toksik etkileri

Özet: Bu çalışmada, alüminyum nanopartiküllerine (Al-NP) ve magnezyum nanopartiküllerine (Mg-NP) önemli maruziyet yollarından olan deri ve ağız yoluyla maruziyetin, yapısı ve görevleri itibarıyla hayati öneme haiz beyin dokusu üzerindeki etkilerinin araştırılması amaçlandı. Çalışma; kontrol grupları (grup 1 ve 2), Al ve Mg NP'lerin 500 mg/kg ve 1500 mg/kg dozlarda ağız yoluyla (grup 3-6) ve 1000 mg/kg ve 2000 mg/kg dozlarda deri yoluyla (grup 7-10) verildiği gruplarda, tek doz uygulamalar yapılarak, 10 grup Wistar-Albino rat üzerinde gerçekleştirildi. Uygulama gruplarından Mg-NP'nin ağız yoluyla 1500 mg/kg dozda uygulandığı grupta beyin dokusu ve serum MDA düzeyleri ile beyin dokusu TNF-α ve IL-6 düzeyleri önemli ölçüde artarken, beyin dokusu GPX ve SOD düzeylerinde önemli azalmalar olduğu gözlendi. Ağız yoluyla Al-NP'nin 1500 mg/kg dozda verildiği grupta beyin dokusu SOD düzeylerinde anlamlı azalmalar ve TNF-α ve IL-6 düzeylerinde ise önemli artışlar tespit edildi. Diğer taraftan, deri yoluyla Mg-NP'nin 2000 mg/kg dozda verildiği grubun beyin dokusu GPX ve SOD düzeylerinde önemli bir azalma olduğu belirlendi. Histopatolojik incelemeler de bulguları destekledi. Çalışma sonucunda, Al ve Mg NP'lerin toksik etkisinin uygulama yoluna, verilen doza ve süreye bağlı olarak değiştiği gözlendi.
Anahtar sözcükler: Alüminyum nanopartikül, beyin toksisitesi, deri, magnezyum nanopartikül, rat.

Introduction

As nanomaterials and nanoparticles (NPs) take more space in many areas of our lives, the number of products manufactured with nanotechnology does also increase day by day. The effectiveness and role of NPs in our lives have increased significantly in recent years since it has begun to be used widely in medicine, molecular biology and engineering, foodstuffs, cosmetics and other industries (12, 25, 26). Various NPs being used in almost everywhere and in various forms have become promising for nanotechnology, but as their area of use increases their negative effects on the human health have also become a topic for debate (26).

Nanoparticles mainly enter the body through oral, respiration and cutaneous. Since they are small in sizes, a NP that has entered the body can easily penetrate into circulatory and lymph system and travel through all body tissues and organs. Some NPs may cause irreversible

damages to cells, based on their composition and size (2, 5).

Nanoparticles affect basic cellular processes, such as proliferation, metabolism and death and many diseases can be associated with the dysfunction of these basic processes. For example, while neurodegenerative diseases are causing early death of cells, non-controllable cellular proliferations are causing cancer (5). Although it is not exactly known which mechanism are used by NPs to induce pro-inflammatory effects, it is asserted that they create reactive oxygen species (ROS) and induce cytokine production (3). Oxidative stress is responsible for cell and DNA damage (24). It is also accepted that severe inflammation is the first step of occurrence of systemic autoimmune diseases (systemic lupus erythematosus, scleroderma and rheumatic arthritis) as a result of being exposed to some NPs, such as silica and asbestos (16, 22).

Aluminum nanoparticles are used widely in various industries, such as ceramics, defence, dyeing and optics as well as in rocket fuels, food, implant, drug and vaccine production and in personal care products. People can be exposed to these particles either by consuming food and water or by using many other products that contain Al (23, 27). On the other hand, Mg-NPs are used to produce antimicrobial food packages, fire resistant ceramics, humidity sensors and syringes as well as in chemicals industry, surface coatings and fuel additives. They are also used as antacids against heartburn, detoxifying agents, for antibacterial purposes and bone regeneration (8, 10, 14). Particularly magnesium oxide (MgO) is a significant NP that attracts a lot of scientific attention due to its ease of synthesis and chemical stability properties and it does also have antibacterial activity. Its antibacterial effect is associated with the production of ROS on the oxide surface. On the other hand, it does also have an important cytotoxic effect; the studies showed its cytotoxic effects on human umbilical vein endothelial cells and cardiac microvascular endothelial cells and its toxicity on lung tissues (8, 14).

Nanoparticles have the potential to improve the environment and the lives of people, but their interaction with the environment is also inevitable. The results of using these particles are not completely defined yet for human health and the ecosystem. However, recent studies have revealed the toxic effects of NPs, to which humans are exposed through various means. Although the exposure to some NPs through respiration and oral has been found in a few studies that were made, the data are conflictive. On the other hand, NPs mainly enter the body through oral, respiration and cutaneous. In this study, it was aimed to research the effects of cutaneous and oral exposure to Al and Mg NPs on the brain tissue, which is vitally important in terms of its structure and functions.

Material and Methods

Chemicals: Al-NPs (Al_20_3) <50 nm (Sigma Aldrich, 702129) and Mg-NPs (MgO) <50 nm (Sigma Aldrich, 549649) were used. 1 mg/ml concentration of the particles in distilled water was prepared in ultrasonic water bath and kept in sonicator for 2 hours before use. Polyethylene glycol (PEG) solution (Sigma Aldrich, P7181) was kept at 2-8°C.

Experiment protocol: This study was performed with 80 adult male Wister-Albino rats, which were 6-9 months old and 230±20 gr weight. All experiments were performed in Sivas Cumhuriyet University. Rats were kept under standard laboratory animal conditions (12 hours of light/dark cycle, 24±2°C, 35-60% humidity) and feed and water were given *ad libitum*. Experiments were made in accordance with the Guide on Caring and using Laboratory Animals (a publication of DHEW (NIH) 8523, 1985). The study was performed with the authorization (date: 04/03/2015, issue: 65202830/25) of Local Ethic Board of Animal Experiments of Sivas Cumhuriyet University (Turkey).

Rats were divided into 10 groups, each of which include 8 rats, such as control groups (groups 1 and 2), groups, to which Al and Mg NPs were applied as 500 mg/kg and 1500 mg/kg orally (groups 3-6) and 1000 mg/kg and 2000 mg/kg cutaneously (groups 7-10) and these dosages were administered as a single dose (18, 19). The design of experimental groups were given in Table 1.

Table 1. Design of experimental groups.

Groups	Application
Group 1 (Control group I)	PEG was applied through gavage to rats
Group 2 (Control group II)	PEG was applied cutaneous (through rubbing) to rats
Group 3 (Al NP-Oral-500)	500 mg/kg dosage of Al-NPs was given within PEG to rats through gavage
Group 4 (Al NP-Oral-1500)	1500 mg/kg dosage of Al-NPs was applied within PEG to rats through gavage
Group 5 (Mg NP-Oral-500)	500 mg/kg dosage of Mg-NPs was given within PEG to rats through gavage
Group 6 (Mg NP-Oral-1500)	1500 mg/kg dosage of Mg-NPs was applied within PEG to rats through gavage
Group 7 (Al NP-Cutan-1000)	1000 mg/kg dosage of Al-NPs was given cutaneous (by rubbing) within PEG
Group 8 (Al NP-Cutan-2000)	2000 mg/kg dosage of Al-NPs was given cutaneous (by rubbing) within PEG
Group 9 (Mg NP-Cutan-1000)	1000 mg/kg dosage of Mg-NPs was applied cutaneous (by rubbing) within PEG
Group 10 (Mg NP-Cutan-2000)	2000 mg/kg dosage of Mg-NPs was applied cutaneous (by rubbing) within PEG

Single dose of NPs was applied to the animals in all application groups. The hairs on 10% of the rat's body (dorsal area) were shaved before apply mentioned dosages of NPs cutaneous and it was ensured that applied NPs stayed for 24 hours on the same area. Then NPs were removed and animals were kept under observation for 14 and 28 days. The application was ended at the end of specified periods. When NPs were applied orally, the application was ended 14 and 28 days after NPs were given in specified dosages to the animals. Blood samples were taken from the anesthetized rats after experiments were completed. Subsequently, the brain tissues were removed carefully through necropsy and while some part of these brain tissues were being kept within 10% formaldehyde solution for histopathological examinations, the remaining parts were kept at -80°C in order for other analyses. The blood samples were centrifuged at 3000 rpm for 15 min and sera were kept at -80ºC until further analyses.

Determination of lipid peroxidation: Lipid peroxidation was determined by malondialdehyde (MDA) content according to the method defined by Ohkawa et al. (20). MDA analyses were made in rat serum and brain tissue samples. MDA, a secondary product of lipid peroxidation was created by incubating with thiobarbituric acid (TBA) and sample at 100ºC and in an aerobic environment, at where pH was 3.4 and lipid peroxidation was detected through spectrophotometer measurement of pink colour at 532 nm, which was created in the form of a complex with MDA done TBA. Identified absorbance value was calculated as nmol/ml from standard curve of MDA. The samples were read against the blind at 532 nm wavelength in spectrophotometer device (Perkin Elmer, Lambda 25 UV/VIS, USA).

Determination of glutathione peroxidase enzymes: Glutathione peroxidase (GPX) activity was determined with ELISA reader (Thermo Multiscan Go, USA) by using a commercially available standard enzymatic kit (YL Biont, YL Biotech Co, Shanghai). The ELISA kits, which are based on biotin double antibody sandwich technology, were used to detect rat GPX. Analyses were made according to the kit procedure, recommended by the manufacturer. The absorbance was read at 450 nm.

Determination of superoxide dismutase enzyme: Superoxide dismutase (SOD) enzyme's activity was determined by ELISA reader in accordance with the procedure recommended by the manufacturer by using a standard enzymatic kit (Fn-test, Fine Biotech Co., China). According to this analysis procedure, kit plates, which were already coated with anti-SOD antibodies, and biotin conjugated anti-SOD antibodies were used as determination antibodies. TMB substrates were used to visualize the reaction of Horse Radish Peroxidase (HRP) enzyme. The absorbance was read at 450 nm.

Determination of tumor necrosis factor alpha and interleukin 6: Tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) levels were determined by using commercially available standard enzymatic kits (YL Biont, YL Biotech Co, Shanghai). The ELISA kits, which are based on biotin double antibody sandwich technology, were used to detect rat TNF-α and IL-6. Absorbance was read at 450 nm.

Histopathological analysis: Rat brain tissue samples, which were kept in 10% formaldehyde solution for histopathological examinations, were embedded in paraffin and blocked after they were washed under tap water overnight and alcohol-xylol series were applied. Then 5 µm of paraffin blocks were taken, placed on glass slide and stained with Haematoxylin-Eosin. *Cornu ammonis* (hippocampus) and *Bulbus olfactorius* regions of cross sections of all stained groups were examined under a light microscope (Zeiss AxioCam ERc5s, Germany) in order to evaluate and score inflammation, necrosis, gliosis, hyperaemia, oedema and demyelination.

Statistical analyses: The data were analysed by using SPSS (Version 23) software. The results were calculated as mean and standard error of mean (SEM). It was determined by Shapiro-Wilk whether the data were distributed normally or not. One way variance analysis (One-way ANOVA) and post hoc analysis Tukey test were used to find the difference between groups. The level of statistical significance was accepted as $P<0.05$.

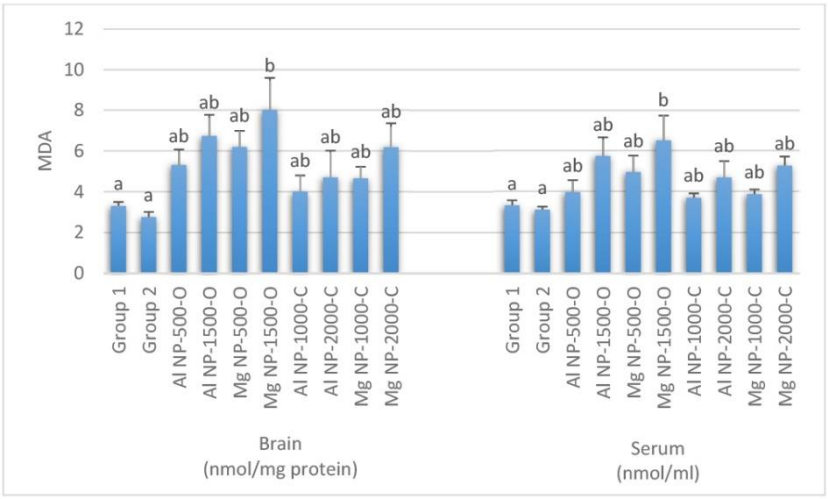
Results

Results of lipid peroxidation: When the brain tissue and serum MDA levels of groups, which orally received 500 mg/kg and 1500 mg/kg doses of Al and Mg NPs, were compared with control group (group 1), it was found that there was a statistically significant increase in group 6 according to control group ($P<0.05$). Brain tissue and serum MDA levels of experimental groups were given in Figure 1.

Glutathione peroxidase results: When brain tissue GPX levels of groups, to which 500 mg/kg and 1500 mg/kg doses of Al and Mg NPs were given orally and control group (group 1), were compared, it was found that there was a statistically meaningful difference between the group 6 and control group ($P<0.05$). In addition, when brain tissue GPX levels of groups, to which 1000 mg/kg and 2000 mg/kg doses of Al and Mg NPs were given cutaneous and control group (group 2), were compared, it was found that there was a statistically significant decrease in group 10 compared to control group ($P<0.05$) (Figure 2).

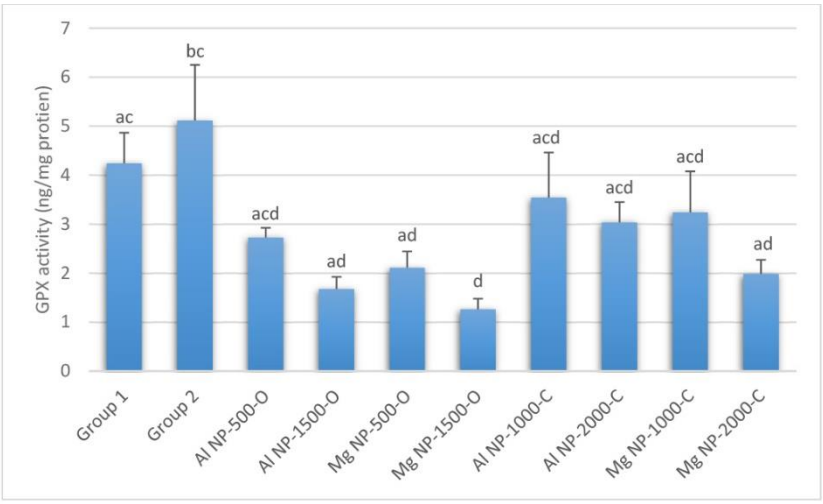
Superoxide dismutase results: When brain tissue SOD levels of experimental groups, to which 500 mg/kg and 1500 mg/kg doses of Al and Mg NPs were given orally and control group (group 1), were compared, it was

found that there was a statistically meaningful decrease in group 4 and group 6 according to control group (P<0.01). Furthermore, when brain tissue SOD levels of groups, which cutaneous received 1000 mg/kg and 2000 mg/kg doses of Al and Mg NPs, were compared with control group (group 2), it was found that there was a statistically meaningful difference between the group 10 and control group (P<0.01). Brain tissue SOD levels of experimental groups were given in Figure 3.



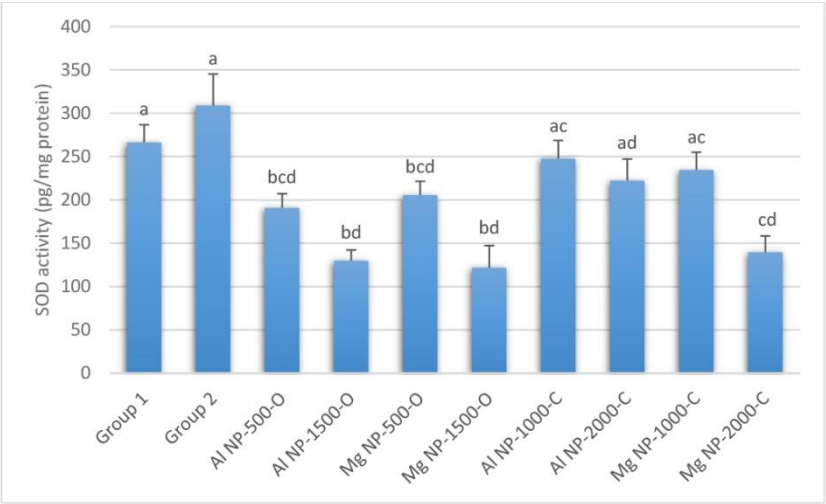
Different letters (a, b) identify the statistical difference between groups (P<0.05).

Figure 1. Brain tissue and serum MDA levels of experimental groups.



Different letters (a, b, c, d) identify the statistical difference between groups (P<0.05).

Figure 2. Brain tissue GPX levels of experimental groups.



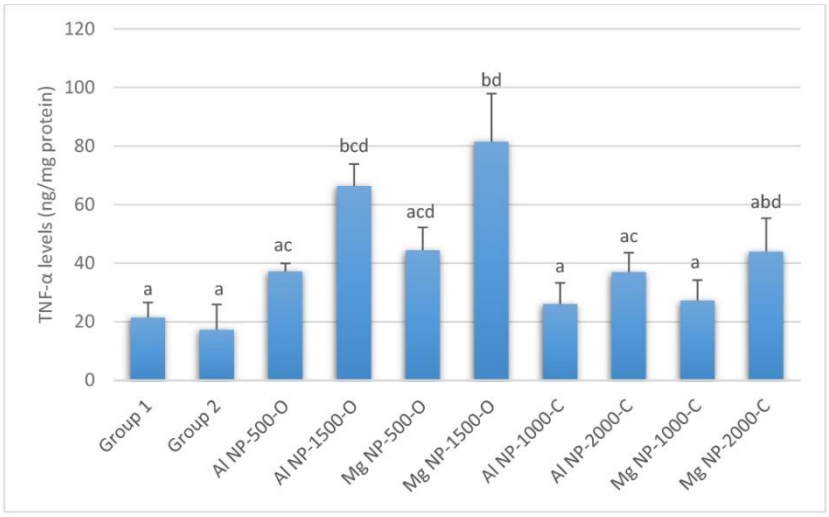
Different letters (a, b, c, d) identify the statistical difference between groups (P<0.05).

Figure 3. Brain tissue SOD levels of experimental groups.

Tumor necrosis factor alpha results: When brain tissue TNF-α levels of groups, to which 500 mg/kg and 1500 mg/kg doses of Al and Mg NPs were given orally and control group (group 1), were compared, it was found that there was a statistically meaningful increase in group 4 and group 6 compared to control group ($P<0.05$, $P<0.01$) (Figure 4).

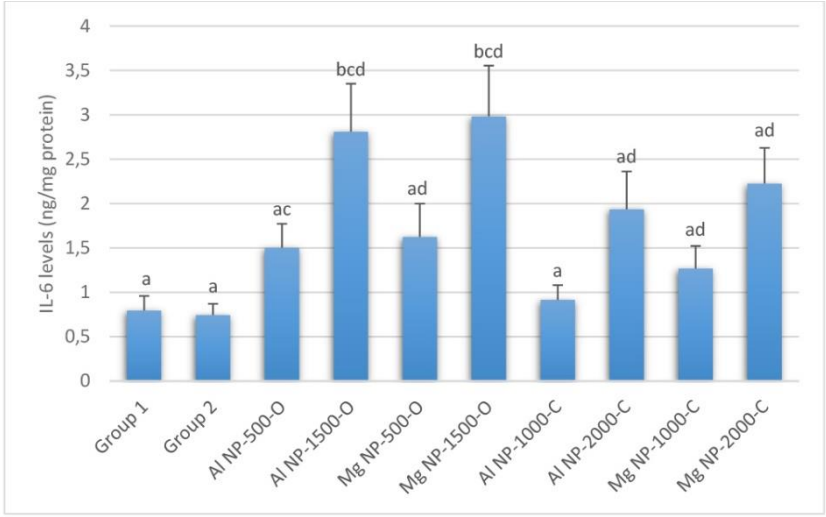
Interleukin 6 results: When brain tissue IL-6 levels of groups, which orally received 500 mg/kg and 1500 mg/kg doses of Al and Mg NPs, were compared with control group (group 1), there was a significant increase in group 4 and group 6 according to control group ($P<0.01$) (Figure 5).

Histopathology results: Cross-sections taken from *Cornu ammonis* (hippocampus) and *Bulbus olfactorius* regions of brain tissue samples of experimental groups were examined in terms of inflammation, necrosis, gliosis, hyperaemia, oedema and demyelination and then histopathological changes were rated and summarized in Tables 2 and 3. No histopathological change was found in cross-sections, taken from samples of control groups (group 1 and 2). While slightly severe and mid-severe demyelination lesions were found in cross sections of *Cornu ammonis* (hippocampus) regions of group 5 and group 6, slightly severe inflammation was found in the group 10. Slightly severe inflammation was found in cross sections of *Bulbus olfactorius* regions of group 5 and group 10 (Figure 6).



Different letters (a, b, c, d) identify the statistical difference between groups ($P<0.05$).

Figure 4. Brain tissue TNF-α levels of experimental groups.



Different letters (a, b, c, d) identify the statistical difference between groups ($P<0.05$).

Figure 5. Brain tissue IL-6 levels of experimental groups.

Table 2. Scoring of histopathological features of cross sections, taken from brain tissue *Cornu ammonis* (hippocampus) regions of experimental groups.

Groups	Histopathological features					
	Inflammation	**Necrosis**	**Gliosis**	**Hyperemia**	**Oedema**	**Demyelination**
Group 1	-	-	-	-	-	-
Group 2	-	-	-	-	-	-
Group 3 (Al NP-Oral-500)	-	-	-	-	-	-
Group 4 (Al NP-Oral-1500)	-	-	-	+	-	-
Group 5 (Mg NP-Oral-500)	-	-	-	++	-	+
Group 6 (Mg NP-Oral-1500)	-	-	-	+++	-	++
Group 7 (Al NP-Cutan-1000)	-	-	-	+	-	-
Group 8 (Al NP-Cutan-2000)	-	-	-	++	-	-
Group 9 (Mg NP-Cutan-1000)	-	-	-	+	-	-
Group 10 (Mg NP-Cutan-2000)	+	-	-	++	-	-

-: none; +: slightly severe; ++: mid-severe; +++: severe.

Table 3. Scoring of histopathological features of cross sections, taken from brain tissue *Bulbus olfactorius* regions of experimental groups.

Groups	Histopathological features					
	Inflammation	**Necrosis**	**Gliosis**	**Hyperemia**	**Oedema**	**Demyelination**
Group 1	-	-	-	-	-	-
Group 2	-	-	-	-	-	-
Group 3 (Al NP-Oral-500)	-	-	-	-	-	-
Group 4 (Al NP-Oral-1500)	-	-	-	-	-	-
Group 5 (Mg NP-Oral-500)	+	-	-	+	-	-
Group 6 (Mg NP-Oral-1500)	-	-	-	++	-	-
Group 7 (Al NP-Cutan-1000)	-	-	-	-	-	-
Group 8 (Al NP-Cutan-2000)	-	-	-	-	-	-
Group 9 (Mg NP-Cutan-1000)	-	-	-	+	-	-
Group 10 (Mg NP-Cutan-2000)	+	-	-	-	-	-

-: none; +: slightly severe; ++: mid-severe.

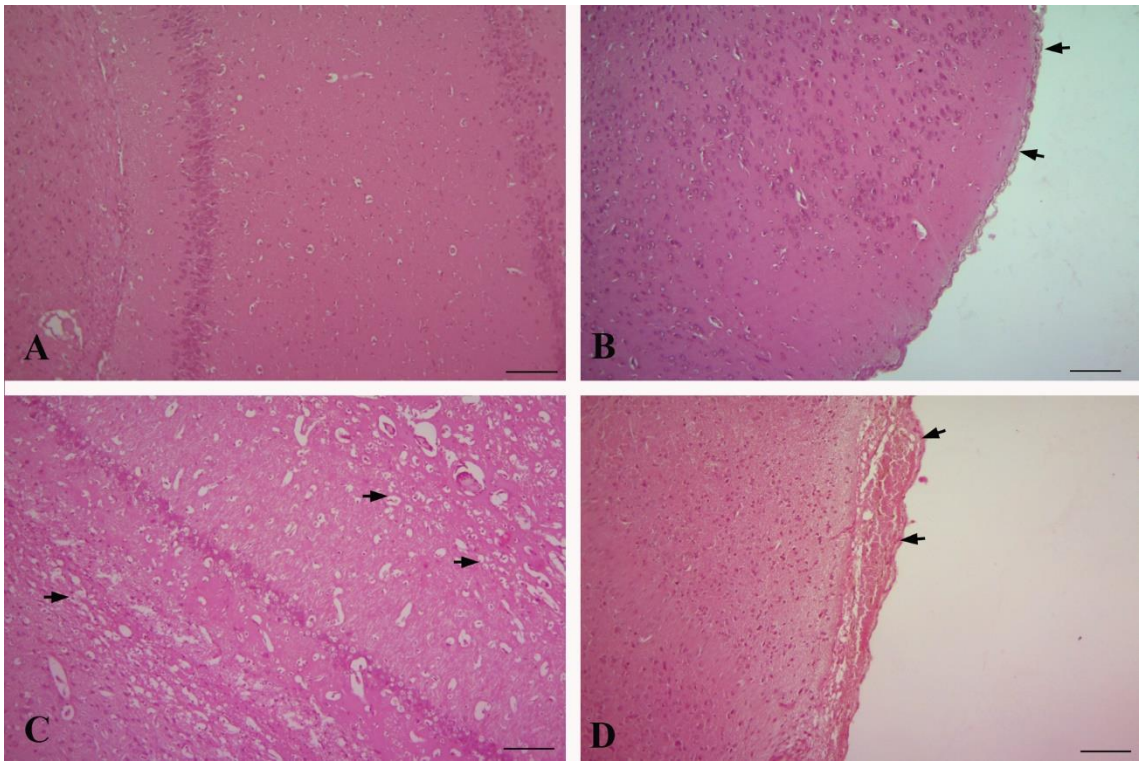


Figure 6. Photos of histopathological staining. **A.** Control groups of cross sections, taken from *Cornu ammonis* (hippocampus) regions, H&E, Bar: 50 µm, **B.** Control groups of cross sections, taken from *Bulbus olfactorius* regions, H&E, Bar: 50 µm, **C.** Slightly severe or mid-severe demyelination lesions in *Cornu ammonis* (hippocampus) (arrow), H&E, Bar: 25 µm, **D.** Slightly to mid-severe *Bulbus olfactorius* hyperaemia and degeneration (arrow), H&E, Bar: 50 µm.

Discussion and Conclusion

When they are properly designed, NPs can show unique and adjustable chemical and physical characteristics and they may have unique biological effects in terms of developing technologies, but it is inevitable that they would interact with the environment. Furthermore, there is only limited literature about their effects on the health of humans, who are exposed to these particles through oral, cutaneous or respiration.

The special structure and vital tasks of brain tissue increase its sensitivity to NPs. It is rich in highly oxidizable poly-unsaturated fatty acids and has a high metabolic rate and weak antioxidant defence system. Therefore, reported to be particularly vulnerable to oxidative stress, and it is also stated that NPs have the ability to penetrate the blood-brain barrier (23).

It is stated that excessive Al dose is connected with some neurodegenerative diseases of humans, such as Alzheimer and Parkinson-dementia; it is also highlighted that it may cause adverse effects, such as inflammatory response or genetic damage, it may cause problems in ROS production and mitochondrial function disorders and it has carcinogenic potential and cytotoxic effects. The mitochondrial disorders and oxidative damages caused by Al on the neural cells lead to neural cells loss resulting in neurotoxic effect. On the other hand, Al does also affect antioxidant enzyme activity and causes oxidative damage. Furthermore, Al-NPs have a tendency to be accumulated in organs for various periods and in various quantities. The studies conducted on experimental animals showed that they are accumulated in all areas of brain after chronic exposure and they are found mostly in hippocampus, the area of memory and learning (15, 21). Mg-NPs may cause a decrease in antioxidant capacity and defence mechanisms, increase in ROS production and/or inhibition or depletion of antioxidant systems through mitochondrial dysfunction. Usually our bodies have a balance in production of free radicals and repairing of damage caused by these free radicals, but exposure to Mg-NPs impair this balance (8, 11).

In this study, increase in brain tissue oxidative stress parameters (MDA levels) and decrease in antioxidant potential (GPX, SOD levels) were found in group 4 and group 10, and particularly in group 6. Prabhakar et al. (23) investigated the effects of Al_2O_3-NPs administered by oral to rats (at doses of 500, 1000 and 2000 mg/kg) on oxidative stress, which is accepted as an important mechanism in carcinogenesis. During the study the samples (liver, kidney, brain, heart) that were taken on the 3rd and 14th days were examined for oxidative stress parameters and it was found that MDA levels were meaningfully high, there was an increase in catalase (CAT) activity due to dosage, there was not any change in GPX and there was a decrease in glutathione (GSH), SOD and glutathione reductase (GR) activities. In another study, the effects of oxidative stress induced by Al on brain cortex neurons and glial cells were investigated and rats were administered Al at a dose of 100 mg/kg orally for 8 weeks. In experimental groups, a significant decrease was found in MDA, SOD, oxidized glutathione (GSSG), GPX, and CAT levels of glial cell fractions, increase in GSH, GR, glutathione-s-transferase (GST) levels, decrease in SOD, CAT, GSH, GSSG, GR, and GPX levels of neuronal cells and increase in MDA and GST levels (9). In a study, conducted by Mangalampalli et al. (14), in which they aimed to examine toxicological potential of MgO-NPs, they evaluated genotoxicity, histological, biochemical, antioxidant and bio-distribution parameters of blood and tissue (liver and kidney) samples, taken respectively 24 and 72 hours after application of low (100 mg/kg), medium (500 mg/kg) and high (1000 mg/kg) dosages of MgO-NPs orally to rats. As a result of the study, it was stated that acute exposure to high doses of MgO-NPs causes significant DNA damage and biochemical changes, the antioxidant analyses (high MDA levels and SOD, CAT, GPX and total antioxidant depletion) have highlighted the oxidative stress and these results were supported with histopathological findings. Considering the findings such as brain tissue oxidative stress parameters (MDA levels), ROS generation, antioxidant potential (GPX and SOD levels) and effect do change at depending on the dose and duration in this study, it was found to be consistent with the results obtained in previous studies.

If an infection occurs, it activates chemical precursors that alert immunity system, such as pro-inflammatory mediators or intercellular cytokines (such as TNF-α, IL-1α, IL-6) (4). Although it was not possible to completely explain induction mechanism of NPs for pro-inflammatory effects, it was asserted that they activate transcription factors, which modulate in-cell calcium concentration, they generate ROS and increase cytokine production (3). At the same time, it was highlighted that NPs can create ROS directly on their surfaces or through macrophage activation and cause an increase in production of oxidative types, antioxidant production and inflammation (6, 13, 17).

It was found in the present study that brain tissue cytokine (TNF-α, IL-6) levels were increased in group 4 and particularly in group 6. Park et al. (21) have examined toxic effects of Al_2O_3-NPs, which was applied orally to mice for 13 weeks (1.5, 3 and 6 mg/kg of doses; 6 times/week). When the results were compared with control group, an increase was observed in IL-6 levels, based on the amount of dosage. On the other hand, when the effects of NPs (Al_2O_3, CeO_2 and SiO_2), which were used frequently in semiconductor industry, as a result of potential professional exposure were examined, it was

found that Al_2O_3-NPs increases pro-inflammatory cytokine levels (TNF-α, IL-6 and IL-1β) (7). In the study conducted by Adamcakova-Dodd et al. (1) in order to examine toxic effects of Al_2O_3 based nanowhiskers, male mice were exposed to these nanomaterials for 2 or 4 weeks (4 hours/day and 5 days/week) in a dedicated exposure room. As a result, it was reported that no significant differences between control group and experimental groups were observed in terms of blood and bronchoalveolar lavage fluid LDH activity and cytokine levels (IL-6, IFN-y, MIP-la, TNF-α and MIP-2). The results of this study were found in conformity with the results of the studies, conducted by Park et al. (21) and Flaherty et al. (7) but not found in conformity with the results of the study, conducted by Adamcakova-Dodd et al. (1).

As a result of this study, it was found that brain tissue oxidative stress parameters (MDA levels) were increased, antioxidant potential (GPX, SOD levels) were decreased and cytokine (TNF-α, IL-6) levels were increased in groups, to which 1500 mg/kg dose of Al-NPs was given orally (group 4) and 2000 mg/kg dose of Mg-NPs was given cutaneous (group 10), particularly including the group, to which 1500 mg/kg dose of Mg-NPs was given orally (group 6). Histopathological examinations have also supported these findings. It was observed that the toxic effect of Al and Mg NPs varies, depending on the application method, dosage and duration.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Vascular endothelial growth factor and epithelial cell adhesion molecule immunoexpression in enzootic nasal adenocarcinoma of goats

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Abstract: Enzootic nasal adenocarcinoma (ENA) is a contagious and viral disease in sheep and goats. The disease leads to the death of the affected animals and causes economic losses in infected small ruminant flocks. Epithelial cell adhesion molecule (EpCAM) is a newly discovered tumor antigen, commonly expressed by several tumors occurring in humans. Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that stimulate the formation of blood vessels and plays an important role in angiogenesis, essential for tumor development. In this study, ENA originating from the nasal mucosa is detected on histopathological and cytopathological examinations, with transmission electron microscopy confirming viral particles in the tumoral cells. The aim of this study was to examine VEGF and EpCAM immunoexpressions using streptavidin–biotin complex immunoperoxidase technique in 24 naturally ENA-affected goats. Sneezing, dyspnea, seromucous or purulent nasal discharge, exophthalmos, and weight loss were commonly observed clinical symptoms. The tumors located in the nasal cavity were generally unilateral and were bilateral in only two cases. Cytological examination of the tumoral masses revealed uniform epithelial cell clusters and abundant inflammatory reaction. On histopathological examination, tubular, papillary, or mixed types of ENA were diagnosed. On ultrastructural examination, intracytoplasmic, spherical, retrovirus-like particles were demonstrated. Immunohistochemically, strong positive reactions were reported for both EpCAM and VEGF in the cytoplasm of the tumor cells. Thus, this study showed that EpCAM and VEGF may have an important role in ENA pathogenesis.

Keywords: Clinicopathology, enzootic nasal adenocarcinoma, pathogenesis, VEGF, EpCAM.

Keçi enzootik nazal adenokarsinomlarında vasküler endoteliyal büyüme faktörü ve epiteliyal hücre adezyon molekülü immunoreaksiyonları

Özet: Enzootik nazal adenokarsinom (ENA) koyun ve keçilerin viral ve bulaşıcı bir hastalığıdır. Hastalık etkilenen hayvanların ölümüne yol açarak enfekte küçük ruminant sürülerinde ekonomik kayıplara neden olur. Epiteliyal hücre adezyon molekülü (EpHAM), insanların birçok tümörü tarafından sentezlenen yeni keşfedilmiş bir tümör antijenidir. Vasküler endoteliyal büyüme faktörü (VEBF), hücreler tarafından üretilen kan damarlarının oluşumunu uyaran, tümör gelişimi için gerekli olan ve anjiyogenezde önemli rol oynayan bir sinyal proteinidir. Bu çalışmada, nazal mukozadan kaynaklanan ENA'da, histopatolojik ve sitopatolojik incelemelerde yapıldı ve viral partiküller tümör hücrelerinde transmisyon elektron mikroskobu ile saptandı. Bu çalışmanın amacı ENA ile doğal enfekte 24 keçide klinik, patolojik, sitolojik, elektron mikroskobik bulgular ile VEBF ve EpHAM ekspresyonlarının immunohistokimyasal olarak streptoavidin-biotin kompleks metoduyla incelenmesidir. Olguların birçoğunda klinik olarak hapşırma, solunum güçlüğü, serömüköz veya purulent burun akıntısı, ekzoftalmus ve kilo kaybı bulguları gözlendi. Tümörler burun boşluğunda genellikle tek taraflı, sadece iki olguda çift taraflı olarak yerleşmişti. Tümöral kitlelerin sitolojik incelemesinde genellikle üniform şekilli epitel hücre kümeleri ve belirgin yangısal reaksiyon dikkati çekti. Histopatolojik incelemede tubuler, papiller veya karışık tipte görünümler saptandı. Elektron mikroskobik incelemede yuvarlak şekilli intrasitoplazmik Retrovirus benzeri partiküller gözlendi. Immunohistokimyasal olarak tümör hücrelerinin sitoplazmalarında hem EpHAM hem de VEGF yönünden güçlü pozitif reaksiyon saptandı. Bu çalışma EpHAM ve VEBF'nin ENA patojenezinde önemli bir rol oynayabileceğini gösterdi.

Anahtar sözcükler: Enzootik nazal adenokarsinom, klinikopatoloji, patojenez, VEBF, EpHAM.

Introduction

Enzootic nasal adenocarcinoma (ENA) is an economically important, contagious, viral, and progressive disease, characterized by neoplastic transformation originating from the ethmoid mucosa of sheep and goats (3, 12, 13, 29-31). Enzootic nasal

adenocarcinoma virus (ENAV), a retrovirus, is known as a potential agent. This tumor is naturally occurring worldwide and has been reported in several countries, including Turkey (2, 3, 12, 13, 30-32). Clinically, the tumor is characterized by chronic and progressive respiratory symptoms. Grossly, the tumor can be localized unilaterally or bilaterally in the nasal cavity (3, 11, 30, 31). Cytological examination of the tumoral masses reveals uniform epithelial cells and abundant inflammatory reaction, the main characteristic of the tumor (34). The tumoral cells are generally uniform with no cellular atypia, and tubular, papillary, acinar, and mixed patterns may be evident on microscopical examination. Genetic, breed, and sex predispositions have not been reported in any published study. ENA leads to death in the affected animals; however, mortality rate may vary from flock to flock (30-32).

To reach a definitive diagnosis, cytological, histopathological, and ultrastructural examinations are necessary (11, 29-31). Transmission electron microscopy (TEM) analysis is an important technique to distinguish ENA from other nasal tumors (30).

Vascular endothelial growth factor (VEGF) is a heparin-binding growth factor in a homodimeric glycoprotein construction, specific to vascular endothelial cells (9, 18, 36). It plays an important role in vasculogenesis, angiogenesis, differentiation of endothelial cells, inflammation, and tumor formation, proliferation, and migration (6, 8, 9, 17, 27). VEGF, proven to be overexpressed in several types of cancers, as a key factor in the regulation of angiogenesis, has been shown to provide the activation of endothelial cells as well as the most specific mitogens known for the endothelial cells (7, 9, 27).

Epithelial cell adhesion molecule (EpCAM) is a surface molecule that is highly expressed in different carcinomas (23, 29). EpCAM acts as a calcium-independent homotypic cell adhesion molecule intracellularly and exhibits adhesion at the stages of cell–cell adhesion, cell aggregation, and inhibition of cell spreading (5, 20). EpCAM and VEGF expressions are not completely defined in animal tumors, and there is no report in ENA. The aim of this study was to examine VEGF and EpCAM expressions in the neoplastic cells and show their possible roles in the pathogenesis of ENA in naturally-infected goats.

Material and Methods

Tissue samples: In this study, tissues were collected from 24 goats, sent for routine diagnosis to the Department of Pathology, Veterinary Faculty, Burdur Mehmet Akif Ersoy University, from Denizli, Burdur, Isparta, and Antalya, between 2010 and 2015. Normal nasal tissue collected from five goats without ENA sent for routine necropsy were evaluated as controls.

Cytological examination: For cytological examination, touch impression smears were prepared from the fresh tumor tissue during necropsy. The slides were dried in air stream, fixed in methyl alcohol, stained with Giemsa method (Giemsa Stain, Catalog no: 1.09204.0500; Merck Millipore, Darmstadt, Germany), and then examined microscopically.

Histopathological examination: For histopathological studies, tumor samples were fixed in 10% neutral buffered formalin. The samples were then routinely processed in an automatic tissue processor equipment (Leica ASP300S, Wetzlar, Germany) and embedded in paraffin wax. Tissue sections were cut into 5-µm-thickness by a rotary microtome (Leica RM2155, Leica Microsystems, Wetzlar, Germany). The sections were stained with hematoxylin–eosin stain (HE), mounted with a coverslip and mounting medium, and examined under a light microscope (24).

Immunohistochemistry: The routine streptavidin–biotin peroxidase complex method was used for the detection of VEGF [Anti-VEGF antibody *(VG-1)* (ab1316)] and EpCAM [Anti-EpCAM (ab71916) antibody]. For the immunohistochemical examination, sections were routinely processed according to the manufacturer's instructions. Expose Mouse and Rabbit Specific HRP/DAB detection IHC Kit (ab80436) was used as a secondary antibody. Primary antibodies were omitted for negative controls. All primary serums and secondary antibodies were purchased from Abcam (Cambridge, UK), and the primary antibodies were used in 1/100 dilution.

Electron microscopy: Samples were also processed for TEM analysis. They were fixed in 2.2% glutaraldehyde and post-fixed in 1% osmium tetroxide (OsO_4) prepared in 0.1 M phosphate buffer solution. Then, the tumor samples were dehydrated in a graded series of alcohol and embedded in Araldite CY212. Ultrathin sections were taken from plastic blocks and stained with uranyl acetate/lead citrate.

Results

Chronic and progressive symptoms were characteristic in the anamnesis. Clinically, mostly purulent or seromucous nasal discharge, dyspnea, snoring, coughing, sneezing, mouth breathing, and less frequently, exophthalmos, and deformations in the skull bones were noted. Findings, such as lacrimation, conjunctivitis, facial asymmetry, and in some goats, head shaking were also detected. Rarely, depigmentation and alopecia were observed around the nostrils due to chronic nasal discharge. Anorexia and cachexia were commonly found in the infected animals. At necropsy, soft to hard tumoral

masses were observed in the nasal cavity (Figure 1). Variable sized tumors were located unilaterally or bilaterally in the ethmoidal region of the nasal cavity in all the animals. The tissues were irregularly structured as pinkish-white polypoid masses (1-2.5 cm in length), sessile (0.5-3 cm in diameter), and covered with seromucous exudate. In only two cases, invasion into the sinus frontalis was observed; however, lymph nodes or internal organ metastases were not detected.

Examination of cytological preparations revealed that the tumoral epithelial cells were generally collected in groups. The cells had marked vesicular nuclei and pale cytoplasm (Figure 2). Cytological examination revealed marked inflammatory reaction characterized by numerous lymphocytes and neutrophils.

Histopathologically, similar and characteristic findings were detected in all cases with ENA. All tumor types (tubular, papillary, and mixed), were observed in our cases. In this study, 9 tubular, 10 papillary, and 5 mixed tumor types were diagnosed. The tumor cells were mostly uniform and cubic, and the nucleus was oval to round and hyperchromatic (Figure 3). Mitotic figures were rarely seen; however, in some cases, pleomorphic cells were identified. In nine of our cases, abundant lymphocyte, plasma cell, and macrophage infiltration was observed.

Immunohistochemistry demonstrated increased VEGF and EpCAM expressions in the tumoral cells compared with those in the normal tissue. It was noticed that the EpCAM activity was high in most of the tumor cells and weak to moderate immunoreaction was seen in some of the tumor cases (Figure 4).

Figure 1. Gross appearance of the tumoral masses (arrows) that completely filled the nasal cavity in a goat.

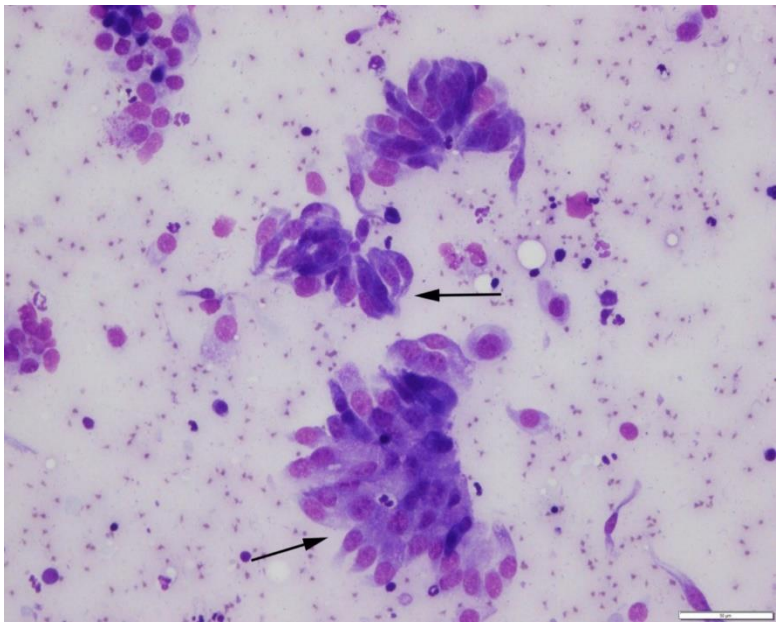

Figure 2. Cytology of the tumoral cells, irregular, grouped cuboid cells (arrows), Giemsa stain, Bar: 50 µm.

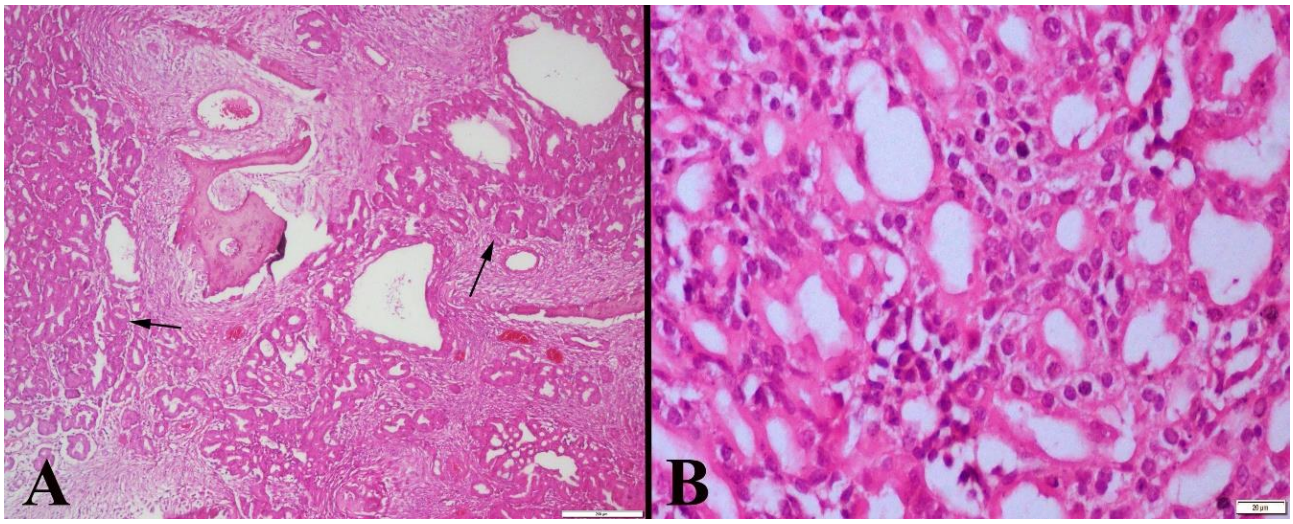

Figure 3. A. Histopathology of the tumoral masses, increase in nasal glands (arrows), H&E, Bar: 200 µm, **B.** Higher magnification of the tumoral cells, H&E, Bar: 20 µm.

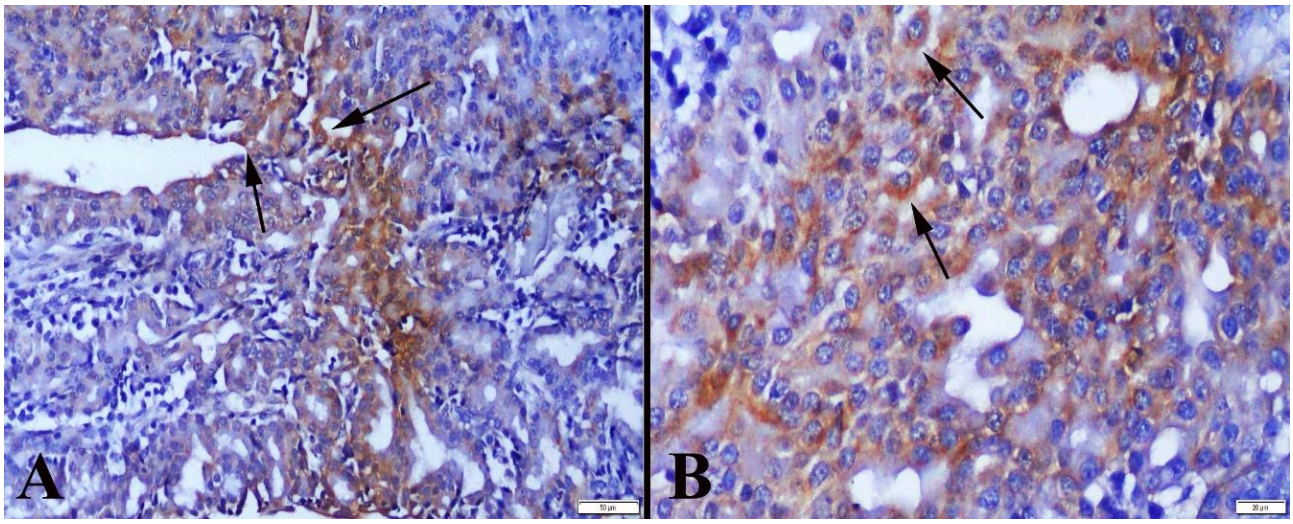

Figure 4. A. Increased EpCAM immunoreaction of the tumoral cells (arrows), Streptavidin-biotin-peroxidase complex method, Bar: 200 µm, **B.** Higher magnification of the EpCAM expressed cells (arrows), Streptavidin-biotin-peroxidase complex method, Bar: 20 µm.

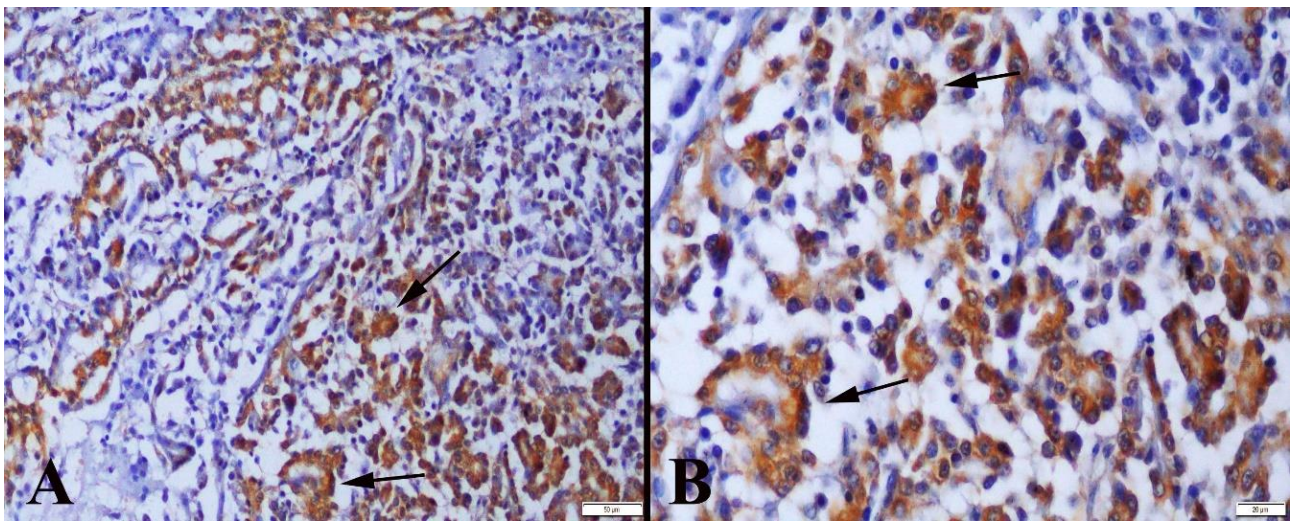

Figure 5. A. Increased VEGF activity in the tumoral cells (arrows), Streptavidin-biotin-peroxidase complex method, Bar: 100 µm, **B.** Higher magnification of the VEGF immune-positive cells, Streptavidin-biotin-peroxidase complex method, Bar: 20 µm.

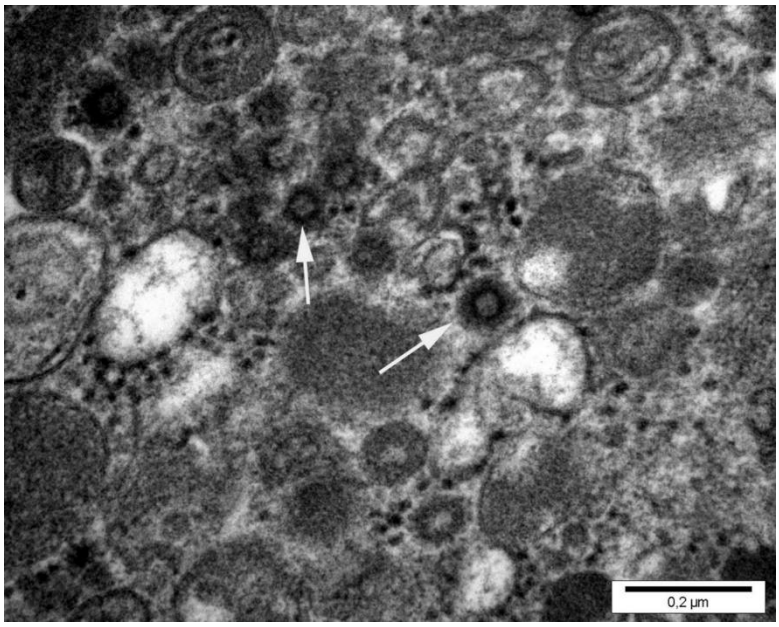

Figure 6. Ultrastructural appearance of the tumoral mass, retroviral particles (arrows) in the cytoplasm of the cells.

In this study, no correlation between EpCAM expression and the subtypes of tumors was found. EpCAM reaction was generally determined in the cytoplasm near the cell membrane. No or slight EpCAM expression was evident in the normal cells. Similar findings were seen with VEGF immunoreactions and the expression increased in the tumoral cells (Figure 5). In this study, increased EpCAM and VEGF immunoreactions were observed in both the tumoral and some interstitial cells.

In electron microscopic examination, in the cytoplasm of the neoplastic cells, numerous electron opaque and round to oval secretory granules surrounded by a membrane, ranging from 0.2 to 1µm in diameter, were observed. Ultrastructurally, intracytoplasmic spherical retrovirus-like particles 70-90nm in diameter, were demonstrated (Figure 6).

Discussion and Conclusion

ENA was first reported in Turkey in 2010 and became an endemic problem in the Burdur province (2, 3, 30-32). Because of the chronic behavior and tumoral masses located in the nasal cavities, the diagnosis of the disease is generally difficult, and an exact incidence is unknown in the goats in Turkey. Another reason for wrong diagnosis is related to a sporadic behavior and few animal losses due to the disease in a short time. However, the illness can cause an important economic loss over years.

Cytological and histopathological examinations are important for the evaluation of the malignancy. However, it is more important to detect the viral particles in the tumoral cells for a definitive diagnosis of ENA (30, 31). To examine the presence of the viral particles, TEM analysis of the tumor samples was performed in this study, and retroviral particles were demonstrated in the cytoplasm of the cells.

EpCAM, a type 1 transmembrane protein, is an epithelial adhesion molecule localized at the basolateral edge of epithelial cells. Studies have shown that EpCAM has an important role in cell interaction and proliferation. EpCAM is highly expressed in human cancer cells, such as those of colorectal, breast, stomach, prostate, ovarian, and lung cancers. It is the first discovered human tumor-associated protein that can be identified with monoclonal antibodies (4). EpCAM expression has been associated with poor prognosis in some types of cancers and good prognosis in other types of cancers. While EpCAM expressions in primary breast cancer are associated with decreased survival, in patients with colorectal cancer its associated with increased survival (25). In renal cell carcinoma (chromophobe and collecting duct origin), EpCAM was found to be an important prognostic marker (19). *In vitro* and *in vivo*, EpCAM expression has been associated with the spread of breast cancer to surrounding tissues (10). In accordance with the literature, this study showed that EpCAM activity increased in the ENA cells, and this protein may play an important role in the ENA pathogenesis.

VEGF is a heparin-binding glycoprotein, synthesized by numerous cells, such as tumoral, smooth muscle, and endothelial cells (16, 28). It is the most important angiogenic factor and commonly expressed in cancers, such as those of the lung and colon (9, 18). Although there are several studies on the expression of VEGF in most types of human cancers, its role in pathogenesis has not been completely understood (7, 26, 33). In our study, VEGF immunoreaction was found to

increase in ENA, and this increase was thought to be related to the possible role of VEGF in tumorigenesis.

Head, neck, cervix, colon, and small-cell lung cancers markedly express VEGF (14). VEGF is reported to affect survival in cases of local and locally invasive colorectal cancers, according to a multivariate analysis (21). Because our study was performed on dead animal materials, there was no link between VEGF and the clinical prognosis.

In solid tumors, tumor growth, invasion, and metastatic ability are found to be significantly correlated with the VEGF levels. It has been suggested that increased levels of VEGF in most of the solid tumors, such as those of the breast, lung, prostate, colorectal, bile duct, and liver, may be associated with poor prognosis (1). In another study, they found a correlation between improvement in human mammary tumors and tumor VEGF expression (18). In a similar study, they found that VEGF expression enhances the angiogenic activity (22). In one study, VEGF expression in patients with breast cancer was found to be a malignancy determining factor (34, 35). In another study, increased VEGF expression was reported in tumor-associated samples with significant lymph node involvement (15).

It is not practical to examine the duration of disease or predict prognosis and survival in farm animals. The main purpose is the early detection of chronic and contagious diseases such as ENA and the removal of the patient from the flock. The present study shows that EpCAM and VEGF may be important molecules that can serve this purpose in suspect surveillance. ENAs in sheep and goats are invasive, low metastatic tumoral diseases, originating from ethmoidal turbinate, with increased prevalence all over the world. In this study, histopathological and immunohistochemical studies were performed on 24 tumor samples, previously diagnosed as ENA. Increased VEGF and EpCAM expressions were observed in the ENA tissues.

The incidence of ENA is increasing among goat flocks. This endemic problem has been threatening the economy indirectly, day by day, in the Mediterranean region including Burdur province and other provinces, due to bad prognosis in goats. These selected markers may facilitate to better understand the malignant behavior of ENA.

To the best of the authors' knowledge, expressions of VEGF and EpCAM markers have not been previously used in determining prognosis of the tumors. Moreover, electron microscopy as well as the methods used in this study provides an additional value in diagnosis, because retroviral particles could not be demonstrated in every previous study. EpCAM, mentioned as a new therapeutic target in human tumors, may be useful for the treatments and vaccinations against retrovirus-induced tumoral progress or the management of ENA.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Comparison of qualitative and quantitative alterations caused by use of various fixatives in the myocardium

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Abstract: In the field of anatomy, preservation techniques used to preserve the integrity of specimens prepared for use as educational and research material are very ancient. It is desirable that these materials be as close as possible to the characteristics of living animal tissues in order to be an effective educational material. One of the most important criteria in determining similarity to fresh cadavers is the color and odor changes in the tissues. Therefore, the aim of this study is to reveal the anatomical and physiological changes on myocardial tissue, fixed by 4 different solutions in which qualitative and quantitative methods such as color analysis, sensory analysis, and microbiological tests. As a result, it was determined that a newly developed enriched saline solution and low concentration formaldehyde solution can be used safely for the preparation, preserve and long term use of educational materials.
Keywords: Colorimeter, fixation, sensory analysis, solution.

Farklı tespit solüsyonları kullanımının miyokard üzerinde neden olduğu nitel ve nicel değişikliklerin karşılaştırılması

Özet: Anatomi alanında, eğitim ve araştırma materyali olarak kullanılmak üzere hazırlanan örneklerin bozulmadan korunabilmesi için kullanılan prezervasyon teknikleri çok eski zamanlara dayanır. Bu materyallerin etkin bir eğitim materyali olabilmesi için mümkün olduğunca canlı hayvan dokularındaki özelliğe yakın olması istenir. Canlı hayvan dokularına benzerliğin belirlenmesindeki en önemli kıstaslarından biri şüphesiz ki dokulardaki renk değişimidir. Bu sebeple bu çalışmada, 4 farklı tespit solüsyonuyla hazırlanmış olan koyun kalplerindeki anatomik ve fizyolojik değişikliklerin, örneklere uygulanan renk analizi, duyusal analiz ve mikrobiyolojik testlerle nitel ve nicel olarak ortaya konulması amaçlandı. Sonuç olarak, yeni geliştirilen zenginleştirilmiş tuzlu su solüsyonu ile zenginleştirilmiş düşük konsantrasyonlu formaldehit solüsyonunun, eğitim materyallerinin hazırlanması, korunması ve uzun vadede kullanılabilir olması için güvenle kullanılabilecek solüsyonlar olduğu belirlendi.
Anahtar sözcükler: Duyusal analiz, kolorimetre, solüsyon, tespit.

Introduction

There are lots of anatomical techniques used for a long time to ensure that animal and human bodies are preserved intact after death as educational and research material. The process, first began with the mummification of corpses in Egypt and Chinchorro (a region of Arica, Chile and Peru coast) cultures in the years 7000-5000 BC and has continued to until development of various methods like as the plastination technique, which was developed by Gunter von Hagens in the late 1970s (2, 6, 12, 24). As written in many articles, cadaver-based education is used not only for the anatomy but also for clinical sciences teaching, especially surgery. It is desirable that these cadavers, which are used for practice, should have a similar quality to fresh cadavers, in order to be an effective educational material, if it is possible (8, 9, 17, 21, 25, 29). However, in recent years, studies on the development of different cadaveric preparation techniques have begun to increase especially with the increase of ethical rules, in order to reduce the use of cadavers prepared from alive animals (26). Although new fixatives have been developed with varied techniques and laboratory process, decomposition is still not completely prevented. Because of the physiological parameters

change when the cells and tissues are taken out from the body. Accordingly, a number of pathological reactions such as putrification and autolysis are formed. In other words, the changes are caused by putrification, which is formed by the bacterial activation and autolysis, which is developed with the release of intracellular autolytic substances, after physiological death occur. When considered at the cellular mechanism, firstly cytoplasmic content and lysosomal enzymes are released outside the cell; necrotic cell death and diffuse tissue damage are observed (19, 20). Because of these reactions, one of the most important criteria of decomposition is the color change that occurs in tissues. The color changes of tissues can be displayed on the X/Y coordinates and can be computed numerically in the CIE (International Lighting Commission) L* a* b* system standards via the usage of the tristimulus color measurement devices (3, 13, 14, 18, 28).

The aim of this study was to quantitatively evaluate the anatomical and physiological changes in sheep hearts, which were fixed in 4 different fixation solutions, by color measurement and microbiological analysis. In addition, the study was qualitatively strengthened with the sensory analysis test applied to semi-educated panelists. Two of the four fixatives used here were newly developed and their effectiveness were demonstrated.

Material and Methods

When determining the sample to be used in the study the Power of test (1-β) was 0.80 calculated by G. Power statistical Packet software. Accordingly, a total of 35 sheep hearts obtained from a licensed slaughterhouse in Ankara Province were used. Cardiac tissue was preferred in the study both it is an organ with high blood content anatomically and it has striated muscles that perform at high capacity physiologically. The samples were initially divided into 5 different groups that one of them would being a control. For this purpose, 4 different fixation solutions were prepared. The whole groups were named according to the content of solutions. The first group is a saline solution (SS) that prepared with low concentration saline (<20%). The second group is an enriched saline solution (INSALT) that prepared with 26.5% saline, ethyl alcohol, polyethylene glycol, and citric acid. The third solution is a 10% formaldehyde solution (FS), a strong disinfectant. The fourth solution is the lower concentration of formaldehyde enriched with ethyl alcohol and polyethylene glycol (EFS). Moreover, in the fifth group, the samples kept in +4 C^o in the refrigerator without any solutions for being control. The salt used in the first and second groups for fixation; was used since it serves to adjust the osmotic structure of the solutions and the acid-base balance. It is also preferred inasmuch as it is the cheapest, non-toxic and most easily accessible chemistry available in fixatives (4, 10). Despite all the known toxic effects and disadvantages, formaldehyde, which is still widely used, was utilized in the third and fourth solutions (5, 6, 11). The ethyl alcohol used in the second and fourth groups was preferred because of its bactericidal, antiviral, antifungal and antimycotic effects as well as its ability to easily penetrate into deep tissues and increase the effectiveness of formaldehyde. Polyethylene glycol was added to the solutions as it increased the disinfectant effect of formaldehyde and reduced mold formation when used together with formaldehyde (2, 6, 15). In addition, the citric acid used in the third solution was added to the mixture due to the known antioxidant and anticoagulant properties (22, 23). Besides, in the fourth group formaldehyde was used at a very low concentration of 0.5% to reduce the toxicity ratio of the solution. A total of 35 sheep hearts, 7 specimens for each group, were obtained from a licensed slaughter house. The organs were brought to the laboratory within post-mortem first hour. Each was then numbered separately. In the study, a colorimeter device (Konica Minolta CR-400) with the capability to obtain results on international L* a* b* measurement standards was utilized to determine the color alteration (14). The measurements were taken from 3 dividual points on the myocardial tissue to increase the accuracy of the results and estimate the averages statistically. While these mentioned three points were determined, the hearts were arranged on the bench floor where the *facies auricularis* would get in touch with. The first landmark; right side of *sulcus interventricularis paraconalis,* the second marker point; left side of *sulcus interventricularis paraconalis* and the third marker point is *apex cordis* of the organ was preferred. Then, all samples were made post-mortem initial measurements from marked-point without any process. Then, the post-mortem initial measurements on the marked-point of organs were received from all samples without any process. All samples were taken in organ storage containers containing divergent fixatives. The samples which were not treated with any solution were put in an airtight and closed container and kept in the refrigerator at +4 ºC for storage. The measurements were received on post-mortem first day and repeated on the 7^{th}, 15^{th}, 30^{th}, 60^{th} days and 1^{st} year in order to observe the time-dependent effect of anatomical and physiological changes. Besides, total bacteria, total fungus and yeast were examined from the specimens collected from surface and deep of cadaver and the fixation solutions at the end of the study for all groups. In addition, the sensorial properties of the samples were determined by 7 semi-trained panelists using scoring test modified from Meilgaard et al. (16) at the end of the experiment. A fresh cadaver sample was given to the panelists as the control sample and they were asked to evaluate the similarities of the samples were

kept in SS, INSALT, FS and EFS in terms of appearance, odor and texture (elasticity) over 9 points compared to the control sample. The panelists rated the samples named A, B, C, D without informing them about the solutions. In the new marking for panelists, letters described the solutions. Accordingly, to 'A' was the saline solution, 'B' was enriched saline solution; 'C' was the formalin solution, and 'D' was enriched formaldehyde solution.

The color changes (L* a* b*) of the five different groups on the different days and sensory analyses data were compared with the repeated measure ANOVA test (1^{st}, 7^{th}, 15^{th}, 30^{th}, 60^{th} days and 1^{st} year). Duncan multiple comparison tests were used for determining which groups were different if groups were identified as important. Statistical analyses were estimated by SPSS v.15 packet program.

Results

The samples in the low concentration saline solution were removed, notwithstanding color change, from the experiment on the 8^{th} day. Because of the perforation and deterioration of the organs, there was a severity bad smell affecting the vomiting center. Similarly, the fifth group which formed the control group of the study, was removed on the 15^{th} day for the same reasons. According to the obtained data; the closest values of the brightness from the white (L*) and the color change from red to green (a*) and from yellow to blue (b*) to the fresh tissue was observed from the enriched formaldehyde solution (EFS) at the end of the 1^{st} year (Table 1). While the 10% formaldehyde solution increased the luminosity (L*) value of the organs by approximately 26.15 units, it reduced the red color by 12.37 units. Therefore, it was observed that the color of organs was changed to the different shades of gray. In addition, it was observed that these organs lost elasticity of the tissues in parallel with they were durable and extremely rigid. The samples waiting in the INSALT solution lost the reddish color approximately 12.69 units and the yellow color by 0.18 units while increased the brightness only by 5.55 units, during the first year (Table 1). However, it was observed that these samples were more vivid than the waiting samples in the 10% formaldehyde solution. When all of the data were obtained, the results were significant on the brink of $P<0.05$ statically (Table 1). In addition; no perforation or malodor formation was observed in specimens waiting in this solution. On the contrary, it was noted that the specimens were so durable, robust and at the same time, they were quite elastic that they could be used in training. According to the results of the sensory analysis performed, sample D was prepared with EFS was the most similar sample to the fresh cadaver (control sample) in terms of appearance, odor and textural (elasticity) characteristics. Sample D was described by the panelists as “almost the same as the control sample”, followed by sample B was prepared with INSALT, C was prepared with FS and A

Table 1. Color change graphic on the myocardium according to days.

		Control (∑±SEM)	SS (∑±SEM)	INSALT (∑±SEM)	FS (∑±SEM)	EFS (∑±SEM)	P
	L*	38.66 ± 1.15^{b}	40.99 ± 0.89^{b}	39.81 ± 0.80^{b}	35.12 ± 0.72^{a}	39.81 ± 1.08^{b}	***
1^{st} d	a*	18.94 ± 0.62	17.79 ± 0.39	17.41 ± 0.46	19.02 ± 0.54	18.28 ± 0.69	-
	b*	12.08 ± 0.71^{b}	12.64 ± 0.54^{b}	11.88 ± 0.37^{b}	9.94 ± 0.68^{a}	12.12 ± 0.56^{b}	*
	L*	34.38 ± 0.74^{a}	49.65 ± 2.82^{c}	39.10 ± 1.04^{b}	47.36 ± 0.66^{c}	39.76 ± 1.36^{b}	***
7^{th} d	a*	16.25 ± 0.76^{c}	7.46 ± 0.51^{b}	5.38 ± 0.20^{a}	8.63 ± 0.21^{b}	17.15 ± 0.59^{c}	***
	b*	5.00 ± 0.20^{a}	10.65 ± 0.41^{b}	13.54 ± 0.67^{c}	10.07 ± 0.67^{b}	12.56 ± 0.94^{c}	***
	L*	36.92 ± 0.65^{a}	-	38.98 ± 1.16^{a}	47.35 ± 0.48^{b}	39.85 ± 1.36^{a}	***
15^{th} d	a*	12.32 ± 0.42^{c}	-	5.36 ± 0.26^{a}	8.61 ± 0.20^{b}	17.26 ± 0.63^{d}	***
	b*	7.55 ± 0.42^{a}	-	13.77 ± 0.49^{c}	9.79 ± 0.60^{b}	12.61 ± 0.86^{c}	***
	L*	-	-	39.45 ± 1.14^{a}	47.27 ± 0.63^{b}	39.03 ± 1.36^{a}	***
30^{th} d	a*	-	-	5.21 ± 0.21^{a}	8.67 ± 0.16^{b}	17.33 ± 0.69^{c}	***
	b*	-	-	13.12 ± 0.70^{b}	10.11 ± 0.57^{a}	13.72 ± 0.80^{b}	**
	L*	-	-	41.11 ± 1.42^{a}	48.00 ± 0.71^{b}	39.20 ± 1.50^{a}	***
60^{th} d	a*	-	-	4.77 ± 0.27^{a}	8.29 ± 0.24^{b}	16.57 ± 0.62^{c}	***
	b*	-	-	12.16 ± 0.67^{b}	10.11 ± 0.65^{a}	13.76 ± 0.71^{b}	**
	L*	-	-	45.36 ± 1.27^{a}	61.27 ± 1.70^{b}	41.36 ± 1.42^{a}	***
1^{st} y	a*	-	-	$4.72 \pm 0,35^{a}$	6.65 ± 0.28^{b}	16.43 ± 0.53^{c}	***
	b*	-	-	11.70 ± 0.58^{b}	9.08 ± 0.57^{a}	12.21 ± 0.64^{b}	**

$^{a, b, c}$: means in a row with different superscript letters are statistically different; d: means days; y: means year; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$, -: $P>0.05$ (insignificant). SS: Saline solution; INSALT: Enriched saline solution; FS: Formalin solution; EFS: Enriched formaldehyde solution.

Table 2. Sensory analyses of the heart samples for all groups.

	A (∑±SEM)	B (∑±SEM)	C (∑±SEM)	D (∑±SEM)
Apperance	0.29 ± 0.49	7.57 ± 1.13	6.43 ± 0.79	8.67 ± 0.52
Odor	0 ± 0	7.29 ± 0.95	5.29 ± 0.95	8.00 ± 071
Texture (Elasticity)	0.29 ± 0.49	8.00 ± 0.89	3.86 ± 0.90	7.14 ± 0.90

*The degree of similarity of the samples to the control sample was evaluated between 0 and 9 points. A: Saline solution (SS); B: Enriched saline solution (INSALT) C: Formalin solution (FS); D: Enriched formaldehyde solution (EFS).

was prepared with SS, respectively. In addition, all panelists stated that A, B, and D did not have a chemical smell, while 5 panelists indicated that sample C had a disturbing chemical smell. The sample A received lowest scores (Table 2) for all properties analysed and all of the panelists indicated that there was no similarity between sample A and the control sample. It has also been noted there was purification in sample A by all the panelists. Besides no pathogenic microorganisms have been detected in the samples keeping in INSALT, FS and EFS solutions analysed for total bacteria, total fungus, and yeasts. However, high levels of bacteria, fungi, and yeast were found in the samples that were control and waiting in SS solution. At the end of the 15th day, in the total bacteriological culture analysis of the samples kept in the SS fixative; *Lactobacillus* spp. and *E. coli*, in the total fungal analysis; *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp. and *Rhizopus* spp., in the total yeast analysis; *Candida* spp. and *Saccharomyces* spp. were isolated and identified. On the contrary, even at the end of the study, no pathogenic microorganisms mentioned above were found in the other groups.

Discussion and Conclusion

Many studies have confirmed that chemical fixation solutions used when preparing cadaver by classical methods have risks in terms of human health (1, 2, 4-6, 10, 12). For this reason, the effects of 4 different fixative solutions that include formaldehyde and non-formaldehyde on heart tissue were studied in this study. When choosing the fixative solutions, the 10% formaldehyde solution, which is known to be toxic and which is still used despite all its disadvantages, and saline solution, which is totally nontoxic and controversial to use alone despite its many advantages, were preferred. In contrast to Bakici et al. (3), fixatives used in the past with high formaldehyde concentrations were not modified in this study. Low concentration formaldehyde and high degree saline solutions were prospered with other chemicals as ethyl alcohol, polyethylene glycol, citric acid directly in this study. Thereby new fixative solutions were developed by this study. These new solutions and other known solutions were compared in terms of the positive and negative aspects of the effects on the hearts. The newly developed fixation solutions were examined in terms of their preparation methods, ease of handling and their impact on myocardial tissue. In the light of this information, the choice of the fixatives was left to the preference of the researchers in all aspects. The theory that saline solution is an unsuccessful fixation solution when used at a concentration lower than 20% as explained by Fricker et al. (7) is confirmed by this study. Similar to the article by Janczyk et al. (10), the salt was utilized in this experiment for targeting develop a non-toxic fixative. However, contrary to Janczyk et al. (10) the salt was used not rate of 23%, was added the mixture at a rate of 26.5%, which would produce a saturated salt water concentration. In contrast to Janczyk et al. (10) instead of 3% ascorbic acid, the 5% citric acid, which due to known anticoagulant and antioxidant properties was supplemented in INSALT solution. Some literature (6, 11, 27) examining the color changes in the tissues have observed that the cadaver was got lost their color, based on fixatives consisting of formaldehyde coagulates the blood rapidly. This situation was confirmed with samples fixed in 10% formaldehyde solution quantitatively by approximately 26.15 units increased brightness degree and 12.37 units changed reddish color to green. On the other hand, as a result of decreasing the formaldehyde ratio to 0.5% in the EFS, it was observed that the samples detected with this solution best preserved the color and were the closest samples to fresh tissue. Thus, it was revealed that EFS can be utilized because being both a less toxic and efficient fixative by this study.

As a result, the deterioration effects of the fixatives in which the tissues and organs prepared to be used as educational material in the field of anatomy were examined in terms of color variance on the sheep heart. These color changes were quantified for the first time, and all obtained data were compared to compose meaningful results between groups statistically (Figure1). As a result of the research, it was concluded that samples which were fixed with the low concentration of saline solutions and kept without any treatment were not possible to use for a long time in education. The microbiological results revealed that Turan et al. (27) encountered *Bacillus* spp.

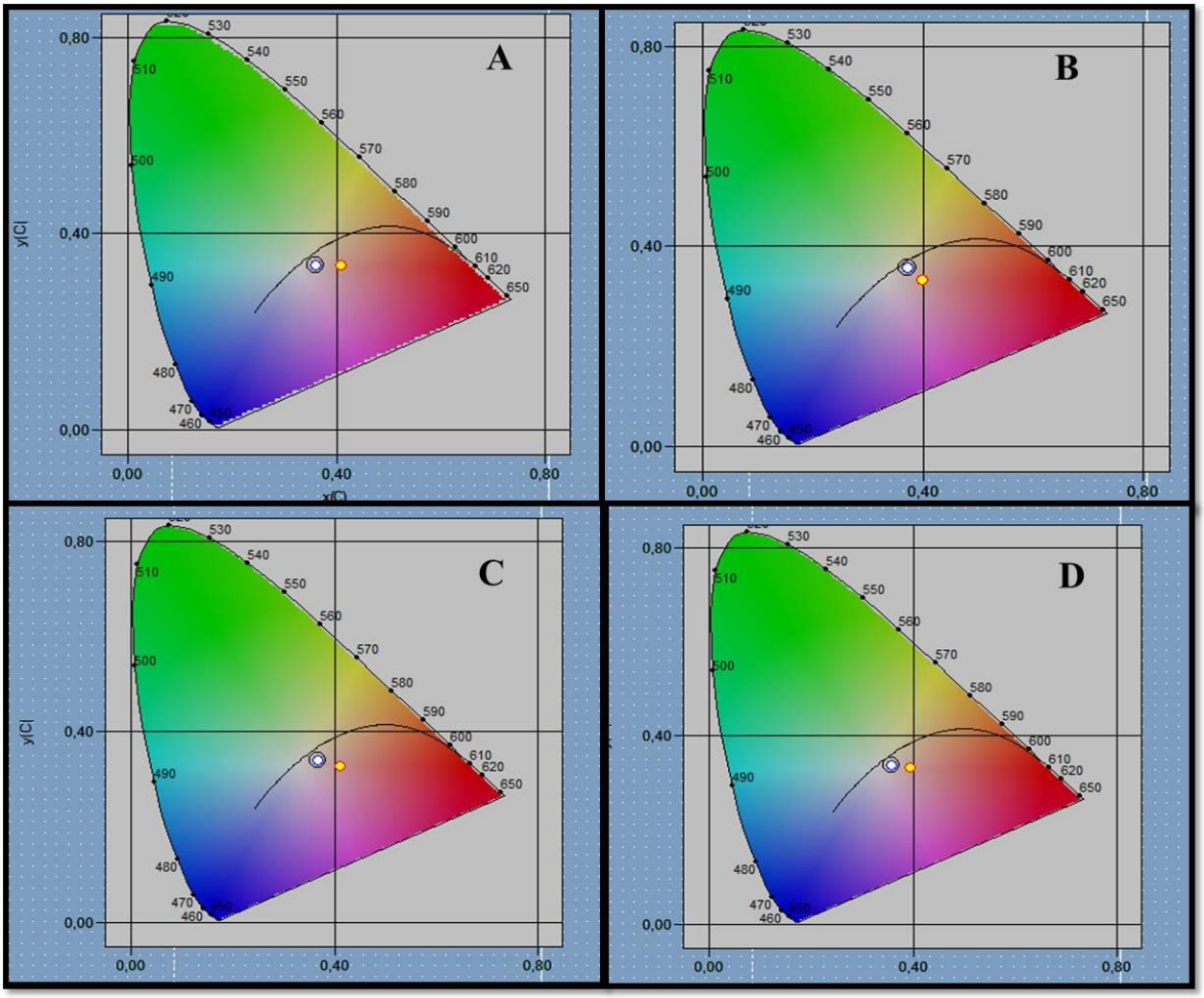

Figure 1. Color change chart; A: Saline solution (SS); B: Enriched saline solution (INSALT); C: Formalin solution (FS); D: Enriched formaldehyde solution (EFS).

rarely. In this study, *Bacillus* spp. and other microorganisms that mentioned in the results were observed in control and SS group. However, no microorganisms including *Bacillus* spp. were found in other groups (FS, EFS, INSALT). Accordingly, EFS and INSALT solution delayed decomposition as FS and could be used during the preparation of training materials. Also, the results of the sensory analyses were found to support these data.

Consequently, it was detected that the INSALT solution can be preferred for the preparation of organs to be used as training material. Because there are many advantages such as being a non-toxic solution, durability, and elasticity of the organs and color change rates in tissues similar to fresh tissue. Despite the fact that the EFS solution contains formaldehyde in low concentrations, it can be safely used for the preparation of organs in anatomy training. Because it has positive effects on the organs likewise the INSALT solution. Based on the data obtained from this study, it is planned to work on the fixation of whole-body cadavers and organs of other mammals and avian with these solutions.

Acknowledgements

The newly developed solution named "Salt Content Cadaver Solution (INSALT)" was registered by the Turkish Patent and Trademark Office owned by Ankara University with reference number 2018/08311 on the date of 11.06.2018.

Conflict of Interest

The authors declared that there is no conflict of interests.

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Prevalence and phylogenetic analysis of gastrointestinal helminths (Nematoda: Trichostrongylidae) in ruminant livestock of northwest Iran

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Abstract: Trichostrongylidae family is considered as a group of gastrointestinal nematodes of ruminants with widespread distribution in the world. The parasites are the major causes of productivity loss in many countries including Iran. This study was carried out to determine the distribution of abomasal Trichostrongylidae among livestock of Qazvin, Iran. Totally, 160 abomasum samples including 83 from sheep, 72 from cattle and 5 from goats were collected from slaughterhouses throughout the Qazvin Province. The ribosomal DNA internal transcribed spacer 2 (ITS2) region was amplified using PCR followed by sequencing 13 different isolates. Phylogenetic analysis of the identified sequences was performed using MEGA 7 software. The prevalence rate of the nematodes was 19.4% among the specimens. Also, two spices including *Marshallagia marshalli* and *Teladorsagia circumcincta* were identified among the isolates with *M. marshalli* at higher frequency. No *Trichostrongylus* spp. was detected in the region. Since livestock plays a major role in ecosystem balance and as some species of nematodes have a zoonotic nature, it is necessary to proceed with more comprehensive epidemiologic studies to clarify the infection rate among the human population living in the region.

Keywords: Iran, ITS2-rDNA region, Phylogenetic analysis, Qazvin, Trichostrongylidae.

Introduction

The family of Trichostrongylidae is considered as the most important gastrointestinal parasites among sylvatic and domesticated ruminants with worldwide distribution (19, 38). Several species of the family were reported such as *Teladorsagia circumcincta*, *Marshallagia marshalli* and *Trichostrongylus* spp. (19, 33, 34, 38).

Human infection by *Trichostrongylus* spp. could occur by ingesting the infective-stage larvae or through skin penetration by larvae (14). Human infections are frequently free of apparent clinical symptoms, although gastrointestinal signs may occur in some patients (31, 39). The importance of veterinary medicine is not seriously taken into account and this has caused reduced production, economic losses, and public health concerns. These nematodes are considered as major causes of veterinary and zoonotic infections (12, 32). Differentiation and improved diagnosis between the species are necessary for control programs as effective control strategies can lead to positive effect over the economy of different countries (3).

Conventional methods based on morphological characters such as spicules, gubernaculum, and caudal bursa in male worms are reliable to detect the species nevertheless, these methods cannot discern the species of female worms (12, 31).

Molecular biology assays are applied for identification and phylogenetic analysis of different species of nematodes (38). Although several ribosomal and mitochondrial regions were used to survey the nematodes, investigating the ribosomal DNA sequencing (particularly the ITS2 region) has been shown to be a useful tool for this purpose as the ITS-2 gene region can clarify the intraspecific homogeneity and interspecific divergence of sequence in the species of gastrointestinal nematodes (5, 20, 31, 36).

It is well documented that Iranian researchers have a long term experience over examining the gastrointestinal nematodes among humans and animals by morphological features (2, 7, 11). Domestically, recent molecular analysis studies are limited to few investigations concerning the diagnosis of *Trichostrongylus* species,

without any data available for other species of the Trichostrongylidae family in the country (13, 30, 31). Due to lack of data on nematodes in the study region, the present research aimed at analyzing the morphological features as well as molecular characterization of ITS2 region of different species of the helminthes.

Material and Methods

Sample collection and specimens examined: Totally, 160 abomasum samples were collected from 83 sheep, 72 cattle and 5 goats during 2017-2018. The animals originated from Qazvin Province, located in northern margin of central Iran and were slaughtered at two local abattoirs of the cities of Qazvin and Takestan (Figure 1). This study was approved by the Medical Ethics Committee of Qazvin University of Medical Sciences (Approval code: IR.QUMS.REC.1396.375). All specimens were examined for infectivity by Trichostrongylidae family through washing the content of each abomasum by passing through 20, 40 and 100 mesh sieves and observed with stereomicroscope. The helminths were collected and cleaned with normal saline followed by addition of lactophenol to observe their morphological features. The specimens were preserved in 70% ethanol and kept at room temperature.

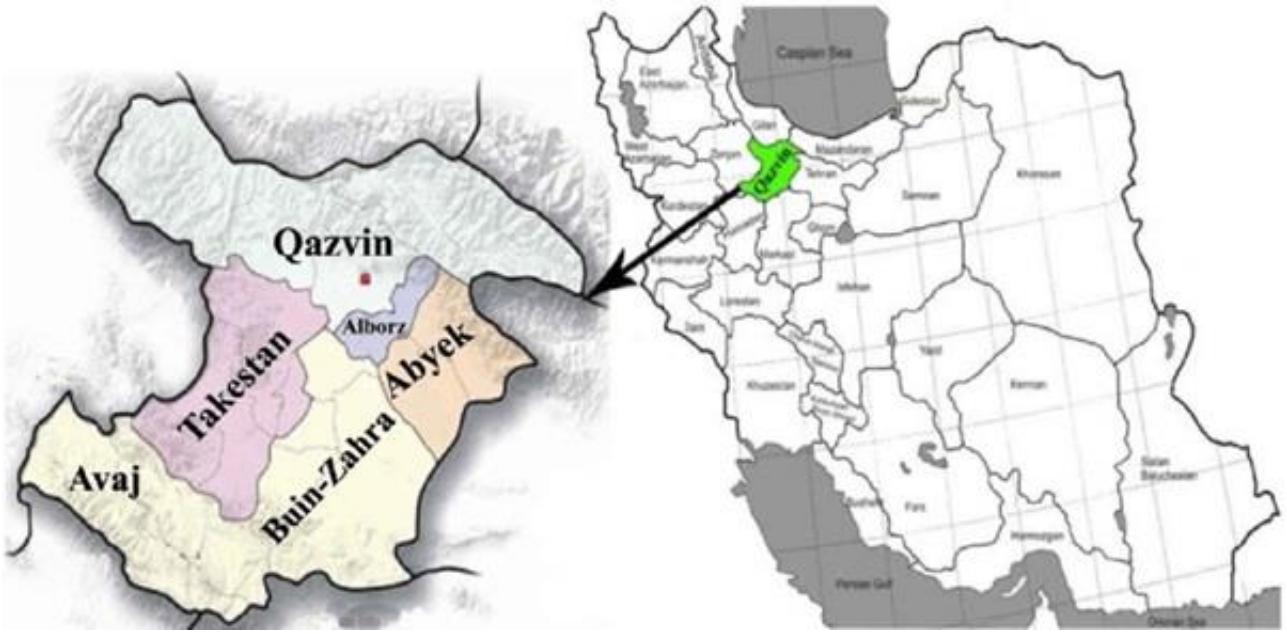

Figure 1. Map of Qazvin Province, located in the northern margin of central Iran.

DNA extraction and PCR assay: Twenty female specimens were randomly selected for DNA extraction. The helminth was minced by 6 rounds of freeze-thaw cycles using 200 mg tissue lysis buffer and the finely minced worm was used the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) for DNA extraction according to the manufacturer's recommended protocol.

Fragments of ribosomal DNA internal transcribed spacer 2 (ITS2) region with approximately 328 bp was amplified with specific primers (30). Thirty microliter reaction volume containing 15 µl of PCR mix including Taq DNA polymerase, dNTPs and $MgCl_2$ (2 x Master Mix RED Ampliqon, Denmark), 11 µl of molecular biology grade water, 1 µl of each primer (10 pmol/µl) and 2 µl of DNA template was applied for each reaction. The PCRs were included the following temperatures, 94°C for 20 s (denaturation), 55°C for 20 s (annealing), 72°C for 30 s (extension) for 35 cycles and a final extension at 72°C for 5 min.

Sequencing and phylogenetic analysis: Following the purification (AccuPrep® PCR/Gel Purification Kit-Bioneer, Korea) of PCR products, each specimen was sequenced using Applied Biosystems ABI 3730xl DNA analyzer sequencing (Bioneer, Korea). The nucleotide sequences were compared with each other and with reference sequences using BioEdit software and adjusted manually (18). Multiple sequence alignment was made with the ClustalW method and further compared with the sequences present in the GenBank sequence database. Phylogenetic tree was constructed using Tamura 3-parameter model of ML (Maximum likelihood) method by the Molecular & Evolution Genetic Analysis software version 7 (MEGA 7.0). Bootstrap value was considered based on 1000 replications. *Necator americanus* sequence was considered as outgroup (Figure 2).

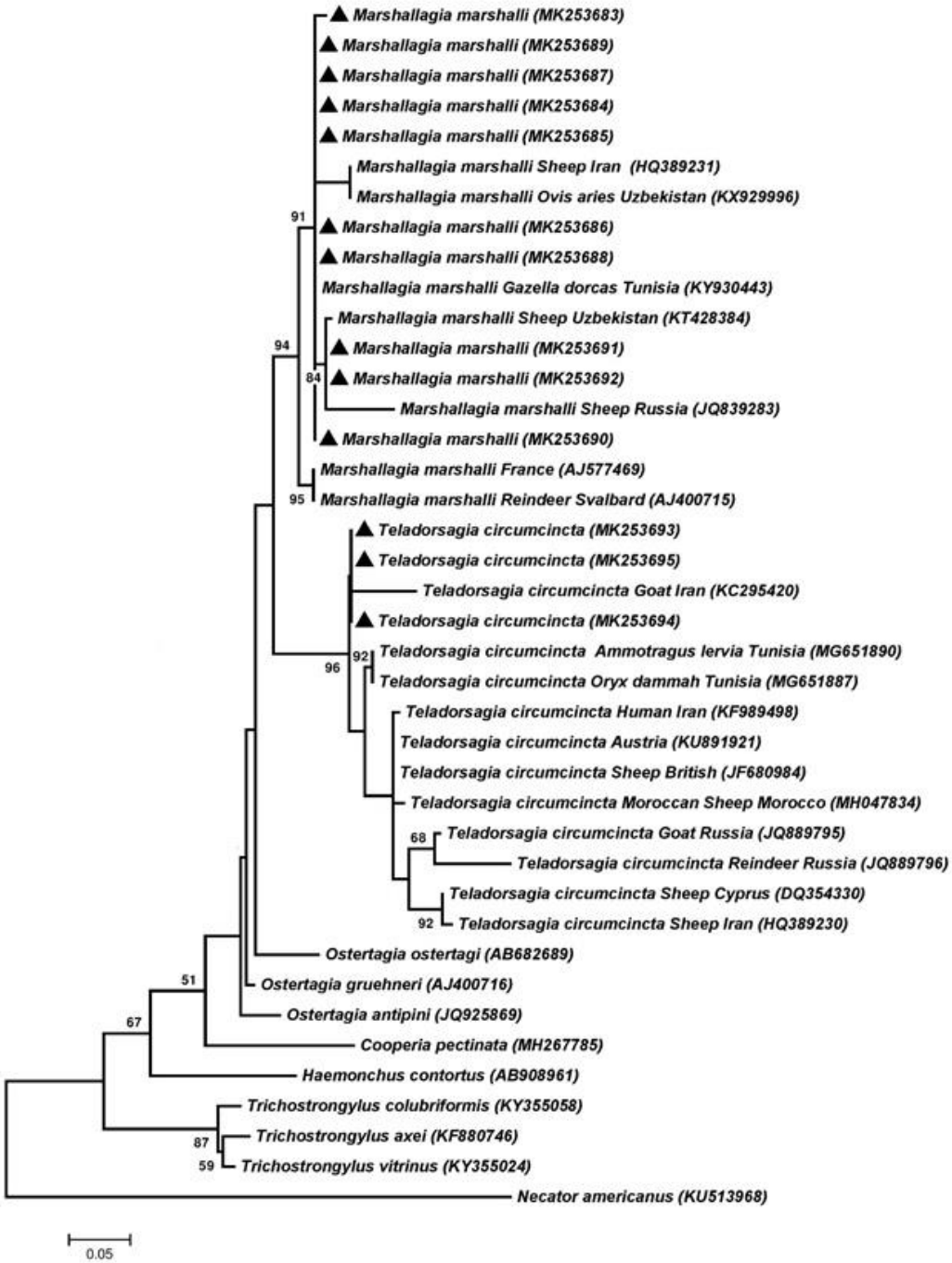

Figure 2. Phylogenetic tree of Trichostrongylidae family obtained in this study (▲) and reference sequences retrieved from GenBank based on ITS2 nucleotide sequences and constructed using the Tamura 3-parameter model in MEGA7 software. *Necator americanus* considered as an out group.

Results

Out of 160 specimens collected from sheep (51%), cattle (45%), and goats (3%) in the present study, 19.4% (31/160) samples were found to be infected with Trichostrongylidae. The prevalences of nematodes were 33.7% (28/83) and 60% (3/5) among sheep and goat, respectively. No infection was found in cattle specimens. Two species including *Marshallagia marshalli* and *Teladorsagia circumcincta* were identified among the isolates by morphological features which was later confirmed by molecular analysis (Figure 3). Infection rate of *M. marshalli* and *T. circumcincta* among sheep and goats are clarified in Table 1. None of *Trichostrongylus* spp. was detected in the study. A total of 13 ITS2-sequences of nematodes were deposited in GenBank, including ten *M. marshalli* (accession numbers: MK253683-MK253692) and three *T. circumcincta* (accession numbers: MK253693-MK253695). In addition,

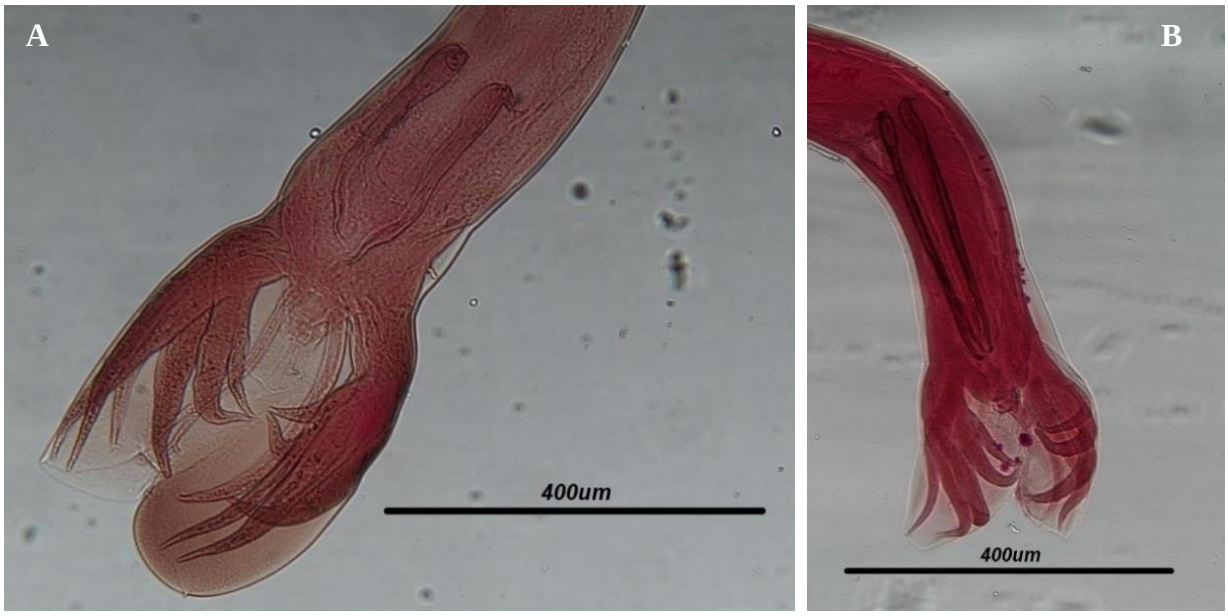

Figure 3. A: Copulatory bursa and spicules of *Marshallagia marshalli;* B: *Teladorsagia circumcincta*.

the molecular survey demonstrated high similarity (more than 95%) between the sequences found in our study and those of *M. marshalli* and *T. circumcincta* in the GenBank sequence database.

Table 1. Trichostrongylidae family species isolated from livestock in Qazvin, Iran.

Host	Number of male worms in abomasum		Total
	T. circumcincta (n/%)	*M. marshalli (n/%)*	
Sheep	5 (6)	78 (93)	83
Goat	5 (25)	15 (75)	20
Total	10 (9.7)	93 (90)	103

A dendrogram based on the phylogenetic analysis revealed that morphological examination can detect the different species of the parasite (Figure 2). The isolates were grouped into two distinct clusters corresponding to the *M. marshalli* and *T. circumcincta* alongside the gene references. Multiple alignments represented that the intra-species similarity for *M. marshalli* and *T. circumcincta* were 98-100% and 100%, respectively.

Discussion and Conclusion

Trichostrongylidae family is a group of significant parasites among sylvatic and domesticated ruminants and this makes the nematode infection as a common disease between the human and ruminant animals in endemic regions. Contamination by Trichostrongylidae nematodes can cause permanent damage to livestock industry, leading to changes in animal body weight and decreased production output. The most frequent species of the family in ruminant are *Nematodirus, Marshallagia, Haemonchus, Cooperia*, *Trichostrongylus* and *Teladorsagia* (4, 6, 10, 21, 28).

The present study is the first report of the nematodes in Qazvin, Iran. This research revealed that the infection rate among sheep and goats were 33.7% and 60%, respectively. Several studies reported from Iran showed higher infection rate in livestock compared to that of the current research. Eslami et al. (7) reported an infection rate as 88% in sheep. Also, Borji et al. (2) described the presence of high infection rate (75.1%) by gastrointestinal helminths in camels. Mashayekhi et al. (25) found an infection rate equal to 44% among the abomasal nematodes of cattle. Unlike the research stated above, few studies have been reported lower infection rate in livestock (8, 27).

Based on morphological and molecular analysis, two species including *M. marshalli* and *T. circumcincta* were found among the domestic ruminants in Qazvin Province, Iran. *Marshallagia marshalli* was the most frequent species (90%), while *T. circumcincta* (9.7%) was less prevalent among the isolates. Aligned with our findings, the most commonly reported species among the livestock in different parts of the country was *M. marshalli* as claimed by Eslami et al. (7) who found *M. marshalli* (93%) and *T. circumcincta* (9.3%) among the wild sheep (*Ovis orientalis*). Borji et al. (2) has also reported *M. marshalli* and *T. circumcincta* in camel isolates in the northeast of Iran. Other studies carried out in different parts of Iran (including the southeastern, northwest, and central parts) determined isolation of the parasite species from sheep and goat (17, 23, 28, 35). Conversely, few

studies have shown the higher frequency of *T. circumcincta* than *M. marshalli* in the ruminants. Nabavi et al. (27) found *T. circumcincta* at higher prevalence (19.3%) than *M. marshalli* (12.2%) in sheep isolates in the northern, central, and southern regions of Iran (Mazanderan, Isfahan and Khuzestan Provinces). Several studies have revealed the presence of *M. marshalli* and *T. circumcincta* among the ruminants in different parts of the world (5, 20, 26, 37).

Considering the extensive use of anti-parasitic drugs in the country, the main cause of high prevalence rate for nematodes is related to drug resistance emerged by the worms or might be due to decreased host immune response (15). The government strategic treatment of local livestock started since 1968 by administration of albendazole, twice a year for a duration of years (8), and subsequently, several species including *M. marshalli*, *T. circumcincta*, *T. colubriformis*, and *H. contortus* exhibited resistance to therapy (15, 28).

In domestic traditional animal husbandry throughout the country, the flocks of sheep and goats are kept and grazed together and this causes the persistence of the parasites in both types of farm animals (28), and the results of our study confirmed the presence of both *M. marshalli* and *T. circumcincta* species in two different groups of animals. It is documented that goats acquire a lower level of immunity against the parasites compared to sheep helminths of ruminants, hence goats experience more severe disease (16), however, the number of goat specimens used in the current research was limited and no further useful suggestion could be made on infection rate among the sheep and goat isolates in the study areas.

The family of Trichostrongylidae is classified as gastrointestinal helminths of ruminants, however, few studies have categorized these parasites according to their infection in human. The recovery of *Haemonchus contortus*, *Ostertagia ostertagi*, and *Marshallagia marshalli* were reported from human cases in Iran (9). Therefore coexistence of human alongside the ruminant animals could lead to human infection.

Iran is considered as a major focus for human and animal trichostrongyliasis in the world (10, 13, 30, 31) and several species of *Trichostrongylus* were reported from different parts of the country including *T. colubriformis*, *T. vitrinus*, *T. axei*, *T. capricola*, *T. probolurus*, *T. longispicularis*, *T. orientalis*, *T. lerouxi*, *T. skrjabini*, and *T. hamatus* (1, 10, 12, 31).

A striking finding of the present research was the lack of *Trichostrongylus* spp. and consistent with our results several studies failed to observe these nematodes among the specimens across the country (8, 17, 23, 27). The nematodes distribution in different geographical regions is depending on climate condition, pasture type, and management (29). It is obvious that the favorable conditions helps the existence of the nematode, therefore the absence of these parasites may be due to the drug sensitivity demonstrated by *Trichostrongylus* spp.

In the current study, the ITS2 region of rDNA was chosen for detecting the species of Trichostrongylidae family as several studies had used the region for molecular analysis (5, 13, 22, 24, 30, 38). The phylogenetic analysis performed for the present study clarified that all *M. marshalli* and *T. circumcincta* isolates were placed in one branch together with other specimens of different parts of the world. BLAST analysis indicated that there was 100% homology between the MK253683, MK253689, MK253687, MK253685, MK253686, MK253690, and MK253688 from sheep *M. marshalli* isolates and the MK253684 recovered from goats and that these isolates also exhibited 100% similarity to *M. marshalli* sequence (KY930443) from *Gazella dorcas* in Tunisia. In addition, there was 100% homology between the MK253691 and MK253692 obtained from the goat and sheep isolates, respectively. These isolates were grouped with sheep isolates from Uzbekistan (KT428384) and Russia (JQ839283). Also, there was 100% homology between the *T. circumcincta* isolates from sheep (MK253695) and those isolated from goats (MK253693 and MK253694). These isolates were grouped with goat (KC295420) isolate from Iran.

In conclusion, the present study demonstrated two species including *M. marshalli* and *T. circumcincta* belonging to the Trichostrongylidae family among the goats and sheep in the study region. In addition, it showed that *M. marshalli* is the predominant species among the local ruminants. The prevalence of nematodes could be related to the geographical condition, ecology, and the use of antiparasitic drugs.

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Conflict of Interest

The authors declared that there is no conflict of interests.

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Microbial contamination in food, food- handlers' hands and surfaces and evaluation of contamination sources by the similarity between isolates

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Abstract: Food can be contaminated with surfaces and food employees during chopping, shredding and serving. Pathogenic microorganisms are transmitted by direct contact with food or indirectly with airborne particles. The aim of this study was to determine the prevalence and the relationship between pathogenic microorganisms isolated from food, kitchen equipment and foodhandler's hands. A total of 212 microbiological samples were collected from surfaces, foods and food handlers' hands at different six canteens inside of the Afyon Kocatepe University campus during the period 2017-2018. Following biochemical tests, identification of *Staphylococcus* species were performed from a specific region of 23S rRNA gene. The genetic relationships between totally 25 *Staphylococcus* spp. and *Proteus mirabilis* isolates were determined. *S.epidermidis* was detected in two samples from knife handle (5%), nine samples from hands (20%) and in one of food sample (1%), too. *S.aureus* was found to be existed in one sample from hands (2.2%), two samples of soujouk (2%) and in one sample pancakes (3%) obtained from the university canteens. Besides from one of food sample (1%) *S.sciuri*, from one of hand sample (2.2%) *S.haemolyticus* and one of the food samples (1%) *S.saprophyticus* bacteria were identified. As a result, foods, food preparation surfaces and foodhandler's hands were contaminated with microorganisms in canteens and they were similar/same to each other. Also, considering the number of isolates, the highest of contamination is the hands of food-handler.

Keywords: Canteen, contamination, food, genotyping, sequence analysis.

Gıdalarda, gıda çalışanlarının ellerinde ve yüzeylerde mikrobiyal kontaminasyon ve kontaminasyon kaynaklarının izolatlar arasındaki benzerlikler ile değerlendirilmesi

Özet: Gıdalar işleme, hazırlama, doğrama, parçalama ve servis sırasında yüzeyler ya da gıda çalışanları aracılığıyla kontamine olabilmektedir. Patojen mikroorganizmalar gıdaya doğrudan temas veya dolaylı olarak havadaki partiküller ile bulaşmaktadır. Bu çalışmanın amacı, gıda, mutfak ekipmanları ve gıda çalışanlarının ellerinden izole edilen patojen mikroorganizmaların prevalansını ve aralarındaki ilişkiyi belirlemektir. Afyon Kocatepe Üniversitesi kampüsünde yer alan altı farklı kantinden 2017-2018 döneminde yüzeylerden, gıda ve gıda çalışanlarının ellerinden toplam 212 örnek toplandı. *Staphylococcus* spp. identifikasyonu biyokimyasal testlerin akabinde 23S rRNA geninin belirli bir bölgesi baz alınarak gerçekleştirildi. Toplam 25 *Staphylococcus* spp. ve *Proteus mirabilis* izolatı arasındaki genetik ilişki belirlendi. İki bıçak sapından (%5), dokuz elden (%20) ve bir gıdadan (%1) *S. epidermidis* tespit edildi. *S. aureus* bir el (%2,2), iki sucuk (%2) ve bir gıda (gözleme) örneğinde (%3) bulundu. Ayrıca, bir gıda örneğinde (%1) *S. sciuri* ve *S. saprophyticus,* bir el örneğinde ise (%2,2) *S. haemolyticus* saptandı. Sonuç olarak, kantinlerdeki gıdaların, hazırlama yüzeyleri ve çalışan ellerinin mikroorganizmalar ile kontamine olduğu ve bu mikroorganizmaların birbirleriyle aynı/benzer yapıda oldukları saptandı. Ayrıca, izolat sayıları dikkate alındığında, en yüksek kontaminasyonun gıda çalışan ellerine ait olduğu tespit edildi.

Anahtar sözcükler: DNA dizi analizi, genotiplendirme, gıda, kantin, kontaminasyon.

Introduction

Food safety is one of the most important perspectives in foodservice operations. However, this issue is generally accepted as the smallest amount of observation and attention (38). Many interrelated determinants are effecting microbial contamination of foods related to the preparation method, the hygienic sanitary situation of catering/canteen facilities, or food handling, storage, and distribution (14, 42). The unsuitable handling of foods by the food service sector has been a major concern in 97% of food poisoning cases (17, 36). Pathogenic microorganisms are spreading through the foodhandlers' hands and lead to cross-contamination (1, 33). *Staphylococcus aureus* is one of the leading causes of food

poisoning by means of enterotoxins (20). This commensal and opportunistic pathogen that is the main resident in humans, is mostly isolated from the nostrils, skin, oropharynx and feces (6, 23). Therefore, human-induced contamination the third most important cause of food-borne diseases in the world (28). Food poisoning has been stated to be a result of infection with enterotoxigenic strains of *S. aureus* and occurred to be 14-20% of outbreaks involving contaminated food (9). Today, Coagulase Negative *Staphylococcus* (CoNS), as general opportunist, reflects one of the major nosocomial pathogens, having a significant effect on human life and health. These microorganisms are found in different parts of the skin and mucous membranes of the host. *S. epidermidis* colonizes the body surface, predominantly existing in moist areas, such as the axillae, inguinal and perineal areas, anterior nares, conjunctiva, and toe webs. *S. haemolyticus* is primarily isolated from axillae and pubic areas, which are high in apocrine glands. *S. saprophyticus* mostly exists in the rectum and genitourinary tract (4, 31). *S. sciuri* is known as an invasive pathogen for wound infections, blood, urine and abscesses in humans. Also, the agent is commonly found in a large variety of hosts and environments and frequent contact with pets and livestock has been stated to cause skin colonization and wounds (16, 37). Also, dust is an important tool in the transport of these bacteria (25, 27).

The *Enterobacteriaceae* group of bacteria is the most challenging bacterial contaminant to raw and processed meat products worldwide (3). *Proteus mirabilis*, a member of this group is known to be other human opportunistic pathogens, isolated from urine, wounds, and other clinical sources. Intestine is the reservoir of such proteolytic microorganisms. A lot of wild and domestic animals are hosts of *Proteus* spp., which are mostly known to play a role of parasites or commensals. *Proteus* spp., generally present in soil or water habitats is usually considered as indicators of faecal pollution, creating a threat of poisoning when the contaminated water or food is consumed (12). According to our best knowledge, an extensive evaluation of a sequence-based PCR method for the identification from different sources of *Staphylococcus* spp. and *Enterobacteriaceae* has not been reported in the literature. This study aims to detect microorganisms important for food safety and public health from foods, surfaces and hands of employees and to determine the relationship between cross-contamination and sources of contamination between these isolates.

Material and Methods

This study was performed with the permission of the Afyon Kocatepe University Animal Ethics Committee (2016-49533702-68).

***Microbiological sampling of surfaces, food equipment, and food handler's hands*:** A total of 212 microbiological samples of surfaces, food tools and food handler's hands were taken different six canteens inside the university campus during the period 2017-2018.

One hundred gram of food samples were taken to sterile bags and brought to the laboratory under the cold chain and analyzed within 1-2 hours. Ten grams of each food samples were taken aseptically, transferred to sterile plastic bags and homogenized with 90 mL of sterile peptone water (1 g / L) for 60 seconds. After the serial dilutions of the samples, they were inoculated in growth media using standard drop-plate method (Table 1).

There were six canteens in the university with 45 food handlers (inclusive of permanent and contract workers). Hand samples were taken from thumb and forefinger through the "press your finger into the petri dishes" method. For this purpose, RODAC petri dishes containing Baird-Parker Agar (Oxoid, CM 0275, Basingstoke, UK) and Chromocult Coliform Agar (Merck 1.10426, Germany) were used and incubated at 37ºC for 24 h. In a sampling of food-contact surfaces (27 cutting board, 40 knives handle) was carried out by using a sterile frame of 15 cm^2 that was used to outline a known area, inside which the swabbing was done. The swab was then placed in a tube containing 10 mL of maximum recovery dilution with 0.05% sodium thiosulfate (MRD, Oxoid CM733, Basingstoke, UK) and stored in an ice container and then analyzed within 2 h. The *Enterobacteriaceae* and *Staphylococcus* spp., counts were obtained after incubation on Chromocult Coliform Agar and Baird-Parker Agar at 37ºC for 24 h. *Enterobacteriaceae* and *Staphylococcus* spp. were determined by biochemical and coagulase tests (5, 7, 10).

Table 1. Culture media, plating techniques and incubation conditions.

Microorganisms	Incubation		Culture technique	Culture media and confirmation tests
	Time (h)	Temperature (ºC)		
Staphylococcus spp.	24	37	Drop plate	Baird-Parker Agar plus egg yolk tellurite emulsion (5% v/v) (Oxoid CM 0275). Black colonies, Gram positive
Enterobacteriaceae	24	37	Drop plate	Chromocult Coliform Agar (Merck, 1.10426). Colorless typical colonies

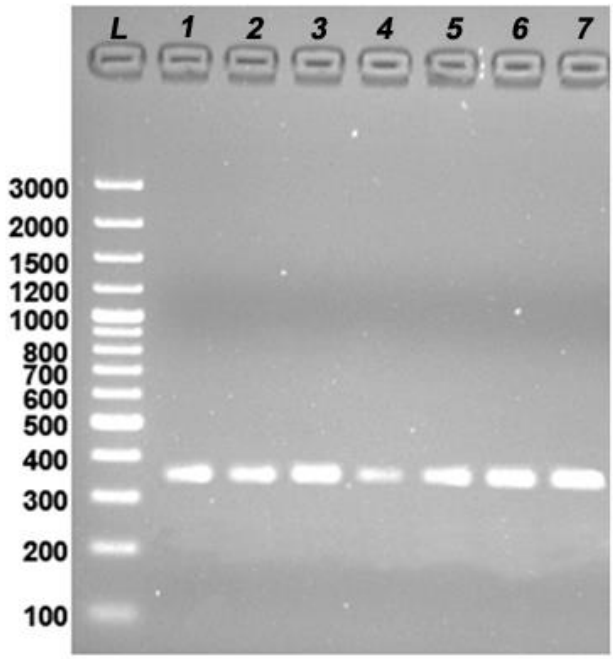

Figure 1. PCR fragment size of bacterial species in samples.Lanes: M, GeneRuler 100 bp Plus DNA Ladder (ThermoFisher Scientific, SM0323, USA); Lane 1, Positive control; Lane 2, Sample A; Lane 3, Sample 6; Lane 4, Sample 30; Lane 5, Sample 43; Lane 6, Sample 54, Lane 7, Sample 67.

Polymerase Chain Reaction and sequencing: Briefly, the total genomic DNA was isolated by using the commercial spin column kit (Thermo Fisher Scientific, K0722, USA) according to the manufacturer's instructions. Primers were designed using the Fast PCR Professional 6.1.2 package program (21). The dimer and hairpin formation between the primers were controlled by the same program. Isolated DNA was amplified using the forward primer: F112 (5'-TTC CGA ATG GGG AAA CCC AGC-3') and reverse primer R472 (5'-GCC TTA GGA GAT GGT CCT CCC A-3'). It was 23S rRNA genes in order to evaluate *Staphylococcus* spp. and *Enterobacteriaceae* strains. The gradient PCR was previously performed to determine the melting temperature (T_m). Totally, 20 µl containing 1xPCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.25 mM forward and reverse primers, 1U Platinium Taq DNA polymerase (Invitrogen, 10966034, USA) and 20 ng template DNA were included into PCR reactions performed in a thermocycler (Applied Biosystems Veriti). The PCR protocol was set to initial denaturation at 95ºC for 2 min, 35 cycles of denaturation 30 sec at 94ºC, 30 sec primer annealing at 60ºC, 1 min extension at 72ºC and, a final extension at 72ºC for 10 min., respectively. Amplification products were analyzed by gel electrophoresis on 2% agarose gel (Figure 1) followed by RedSafe (INtRON, 21141, South Korea) staining. Subsequently, all of the PCR products were purged with Exonuclease I (Thermo Fisher Scientific, EN0581, USA) and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, EF0652, USA).

Sequencing PCR was performed according to BigDyeTherminator 3.1 kit protocol. The products obtained at the end of the sequenced PCR were purified by ethanol / EDTA / sodium acetate precipitation method. Hi-Di formamide was added to 15 µl to each well and loaded onto the DNA Sequence Analyzer (ABI 3500).

Statistical analyses: All sequences were edited (GeneCode, Sequencher 5.4.6), aligned with the BioEdit Sequence Alignment Editor Analysis Program (18) and amplification product was read 360 bp. Genotypic results were compared and similarity searches were performed using MEGA 4. (40).

Results

Sequence analysis of the 23S rRNA, a highly conserved region present in all bacteria, has been implemented in laboratories to identify CoNS species. All analysis of *Staphylococcus* spp. and *Enterobacteriaceae* strains were performed using one primer set and generated PCR products were 360 bp in size. Most of strains revealed visually same fingerprint profiles (Table 2). The relationship between isolates are shown on the dendrogram (Figure 2). The present study determined the genetic relationships between 19 *Staphyloccocus* spp. and 6 *P. mirabilis* isolates obtained from food, food handlers' hand and food equipment by genotyping characterization. For this purpose, *Staphylococcus* spp. and *P. mirabilis* genes were evaluated by sequence analysis.

Most isolates were harvested from hands (52%) and food samples (36%) while few isolates (12%) were obtained from kitchen equipment. Sequence analysis revealed a correlation between some strains and the source of the isolates. In our study, the prevalence of food pathogens were determined in foods, hands and kitchen equipment. According to this; *P. mirabilis* was isolated from three samples of food (3%), one sample of hands (2.2%) and one sample of knife handle (2.5%), also *S. aureus* was isolated one sample of hands (2.2%), two of soujouk samples and one of the pancakes (3%) in the university canteens. Besides, *S. epidermidis* has been detected from two of knife handle samples (5%), nine of hand samples (20%) and one of the food sample (1%) (chicken skewers). Also, from one of the food sample (1%) *S. sciuri*, from one sample of hand (2.2%) *S. haemolyticus* and from one of food samples (1%) *S. saprophyticus* were isolated. According to these results this; *S. aureus* isolates existed from food handlers' hand and soujouk, also *S. epidermidis* isolates obtained from knife handle and foodhandlers' hands were revealed 100% similar in blast comparison in 1st canteen. Correlatively, *S. epidermidis* isolates obtained from food handlers' hands and chicken skewers have been identified as 100% similar

Table 2. Identification of 25 food, food handler's and food contact surface isolates by using DNA sequencing.

Sample (Source)	Similarity 100%	GenBank No (26)	Definitive Identification	Canteen Number
A (KnifeHandle)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 1
9 (Hand)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 1
41 (KnifeHandle)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 1
43 (Hand)	*S. aureus* strain PCFH-226	CP035005.1	*S. aureus*	Canteen 1
52 (Soujouk)	S. *aureus* strain PCFH-226/ FORC-061	CP035005.1	*S. aureus*	Canteen 1
6 (Hand)	*S. epidermidis* strain NCTC4133	LR134242.1	*S. epidermidis*	Canteen 2
8 (Hand)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 3
47 (Hand)	*P. mirabilis* strain NCTC4199	LR134205.1	*P. mirabilis*	Canteen 4
13 (Hand)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 4
14 (Hand)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 5
19 (Hand)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 6
18 (Hand)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 6
20 (Hand)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 6
30 (Hand)	*S. haemolyticus* strain SGAir0252	CP025031.1	*S. haemolyticus*	Canteen 6
31 (Hand)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 6
42 (Hand)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 6
54 (Vegetable mix)	*S. sciuri* strain NCTC12103	LS483305.1	*S. sciuri*	Canteen 6
61 (Chickenskewers)	*S. epidermidis* strain CDC121	CP034115.1	*S. epidermidis*	Canteen 6
63 (Pancakes)	*S. aureus* strain PCFH-226	CP035005.1	*S. aureus*	Canteen 6
67 (Urfa Kebab)	*S. saprophyticus* strain NCTC7666	LR134089.1	*S. saprophyticus*	Canteen 6
58 (Adana Kebab)	*P. mirabilis* strain NCTC4199	LR134205.1	*P. mirabilis*	Canteen 6
60 (Adana Kebab)	*P. mirabilis* strain NCTC4199	LR134205.1	*P. mirabilis*	Canteen 6
46 (Hand)	*P. mirabilis* strain NCTC4199	LR134205.1	*P. mirabilis*	Canteen 6
38 (KnifeHandle)	*P. mirabilis* strain NCTC4199	LR134205.1	*P. mirabilis*	Canteen 6
66 (Meatball)	*P. mirabilis* strain NCTC4199	LR134205.1	*P. mirabilis*	Canteen 6

Figure 2. The relationship between isolates with dendrogram.

isolates in the number 6th canteen, also coagulase negative staphylococci such as *S. haemolyticus, S. sciuri, S. saprophyticus* found in human skin and mucosa were identified in foods. In the same canteen, 100% similar *P. mirabilis* isolates were determined from food, food handlers' hands and knife handles. In 2nd, 3rd, 4th and 5th canteens, *P. mirabilis* and *S. epidermidis* isolates were found to be the same as those obtained from other canteens. As shown in the dendrogram, bootstrap values are generally high (78, 96, 98 etc.) and reliable, some values are low (26, 40, etc.). This is because; sample 6, for example, although *S. epidermidis* isolates, the other *S. epidermidis* is similar to 40% due to differences in 13 different nucleotides. Samples 38, 66 and 46, 47, 58, 60, identified as *P. mirabilis* are 100% similar, although they are separated from each other in terms of two nucleotides. However, 38 and 66 *P. mirabilis* isolates are 20-47% similar to other *Staphylococcus* spp. isolates.

The Neighbor-Joining method was opted to deduct the evolutionary relationship of isolates (32). The optimal tree with the sum of branch length = 0.46534862 is shown in Figure 2. The branches designate the percentage of replicates of acquainted taxa clusters obtained during bootstrap test (1000 replicates) (15). To develop the phylogenetic tree same units of branch lengths were used as those of evolutionary distances when drawing to the scale. The evolutionary distances were calculated by following the Maximum Composite Likelihood method and the number of base substitutions per site was used as units (41). All positions from data set were cleared by using complete deletion option for prevalent gaps and absent values. A total of 297 positions in the final dataset were obtained. Phylogenetic assessments were performed in MEGA 4 (40).

Discussion and Conclusion

In this study, food samples consisted of ready-to-eat and freshly prepared foods. The food contact with equipment harboring pathogens contributed as a most significant factor during a processing of food. Another reason which was among the leading cause of food contamination were the peeling trimming, slicing, milling, shredding, mechanical abrasion and diverse disintegration processes which could bring pollutants from related equipment (24, 34). Especially, the contaminated cutting boards used during cutting of raw meat, and poultry etc. when coming in contact with other foods, are among the major sources of contamination (44). The infected food handlers and their non-hygienic applications during food processing and preparation contribute another to contamination (19). Human (skins and nostrils, mucosae's and cuts, open wounds, or an infected wound) can function as major reservoirs of pathogens, such as *S. aureus* and *Proteus* spp., and act as vectors and contaminated food under hygienic conditions, especially unwashed hands (8, 13, 39). The results of following study clearly outline the reasons for food contamination with *E. coli, Klebsiella* and *Citrobacte*r which are likely in a food service setting and food handlers' hands. It is possible that different populations of employees in different localities and during different times of the year could carry variable hand carriage rates for vulnerable pathogenic species and more frequently infects the surfaces or foods (11). In another study using various products containing 238 retail samples, 137 samples of intact and raw vegetables, and 159 samples of fresh products were analyzed for different microorganisms and researchers were able to isolate *S. aureus* from two samples of raw vegetables and five kinds of cooked foods (22). *S. aureus* (16%) and *Proteus* spp. (12%) was detected from 55 analyzed food samples (39), *P. mirabilis* (6.3%), *S. aureus* (3.2%) from 252 samples (24), from 12 samples *Proteus* spp. (22.7%) were significant and accompanied by *S. aureus* (13.6%) (29), in 48 ready to eat foods and chicken derivative products *S. aureus* was the predominant organisms with 30% and *Proteu*s spp. (12%) (30). Wang et al. (43), showed that the handlers and helpers of cook in the kitchen and waiters were among the sources who carried infectious agents, and he described that pork balls were contaminated with *Proteus* spp. Also, a confirmation was recorded that the waiter and cook handler had previously contacted the pork balls directly without washing hands 4 h before the dinner. Although studies are generally related to food, no similar study has been found to determine the relationship between the isolates obtained from food, hands and food contact surfaces. According to our results; the same or similar isolates determined from food, food surfaces and food handlers' hand indicate that there is cross contamination in the same canteen (26). In this study, workers in contact with food are seasonal, part-time, rotated and some of them are citizens of different countries. Studies suggest that with the help of microbial indicators, it is easy to pinpoint the inadequacy in the food quality (35, 42). The practices such as the habit of touching contaminated surface while wearing gloves, the phenomenon of sneezing or coughing into a gloved hand, exclusive habit of not wearing gloves are major sources of food contamination other than actual contamination during food processing and preparation. Generally, the food handlers who lack the habit of wearing gloves usually escalate Staph bacteria to food (13). Besides, roughly 71.4% of food handlers had not known *S. aureus* is the causative agent of foodborne infections (2). The information obtained from this study is a strong evidence of the presence of cross-contamination of personnel and inadequate hygiene practices at mass consumption sites as well as it may be used to formulate the necessary measures to protect against foodborne infections and intoxications.

In order to maintain and assure the availability of good quality meals and foods for students, strict and comprehensive regulations notified by the authorities must be followed by catering services. Despite the fact that food safety legislation do exist, yet the administration of the school, cafeteria and canteen during meal preparation fails to justify the food safety needs and sometimes even does not take into account the specific hygienic requirements needed for each stage of food preparation. Consequently, during the whole production chain, food is constantly exposed to microbial contamination. The genotypic results of the present study may help to better understand the distribution of microorganisms between food, food handler and food contact surfaces and provide information on foodborne infection pathways.

As a consequence, food handlers should have basic understanding and a good attitude about personal hygiene, hand washing and proper cleaning and strict food hygiene practices. Health hazards associated with catering facilities can be nullified by avoiding poor handling. Similarly, self-consciousness about personal hygiene and safeties during food preparation and storage could help to combat foodborne diseases. Periodic sanitary-hygienic evaluation, proper monitoring of catering facilities and strict quality control of food should be enforced in order to facilitate disease free processed food and to significantly minimize the public health hazards acquainted with food-borne infectious agents.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Effects of supplementation of sepiolite and humate to beef cattle concentrate on pellet quality characteristics

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Abstract: The aim of this study was to evaluate some pelleting characteristics of beef cattle concentrate with added sepiolite and potassium humate alone or combined. For this purpose, one control group (basal concentrate) with no additive and five treatment groups with 1% sepiolite (S), 0.25% potassium humate (H-25), 0.50% potassium humate (H-50), 1% sepiolite + 0.25% potassium humate (SH-25) and 1% sepiolite + 0.50% potassium humate (SH-50) added as top-dressed into mixer were used. For each experimental group, a total of 36 tons of pellet concentrates were produced in six batches with 6 tons per batch. The basal pelleted concentrate included 90.29% dry matter, 14.51% crude protein, 11.76% crude fibre and 3.14% ether extract. Results showed that adding sepiolite up to 1% in beef cattle concentrate as top-dressed in mixer reduced moisture losses and improved the durability of pelleted feed. Sepiolite addition also lowered pellet water activity during storage. Adding potassium humate alone up to 0.50% was not enough to improve the pellet quality in terms of durability or water activity. Potassium humate supplementation alone or with sepiolite presented with lower moisture losses than control, and it was more evident during storage. In conclusion, the use of sepiolite in beef cattle concentrates as pellet binder seemed to be providing more durable feed pellets with lower water activity. Promising results were obtained from both the supplements in limiting the moisture losses post- pelleting and storage. Regarding the role of potassium humate as pellet binder in animal feed industry further research is needed.
Keywords: Beef cattle, concentrate feed, pellet quality, potassium humate, sepiolite.

Besi sığırı konsantre yemlerine sepiyolit ve humat ilavesinin pelet kalite özellikleri üzerine etkileri

Özet: Bu araştırmanın amacı, besi sığırı yemlerinde sepiyolit ve potasyum humatın tek başlarına ve birlikte kullanımının pelet kalite özelliklerine olan etkilerini belirlemektir. Araştırmada katkı yapılmayan kontrol grubu yemi (bazal kesif yem) ve beş deneme grubu yemi hazırlanmıştır. Deneme grup yemlerini oluşturmak için bazal yemin üzerine karıştırıcıda sırasıyla %1 sepiyolit (S), %0,25 potasyum humat (H-25), %0,50 potasyum humat (H-50), %1 sepiyolit + %0,25 potasyum humat (SH-25) ve %1 sepiyolit + %0,50 potasyum humat (SH-50) katılmıştır. Denemedeki grupların her biri için altı kez altışar tonluk pelet yem üretimi yapılmıştır. Dolayısıyla her bir grup için toplam da 36 ton pelet yem üretilmiştir. Peletlemede kullanılan besi sığırı kontrol yemi %90,29 kuru madde, %14,51 ham protein, %11,76 ham selüloz ve %3,14 ham yağ içermektedir. Sonuçlar besi sığırı konsantre yemlerine %1'e kadar sepiyolit eklenmesinin nem kayıplarını azalttığını ve pelet dayanıklılık indeksi değerlerini iyileştirdiğini göstermiştir. Ayrıca sepiyolitin eklenmesi depolanma sırasında pelet su aktivitesini azaltmıştır. Potasyum humatın yemlere tek başına %0,50'ye kadar katılması; pelet dayanıklılığı ve su aktivitesini iyileştirmesi açısından yeterli olmamıştır. Potasyum humatın tek başına veya sepiyolit ile birlikte kullanılması özellikle depolama süresince kontrol grubu yemine kıyasla nem kaybının daha düşük olmasını sağlamıştır. Bu nedenle besi sığırı konsantre yemlerine sepiyolitin pelet bağlayıcı olarak katılmasının düşük su aktivitesine sahip daha dayanıklı pelet yemin üretiminde etkili olacağı sonucuna varılmıştır. Her iki yem katkısının pelet üretim süresince ve pelet depolama esnasında su kaybını azaltması da çalışmanın önemli sonuçlarındandır. Yem sanayisinde potasyum humatın pelet bağlayıcı olarak kullanımının daha iyi belirlenebilmesi için çalışmalar yapılmalıdır.
Anahtar sözcükler: Besi sığırı, kesif yem, pelet kalitesi, potasyum humat, sepiyolit.

Introduction

Pelleting was first introduced into the feed industry in the 1920s (24) and since that time its usage has increased and improved steadily. Nowadays the pelleting process, due to its physical and nutritional benefits, is widely used in commercial feed production. The potential

physical benefits involve improved ease of handling, reduced ingredient segregation, less feed wastage, and increased bulk density whereas the improved animal performance and increased animal products with higher quality are one of the well-known nutritional benefits of feeding pelleted feed. The improvements in animal performance with the feeding of pelleted feed have been attributed to many factors such as better feed utilization, destruction of pathogenic microorganisms, thermal modification of starch & protein and enhanced palatability (8). All these modifications in feed characteristics make the pellet quality a high priority for the feed manufacturers.

The physical form (mash or pelleted) in which the concentrate feeds are fed to the cattle is of great importance. Bertipaglia et al. (10) proposed three possible characteristics by which the pelleted concentrates may affect rumen fermentation in intensively reared beef cattle. The first two pelleting features favor the fermentation process via a reduction in the particle size and starch gelatinization, however, the third feature involving the compactness and hardness of pelleted feed would reduce its breakdown, thus counterbalancing the former effect and delaying the fermentation process (10). A lot of efforts have been made to improve this compactness of the pelleted feed without compromising its quality.

The pellet durability index (PDI) is frequently used as a predictor of pellet fines produced during production and mechanical handling of feed pellets. Fines in the feeders result in feed wastage, animal refusals and increased management (9). Several factors including type of feed materials, their proportions, nutrient content (especially fat, fibre and minerals), particle size, presence of binders and factors related to the pelleting process such as conditioning, the distance between the roller and die, hole compression etc. affect PDI value of feeds (3, 11, 16, 22, 28). The addition of high levels of fats before pelleting process results in decreased pellet quality, however, the addition of protein and fibrous ingredients improves pellet quality (9, 19, 23). Other than these factors, a lot of alternative feed additives such as sepiolite, bentonite, carboxymethylcellulose, and lignosulfonates have been investigated for their role in improving the pellet quality (21).

Sepiolite is a hydrated magnesium silicate. It can be used as a binder and an anti-caking agent up to 2% in feeds for different animal species (13). Sepiolite reduces dust losses and increases pellet durability and quality (30, 32, 34).

Potassium humate, the potassium salt of humic acid, is commercially produced from leonardite, an oxidation product of lignite coal. Leonardite has a high humic acid content compared with other organic materials (2, 6, 14). They are colloidal substances and act as clay (1). Humic acid has a chemically heterogeneous structure which includes functional groups such as carboxyl (COOH), hydroxyl (OH) and amine (NH2). By replacing hydrogen ions in carboxyl and hydroxyl groups with metal ions or chemically cross-linking, humic acid is rendered insoluble in water and can be used in different areas (2). It improves water retention properties of the soil, as well as prevents bacterial and fungal growth, thus reducing the level of mycotoxin in the feed.

To best of our knowledge, no published data is available investigating the pelletizing of beef cattle concentrate with added potassium humate alone or combined with sepiolite. The present study, therefore, was proposed to evaluate some pelleting characteristics of beef cattle concentrate with added sepiolite and potassium humate alone or combined.

Material and Methods

A commercial concentrate for the beef cattle was used in this study and pellet feeds were produced in a commercial feed factory in Turkey. The concentrate comprised mainly wheat bran (350 kg/t), barley (100 kg/t), wheat (100 kg/t), corn (100 kg/t), sunflower seed meal (70 kg/t), lentil bran (70 kg/t) and molasses (35 kg/t). The present study consisted of a total of six experimental groups; a control group with no added sepiolite or potassium humate in the basal diet, and five treatment groups having 1% sepiolite (S), 0.25% potassium humate (H-25), 0.50% potassium humate (H-50), 1% sepiolite + 0.25% potassium humate (SH-25) and 1% sepiolite + 0.50% potassium humate (SH-50) in the basal diet added as top-dressed into the mixer. Before addition to the basal diet, potassium humate was first mixed in water in a ratio of 1:1. The potassium humate (TKİ Hümas-Turkey) used in this study was produced from leonardite reserves in Turkey. It consisted of 5% organic matter (w/w), 12% humic acid + fulvic acid, 3% water-soluble potassium oxide and had a pH between 11 and 13 (27). The sepiolite (Exal T, Tolsa-Turkey) used consisted of 73% clay minerals (65% sepiolite), 21% dolomite and 6% calcite as reported by Yalçın et al. (32).

Mixer capacity of the feed mill was 2 tons. For each experimental group, a total of 36 tons of pellet concentrates were produced in six batches with 6 tons per batch. The disc used was 5 mm in diameter and 60 mm in hole length. The parameters for the pellet manufacturing process are presented in Table 1.

During each production batch, the feed samples (for every mill run; 2 tons/run) were taken from the mixer blend, post-conditioning & post-pelleting (cooled pellets), and later subjected to the moisture analysis (5). Moisture losses between the feed blend in the mixer and pelleted feed after cooling were also calculated. Additionally, for all the production batches, pelleted feed samples from the control group were analyzed for crude protein, crude fibre, ether extract, ash and starch (5) as well as for NDF and ADF values (17).

Table 1. Some production parameters for pelleting process.

Parameters	Control group	Treatment groups				
		S	H-25	H-50	SH-25	SH-50
Production, t	36	36	36	36	36	36
Mixer capacity, t	2	2	2	2	2	2
Disc hole diameter, mm	5	5	5	5	5	5
Disc hole length, mm	60	60	60	60	60	60
Sepiolite addition (%)	-	1	-	-	1	1
Potassium humate addition (%)	-	-	0.25	0.50	0.25	0.50

S: 1% sepiolite; H-25: 0.25% potassium humate; H-50: 0.50% potassium humate; SH-25: 1% sepiolite and 0.25% potassium humate; SH-50: 1% sepiolite and 0.50% potassium humate.

The PDI values for the pelleted feed after cooling were measured with a Pfost Box equipment using the sieve with a hole diameter of 4 mm (7). Four measurements were made for each feed sample. The water activity values of all the mash and pelleted feed samples collected were measured using LabSwift-aw (Novasina) water activity device. Furthermore, the pelleted feeds were stored in 50 kg bags at room temperature for 20 days, and later, their moisture content and water activity level were determined as described above. Moisture losses between post-pellet and post storage were also calculated.

Statistical analysis was conducted using the ANOVA procedure of the SPSS 23.0 (SPSS Inc., Chicago, IL, USA). The experimental unit was 18 (6 batches with 3 samples each). The normality of data distribution was tested using the Kolmogorov-Smirnov test. One-way ANOVA was performed to examine the differences among groups. The significance of mean differences among groups was tested by the Tukey test. A value of $P<0.05$ was considered statistically significant (12).

Results

The average nutrient composition of the pelleted basal beef cattle concentrate is presented in Table 2. Effects of addition of sepiolite and potassium humate on moisture content, moisture losses and PDI values of concentrate feeds are shown in Table 3. No significant differences were observed among experimental groups for the moisture content of concentrate feed-blends in the mixer, however, post-conditioning, the moisture content of the SH-50 feed group was significantly higher than that of other groups ($P<0.001$). Post-pelleting, the moisture loss in the H-25 group was lowest among all groups ($P<0.001$). PDI values for the S, SH-25, SH-50 feed groups were significantly higher ($P=0.002$) when compared to the control group as well as H-25 and H-50 feed groups.

Table 2. Nutrient composition of the pelleted basal concentrates (mean ± standard error).

Nutrient	%
Dry matter	90.29±0.38
Ether extract	3.14±0.08
Crude protein	14.51±0.15
Crude fibre	11.76±0.12
Ash	6.24±0.28
Starch	24.36±0.41
NDF	28.35±0.44
ADF	14.04±0.41

Table 3. Moisture content and moisture losses of concentrate feeds during pellet production and PDI values of pelleted concentrates.

Group	Moisture content (%)			Moisture loss (%)	PDI (%)
	Mixer blend	Post-conditioning	Post-pelleting	Mixer to Post-pelleting	
Control	11.14	13.10^{b}	10.02^{b}	10.05^{a}	94.65^{b}
S	11.06	13.44^{b}	10.49^{a}	5.15^{cd}	95.57^{a}
H-25	11.14	13.15^{b}	10.69^{a}	4.04^{d}	93.90^{b}
H-50	11.56	13.32^{b}	10.60^{a}	8.30^{ab}	93.65^{b}
SH-25	11.38	13.29^{b}	10.49^{a}	7.82^{ab}	94.95^{a}
SH-50	11.26	14.04^{a}	10.48^{a}	6.93^{bc}	95.32^{a}
SEM	0.052	0.067	0.056	0.319	0.166
P	0.074	<0.001	0.003	<0.001	0.002

^{a,b,c,d}: Means within a column followed by different letters differ significantly ($P<0.05$); S: 1% sepiolite; H-25: 0.25% potassium humate; H-50: 0.50% potassium humate; SH-25: 1% sepiolite and 0.25% potassium humate; SH-50: 1% sepiolite and 0.50% potassium humate; SEM: Pooled standard error of mean; PDI: Pellet durability index.

Table 4. Moisture content and total moisture losses of pelleted concentrate after storage.

Group	Moisture content (%)	Moisture loss (%)
	Post-storage	Post-pellet to Post-storage
Control	9.42[b]	5.99[a]
S	10.28[a]	2.00[b]
H-25	10.34[a]	3.27[b]
H-50	10.24[a]	3.40[b]
SH-25	10.16[a]	3.15[b]
SH-50	10.26[a]	2.10[b]
SEM	0.073	0.313
P	<0.001	<0.001

[a,b]: Means within a column followed by different letters differ significantly (P<0.05); S: 1% sepiolite; H-25: 0.25% potassium humate; H-50: 0.50% potassium humate; SH-25: 1% sepiolite and 0.25% potassium humate; SH-50: 1% sepiolite and 0.50% potassium humate; SEM: Pooled standard error of mean.

Table 5. Water activity levels of mash/pelleted concentrates.

Group	Water Activity			
	Mixer blend	Post-conditioning	Post-pelleting	Post-storage
Control	0.60[ab]	0.66[b]	0.54[b]	0.61[a]
S	0.58[b]	0.66[b]	0.54[b]	0.56[b]
H-25	0.61[ab]	0.66[b]	0.57[ab]	0.59[ab]
H-50	0.63[a]	0.67[b]	0.59[a]	0.62[a]
SH-25	0.64[a]	0.68[ab]	0.58[a]	0.60[a]
SH-50	0.62[ab]	0.70[a]	0.59[a]	0.61[a]
SEM	0.004	0.003	0.004	0.005
P	0.003	0.002	<0.001	<0.001

[a,b]: Means within a column followed by different letters differ significantly (P<0.05); S: 1% sepiolite; H-25: 0.25% potassium humate; H-50: 0.50% potassium humate; SH-25: 1% sepiolite and 0.25% potassium humate; SH-50: 1% sepiolite and 0.50% potassium humate; SEM: Pooled standard error of mean.

The moisture content of pelleted feeds after 20 days of storage along with the moisture losses between post-pellet and post-storage period is shown in Table 4. After 20 days, the moisture loss in the control group was highest than all supplemented groups (P<0.001).

Effects of usage of sepiolite and humate in beef cattle concentrate on water activity levels (%) at a temperature of 22-24ºC are shown in Table 5. In the mixer, the water activity levels of concentrate feed supplemented with sepiolite or/and humate didn't differ significantly from that of the control group. However, when compared to the control group the SH-50 feed group post-conditioning (P=0.002) whereas H-50, SH-25 and SH-50 feed groups post-pelleting (P<0.001) presented with significantly higher water activity values. Post-storage, feed group with added sepiolite (S) only presented with the lowest water activity (P<0.001) when compared to others.

Discussion and Conclusion

Feeding pelleted feed alone may not be enough to ensure improved animal performance; therefore, pellet quality should always be considered for better results. Pellet quality is affected by many factors such as diet formulation, feed composition, ingredient particle size, the inclusion of binder additives and pelleting conditions. Considering the importance of binders in pellet production, the objective of this study was to evaluate the pellet quality of beef cattle concentrate with added sepiolite and potassium humate alone or combined.

In the present study, all the experimental runs were completed with the same production parameters including diet formulation, amounts of pellets produced, mixer capacity, disc hole diameter, and disc hole length. As both the feed additives are considered of no nutritive value, therefore, the pelleted feed samples from the control group only were subjected to the compositional analysis. The crude protein, ether extract and crude fiber contents of the pelleted basal beef cattle concentrate were 14.51, 3.14 and 11.76%, respectively.

The moisture content of the mash feed pre- and post-conditioning is a very important factor affecting the pellet quality as well as production efficiency. Stable moisture content post-pelleting is also important especially from manufacturer's point of view, concerning economic

consequences with the total loss in weight in the finished product between packing and delivery to the customers. Results from the present study indicated that post-conditioning the moisture retention in the feed group added with 1% sepiolite and 0.50% potassium humate (SH-50) was highest among all experimental groups ($P<0.001$). Although non-significant data also suggested a relatively small increase in the moisture level of other treatment groups post-conditioning when compared to the control group. Furthermore, among experimental groups, the control group presented with the highest moisture loss from mixer blend to post-pelleting. The moisture losses of S, H-25 and SH-50 feed groups were significantly lower than that of control group feed ($P<0.001$). Considering the statistically non-different moisture level of all feed blends in mixer and similar conditioning environment, it can be inferred that addition of sepiolite or potassium humate alone or combined might have increased the porosity and water absorption capacity of the feed mash post-conditioning, which ultimately rendered in lower moisture losses in these feed groups post-pelleting. In accordance with this view, Yalçın et al. (32) also reported a linear increase in the moisture content of the dairy concentrate post-conditioning as well as post-pelleting when supplemented with increasing doses of sepiolite. In another study, also with dairy cattle concentrate added with 1% sepiolite, Yalçın et al. (33) found a marked rise in feed moisture level post-conditioning and post-pelleting. For potassium humate, no scientific study was available determining its role in quality pellet feed production. Griban et al. (18) reported that humic acid can improve the water retention and water holding capacity of materials. Data on moisture losses, especially post-storage, showed that addition of sepiolite and potassium humate alone or combined significantly reduced moisture losses as compared to the control group ($P<0.001$). Sepiolite alone resulted in the lowest storage losses in terms of moisture (2%).

A reduction in fine particles is beneficial in terms of feed losses, animal performance and the farmer profit (30). In the present study, pellet durability results were lower for potassium humate alone either 0.25 or 0.50%, however, when added with sepiolite it significantly improved the PDI values than that of the control group. Sepiolite alone topped the PDI test among all groups. High temperature and moisture levels are very critical in the activation of natural binders in the material including protein and starch, thus, improving the pellet quality (15, 26). In this study, more moisture retention post-conditioning and possible higher heat of friction by sepiolite particles at the die might be reasons for better pellet quality. Other studies also reported improvement in PDI values of pelleted feeds with sepiolite addition (3, 4, 29-33).

Potassium humate had not been tested before in animal diets for its binding properties. In this study, no positive effect of potassium humate addition was observed on pellet PDI values when compared to the control group, however, in iron ore pelletization humic acid has been reported to have promising results as a binder (20, 35). The different raw materials to be pelleted might be the reason for such results.

Water activity level serves as an indicator of the volume of free water. The higher the free water present, the better the conditions for unwanted mould development. A water activity level of 0.65 is often referred to as the limit for safe storage of foods, below which microbiological growth is unlikely to occur (25). In the present study, no explicit effects on water activity were observed when concentrate feeds were supplemented with different additives either alone or combined. Although data showed that sepiolite addition alone might be beneficial in reducing the water activity during storage, it is difficult to conclude that whether the addition of sepiolite or potassium humate were or were not effective in limiting water activity. In an experiment, Yalçın et al. (33) reported no significant difference between water activity values of pelleted concentrates of dairy cattle either from control or sepiolite added group.

The overall conclusion from the present study was that adding sepiolite up to 1% in the beef cattle concentrate as top-dressed in mixer could help in reducing the moisture losses and improving the PDI values thus enhancing the pellet quality. Sepiolite addition also lowered the pellet deterioration and water activity during storage. On the other hand, the addition of potassium humate (0.25 and 0.50%) alone was not enough to improve the pellet quality in terms of PDI or water activity. However, for moisture losses especially during storage, humate supplemented groups either alone or with sepiolite presented with lower moisture losses than the control group. Further research is needed in evaluating the role of potassium humate as a pellet binder in the animal feed industry.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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The evaluation of the oxidative stress index of reproductive tissues and serum thiol/disulphide homeostasis during estrous cycle in bitches

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Abstract: The aim of this study was firstly to determine the relationship between estrous cycle and oxidative stress in reproductive tissues in bitches. This research was performed in twenty-nine healthy bitches from different breeds and of varied ages (the range was 2-5 years) that were brought to the clinic for routine ovariohysterectomy. The stages of estrous cycle were detected using by vaginal cytology, blood progesterone level and histological findings. Ovarian, oviduct and uterine tissues were taken into Eppendorf tubes for oxidative stress index and stored at -80°C until analyses. Also, another part of these tissues were fixed in 10% formalin solution. There were no significant differences ($P > 0.05$) among total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) values in the reproductive tissues in concerning estrous cycle stages. However, there were remarkable correlations between oxidative stress parameters and reproductive tissues in different estrus stages in bitches. In conclusion, the physiological values of TAS and TOS concentration, and OSI in the ovarian, oviduct and uterine tissues during estrous cycle were firstly defined in this article. The serum thiol/disulphide homeostasis was also determined in the estrous cycle of bitches. We have found that there are remarkable variations of oxidative stress balance in the ovary, oviduct and uterus concurrently, during estrous cycle in the bitches. Besides, significant correlations between oxidative stress parameters and estrous cycle stages in the reproductive tissues were observed in the present study.
Keywords: Bitch, estrous cycle, oxidative stress index, progesterone, thiol/disulphide.

Köpeklerde östrus siklusu boyunca reprodüktif dokularda oksidatif stres indeksi ve serum tiyol/disülfit homestazının değerlendirilmesi

Özet: Bu çalışmada, köpeklerde östrus siklusu ile reprodüktif organlardaki oksidatif stres arasındaki ilişkinin ilk olarak ortaya konulması amaçlandı. Bu araştırma, rutin ovaryohisterektomi için kliniğe getirilen farklı ırklardan ve değişik yaşlardan (2-5 yıl arasında) sağlıklı yirmi dokuz köpekte gerçekleştirildi. Östrus siklusunun evreleri vajinal sitoloji, kan progesteron seviyesi ve histolojik bulgular ile tespit edildi. Ovaryum, ovidukt ve uterus dokuları, oksidatif stres indeksinin belirlenmesi için Eppendorf tüplerine alındı ve analiz edilene kadar -80°C'de saklandı. Ayrıca, bu dokuların diğer bir kısmı %10 formalinde fikse edildi. Reprodüktif organlardaki total antioksidan durum (TAS), total oksidan durum (TOS) ve oksidatif stres indeksi (OSI) düzeylerinde östrus siklusu evreleri bakımından istatistiksel bir fark bulunmadı ($P > 0,05$). Ancak östrus siklusunun farklı evrelerinde oksidatif stres parametreleri ile reprodüktif dokular arasında dikkat çekici korelasyonlar belirlendi. Sonuç olarak, bu araştırma ile köpeklerde östrus siklusu boyunca ovaryum, ovidukt ve uterus dokusundaki TAS, TOS ve OSI'nin fizyolojik değerleri ilk kez ortaya konuldu. Ayrıca köpeklerde östrus siklusunun evrelerine göre serum thiol/disülfit homeostazisi de belirlendi. Bununla birlikte, reprodüktif dokulardaki oksidatif stres parametreleri ile östrus siklusunun evreleri arasında önemli korelasyonların olduğu gözlendi.
Anahtar sözcükler: Dişi köpek, oksidatif stres indeksi, östrus siklusu, progesteron, tiyol/disülfit.

Introduction

Domestic canids are typically classified as monoestrus, and have three estrous cycle every two years. However, in domestic bitches, most females display two estrous cycle in one year. The estrous cycle of bitches is much longer than those of other animals, and consists of proestrus, estrus, diestrus and anestrus stages (22). Endocrine mechanism of canine cycle differs from other mammalian species; ovulation occurs 2-3 days after LH surge at the end of proestrus or at the beginning of estrus

phase. The bitch becomes receptive to the male in estrus phase during rising progesterone levels. Unlike those of other mammalians, estrus stage proceeds under the influence of progesterone hormone in bitches (2, 5). The changes in the reproductive organs in relation with steroids and gonadotrophins are ever-repeating process. During estrous cycle, preantral follicles grow, granulosa cells in the antral follicles transform into the luteal cells, ovulation occurs, and corpora lutea develops and then regress in the ovary of bitches. In addition to this, proliferative, regressive, and apoptotic changes occur in the endometrium of uterine tissue and vagina synchronously with ovarian changes (21, 28).

Oxidative stress is defined as a result of an increased number of lipid and protein oxidation products, and decreased number of antioxidants. Under normal conditions, pro-oxidants and antioxidants remain in balance in aerobic cells (1). The oxidative stress balance not only plays a crucial role for healthy body, but it is essential also for reproductive system physiology. It is well known that the reproductive physiology is a continuous process with follicular growth, preovulatory luteinizing hormone (LH) surge, oocyte maturation, ovulation, formation of corpus luteum, steroidogenesis, luteolysis, endometrial proliferation and regression, fertilization, embryonic growth, and pregnancy (1, 18, 20). Reactive oxygen species (ROS) are markers of oxidative stress, and ROS are both by-products and influencers of normal reproductive metabolism, especially for steroidogenic cells (24). However, in some cases ROS production may be increase, and enzymatic or non-enzymatic antioxidants such as superoxide dismutase, catalase, vitamin E, vitamin C, selenium, zinc, glutathione fail to reduce ROS excess; then, oxidative stress causes damage in cells and tissues. In female reproduction, insufficient antioxidant defense mechanism and/or excessive production of ROS may play an important role on infertility, repeat breeding, inadequacy of oocyte maturation or embryo development, cystic ovarian diseases, endometriosis, preeclampsia, abortions (13-15, 19). The levels of antioxidant and oxidant components influence oxidative balance in the aerobic cells. Individual metabolites of ROS, which are superoxide, hydrogen peroxide and hydroxyl, may not necessarily reflect the whole condition of oxidative stress in the organism. Therefore, the measurement of TAS, TOS and the calculation of OSI reflect the relation between ROS formation and antioxidant activity (9).

Dynamic thiol/disulphide homeostasis plays a critical role in antioxidant protection similarly to enzymatic and non-enzymatic antioxidants both in physiologic and pathological conditions such as antioxidant protection, detoxification, signal transduction, apoptosis, regulation of enzymatic activity and transcription factors and cellular signaling mechanisms. Cysteine, cysteinylglycine, glutathione, homocysteine, γ-glutamylcysteine, and β-mercaptoethanol are most known thiol compounds (4, 6, 10). Some of these thiol compounds such as cysteine, cysteamine and β-mercaptoethanol are known to improve oocyte maturation *in vitro* and promote late embryonic development (12, 25). In the light of these scientific findings, the aim of the present study was to firstly evaluate TAS, TOS levels of the ovarian, oviduct and uterine tissues and OSI, and serum thiol/disulphide homeostasis in bitches during estrous cycle.

Material and Methods

The present study was maintained in accordance with the directions of Guide for the Care and Use of Animal in Research (AKUHADYEK-24-18). This research was performed in 29 healthy bitches from different breeds and of varied ages (between 2 and 5 years) that were brought to the Afyon Kocatepe University Veterinary Health Application and Research Center for routine ovariohysterectomy. Research material was formed obtained blood, ovarian, oviduct and uterine tissues from these bitches. For this purpose, general clinical examination of bitches was performed. The stages of estrous cycle were determined using by vaginal cytology, serum progesterone level and histological findings of reproductive organs.

Vaginal cytology: After asepsis of the vulva, the vagina was inspected (appearance, shape, color of the vagina and the presence of discharge). Following this process, vaginal smear samples were collected to investigate the estrous cycle. A moistened cotton bud swab was inserted into vagina. The cells from the vaginal lumen and walls were gently taken and transferred to a glass slide. After the samples were allowed to air-dry, it was stained with Giemsa stain and then evaluated under the light microscope. The phases of estrous cycle were classified as proestrus (presence of erythrocytes, gradually increase in cornified cells, decrease in polymorphonuclear leukocyte cells, intermediate and parabasal cells), estrus (80 percent of the cells were cornified and anuclear, no polymorphonuclear leukocyte cells), early diestrus (non-cornified cells, large number of polymorphonuclear leukocyte cells, epithelial cells engulfing a polymorphonuclear leukocyte cells and the foam cell), late diestrus (primarily polymorphonuclear leukocytes and a few epithelial cells) and anestrus (non-cornified cells and rare polymorphonuclear leukocyte cells) (17).

The histopathological evaluation of ovarian, oviduct and uterine tissues: The samples were removed immediately, and transferred into 10% neutral formalin solution for fixation. The tissues were put into the cassettes and processed using automated tissue processor

(Leica, TP1020, Germany), subsequently embedded in paraffin and 4 µm thickness sections were obtained for microscopic examination. The slides were stained with Hematoxylin & Eosin (H & E). Each H&E slide was examined blindly under a light microscope (LeicaDM4000, Germany). Microscopically, all samples were classified as proestrus, estrus, diestrus, anestrus according to Sato et al. (21).

Biochemical analyses: The progesterone and thiol/disulphide homeostasis analyses were performed on serum samples, and TAS and TOS levels in reproductive tissues were determined.

Blood specimen collection: Blood samples were collected preoperatively from the *vena cephalica antebrachii* into plain tubes (Vacutainer® SST™ II Advance Serum Tubes) for the determination of serum biochemical parameters and kept at 4°C for approximately 1 hour. The blood samples were centrifuged at 3000 rpm for 15 min and sera were stored at -80°C in a freezer until analysis. The progesterone and thiol/disulphide homeostasis levels were analyzed.

The determination of progesterone level: Serum progesterone levels were assayed by Enzyme-Linked Immunosorbent Assay (ELISA; VersaMax™ ELISA Microplate Reader) using a Canine Progesterone, PROG ELISA kit of Cusabio® and the results were given as ng/ml. The progesterone data was confirmed with findings of vaginal cytology. Animals with progesterone level 0.5 - 2 ng/ml were classified as being in proestrus. Animals with progesterone levels 2 - 15 ng/ml were classified as being in estrus. Bitches were classified in early diestrus when progesterone levels were above 15 ng/ml. Animals with progesterone levels lower than 10 ng/ml and higher than 0.5 ng/ml were classified as being in late diestrus. The bitches with progesterone levels ≤0.5 ng/ml were in anestrus (28).

Serum thiol/disulphide homeostasis: Serum thiol/disulphide homeostasis was identified by newly developed automatic method (10). Dynamic and degradable disulphide bonds (–S–S) were decreased to free functional thiol groups (–SH) by sodium borohydride ($NaBH_4$). Unused reductant sodium borohydride was eliminated with formaldehyde and removed from the environment. All thiol groups including reduced and native thiol were analyzed after reaction with DTNB (5,5'-dithiobis-2-nitrobenzoic acid). Native thiol (–SH) and total thiol (–SH+–S–S) levels were spectrophotometrically measured. Half of the difference between total thiol and native thiol exhibited the dynamic disulphide amount. Subsequently, total thiol amount, native thiol / total thiol ratio, disulphide / total thiol ratio and disulphide / native thiol ratio were calculated.

Preparation of tissues homogenate: The whole ovarian, oviduct and uterine tissues were homogenized in phosphate saline buffer (50 mM; pH 7.4) and then centrifuged (3000 rpm, 5 min, 4°C). The supernatant was separated and used for biochemical analysis.

Measurement of TAS and TOS levels and calculation of OSI: Total antioxidant-oxidant levels in the homogenized ovarian, oviduct and uterine tissues were determined using a novel automated measurement method (Rel Assay kit, Turkey; 8, 9). The antioxidative effect of the sample against the potent free radical reactions, which is initiated by the produced hydroxyl radical, is measured. This assay relies on the ability of antioxidants in the sample to inhibit the formation of 2,2'-azino-bis [3-ethylbenz-thiazoline-6-sulfonic acid (ABTS)]. The values of TAS are expressed as mmol Trolox equivalent /L (8).

The colour intensity is related to the total amount of oxidant molecules present in the sample. The test is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidative species in acidic medium and the measurement of the ferric ion by xylenol orange. The assay is calibrated with hydrogen peroxide (H_2O_2). The results are expressed in terms of µmol H_2O_2 equivalent /L (9).

The OSI was defined as the ratio of the TOS level to TAS level. TAS levels were converted to µmol. Specifically, OSI (arbitrary unit) = TOS (µmol H_2O_2 equivalent/L)/TAS (µmol Trolox equivalent /L) x 100.

Statistical analysis: Statistical analyses were performed by using SPSS 14.01 for Windows (SPSS, Inc., Chicago, IL, USA). Descriptive statistics for each variable were calculated and presented as "Mean ± Standard Error of Mean". Shapiro Wilk test for normality and Levene test for homogeneity of variances as parametric test assumptions. Kruskal Wallis analysis was used to evaluate the difference between variables and estrous cycle stages. Pearson correlation coefficient and Spearman's rho were used for calculation of the relationship between variables. Statistical significance was assumed at a level of $P < 0.05$.

Results

The determination of cycle stage: The stages of the estrous cycle were determined using by vaginal cytology, serum progesterone level and histologic findings. According to cytological findings, 9 bitches were in proestrus, 8 in estrus, 10 in diestrus (5 in early diestrus, 5 in late diestrus) and 2 in anestrus. These data were confirmed with serum progesterone and histological analysis.

Serum progesterone level: Mean level of progesterone was 1.24 ng/ml (min: 0.6-max: 1.85) in proestrus, 9.71 ng/ml (min: 3.88-max: 15.9) in estrus, 14.34 ng/ml (min: 3.01-max: 30.46) in diestrus (early diestrus 22.85 ng/ml; late diestrus 5.82 ng/ml), and 0.39 ng/ml (min: 0.30-max: 0.49) in anestrus stages.

Bright-field microscopy analysis: In proestrus, cortex of the ovary had several antral follicles. The endometrial stroma of the uterine tissue showed oedema and increased luminal crypts and basal glands. In estrus, there were tertiary follicles in ovarian tissue. Granulosa cells were changed to luteal cells. After ovulation, endometrial stromal oedema decreased and number of hypertrophic glands increased. In diestrus, the ovaries had large corpora lutea. Endometrium and myometrium layers exhibited the thickness and cellular density. During anestrus phase, corpora lutea gradually regressed and showed vacuole and had irregular size deeper in the medulla of ovary. The endometrium and myometrium were atrophic and endometrial glands are smaller in the uterus. The oviduct showed ciliated columnar epithelium with equal height secretory cells in proliferative phase. The lower ciliated cells have clear cytoplasm in luteal phase (Figure 1).

TAS, TOS, and OSI values, and correlations: Descriptive analyses of TAS, TOS and OSI values in tissues are shown in Table 1. There was no significant difference ($P > 0.05$) among TAS, TOS and OSI values in the reproductive tissues in relation to estrous cycle stages (Table 2). Correlation coefficient between variables are shown in Table 3, and between variables and estrous cycle stages are presented in Table 4. Correlation coefficient could not be analysed in anestrus stage due to the inadequate number of samples. Positive correlation was found ($P < 0.05$) between TOS in the uterine tissue and OSI in the oviduct tissue, and negative correlation was found ($P < 0.05$) between TAS in the oviduct and TAS in the ovary, TOS in the oviduct and the progesterone, OSI in the oviduct and progesterone, TOS in the uterine tissue and TAS in the oviduct, independently of estrous cycle stages. Positive correlation was found ($P < 0.05$) between TAS in the oviduct and TOS in the ovarian tissue in estrus stage, TOS in the uterine tissue and TAS in the ovarian tissue in late diestrus stage, and a negative correlation was found ($P < 0.05$) between TAS in the uterus and progesterone in proestrus stage, TAS in the uterus and TOS in the oviduct in early diestrus stage, TAS in the uterus and OSI in the oviduct in early diestrus stage.

Serum thiol/disulphide homeostasis status: The correlation coefficients between total thiol, native thiol, thiol disulphide and progesterone levels were calculated and positive correlation was found ($P < 0.05$) between thiol disulphide and total thiol ($r_p = 0.950$), progesterone and native thiol ($r_p = 0.603$).

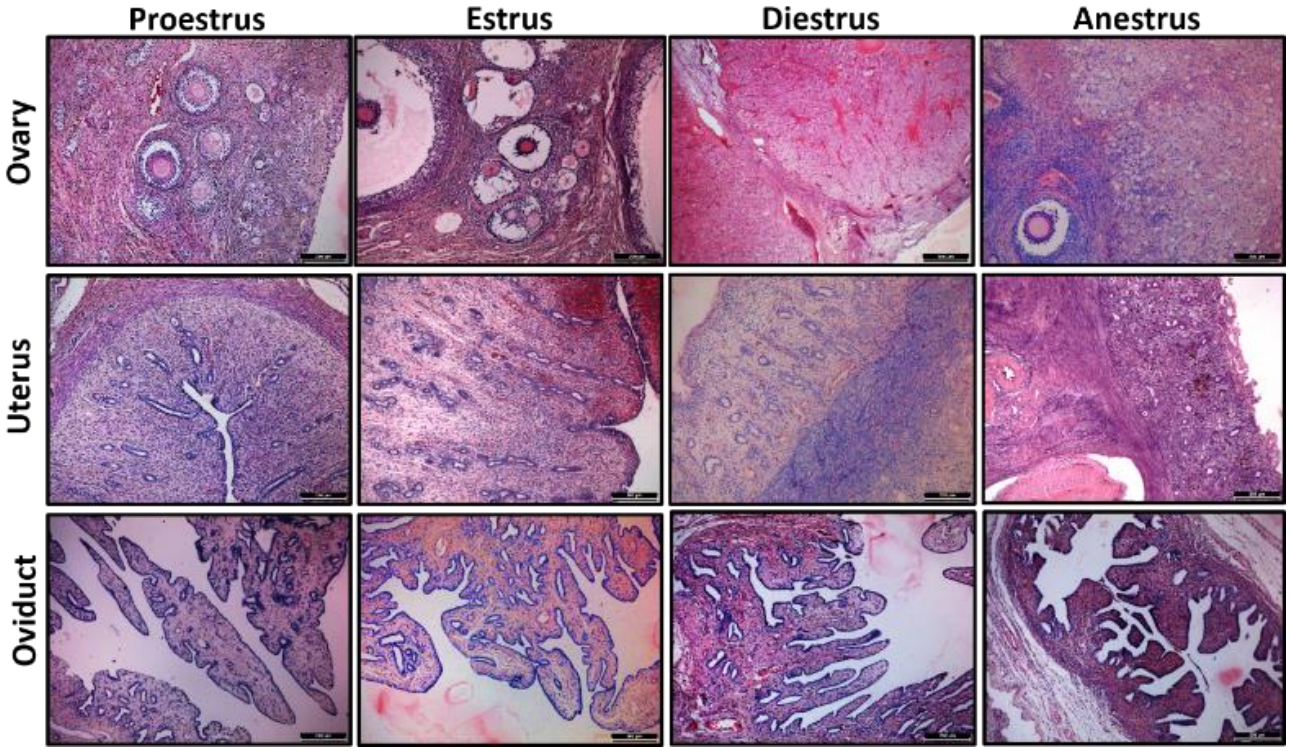

Figure 1. The histological views of ovary, uterus and oviduct tissues in different stages of estrous cycle in bitches. The scale bars: 200 µm.

Table 1. Descriptive statistics for variables.

Variables	N	Mean	S.E.M	Median	Min	Max
Progesterone	29	8.12	1.59	3.88	0.3	30.46
TAS Ovary	29	1.15	0.08	1.06	0.46	2.04
TOS Ovary	29	9.44	1.07	8.52	1.18	22.21
OSI Ovary	29	0.84	0.09	0.74	0.12	2.27
TAS Oviduct	29	1.36	0.07	1.39	0.47	2.00
TOS Oviduct	29	12.74	127	10.29	3.29	25.87
OSI Oviduct	29	1.08	0.19	0.79	0.25	5.46
TAS Uterus	29	1.44	0.10	1.47	0.47	2.19
TOS Uterus	29	13.98	1.23	15.07	3.23	25.92
OSI Uterus	29	1.23	0.22	0.80	0.27	5.09

S.E.M: Standard Error of the Mean.

Table 2. The results of differences between variables.

Variables	Estrous cycle	n	Mean	S.E.M	Min.	Max.	P
TAS Ovary	Proestrus	9	0.93	0.1	0.46	1.48	0.334
	Estrus	8	1.36	0.15	0.71	2.04	
	Early Diestrus	5	1.14	0.26	0.62	1.91	
	Late Diestrus	5	1.20	0.24	0.56	1.94	
TOS Ovary	Proestrus	9	7.14	1.27	1.78	13.50	0.219
	Estrus	8	12.41	1.78	5.60	18.64	
	Early Diestrus	5	12.22	3.39	5.89	22.21	
	Late Diestrus	5	6.69	2.50	1.18	14.40	
OSI Ovary	Proestrus	9	0.74	0.10	0.29	1.19	0.262
	Estrus	8	1.04	0.23	0.4	2.27	
	Early Diestrus	5	1.05	0.10	0.65	1.19	
	Late Diestrus	5	0.56	0.18	0.12	1.11	
TAS Oviduct	Proestrus	9	1.43	0.10	1.07	1.95	0.700
	Estrus	8	1.43	0.16	0.81	2.00	
	Early Diestrus	5	1.28	0.17	0.64	1.59	
	Late Diestrus	5	1.31	0.26	0.47	1.90	
TOS Oviduct	Proestrus	9	14.89	2.44	4.03	25.14	0.305
	Estrus	8	9.76	1.52	4.77	18.46	
	Early Diestrus	5	9.65	2.39	3.69	17.21	
	Late Diestrus	5	16.33	4.34	3.29	25.87	
OSI Oviduct	Proestrus	9	1.04	0.17	0.29	1.78	0.402
	Estrus	8	0.74	0.16	0.41	1.77	
	Early Diestrus	5	0.97	0.44	0.25	2.71	
	Late Diestrus	5	1.76	0.94	0.34	5.46	
TAS Uterus	Proestrus	9	1.42	0.19	0.47	2.08	0.233
	Estrus	8	1.31	0.19	0.55	2.12	
	Early Diestrus	5	1.13	0.23	0.54	1.77	
	Late Diestrus	5	1.79	0.18	1.25	2.19	
TOS Uterus	Proestrus	9	12.46	1.98	5.56	23.65	0.709
	Estrus	8	14.92	2.50	4.71	23.19	
	Early Diestrus	5	12.64	4.45	3.23	25.92	
	Late Diestrus	5	17.06	2.15	11.12	23.50	
OSI Uterus	Proestrus	9	1.26	0.49	0.27	5.09	0.747
	Estrus	8	1.28	0.34	0.56	3.58	
	Early Diestrus	5	1.50	0.84	0.34	4.84	
	Late Diestrus	5	1.05	0.23	0.51	1.61	

*****Difference in anestrus stage could not be analysed in anestrus stage due to the inadequate number of samples.

Table 3. Correlations between variables.

	P4	TAS Ovary	TOS Ovary	OSI Ovary	TAS Oviduct	TOS Oviduct	OSI Oviduct	TAS Uterus	TOS Uterus	OSI Uterus
P4	1									
TAS Ovary	0.166	1								
TOS Ovary	0.251	**0.503***	1							
OSI Ovary	0.235	-0.108	**0.754***	1						
TAS Oviduct	0.057	**-0.426***	0.035	0.289	1					
TOS Oviduct	**-0.395***	0.006	-0.080	-0.149	0.035	1				
OSI Oviduct	**-0.369***	0.005	-0.138	-0.183	-0.246	**0.922***	1			
TAS Uterus	-0.290	-0.241	-0.339	-0.178	-0.112	-0.189	-0.136	1		
TOS Uterus	0	0.233	0.210	0.083	**-0.374***	0.214	**0.410***	0.019	1	
OSI Uterus	0.038	0.296	0.207	0.052	-0.241	0.208	0.232	**-0.368***	**0.872***	1

*P < 0.05; P4: Progesterone.

Table 4. Cross correlations for variables according to estrous cycle stages (n= 29).

		P4	TAS Ovary	TOS Ovary	OSI Ovary	TAS Oviduct	TOS Oviduct	OSI Oviduct	TAS Uterus	TOS Uterus	OSI Uterus
Proestrus (n= 9)	P4	1									
	TAS Ovary	0.418	1								
	TOS Ovary	0.234	**0.700***	1							
	OSI Ovary	0.025	0.250	**0.833***	1						
	TAS Oviduct	0.259	-0.217	0.083	0.333	1					
	TOS Oviduct	0.301	-0.15	-0.267	-0.200	0.283	1				
	OSI Oviduct	0.075	-0.317	-0.533	-0.450	0.050	**0.933***	1			
	TAS Uterus	**-0.795***	-0.467	-0.267	0	0.233	-0.417	-0.283	1		
	TOS Uterus	0.343	-0.133	-0.333	-0.233	-0.150	0.200	0.250	-0.350	1	
	OSI Uterus	0.577	0.167	-0.117	-0.183	-0.317	0.433	0.433	**-0.783***	**0.783***	1
Estrus (n= 8)	P4	1									
	TAS Ovary	0.214	1								
	TOS Ovary	-0.381	-0.310	1							
	OSI Ovary	-0.405	**-0.714***	**0.833***	1						
	TAS Oviduct	-0.071	-0.071	**0.833***	0.667	1					
	TOS Oviduct	-0.333	0.452	0.190	0.048	0.286	1				
	OSI Oviduct	-0.262	0.190	-0.476	-0.381	-0.619	0.500	1			
	TAS Uterus	0.024	-0.119	-0.619	-0.214	-0.500	-0.071	0.238	1		
	TOS Uterus	-0.405	-0.476	-0.024	0.238	-0.381	-0.143	0.190	0.548	1	
	OSI Uterus	-0.500	-0.119	0.333	0.286	-0.024	0.190	0.071	0.167	**0.810***	1
Early Diestrus (n= 5)	P4	1									
	TAS Ovary	0.300	1								
	TOS Ovary	-0.100	0.600	1							
	OSI Ovary	-0.100	-0.100	0.700	1						
	TAS Oviduct	-0.100	-0.600	-0.200	0.300	1					
	TOS Oviduct	0.600	0.100	0.300	0.500	-0.300	1				
	OSI Oviduct	0.600	0.100	0.300	0.500	-0.300	**1**	1			
	TAS Uterus	-0.700	-0.300	-0.500	-0.600	0.100	**-0.900***	**-0.900***	1		
	TOS Uterus	-0.200	0.700	0.500	-0.100	**-.900***	0.100	0.100	0	1	
	OSI Uterus	-0.400	0.500	0.700	0.300	-0.700	0.200	0.200	-0.100	**.900***	1
Late Diestrus (n= 5)	P4	1									
	TAS Ovary	-0.200	1								
	TOS Ovary	0.100	0.500	1							
	OSI Ovary	0.100	-0.100	0.800	1						
	TAS Oviduct	-0.500	-0.700	-0.400	0.100	1					
	TOS Oviduct	-0.500	0.300	0.600	0.600	0	1				
	OSI Oviduct	-0.500	0.300	0.600	0.600	0	**1**	1			
	TAS Uterus	0.700	-0.700	0.100	0.500	0.200	-0.300	-0.300	1		
	TOS Uterus	-0.500	**0.900***	0.300	-0.200	-0.500	0.500	0.500	**-0.900***	1	
	OSI Uterus	-0.700	0.700	-0.100	-0.500	-0.200	0.300	0.300	**-1**	**0.900***	1

*P < 0.05; P4: Progesterone. Correlation coefficient could not be analysed in anestrus stage due to the inadequate number of samples (n= 2).

Discussion and Conclusion

The estrous cycle of bitches is classically divided into 4 recurring stages: proestrus, estrus, diestrus, and anestrus. These stages reflect the change of steroid hormone, and LH surge. Estrogen levels rise in follicular stage, progesterone levels increase with preovulator luteinisation in the initial stage of luteal period following LH surge, estrogen levels decrease immediately, ovulation occurs, progesterone declines slowly after 20-35 days of cycle, thereafter going below 1 ng/ml, respectively (5, 17). The determination of estrous cycle in dog breeding has a crucial role because of monoestrus pattern. Therefore, many diagnostic methods are developed for the detection of estrous cycle in dogs. For this purpose, progesterone and LH assay, vaginoscopy, vaginal cytology, behavioural and physical changes, ultrasonographic view of ovaries, electrical impedance of vagina, glucose concentration in vaginal fluid, ferning of vaginal fluid (11, 16, 28) and histological evaluation of reproductive tissues (21) are used. However, vaginoscopy, vaginal cytology and progesterone analysis are mostly preferred in clinical practice (3). In the present study, the stages of estrous cycle were characterized by vaginal cytology and serum progesterone level. Also, these results were confirmed with bright microscopic view of ovarian, oviduct and uterine tissues. Histological data were verified by our findings of vaginal cytology and progesterone analysis.

The estrogen concentration increases in progressive process, and accordingly oxidative balance shows an alteration due to antioxidative effect of estrogen (7, 26, 27). With the development of follicles, an increase in antioxidant substances is observed in the follicular fluid. Thus, granulosa cells, oocytes and steroidogenesis process are preserved. However, the ROS concentration begins to increase following preovulatory LH surge in the estrus stage. It is known that severe lipid peroxidation occurs in the preovulatory graafian follicle. Increased PMN and leucocytes, which are a part of the process of acute inflammation, due to LH peak during ovulation lead to increased ROS concentration (1, 20, 23). The result of TAS, TOS and OSI in different reproductive tissues in our study revealed that some variations about oxidative stress parameters were going on in the ovary, oviduct and uterine tissue concurrently. In the proestrus stage, uterine TAS and progesterone were negatively correlating, and TAS level in the oviduct tissue was positively correlating with TOS in the ovarian tissue. After all, TAS level in the uterus had a negative correlation with oviduct tissue TOS and OSI level in early diestrus stage. These findings suggested that oviduct was prepared with elevated TAS level to prevent oocyte after ovulation while ovary was in the periovulator/ovulator period with increased TOS level in estrus stage. The uterine antioxidant status was negatively affected while oxidative stress was being in raised in the oviduct tissue during early diestrus stage probably due to increased steroidogenesis and progesterone synthesis. Because, ROS plays a role as both a by-product and an influencer in progesterone synthesis in this period. In relation with thiol/disulphide homeostasis, there was a relationship between native thiol and progesterone levels in the present study. According to this finding, it was though that there might be an antioxidative protection mechanism with elevated native thiol level, synchronously with increased progesterone concentration during estrus and early diestrus stage.

In conclusion, the physiological values of TAS and TOS concentration, and OSI in the ovarian, oviduct and uterine tissues during estrous cycle were firstly defined in this article. The serum thiol/disulphide homeostasis was also determined in the estrous cycle of bitches. We have found that there is a remarkable variations of oxidative stress balance in the ovary, oviduct and uterus concurrently, during estrous cycle in the bitches. Besides, significant correlations between oxidative stress parameters and estrous cycle stages in the reproductive tissues were observed in the present study. According to results of present study, it was thought that follicular structure and fertility could be affected by oxidant/antioxidant profile changes in reproductive tissues. It was concluded that further studies should be conducted on this issue in dogs and other animal species.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Nutrient content and *in vitro* digestibility of kermes oak (*Quercus coccifera* L.) growing in the provincial borders of Burdur

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Abstract: The purpose of this study was to investigate the *in vitro* dry matter digestibility (IVDMD), nutrient content, cell wall components, total and condensed tannin content of kermes oak (*Quercus coccifera* L.) which is the main feed source for goat herds. Kermes oak was exemplified in 3 different stations during a year (12 months). Sampling was made to include leaves, some branches, acorns and young shoots, if any based on season, which were consumed by the goats. The amount of dry matter (DM), crude ash (CA), crude protein (CP), ether extract (EE), crude fibre (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF), acid detergent lignin (ADL), total tannin and condensed tannin content, and IVDMD (by using two-stage technique) were determined on the samples (12 × 3 = 36). Crude protein values were not statistically significant in terms of seasonal averages. On the other hand, it was found that the CF in spring and summer was significantly lower than the CF in winter ($P<0.05$) and the highest EE during the year was in the autumn ($P<0.05$). IVDMD of winter season was significantly lower than summer and autumn. ($P<0.05$). In terms of total tannin content, it was revealed that autumn was significantly lower than winter and spring ($P<0.05$). It was concluded that the nutritional values and IVDMD of kermes oak were low in the winter season.

Keywords: *In vitro*, kermes oak, nutrient composition.

Burdur il sınırları içinde yetişen kermes meşe (*Quercus coccifera* L.) 'nin besin madde içeriği ve *in vitro* sindirilebilirliği

Özet: Bu çalışmanın amacı, keçi sürüleri için ana yem kaynağı olan kermes meşesinin (*Quercus coccifera* L.) *in vitro* kuru madde sindirilebilirliği (IVKMS), besin maddesi içeriği, hücre duvarı bileşenleri, toplam ve kondanse tanen içeriğini belirlemektir. Kermes meşesi yıl boyunca (12 ay) üç farklı istasyondan örneklenmiştir. Örnekleme, keçiler tarafından tüketilen yaprakları, bazı dalları, palamutları ve mevsime göre eğer varsa bazı genç sürgünleri içerecek şekilde yapılmıştır. Örneklere ait kuru madde (KM), ham kül (HK), ham protein (HP), ham yağ (HY), ham selüloz (HS), asit deterjan fiber (ADF), nötral deterjan fiber (NDF), asit deterjan lignin (ADL), toplam ve kondanse tanen içeriği ile IVKMS değerleri (iki aşamalı teknik kullanılarak) belirlenmiştir (12 × 3 = 36). Ham protein değerlerinin, mevsim ortalamaları açısından istatistiksel önem taşımadığı belirlenmiştir. Diğer taraftan ilkbahar ve yaz HS değerinin kışa kıyasla önemli düzeyde düşük ($P<0.05$) ve yıl boyunca en yüksek HY'nin sonbaharda olduğu ($P<0.05$) belirlenmiştir. Kış mevsimi IVKMS, yaz ve sonbahara kıyasla önemli derecede düşük bulunmuştur ($P<0.05$). Toplam tanen içeriği açısından, sonbaharın kış ve ilkbahardan önemli düzeyde düşük olduğu belirlenmiştir ($P<0.05$). Araştırmada kermes meşesinin kış mevsiminde besin değerlerinin ve IVKMS'nin düşük olduğu sonucuna varılmıştır.

Anahtar kelimeler: Besin maddesi, *in vitro*, kermes meşesi.

Introduction

Mediterranean phytogeographical region has a vegetation which includes the evergreen, flaky shrub or shrubs, and kermes oak (*Quercus coccifera* L.) which is the dominant species that constitutes this vegetation (21). Hot and arid summer causes drying of superficially rooted plants; however, deep-rooted plants such as bushes can reach water and remain green (2). It can be considered that it is an important source of roughage for goats, especially if the pasture areas of sufficient quality and quantity are limited. Türkoğlu et al. (33) reported that there is a very strong positive relationship between growth performance of grazing hair goats and the feed mass of the kermes oak. Kamalak et al. (13) reported that oak leaves are rich in CP, CA, NDF, ADF and condensed tannin. However, they are weaker than acorns in terms of *in vitro* organic matter digestibility. Besides the plant parts of oak, the species of oak also affect nutritional properties of plant. For example,

Kökten et al. (16) reported that IVDMD of acorns of *Quercus patreae, Quercus libani, Quercus infectoria, Quercus cercis, Quercus macrolepis, Quercus vulcanica* and *Quercus coccifera* range between 74.67-85.23%. They stated that one of the lowest digestible species is *Quercus coccifera.*

There are some studies, limited in number though, which evaluated the effects of various oak species on the digestibility, feed consumption and fattening performance of goats in the period June - August (19, 34). However, the studies worked on different oak species on dry matter digestibility without concerning seasonal variation (13, 27) and on the nutritive value with ADF and NDF (13, 24). Besides, there are several studies that focused on the nutrient content (10, 13, 15, 26), fatty acid profile (6, 11, 20, 23, 29) and *in vitro* digestibility (10, 25-27, 34) of different species and parts of oak. A study (22) that was encountered in the relevant literature sampled on the main and lateral branch and fresh twigs with leaves of kermes. In the present study, however, samples contained leaves, some branches, acorns and young shoots (if any based on season) all together. Besides, *in vitro* dry matter digestibility of samples were processed by using two-stage digestion method. On the other hand, several fatty acid levels of collected samples were determined. Accordingly, the aim of the study is to evaluate the changes of *in vitro* dry matter digestibility (IVDMD), nutrient content, cell wall components, condensed and extractable tannin content of the samples in order to monitor the changes of nutritional value throughout the year in Burdur Province.

Material and Method

Kermes oaks were sampled from three different bush location within the borders of the Burdur Province. The coordinates of determined stations were 37˚40’33.9”N 30˚19’23.6”E – 37˚40’34.3”N 30˚19’36.0”E and 37˚40’36.4”N 30˚19’22.5”E. Management protocols of the research were made in accordance with approved local ethical committee of Burdur Mehmet Akif Ersoy University (2017-319). Kermes oak samples were collected for 12 months as of January 2017. Winter samples were composed of collected samples from three different station in December 2017, January 2017 and February 2017 (3 × 3 = 9); similarly, the others were composed the samples of each 3 consecutive months of the other seasons of 2017 (9 × 4 = 36 sample). Samples included all parts of plant which was consumed by goats as shown by shepherds. These parts are mixture of leaves, some branches and young shoots, if any based on season. Collected samples were dried in incubator at 65˚C for 48 h (Memmert UE500, F. NR. C593.0011, Memmert GMBH and CO. KG, Germany). Dried kermes oak was milled for analysis in the forage mill (Retsch SK100, 70511012, Retsch GMBH, Germany). Nutrient analysis (DM, CA, CP and EE) of oak samples was performed according to the methods reported in AOAC (3); CF, ADF, NDF and ADL analyses (crude ash free) were determined by using the methods of Crampton and Maynard (7) and Goering and Van Soest (12), respectively. For the analysis of total tannin and condensed tannin contents, oak samples were extracted based on the methods of Terrill et al. (30). Total soluble tannin content was determined by using the method of Lowry et al. (17) and condensed tannin content was determined according to Bate-Smith (5).

Two-stage digestion method was used to determine the changes in dry matter digestibility of oak samples during the seasons. Phosphate-bicarbonate buffer solution and acid-pepsin solution were used at the first and second steps of the method respectively (31). Rumen fluid used in the experiment was obtained from three healthy cow which consumed corn silage and commercial concentrate. The type and amount of fatty acids were determined by gas chromatography (GC/MS, Agilent 5975C, 7890A, Agilent, USA) using the method of Bardakçı and Seçilmiş Canbay (4). For this purpose, the solvent of the oil sample obtained was eliminated by using nitrogen flow and the residue was dissolved in 2 ml of toluene. 5 ml of 1% methanol H_2SO_4 was added and mixed well (8). 5 ml of 2% NaCl solution was added to the mixture which was esterified in water bath at 50ºC for 12 hours. The released fatty acid methyl esters were extracted with hexane. The methyl ester mixture was washed with 5 ml of 2% $KHCO_3$ and dried over anhydrous sodium sulfate. Subsequently, methyl esters were dissolved in 2 ml hexane and analyzed using a gas chromatography. Meteorological records (18) of Burdur province in 2017 were obtained from Burdur Provincial Directorate of Meteorology. According to the climate data, the average temperature was the highest in July (41˚C) and the lowest in January (11.3˚C). The highest precipitation in the year was 60.2 mm in May.

A package program of SPSS was used for statistical analysis (28). Seasonal differences of the investigated parameters were examined through One-Way ANOVA. The significance of mean differences between the seasons was controlled by using Duncan test (14). The results of the investigated parameters were given as marginal means and their standard error of mean. $P<0.05$ was determined for the level of significance (9).

Results

Chemical composition of Kermes oak (*Quercus coccifera* L.) can be seen in Table 1. *In vitro* DM digestibility (IVDMD) and total soluble tannin and condensed tannin levels are presented in Table 2. Fatty acid compositions of samples are provided in Table 3. In

terms of CA values, autumn (3.58%) was found to be significantly lower than summer and winter (P<0.05). On the other hand, there was no statistical difference observed between the mean CA values of spring, summer and winter. It was determined that CP values of samples were ranged from 6.93 to 7.58% throughout the year. The EE content of autumn was determined as the highest (5.54%) when comparing with other seasons (P<0.05). While CF of winter and autumn samples (23.37% and 22.00%) were similar, they were significantly higher than spring and summer averages (P<0.05). In terms of NDF values, the summer is the lowest (47.71%) among the other seasons (P<0.05). Besides, ADF values of summer was higher than the other seasons (P<0.05), while ADF ratio of the spring and autumn were the lowest (P<0.05). There was no statistical significance observed on ADL among the seasons. IVDMD value of winter was lower than summer and autumn (P<0.05). In the autumn, the total soluble tannin content was lower than winter and spring (P<0.05). However, in terms of condensed tannin, there was no difference observed among the seasons. Linoleic acid levels of samples in summer and winter were determined similar, on the other hand, these two season levels were determined higher than spring and autumn (P<0.05).

Table 1. Chemical composition of kermes oak (*Quercus coccifera* L.) samples (% of DM).

Parameters (%)	Winter	Spring	Summer	Autumn	P value
DM	59.10 ± 1.37	57.09 ± 1.22	55.50 ± 1.50	56.77 ± 0.62	0.15
CA	4.37 ± 0.11^{a}	4.03 ± 0.09^{ab}	4.22 ± 0.18^{a}	3.58 ± 0.21^{b}	0.01
CP	7.51 ± 0.26	7.58 ± 0.29	6.93 ± 0.11	7.16 ± 0.35	0.31
EE	3.04 ± 0.28^{c}	3.38 ± 0.27^{c}	4.20 ± 0.20^{b}	5.54 ± 0.33^{a}	0.01
CF	23.37 ± 0.52^{a}	21.00 ± 0.60^{b}	21.58 ± 0.41^{b}	22.00 ± 0.53^{ab}	0.02
NDF	69.28 ± 1.69^{a}	62.44 ± 0.89^{b}	47.71 ± 0.75^{c}	66.58 ± 0.91^{ab}	0.01
ADF	52.23 ± 1.30^{b}	48.37 ± 1.16^{c}	64.13 ± 0.86^{a}	47.62 ± 1.08^{c}	0.01
ADL	34.14 ± 3.71	27.85 ± 1.46	29.48 ± 2.23	27.72 ± 2.07	0.25

n= 9; DM: Dry matter; CA: Crude ash; CP: Crude protein; EE: Ether extract; CF: Crude fibre; NDF: Neutral detergent fibre; ADF: Acid detergent fibre; ADL: Acid detergent lignin; ^{a,b,c}: Means in a row with different superscripts are significantly different (P<0.05).

Table 2. *In vitro* dry matter digestibility (%), total and condense tannin (g/kg) contents of Kermes (*Quercus coccifera* L.) (% of DM).

Parameters	Winter	Spring	Summer	Autumn	P value
IVDMD	22.55 ± 0.95^{b}	24.32 ± 0.98^{ab}	27.36 ± 1.58^{a}	27.01 ± 0.70^{a}	0.01
Total tannin	58.59 ± 2.27^{a}	56.91 ± 1.55^{a}	54.86 ± 1.59^{ab}	51.05 ± 1.42^{b}	0.03
Condanse tannin	15.06 ± 2.42	14.33±1.32	12.92±1.27	17.13± 2.03	0.44

n=9; IVDMD: *In vitro* dry matter digestibility; $^{a, b}$: Means in a row with different superscripts are significantly different (P<0.05).

Table 3. Fatty acid composition of Kermes (*Quercus coccifera* L.) oak (% of total methyl esters of fatty acids).

Parameters (%)	Winter	Spring	Summer	Autumn	P value
Myristic acid	5.87 ± 0.12	6.35 ± 0.24	6.40 ± 0.17	6.52 ± 0.18	0.09
Palmitic acid	30.66 ± 0.17	31.46 ± 0.45	31.66 ± 0.46	30.45 ± 0.18	0.05
Palmitoleic acid	2.15 ± 0.05	1.93 ± 0.07	1.87 ± 0.09	1.91 ± 0.11	0.14
Stearic acid	7.69 ± 0.32	8.57 ± 0.37	7.59 ± 0.17	8.54 ± 0.16	0.20
Oleic acid	41.15 ± 0.13	40.65 ± 0.46	40.73 ± 0.38	41.38 ± 0.29	0.39
Linoleic acid	8.57 ± 0.19^{a}	6.82 ± 0.18^{b}	8.07 ± 0.03^{a}	7.07 ± 0.29^{b}	0.01

n= 9; $^{a, b}$: Means in a row with different superscripts are significantly different (P<0.05).

Discussion and Conclusion

In the present study a parallelism was observed between CF and NDF values in terms of 2017 seasons. Although there were seasonal differences, the results of present study were similar to the report of Alatürk et al. (2) who state that NDF and ADF increase with plant maturation. Parlak et al. (22) declared that the level of NDF was about 60% except in April (29.8%), and that the levels of ADF and ADL were about 45% and 19%, respectively during the year. In the present study seasonal ADL levels were determined higher than Parlak et al. (22); on the other hand, generally, the results of ADF and NDF levels were found similar. Moreover, Roukos (25) who worked with the leaves of kermes oak found that the amounts of NDF, ADF and ADL (582, 366 and 132 g/kg dry matter, respectively) was the least in level in August and September when compared to the present study. These differences between the results of investigations may be due to different plant parts with the proportions of the samples. On the other hand, these results confirm that the shrubs consumed by the goats in Burdur, Isparta and its vicinity are rich in cell wall components.

The results of CP levels of kermes oak samples in the present study were similar to Parlak et al. (22) who declared that CP of kermes oak remained approximately 7.5% throughout the year. On the other hand, Roukos (25) found that CP levels of kermes oak leaves were between 14.3% and 14.7% in April and May. It is clear that grazing goats should be fed with additional energy in spring and with additional CP in summer throughout the year in the Mediterranean climate conditions.

It is revealed that CP of sun-drenched twigs and leaves of kermes oak was higher than sunless parts when the plant development was fast (April) (15). Tolunay et al. (32) noted that kermes oak accelerates its spread in March and sprouts in April. They stated that its leaves from the previous season begin to fall in the middle of June. However, we did not find any significant difference among the seasons in the present study. This may be because of the sampling method and the number of the samples. The findings of this study is similar to Alatürk et al. (2) who declared that CP levels of kermes oak are the lowest in summer and the highest in winter and spring. Kamalak et al. (13) found that the CP values of leaves and acorns of kermes oak were 9.17% and 4.23%, respectively. These differences were not seen in the present study because of the sampling method. In conclusion, these studies show that CP levels of different parts or the mixture of all parts of kermes oak are generally low in crude protein.

The EE levels of kermes oak samples in the study were determined as under 5.54% during the year and this result is similar to Alatürk et al. (2) who stated that EE levels of bush species consumed by goats in Isparta Province does not exceed 7.50% seasonally on average.

In vitro dry matter digestibility of Kermes oak was not statistically significant in the spring, summer and autumn while the average of summer and autumn was significantly higher than winter. There was no statistical difference in terms of IVDMD during spring and winter. The fact that the NDF level in the summer was statistically lower compared to the other seasonal averages may have caused the IVDMD to be higher but not statistically significant. However, Roukos (25) revealed that the IVDMD of oak leaves and twigs was significantly lower between August and September compared with the other mounts of year. Parlak et al. (22) declared that dry matter digestibility of kermes oak (Calculated by N and ADF content) was the highest in April (70%), and was between 43.6 to 51.4% in other mounts of year. These results may be due to the use of the difference of methods.

Roukos et al. (26) declared that cell wall digestibility of acorns placed in low altitude and warm climate conditions was higher than placed in high altitude and cold climate conditions. IVDMD of samples in winter was significantly lower than other seasons of the year. This may be caused by high levels of CF and NDF in winter.

Natis and Malechek (19) declared that with the ratio of 46.7%, IVDMD of *Quercus gambelii* was higher than that of the samples in the present study. This difference may be caused by the species of oak. Beside this, Kamalak et al. (13) reported that the leaves of *in vitro* organic matter digestibility of *Quercus coccifera* was 47.05% and this ratio was higher than the present study. It is clear that the difference between the results was caused by the part of plant. On the other hand, similar to the present study, Sidahmed et al. (27) declared that IVDMD of red oak was determined as 29.4%.

It can be concluded that the CP of *Quercus coccifera* is lower than *Quercus persica*, *Quercus infectoria* and *Quercus libani*, and NDF and ADF content of *Quercus coccifera* is similar when these four oak species were compared. Elahi and Rouzbehan (10) stated that the CP levels of *Quercus libani* was 12.3%, ADF levels of *Quercus persica* was 53.2%, and lignin levels of *Quercus infectoria* was 10.3%.

In vitro dry matter digestibility of *Quercus coccifera* did not exceed 27.36% during the 2017 in Burdur province. However, Raju et al. (24) reported that the goats which consume high tannin content have lower IVDMD compared to the goats which consume green grass. Alatürk et al. (2) showed that the highest level of tannins was in the species they examined in sea grape (2.18%) and the lowest in spartium (0.11%). In the present study, condensed tannin content of samples were determined between 12.92 to 17.13 g/kg throughout the year. Kamalak et al. (13) declared that the condensed tannin level of the

leaves of *Quercus coccifera* was 9.22%. Akbağ (1) reported that the condensed tannin content of berm oak in the spring, summer and autumn averages were 19.16; 17.87 and 18.82 g/kg, respectively.

The literature related to the fatty acid composition of oak species focused on the acorns of the plant. When comparing the levels of saturated fatty acids of oak species, the samples of *Quercus coccifera* collected at the present study had more stearic acid levels than the acorns of several oak species. Studies on *Quercus rotundifolia* (29), *Quercus ilex* and *Quercus suber* (6), *Quercus pontica*, *Quercus robur* ssp., *Quercus vulcarinica*, *Quercus pubescens*, *Quercus ithaburensis* ssp. *macrolepis*, *Quercus brantii*, *Quercus libani* and *Quercus trojana* (20), *Quercus robur* L. and *Quercus cerris* L (23), *Quercus suber*, *Quercus rotundifolia* and *Quercus pyrenaica* (11) showed that palmitic acid level was between 12.72-22.8%; stearic acid level was between 0.9-3%; oleic acid level was between 16.9-65.05%; and linoleic acid level was between 13.6-49.1%. It was observed that palmitic and stearic acid ratios of the obtained fatty acids were higher than those of oak samples; oleic acid ratios were similar and linoleic acid ratios were lower. This difference may be due to the fact that the research samples contain all parts of the plant that are consumed by goats.

It was concluded that CF content of kermes oak increased as of summer and reached the highest level in winter. Because of the low IVDMD were determined in winter and the highest NDF content were seen in autumn and winter, it can be said that kermes oak completes its vegetation at the winter in Burdur climate. On the other hand, the plant has the most suitable tannin content for the animals in the autumn months. Because of kermes oak is the main source of feed throughout the year in Burdur, the nutrient requirements of mountain grazing goats are needed to be supported especially in winter and spring months.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Case Report / *Olgu Sunumu*

Spontaneous toxoplasmosis in a chicken

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Abstract: The carcasses of three 1-year-old, Leghorn chickens were presented for necropsy; one of the chickens was told to have shown neurological signs before death. At necropsy, meningeal and cerebral hyperemia were observed in the carcass that had neurological symptoms, and diarrhea was evident in the other two chickens. Histopathological examination revealed nonsuppurative meningoencephalitis, myositis in skeletal and cardiac muscles, and focal nonsuppurative hepatitis, and nephritis. Bradyzoites and tachyzoites were present in the meninges and brain. The present case is the first report of *Toxoplasma gondii* infection in a chicken in Turkey.
Keywords: Chicken, histopathology, *Toxoplasma gondii.*

Bir tavukta doğal toksoplazmozis olgusu

Özet: Bu çalışmanın materyalini nekropsi yapılması amacıyla getirilen ve bir tanesi sinirsel semptom göstererek ölen, bir yaşlı üç adet beyaz Leghorn ırkı ölü tavuk oluşturdu. Nekropside sinirsel semptom göstererek ölen tavukta meninksler ve beyinde hiperemi, diğer iki tavukta diyare dışında herhangi bir patolojik bulguya rastlanmadı. Dokulardan alınan örneklerin histopatolojik muayenesinde nonsuppuratif meningoensefalitis, iskelet ve kalp kasında myozitis, fokal nonsuppuratif hepatitis ve nefritis görüldü. Meninksler ve beyinde bradizoit ve serbest takizoitler gözlendi. Histopatolojik incelemeler sonucunda toksoplazmozis olarak tanımlanan bu olgu Türkiye'de tavuklarda bildirilen ilk vakadır.
Anahtar sözcükler: Histopatoloji, tavuk, *Toxoplasma gondii.*

Toxoplasmosis is a systemic protozoal disease caused by the intracellular protozoan *Toxoplasma gondii* that infects multiple mammalian, poultry and wild bird species (9, 14, 18, 19). In addition to naturally occurring toxoplasmosis cases in domestic birds, experimental studies have been carried out in many species such as white quails, Japanese quails, chickens, broilers, pigeons, turkeys, and pheasants (2, 3, 7, 8, 14). *Toxoplasma gondii* has a subclinical course in many avian species. The prevalence of *T. gondii* in naturally infected poultry varies greatly (5, 10, 12, 18). Studies reported that the prevalence in caged and free-range chickens were 30-50% and 100%, respectively (10, 20). Soil contamination with oocysts excreted by infected cats is an important factor in the development of disease in free-range poultry (10). Several seroprevalences (1, 16) and experimental studies (2, 3, 8, 14) have been conducted with domestic and wild avian species in Turkey. In this article, we describe the histopathological features of a *T. gondii* infection in a chicken from a family backyard farm consisting of 15 free-range Leghorn chickens.

Carcasses of three 1-year-old, Leghorn chickens were presented for a necropsy. One of the chickens had shown partial hind limb paresis and torticollis before death and the other two had shown non-specific clinical signs, including diarrhea. Additionally, presence of cats and their contact with chicken in the family backyard farm was reported by the animal owner. At necropsy, no macroscopic lesion was observed except meningeal and cerebral hyperemia in the carcass with neurological symptoms. Necropsy of the two other chickens revealed watery intestinal content. Collected tissue samples were fixed in 10% neutral formalin. After 48-hour fixation, tissue specimens were processed routinely and sectioned at 5 μm. After staining with hematoxylin and eosin (H&E), slides were examined under a light microscope (Olympus, BX51). Histological examination of the brain revealed edema, hyperemia and hemorrhages while

lymphocytic infiltration and fibrin deposition were seen over meninges (Figure 1A, 1B). There were irregularly-shaped necrotic areas in the substantia grisea, particularly in the occipital and temporal lobes. Edema, gliosis large haemorrhage areas (Figure 1C), and perivascular cuffing by lymphocytes (Figure 1D) were also observed in the brain parenchyma. Approximately 2x5 µm, elongated, banana-shaped structures resembling *T. gondii* tachyzoites (Figure 2A, 2B) and perivascular and meningeal-located bradyzoites were present in the surroundings of necrotic foci (Figure 2C-F). The presence of lymphocyte-rich mononuclear cell infiltration was noted among myocardial and skeletal muscle fibers (Figure 3A, 3B). Focal hemorrhage and hyperemia of the liver and kidneys accompanied mononuclear cell infiltrations, predominantly composed of lymphocytes (Figure 3C, 3D). The other two chickens were examined microscopically and no pathological findings associated with toxoplasmosis were observed.

Although clinical disease is rarely seen in chickens, they are one of the most sensitive domestic species for *T. gondii* infection (6, 11). Contamination occurs by ingesting sporulated oocytes that are spread to the environment by the feces of cats (17). Due to their ground-feeding behaviour, free-range chickens are more susceptible to contaminated food and water than caged chickens (15). In this report, we describe histopathologically diagnosed toxoplasmosis in a Leghorn chicken referred for necropsy. Researchers have reported that contact with the feces of infected cats in the small backyard farms may increase the possibility of contamination with oocysts (15, 17). In the present case, the information from the animal owner suggests that toxoplasmosis in chickens might have been transmitted from cat feces.

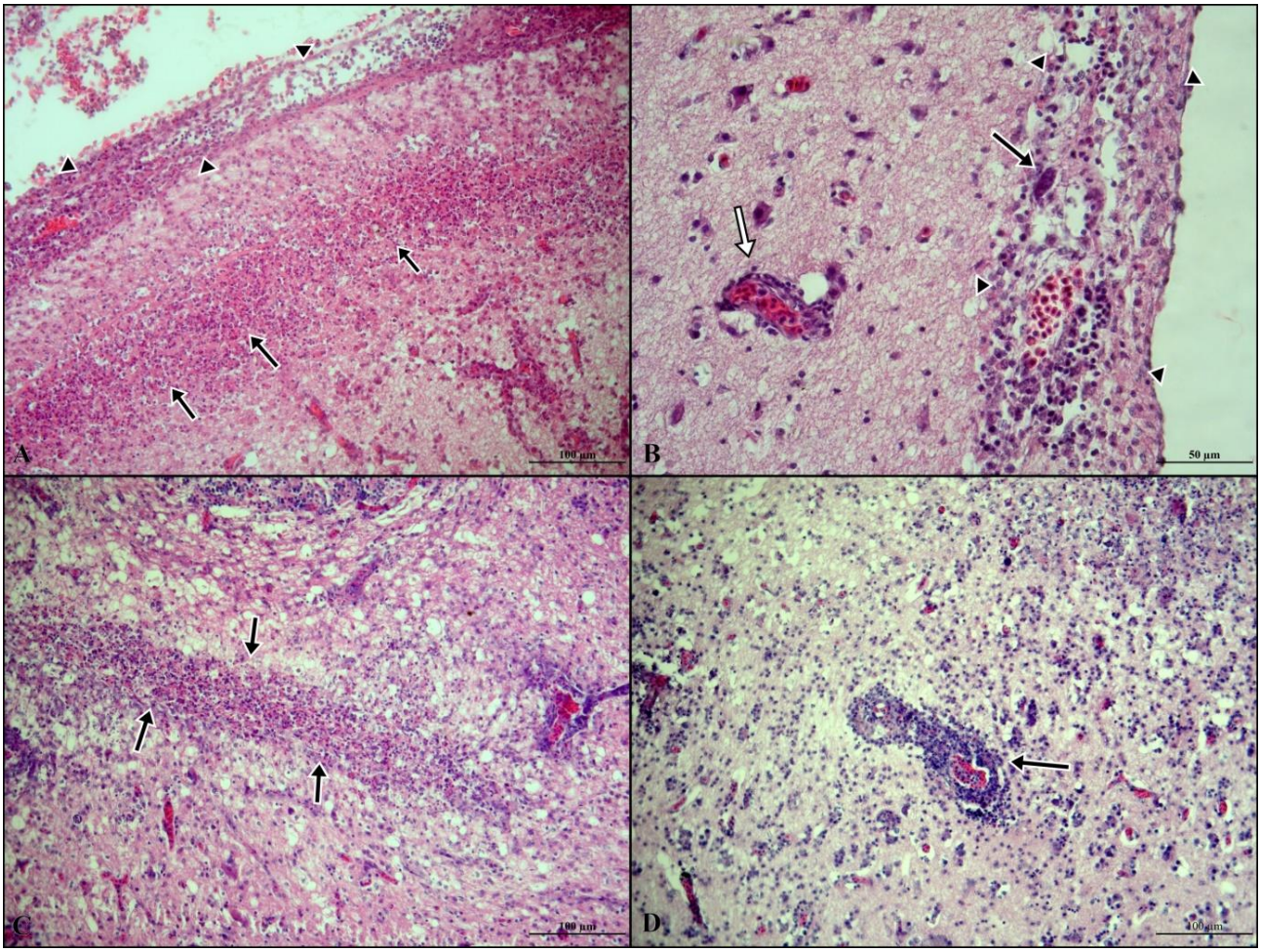

Figure 1. A. Large necrosis areas (arrows) in the brain parenchyma and meningitis (arrowheads), H&E, Scale bar: 100µm **B.** Perivascular cell infiltration (white arrow), meningitis (arrowheads) and bradyzoite cysts (black arrow) H&E, Scale bar: 50µm, **C**. Edema and necrotic areas in the brain parenchyma (arrows) H&E, Scale bar: 100µm, **D.** Perivascular lymphocytic cell infiltrations (perivascular cuffing formation) in the brain parenchyma (arrow), H&E, Scale bar: 100µm, chicken.

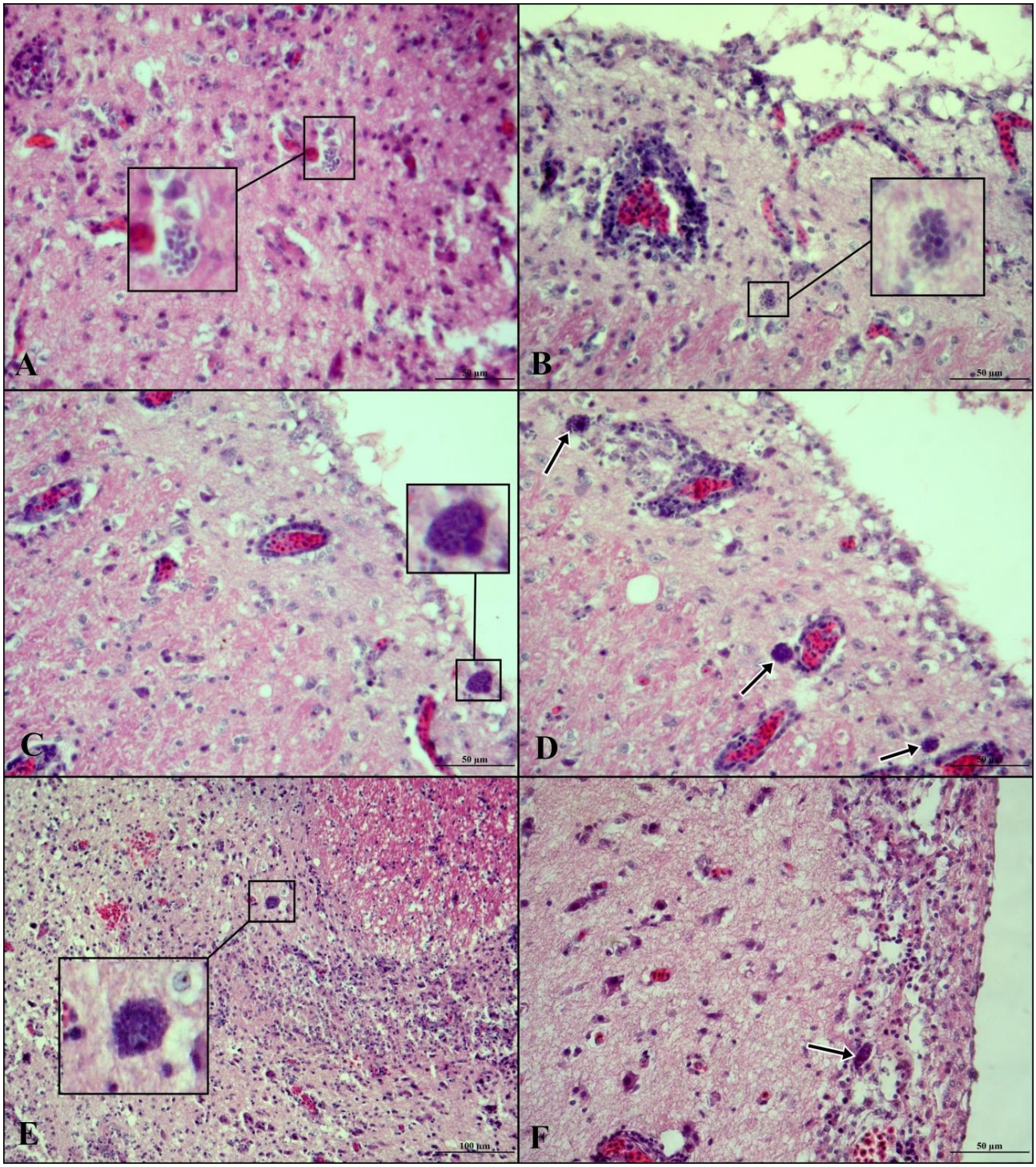

Figure 2. A. The appearance of free tachyzoites in the brain parenchyma, H&E, Scale bar: 50µm; **B.** The appearance of bradyzoites in the brain parenchyma, H&E, Scale bar, 50µm, **C.** The appearance of bradyzoites in brain parenchyma, H&E, Scale bar: 100µm, **D.** Perivascular forms of bradyzoites in the brain parenchyma (arrow), H&E, Scale bar: 50µm, **E.** Bradyzoites in the periphery of the necrotic area in brain parenchyma, H&E, Scale bar: 100µm, **F.** The appearance of bradyzoites (arrow) in meninges, HxE, Scale bar: 50µm.

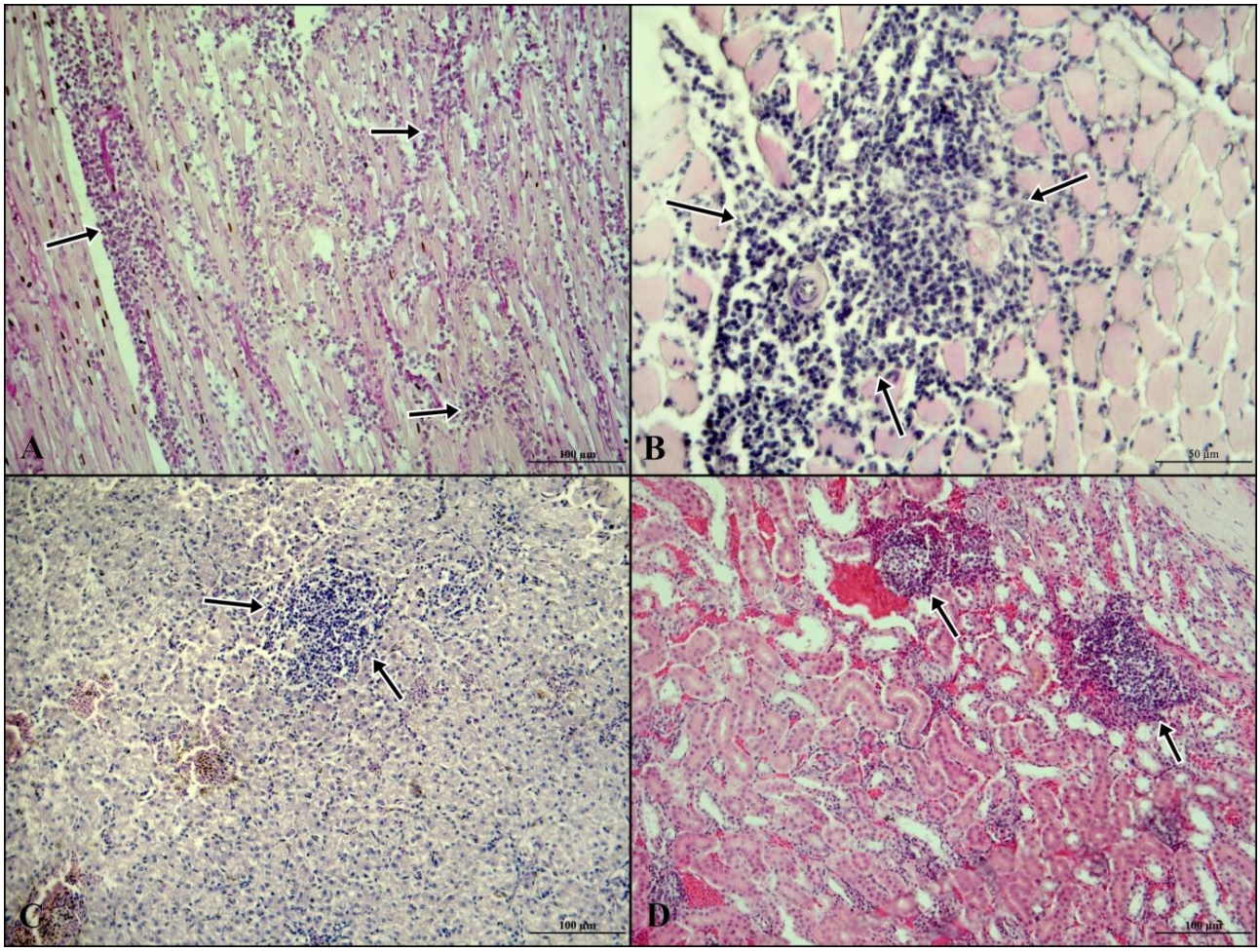

Figure 3. Intense lymphocytic cell infiltration among heart (A. Scale bar: 100µm) and skeletal muscle fibers (B. Scale bar: 50µm) (arrows); the areas of focal lymphocytic cell infiltration in liver (C. Scale bar: 100µm) and kidney (D. Scale bar: 100µm) (arrows), H&E.

Nonspecific clinical findings including diarrhea were present in two of the three dead chickens in our case and it was similar with previous reports (2, 13). Immunohistochemistry and histopathological examination are important techniques to confirm the diagnosis of toxoplasmosis (13). Mostly, immunohistochemistry is required in cases where toxoplasmosis cannot be diagnosed histopathologically. An immunohistochemical examination could not be performed in this case because we did not have chicken compatible anti-*T. gondii* antibody, but histopathological examination revealed bradyzoites and tachyzoites of *T. gondii* in brain sections. For the diagnosis of *T. gondii*, organs such as brain, liver, lung, myocardium, small intestine, and spleen (4, 13) should be sampled, and observation of protozoal cysts and tachyzoites is important in definitive diagnosis (4, 6). Necrosis and cell infiltrations in toxoplasmosis are related to inflammation, and necrotic changes have been associated with intracellular proliferation of the parasite (6). In this report, the observation of *T. gondii* bradyzoites and tachyzoites in the brain parenchyma and the presence of necrotic areas and perivascular lymphocytic cuffings enabled us to consider this case as a *T. gondii* infection. The results were consistent with the findings of other researchers who reported meningoencephalitis, myositis, focal hepatitis, and nephritis (4, 6, 13).

In conclusion, this is the first spontaneous case of toxoplasmosis detected during histopathological examination in a one-year-old Leghorn chicken in Turkey. The animal showed no clinical signs and was reported to be in contact with cats before death. Therefore, we aim to drew attention to chickens in contact with cats regarding toxoplasmosis.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Review / *Derleme*

Pharmacotherapy of canine atopic dermatitis - current state and new trends

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Abstract: This review offers a concise overview of current treatment options for canine atopic dermatitis and provide an outline of two promising new treatment options (phosphodiesterase 4 and histamine H4 receptor inhibitors). Glucocorticoids have been one of the first successful treatment options and are still part of the treatment regime. Ciclosporin was introduced more than 15 years ago and is also a main pharmacological treatment option. In 2013, the Janus kinase inhibitor oclacitinib was introduced as a first in class, which is then followed by the anti-canine IL-31 antibody lokivetmab in 2016. Thus, exciting new treatment options have found their way into clinical practice. Apart from these substance classes, antihistamines, essential fatty acids and lipid substitution will be discussed as add-on treatments.

Keywords: Atopic dermatitis, dog, pharmacotherapy.

Introduction

Atopic dermatitis (AD) is a chronic recurrent inflammatory skin disease that is clinically characterized by extreme itching and a typical eczematous morphology and body distribution. AD is a common skin disease, particularly in dogs, as almost 10% of the dog population is affected by this hypersensitivity (15). In other species, such as horses and cats, it plays a less substantial role. In the following, we will therefore focus on atopic dermatitis in dogs, as the diagnosis and treatment outcome is best characterized for the dog. AD is a genetically predisposed disease that for the majority of dogs occurs for the first time between the ages of 6 months and 3 years. The symptoms in dogs are typically accompanied by localized dermatitis (face, ears, paws, abdomen, armpits, inner thighs) and often severe itching. The severe itching and cutaneous hyperreactivity lead to a vicious circle of self-injury due to scratching, destruction of the skin barrier, penetration of bacteria and allergens followed by a dysregulation of cytokine release (31). Complications include secondary pyoderma and dermatitis induced by Malassezia. The exact pathogenetic mechanisms that contribute to the establishment of this allergic disease are still only partially understood. Recent research indicates that genetic and environmental factors are involved in determining susceptibility to clinical disease. Dogs might be sensitized to environmental allergens but also to food allergens. However, microbial and even insect antigens might also be the source of trigger that leads to inflammatory cell infiltration into the skin. Due to their dominance in cellular infiltrates in lesioned skin, there are indications for an important role of antigen-presenting (dendritic) cells and T-cells (24). In addition, other pathogenetic factors such as keratinocyte dysfunction or skin barrier dysfunction play a role, as well. Intercellular lipids are important for an intact barrier function. The lipids extractable from normal skin are composed of ceramides, cholesterol and free fatty acids in nearly equimolar proportions. An imbalance of the lipid metabolism can lead to a deficiency of stratum corneum ceramides and the disturbance of the barrier function in atopic dermatitis. This has also been shown for the atopic dog (8). In the *stratum corneum* of atopic dogs, the contents of some ceramides are lowered, while the cholesterol content is increased. These lowered ceramide levels may also be responsible for a disturbed barrier function (8, 27), which might favor the penetration of typical AD triggering allergens (e.g. antigens from grass pollen or house dust mite). In this review, current treatment options for canine atopic dermatitis are summarized and updated for a former review in German (3).

Pharmacotherapy of atopic dermatitis

Glucocorticoids: Although glucocorticoids are one of the oldest forms of therapy, they still play an important role in the pharmacotherapy of AD despite undesirable drug effects like polyuria, polydipsia, muscle atrophy, behavioral changes, bacterial and fungal infections and, especially after topical administration, skin atrophy. They are characterized by both anti-inflammatory and antipruritic effects, whereby the mechanism of action is mainly based on the anti-inflammatory effect. In addition, glucocorticoids also have an influence on the expression and secretion of a number of itching mediators. They are administered both topically and systemically and the clear advantage is their fast onset of action (31).

Systemic glucocorticoid therapy: Prednisolone has been the drug of choice for systemic therapy for decades. Initially the dosage should be 0.5 - 1 mg/kg. Once the itching is significantly reduced, the therapy interval is extended to every 48 hours. A maintenance dose of 0.25-0.5 mg/kg/48h can be achieved (varies in individuals), so that side effects can be reduced.

Topical glucocorticoids: As a topical therapeutic option, a 0.0584% hydocortisone aceponate spray has been approved in Europe for the treatment of canine AD. A published clinical study on efficacy in the treatment of AD showed a significant reduction in inflammation (skin lesions) and itching 28 days after treatment compared to the placebo group. The treatment was well-tolerated and there were no stronger adverse effects (e.g. cortisol suppression), suggesting low absorption and/or rapid metabolism of hydocortisone aceponate (21). More recently, it has been demonstrated that the topical treatment with hydocortisone aceponate is suitable for long-term maintenance therapy (18) in a pro-active manner, i.e. the glucocorticoid is administered in lesion free periods (e.g. twice a week) and time to relapse is significantly extended by this treatment schedule.

Ciclosporin: The calcineurin inhibitor ciclosporin was originally used in human medicine as an immunosuppressant to prevent transplant rejection. In human medicine, ciclosporin is also approved for the treatment of severe forms of atopic dermatitis that is otherwise therapy-resistant. Cyclosporin works by binding to cyclophilin in the cytoplasm of lymphocytes (and keratinocytes) and thus inhibits the translocation of the nuclear factor of activated T cells (NF-AT) to the nucleus. This ultimately leads to a reduced synthesis of cytokines like IL-2 and IFN-γ. Apart from its effect on the function of lymphocytes, it also leads to a modulation of dendritic cell function (especially Langerhans cells). In addition, the function of other inflammatory cells (mast cells, macrophages) is impaired and the activation of keratinocytes is inhibited (2).

Ciclosporin has been successfully used in veterinary medicine for the treatment of canine AD for several years now. In clinical studies, ciclosporin A shows an anti-inflammatory and itch-reducing effect comparable to that of prednisolone (23, 25). Ciclosporin is administered orally at a dose of 5 mg/kg/day until the symptoms are controlled, then reduced to the lowest effective maintenance dose. Compared to glucocorticoids, the onset of action is delayed and the patient owner has to be informed that an optimal reduction of lesions (and itch) might take up to 4 to 6 weeks. Although ciclosporin is generally considered safe for long-term administration, adverse effects, including nausea, vomiting and diarrhoea as well as gingiva hyperplasia can occur (31). Often, side effects are observed especially during the initiation of treatment and might be controllable in the long term.

Antihistamines: The first generation of H1 antihistamines is currently used for the treatment of canine AD, because they have a pronounced sedative effect and often also an anticholinergic effect. In dogs, diphenhydramine (p.os 2-5 mg/kg 1-2x daily) or chlorphenamine (p.os 4-8 mg/kg 2-3x daily) are used. Diphenhydramine might not work in the recommended doses, as we did not see an inhibition of histamine-induced weal or flare reactions in laboratory dogs (11). For hydroxyzine, a reliable PK/PD modelling exists for histamine and anti-IgE induced skin reactions. It was demonstrated that 2 mg/kg hydroxyzine can reduce the skin reaction significantly (6). If ineffective, a change of antihistamine can be successful. Nevertheless, the data on efficacy (reduction of itching) vary from 10% to a maximum of 50% (23, 25). Therefore, the administration of H1 antihistamines can only be supportive but can be encouraged, as side effects are rare and tolerable (e.g. sedation after administration of first generation antihistamines). Often, there is at least a drug sparing effect (e.g. reduction in prednisolone dose by co-administration of an anti-histamine).

Essential fatty acids: Numerous studies on the supplementation of essential fatty acids in dogs with AD have now been published. Many studies indicate that the clinical progression (itching and inflammation) can be positively influenced. The n-6 fatty acids, such as linoleic acid, are naturally present in the epidermis, where they are incorporated into ceramides. Since ceramides are important for epidermal barrier function, n-6 fatty acid supplementation may be preferable, at least for the restoration of barrier function. Many studies recommend a ratio of 6- and 3-fatty acids between 5:1 and 10:1. However, essential fatty acids are rather not suitable as a monotherapy of AD, since a randomized cross-over study also comes to the conclusion that, despite a significant improvement in clinical signs, supplementation can only be a supportive measure in most cases (5). Furthermore,

the ideal fatty acid composition of these supplements and the dosage regimen required to achieve these goals remain unclear. However, as essential fatty acid supplementation is generally considered to be safe, it can be recommended as an add-on therapy for long-term management of AD (31).

Substitution of epidermal lipids

A relatively new approach to the therapy of AD is to substitute the lipids present in the stratum corneum of skin-healthy patients in a lamellar structure as this structure is disorganized in atopic patients (26). This leads to a reorganization of the skin lipids in the stratum corneum of atopic dogs and could thus contribute to an increased barrier function. This approach is supported by several published studies in which it was shown that even in non-lesional skin of dogs with AD, the lipid pattern is disturbed (33). As mentioned in the introduction, the ceramide pattern is altered in AD dogs, which leads to a disorganization of the lamellar structure. This modified ceramide content is therefore held responsible for a disturbed barrier function (8, 27). Nevertheless, the efficacy of local substitution with regard to the reduction of pruritus and reduction of skin lesions is not very impressive overall (31).

Janus kinase inhibitor (oclacitinib): The Janus kinase (JAK) inhibitor has been approved for several years as an interesting new treatment strategy ("first in class") for the treatment of canine AD. In contrast to ciclosporin A and glucocorticoids, which mainly inhibit the synthesis of inflammatory and itching mediators, JAK inhibitors inhibit the signal transduction of cytokines. Since some cytokines, e.g. interleukin (IL-) 31, also induce itching, the rapid onset of action (especially reduction of itch within hours) was explained by the inhibition of IL-31-induced neuronal activation. However, it is now known that oclacitinib also inhibits IL-31 independent itching (e.g. via histamine or serotonin) probably by inhibiting the calcium channel TRPV1 (13). Controlled studies have shown that oclacitinib is comparably effective to prednisolone or ciclosporine A (9, 10). The initial dosage of 0.4–0.6 mg/kg orally twice daily is recommended to be reduced to once daily after the first 2 weeks (9, 10, 14). Adverse effects seem to be uncommon, but include anorexia, vomiting and diarrhea. However, only long-term experiences will determine the safety of this quite new class of immune modulators.

Anti-canine IL-31-antibody (Lokivetmab): Based on the findings related to oclacitinib, a specific caninized antibody against the Th-2 and pruritus inducing cytokine IL-31 (Lokivetmab) was recently launched. In a comparative study with cyclosporine A, lokivetmab was not inferior in reducing itching and lesions (20). However, some dogs were "nonresponders" with regard to the lesions. This indicates that this very narrowly focused therapy does not lead to the desired success in all dogs, as the pathogenesis of canine AD is probably too multifactorial. Lokivetmab (administered at 1 mg/kg s.c.) seems to be well tolerated, only some local reactions at the injection site or general hypersensitivity reactions are reported as adverse effects.

New therapeutic approaches

Although most dogs suffering from atopic dermatitis can be treated successfully with the above-mentioned drugs, there are still dogs that do not respond sufficiently to current therapeutics. Thus, there is still a need for new therapeutics with novel mechanism of action. Two newer treatment strategies will be briefly discussed here as examples:

Phosphodiesterase-4 inhibitors (PDE4 inhibitors): Phosphodiesterase 4 (PDE4) is a central cAMP-inactivating enzyme in almost all inflammatory and immune cells. Inhibition of PDE4 leads to immunosuppressive signals in these cells (e.g. inhibition of pro-inflammatory cytokines, reactive oxygen species and inhibition of chemotaxis of immune cells). PDE4 inhibitors therefore show a distinct anti-inflammatory and immunomodulatory potential in several animal models and some clinical studies, which also makes this group of drugs interesting for the pharmacotherapy of canine atopic dermatitis. There is one clinical study in atopic dogs with the rather non-selective PDE4 inhibitor arofylline. Arofylline has an antipruritic and anti-inflammatory effect comparable to prednisone. However, the strong emetic effect of systemically administered arofylline requires to limit the dose (12). At this point in time, a comprehensive benefit-risk assessment cannot yet be provided. After systemic administration of PDE4 inhibitors, the undesired effects like nausea, vomiting and increased gastric juice production can be dose limiting. Therefore, topical application of PDE4 inhibitors with a higher therapeutic window can be a reasonable approach (1). Recently, the first topically active PDE4 inhibitor has been approved for human AD. Since 2016, crisaborol cream is on the market for the topical treatment of mild to moderate atopic dermatitis in humans (7). However, the clinical outcome is modest and thus only studies performed in dogs can tell us the therapeutic value of topically (or systemically) administered PDE4 inhibitors.

The histamine-4 receptor as a target for the treatment of allergic skin diseases: With the discovery of the histamine-4-receptor (H4R) in the year 2000, the involvement of histamine in (allergic) inflammation and itching has to be revisited (see only limited efficacy of H1 antihistamines). The H4R is mainly expressed by hematopoietic cells, such as mast cells, eosinophils, basophils, dendritic cells and T-cells. In addition, our

working group showed for the first time that the H4R is functionally expressed on sensory neurons in the skin (28). This expression profile indicates the central importance of H4R in the inflammatory process and in the course of the immune response. Due to the co-expression of H1R and H4R on many immune cells, a combination of H1R and H4R antagonism is suggested as a new strategy for the treatment of allergic inflammatory diseases (34). A highly selective H4R antagonist (JNJ7777120) was investigated in a murine models of allergic contact dermatitis (30). While it was possible to inhibit hapten-induced itching considerably, the effects on inflammation were only moderate (30). However, more recent studies of our own indicate that H4R antagonism has anti-inflammatory effects if the allergic eczema is chronic (29). Interestingly, our own studies also support the use of combined H1R and H4R antagonism in a mouse model of AD (17). In an acute dog model of AD in maltese-beagles, the H4R antagonists JNJ7777120 and JNJ 28307474 had no effect on lesions. However, at that time, itch reaction could not yet be determined (4).

Taken together, only long-term studies and combination studies (H1R and H4R antagonists) can provide us the therapeutic value of inhibiting the H4R. In human medicine, a very promising clinical study has been performed with the H4R antagonist ZPL-3893787. ZPL-3893787 could significantly reduce the lesions in AD patients compared to placebo group (35).

Outlook

After years of very few innovative approaches, there has been an interesting and exciting development within the last 5 years. With oclacitinib, a first in class was introduced to veterinary medicine before a similar drug was approved for human medicine (actually, there are promising clinical phase III studies in human medicine, however, no licensed Janus kinase inhibitor for the treatment of human AD). The circumstances are similar in terms of the monoclonal antibody lokivetmab, where again, a similar approach is in clinical trials for human medicine, although it more precisely targets the IL-31 receptor (Nemolizumab), not the IL-31 itself (16). The clinical data concerning the efficacy of Janus kinase inhibitors and the anti-IL-31 approach are very comparable between human and canine AD, which is a further indication that dogs with naturally occurring AD can serve as a translational model for human AD (19). Apart from PDE4 inhibitors and H4R antagonists, further monoclonal antibodies might find their way into clinical trials, as e.g. an antibody against the interleukin-4 receptor alpha (dupilumab) shows fairly impressive efficacy in human patients suffering from moderate to severe AD (32).

As far as a treatment strategy is concerned, there is an interesting, recently published position paper Olivry and Banovic (22) to suggest that it makes sense to start treatment with a broad-acting anti-inflammatory agent like a glucocorticoid to induce fast clinical remission. Once clinical remission is achieved, the authors suggest to switch to a JAK inhibitor like oclacitinib, or to administer it in combination. In the next step, when lesions are well controlled, a proactive treatment with e.g. a topical steroid is recommended (see description in glucocorticoid section above). It might make sense to add a targeted therapy like lokivetmab at this point of treatment. In case of flare-ups, again, systemic glucocorticoids (and a JAK inhibitor) should be started until the resolution of lesions (22).

In conclusion, there is an exciting development in new treatment options as well as treatment schedules for clinical management of canine AD and further candidates might find their way to clinical trials pretty soon.

Conflict of Interest

The author declared that there is no conflict of interests.

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   Lamont LA, Bulmer BJ, Sisson DD, et al. (2002): *Doppler echocardiographic effects of medetomidine on dynamic left ventricular outflow tract obstruction in cats.* J Am Vet Med Assoc, **221**, 1276-1281.
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