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RESEARCH ARTICLE

The Ameliorative Effects of Propolis against Cyclosporine A Induced Hepatotoxicity and Nephrotoxicity in Rats [1] [2]

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[2] *This study had been presented at VI. National Congress of Veterinary Pharmacology and Toxicology (11-14 September 2013, Elazig - TURKEY)*

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Summary

This study was planned to determine the effects of propolis in rats applied Cyclosporine A (CsA). In this study, 24 male Sprague-Dawley rats were used. Rats were randomly divided into 4 groups including control and 3 treatment groups. Group 1 (Control) were no supplement; CsA (group 2) were given as s.c. 15 mg/kg body weight (BW) every day; Propolis (group 3) were given by gavage 100 mg/kg BW every day; CsA+Propolis (group 4) were given as s.c. 15 mg/kg BW of CsA and by gavage 100 mg/kg BW of propolis every day. The feed intake were significantly higher (P<0.01) in Control and Propolis groups than CsA and CsA+Propolis groups within time period of 21 days. Further, body weight was significantly lower (P<0.01) in groups administrated with CsA (Group 2 and 4) than the other groups. Cortisol, AST, ALT and urea levels in serum of Control, Propolis and CsA+Propolis groups were found significantly lower (P<0.01) than those of CsA group. Malondialdehyde levels in kidney and liver tissues were significantly higher (P<0.01) than in the CsA groups compared to other groups. The catalase and reduced glutathione activities in kidney tissue of CsA+Propolis group were significantly higher (P<0.01) than those of CsA group. The present study demonstrated that propolis provided amelioration in terms of hepatotoxicity and nephrotoxicity consisting rats applied to CsA.

Keywords: *Cyclosporine A (CsA), Propolis, Hepatotoxicity, Nephrotoxicity, Rat*

Ratlarda Hepatotoksisite ve Nefrotoksisite Oluşturan Siklosporin A'ya karşı Propolisin İyileştirici Etkileri

Özet

Bu çalışma, Siklosporin A (CsA) uygulanan ratlarda propolisin etkilerini belirlemek amacıyla planlanmıştır. Çalışmada 24 adet Sprague-Dawley erkek rat kullanılmıştır. Ratlar tesadüfi olarak kontrol ve 3 muamele grubuna ayrılmıştır. Grup 1 (Kontrol)'e katkı yapılmadı; CsA her gün canlı ağırlığa (BW) 15 mg/kg s.c. olarak verildi (grup 2); Propolis her gün 100 mg/kg BW gastrik gavajla verildi (grup 3); CsA+Propolis her gün 15 mg/kg BW CsA s.c. olarak ve 100 mg/kg BW propolis gastrik gavajla verildi (grup 4). 21 günlük peryottaki yem tüketimi, Kontrol ve Propolis gruplarında CsA ve CsA+Propolis gruplarından önemli derecede daha yüksek oldu (P<0.01). Ayrıca, canlı ağırlık CsA uygulanan gruplarda (Grup 2 ve 4) diğer gruplardan önemli derecede daha düşüktü (P<0.01). Kontrol, Propolis ve CsA+Propolis gruplarında serum kortizol, AST, ALT ve üre düzeyleri, CsA grubundan önemli derecede düşük bulundu (P<0.01). Böbrek ve karaciğer dokularının malondialdehid düzeyleri CsA gruplarında, diğer gruplarla karşılaştırıldığında önemli derecede yüksekti (P<0.01). CsA+Propolis grubunun böbrek dokusu katalaz ve redükte glutatyon aktiviteleri CsA grubununkinden önemli derecede daha yüksek oldu (P<0.01). Bu çalışma, propolisin CsA uygulanan ratlarda oluşan hepatotoksisite ve nefrotoksisite açısından iyileşme sağladığını gösterdi.

Anahtar sözcükler: *Siklosporin A (CsA), Propolis, Hepatotoksisite, Nefrotoksisite, Rat*

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INTRODUCTION

Cyclosporine A (CsA) is an immunosuppressive drug that has considerably improved the survival of transplant patients in recent years [1-3]. However, several side effects have been associated with CsA treatment, such as hypertension, nephrotoxicity and neurotoxicity [4]. All alterations in mitochondrial functions, covalent binding of CsA metabolites to proteins, elevated thromboxane synthesis, and lipid peroxidation have been implicated in the CsA-mediated cell damage. Whereas its precise toxic mechanisms remain to be investigated, lipid peroxidation ascribable to oxygen radicals produced in the kidney has been proposed to be one of the major mechanisms for CsA nephrotoxicity and cell injury, which are partly reversed by some antioxidants [5].

The antioxidant serves as a defensive factor against free radicals in the body. Enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px**)** and non enzymatic antioxidant such as reduced glutathion (GSH) are the main system that opposes oxidation. If production free radicals overwhelm the capacity of enzymatic system, the second line of defense (vitamins) may come to action [6,7]. Antioxidants such as vitamin C and E extinguish free radicals and become oxidized and non-active [8,9]. Propolis contains about 300 constituents. In these days, propolis has gained popularity in connection with oxidative stress [10] and used widely as a food additive to improve health and prevent diseases such as inflammation, heart disease, diabetes and even cancer [11,12]. Flavonoids of propolis are one of the most important compounds. Compounds of propolis are being used for many biological and pharmacological activities including anticancer, anti-inflammatory, antimicrobial and antioxidant [10,13]. Flavonoids and various phenolics in propolis have been appeared to be capable of scavenging free radicals and thereby defending lipids and other compounds such as vitamin C from being oxidized or break down during oxidative stress [7]. Propolis widely began to attract the attention of scientists. The results of many animal researches showed that propolis may relieve the negative effects of oxidative stress on the body's defense system [10,14,15].

This study was planned to determine the effects of propolis on feed intake (FI), body weight (BW), body weight change (BWC), some blood parameters and antioxidant status in rats applied CsA which induced neprotoxicity and hepatotoxicity.

MATERIAL and METHODS

Drugs

CsA (Sandimmun® enj. sol., 50 mg/ml, Novartis) and propolis (Ari Dunyasi Firm, Istanbul-Turkey) were both dissolved in ethanol. CsA was injected as sub-cutaneous (s.c.) 15 mg/kg and propolis was given by gavage daily 100 mg/kg during the experimental period (for 21 days). CsA and propolis doses have been chosen, respectively, according to Rezzani et al.[3] and Seo et al.[16].

Animals, Diet and Treatment

Twenty-four healthy adult male Sprague-Dawley rats (8-10 weeks old, 280-300 g BW) were used in this study. The animals were obtained from Firat University, Experimental Research Centre (Elazig, Turkey) and were housed in stainless steel cages under standard laboratory conditions (24±3°C, 40-60% humidity, 12 h dark/light cycle). A standard commercial pellet diet (Elazig Food Company, Elazig/Turkey) containing 23% crude protein and 2.650 kcal/kg metabolic energy, and fresh drinking water were given *ad libitum*. The protocol for the use of animals was approved by the National Institutes of Health and Local Committee on Animal Research. This study was approved by the Animal Ethical Committee of Firat University (18.04.2012/57).

Rats were randomly divided into the Control and 3 treatment groups. Rats were housed in individual cages. During a 21 days period, while Control group: No supplement, group 2: CsA were given s.c. 15 mg/kg BW of CsA every day; group 3: Propolis were given by gavage 100 mg/kg BW of propolis every day; group 4: CsA+Propolis were given by s.c. 15 mg/kg BW of CsA and 100 mg/kg BW of propolis were given by gavage every day. Rats were individually weighed initially and then weekly to monitor the BW. In addition, FI and BWC at 7, 14 and 21 days of the experiment were determined. No rat died during experimental period.

Sample Collection

After 24 h of last application, rats were anaesthetized by light inhalation of diethyl ether and were decapitated, then 1.5 ml blood sample from each rat was collected for biochemical analysis. The kidney and liver tissues were removed for biochemical analysis. These samples were stored at -20°C until further analysis.

Biochemical Analysis

Serum cortisol, glucose, albumin, globulin, total protein, urea, triglycerides, HDL, VLDL, LDL, total cholesterol, creatinine, AST and ALT values were determined using autoanalyzer.

Extraction Procedure of Propolis

0.1 g sample was extracted, in 3 parallels, with 25 mL 60% ethanol and incubated for 6 days, vortexing every day. At the end of the 6th day of incubation, the extracts were sonicated for 10 min and then centrifuged for 10 min at 4.000 rpm and 4°C [17]. The extracts were then used for the spectrophotometric analysis of total phenolic content,

total flavonoid content, and total antioxidant capacity. HPLC analysis was also performed to determine the phenolic profile of the propolis sample.

Spectrophotometric Assays

Analysis of Total Phenolics: The amount of total phenolics in extracts was determined with the Folin-Ciocalteau reagent using the method of Velioglu et al.[18]. To 0.1 mL of each sample (three replicates), 0.75 mL 0.1 N Folin-Ciocalteau reagent and 0.75 mL Na_2CO_3 (6%, w/v) were added. After 1.5 h, the absorbance was measured at 725 nm using spectrophotometer. Results were expressed as mg gallic acid equivalent (GAE)/g fresh weight sample *(Table 1)*.

Analysis of Total Flavonoids: The total flavonoid content was determined using the colorimetric method reported by Kim et al.[19]. 1 mL extract was mixed with 0.3 mL 5% $NaNO_2$ at t=0 min. Then 0.3 mL 10% $AlCl_3$ was added at t=5 min. After 6 min, 2 mL 1 N NaOH was added and the solution was mixed. The absorbance was measured against prepared water blank at 510 nm. Total flavonoid content was expressed as mg quercetin equivalents (QE)/g fresh weight sample *(Table 1)*.

Analysis of Total Antioxidant Capacity - DPPH Method: The antioxidant activity of the propolis extracts were assessed on the basis of the radical scavenging effect of the stable DPPH free radical [20]. 0.1 mL extract was added to 2 mL 0.1 mM DPPH in methanol solution in a test tube. After incubation for 30 min at room temperature, the absorbance of each solution was determined at 517 nm against blank (methanol). The results were expressed as mg trolox equivalent antioxidant capacity (TEAC)/g fresh weight sample *(Table 1)*.

Analysis of Total Antioxidant Capacity - CUPRAC Method: The CUPRAC (Cupric Reducing Antioxidant Capacity) method was utilized using the method described by Apak et al.[21]. First, 1 mL of 0.01M copper (II) chloride ($CuCl_2$), 1 mL of 0.0075 M neocuproine (Nc), 1 mL of ammonium acetate (NH_4Ac) buffer (pH 7.0) was mixed in a test tube. Subsequently, 0.1 mL of sample extract or Trolox was added to this mixture. Lastly, 1 mL of MQ water was included to make the final volume 4.1 mL. After 1h reaction time, absorbance was measured at 450 nm. The results were expressed as mg TEAC/g fresh weight sample *(Table 1)*.

Determination of Phenolic Profile by HPLC Analysis

Filtered extracts were analysed using a W600 Waters HPLC system coupled to a Waters 996 photodiode array (PDA) detector as described previously [22,23]. Compounds were separated using a C18 column (150x4.6 mm, 3 µ) and applying a gradient from 95% to 25% MQ and a 5-75% acetonitrile, both in 0.1% trifluoroacetic acid (TFA) (1 mL/min flow rate) across a period of 50 min. Phenolics of propolis were detected at 280, 312, and 360 nm. For quantification, dose-response curves of available pure standards (0-500 µg/mL) were used as reference *(Table 2)*.

Lipid Peroxidation

The levels of MDA were measured as described by Candan and Tuzmen [24]. One gram sample was homogenized in 4 ml of 20 mM phosphate buffer (pH 7.4). Then the homogenate centrifuged at 15.000 x g for 15 min. The supernatant was used for analysis. Tissue lipoperoxides were hydrolyzed in dilute sulfuric acid (H_2SO_4, 1%) and then by boiling in phosphoric acid (H_3PO_4). MDA is reacted with thiobarbituric acid (TBA) to form MDA-TBA. Tissue proteins are precipitated with methanol and removed from the reaction mixture by centrifugation. HPLC analysis was performed using Scimadzu LC-20AT HPLC system. A mobile phase consisted of 40:60 (v/v) methanol-KH_2PO_4. The C_{18} column (150x4.6 mm, 5 µm, Fortis) was used with a flow rate of 0.6 ml/min (30°C), sample run was 10 min, injection volume was 20 µl and fluorescence detector wavelengths were set at 532 nm (excitation) and 553 nm (emission). Results were expressed µg/ml homogenate.

Reduced Glutathione

The GSH levels were measured spectrophotometrically at 412 nm using the method of Ellman [25]. The protein content in the kidney and liver was measured using by method of Lowry et al.[26] with bovine serum albumin as the standard.

Table 1. *The total phenolic content, total flavonoid content and total antioxidant capacity values of propolis*
Tablo 1. *Propolisin toplam fenolik madde, toplam flavonoid madde ve toplam antioksidan kapasite değerleri*

Content of Propolis	Amount in 1 g Propolis *
Total Phenolics	139.1±1.8 mg GAE
Total Flavonoids	397.6±1.2 mg QE
Total Antioxidant Capacity - DPPH	269.5±0.4 mg TEAC
Total Antioxidant Capacity - CUPRAC	494.5±1.3 mg TEAC

** Values are given as mean ± standard deviation of the values found for three replicates;* ***GAE:*** *Gallic Acid Equivalent;* ***QE:*** *Quercetin Equivalents;* ***TEAC:*** *Trolox Equivalent Antioxidant Capacity*

Table 2. *Phenolic substances and quantities defined in propolis*
Tablo 2. *Propoliste tanımlanan fenolik maddeler ve miktarları*

Phenolics	Amount (mg/g) *
Pinobanksin	8.9±0.5
Pinostrobin	5.5±0.3
Pinocembrin	3.4±0.2
Chrysin	2.7±0.1
Galangin	2.2±0.2
Apigenin	1.7±0.1
Kaempferol	0.7±0.0
Luteolin	0.6±0.0

** Values are given as mean ± standard deviation of the values found for three replicates*

The GSH level was expressed as nmol/mg protein.

Catalase

The kidney and liver tissue CAT activity was determined according to the method of Aebi [27]. The principle of the method is based on the determination of the rate constant (*k*) for the H_2O_2 decomposition rate at 240 nm. Results were expressed as k/g protein.

Statistical Analysis

All values were presented as mean±SD. Differences were considered to be significant at *P<0.05*. Statistical analysis was performed using one-way ANOVA and *post hoc* Duncan's significance difference test by SPSS 21 [28] program.

RESULTS

The FI were significantly higher in Control and Propolis groups than CsA and CsA+Propolis groups in period of 21 days (*P<0.01*) *(Table 3)*. FI of Control, CsA, Propolis and CsA+Propolis groups were found as 4.11, 3.79, 4.22 and 3.83 g/day/animal in period of 21 days, respectively (*P<0.01*). The BW were significantly lower in groups administrated with CsA than other groups (*P<0.01*). Body weight of Control, CsA, Propolis and CsA+Propolis groups were found as 330.20, 257.50, 338.11 and 280.33 g/animal in period of 21 days, respectively (*P<0.01*) *(Table 3)*. The decrease of BW were significantly highest in CsA group compared with the other groups (*P<0.01*). Further, BWC of Control, CsA, Propolis and CsA+Propolis groups were found as 1.95, -1.58, 1.79, -0.83 g within a period of 21 days, respectively (*P<0.01*). The results indicate that CsA had negative effects on the FI, BW and BWC. Based on the BWC values, the negative impact of CsA on BWC was decreased by oral administration of propolis *(Table 3)*. Cortisol, HDL, LDL, VLDL, total cholesterol, AST, ALT and urea levels of Control group were significantly lower than those of CsA group (*P<0.01*) *(Table 4)*. Cortisol, AST, ALT and urea levels of Control, Propolis and CsA+Propolis groups were found significantly lower than those of CsA group (*P<0.01*) *(Table 4)*. Furthermore, MDA levels in kidney and liver were significantly the highest in the CsA groups compared to Control, Propolis and CsA+Propolis groups (*P<0.01*) *(Table 5)*. The CAT and GSH activities of CsA+Propolis groups in kidney were significantly found higher than those of CsA group (*P<0.01*). GSH activity of CsA+Propolis groups in liver was determined significantly higher than that of CsA group (*P<0.05*) *(Table 5)*.

DISCUSSION

Cyclosporine A (CsA) is a drug most frequently used in transplant surgery because of its potent immunosuppressive action. However, its clinical use is accompanied by adverse side effects such as hypertension, nephrotoxicity and hepatotoxicity [29]. Previous studies established that reactive oxygen species production and oxidative stress situation are involved in CsA hepatotoxicity [30,31]. The present work investigated the effect of propolis supplementation on the severity of CsA-induced oxidative stress, nephrotoxicity and hepatotoxicity.

The chemical composition and biological activities of propolis depend mainly upon the local flora, the geographic region, and the climate. Thus, development of analytical methods to evaluate the antioxidant capacity and to discriminate the floral origin of propolis is necessary. There are numerous methods for determining the antioxidant capacity of soluble natural extracts as well as for insoluble food components [32].

Table 3. *Effects of propolis on feed intake (FI), body weight (BW) and body weight change (BWC) of experimental groups (g)*
Tablo 3. *Deneme gruplarının, yem tüketimi (FI), canlı ağırlığı (BW) ve canlı ağırlık değişimi (BWC) üzerine propolisin etkileri (g)*

Performance	Days	Control	CsA	Propolis	CsA+Propolis	P
FI	1-7	3.96±0.09^{a}	3.54±0.08^{b}	4.02±0.14^{a}	3.57±0.09^{b}	**
	8-14	4.06±0.06^{a}	3.77±0.11^{b}	4.04±0.04^{a}	3.81±0.05^{b}	**
	15-21	4.31±0.06^{b}	4.01±0.10^{c}	4.58±0.04^{a}	4.08±0.10^{bc}	**
	1-21	4.11±0.05^{a}	3.79±0.08^{b}	4.22±0.06^{a}	3.83±0.05^{b}	**
BW	IW	291.20±6.35	289.16±8.76	302.17±6.31	297.00±6.61	NS
	7	302.80±5.22	279.25±9.99	311.33±8.15	289.78±7.61	NS
	14	316.90±4.99^{a}	268.67±5.71^{b}	324.67±7.39^{a}	281.98±5.29^{b}	**
	21	330.20±4.82^{a}	257.50±4.26^{b}	338.11±7.59^{a}	280.33±7.10^{b}	**
BWC	1-7	1.93±0.56^{a}	-1.65±0.49^{b}	1.52±0.32^{a}	-1.20±0.88^{b}	**
	8-14	2.01±0.43^{a}	-1.51±0.67^{b}	1.90±0.41^{a}	-1.11±0.86^{b}	**
	15-21	1.90±0.40^{a}	-1.59±0.51^{c}	1.92±0.37^{a}	-0.43±0.37^{b}	**
	1-21	1.95±0.34^{a}	-1.58±0.28^{c}	1.79±0.13^{a}	-0.83±0.19^{b}	**

IW: *Initial weight;* ^{a,b,c} *Mean values with different superscripts within a row differ significantly;* ***NS:*** *Non significant; ** P<0.01*

Table 4. *Effects of propolis on some blood parameters of experimental groups*
Tablo 4. *Deneme gruplarının, bazı kan parametreleri üzerine propolisin etkileri*

Parameter	Control	CsA	Propolis	CsA+Propolis	P
Glucose (mg/dL)	105.67±3.34	108.33±5.23	105.50±6.25	105.33±3.82	NS
Cortisol (ug/dL)	0.47±0.06^{b}	0.93±0.04^{a}	0.53±0.09^{b}	0.60±0.07^{b}	**
HDL (mg/dL)	13.97±0.55^{c}	17.43±0.49^{a}	14.78±0.40^{bc}	16.75±1.16^{ab}	**
LDL (mg/dL)	5.00±0.37^{b}	12.00±1.75^{a}	7.33±0.61^{b}	11.67±0.42^{a}	**
VLDL (mg/dL)	10.00±1.63^{b}	14.83±2.02^{a}	9.50±0.99^{b}	11.50±1.61^{ab}	**
Total Cholesterol (mg/dL)	29.03±1.82^{b}	44.30±2.57^{a}	31.62±1.72^{b}	39.88±1.44^{a}	**
Triglyceride (mg/dL)	50.33±8.41^{ab}	74.33±10.19^{a}	47.50±5.09^{b}	57.33±7.92^{ab}	*
AST (U/L)	201.17±18.81^{c}	398.50±38.51^{a}	189.83±16.64^{c}	290.16±17.69^{b}	**
ALT (U/L)	72.00±6.32^{b}	96.83±2.95^{a}	69.84±3.78^{b}	80.16±3.70^{b}	**
Total Protein (g/dL)	6.13±0.06^{a}	5.55±0.09^{c}	5.97±0.07^{ab}	5.73±0.09^{bc}	**
Albumin (g/dL)	3.75±0.06^{a}	3.23±0.08^{b}	3.68±0.06^{a}	3.53±0.07^{a}	**
Urea (mg/dL)	58.00±4.21^{b}	84.16±6.37^{a}	55.33±1.76^{b}	60.50±4.99^{b}	**
Creatinine (mg/dL)	0.25±0.02	0.24±0.01	0.27±0.02	0.27±0.01	NS
Globulin (g/dL)	2.38±0.06	2.32±0.03	2.28±0.04	2.40±0.05	NS

^{a,b,c} Mean values with different superscripts within a row differ significantly; ***NS:*** *Non significant; * P<0.05; ** P<0.01*

Table 5. *Effects of propolis on MDA (µg/ml homojenat), CAT (k/g protein) and GSH (nmol/mg protein) activities of experimental groups*
Tablo 5. *Deneme gruplarının, MDA (µg/ml homojenat), CAT (k/g protein) ve GSH (nmol/mg protein) aktiviteleri üzerine propolisin etkileri*

Tissues		Control	CsA	Propolis	CsA+Propolis	P
Kidney	MDA	1.15±0.00^{4}c	3.03±0.02^{a}	1.18±0.002^{c}	2.26±0.06^{b}	**
	CAT	5.22±1.^{3}4a	3.24±0.12^{c}	4.60±0.39^{ab}	4.18±0.49^{b}	**
	GSH	64.38±1.58^{a}	31.38±2.11^{b}	59.12±7.29^{a}	49.93±9.21^{a}	**
Liver	MDA	1.14±0.002^{c}	2.43±0.13^{a}	1.16±0.009^{c}	1.90±0.016^{b}	**
	CAT	4.43±0.41^{a}	2.72±0.12^{c}	4.13±0.42^{b}	3.64±0.28^{bc}	**
	GSH	62.30±2.08^{a}	47.17±1.61^{b}	61.38±2.93^{a}	55.94±2.64^{a}	*

*^{a,b,c} Mean values with different superscripts within a row differ significantly; * P<0.05; ** P<0.01*

In this study, two methods (CUPRAC and DPPH) were used to determined total antioxidant capacity for propolis. Total antioxidant capacity were investigated to the two different methods which were contained polyphenols (quercetin, catachin, naringenin, ferulic acid, caffeic acid), vitamins (ascorbik acid, α-tocopherol), thiols (glutathione cysteine), plasma antioxidants (uric acid and bilirubin), and synthetics (butylated hydroxy, anisole, tert- butyl, hydroquinone) CUPRAC assay which is based on reduction of Cu^{+2} to Cu^{+} by antioxidants. This method is simultaneously cost-effective, rapid, stable, selective and suitable for a variety of antioxidants regardless of chemical type or hydrophilicity [33].

The DPPH method, though simpler and of lower cost, has been reported to be much influenced by light, air oxygen, pH and type of solvent. Since DPPH is essentially soluble in organic solvent media. Flavonoids and other complex phenols generally exhibit moderate-to-slow reaction with DPHH. In this study, it was found higher total antioxidant capacity of propolis by the CUPRAC method than DPHH method *(Table 2)*. This may be due to DPPH assay is brings an important limitation to the determination of hydrophilic antioxidants [34].

In this study, FI and BW of rats applied CsA decreased significantly in comparison with that of Control in periods of 21 days (*P<0.01*) *(Table 3)*. This might be caused due to anorexia as aside effect of CsA [35]. Supplementation of rats with antioxidant compounds would attenuate partially the side effects of CsA-induced-oxidative stress [3]. The present study demonstrated that, FI and BW with propolis supplementation in rats applied CsA increased numerically, besides BWC in CsA+Propolis group ameliorated significantly in comparison with that of CsA group (*P<0.01*). The other a study [36] demonstrated that the CsA-treated animals lost the BW compared to those treated with control. The decrease in BW was certainly because of a parallel reduction in food intake following CsA administration. Similar finding has been reported in previous publications [37,38]. These results are in agreement with the present study. However, an increased metabolic

rate caused by the catabolic effect of CsA could not be ruled out because other studies have also reported a decreased BW in CsA-treated rats although the amount of food intake remained unaltered [36,39,40]. Propolis has delicious substances like resin, wax, honey and vanillin [41]. In the present study, the attenuate in BWC of CsA+Propolis group could be connected to the tasty characteristic and flavonoid content of propolis *(Table 1, Table 2)*. It could be linked to flavanoids show antioxidant characteristics by chelating with trace elements or radicals [42,43].

Nephrotoxicity and hepatotoxisity can be determined via changes in serum biochemical parameters. Hirano et al.[44] reported that in nephrotic syndrome patients, serum total and LDL cholesterol concentrations were significantly higher than those of 15 healthy subjects (*P<0.005*). We observed that serum cholesterol parameters (HDL, LDL, VLDL, total cholesterol) *(P<0.01)* and triglyceride *(P<0.05)* of CsA administration in rats increased significantly more than those of Control group [44]. Glucose and globulin values were similar between all of groups. In this study, CsA induced hepatotoxicity characterized by increased biochemical parameters such as AST and ALT that are indicators of liver toxicity which is in accordance with our study *(Table 4)*. The transaminase enzymes such as AST and ALT are the most sensitive markers that play a majör role in the diagnosis of the liver injury. The changes in the levels of transaminases are the indicators of impaired liver function state [45,46]. Kim et al.[47] have suggested that the significant increase in the activities of hepatic marker enzymes such as AST, ALT and ALP manifested by CsA induced hepatocellular damage. Administration of propolis in rats significantly decreased the activities of AST and ALT (*P<0.01*) *(Table 4)*, suggesting that they offer protection by preserving the structural integrity of the hepatocellular membrane against CsA [48]. Similarly our study, other researchers showed that the protective effects of caffeic acid phenethyl ester is an active component of propolis obtained from honeybee hives on hepatotoxicity induced by lead acetate [49] and nephrotoxicity induced by CsA [36]. Similarly with present study, other authors [29,46] suggested that a significant decrease in serum total proteins associated with a significant elevation in hepatic thiobarbituric acid reactive substances and a decline in GSH, GSH-Px and CAT concentrations. Urea is a waste product made when protein is broken down and it is made in the liver. It is well known that blood urea nitrogen (BUN) measures the amount of urea in blood and increased BUN levels show kidney dysfunction in clinical practices [50]. We showed that CsA administration caused renal damage, which was reflected by a significant increase in serum urea levels in the CsA group in comparison with Control, Propolis and CsA+Propolis groups (*P<0.01*). In this study, propolis supplementation restored the normal values of some blood parameters (Cortisol, AST, ALT, albumine, urea) which were deteriorated after inoculation of CsA *(Table 4)*, similarly to caffeic acid supplementation [36].

We observed that the MDA levels in the kidney and liver tissues were significantly higher in the CsA group compared to the Control group (*P<0.01*). Whereas, CsA signeficantly increased kidney and liver (*P<0.01*) MDA, and decreased kidney (*P<0.01*) and liver GSH (*P<0.05*) as well as their CAT (*P<0.01*) contents [36,45,51]. Furthermore, GSH activities in kidney and liver along with CAT activity in kidney of CsA+Propolis groups were found significantly higher than CsA group (*P<0.01*). Propolis treatment partially ameliorated the CsA-induced lesions in hepatic and renal tissues. Flavonoids and various phenolics in propolis have been appeared to be capable of scavenging free radicals and thereby defending lipids and other compounds such as vitamin C from being oxidized or destroyed during oxidative damage *(Table 3)* [7,10]. Additionally, flavonoids inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, and the activity of enzyme systems including cyclooxygenase and lipoxygenase [52]. These results clearly demonstrate the important role of oxidative stress and its relation to renal dysfunction and hepatic toxisity and also point out the protective potential of propolis against CsA nephro and hepatic toxicities. At least in part, the protection afforded by propolis is mediated through inhibiting renal and liver lipid peroxidation and increasing or maintaining the GSH and CAT contents in that tissues.

Together, it can be concluded that CsA administration in rats decreases BW and increases oxidative stress in blood and tissues. Propolis appeared to improve reduction in BW and ameliorate the toxicity of CsA by scavenging the free radicals and increasing the antioxidant activities. Therefore, propolis as an antioxidant compound administration might be appropriate to prevent CsA-induced renal and hepatic toxicity in proper dose.

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RESEARCH ARTICLE

Investigation of Polymorphisms on ABCG2, AA-NAT and FABP3 Genes in the Kıvırcık Sheep Reared in Three Different Provinces of Turkey [1]

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Summary

In this study mutations located in intron 5 of *ABCG2,* exon 3 of *AA-NAT* and exon 2 of *FAB3* genes were investigated by PCR based methods in the Kıvırcık sheep sampled from Bursa, Manisa, and İstanbul provinces of Turkey. All loci investigated were found as polymorphic. While in the *ABCG2* and *FABP3* loci two alleles and three genotypes were found, in the *AA-NAT* locus two alleles and two genotypes were detected. In *ABCG2* locus allele "- ", in *AA-NAT* allele A, in *FABP3* locus allele G were observed as predominant. The frequency values of the predominat alleles at *ABCG2, AA-NAT* and *FABP3* loci were found 0.60, 0.87 and 0.58, respectively. While the whole population investigated exhibits deviation from the Hardy- Weinberg equilibrium for *ABCG2* and *FABP3* loci, was found at Hardy-Weinberg equilibrium for *AA-NAT* locus.

Keywords: *ABCG2, AA-NAT, FABP3, Polymorphism, Sheep*

Türkiye'nin Üç Faklı İlinde Yetiştirilen Kıvırcık Koyunlarda ABCG2, AA-NAT ve FABP3 Genlerindeki Polimorfizmlerin İncelenmesi

Özet

Bu çalışmada Türkiye'nin Bursa, Manisa ve İstanbul illerinden örneklenen Kıvırcık koyunlarında *ABCG2* geninin 5.intron, *AA-NAT* geninin 3. ekzonunda ve *FABP3* geninin 2. ekzonunda bulunan mutasyonlar PCR tabanlı yöntemlerle incelenmiştir. İncelenen tüm lokuslar polimorfik bulunmuştur. *ABCG2* ve *FABP3* lokuslarında iki allel ve üç genotip belirlenirken, *AA-NAT* lokusunda iki allel ve iki genotip belirlenmiştir. *ABCG2* lokusunda "- " alleli, *AA-NAT* lokusunda A alleli ve *FABP3* lokusunda G allellinin predominant olduğu gözlenmiştir. *ABCG2, AA-NAT* ve *FABP3* lokuslarındaki predominant allelerin frekans değerleri sırasıyla 0.60, 0.87 ve 0.58 olarak bulunmuştur. İncelenen populayonun tamamı *ABCG2* ve *FABP3* lokusları bakımından Hardy-Weinberg dengesinden sapma gösterirken *AA-NAT* lokusu bakımından dengede bulunmuştur.

Anahtar sözcükler: *ABCG2, AA-NAT, FABP3, Polimorfizm, Koyun*

INTRODUCTION

Kıvırcık is the one of the most important native sheep breed of Turkey that constitutes almost seven percent of total sheep population. It is a multipurpose breed and it has higher meat quality when compare with the other native sheep breeds in the country [1].

In Turkey some efforts have been began by General Directorate for Agricultural Research and Policies (GDAR) to improve yield characteristics of Kıvırcık sheep in 2005 with Communiqué 2005/13 based on Cabinet Decision with number 2005/8503. It is well known that genetic improvement of the livestock species is an expensive and time consuming process. To increased genetic gain by selection a gene has major affect can be used for selection criteria in the breeding schema. Genetic polymorphisms are used by selection of carrier animals of causal mutations with desirable effects on the economic traits in farm animals such as Broola mutation for reproduction or PrP

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gene for scrapie resistant in sheep [2]. In goat predicted advantage of casein assisted selection over traditional selection based on performance was 18% for protein content [3]. In Turkey lack of production records is a limiting factor to analyze this kind of associations. On the other hand investigations of allelic frequencies of economically important traits loci have been increased among Turkish native livestock species as cattle, goat and sheep, in recent years [4-6]. It is assumed that mutations occur on *ABCG2*, *AA-NAT* and *FABP3* genes have effects on some economically important traits and can be thought as molecular markers.

The ATP-binding casette sub-family G member 2 (*ABCG2*) is a member of a protein family responsible for transport of various molecules across cell membranes [7,8]. *ABCG2* is expressed several tissue including mammary gland and it is reported that the level of expression is elevated in lactation period in some monogastrics, goats and dairy cows [9-11]. Due to its function and near chromosomal location to an important QTL region effects on milk production traits in cattle [12,13], researches have been focused on associations between mutations occur in the *ABCG2* gene and milk production traits. Thus several relationships between mutation located in this gene and milk yield, protein and fat percentage, and somatic cell score (SCC) in dairy cows were revealed [14-17]. Number of studies carried out in ovine is very limited when compare with studies focused on bovine. Relationships between a microsatellite locus located with *ABCG2* on ovine chromosome 6 and some production traits were highlighted [18]. In a recent study [19] mutations in ovine *ABCG2* gene and their relationship with some economically important traits were investigated and 13 SNPs and a single 35 bp insertion/deletion were revealed in different region of the gene. They also reported significant relationship with a T→C transition and protein percentage as well, 35 bp insertion/deletion and SCC.

Arylalkylamine N-acetyltransferase (*AA-NAT*) is an important enzyme in Melatonin (MLT) biosynthesis by acting on serotonin for MLT regulating the animal seasonal breeding [20-23]. Due to its critical role in melatonin production it has been suggested that genetic mutation of the gene may also important in seasonal reproduction in sheep [22]. Researches on *AA-NAT* polymorphism have been focused on human [24-26]. Although *AA-NAT* is very important for animal reproduction, there are a limited number of studies carried out on *AA-NAT* in farm animals. Majority of these studies have been realized on associations between *AA-NAT* gene polymorphisms and meat production traits in cattle [27,28]. Recently 1142 bp of ovine *AA-NAT* gene has been investigated to reveal mutations occurred in this gene and their relationship with seasonality in Chinese sheep breeds [22]. The authors reported an A→G transition located at exon 3 and important differences in distribution of genotype frequencies among seasonal and non-seasonal sheep breeds [22]. The GG genotype was higher in non-seasonal breeds while the GA genotype was higher in seasonal sheep breeds [22]. Mutations, occur in goat *AA-NAT* gene, were also reported that may be used for improvement of litter size [23].

Fatty acid-binding proteins (FABPs) involve in fatty acid transport from the plasma membrane to the sites of ß-oxidation and triacylglycerol or phospholipid synthesis [29]. Among FABPs the *FABP3*, also known as Heart FABP (H-FABP), is mainly expressed in cardiac and skeletal muscle [30]. Fatty acid-binding protein (*FABP*) gene is an important candidate gene for both meat quality and milk product properties as cheese making because of its possible effects on milk fat content [31]. It is reported that *FABP3* genetic variants affect on intramuscular fat content both sheep and pigs [30-32]. Ovine *FABP3* gene and its chromosomal location were analyzed at 2002 [33]. 13 SNPs, one CTC insertion/deletion and a variable polyA tract were detected. Afterward two of these SNPs located in exon 2 and intron 13 were analyzed for association studies and heterozygous genotypes for both SNPs were found related to milk fat content.

Due to their possible affects on economically important traits they can be thought as molecular markers in breeding scheme after verify the associations. In order to realize this kind of selection it should be known frequencies of the molecular marker. This study aimed to investigate distribution of allele frequencies of *ABCG2*, *AA-NAT* and *FABP3* genes in native Kıvırcık Sheep from breeding populations.

MATERIAL and METHODS

The study was approved by the Ethics Committee of Uludag University (UÜHADYEK), (approval date: 10.07.2012; no: 2012-08/5).

In this study 100 animals from Kıvırcık sheep breed investigated for polymorphisms located in *ABCG2* and *FABP3* gene. Due to some analytic problems *AA-NAT* polymorphism was analyzed in 98 sheep. Blood samples were collected from six distinct farms in Bursa (n=30), six distinct farms in Manisa (n=34) and only single farm (this flock was constitute different flocks from Thrace at different time) in İstanbul (n=36) provinces. Total DNA was extracted using a genomic DNA purification kit (K0512, Fermentas, Lithuania) according to the instructions provided in the manual.

While PCR-RFLP analyzes to investigate polymorphisms of *FABP3* [33] and *AA NAT* [22] loci, PCR was performed to genotyping *ABCG2* locus [19]. Primers and restriction enzymes used in the study are given in *Table 1*. The restriction fragments were directly analyzed by electrophoresis in 2% and 2.5% agarose gels in 1 TBE buffer, stained with ethidium bromide, and visualized under UV light.

Table 1. *Primers and restriction enzymes used in the study*
Tablo 1. *Çalışmada kullanılan primerler ve restriksiyon enzimleri*

Locus Name	Genomic Region	Primers (5′→ 3′)	Enzymes
ABCG2	Intron 5	GCCTCTTCTCCCATACGTC AAAC CAGTTGTGGGCTCATC	-
AA-NAT	Exon 3	AGCGTCCACT GCCTGAAAC GGGATGGAAGCCAAACCTC	*Sma*I
FABP3	Exon 2	GGTTTTGCTACCAGGCAGGT TTCCCTATTCCCCTTCAGGG	*BsaJ*I

Direct counting was used to estimate genotype and allele frequencies of the genetic variants for all loci. The chi-square test ($\chi 2$) was used to check whether the populations were in Hardy-Weinberg equilibrium. All calculations and the $\chi 2$ analyses were carried out using PopGene32 software [34].

RESULTS

Electrophoretic analysis revealed two alleles and three genotypes for *ABCG2* and *FABP3* loci while two alleles and two genotypes detected at the *AA-NAT* locus. PCR products and RFLP fragment size of alleles are given in *Table 2* and electroforograms are shown in *Fig. 1*.

Table 2. *Sizes of PCR products and RFLP fragments obtained from electrophoretic analysis*
Tablo 2. *Elektroforetik analizlerden elde edilen PCR ürünleri ile RFLP fragmanlarının büyüklükleri*

Locus Name	PCR Product	Allele Size	Allele Name
ABCG2	267 bp (if deletion not present) 232 bp (if deletion present)	267 bp	+
		232 bp	-
AA-NAT	1142 bp	255 bp, 371 bp and 516	A
		183 bp, 255 bp, 333 bp and 371 bp	G
FABP3	222 bp	186, 36 bp	A
		143, 43, 36 bp	G

Allele and genotype frequencies at each locus for each Kıvırcık population from different provinces and overall Kıvırcık population are given in *Table 3*. Observed and expected heterozygosity values for all loci in investigated populations also given in *Table 4*.

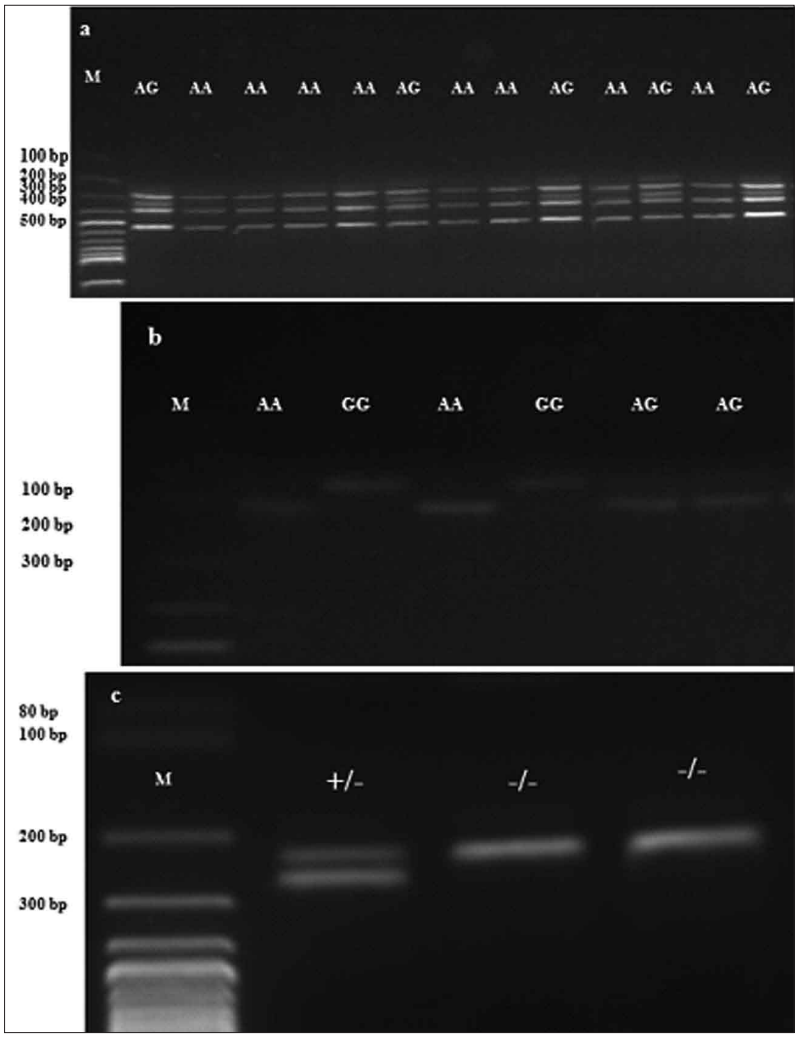

Fig 1. a- Electrophoretic image of *AA-NAT* genotype (M: Bio Basic MSM34DNA ladder), **b-** Electrophoretic image of *FABP3* genotype (M:Bio Basic MSM34 DNA ladder), **c-** Electrophoretic image of *ABCG2* genotype (M: Fermentas SM0243, GeneRuler)

Şekil 1. a- *AA-NAT* genotiplerinin elektroforetik görüntüsü (M: Bio Basic MSM34DNA ladder), **b-** *FABP3* genotiplerinin elektroforetik görüntüsü (M:Bio Basic MSM34 DNA ladder)**, c-** *ABCG2* genotiplerinin elektroforetik görüntüsü (M: Fermentas SM0243, GeneRuler)

Table 3. *Distribution of allele and genotype frequencies among populations, overall population and χ2 values*
Tablo 3. *Populasyonlar arasında, populasyonlar genelinde allel ve genotip frekanslarının dağılımı ile χ2 değerleri*

LOCUS	Alleles	Genotypes	KIV-BURSA			KIV-MANİSA			KIV-İSTANBUL			OVERALL		
			Genotype Freq. (%)	Allele Freq.	χ2	Genotype Freq. (%)	Allele Freq.	χ2	Genotype Freq. (%)	Allele Freq.	χ2	Genotype Freq. (%)	Allele Freq.	χ2
ABCG2	-	-/-	50.0	0.65	3.86*	41.7	0.67	0.45	35.3	0.5	6.19*	42.0	0.60	5.34*
	+	+/-	30.0	0.35		50.0	0.33		29.4	0.5		37.0	0.40	
		+/+	20.0			8.3			35.3			21.0		
AA-NAT	A	AA	79.3	0.90	0.32	80.0	0.90	0.37	64.7	0.82	1.41	74.5	0.87	2.00
	G	AG	20.7	0.10		20.0	0.10		35.3	0.18		25.5	0.13	
FABP3	A	AA	23.4	0.40	3.13	19.0	0.33	5.49*	47.0	0.53	20.63**	30.0	0.42	26.27**
	G	AG	33.3	0.60		28.0	0.67		11.8	0.47		24.0	0.58	
		GG	43.3			53.0			42.2			46.0		

** significant, P<0.05; ** significant, P<0.01*

Table 4. *Observed and expected heterozygosity values for all loci in investigated populations*
Tablo 4. *İncelenen populasyonlardaki beklenen ve gözlenen heterozigotluk değerleri*

LOCUS	KIV-BURSA		KIV-MANİSA		KIV-İSTANBUL		OVERALL	
	Obs-Het	Exp-Het	Obs-Het	Exp-Het	Obs-Het	Exp-Het	Obs-Het	Exp-Het
ABCG2	0.3000	0.4627	0.5000	0.4507	0.2941	0.5075	0.3700	0.4804
AA-NAT	0.2069	0.1887	0.2000	0.1826	0.3529	0.2950	0.2551	0.2237
FABP3	0.3333	0.4881	0.2778	0.4507	0.1176	0.5057	0.2400	0.4896

DISCUSSION

All loci investigated were found as polymorphic in Kıvırcık population. There are few number of studies carried out with these mutations investigated in the present study [19,22,31-33]. Allelic frequencies obtained from analysis are not consistence with the previous studies carried out for *ABCG2* [19] while frequency distributions of the *FABP3* and *AA-NAT* alleles are found similar to those of some Spanish sheep breeds and Chinese seasonal reproduction breeds, respectively [22,33]. For *FABP3* locus the G allele was also found as predominant majority of breeds investigated and frequencies of A allele was ranged from 1 (in Mouflon) and 0.26 (in Raza Aragonesa) [33]. The authors suggested that the wild allele of the *FABP3* locus investigated could be A allele and a subsequent mutation followed by selection may have increased the frequency of the G allele in domestic sheep breeds [33]. In the later study of the same authors heterozygous genotypes for this mutation was found related to milk fat content and it seems as a candidate gene for the marker assisted selection studies [19].

At *ABCG2* locus the "–" allele was found as predominant and its frequency differed from 0.50-0.65 in the present study and for all over the population it was found as 0.60 *(Table 3)*. On the contrary the "+"allele was found as predominant in a previous study [19]. Otherwise they found to be related the "–" allele and higher SCC. The high frequency of this allele is an unfavorable event in Kıvırcık breeding populations.

In the case of *AA-NAT* it can be said that our findings are concordance with the results obtained from Chinese sheep breeds [22]. The study carried out in these Chinese sheep breeds revealed that frequencies of the *AA-NAT*-G are higher in non-seasonal reproduction breeds while found quite lower in the seasonal reproduction breeds [22]. It is well known that Turkish sheep breeds are generally seasonal reproduction breeds thus in our study G allele frequencies showed quite low. On the other hand G allele frequency obtained Kıvırcık populations was lower than allelic frequency of G allele frequencies obtained Chinese seasonal reproduction breeds. In the present study we are not found any heterozygous genotypes for this locus which may a negative situation when the locus is needed for marker assisted selection.

Furthermore chi-square test (χ2) revealed that allelic frequencies in *ABCG2* loci Kıvırcık populations from Bursa and İstanbul are in disequilibrium ($P<0.05$) and in the *FABP3* locus Kıvırcık- Manisa ($P<0.05$) and Kıvırcık- İstanbul the frequencies are also in disequilibrium ($P<0.01$). It should be kept in mind that these populations are breeding populations and there may strong selection pressures across populations and this situation probably due to selection acting on these loci in these populations, which are being selected for milk production or resistance to mastitis.

Genetic improvement of the livestock species is an expensive and time consuming process. Instead of or beside of classical selection methods for economically important traits they may be used genes have major effect on the traits for selection criteria. Kıvırcık is one of the most important sheep breed in animal production of Turkey. Molecular markers may be used in studies on genetic improvement of Kıvırcık sheep breed when efforts are continued in this field. Studies should be increased to reveal genetic structure of this breed for genes affect on economically important traits. On the other hand frequencies of these kinds of genes investigated routinely and further investigations should be carried out to ensure the relationships with the genes and the traits.

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RESEARCH ARTICLE

Seasonal Activity of Tick Infestation in Goats and Buffalo of Punjab Province (District Sargodha), Pakistan

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Summary

The purpose of the present study was to determine the prevalence of tick infestation in buffalo and goats of Punjab Province (District Sargodha), Pakistan. There were a total of twelve hundred buffalo and goats examined from October 2012 to September 2013 to determine the prevalence of ticks infestation. The results showed that the prevalence of tick infestation in buffalo was 84.33% (1012/1200). The ticks were collected and identified. In buffalo, the higher prevalence rate was *Hyalomma aegyptium* (37.91%) followed by *Rhipicephalus (Boophilus) microplus* (22.59%), *Rhipicephalus (Boophilus) annulatus* (17.15%), *Hyalomma marginatum* (9.45%), H*yalomma anatolicum* (9.03%) and *Rhipicephalus sanquineus* (3.84%) respectively. The breed wise prevalence was in Chenab breed (84.59%), Ravi breed (79.10%) and Hybrid breed (91.66%) respectively. The sex wise prevalence was in male (87.71%) and in female (83.53%). The prevalence based on age showed that the rate of infestation in buffalo having age group (1-4 year) was 85.48%, (4-8 year) was 86.02% and (>8 year) was 66.66%, respectively. The prevalence based on grazing pattern showed that rate of infestation in intensive grazing 85.28%, extensive grazing 86.41% and both (intensive and extensive) was 81.94%.While in case of goats the tick infestation was 86.50% (1038/1200). In goats, the higher prevalence was *Hyalomma anatolicum* (31.56%), *Rhipicephalus* spp. (25.95%), *Haemophysalis* spp. (21.07%), *Ixodes* spp. (15.46%), and *Ambylomma* spp. (5.93%) respectively. The breed wise prevalence was in Desi breed (91.12%), Taidi breed (90.97%), Lail Poori breed (91.78%) and Hybrid breed (66.66%) respectively. The sex wise prevalence was in male (79.16%) and in female (88.81%). The prevalence based on age showed that the rate of infestation in goats having age group (1-4 year) was 86.17%, (4-8 year) was 91.66%, respectively. The prevalence based on grazing pattern showed that rate of infestation in intensive grazing 86.71%, extensive grazing 85.97% and both (intensive grazing and extensive grazing) was 87.12%.The present study showed that these epidemiological factors have a significant effect on the prevalence of tick infestation in buffalo and goats of Sargodha district, Pakistan. The results of this survey showed that the ticks are active throughout the year except January but highly active from June to August. It would be very helpful in devising the future strategies towards the eradication and control of ticks in other endemic areas of Pakistan.

Keywords: *Ticks, Intensive grazing, Extensive grazing, Prevalence, Epidemiological factors, Pakistan*

Pakistan'ın Sargodha Bölgesi Pencab Eyaletinde Keçi ve Yaban Sığırlarında Kene Enfestasyonunun Mevsimsel Aktivitesi

Özet

Bu çalışmanın amacı Pakistan'ın Sargodha Bölgesi Punjab Eyaletinde keçi ve yaban sığırlarında kene enfestasyonunun prevalansını belirlemektir. Kene prevalansını tespit etmek amacıyla toplam Her birinden 1200'er yaban sığırı ve keçi Ekim 2012 ile Eylül 2013 yılları arasında incelendi. Yaban sığırlarında kene enfestasyonu %84.33 (1012/1200). Keneler toplandı ve tanımlandı. Yaban sığırlarında en yüksek prevalans *Hyalomma aegyptium* (%37.91) iken bunu sırasıyla *Rhipicephalus (Boophilus) microplus* (%22.59), *Rhipicephalus (Boophilus) annulatus* (%17,15), *Hyalomma marginatum* (%9.45), *Hyalomma anatolicum* (%9.03) ve *Rhipicephalus sanquineus* (%3.84) takip etti. Türlere göre prevalans Chenab (%84.59), Ravi (%79.10) ve Hibrit (%91.66) olarak tespit edildi. Cinsiyete göre prevalans erkeklerde %87.71, dişilerde %83.53 olarak saptandı. Yaş gruplarına göre yaban sığırlarında enfestasyon oranları 1-4 yaş arası %85.48, 4-8 yaş arası %86.02 ve 8 yaş üzeri olanlarda %66.66 olarak bulundu. Otlama şekillerine göre prevalans yoğun otlatılanlarda %85.28, seyrek otlatılanlarda %86.41 ve her iki şekilde birden olanlarda %81.94 olarak tespit edildi. Keçilerde kene enfestasyonu %86.50 (1038/1200) olarak belirlendi. Kene prevalansları türlere göre sırasıyla %31.56 *Hyalomma anatolicum*, %25.95 *Rhipicephalus* spp., %21.07, *Haemophysalis* spp., %15.46 *Ixodes* spp. ve %5.93 *Ambylomma* spp. olarak tespit edildi. Keçi türlerine göre %91.12 Desi, %90.97 Taidi, %91.78 Lail Poori ve %66,66 Hibrit olarak belirlendi. Cinsiyete göre prevalans erkeklerde %79.16 dişilerde %88.81 olarak saptandı. Yaş gruplarına göre keçilerde enfestasyon oranları 1-4 yaş arası %86.17, 4-8 yaş arası %91.66 olarak bulundu. Otlama şekillerine göre prevalans yoğun otlatılan keçilerde %86.71, seyrek otlatılanlarda %85.97 ve her iki şekilde birden olanlarda %87.12 olarak tespit edildi. Bu çalışma Pakistan'ın Sargodha bölgesinde epidemiyolojik faktörlerin yaban sığırı ve keçilerde kene enfestasyonunun prevalansına önemli etkileri olduğunu göstermiştir. Bu taramanın sonuçları kenelerin Ocak ayı hariç tüm yıl boyunca özellikle de Hazirandan Ağustas ayına kadar oldukça aktif olduklarını ortaya koymuştur. Pakistan'ın diğer endemik bölgelerinde kenelerin eradikasyon ve kontrolüne yönelik planlamalar yapılmasının yaralı olacığa görüşündeyiz.

Anahtar sözcükler: *Kene, Yoğun otlatma, Seyrek otlatma, Prevalans, Epidemiyolojik faktörler, Pakistan*

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INTRODUCTION

Ticks are very important and most common ecto-parasites of mammals, birds and reptiles worldwide [1,2]. They are dioecious having separate sex [3]. Based on the number of hosts required to complete their as one-host, two-host and three-host ticks [4].

Ticks also have adverse effect on livestock in several ways and parasitize a wide range of vertebrate hosts and transmit a wide variety of pathogenic agents than any other group of arthropods [5]. They transmit protozoa, bacterial, rickettsial and viral diseases [6]. They down grade hide and skins quality and reduce milk and wool production, reduce productivity and increase susceptibility to the other diseases [7]. Due to economic and veterinary importance of ticks, their control and the transmission of tick borne diseases remain a challenge for the cattle industry in tropical and subtropical areas of the world and it is a priority for many countries in tropical and subtropical regions [8].

Tick infestation has adverse effects throughout the world particularly in the livestock. The 80% of cattle population is suffering with tick infestation in the world. Tick-borne diseases (TBDs) around the world have been associated with US$ 13.9 to US$ 18.7 billion annually loss in productivity [7,9-11]. More than US$1.0 billion and US$1.0 million annually losses by a single tick *Boophilus microplus* are reported in South America and Australia respectively [12]. Acricidal activities against diseases in order to control prevailing conditions enhance the production cost for the owner [13].

Pakistan owns a large number of animals. These animals are playing important role to meet the ever increasing demand of animal protein and milk for mankind. Ticks are cosmopolitan in distribution, but occur principally in tropical and subtropical regions [1]. Pakistan being a tropical country provides optimal climatic conditions for growth and multiplication of ticks. Tick fauna of Pakistan is rich in number of genera and species [14]. The impact of ticks and tick borne diseases on the individual and national economics warrants application of appropriate tick control strategies on priority basis [15]. Most of the investigations on prevalence of tick species in Pakistan are more than a decade old [16-19], whereas periodical monitoring of tick infestation is an essential component for formulating effective control recommendations.

Due to high prevalence of this disease around the world and Pakistan keeping in mind the importance of livestock, above described facts and figures the present study was designed With following Objectives.

The objectives of the present study were to determine the;

- Seasonal fluctuation in population of ticks with respect to epidemiological factors like breed, herd size and sex etc.

- Correlation of tick population with intensive and extensive grazing pattern.

MATERIAL and METHODS

Location

Punjab is the Pakistan's second largest province at 205.344 km^2 (79.284 sq^2 miles) after Balochistan and is located at the northwestern edge of the geologic Indian plate in South Asia. The geographical location of the Sargodha is 32° 5′ 1″ North, 72° 40′ 16″ in Punjab Province, Pakistan.

Topography

The Punjab province is bordered by Kashmir (Azad Kashmir, Pakistan and Jammu and Kashmir, India) to the north-east, the Indian states of Punjab and Rajasthan to the east, the Pakistani province of Sindh to the south, the province of Baluchistan to the southwest, the province of Khyber Pakhtunkhwa to the west, and the Islamabad Capital Territory to the north. Undivided Punjab is hometo six rivers, of which five flows through Pakistani Punjab. From west to east, these are: the Indus, Jhelum, Beas, Chenab, Ravi and Sutlej. Nearly 60% of Pakistan's population lives in the Punjab. It is the nation's only province that touches every other province; it also surrounds the federal enclave of the national capital city at Islamabad. This geographical position and a large multi-ethnic population strongly influence Punjab's outlook on National affairs and induces in Punjab a keen awareness of the problems of the Pakistan's other important provinces and territories. The landscape is amongst the most heavily irrigated on earth and canals can be found throughout the province. Weather extremes are notable from the hot and barren south to the cool hills of the north. The foothills of the Himalayas are found in the extreme north as well.

Study Area

The present study was conducted in the district Sargodha, Punjab, Pakistan. The data was collected from five tehsils of Sargodha, Sahiwal, Silanwali, Kotmomin and Shahpur. The area is located 550 to 650 feet above the sea level. The area has a climate of extreme heat and cold. The maximum temperature reaches 50°C (122°F) in the summer while the minimum temperature recorded is low as freezing point in the winter [20].

Data Collection

The study was conducted from October 2012 to September 2013. Data was collected on monthly basis

from Sargodha and Silanwali, Shahpur and Sahiwal and Kotmomin.

Tick specimens were collected using forceps without damaging their mouthparts and preserved in 70% ethyl alcohol. Complete record was maintained for each tick specimen for their origin regarding species of the host. Permanent mounts of the tick specimens were prepared. Morphological characterization of ticks was carried out using a stereoscopic microscope according to the instructions given by [1].

Statistical analysis

The Statistical analysis (Chi-square) was done by using the statistical package SPSS version 20.0 for Windows 2007.

RESULTS

Buffalo

The present study was conducted in five tehsils of Sargodha district to determine the prevalence of tick infestation in the buffalo from October 2012 to September 2013. The results of present study revealed that the rate of infestation in buffalo was 84.33% (1012/1200). The ticks were observed mostly on the udder parts on the body dorsal side and ventral side of the infested buffalo. The ticks started to appear by the start of February and highly active from end of May to August *(Fig.1)*. The species collected from infested buffalo were identified as *Hyalomma aegyptium* 37.91% followed by *Rhipicephalus (Boophilus) microplus* 22.59%, *Rhipicephalus (Boophilus) annulatus* 17.15%, *Hyalomma marginatum* 9.45%, *Hyalomma anatolicum* 9.03% and *Rhipicephalus sanquineus* (3.84%).

The buffalo of three breeds (Chenab breed, Ravi breed, Hybrid breed) were examined on monthly basis. The prevalence in Chenab breed was 84.59% (851/1006), Ravi breed 79.10% (106/134) and Hybrid breed 91.66% (55/60) respectively *(Fig. 2)*.

The tehsil wise prevalence was determined in the present study. It was in tehsil Kotmomin 86.20% (300/348), Shahpur 81.66% (147/180), Sargodha 79.41% (162/204), Silanwali 86.84% (198/228) and Sahiwal 85.41% (205/240) were respectively *(Fig. 3)*. The statistical analysis has showed no significant differences (P<0.13) in the prevalence of tick infestation in different tehsils of district Sargodha Punjab Province, Pakistan.

The results showed that female 83.53% and male 87.71% buffalo were infested *(Fig. 4)*. The prevalence in buffalo having age group (1-4 year)was 85.48% (636/744), in age group (4-8 year) was 86.02% (320/372) and the age group (>8 years) was 66.66% (56/84) were infested *(Fig. 5)*. The results showed that younger buffalo were more infested as compared to older buffalo.

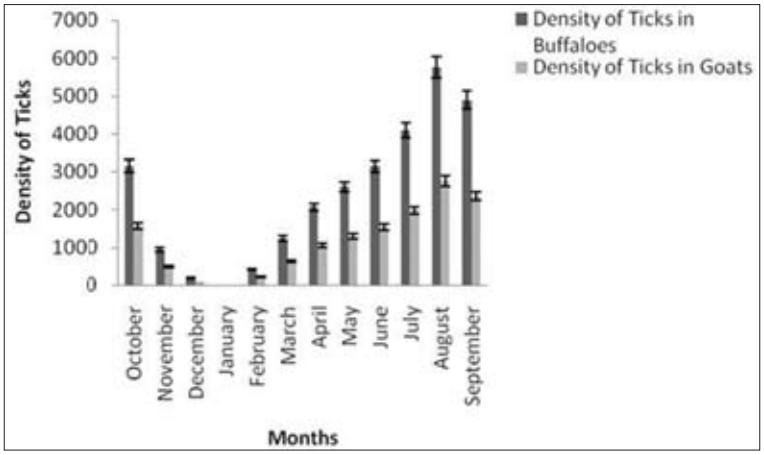


Fig 1. Showing the fluctuation of tick density on the goats and buffalo examined during study. Tick density was highest in August followed by September and was least in January

Şekil 1. Çalışma boyunca incelenen yaban sığırı ve keçilerde kene yoğunluğunun aylara göre dağılımı. Kene yoğunluğu en yüksek Ağustos ayında gözlendi, bunu Eylül ayı izledi. En düşük Ocak ayında tespit edildi

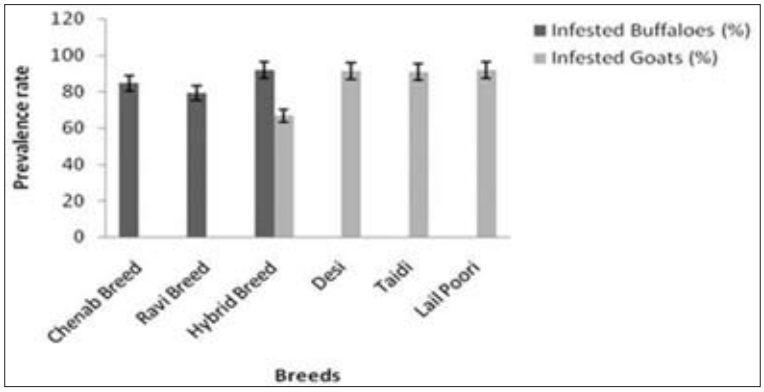


Fig 2. Showing breed wise prevalence of tick infestation in goats and buffalo

Şekil 2. Yaban sığırı ve keçi türlerine göre kene enfestasyonunun prevalansı

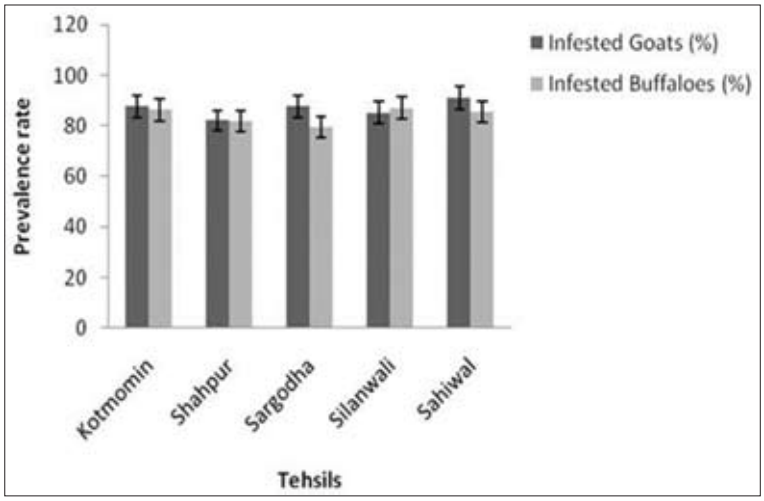
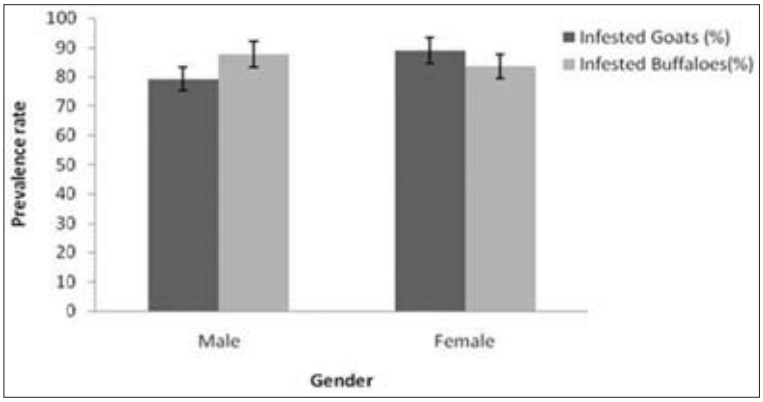


Fig 3. Showing the tick infestatıon of goats and buffalo in different tehsils

Şekil 3. Değişik bölgelere göre keçi ve yaban sığırlarındaki kene enfestasyonu

Fig 4. Showing sex wise prevalence in goats and buffalo

Şekil 4. Cinsiyete göre keçi ve yaban sığırlarındaki kene prevalansı



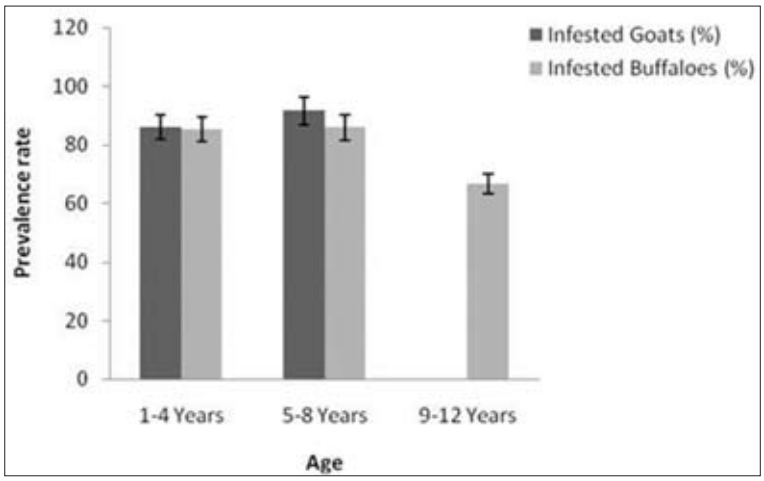


Fig 5. Showing age wise prevalence of tick infestatıon in goats and buffalo

Şekil 5. Keçi ve yaban sığırlarındaki yaşa göre kene enfestasyonu prevalansı

The statistical analysis showed that there is no significant difference between infested and non-infested buffalo in all age groups *(Table 1)*. The prevalence rate in animals reared under intensive grazing system was 85.28% (429/503), extensive grazing 86.41% (229/265) and both (intensive and extensive) grazing was 81.94% (354/432) respectively *(Fig. 6)*.

Goats

The results of present study revealed that the rate of infestation in goats was 86.50% (1038/1200). The ticks started to appear by the start of February and highly active from end of May to August *(Fig. 1)*. The species collected from infested goats were identified as *Hyalomma anatolicum* 31.56%, *Rhipicephalus* spp. 25.95%, *Haemophysalis* spp. 21.07%, *Ixodes* spp. 15.46%, and *Ambylomma* spp. 5.93%.

The goats of four breeds (Taidi breed, Desi breed, Lailpoori and hybrid breed) were examined in the present study on monthly basis. The statistical analysis shows

Table 1. *Showing the statistical analysis of different epidemiological factors on the prevalence of tick infestation in buffalo Sargodha district of Punjab Province, Pakistan*

Tablo 1. *Pakistan'ın Sargodha Bölgesi Pencab Eyaletinde yaban sığırlarında kene enfestasyonu prevalansı üzerine değişik epidemiyolojik faktörlerin istatistiksel analizi*

S. No	Factors	Groups	Prevalence in Buffalo		Statistical Analysis (Chi-square)
			Infested	Non Infested	
1	Age	1-4 Years	636 (85.48%)	108 (14.51%)	χ^2=21.39 d f=2 P=0.000
		5-8 Years	320 (86.02%)	52 (13.97%)	
		9-12 Years	56 (66.66%)	28 (33.33%)	
2	Breed	Chenab	851(84.59%)	155 (15.40%)	χ^2=5.266 df=2 P=0.072
		Ravi	106 (79.10%)	28 (20.89%)	
		Hybrid	55 (91.66%)	5 (8.33%)	
3	Gender	Male	200 (87.71%)	28 (12.28%)	χ^2=2.443 df=1 p=0.069
		Female	812 (83.53%)	160 (16.46%)	
4	Tehsils	Kotmomin	300 (86.20%)	48 (13.79%)	χ^2=6.933 df=4 P=0.139
		Shahpur	147 (81.66%)	33 (18.33%)	
		Sargodha	162 (79.41%)	42 (20.58%)	
		Silanwali	198 (86.84%)	30 (13.15%)	
		Sahiwal	205 (85.41%)	35 (14.58%)	
5	Grazing Patterns	Intensive Grazing	429 (85.28%)	74 (14.71%)	χ^2=3.082 df=2 P=0.214
		Extensive Grazing	229 (86.41%)	36 (13.58%)	
		Both (intensive and extensive grazing)	354 (81.94%)	78 (18.05%)	

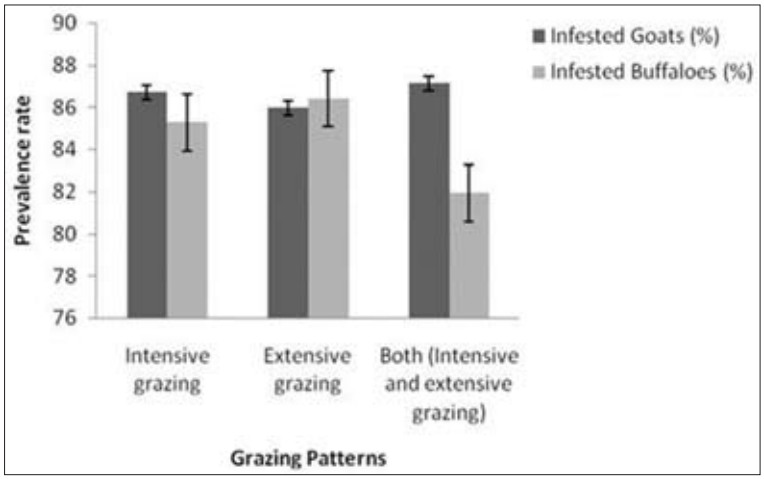


Fig 6. Showing grazing pattern based prevalence of tick infestatıoin in buffalo and goats

Şekil 6. Keçi ve yaban sığırlarındaki otlama şekillerine göre kene enfestasyonu prevalansı

that prevalence in Desi breed was 91.12% (688/755) Taidi breed 90.97% (131/144) Lail poori 91.78% (67/73) and Hybrid breed 152/228 (66.66%), respectively. Among all four breeds the highest infestation was observed in Lailpoori breed 91.78% *(Fig. 2)*.

The tehsil wise prevalence was determined from the Sargodha district. The prevalence in Sargodha district according to tehsil Kotmomin 87.50% (210/240), Shahpur 81.94% 177/216), Sargodha 87.50% (252/288), Silanwali 85.14% (235/276) and Sahiwal 91.11% (164/180) were respectively *(Fig. 3)*. The statistical analysis has showed the no significant differences (P<0.091) in the prevalence of tick infestation in different tehsils of district Sargodha Punjab Province, Pakistan.

The results showed that the infestation rate was 88.81% (810/912) female and 79.16% (228/288) in male goats *(Fig. 4)*. The prevalence in goats having age group (1-4 year) was 86.17% (972/1128) and in age group (4-8 year) was 91.66% (66/72) *(Fig. 5)*. The results showed that older goats were more infested as compared to younger goats. The statistical analysis showed that there is no significant difference between infested and non-infested animals in all age groups *(Table 2)*. The results showed that prevalence in the intensive grazing 86.71% (385/444), extensive grazing 85.97% (423/492) and both (intensive and extensive) grazing was 87.12% (230/264) respectively *(Fig. 6)*.

Table 2. *Showing the statistical analysis of different epidemiological factors on the prevalence of tick infestation in goats Sargodha district of Punjab Province, Pakistan*

Tablo 2. *Pakistan'ın Sargodha Bölgesi Pencab Eyaletinde keçilerde kene enfestasyonu prevalansı üzerine değişik epidemiyolojik faktörlerin istatistiksel analizi*

S. No	Factors	Groups	Prevalence in Goats		Statistical Analysis (Chi-square)
			Infested	Non Infested	
1	Age	1-4 Years	972 (86.17%)	156 (13.82%)	X^2=1.751 df=1 P=0.186
		5-8 Years	66 (91.66%)	6 (8.33%)	
2	Breed	Desi	688 (91.12%)	67 (8.87%)	X^2=94.847 df=3 P=0.000
		Lail Poori	67 (91.78%)	6 (8.21%)	
		Taidi	131 (90.97%)	13 (9.02%)	
		Hybrid	152 (66.66%)	76 (33.33%)	
3	Gender	Male	228 (79.16%)	60 (20.83%)	X^2=17.451 df=1 P=0.000
		Female	810 (88.81%)	102 (11.18%)	
4	Tehsils	Kotmomin	210 (87.50%)	30 (12.50%)	X^2=8.002 df=4 P=0.091
		Shahpur	177 (81.94%)	33 (18.05%)	
		Sargodha	252 (87.50%)	36 (12.50%)	
		Silanwali	235 (85.14%)	41 (14.85%)	
		Sahiwal	164 (91.11%)	16 (8.88%)	
5	Grazing Patterns	Intensive Grazing	385 (86.71%)	59 (13.28%)	X^2=0.220 df=2 P=0.896
		Extensive Grazing	423 (85.97%)	69 (14.02%)	
		Both (intensive and extensive grazing)	230 (87.12%)	34 (12.87%)	

DISCUSSION

The results showed that the prevalence of tick infestation in buffalo and goats of Sargodha district was 84.75% and 86.50% respectively. Our results correlates as 85.6% cattle were infested [21]. The spp. wise prevalence was *Rhipicephalus microplus* (22.59%), *Rhipicephalus annulatus* (17.15%), *Hyalomma marginatum* (9.45%), *Hyalomma anatolicum* (9.03%) and *Rhipicephalus sanquineus* (3.84%) respectively. Similar observations of tick spp. were reported on different genera of ticks on Friesian cattle in district Kasur, Punjab, Pakistan [22].

The results showed that the peak of population of ticks from June to August which is due to the high temperature and humidity [22]; the minimum population observed in January is due to low temperature and less humidity and short day length in buffalo and goats, respectively. The *Fig. 6* describe grazing pattern in buffalo and goats. According to this in buffalo, prevalence rate of tick infestation is higher in intensive grazing than extensive and the buffalo which possess the both types of grazing have high prevalence. While in case of goats prevalence rate is higher in extensive than intensive grazing and the goats which posses the both types of grazing have low prevalence. Similar observations were reported in case of parasitic disease like WFI, where the prevalence of disease was higher in intensive-extensive management grazing [23].

In case of extensive grazing the animals have to walk for food in the fields here there is little chance of attachment of ticks on the animal body due to more light exposure. When organism walks the more light falls on the dorsal side of body which increases the temperature and specify the under sites for attachment of ticks [24] that affect the reproductive activity of ticks and hence the prevalence of tick infestation decreases.

Most of the ticks were found on the udder, under tail and a small number on the chest and neck areas of the buffalo, while in case of goats mostly ticks were found on the ears and udder parts of the body [25]. It might be that the parts of the animals where ticks are found posses the soft tissues. This is the advantage for ticks that they can easily attach with the soft tissues and make a contact with the blood capillaries of the animal. Here female ticks can easily feed on the blood and get ready for the reproduction by leaving the host body part for laying the eggs. The other advantage for the ticks by attaching with the soft tissue is that they get protection from the predators such as birds. If they are exposed on the animal's body predators can easily get to them, will consume them and in this way the population of the ticks could be decrease.

The climatic determinants like temperature of the study area are very important in the prevalence of ticks in domestic ruminants [26]. The poor husbandry practices of small holder dairy farmers may be a determinant making the animals more prone to tick infestation. Moreover, a

stress should be given to practice a routine preventive therapy against ticks rather than treating the animals at the cost of lowered milk production [27]. Our study result shows that the prevalence of ruminant tick infestation is much higher in the developing countries of Asia [16,19,28-31] and Africa [32-35], followed by Australia [36], Europe [37-40], and the Americas [41-42].

After correlating temperature with that of population of ticks in multi angels it is concluded that density of the ticks fluctuates throughout the study period on goats and buffalo in district Sargodha Punjab province Pakistan. Climatic determinants of the study area greatly affect the prevalence of tick infestation. The population fluctuation depends on number of factors e.g. temperature, humidity, rain fall, gender and breed etc. The poor husbandry practices of small holder dairy farmers may be a determinant making the animals more prone to tick infestation. Keeping in view the results of this study, the farmers of the study area should be educated about the significance of the disease through local extension programs. Moreover, a stress should be given to practice a routine preventive therapy against ticks rather than treating the animals at the cost of lowered meat and milk production.

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RESEARCH ARTICLE

Assessment of Gastric *Helicobacter* spp. in Fresh Gastric Samples of Naturally Infected Dogs by Scanning Electron Microscopy

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Summary

Different species of gastric *Helicobacter*-Like Organisms (GHLO) reported from dogs' stomach. The aim of present study was to morphological evaluation of gastric *Helicobacter* spp. in fresh gastric samples of naturally infected dogs. Thirty two gastric samples of the stray dog were taken at necropsy. The specimens were used for rapid urease test, light microscopy, scanning electron microscopy (SEM) and polymerase chain reaction (PCR). Light microscopy examination confirmed the presence of GHLO in 90.5% of stray dogs. 87.5% and 94% of gastric samples were positive in rapid urease test and PCR, respectively. Four distinguishable *Helicobacter* organisms were confirmed by SEM. Three strains of these organisms were inditified as *H. felis,* candidatus *H. heilmanii* and *H. bizzozeronii* because of their apparent morphological differences and PCR results. The last strain of these bacteria was not distinguishable with routine studies. The large-scale studies with fast and simple recognition methods are recommended to confirm the different types of canine gastric *Helicobacter*. The results of present study showed further investigation in canine GHLO is required because new species of *Helicobacter* reported.

Keywords: *Canine Helicobacter, SEM, PCR, Smear*

Doğal Enfekte Köpeklerin Taze Mide İçeriği Örneklerinde Gastrik *Helicobacter* Türlerinin Taramalı Elektron Mikroskopi İle Değerlendirilmesi

Özet

Köpeklerin midesinde Gastrik *Helicobacter*-benzeri Organizmalar (GHBO)'ın üç farklı türü olduğu bilinmektedir. Bu çalışmanın amacı, doğal enfekte köpeklerin taze mide içeriklerinde gastrik *Helicobacter* türlerinin morfolojik özelliklerinin belirlenmesidir. Sokak köpeklerinden nekropsi sırasında otuz iki adet mide içeriği örneği alındı. Örnekler Hızlı Üreaz Testi, Işık Mikroskopisi, Taramalı Elektron Mikroskopisi (SEM) ve Zincirleme Polimeraz Reaksiyonu (PCR) ile incelendi. Işık Mikroskopisi ile köpeklerin %90.5'inde Gastrik *Helicobacter*-benzeri Organizmaların varlığı belirlendi. Köpeklerin %87.5'i ve %94'ü sırası ile Hızlı Üreaz Testi ve PCR pozitif bulundu. Dört adet *Helicobacter* bakterisi SEM ile tespit edildi. Bu bakterilerden 3 suş, PCR ve morfolojik farklılıklarına göre *H. felis,* candidatus *H. heilmanii* ve *H. Bizzozeronii* olarak tanımlandı. Bakterinin son suşu ise rutin metodlar ile identifiye edilemedi. Köpeklerin Gastrik Helicobacterlerinin tiplerini belirlemek için hızlı ve basit tanı metodları üzerinde yapılacak geniş çaplı çalışmalara ihtiyaç vardır. Bu çalışmanın sonuçları yeni *Helicobacter* türlerinin varlığının bildirilmesinden dolayı köpeklerin GHBO'nın daha detaylı araştırılması gereğini ortaya koymaktadır.

Anahtar sözcükler: *Köpek, Helicobacter, SEM, PCR, Sürme preperat*

INTRODUCTION

Helicobacter-Like Organisms (HLO) are live in stomachs of dogs, cats, pigs and other carnivores [1-8], and this genus contains several species from a wide range of hosts [8-10]. HLO are assigned to cause gastric disease in humans

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and animals [1,8,11,12], but the exact role of these fastidious bacteria is not confirmed yet [2,7,8]. Until now, different *Helicobacter* species have been isolated from canine stomachs [2,4,13-15]; but it is not known whether these species are representative of all canine gastric helicobacters or which of them are most common [8,14]. Different studies have reported dissimilar ranges of contamination [3-6,16] and some of these reports were showed high prevalence (over 90%) of contaminations [17-19].

In spite of possibility to culture of canine gastric helicobacters, some of these organisms are not cultivable [8]; or we are not able to culture them. Candidatus *H. heilmanii* is a zoonotic microorganism which is a common cause of the chronic gastric inflammation in human (0.2- 6%) [8,11,20]; but the definitive culture of this organism has not been achieved to date and only two *H. Heilmannii*-like strains *have been* cultured from *human* gastric tissue [8]. The identification of these fastidious requires specific culture techniques and due to presence of non-cultivable species further diagnostic methods are needed [8,21].

The aim of present study was to *evaluate the morphological* characteristics of canine gastric *Helicobacter* spp. and investigate the presences of atypical *Helicobacter* strain(s) in fresh gastric samples of naturally infected dogs.

MATERIAL and METHODS

Admission and Selection of Dogs

Thirty two dogs were randomly selected among the stray dogs that were euthanized in dog population control program which was organized by municipal employees in Tabriz city (East Azerbaijan province, Iran). All dogs of *both sexes* were seven months of age or older and lived in different locations of the city.

Sample Collection

Gastric *samples were taken at necropsy immediately after death* (between April and October, 2010). Four gastric samples were used in diagnostic tests. First sample was immediately fixed and used in cytological study; second sample was placed in normal saline and stored in -20°C for Polymerase Chain Reaction (PCR) assessment; Third sample was used for Rapid Urease Test (RUT) and the fourth sample was placed on microtubule for Scanning Electron Microscopy (SEM).

Impression Smear and Urease Test

Impression smears of gastric mucosa were prepared on an air-dried slide which followed by methanol fixation and stained by Giemsa (Merck, Germany) for detection of GHLO's at 1000 × magnifications. The rapid urease test (Difco, USA) was read within 12 h.

Scanning Electron Microscopy

SEM was performed on fresh samples of canine gastric mucosa. Gastric samples were immediately fixed in 2.5% glutaraldehyde phosphate-buffered solution (pH 7.2) For SEM examination. Samples were dehydrated in a graded ethanol series. After vacuum drying and gold coating, the samples were studied by using a Leo-440i-SEM (Cambridge, UK) at Islamic Azad University in Tehran, Iran.

PCR Amplification of 16S rRNA

Gastric samples were investigated by PCR amplification based on 16S rDNA sequences. The samples were thawed and DNA was extracted by using the DNP™ KIT (Cinna Gen, Iran). PCR analysis on the 16S rRNA gene was performed in an Eppendorf Mastercycler (Bacteriology Laboratory of Veterinary Faculty, Islamic Azad University, Tabriz, Iran) using specific primers *(Table 1)*. Finally, PCR products were examined using agarose gel electrophoresis.

RESULTS

Impression Smears

Presence of canine Gastric *Helicobacter*-like organisms in 29 of 32 stray dogs (90.5%) was confirmed by Light

Table 1. *Oligonucleotide primers which were used for PCR amplification and sequencing of 16S rRNA*
Tablo 1. *PCR amplifikasyonu ve 16S rRNA dizin analizi için kullanılan oligonükleotid primerler*

Target Gene	Reference	Primer Sequence (5'…….. 3')	Amplified Fragment
16Sr RNA genes of *Helicobacter* spp.	Germani [22]	(f): AAC GAT GAA GCT TCT AGC TTG CTA (r): GTG CTT ATT CGT GAG ATA CCG TCA T	399 bp
ure B gene of *H. felis*	Germani [22]	(f): GTG AAG CGA CTA AAG ATA AAC AAT (r): GCA CCA AAT CTA ATT CAT AAG AGC	241 bp
ureB gene of *H. heilmannii*	Neiger [23]	(f): GGG CGA TAA AGT GCG CTT G (r): CTG GTC AAT GAG AGC AGG	580 bp
ureC gene of *H. pylori*	Labinge [24]	(f): GGA TAA GCT TTT AGG GGT GTT AGG GG (r): GCT TAC TTT CTA ACA CTA ACG AGG	294 bp
ureB gene of *H. bizzozeronii*	Neiger [23]	(f): ACT AGG CGA TAC CAA CCT GAT TT (r): TTC TTC AGC TGC GCG GAG CAT GC	499 bp

(f): *Forward sequence,* ***(r):*** *Reverse sequence,* ***bp:*** *base pairs*

microscopy. In most cases, large resemble of GHLO was related to *H. felis*, candidatus *H. heilmannii*; but the size of one of these bacteria was much smaller.

Rapid Urease Test

87.5% (n=26 dogs) among all necropsy samples were positive in RUT.

16S rRNA Sequencing

About 94% (n=30 dogs) of gastric samples were positive in PCR. The presence of *H. felis*, candidatus *H. heilmannii* and *H. bizzozeronii* *(Fig. 1, 2, 3)* was confirmed by PCR. One of the strains was not distinguishable as a common canine gastric *Helicobacter* organism; but it was identified as a *Helicobacter* strain because of its positive 16S rRNA and the positive results of RUT *(Fig. 4)*.

The results of PCR indicated that candidatus *H. heilmannii* was most recognized *Helicobacter*-like organisms (n=16) and other common recognized strains were *H. felis* (n=10) and *H. bizzozeronii* (n=6), respectively.

Scanning Electron Microscopy

Four different *helicobacter* organisms were confirmed by scanning electron microscopy. Three of these organisms were confirmed as *H. felis*, candidatus *H. heilmanii* and *H. bizzozeronii* *(Fig. 5, 6, 7)* by PCR and SEM. The fourth strain *(Fig. 8)* typically varied from others by its small size and different shape (with 2-3 helixes). This strain morphologically was similar to *H. canis* and H. pylori but the RUT of it was positive. Furthermore, there were no positive PCR results for *H. pylori* [16].

The immunological reactions of the gastric mucosa in some gastric samples were notable and they were detected by SEM *(Fig. 9)*.

DISCUSSION

The high incidence of *Helicobacter* organisms in dogs' stomach confirmed by different studies [2,4,7,13,17-19]. In spite of confirmation of *H. pylori* as a main cause

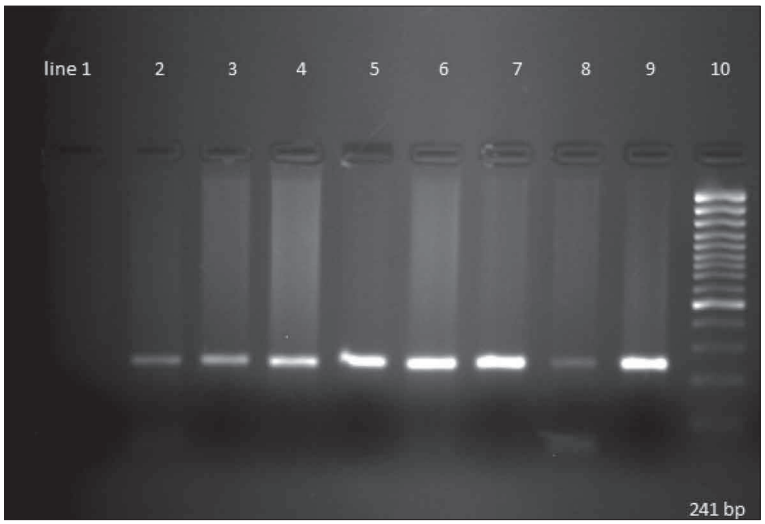
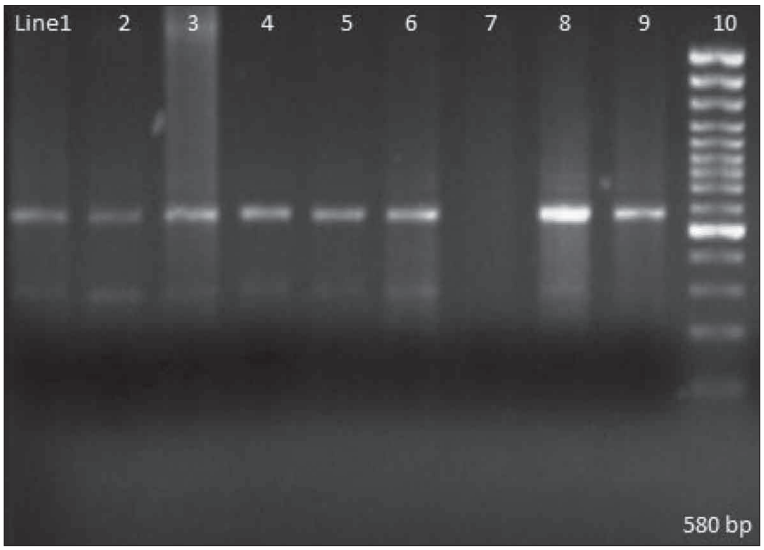


Fig 1. PCR detection of *H. felis*, Lane 1: Negative control, Lane 2-8: DNA samples, Lane 9: positive control, Lane 10: 100 bp DNA marker

Şekil 1. *H. felis*'in PCR tespiti, Şerit 1: Negatif kontrol, Şerit 2-8: DNA örnekleri, Şerit 9: pozitif kontrol, Şerit 10: 100 bp DNA marker

Fig 2. PCR detection of candidates *H. heilmanii*, Lane 1-6: DNA samples, Lane 7: negative control, Lane 9 and 10: positive control, 100 bp DNA marker

Şekil 2. *H. heilmannii*'nin PCR tespiti, Şerit 1-6: DNA örnekleri, Şerit 7: negatif kontrol, Şerit 9 ve 10: pozitif kontrol, 100 bp DNA marker



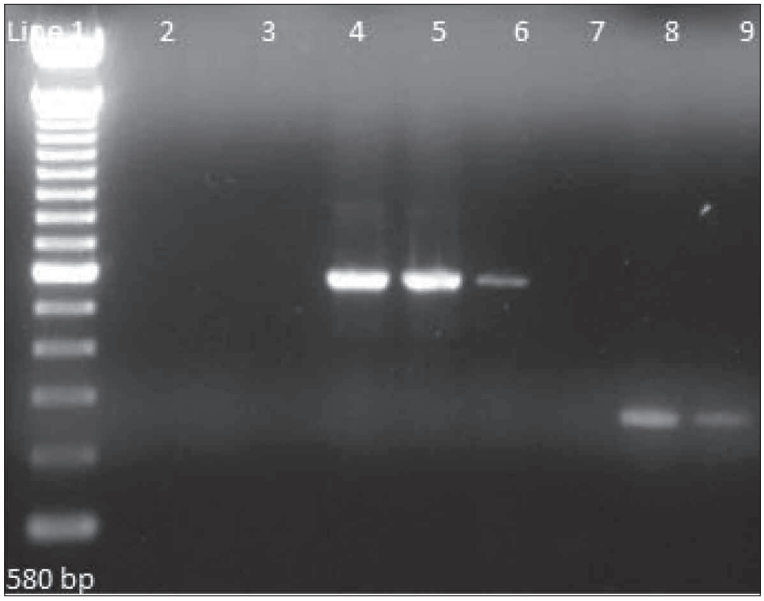
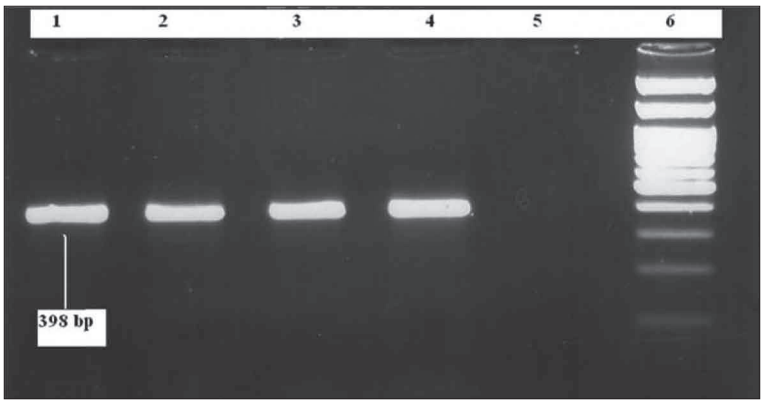


Fig 3. PCR detection of *H. bizzozeronii*, Lane1: 100 bp DNA marker, Lane 2 and 3: negative control, lane 4: positive control, lane 5-9: DNA samples

Şekil 3. *H. bizzozeronii*'nin PCR tespiti, Şerit 1: 100 bp DNA marker, Şerit 2 ve 3: negatif kontrol, Şerit 4: pozitif kontrol, Şerit 5-9: DNA örnekleri

Fig 4. PCR detection of 16S rRNA in gastric biopsy specimens. Lane 1: positive control, Lane 2-4: DNA samples, Lane 5: Negative control, Lane 6: 100 bp DNA marker

Şekil 4. Mide biyopsi örneklerinde 16S rRNA PCR tespiti. Şerit 1: pozitif kontrol, Şerit 2-4: DNA örnekleri, Şerit 5: negatif kontrol, Şerit 6: 100 bp DNA marker



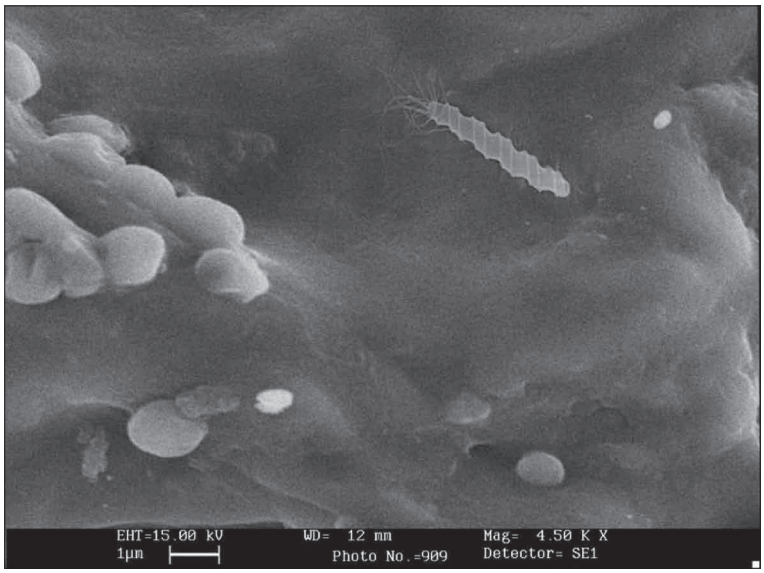


Fig 5. Electron microscopy scanning of *Helicobacter felis* in canine gastric mucosa

Şekil 5. Köpek mide mukozasında *Helicobacter felis*'in elektron mikroskobu taraması

of human chronic gastritis and gastric malignancy, the exact role of gastric helicobacters in dogs has not been established yet [2,7,8]. Six species of helicobacters including *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. bilis*, *H. rappini* (*Flexispira rappini*) and *H. cynogastricus* were cultivated in dogs [2,4,8,25-27]. It is unknown whether these species are representative of all canine gastric helicobacters or not [14]. Furthermore, some studies showed new cultivable *Helicobacter* strains in canine stomach [19,20].

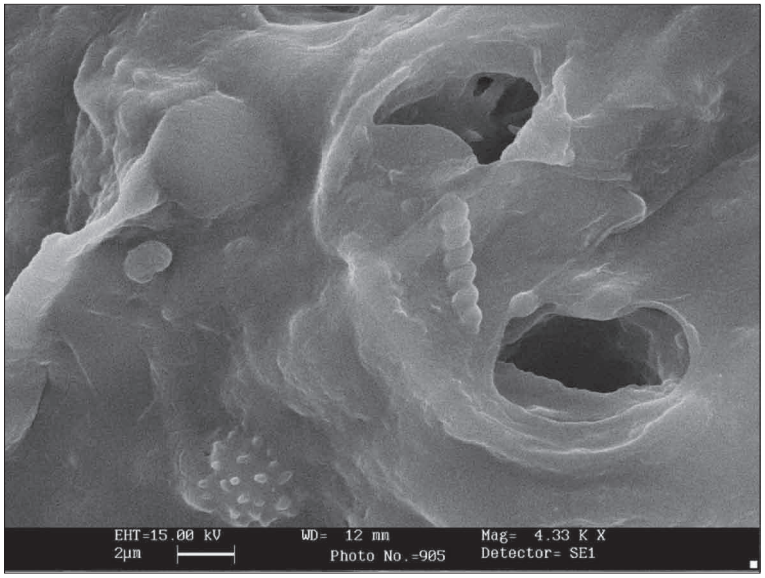
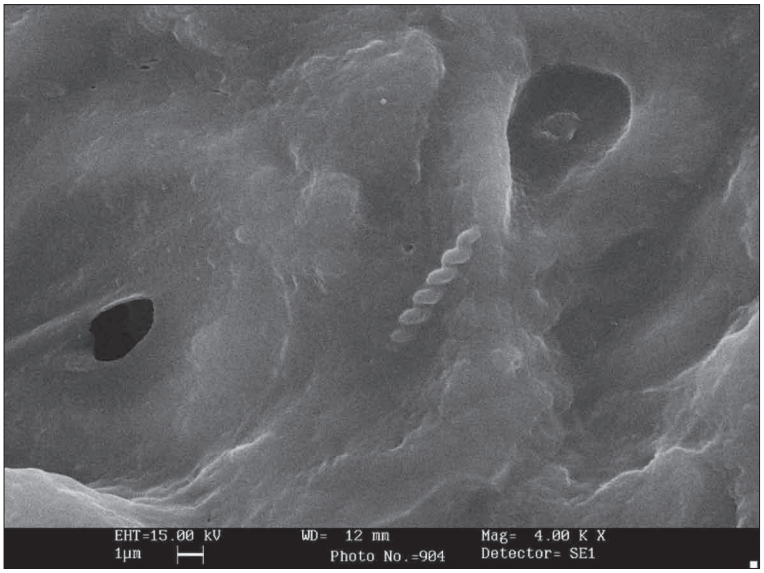


Fig 6. Electron microscopy scanning of candidatus *Helicobacter heilmannii* in canine gastric mucosa

Şekil 6. Köpek mide mukozasında candidatus *Helicobacter heilmannii*'in elektron mikroskobu taraması

Fig 7. Electron microscopy scanning of *Helicobacter bizzozeronii* in canine gastric mucosa

Şekil 7. Köpek mide mukozasında *Helicobacter bizzozeronii*'nin elektron mikroskobu taraması



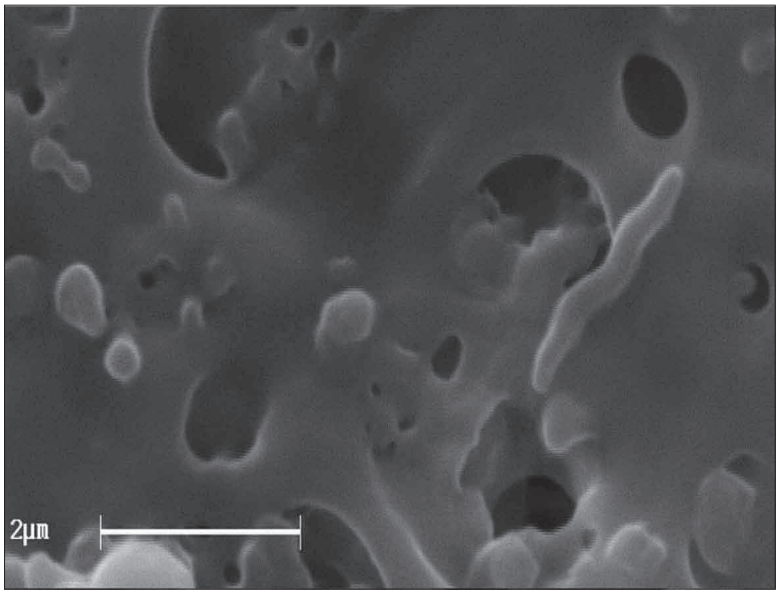


Fig 8. Electron microscopy scanning of Atypical *Helicobacter* in canine gastric mucosa

Şekil 8. Köpek mide mukozasında atipik *Helicobacter*'in elektron mikroskobu taraması

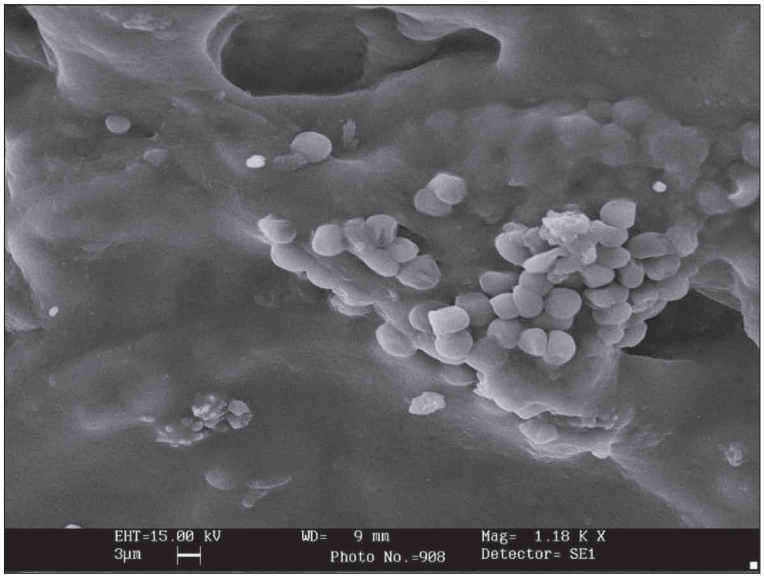


Fig 9. Electron microscopy scanning of canine gastric mucosa and infiltrations of immune cells

Şekil 9. Köpek mide mukozası ve bağışıklık hücreleri sızmasının elektron mikroskobu taraması

In spite of possibility to culture of canine gastric helicobacters, some of GHLO's are not cultivable [8,18]. The rates of success cultures of these organisms are quite low except *H. pylori* [18,21]. The culture of these organisms is important for better differential diagnosis of *Helicobacter* strains, phenotype description and whole cell protein profiles [27]. Additionally, culture of these organisms is essential for assessments of their sensitivities against different antibiotics; especially in recurrent infections. Because of difficulty in culture of these bacteria, PCR and DNA sequencing are being used for detection of various helicobacters [21].

SEM and Transmission Electron Microscopy (TEM) are useful methods for structural analysis of different gastric helicobacters. TEM can reveal better information about germs' ultra- structures; but it needs to consume more time and progressive method. There are a few researchers which are concerned with the morphological study of *Helicobacter* spp. in gastro- enteric specimens [21]. In addition, some studies showed the presence of various cultivable canine gastric helicobacters [19,25-28]. In some studies, obvious differences reported between dissimilar *Helicobacter* organisms [21,27]; but some researchers believe the differences are indistinguishable [26]. The studies indicated a need for accurate investigation about canine gastric helicobacters.

Cytological examination is a fast, cheap and available method only for identification of *Helicobacter* presences in gastric samples. The motility of helicobacters at the fixation time and similarity of *Helicobacter* strains (especially canine GHLO's) caused a hard and accurate diagnosis of these bacteria simply by light microscopy. Therefore, accurate detection of different *Helicobacter* strains can be achieved by combination of impression smears with other diagnostic methods.

Our SEM investigation on fresh gastric specimens showed the presence of four different strains of GHLO's which were distinguishable because of their apparent morphological differences. The ultra structural morphology of these bacteria indicated that *H. felis* was quite distinguishable because of its unique morphology and fibrils *(Fig. 5)*. Candidatus *H. heilmanii* and *H. bizzozeronii* are quite similar. These bacteria are distinguishable because of tight, bluntly and fatty helical structure. Morphologically, *H. bizzozeronii* has more space between its helices *(Fig. 7)*; meanwhile candidatus *H. heilmanii* is more compressed with closed helices *(Fig. 6)*. Based on our results, morphological comparison of different *Helicobacter* species can be an indicator for an accurate detection of different types of gastric helicobacters. It seems that SEM is a fast, available and cheap method for determination of these organisms in the fresh gastric samples.

Difficulties in isolation of some helicobacters can be a reason of not recall for culturing of all gastric helicobacters. Therefore, there is a need for diagnosis of all canine GHLOs' and also their effects and pathogenesis in canine and feline gastric mucosa [25]. It is recommended that large-scale studies with fast and simple methods for recognition and confirmation that would differentiate between dissimilar species of helicobacters (especially in fresh samples of naturally infected animals) are recommended.

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RESEARCH ARTICLE

Osteopontin Expression in Polarized MDCK Cells [1]

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[1] *Preliminary works on this study, presented at 32nd FEBS Congress, 7-12 July, 2007, Vienna - AUSTRIA*

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Summary

The aim of this study is to indicate expression of osteopontin (OPN) in Madin-Darby Canine Kidney (MDCK) cells with different confluences (10, 50, 90, 100%). OPN expression were investigated by western blotting. An increase in OPN expression was observed due to the increased confluency and subsequent initiation of polarization. Expression profiles of flotillin-2 in the same cells were used as a control, since this protein is ubiquitously produced in MDCK cells and its expression rates are independent of confluence and / or polarization. Intracellular distribution of OPN was also monitored by confocal microscopy on preparations immunolabeled with anti-OPN antibodies. Staining patterns have also confirmed increased OPN expression, especially mannose rich isoforms showed an increase dependent on confluency and polarization in MDCK cells. The results were concluded that increased OPN expression during confluency and polarization in MDCK cells. The state of confluence and polarization have significant effects on the mannose rich OPN expression profile.

Keywords: *Osteopontin, Polarization, MDCK cells, Western Blotting*

Polarize MDCK Hücrelerinde Osteopontin Ekspresyonu

Özet

Çalışmada, farklı hücre yoğunluklarındaki (%10, 50, 90 ve 100) Madin-Darby Canine Kidney (MDCK) hücrelerinde, osteopontin (OPN) ekspresyonunun araştırılması amaçlandı. OPN ekspresyonu, western blotting ile araştırıldı. Polarizasyonun başlamasını takiben ve hücre yoğunluktaki artışa bağlı OPN ekspresyonunda artış gözlendi. Hücrelerdeki flotillin-2'nin ekspresyon profili kontrol olarak kullanıldı. Flotillin-2 proteininin ekspresyon hızı, hücre yoğunluğu ve/veya polarizasyona bağımlı olmadan MDCK hücrelerince üretilmektedir. OPN'nin hücre içi dağılımı anti-OPN antikor ile immun işaretleme yapılarak konfokal mikroskopta görüntülendi. MDCK hücrelerindeki hücre yoğunluğuna bağlı artan OPN ekspresyonu, görüntülenen bantlarla teyit edildi. Özellikle OPN'nin mannozdan zengin izoformlarının ekspresyonunun uyarıldığı tespit edildi. Sonuçlara göre, hücre yoğunluğu ve polarizasyona bağlı olarak, MDCK hücrelerinde OPN ekspresyonunda artış gözlendi. Hücre yoğunluğu ve polarizasyon durumu, mannozdan zengin OPN'nin ekspresyon profili üzerine önemli etkisi bulunmaktadır.

Anahtar sözcükler: *Osteopontin, Polarizasyon, MDCK hücreleri, Western Blotting*

INTRODUCTION

Osteopontin (OPN) is a secreted glycoprotein with a multidomain structure and functions characteristic of a matricellular protein [1]. It is highly phosphorylated sialoprotein. OPN interacts with cell surface receptors via arginine-glycine-aspartate sequence (RGD) and non-RGD containing adhesive domains, in addition to binding to components of the structural extracellular matrix. While normally expressed in bone, teeth, kidney and epithelial lining tissues, OPN levels are elevated under conditions of injury and disease like wound healing and inflammation in most tissues studied to date [1,2].

It is expressed at high levels in bony structures of the body. Thus, OPN closely associates with calcified deposits both in normal bone and also in pathologies of ectopic calcification [2,3]. OPN appears to play roles in both the promotion of calcification and mineralization and in the inhibition of calcification. It is found at high levels in

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calcified vascular tissues but in contrast it also acts as an inhibitor of mineralization of bovine aortic smooth muscle cells *in vitro* [4] and ectopic calcification *in vivo* [5].

OPN, also called, early T cell activation gene 1, is a negatively charged acidic hydrophilic protein that is produced by various cell types and participates in diverse physiological and pathological processes, including bone mineralization, oxidative stress, remyelination, wound-healing, inflamation and immunity [6]. OPN has been associated with the progression of numerous types of cancer and this admitted as a marker for cancer malignancy [7-9].

The aim of this study is to indicate expression of OPN in MDCK cells with different confluences.

MATERIAL and METHODS

Cell Culture

Renal epithelial cells of the Madin-Darby Canine Kidney (MDCK) line, type II were used. Cells were grown in Dulbecco-modified Eagle's medium (DMEM) containing 1 g/L glucose, 10% calf serum (FCS) and 1% penicillin-streptomycine at 37°C in a CO_2 incubator. MDCK cells were grown in different cellular confluences (10, 50, 90, 100%).

Reverse Transcription-PCR Analysis

RNA was prepared from confluent MDCK. RNA was reverse-transcribed, and for PCR amplification, 0,5 µl of cDNA was used in 50 µl reactions. The cycling parameters were 95°C for 4 min, 55°C for 1 min, and extension at 72°C for 2 min for 30 cycles, with a final extension period of 2 min at 72°C. 10-µl aliquot of each reaction was electrophoresed through a 2% agarose gel, and the DNA was visualized by ethidium bromide staining under UV light transillumination. The oligonucleotide primers for dog OPNF: GGCATTGCCTACGCCATTCCGA and OPNR: GAGGTGCCTCTCACTGTCCGGGAA were used (SigmaGenosys 2006-07-14).

Confocal Microscopy

MDCK cells grown on cover slips and stained with immunofluorescence method as described [10] for OPN was examined, photographed and confocal imaging on confocal laser scanning microscope (CLSM; Leica, Hannover, Germany). Localization of OPN in MDCK cells by immuno-fluorescence microscopy demonstrated.

Western Blotting of Cell Lysates

Cell lysates were prepared from cell cultures (grown to 10%, 50%, 90% and 100% confluency on 100 mm dishes) by washing each culture dish twice with cold phosphate buffered saline, followed by the addition of 1.000 µl cold lysis buffer (1% Triton-x 100, 1 mM PMSF, 4 mg/ml leupeptin, 4 mg/ml aprotinin, 1 µg/ml pepstatin, 5 µg/ml antipain). Each cell lysate was scraped from the dish, pipetted up and down to complete lysis, and spun at 16.000 g for 10 min to remove in soluble material. Each supernatant was collected and total protein concentration determined by Bradford Protein Assay [11]. Fourty µg of total protein from each cell lysate was used for SDS-PAGE and immunoblotting. Protein gel electrophoresis was done by standard SDS-PAGE methods [12] and immunoblotting by the enhanced chemiluminescence system (Amersham). Cell lysates or conditioned media were fractionated on a denaturing SDS-PAGE gel, electro-phoretically transferred to nylon membrane using a tank blotting system (Hybond P PVDF Transfer Membrane, Amersham) and detected with polyclonal antibody (Biotrend, 100-401-404, raised against human recombinant OPN). The enhanced chemiluminescence detection system (Amersham Corp.) was used to detect immune-reactive bands. Film exposure time was 5 min.

RESULTS

Expression of OPN and flotillin-2 in MDCK cells in different cellular confluences (10%, 50%, 90% and 100%) were shown in *Fig. 1*. Expression profiles of flotillin-2 in the same cells was used as a control, since this protein is ubiquitously produced in MDCK cells and its expression rates are independent of confluence and/or polarization.

Complex-1 and complex-2 forms were same all the cells in different cellular confluency but mannose rich isoforms showed an increase dependent on confluency and polarization. Endo-F and Endo-H tests for isoform of OPN and localization of in MDCK cells were shown in *Fig. 2* and *Fig. 3*, respectively.

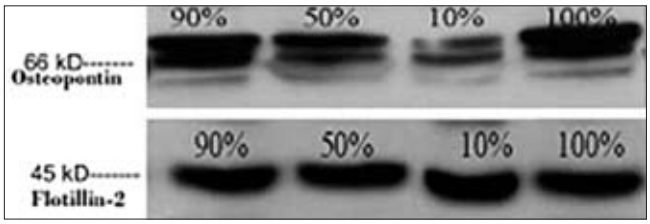

Fig 1. Expression of OPN and flotillin-2 in MDCK cells

Şekil 1. Farklı hücre yoğunluklarındaki MDCK hücrelerinde OPN ve flotillin ekspresyonu

DISSCUSSION

In this study, OPN expression in MDCK cells in different cellular confluences were investigated. Endo-F and Endo-H tests for discovering isoforms of OPN were done *(Fig. 2)*. 2nd band is predominant form (complex-1) and was observed at 66 kDa. 1st band is mannose rich form, 3rd band is complex-2 form. It is thought that complex 1 and 2 isoforms are phosphorylated forms and/or includes O-linked oligosaccharides.

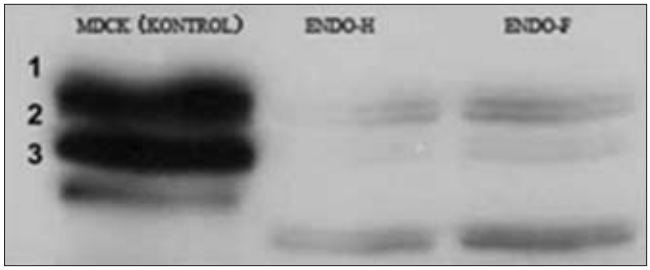

Fig 2. OPN isoforms in different cellular confluences (with Endo-H and Endo-F)

Şekil 2. Farklı hücre yoğunluklarındaki OPN izoformları (Endo-H ve Endo-F ile)

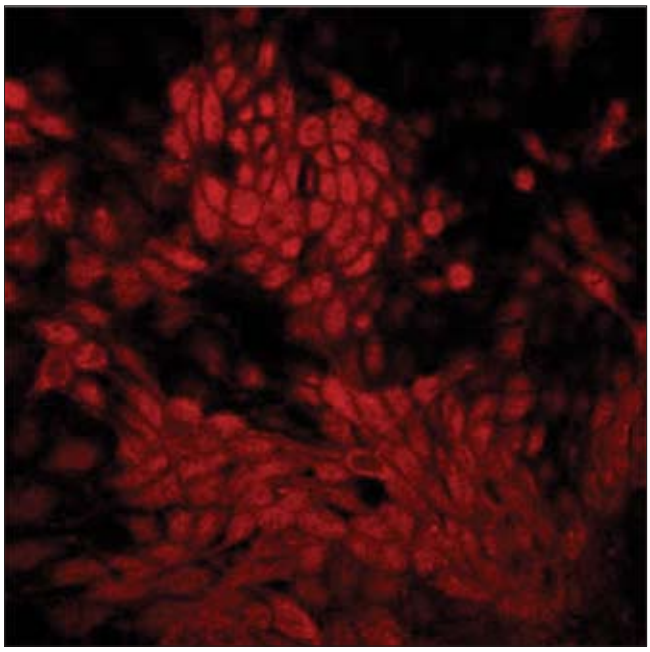

Fig 3. Localization of OPN in MDCK cells

Şekil 3. MDCK hücrelerinde OPN lokalizasyonu

The phosphorylation and dephosphorylation of OPN is an important regulatory mechanism, particularly with regard its role ossification processes. OPN can either faciliate or inhibit ossification depending on phosphorylation state of the protein [13,14].

Malyankar et al.[15] tested the effects of angiotensin 11, basic fibroblast growth factor (bFGF), transforming growth factor/31 (TGF/31), epidermal growth factor (EGF) and insulin like growth factor (IGF), important renal cytokines, on osteopontin regulation in cultured NRK52E cells, a rat renal epithelial cell line. They found that NRK52E cells constitutively express low levels of OPN mRNA and protein. TGF1 and EGF are potent inducers of OPN mRNA and protein in these cells. mRNA stability and nuclear run on assays suggest that induction of OPN expression by TGF1 and EGF is increased via transcription of the OPN gene. The predominant form of osteopontin observed under these conditions had an apparent molecular weight of 66 kDa, consistent with the size previously reported in rat kidney and smooth muscle cells. However, in the TGFf31 treated cells, an additional immunoreactive band was observed. According to Malyankar et al.[15], this band is represent a differentially phosphorylated, glycosylated or spliced isoform of OPN.

Ruutu et al.[16] indicated that numerous genes expression were altered in different confluence states.

At *Fig. 1*, OPN expressionin different cellular confluences were demonstrated. Although, predominant and complex-2 forms are same all the cells in different cellular confluency, mannose rich isoforms showed an increase dependent on confluency and polarization.

In the same cells sometimes OPN can increase some substances but sometimes it can decrease. OPN indicates opposite effects *in vivo* or *in vitro* conditions. This event may depend on transcription of different isoforms of OPN.

The addition of the protein OPN resulted in an increase in the deposition of calcium oxalate [17] or *in vitro* evidence implicates OPN as one of several macromolecular inhibitors of urinary crystallization with potentially important actions at several stages of CaOx crystal formation and retention [18].

OPN originating from different cellular sources may have differential post-translational modifications and/or may be differentially cleaved, suggesting possible differential functions [19]. OPN is normally found in bone, teeth, kidney and epithelial lining tissues. It is expressed at high levels in bony structures of the body, but also in many tissues, the expression of OPN increased under conditions of injury and disease [2,3].

The results were concluded that increased OPN expression during confluency and polarization in MDCK cells. The state of confluence and polarization have significant effects on the mannose rich OPN expression profile.

In order to explain functions of OPN, it is necessary that making more researches about structures and modifications of OPN isoforms. It is necessary to determine the relation between OPN isoforms and diseases.

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RESEARCH ARTICLE

Association of Calpastatin (CAST) Gene Polymorphism with Weaning Weight and Ultrasonic Measurements of Loin Eye Muscle in Kıvırcık Lambs [1]

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Summary

This study was to investigate the association of Calpastatin (CAST) gene with carcass quality characteristics in Kıvırcık lambs, which are important in Turkey in terms of meat production and quality. It was found that allele M of Calpastatin locus was the most common allele. MM, MN and NN genotype frequencies were 72.91%, 22.66% and 4.43%, respectively. This SNP was associated with backfat thickness and skin+backfat thickness values of loin eye muscle (*Musculus longissimus thoracis et lumborum*-MLD) and average daily gain (P<0.05). Live weight, average daily gain, backfat thickness and skin+backfat thickness mean values were found to be lower in animals with NN genotype when compared to those with MM and MN genotype. The results showed that Calpastatin gene affected back fat and skin+backfat and that they had less fatty carcass than those with NN genotype.

Keywords: *Kıvırcık, CAST, Ultrasonic measurement, MLD, Weaning weight*

Kıvırcık Kuzularda Calpastatin Gen Polimorfizmi ve Sütten Kesim Ağırlığı ve Ultrasonik Göz Kası Ölçümleri İle İlişkisi

Özet

Bu çalışmada Türkiye'de önemli bir et tipi koyun olan Kıvırcık ırkı kuzularda karkas kalite karakteristikleri ile Calpastatin geninin ilişkisi araştırılmıştır. Kıvırcık kuzularda Calpastatin lokusunun M allelinin en yaygın allel olduğu tespit edilmiştir. MM, MN ve NN genotipleri için genotip frekansları sırasıyla %72.91, %22.66 ve %4.43 olarak bulunmuştur. Bu tek nokta mutasyonunun (SNP) bel gözü kasına (*Musculus longissimus thoracis et lumborum*-MLD) ait yağ kalınlığı ve deri+yağ kalınlığı değerleri ve günlük canlı ağırlık artışı ile ilişkili olduğu ortaya konmuştur (P<0.05). Canlı ağırlık, ortalama günlük canlı ağırlık artışı, yağ kalınlığı ve deri+yağ kalınlığı ortalamaları NN genotipine sahip hayvanlarda MM ve MN genotipine göre daha düşük bulunmuştur. Elde edilen bulgular yağ kalınlığı ve deri+yağ kalınlığı üzerine Calpastatin allelerinin etkili olduğunu ve NN genotipi taşıyanların diğerlerine göre daha yağsız karkasa sahip olduğunu göstermektedir.

Anahtar sözcükler: *Kıvırcık, CAST, Ultrasonik ölçüm, MLD, Sütten kesim ağırlığı*

INTRODUCTION

Kıvırcık lamb is known for its meat quality in Turkey. Although there is a limited body of research to scientifically support this information, in a study carried out by Ekiz et al.[1], Turkish Merino, Ramlıç, Kıvırcık, Sakız and Imroz breeds were compared in terms of meat quality. The findings of the study revealed that Kıvırcık breed was superior to other breeds in terms of various meat quality characteristics.

Today lean meat is highly demanded in the market. For this reason, ultrasound technology that is developed to identify carcass condition, in other words, carcass composition and quality in animals that will be marketed, allows for the identification of carcass characteristics in live animals in a rapid and economic manner without giving the animal any harm. Ultrasound measurements in live animals have a practical value to provide selection of

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certain carcass characteristics according to measurement criteria for breeding and to estimate the optimum slaughter or marketing period [2-6].

In recent years certain highly effective genes that affect meat yield and quality were identified. Calpastatin gene is one of these genes. Calpastatin (CAST), is the endogenous and specific inhibitor of Calpains, inhibits the calpain activity in post-mortem tissue and thus regulates the rate and extent of post-mortem meat tenderization. Therefore, CAST might be a potential candidate gene to control the development of farm animals [7]. This gene is located on the fifth chromosome of sheep genome. Calpastatin and Calpain were shown to have significant effects on live weight and meat quality. CAST gene was identified in sheep genome for the first time by Palmer et al.[8]. The study based on PCR-RFLP found that Dorset sheep had two different alleles (M and N) of CAST gene. Previous studies reported that Calpastatin gene affected growth characteristics and meat hardness [9-12].

This study analyzed the effect of CAST gene on live weight, average daily gain and some ultrasonic measurements in loin eye muscle *(Musculus longissimus thoracis et lumborum-MLD)* (backfat thickness, skin+backfat thickness, muscle depth, muscle width). A review of the literature was found no study to associate Calpastatin gene with carcass quality characteristics in Turkish sheep breeds. This study will significantly contribute to genomic selection works.

MATERIAL and METHODS

All procedures were approved by local ethical committee of Adnan Menderes University (124-HEK/2009/53 Date: 02.09.2009).

The animal population was consisted Kıvırcık lambs (n=203) in 10 integrated flocks within the scope of TÜBİTAK-KAMAG 1007 project (Project No. 109G014). Sampling location and sample size of Kıvırcık lambs are given in *Table 1*.

Blood samples were collected from the animals into vacuumed tubes containing K3-EDTA. The samples were stored at -20°C until utilization. The DNA was isolated with DNA isolation kit (Applied Biological Materials Column-Pure Blood Genomic DNA Kit, Canada) from blood samples according to manufacturer's instructions in the Adnan Menderes University Faculty of Agriculture Department of Animal Science, Genetic Labaratory, Aydın. Quantity and quality of the DNA were checked with NanoDrop 2000 spectro-photometer (Thermo Scientific, USA).

Genotypes were identified in terms of Calpastatin gene using the Restriction Fragment-Length Polymorphism (PCR-RFLP) method and employing primer pair reported by Khederzadeh [13] (CAST F: 5'-CCTTGTCATCAGACTTCACC-3', CAST R: 5'-ACT GAG CTT TTA AAG CCT CT-3'). A PCR mixture containing dNTP (0.2 mM), $MgCl_2$ (2.0 mM), primers (0.25 µM), PCR buffer (1X) and Taq DNA polymerase and 100 genomic DNA and ddH_2O with a total volume of 25 µl was prepared for Polymerase Chain Reaction.

Table 1. *Sampling location and sample sizes for Kıvırcık lambs*
Tablo 1. *Kıvırcık kuzularda örnekleme lokasyonları ve örnek sayıları*

Farm ID	Location	N
1	Kıran/Eşme/Uşak	28
2	Ahmetler/Eşme/Uşak	23
3	Yeleğen/Eşme/Uşak	17
4	Yeleğen/Eşme/Uşak	30
5	Ahmetler/Eşme/Uşak	30
6	Ahmetler/Eşme/Uşak	15
7	Güllübağ/Eşme/Uşak	13
8	Yeleğen/Eşme/Uşak	16
9	Oymalı/Eşme/Uşak	16
10	Güllübağ/Eşme/Uşak	15
Total		**203**

The PCR cycling condition was a preliminary denaturizing at 95°C for 2 min, followed by 1 cycle, denaturing at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 2 min followed by 35 cycles and 10 min at 72°C as a final extension. The PCR reactions were performed on the ABI Veriti thermocyler. The corresponding PCR products were amplified 565 bp fragments.

Amplified DNA regions were digested with *Msp*I restriction enzyme (Fermentas) for genotyping. For restriction digestion, 3 µl of 10X Buffer Tango, 0.50 µl of ddH_2O and 1.50 µl of *Msp*I (Fermantas) enzyme were added to the PCR products (25 µl) and this mix were incubated at 37°C for at least 6 h. DNA fragments were separated in 2% agarose gel. The fragments were imaged and genotypes were identified.

The lambs were monitored form the time of birth to the time of weaning (mean age 3.5 months). Live weight of the lambs were determined using electronic scale with a sensitivity of 50 g in the time of marketing and average daily gain increase was calculated until the time of birth to the time of weaning. Measurements on the characteristics of MLD were conducted on the area between 12. and 13. ribs using a linear probe (8 MHz) with a scanning area of 6 cm in an ultrasound device (Pie Medical Falco 100). The characteristics of MLD were determined to be backfat thickness (BFT), skin+backfat thickness (S+BFT), muscle depth (MD) and muscle width (MW) *(Fig. 1)*.

Allele and genotype frequency analysis and chi-square ($\chi 2$) test were carried out using GenAlEx [14] and Popgene32 [15]

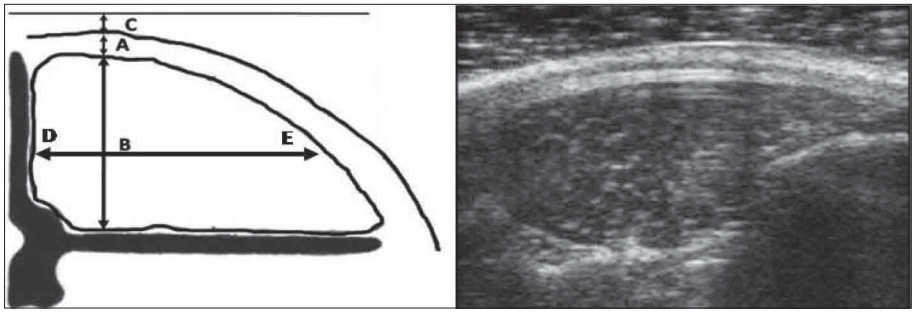

Fig 1. Measured properties belong to MLD (A: Backfat thickness, B: Muscle depth, A+C: Skin + Backfat thickness, D-E: Muscle width) and ultrasound imaging of MLD

Şekil 1. Ölçülen MLD kasına ait özellikler (A: Yağ Kalınlığı, B: Kas Derinliği, A+C: Deri+Yağ Kalınlığı, D-E: Kas Genişliği) ve ultrason görüntüsü

programs. Analysis of variance was conducted to investigate effects of genotypes on Weining Weight (WW), Avarage Daily Gain (ADG), Backfat Thickness (BFT), Skin with Backfat Thickness (S+BFT), Muscle Depth (MD) and Muscle Width (MW). Least Squares means and standart errors estimated using the GLM (Generalized Linear Models) procedure of SAS [16] according to followning linear models:

Model for weaning weights of lambs:

$$y_{ijkl}=\mu+a_i+b_j+c_k+b_1(X_{ijkl}-X)+e_{ijkl}$$

Model for ultrasonic measurements

$$y_{ijkl}=\mu+a_i+b_j+c_k+b_1(Q_{ijkl}-\bar{Q})+e_{ijkl}$$

Where

a_i = Fixed effect of genotype (i=MM, MN and NN)

b_j = Fixed effect of bith type (j=single, twin and triplets)

c_k = Fixed effect of gender (k=male and female)

b_1 = Regression coefficient of lamb age at weaining on weaning weight

b_2 = Regression coefficient of weaning weight on ultrasonic measurements

X_{ijkl} = Age of lamb at weaning

$\bar{X}$ = Mean lamb age at weaning

Q_{ijkl} = Weaning weight of lamb

$\bar{Q}$ = Mean weaning weight of lambs

e_{ijkl} = Random errors with the assumption of N (0, σ^2)

RESULTS

The amplified region of calpastatin gene produced a 565 bp DNA fragment using MspI enzyme in RFLP method. DNA bands obtained from PCR-RFLP were imaged by separation in 2% agarose gel *(Fig. 2)*. Two alleles (M and N) and subsequently three genotypes observed. Bands with different lengths produced as a result of a single point mutation (CCGG → CCAG) in the calpastatin gene, which removes the MspI restriction cut site (...C▼CGG...). The cut area disappeared and no cut took place due to point mutation that occurred in cut region of the enzyme. Thus, in individuals with this situation, only a single band at 565 base pairs was observed and these individuals were genotyped as NN. Due to a mutation only in one of the alleles the individuals showing three bands at the length of 565, 306 and 259 base pairs were genotypes as MN, while the individuals showing two bands at the length of 306 and 259 were genotypes as MM.

Allele frequencies, genotype frequencies, observed (Ho) and expected heterozygosity (He) values obtained from the study and the results of Chi-square test performed for Hardy-Weinberg equilibrium are presented in *Table 2*.

The frequency of alleles M and N in Kıvırcık lambs were found to be 84.24% and 15.76% respectively. This result indicated that allele M was more common in the populations. Although all three of MM, MN and NN genotypes were observed in Kıvırcık breed. It was found that MM genotypes had the highest genotype frequency (72.91%). Observed heterozygosity (Ho) and expected heterozygosity (He) values of Calpastatin gene were found to be 0.227 and 0.266 respectively. Furthermore, it

Fig 2. Genotyping for CAST gene by the PCR-RFLP analysis

Şekil 2. PCR-RFLP yöntemi ile CAST geninin genotiplenmesi

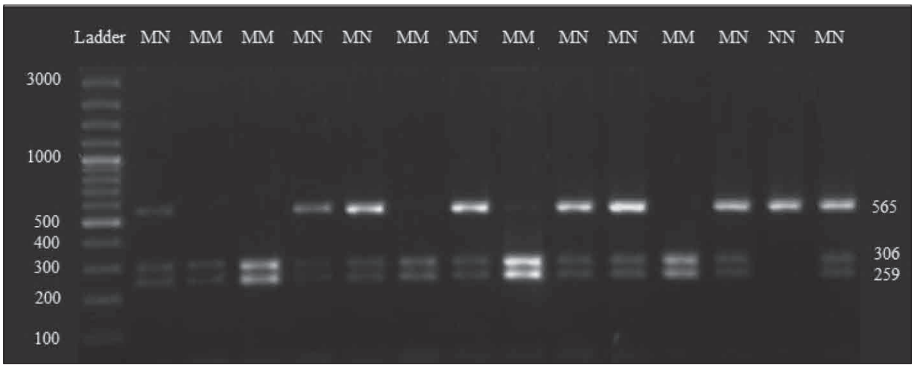

Table 2. *Allele and genotype frequencies, observed (Ho) and expected heterozygosity (He) and Chi-Square test values for the Hardy Weinberg equilibrium belong to Calpastatin locus*

Tablo 2. *Calpastatin lokusuna ait allel ve genotip frekansları, gözlenen (Ho) ve beklenen heterozigotluk (He) ve Hardy Weinberg dengesi için Ki-kare test değerleri*

Locus	N	Allele Freq (%)		Genotype Freq (%)			Heterozygosity		
		M	N	MM	MN	NN	Ho	He	χ^2
CAST	203	84.24	15.76	72.91	22.66	4.43	0.227	0.266	4.372*

** P<0.05*

Table 3. *Least square means and standard errors for ultrasound measurements of MLD*

Tablo 3. *MLD kasının ultrasonik ölçümlerine ait en küçük kareler ortalamaları ve standart hataları*

Genotype	N	WW (kg)	ADG (kg)	BFT (cm)	S+BFT (cm)	MD (cm)	MW (cm)
		P=0.595	P=0.036	P=0.022	P=0.031	P=0.270	P=0.266
MM	148	25.52±0.502	0.22±0.002	0.21±0.007	0.54±0.010	1.75±0.020	3.63±0.031
MN	46	25.94±0.765	0.22±0.004	0.22±0.011	0.56±0.015	1.80±0.030	3.71±0.047
NN	9	24.13±1.673	0.20±0.008	0.15±0.024	0.46±0.032	1.75±0.065	3.66±0.101
BirthType		P=0.032	P=0.008	P=0.487	P=0.270	P=0.080	P=0.760
1	84	26.51±0.747	0.21±0.004	0.18±0.011	0.51±0.014	1.79±0.029	3.65±0.046
2	100	24.69±0.716	0.20±0.003	0.19±0.01	0.51±0.013	1.73±0.027	3.68±0.042
3>	19	24.40±1.214	0.22±0.006	0.21±0.018	0.54±0.023	1.78±0.048	3.67±0.074
Gender		P=0.000	P=0.199	P=0.838	P=0.108	P=0.014	P=0.403
Male	112	26.43±0.740	0.21±0.004	0.19±0.011	0.51±0.014	1.73±0.029	3.65±0.045
Female	91	23.97±0.766	0.21±0.004	0.20±0.011	0.53±0.015	1.80±0.030	3.68±0.047
Reg. Linear			P=0.000	P=0.000	P=0.000	P=0.000	P=0.000
WW		-	0.007±0.000	0.008±0.001	0.011±0.001	0.024±0.002	0.025±0.004
		P=0.000					
Age		0.204±0.025					
General	203	25.20±0.669	0.21±0.003	0.19±0.01	0.52±0.013	1.77±0.026	3.67±0.041

WW: *Weaning weight,* ***ADG:*** *The average daily gain,* ***BFT:*** *backfat thickness,* ***S+BFT:*** *Skin with backfat thickness,* ***MD:*** *Muscle depth:* ***MW:*** *Muscle width*

was observed that the population which was analyzed in terms of this gene was not at Hardy-Weinberg equilibrium. Least squares mean and standard errors obtained from marketing and weaning live weight, average daily gain and ultrasonic measurements of MLD are presented in *Table 3*.

It was found that there was a statistically significant difference (P<0.05) between the genotypes in terms of average daily gain (ADG) and backfat thickness (BFT), skin+backfat thickness (S+BFT) values among ultrasound criteria. There was no statistically significant difference between the genotypes for WW, MD and MW.

There was a statistically significant difference between types of birth in terms of weaning weight (WW) (P<0.05) and ADG (P<0.01). However, there was no statistically significant difference in terms of other characteristics (P>0.05). It was found that lamb age, which is considered as a covariate had a significant effect on lamb weaning weight (P<0.01). Lamb weaning weight had a significant effect on ultrasound measurement parameters.

DISCUSSION

It was found that birth type and sex had a significant effect on weaning weight. Only birth type was found to have a significant effect on live weight gain. Gender had a statistically significant effect on muscle depth. These results are consistent with literature data [3,17].

Our findings showed that allele N had rather low frequencies while allele M had high frequencies. It was observed that 15.76% value we obtained for allele N was slightly lower than the values obtained in Atabi (19%), Kajli (19%), Mutton (19%), Dalagh (20%), Karakul (21%), Polish Merino (24%), Lori (36%) and Zel (25%) breeds [13,18-23]. However, this value was higher than the values obtained from Valachian (3%), Ile de France (5%), Berrichondu Cher (7%), Tsigai (9%), Tsigai x Lacaune (10%), Thalli (10%), Balkhi (12%), Lohi (13%), Kajli (14%) and Arabic (15%) sheep [9,22-25]. On the other hand, in a study carried out on Lacaune and Eastern Friz breed, no allele N was found [24].

Analysis of the results on genotypes showed that NN genotypes had rather low frequencies while MM genotype was more common in populations. In a study carried by Ata and Cemal [26] which analyzed Calpastatin gene polymorphism in Çine Çaparı and Karya breeds, the frequency of MM, MN and NN genotypes were found to be 0.543, 0.388 and 0.069 in Çine Çaparı and 0.296, 0.496 and 0.208 in Karya sheep respectively. Allele and genotype frequencies we obtained showed slight variations when compared to other studies. These variations can be attributed to the use of different breeds in the studies.

Our findings raise suspicion that there is a selection process against NN genotype. Considering this situation, the fact that this population is not at Hardy-Weinberg equilibrium in terms of Calpastatin gene appears as a natural result. Least squares means of ADG, BFT and S+BFT values revealed that the animals with NN genotype had lower values than other genotypes. This appears as a concrete indicator of the process against NN genotype.

Research in this subject mainly concentrated on identification of Calpastatin gene in populations. A review of the literature also found studies associating the gene with phenotypic characteristics [25]. Those studies reported that Calpastatin gene affected live weight and average daily gain values [25,27]. The results of previous studies on live weight and average daily gain showed that the distinction between the genotypes was statistically different and that the animals with MM and MN genotype showed a better performance than those having NN genotype. Studies on other breeds reported that there was a statistically significant difference between Calpastatin genotypes in terms of live weight, weaning weight and average daily gain [25,27,28]. Similarly, our results are consistent with the findings in the study of Sutikno et al.[28] on Indonesia local sheep breed. However, in a study carried out on Romney sheep, it was reported that Calpastatin gene did not affect average daily gain [7].

At physiologic level, Calpastatin gene is an endogenous inhibitor of calpains. Page et al.[29] reported that calpains played an initial role on hardness degree of meat during rigor mortis after the slaughter by destructing myofibrillary proteins. Various researchers who studied meat hardness quality especially in cattle, analyzed physiologic role of Calpastatin on meat hardness together with Calpastatin gene and reported that Calpastatin gene affected the tenderness of meat [11,30-34].

According to the results from this study the investigated population showed a low degree of genetic variability in terms of Calpastatin alleles. This might be explained by only a few rams used as sires in the flock.

A review of literature found no study on the association between MLD characteristics and CAST gene in evaluated lambs. The results showed that Calpastatin alleles affected back fat and skin+backfat and that they had less fatty carcass than those with NN genotype.

There has no study that associated Calpastatin gene and yield characteristics in domestic sheep populations in Turkey. Therefore, this study will make a significant contribution to the literature. The study analyzed the effects of Calpastatin gene on weaning weight, average daily gain and MLD characteristics in Kıvırcık lambs which are important in Turkey in terms of meat quality.

Studies on Calpastatin gene using ultrasonic measurements, that allow for doing selection according to measurement criteria to breed carcass characteristics in live animals, will enhance accuracy of genetic parameters. In this context, the findings reveal that this gene can be an important major gene that can be used in selection programs for meat quality and that they can be reliably used in selection indexes.

Investigation of the region of this gene in a large material using DNA sequence analysis and association of the phenotype data of meat yield and quality characteristics of the emerging polymorphism will significantly contribute to future genomic selection studies.

Further studies on development characteristics, meat quality and genetic analyses in Kıvırcık breed, which are of great importance in terms of meat quality, will provide more concrete data on the functioning mechanism of this gene.

Acknowledgement

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RESEARCH ARTICLE

Survey for the Presence of *Mycobacterium avium* subsp. *paratuberculosis* in the Bull Frozen Semen Samples and Blood Samples of Cattle, Sheep and Camel by Nested-PCR

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Summary

Mycobacterium avium subsp. *paratuberculosis* (MAP) is an obligate, Gram positive, acid-fast bacterium in the genus *Mycobacterium*. It is the causative agent of Johne's disease in some animals and perhaps the human disease Crohn's disease. The aim of this study was to use nested- PCR as an exact and fast technique to trace MAP in cattle, camel and sheep from each samples. Blood samples were collected from 144 cattle, 110 sheep and 95 camels and 83 frozen semen samples were obtained from bulls were obtained and DNA was extracted. At that time, nested-PCR was performed by specific primers for IS900 gene of MAP. The PCR products with 230 bp length were estimated as a positive. The occurrence of MAP detected in 8 of the 83 (9.638%) frozen semen samples of bull's that used for artificial insemination, and was detected in 11 of the 144 (7.638%), 7 of the 95 (7.368%) and 16 of the 110 (14.545%) blood samples of cattle, camel and sheep in Iran, respectively. The results of this study were displayed nested-PCR is a good technique with high efficiency for detection of intracellular bacteria such as MAP in cattle, camel and sheep samples. Consequently, more attention to Johne's disease in cattle, camel and sheep to find MAP quickly is essential.

Keywords: *Blood Johne's disease, Mycobacterium avium subsp. paratuberculosis, Polymerase chain reaction (PCR), Ruminant, Semen*

Mycobacterium avium subsp. *paratuberculosis* Varlığının Dondurulmuş Boğa Semen Örneklerinde ve Sığır, Koyun ve Deve Kan Örneklerinde Nested-PCR İle Taranması

Özet

Mycobacterium avium subsp. *paratuberculosis* (MAP) *Mycobacterium* cinsi içinde zorunlu, Gram pozitif, aside dirençli bir bakteridir. Bu bazı hayvanlarda "Johne's" hastalığı ve belki de insanlardaki "Crohn's" hastalığı etkenidir. Bu çalışmada nested-PCR ile sığır, deve ve koyunların herbir örneğinde MAP'ın belirlenmesi amaçlanmıştır. Kan numuneleri 144 sığır, 110 koyun ve 95 deveden toplandı ve 83 dondurulmuş semen örneği boğalardan elde edildi, takiben DNA izolasyonu gerçekleştirildi. Bu sırada, nested-PCR MAP'ın IS900 geni için özel primerler ile gerçekleştirildi. İkiyüz otuz bp uzunluğuna sahip PCR ürünleri, pozitif olarak tahmin edildi. İran'da suni tohumlamada kullanılan boğalardan elde edilen 83 semen örneğinin 8'inde (%9.638) ve sığır, deve ve koyunlardan alınan sırasıyla 144'te 11 (%7.638), 95'te 7 (%7.368) ve 110'da 16 (%14.545) kan örneğinde MAP belirlendi. Bu çalışmanın sonuçları, nested-PCR'ın sığır, deve ve koyunlarda MAP gibi hücre içi bakterilerin belirlenmesinde yüksek verimlilikli iyi bir teknik olduğunu göstermektedir. Bu sonuçlar sığır, deve ve koyun "Johne's" hastalığında MAP'in hızlı teşhisi için daha fazla dikkatin gerekli olduğunu ortaya koymaktadır.

Anahtar sözcükler: *Kan, Johne's hastalığı, Mycobacterium avium subsp. paratuberculosis, Polimeraz zincir reaksiyonu (PCR), Ruminant, Semen*

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (*MAP*) is most common and economically significant disease of ruminants. It is the causative agent of paratuberculosis or Johne's disease, a gastro intestinal inflammatory disorder

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and a chronic granulomatous enteritis in ruminants such as cattle, goats, sheep, deer and camelids and otherwise animals, and too possibly the human disease Crohn's disease (CD) [1-5]. *MAP* is recognized as a multi-host mycobacterial microorganism by a longtime special skill to start and keep general and chronic infection of the intestine of a variety of histopathological types in many animals, with primates [6].

Though animals by clinical infection are commonly picked from the herd, animals via sub clinical paratuberculosis may make happen cost-effective losses since of poor reproductive and lessened milk production show [7]. Johne's disease causes foremost cost-effective losses to the dairy farm industries and farmers. The range of infection in a herd rises overly time and if the disease is left unmanaged, the economic result of bovine Johne's disease becomes more and more necessary [8]. The infection is dispersed global and Iran is one among those nations that are bare to the disease [9,10]. The results of *MAP* on dairy farm operations were estimated at nearby $200 to $250 million a year in the USA [11].

Rapid weight loss, a protein enteropathy and diarrhea are the three chief signs of Johne's disease. Diarrhea may be fewer common in certain species of animals like goats and sheep [4,12,13]. Generally, the larger the herd the more probably it is to have animals infected by Johne's disease. Later the long incubation period, the chief clinical sign seen in infected animals are plentiful, long-standing watery diarrhea, occasionally, noticeable weight loss and sporadic fever. Johne's infected cows go on to eat even by serious diarrhea. Typically has no mucous or blood in the diarrhea. Therefore the clinical signs of Johne's disease are generally vague and should be make happen via various extra agents. Repeatedly, even in severe clinical cases, Johne's disease is not known and the animals are primarily showed to slaughter without worrying for the primary reason for the disease. Inside any infected herd, single a few infected cows will have diarrhea at any one time [4,13]. The seroprevalence of Johne's disease in Georgia beef and dairy farm cull cattle in United States in 2000 was calculable and in dairy cattle was 9.58%, in beef cattle it was 3.95% and in cattle of unknown breed it was 4.72% [8].

Notwithstanding the development of contemporary techniques and methods for isolation and identification of Johne's disease, its management remains a serious veterinary problematic [14]. One of the difficulties associated by the disease is that signs seem recently and after a long time [15]. Furthermore, identification and isolation procedure is awkward, time consuming and may take several months [16]. Serologic tests, e.g. agar gel immunodiffusion (AGID) test, ELISA and fecal culture are suggested to approve the diagnosis of paratuberculosis in a clinically affected animal or in an infected herd. Indirect diagnostic techniques based on immunological methods for example complement fixation test, skin testing by john in, interferon test, and enzyme linked immunosorbent assay have shown low specificity or sensitivity specificity [17]. Serological methods could too be used to find an earlier exposure to the disease however not an active infection. Nucleic acid hybridization methods were useful effectively for the detection of the disease. Polymerase chain reaction (PCR) provides speedy, sensitive and specific detection for an early identification of the disease [18].

The infection is especially limited to the small intestinal tract and its draining lymph nodes. Infection may spread to more intestinal sites as shown via effective cultural isolation of the organism from milk, lung, semen and fetus. Other the intestinal tract, these other organs does not elicit a characteristic inflammatory reply to the presence of the organism [17].

In the past research describe the isolation of *MAP* from semen in rams and semen and male accessory genital organs in bulls. The usage of molecular techniques for the detection of *MAP* in milk and different matrices were made probably via the detection of specific DNA sequences, particularly IS900 [19,20], however whether semen can send the disease via uterus is not fully investigated [21]. The detection of the IS900 insertion sequence within the *MAP* genome has offered a unique for the fast detection of the bacterium DNA in clinical samples [22].

The aims of this study were to differentiate the possible of PCR for the rapid, to develop and to evaluate for detection of *M. avium* subsp. paratuberculosis in semen and blood samples from Iranian cattle, camel and sheep and also to determine the frequency of this infection in cattle, camel and sheep.

MATERIAL and METHODS

Sampling and DNA Isolation

In this study, a total number of 432 samples of cattle, camel and sheep, were collected randomly from 83 bulls frozen semen and 144 cattle, 95 camel blood and 110 sheep blood between January 2013 and March 2013. Samples are removed aseptically with sterile instruments. Semen and blood samples were sent to the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in a cooler with ice packs. Each of the specimens was stored -20°C for further use. Genomic DNA was extracted from specimens using DNA extraction kit (Qiagen, Germany), according to the manufacturer's protocol. The overall DNA was measured at 260 nm optical density according to the technique described by Sambrook and Russell [23]. The extracted DNA of each sample were kept frozen (−20°C) until analysis.

Gene Amplification

The PCR amplification was done with two sets of

primers: the outer oligonucleotide primers were ISo-1 F: 5´- GTTCGGGGCCGTCGCTTAGG-3´ and ISo-1R: 5´GAGGTCG ATCGATCGCCCACGTGA-3´ and the inner oligonucleotide primers were ISi-2F: 5´- CCGCTAATTGAGAGATGCGAT TGG-3´andISi-2R:5´-AATCAACTCCAGCAGCGCGGCCTCG-3´. The target sequence was amplified in a 50 µl reaction volume containing 100 ng of genomic DNA, 0.2 mM dNTPs, 1X Taq buffer, 2 mM $MgCl_2$, 100 ng of each primer and 1 unit of Taq DNA polymerase (Fermentas, Germany).

The first round of PCR was performed in a DNA thermal cycler at a denaturation temperature of: first denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a last extension for 5 min at 72°C. Two to five µl from the first round amplicon was used as a template for the second round PCR with the same PCR program by inner oligonucleotide primers.

Analysis of PCR Products

Amplified samples were studied by electrophoresis (120 V/208 mA) in 1.5% agarose gel. Negative and positive PCR controls were run with each series of amplifications. The gel was stained by 0.1% ethidium bromide (0.4 g/mL) and were images obtained in UVIdoc gel documentation systems (Uvitec, UK).

In the current study, 83 frozen semen specimens of bulls and 349 blood samples of cattle, camel and sheep were tested for *MAP* by a nested PCR assay. The nested PCR assay used in current study enabled the detection of IS900 gene of *MAP*. Nested PCR amplification of the *MAP* in specific insertion sequence IS900 and later agarose gel analysis of the amplified products showed a single band of 230 bp for the positive samples *(Fig. 1)*.

Statistical Analysis

Data were analyzed by using SPSS (version 15) software.

RESULTS

The presence of *MAP* DNA was detected by nested PCR in each samples were from 42 out of 432 samplings (9.722%). *MAP* infection was detected 11 of the 144 (7.638%) blood samples of cattle, 16 out of the 110 (14.545%) sheep blood samples and 7 out of the 95 (7.368%) camel blood samples were positive for the *MAP* DNA and 8 out of 83 (9.638%) bulls frozen semen samples are infected. The Chaharmahal va Bakhtiary province had the highest prevalence of *MAP* in cattle (8.695%) while The Isfahan province had the lowest prevalence of *MAP* in cattle (5.769%). The Chaharmahal va Bakhtiary province had the lowest prevalence of *MAP* in sheep (13.235%) while The Isfahan province had the highest prevalence of *MAP* in sheep (16.666%). Gender wise differences out of the 25 number positive were females and 17 numbers positive were males. Sheep is a highest occurrence of *MAP* while, camel is a lowest occurrence of *MAP*. These finding suggested that control and eradication programs for *MAP* infection it seems to be necessary in Iranian cattle, camel and sheep. The results of the prevalence of *MAP* infection in cattle, camel and sheep are shown in *Table 1*.

DISCUSSION

MAP is a microorganism that is the cause of Johne's disease, was isolated from the feces of a giver bull in an artificial insemination stud [19]. *MAP* can alive in animals for years lacking inevitably inflicting clinical disease [24]. *MAP* may have a role within the development of Crohn's disease in humans via the consumption of contaminated milk and milk products [9,25]. Milk and milk products resulting from cows via clinical or different suspected para-tuberculosis are not usable even later pasteurization [24]. The signs of this infection in human are bowel disease and a chronic inflammatory that may be severe, extended and

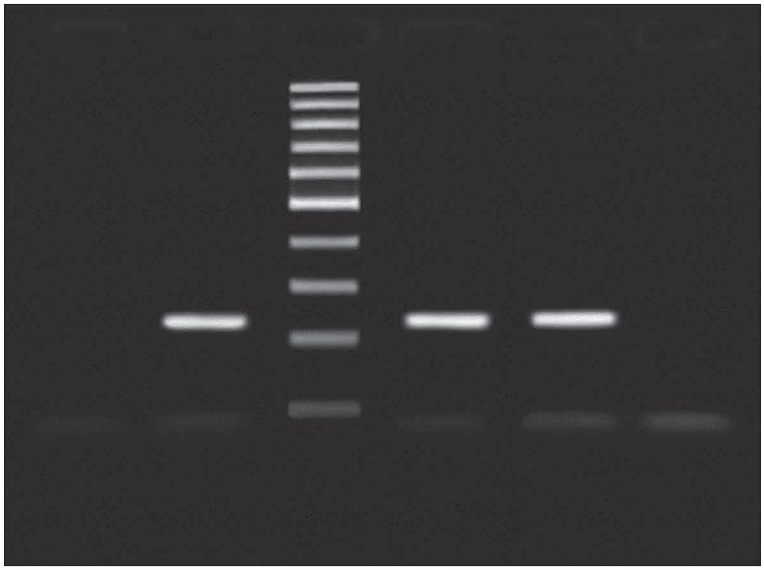

Fig 1. Agarose gel electrophoresis of PCR products (230 bp) for detection of *MAP* DNA in samples after PCR amplification. Lane 3: 100 bp DNA ladder (Fermentas, Germany); lanes 2, 4 and 5: positive samples (230 bp); lanes 1, 6: negative samples

Şekil 1. Örneklerde *MAP* DNA tespiti için PCR ürünlerinin (230 bp) agaroz jel elektroforezi. Şerit 3: 100 bp DNA merdiveni (Fermentas, Almanya); Şeritler 2, 4 ve 5: pozitif örnekler (230 bp); Şerit 1, 6: negatif örnekler

Table 1. *The results of the prevalence of MAP in each specimens*
Tablo 1. *Her bir örnekte MAP yaygınlığının sonuçları*

Variables	Species							
	Blood of Cattle		Blood of Camel		Blood of Sheep		Frozen Semen of Bull	
	N	Prevalence: Positive Samples (n) (%)	n	Prevalence: Positive Samples (n) (%)	n	Prevalence: Positive Samples (n) (%)	n	Prevalence: Positive Samples (n) (%)
Region								
Chaharmahal va Bakhtiary	92	8 (8.695%)	0	0 (0%)	68	9 (13.235%)	Unknown	Unknown
Isfahan	52	3 (5.769%)	95	7 (7.368%)	42	7 (16.666%)	Unknown	Unknown
Sex								
Female	108	9 (8.333%)	28	4 (14.285%)	75	12 (16%)	0	0 (0%)
Male	36	2 (5.555%)	67	3 (4.447%)	35	4 (11.428%)	83	8 (9.638%)
Total	144	11 (7.638%)	95	7 (7.368%)	110	16 (14.545%)	83	8 (9.638%)

incapacitating [3,7]. The identification of Johne's disease is very difficult, especially within the primary phases. This is payable to the long incubation period, the variable log phase associated via bacterial proliferation, and the multifocal distribution of bit via bit developing lesions [26].

There are 2 techniques for transmission of *MAP* is considered and contain direct faecal-oral cycle and indirect transmission, for instance through manure contamination of machinery used for feed sending and water bowls [9]. The diagnostic techniques contain bacteriological cultures and serological. Highest in number of the serological reactions quickly detect antibodies to *MAP* nevertheless not active infections [14].

Improvement diagnostic techniques are presently used in several research laboratories all over the globe. These techniques are based on molecular characterization of the specific microorganism isolates. These diagnostic exams use DNA probes, Restriction Fragment Length Polymorphisms (RFLPS), cloning and PCR [14,18].

PCR based on IS900 has been used for direct detection of *MAP*, lacking primary culture, from faecal specimens, milk, semen and human intestinal tissue and workers have been able to detect the presence of paratuberculosis DNA in intestinal tissue from patients by Crohn's disease and semen samples from bulls. For the goal that the clinical symptoms of Crohn's disease carefully imitator those found in animals via Johne's disease [3,9,27]. A sensitive and quick PCR-exam would help to check *MAP* in semen prior to artificial insemination [17,28], to induce high responsiveness of detection of *MAP* or different pathogens via IMS-PCR on faeces and milk [29-31].

Numerous researches have been focused on the association of Crohn's disease by *MAP*. Publications dealing by the culture detection of *MAP* in milk and milk products have too been increasing in number over the last ten years [13,32]. Larsen et al.[19] detached these microorganisms from the semen and genital organs of bulls, and showed eight of thirty one semen samples are infected. There have been data of detection of *Map* in the tissues [33-36], blood [37] of patients with CD or irritable bowel syndrome extra regularly than in control patients. The occurrence of *MAP* in bulk-milk samples using tracing of IS900 gene in Switzerland was 19.7% and indicated *MAP* can so often be transferred to humans via raw milk consumption [38]. These results largely identical to the outcomes of current study. Claus et al.[17] showed that *M. avium* subsp. paratuberculosis may give hematogenously to the semen and male reproductive tract as an extra intestinal place and agent reservoir.

Map was detected via culture in the intestinal lymph nodes or faeces of 34% of healthy dairy cows and 3% of healthy beef cows obtainable for slaughter [39]. *Map* was too cultured from the liver of 11.1% of the dairy cows and 0.7% of the beef cows tested [40].

Nebbia et al.[4] done molecular identification route for detection of mycobacterium in sheep and goat milk. *MAP* was especially found in 4 out of 14 seronegative and 9 out of 15 seropositive animals. Moreover, *MAP* DNA was sporadically recovered in milk samples from 13 out of 29 animals. A study of 200 retail ground beef samples were check in the USA via Jaravata et al.[41] and *Map* was not detected by PCR (IS900) and conventional culture techniques. In an examination of 133 minced beef samples found from a meat processing plant in the Republic of Ireland, no practical *Map* was detected [42].

Map-contaminated water and contact via infected animals or people have been recommended as vehicles of transmission to humans [43]. *Map* DNA was detected in 15% of the Canadian samples [44] and 9.8% of Irish pasteurised milk samples [45]. *Map* was detected in 11.6% of the pasteurized milk samples in a UK study [46]. Even though most studies have attentive on cattle milk, *Map* DNA has been detected in raw goats and sheep milk [47]. Researches

in India displayed high prevalence of *MAP* in many animals; for example: domestic (cattle, goats, buffaloes and) and wild (blue-bulls, hog deer, bison, etc) ruminants, other animals (camels, rabbits, etc), primates and human beings [48].

The results of present study show that the each samples in cattle, camel and sheep served as a reservoir of disease in Iran. Consequently it could be stated that the animal reservoirs increase the risk of the potential spread of disease to other animals and specially humans, and this deserves special attention.

In conclusion, the results presented high occurrence of *MAP* infection in cattle, camel and sheep samples and suggested that control and eradication programs for prevent and cut of economic loses of *MAP* infection it seems to be necessary. Study in order to knowledgeable about the physiological ecology of mycobacteria in the environment and wildlife is necessary to fully find out the effects that mycobacteria have on human health and to agree to new methods for controlling of their environmental and wildlife reservoirs. It is important to buy bulls, their semen and replacement heifers only from farms/regions which are declared paratuberculosis free. Evading several source of infection from manure contaminated feed, water, soil and a full comprehending of potential reservoirs in wild is significant in developing an effective control programme. Though it is not known whether *M. paratuberculosis* causes disease in populate, livestock diseases that are transmissible to human beings are presently affecting the confidence of consumers principally. So paratuberculosis infection in food animals should be controlled as a safety measures. Furthermore, the results of the current study suggest that PCR was highly sensitive and specific for identification and differentiation of *MAP* and that it could be a suitable tool for diagnosis of *MAP*. Thus, it is essential to screen in all area regularly to prevent the spread of the disease and laboratory support is a significant tool in the identification of the disease. Seemingly, PCR is one of the best ways to detect and characterize *MAP* as fast, less hazardous and sensitive method.

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RESEARCH ARTICLE

Karaciğer ve Dalak Laserasyonları İle Birlikte Bulunan İntraperitoneal Mesane Rüptürünün Ayırıcı Tanısında Intravenöz Fluoresceinin Etkinliğinin Araştırılması: Tavşan Modelinde Deneysel Bir Çalışma

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Özet

Bu çalışmada mesane rüptürü ile birlikte karaciğer ve dalak laserasyonu oluşturulan tavşanlara intravenöz yolla fluorescein verilerek parasentez yöntemiyle mesane rüptürünün tanısının ve ayırıcı tanısının saptanması amaçlandı. Çalışmada 24 adet erkek New Zealand ırkı tavşan kullanıldı. Tavşanlar 8'erli 3 gruba ayrıldı. Grup 1'e iatojenik mesane perforasyonu oluşturulan tavşanlar, Grup 2'ye iatrojenik karaciğer ve dalak laserasyonu oluşturulan tavşanlar ve Grup 3'e ise iatrojenik mesane rüptürü, karaciğer ve dalak laserasyonu oluşturulan tavşanlar dahil edildi. Tüm tavşanlara vena auricularis magnadan intravenöz yolla fluorescein verilerek, rüptüre mesane içerisindeki fluoresceinin karın boşluğuna karışıp karışmadığı parasentez yöntemiyle araştırıldı. Her 3 gruptaki abdominal boşluğa birikmiş fluoresceinli mayilerin renk tonları ayrı ayrı gözlemlendi. Parasentez sıvısında çıplak gözle fluorescein gözleniyorsa F (+), gözlenmiyorsa F (-) olarak kaydedildi. Grup 1'de yapılan sistoskopik muayenede, mesane rüptürü sonrasında fluorescein ile boyanmış idrarın abdominal boşluğa geçtiği gözlendi. Eşzamanlı yapılan parasentezde, Grup 1'deki fluoresceinli idrarın enjektör içerisine geldiği görüldü. Grup 2'de karaciğer ve dalak laserasyonu sonrasında, fluoresceinin abdominal boşluktaki organları boyadığı gözlendi. Çıplak gözle hemorajik mayinin fluorescein ile boyanması net olarak fark edilemedi. Grup 3'e yapılan işlemler sonrasında, hemorajik ve idrar ile karışmış fluorescein net olarak belirlenebildi. Bu çalışma ile intraperitoneal mesane rüptürlerinde intravenöz yolla fluorescein uygulamasının pratikte tanısal bir değere sahip olduğu belirlenmiştir.

Anahtar sözcükler: *Fluorescein, İntraperitoneal mesane rüptürü, Ekstraperitoneal mesane rüptürü, Tavşan, Karaciğer laserasyonu, Dalak laserasyonu, Abdominal travma*

Investigation of Efficacy of Intravenous Fluorescein in the Differential Diagnosis of Intraperitoneal Bladder Rupture with Liver and Spleen Lacerations: An Experimental Study on Rabbit Model

Summary

To evaluate the efficacy of fluorescein for the differential diagnosis of bladder ruptures by parasynthesis after intravenous administration of fluorescein in rabbits with bladder ruptures, liver and spleen lacerations. A total of 24 male New Zealand rabbits were used. They were divided into 3 groups which involved 8 rabbits. Group 1 involved rabbits with iatrogenic bladder ruptures, Group 2 involved rabbits with iatrogenic liver and spleen lacerations and Group 3 involved rabbits with bladder ruptures, liver and spleen lacerations together. We administered IV fluorescein to all rabbits via vena auricularis manga and investigated the presence of fluorescein in the peritoneum by parasynthesis. All the collected fluorescein in the peritoneum of 3 groups were recorded according to the colours. If parasynthesis fluid was observed with naked eye, it was recorded as F (+); if not, it was recorded as F(-). Cystoscopy of Group 1 revealed that urine coloured with fluorescein passed into the peritoneum after bladder rupture. In parasynthesis which was performed simultaneously, in Group 1 urine coloured with fluorescein was aspirated into the injector. In Group 2, it was observed that fluorescein coloured abdominal organs after liver and spleen laceration; but the hemoragic fluid colured with fluorescein could not be clearly observed with naked eye. Fluorescein that mixed with hemoragic fluid and urine could easily be seen with naked eye in Group 3 after interventions. This study revealed that intavenous administration of fluorescein can be used for diagnosis of intraperitoneal ruptures in daily practice.

Keywords: *Fluorescein, Intraperitoneal bladder rupture, Extraperitoneal bladder rupture, Rabbit, Liver injuries, Spleen injuries, Abdominal trauma*

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GİRİŞ

Mesane abdominal boşlukta yer aldığından, genellikle travmalara karşı iyi korunur. Mesane yaralanmaları küt yaralanmalara ve düşmelere bağlı olabileceği gibi, en çok motorlu araç çarpışmalarında hızlı deselarasyona bağlı olarak şekillenebilir. Diğer önemli sebepler ise, penetran yaralanmalar, iatrojenik cerrahi yaralanmalardır [1]. Ayrıca, pelvis kırıklarına bağlı olarak kırılan kemik parçaları mesane rüptürüne neden olabilir.

Mesane yaralanması 2 şekilde olabilir: İntraperitoneal veya ekstraperitoneal. İntraperitoneal yaralanmalarda idrar sadece mesaneyi örten visseral periton içerisine birikirken, ekstraperitoneal yaralanmalarda ise visseral periton içinde idrar birikimi gözlenmez. İdrarın mesane ve visseral periton arasında toplandığı belirlenir. Mesane yaralanmasının teşhisinde opak madde ile retrograd sistografi yöntemleri tanıyı yüksek oranda kesinleştirir. Ancak, bilgisayarlı tomografinin (BT) kullanıma girmesiyle BT retrograd sistografi günümüzde tanı amacıyla daha çok kullanılan bir yöntem olmuş ve tanıda daha başarılı sonuçlar vermiştir [1].

Günümüzde fundus floresan anjiografisinde rutin olarak kullanılan fluorescein 376.67 molekül ağırlıklı organik bir boyadır. Zayıf dibazik bir asittir. Genellikle bu asitin sodyum tuzu kullanılır. Kendisi ile eşit ağırlıktaki sodyum fluorescein, kristal tabiatta olup, aköz solüsyonunda sarı kırmızı yeşil bir renk alır. Bu boya maddesi floresans özellikleri gösterip, absorbe ettiği ışığı %100 oranında floresans ışığa çevirir. Moleküler ağırlığı düşük olup, vücut sıvılarına difüzyonu çok hızlıdır. İntravenöz olarak verildiğinde, fluorescein %60-80 oranında plazma proteinlerine özellikle de albümine bağlanırken %20'si serbest olarak dolaşır. Uygulama dozu genelde 5 ml %10'luk konsantrasyonda olacak şekildedir [2].

Bu çalışmada mesane rüptürü ile birlikte karaciğer ve dalak laserasyonu oluşturulan tavşanlarda intravenöz yolla fluorescein verildikten sonra parasentez yöntemiyle mesane rüptürü tanısının koyulması ve ayırıcı tanısının yapılması amaçlanmıştır.

MATERYAL ve METOT

Çalışma, Kafkas Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'ndan onay alınarak yürütüldü (Onay No: KAÜ-HADYEK 2012-38). Çalışmamızda 24 adet ağırlıkları 1.600 ile 2.100 g arasında değişen erişkin, erkek New Zealand ırkı tavşan kullanıldı. Tavşanlar 8'erli 3 gruba ayrıldı. Grup 1'e iatrojenik mesane perforasyonu oluşturulan tavşanlar, Grup 2'ye iatrojenik karaciğer ve dalak laserasyonu oluşturulan tavşanlar ve Grup 3'e ise iatrojenik mesane rüptürü ile birlikte karaciğer ve dalak laserasyonu oluşturulan tavşanlar dahil edildi. Tüm cerrahi işlemlerde sedasyon için 5 mg/kg Ksilazin HCl (Rompun %2; Bayer; Toronto-Kanada) ve anestezi için 35 mg/kg Ketamin HCl (Ketasol %10; İnterhas; Wels-Avusturya) IM yolla kullanıldı.

Grup 1'deki tavşanlar V/D pozisyonda yatırıldı ve proksimal ucu 7.5 F kalınlıkta olan üreterorenoskopa (Richard Wolf Endoscopy; Almanya), 100 g lokal anestezikli kayganlaştırıcı jel (Cathegell, Taymed; Montavit-Avusturya) sürülerek mesane sistoskopik olarak değerlendirildi. Üreterorenoskop mesanede iken, 0.5 ml fluorescein (%10 AlconCusi, İspanya) v. auricularis magna'dan IV yolla enjekte edildi ve mesane mukozasında fluorescein varlığının saptanana kadar mesane içerisi sistoskopi ile gözlendi. Fluoresceinli tavşan idrarının mesaneyi doldurduğu gözlendikten sonra mesane arka duvar orta hattı, üreterorenoskoptan geçirilen 4F kalınlığındaki yabancı cisim forsepsi ile yaklaşık 1 cm perfore edildi ve perforasyon hattından fluoresceinli idrarın abdominal boşluğa geçtiği teyit edildi. Daha sonra 10 G iğne ve 10 ml'lik enjektör ile parasentez yapılarak abdominal boşlukta fluorescein varlığı kesinleştirildi. Sonrasında ise, orta hattan cerrahi insizyon ile laparotomi yapılarak abdominal boşluk açılarak mesane rüptürü ve batın içerisindeki fluoresceinli idrar teyit edildi.

Grup 2'ye sistoskopi yapılmaksızın orta hat insizyonuyla laparotomi yapıldı. Takiben karaciğer ve dalakta, disseksiyon makası kullanılarak laserasyon oluş turuldu ve abdominal boşluğa kan akışı gözlendi. Eş zamanlı olarak v. auricularis magna'dan 0.5 ml %10'luk fluorescein IV yolla verildi. İşlem sonrası abdominal boşluk gözlemlendi. Abdominal boşlukta biriken kan hem direkt olarak hem de enjektöre çekilerek çıplak gözle incelendi. Günümüzde batın içi organların travma derecelendirmesinde en sık kullanılan sınıflama Amerikan Cerrahi Derneği tarafından geliştirilen sınıflamadır. Bu sınıflamada etkilenen batın içi organdaki hematom ve laserasyona bağlı olarak, dereceler göreceli olarak artar. Buna göre derece 1 laserasyon, en az 1 cm derinlikte olan kanamayan kapsüler hasardır. Derece 2 laserasyon ise 1-3 cm derinliğinde olan ve 10 cm'den kısa olan aktif kanamalı kapsüler hasarlardır. Derece 3 lasearsyonlarda etkilenen organ parankimlerinde 3 cm'den daha derin hasar mevcuttur. Derece 4 laserasyon, karaciğer ve dalağın parankiminin %50'sinin bozulmasına yol açan hasarları kapsamaktadır. Çalışmamızda yapılan laserasyon derecesi karaciğer ve dalak için derece 3'tü.

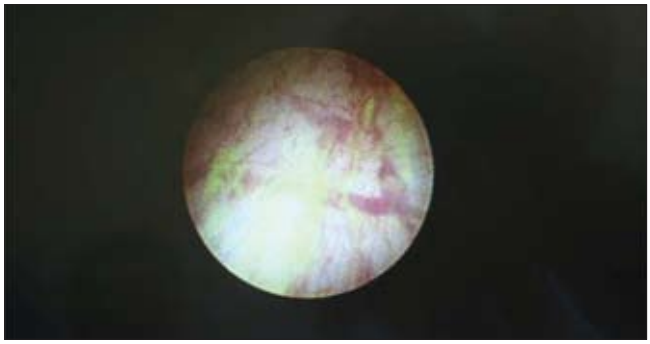
Grup 3'teki tavşanlara Grup 1'deki gibi sistoskopi ve mesane rüptürü prosedürü uygulandı. Ek olarak laparotomi sonrasında karaciğer ve dalakta disseksiyon makası ile derece 3 laserasyon oluşturuldu. İntraperitoneal hemorajinin, batın içine dağılan fluoresceinli idrar içeriği ile karışıp karışmadığı gözlendi. Sonrasında ise, bu heterojen karın boşluğu içi sıvısı bir enjektöre çekilerek çıplak gözle gözlendi.

Her 3 grupta abdominal boşluk içine birikmiş mayiler çıplak gözle izlendiğinde sarı yeşil renkli fluorescein gözleniyorsa F (+), gözlenmiyorsa F (-) olarak kaydedildi.

Bu işlemlerin sonrasında tavşanlara intrakardiak sodyum pentobarbital 100 mg/kg uygulanarak uyutulup sakrifiye edildi.

BULGULAR

Grup 1 ve 3'te sistoskopi eşliğinde intravenöz yolla fluorescein uygulamasını takiben 10 sn içerisinde mesane mukozasının fluorescein ile sarı yeşil renkte boyandığı ve mesane içerisine fluoresceinin toplandığı saptandı *(Şekil 1)*. Grup 1'de yapılan sistoskopide, mesane rüptürü sonrasında fluorescein ile boyanmış idrarın abdominal boşluğa geçtiği gözlendi. Eş zamanlı yapılan parasentezde fluoresceinli idrarın enjektör içerisinde sarı yeşil renkte geldiği tespit edildi ve F (+) olarak kaydedildi *(Şekil 2)*. Grup 2'de karaciğer ve dalak laserasyonu sonrasında çıplak gözle bakıldığında batın içi organların fluoresceinle boyandığı fark edildi *(Şekil 3)*. Ancak hemorajik mayinin fluorescein ile boyanması net olarak gözlenmedi ve F (-) olarak kaydedildi. Grup 3'e yapılan işlemler sonrasında, hemorajik mayi ile karışmış fluoresceinli idrar parasentez sonrasında enjektörde çıplak gözle sarı yeşil renkte bariz fark edildi ve F (+) olarak kaydedildi *(Şekil 4)*.



Şekil 1. Fluoroscein verildikten sonraki mesanenin sistoskopi ile görünümü

Fig 1. Cystoscopic vision of the bladder after adminesteration of fluorescein

Şekil 2. İzole mesane rüptürü oluşturulan tavşanda parasentez mayinin sarı yeşil renkte görünümü

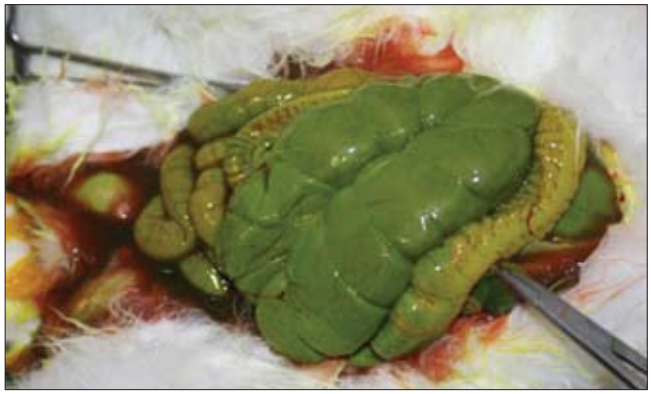
Fig 2. Yellow green coloured parasynthesis fluid that was obtained from the rabbit with isolated bladder rupture

TARTIŞMA ve SONUÇ

Küt travmalarda izole mesane yaralanması nadirdir ve %80-94 hastada üriner sistemi kapsamayan yaralanmalarla birlikteliği görülür [3]. Pelvis kırıklarının %83-95'ine mesane yaralanmaları eşlik eder [4]. Mesane rüptürlerinin %58'i ekstraperitoneal, %34'ü intraperitoneal ve %8'i ise hem intraperitoneal hem de ekstraperitonealdir [5]. Ekstra-

Şekil 3. İzole karaciğer, dalak laserasyonu oluşturulmuş tavşanda batın içi organlar ve hemorajik mayinin görünümü (hemorajik mayide sarı yeşil renkte fluoroscein boyasının olmadığı görülmekte)

Fig 3. Image of hemorhagic fluid and intraperitoneal organs of the rabbit with isolated liver/spleen laceration (yellow green coloured fluorescein was not seen in hemorhagic fluid)



Şekil 4. Karaciğer, dalak laserasyonu ile birlikte mesane rüptürü oluşturulan tavşanda parasentez mayinin sarı yeşil renkte görünümü

Fig 4. Yellow green coloured parasynthesis fluid in the rabbit with liver/spleen laceration and bladder rupture

peritoneal mesane rüptürleri sıklıkla pelvis kırıkları ile birliktedir; ancak intraperitoneal rüptürlerle de birliktelik gösterebilir. Penetran ve küt travmalara bağlı olarak oluşan intraperitoneal rüptürler, mesane dolu olduğunda daha sık gözlenir. Bu durum tanı ve tedavi seçiminde önemlidir [1]. Küt mesane travmalarında kateterizasyon önerilir ve hematüri görülmesi tanıda önemlidir [6]. Kateterizasyon yapılamazsa üretral travma akla gelmelidir; ama bu durumda %10-29 oranında mesane travması ile birliktelik gösterebilir [7]. Travmatik pelvis kırığı olan olguların %0.7-25'inde üriner sistem yaralanmaları da görülür [8]. Yapılan bir çalışmada, çoğunluğu künt travmaya bağlı mesane rüptürlü olguların %62'sinde ekstraperitoneal rüptür ve bunların da %95'inde de pelvis kırığı tespit edilmiştir [9].

Mesane rüptürlerinde en doğru tanı yöntemi retrograd sistografi tetkikidir ve rüptürlerin %85-100'ünü gösterebilir [10]. Ancak, BT sistografinin tanıdaki başarısı daha yüksektir [1]. Bu çalışmada, sistografiye alternatif bir yöntem olabilecek intravenöz fluorescein uygulamasının pratikteki kullanılabilirliği araştırılmıştır. Daha önce yapılan bir çalışmada fluorescein, mesane mukozasındaki tümörleri göstermek amacı ile kullanılmıştır [11].

Bu çalışmada fluoresceinin tanı amaçlı çabuk ve

kolay uygulanabilir olması ve ciddi bir ekip ve ekipman gerektirmemesi sistografi tetkikine göre avantaj olarak kabul edilmiştir. Tanısı gecikmiş intraperitoneal mesane rüptüründe, üroasit ve peritonit gelişmektedir [5]. Fluorescein yöntemi ile mesane rüptürü şüphesi olan olgulara bir doz fluorescein uygulanıp, ardından yapılan intraperitoneal parasentezle tanı konulabileceğini tespit ettik. Kısa tanı süresinin de fluoresceinin avantajı olduğunu düşünmekteyiz. Yapılan bir çalışmada deneysel olarak intraperitoneal mesane rüptürü oluşturulan tavşanlarda tanı amacıyla fluoresceininin etkinliği araştırılmıştır. Hem retrograd hem de intravenöz yolla verilen fuoresceinin mesane rüptürlerinin tanısında kullanılabileceği ancak, üretra taşı ya da darlığı gibi durumlarda retrograt yolun kullanılamayacak olması dezavantaj olarak sunulmuştur [12]. Biz bu çalışmadan farklı olarak sadece idrar kesesi rüptürlerinde değil eş zamanlı olarak yaygın biçimde saptanan karaciğer ve dalak laserasyonlarının, mesane rüptürlerinden ayırıcı tanısında da fluoresceinin kullanılabilirliğini araştırdık.

Ekstraperitoneal rüptürlerin şekillendiği bölgeye anatomik olarak parasentez yapılmamaktadır. Bu nedenle sunulan bu çalışmada ilgili rüptürün tanısı parasentez yoluyla konulamadı. Mesane rüptürlerinin %34'ünün ekstraperitoneal, %8'inin ise intraperitoneal şekillendiği [13-15] göz önüne alındığında geliştirdiğimiz tanı yönteminin ekstraperitoneal rüptürlerde yetersiz kalması ciddi bir dezavantaj olarak değerlendirildi.

Mesane rüptürü ile beraber olan iç organ yaralanmalarının ayırdedilmesinde çalışmamızda kullanılan tanı yönteminin tanısal değerine bakıldı. Grup 2 ve Grup 3 deney hayvanları kontrol grubu olarak hazırlandı ve yapılan karşılaştırmada, parasentez mayisinin Grup 1 ve Grup 3'te çıplak gözle bakılmasında tamamının sarı-yeşil renk aldığı ve mesane rüptürünü tespit ettiğini fark ettik. Diğer yandan izole karaciğer ve dalak laserasyonlarının değerlendirildiği Grup 2'de, parasentez mayisinin Grup 1 ve 3'deki gibi sarı-yeşil renk almadığını saptadık. Mesane rüptürü olmaksızın sadece iç organ yaralanması bulunan hastalarda fluoresceinin bu organ yaralanmalarını tespit etmediğini ancak, mesane rüptürlerinin eş zamanlı iç organ yaralanmaları ile birlikte bulunduğu komplike olgularda mesane rüptürü tanısına katkı sağladığı görülmüştür.

Çalışmamızda mesane rüptürü tanısı için kullanılan fluoresceinin bazı yan etkilerinin olması, bu yöntemin diğer bir dezavantajı olduğunu düşünmekteyiz. Fundus fluorescein uygulamasında insan hayatını tehdit edebilecek akciğer ödemi, kardiyovasküler sorunlar, ürtiker, alerjik rinit ve tromboflebit gibi komplikasyonlar bildirilmiştir [16]. Hafif ve orta dereceli komplikasyonlar 1/63-1/1900 oranında görülürken, ciddi komplikasyonlar ise 1/222000 oranında görülmektedir [17]. Mesane rüptürü tanısının koyulmasında fluorescein kullanıldığında, bu tür komplikasyonların gelişebileceği göz önünde bulundurulmalıdır.

Bu deneysel çalışma, intraperitoneal mesane rüptürlerinde intravenöz fluorescein kullanımının pratikte tanısal bir değere sahip olduğunu göstermiştir. İleriki zamanlarda yapılacak olan uygun klinik çalışmalarla desteklendiğinde, sistografiye göre daha pratik, kolay ve tüm hekimlerin uygulayabileceği bir ön tanı testi olabileceğini düşünmekteyiz. Ancak ekstraperitoneal mesane rüptürlerinde tanısal değerinin olmaması ve intraperitoneal mesane rüptürü ile birlikte olan diğer batın içi organ yaralanmalarının ayırt edilememesi, bu tanı testinin ciddi bir dezavantajı olarak düşünülmektedir. Bu maddenin klinik kullanımında, son derece düşük oranda olsa bile; akciğer ödemi, kardiyovasküler sorunlar, ürtiker, alerjik rinit ve tromboflebit gibi [18] yan etkilerinin de bulunabileceği akılda tutulmalıdır.

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RESEARCH ARTICLE

Occurrence of *Aeromonas hydrophila* in Fish, Shrimp, Lobster and Crab in Iran

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Summary

This study was conducted to determine the prevalence rate and antimicrobial resistance of *Aeromonas hydrophila* in fish, shrimp, lobster and crab caught off the south coast of Iran. A total of 541 samples including, 133 freshly caught fish of 4 different types including *Otollithes ruber, Pamous argenteus, Parastromateus niger* and *Psettodes erumel,* 240 shrimp of 4 different species including *Penaeus monodon, P. semisulcatus, P. indicus,* and *P. merguiensis,* 108 lobster (*Panulirus homarus*) and 60 crab (*Panulirus homarus*) were collected in 3 provinces along Persian Gulf in the south coast of Iran. Samples were collected at the end of each month from September 2011 to May 2012. Using conventional bacteriological techniques, 66 *A. hydrophila* isolates were identified in which 62 strains were confirmed by PCR assay targeting *16S rDNA* gene of *A. hydrophila*. Using PCR assays targeting the *A. hydrophila* cytolytic enterotoxin gene, 57 (10.5%) isolates were positive. The highest prevalence of *A. hydrophila* was found in fish (19.5%), followed by shrimp (9.2%), lobster (9.3%) and crab (6.7%). The highest prevalence of A. hydrophila occurred in summer (21.3%) followed by fall (12.0%), spring (10.8%), and winter (5.6%). To our knowledge, the present study is the first report of the isolation of *A. hydrophila* from fish, shrimp, lobster and crab in Iran.

Keywords: *Aeromonas hydrophila, Seafood, Seasonal variation, Virulence factors, Fish, Shrimp, Lobster, Crab*

İran'da Balık, Karides, Istakoz ve Yengeçlerde *Aeromonas hydrophila* Mevcudiyeti

Özet

Bu çalışma İran'ın güney kıyılarında yakalanan balık, karides, ıstakoz ve yengeçlerde *Aeromonas hydrophila* prevalansını ve etkenin antimikrobiyal dayanıklılığını belirlemek amacıyla yapılmıştır. Çalışmada 4 ayrı türden, *Otollithes ruber, Pamous argenteus, Parastromateus niger* ve *Psettodes erumel,* toplam 133 balık; 4 ayrı türden, *Penaeus monodon, P. semisulcatus, P. indicus,* ve *P. merguiensis,* toplam 240 adet karides; 108 ıstakoz (*Panulirus homarus*) ve 60 yengeç (*Panulirus homarus*) İran'ın güney sahillerinde İran Körfezi boyunca 3 bölgeden olmak üzere toplam 541 örnek toplanmıştır. Örnekler Eylül 2011 ile Mayıs 2012 arası her ayın sonunda toplanmıştır. Rutin bakteriyolojik metot ile 66 *A. hydrophila* izolatı belirlenmiş ve bunların 62'si PCR ile *A. hydrophila 16S rDNA* gen hedefi ile teyit edilmiştir. *A. hydrophila* sitotoksik enterotoksin gen hedefi ile uygulanan PCR metodu ile 57 (%10.5) izolat pozitif olarak tespit edilmiştir. *A. hydrophila* prevalansı en yüksek olarak balıklarda (%19.5), sonrasında ise sırasıyla ıstakozlarda (%9.3), karideslerde (%9,2) ve yengeçlerde (%6.7) belirlenmiştir. Mevsimsel olarak en yüksek *A. hydrophila* prevalansı yaz ayında (%21.3) olurken bunu sırasıyla sonbahar (%12.0), ilkbahar (%10.8) ve kış (%5.6) izledi. Bizim bilgimiz kapsamında bu çalışma İran'da balık, karides, ıstakoz ve yengeçlerde *A. hydrophila* izolasyonunun rapor edildiği ilk çalışmadır.

Anahtar sözcükler: *Aeromonas hydrophila, Deniz ürünü, Mevsimsel varyasyon, Virulans faktörleri, Balık, Karides, Istakoz, Yengeç*

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INTRODUCTION

Genus *Aeromonas* has emerged as an important human pathogen because of suspected food-borne outbreaks [1,2] and the increased incidence of its isolation from patients with traveller's diarrhea [3,4]. Among the 14 species of *Aeromonas* known to date *A. hydrophila, A. caviae*, and *A. veronii* biotype sobria have most commonly been involved in human infections and have been found to produce a variety of virulence factors such as hemolysins, cytotoxins, enterotoxins, proteases, leukocidin, phospholipases, endotoxins, outer membrane proteins, and fimbriae [5]. A number of *Aeromonas* spp. are able to grow in raw, cooked, and processed foods, at refrigeration temperature, under modified atmosphere and under modified growing conditions [6,7].

Aeromonas can be found in soil, fresh and saline water, drinking water and animal faeces [8]. Also several investigations have shown that members of the genus *Aeromonas* are also widely distributed in various foods such as meat [9,10], sea food [11-14], and vegetable [15]. Thus, foods have been suggested as a vector in the dissemination of this pathogen. The potential role of *A. hydrophila* in human gastrointestinal infections is noted by Kirov [1]. The majority (>85%) of gastroenteritis cases are attributed to three *Aeromonas* species, one of them is *A. hydrophila* [4].

Currently, there is limited information regarding the prevalence of *A. hydrophila* in seafood in Iran. This study was conducted to determine the prevalence rate and virulence genes of *A. hydrophila* in fish, shrimp, lobster and crab caught off the south coast of Iran.

MATERIAL and METHODS

Sample Collection

A total of 133 freshly caught fish of 4 different species including *Otollithes ruber* (n=39), *Pamous argenteus* (n=37), *Parastromateus niger* (n=28) and *Psettodes erumel* (n=29), 240 freshly caught shrimp of 4 different species including *Penaeus monodon* (n= 60), *P. semisulcatus* (n= 60), *P. indicus* (n= 60), and *P. merguiensis* (n= 60), 108 freshly caught lobster (*Panulirus homarus*) and 60 freshly caught crab *(Porpunus pelagicus)* were collected in 3 provinces (Bushehr, Hormozgan and Khuzestan) along Persian Gulf in the south coast of Iran. Samples were collected at the end of each month from September 2011 to May 2012, placed in separate sterile plastic bags to prevent spilling and cross contamination, and immediately transported to the laboratory in a cooler with ice packs.

Microbiological Analysis

The samples were processed immediately upon arrival using aseptic techniques. All the specimens were rinsed with sterile water to remove the adhering particles. Twenty-five grams of the fish samples were homogenized with 225 mL alkaline peptone-water (APW).The homogenate was incubated for 6 h at 37°C. Whole shrimp, lobster and crab were dipped into screw cap bottles containing APW so as to transfer the bacterial load into APW. Samples were removed from the bottles after dipping for 2 min. After incubation, a loopful of the APW culture was streaked on starch ampicillin agar medium (Himedia, Mumbai, India) and incubated at 37°C for 18-24 h as described by Vivekanandhan et al.[11]. The plates were then flooded with approximately 5 ml of Lugol's iodine solution and amylase positive yellow to honey coloured colonies were isolated. The isolated cultures were then purified by repeated streaking on nutrient agar and maintained in nutrient agar slants. Tubes with alkaline slant and acid butt after 24 h at 37°C were considered as presumptive positive for *A. hydrophila*. The presumptive isolates were confirmed as *A. hydrophila* based on the following reactions: motile, Gram-negative, cytochrome oxidase positive, glucose fermentation positive, arginine dihydrolase positive, ornithine decarboxylase negative, ONPG positive, H_2S from cystein, acetoin from glucose, gas from glucose, l-arabinose utilization and fermentation of salicin [11]. We have used a type strain of *A. hydrophila* (ATCC 7966), as reference strain to compare the results.

Detection of A. hydrophila from Pure Culture

One milliliter pure culture of *A. hydrophila*, identified by biochemical tests, was centrifuged at 13.000 g for 5 min at room temperature. Purification of DNA was achieved using a Genomic DNA purification kit (Fermentas, GmbH, Germany) according to the manufacturer's instructions and the total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell [16].

The PCR procedures used in this study have been described previously [17]. Two genes selected for the identification of the *A. hydrophila* and *A.* hydrophila cytolytic enterotoxin gene as a multivirulence gene causing lethality in mice, haemolysis, cytotoxicity and enterotoxigenicity were the 16S rDNA gene [18], and the Aero gene [19], respectively. The sequences of the three sets of primers used for gene amplification are presented in *Table 1*. All oligonucleotide primers were obtained from

Table 1. *Primer sequences and predicted lengths of PCR amplification products*
Tablo 1. *Primer sekansları ve tahmini PCR amlifikasyon ürün boyutları*

Primer	Oligonucleotide Sequence (5-3)	Fragment Size (pb)	Reference
16S rDNA1	GAAAGGTTGATGCCTAATACGTA	462	[5]
16S rDNA1	CGTGCTGGCAACAAAGGACAG		
Aero1	CTCAGTCCGTGCGACCGACT	685	[16]
Aero2	GATCTCCAGCCTCAGGCCTT		

a commercial source (Cinna Gen, Iran). PCR amplification was performed using a DNA thermal cycler (Master Cycler Gradiant, Eppendrof, Germany) in a total volume of 50 µl. The reaction mixture consisted of 5 µl of template DNA, 5 µl 10x PCR buffer (+$MgCl_2$) (Roche Applied Science, Germany), 4 µl of deoxyribonucleoside triphosphates (2.5 mmol L-1 each of dATP, dTTP, dGTP and dCTP), 0.5 µl of each primer, and 0.25 µl (0.5 U µl-1) of Taq DNA polymerase (Roche Applied Science, Germany), with 50 µl sterile water added. Thirty PCR cycles were run under the following conditions; denaturation at 94°C for 2 min, primer annealing at 56°C for 2 min, and DNA extension at 72°C for 2 min in each cycle.

Detection of A. hydrophila form Enrichment Broth

One millimeter enrichment broth from each shrimp sample was centrifuged at 13.000 g for 5 min at room temperature. The cell pellets were subjected to DNA extraction as described above. A 5 µl aliquot of each sample was used for PCR amplification. All reactions were performed in triplicate. The PCR-amplified products were examined by electrophoresis in a 1.5% agarose gel, stained with a 1% solution of ethidium bromide, and examined under UV illumination. In the present study, *A. hydrophila* (ATCC 7966) were used as the positive control and DNase free water was used as the negative control, respectively.

Statistical Analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA), chi-square test and fisher's exact two-tailed test analysis were performed and differences were considered significant at values of $P<0.05$.

RESULTS

Using conventional bacteriological techniques, 66 *A. hydrophila* isolates were identified. A PCR assay targeting *16S rDNA* gene of *A. hydrophila* confirmed 62 strains as *A. hydrophila* (*Fig. 1*). Using PCR assays targeting the *A. hydrophila* cytolytic enterotoxin gene, 57 (91.9%) isolates were positive (*Fig. 2*). The PCR assays were performed in triplicates and no variability in the results was present. The highest prevalence of *A. hydrophila* was found in fish (19.5%), followed by shrimp (9.2%), lobster (9.3%) and crab (6.7%) (*Table 2*). There were significant differences ($P<0.05$) in the level of contamination with *A. hydrophila* between different types of seafood samples; however, no significant differences ($P>0.05$) were found between different shrimp and fish species. No significant differences in the prevalence rates ($P>0.05$) were observed between seafood isolated in Bushehr, Hormozgan and Khuzestan.

The PCR assays performed on enrichment broth from each sample gave positive results for *A. hydrophila* in 73 (13.5%) samples. *A. hydrophila* cytolytic enterotoxin gene

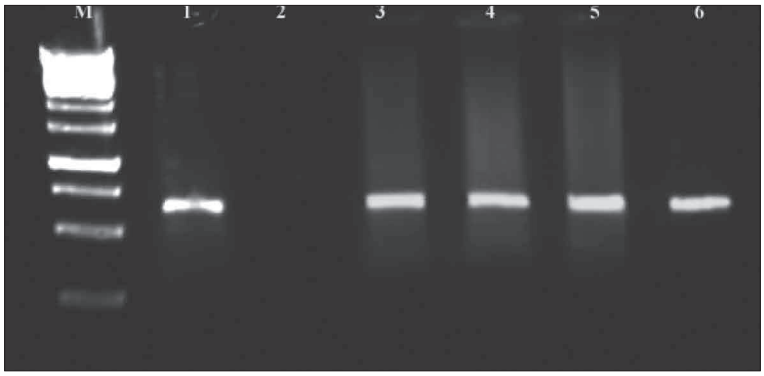

Fig 1. Electropherogram of the amplification products of the polymerase chain reaction (PCR) assay. M, 100 bp DNA ladder; lane1, positive control; lane 2, negative control; lanes 3 to 6, *Aeromonas hydrophila* positive samples from fish, shrimp, lobster and crab

Şekil 1. Polimeraz zincir reaksiyonu ile amplifiye edilen ürünlerin elektroferogramı. M, 100 bp DNA merdiveni; sütun1, pozitif kontrol; sütun 2, negatif kontrol; sütun 3-6, balık, karides, ıstakoz ve yengeçlerde *Aeromonas hydrophila* pozitif örnekler

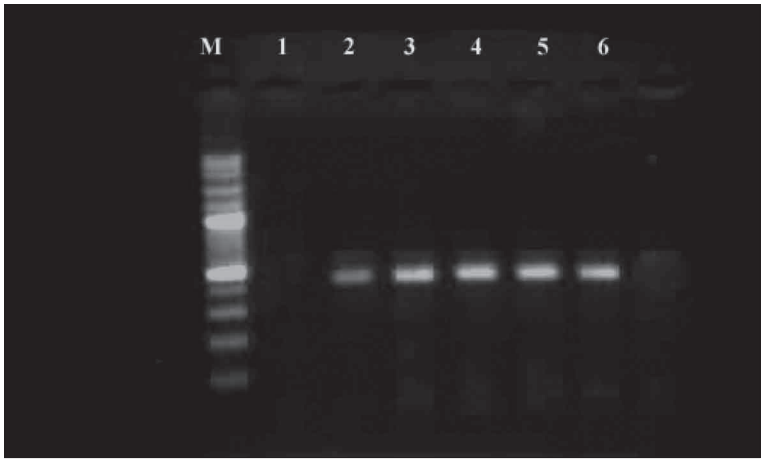

Fig 2. Electropherogram of the amplification products of the polymerase chain reaction (PCR) assay. M, 100 bp DNA ladder; lane1, negative control; lane 2, positive control; lanes 3 to 6, *A. hydrophila* cytolytic enterotoxin gene positive samples

Şekil 2. Polimeraz zincir reaksiyonu ile amplifiye edilen ürünlerin elektroferogramı. M, 100 bp DNA merdiveni; sütun1, negatif kontrol; sütun 2, pozitif kontrol; sütun 3 - 6, *A. hydrophila* sitolitik enterotoksin gen pozitif örnekler

Table 2. *Distribution of the 16S rDNA, and cytolytic enterotoxin genes possessing A. hydrophila isolates detected by PCR in different fish, shrimp, lobster and crab species*
Tablo 2. *Balık, karides, ıstakoz ve yengeçlerde PCR ile tespit edilen A. hydrophila 16S rDNA ve sitolitik enterotoksin gen dağılımı*

Sample	No. of Samples	Pure Culture (%)		Enrichment Broth (%)	
		16S rDNA	Cytolytic Enterotoxin	16S rDNA	Cytolytic Enterotoxin
Fish					
Otollithes ruber	39	7 (17.9%)	7 (17.9%)	9 (23.1%)	9 (23.1%)
Pamous argenteus	37	3 (8.1%)	3 (8.1%)	5 (13.5%)	4 (10.8%)
Parastromateus niger	28	5 (17.9%)	5 (17.9%)	7 (25.0%)	7 (25.0%)
Psettodes erumel	29	11 (37.9%)	11 (37.9%)	12 (41.4%)	12 (41.4%)
Shrimp					
Penaeus monodon	60	9 (15.0%)	7 (11.7%)	9 (15.0%)	7 (11.7%)
Penaeus semisulcatus	60	3 (5.0%)	3 (5.0%)	4 (6.7%)	4 (6.7%)
Penaeus indicus	60	5 (8.3%)	5 (8.3%)	6 (10.0%)	6 (10.0%)
Penaeus merguiensis	60	5 (8.3%)	4 (6.7%)	7 (11.7%)	6 (10.0%)
Lobster					
Panulirus homarus	108	10 (9.3%)	8 (7.4%)	10 (9.3%)	8 (7.4%)
Crab					
Porpunus pelagicus	60	4 (6.7%)	4 (6.7%)	4 (6.7%)	4 (6.7%)
Total	541	62 (11.5%)	57 (10.5%)	73 (13.5%)	67 (12.4%)

Table 3. *Prevalence of Aeromonas hydrophila isolated from fish, shrimp, lobster and crab*
Tablo 3. *Balık, karides, ıstakoz ve yengeçlerde Aeromonas hydrophila prevalansı*

Season	Seafood Sample				Total
	Fish	Shrimp	Lobster	Crab	
Winter	4/41(9.8)*	2/60 (3.3)	0/27 (0.0)	0/14 (0.0)	6/142 (4.2)
Spring	5/31 (16.1)	5/60 (8.3)	3/29 (10.3)	2/18 (11.1)	15/138 (10.9)
Summer	12/37 (32.4)	10/60 (16.7)	5/24 (20.8)	2/15 (13.3)	29/136 (21.3)
Fall	5/24 (20.8)	5/60 (8.3)	2/28 (7.1)	0/13 (0.0)	12/125 (9.6)

** Results expressed as the number of Aeromonas-positive samples/number of samples analyzed (%)*

was detected in 67 (91.8%) isolates. Overall, the observed difference in the frequency of detection of the *A. hydrophila* cytolytic enterotoxin genes from the pure culture versus the enrichment broth was not statistically significant.

Table 3 shows the seasonal prevalence of *A. hydrophila* in fish, shrimp, lobster and crab caught off the south coast of Iran. Overall, the highest prevalence of *A. hydrophila* in seafood samples occurred in summer (21.3%) which was significantly ($P<0.05$) higher than spring (10.9%), fall (9.6%) and winter (4.2%); however, the difference in the prevalence rates of *A. hydrophila* between fall and spring was not statistically significant.

DISCUSSION

To our knowledge, the present study is the first report of the isolation of *A. hydrophila* from four different types of fish, four species of shrimp including *P. monodon*, *P. semisulcatus*, *P. indicus*, and *P. merguiensis*, lobster and crab caught off the south coast of Iran. Out of 133 fishes analysed, 26 (19.5%) fishes were found to be contaminated with *A. hydrophila*. These findings are comparable with those reported from Malaysia [20] and Taiwan [21], New Zealand [22] and Turkey [23]; however, are higher than the prevalence reported from India [11] and of Switzerland [24].

Overall, 9.9% (22 of 240) of all shrimp samples were contaminated with *A. hydrophila*. The prevalence of *A. hydrophyla* in different shrimp species such as *P. monodon*, *P. semisulcatus*, *P. indicus*, and *P. merguiensis* was found to be 15%, 5%, 8.3% and 8.3% respectively. The prevalence of different shrimp species in different shrimp species observed in this study is similar to a recent report in different shrimp species that showed a prevalence of different shrimp species of 16.58%, 13.20% and 25.52% in *P. indicus*, *P. monodon*, and *P. semisulcatus*, respectively [10].

Also this is in agreement with findings of Tsai and Chen [21] and Colakoglu et al.[25]. In another study conducted in coastal South India, *A. hydrophila* was identified in 35.6% of shrimp samples [26]. No previous report could be found on the occurrence of *A. hydrophila* on the lobster and crab.

The prevalence of cytolytic enterotoxin gene carrying *A. hydrophila* isolates reported in our study are comparable with those reported from Malaysia [9,13]. However, our results are higher than the results of a study conducted in India [27].

Variation in the prevalence of *A. hydrophila* isolates from raw fish and shrimp, samples reported in different studies may be a result of different sampling techniques employed, seasonal effects and/or laboratory methodologies employed in different studies (bacteriological and biochemical testing vs. PCR assays) [11,16]. Furthermore, a higher prevalence rate of *A. hydrophila*-positive in seafood could be due to cross-contamination during manual processing or insufficient hygiene during storage and transport in the seafood markets.

The overall prevalence levels in shrimp, lobster and crab were much lower than those recorded in fishes ($P<0.05$). This is in agreement with findings of Tsai and Chen [21] and Vivekanandhan et al.[11]. The chitinous shell of the prawns may not be that conducive for proliferation of the *A. hydrophila*, as the moisture rich body surface of fish [11].

The prevalence of *A. hydrophila* isolated from fish, shrimp, lobster and crab samples in this study was significantly ($P<0.05$) higher in summer (21.3%) than spring, fall and winter. This finding is in agreement with other studies that reported peak prevalence rate of *A. hydrophila* in seafood in the warmer months [10,25]. This could be due to the increased coastal water pollution resulting from land run off, municipal sewage outflows and storm water surge during the monsoon season [11]. However, in some studies no apparent pattern in the seasonality of *A. hydrophila* prevalence was observed in shrimp samples [2].

In this study, *A. hydrophila* was more detected by the PCR assays than the cultural method. This could be due the higher analytical and diagnostic sensitivities of the PCR assays. PCR is capable of detecting culturable and also non-culturable but viable cells, which increases its sensitivity as a detection method. The high throughput and cost-effective m-PCR system developed in this study could provide a powerful addition to conventional methods for more accurate risk assessment and monitoring of pathogenic strains of the *A. hydrophila*. The PCR method identified potential pathogenic *A. hydrophila* strains in <8 h. In addition, the method had advantages in terms of its specificity, easy of use and cost, compared to biochemical and DNA hybridization methods [17].

This study shows the importance of fish, shrimp, lobster and crab as potential sources of *A. hydrophila* infection in people. *Aeromonas* spp. is being considered as a pathogen of emerging importance due to its special features such as ubiquitous presence in the aquatic environment, multiplicity of virulence factors and psychrotrophic nature. Though the occurrence of foodborne infections due to *Aeromonas* has not been recognized in Iran, it has been suggested in other countries in association with consumption of various foods. In Iran, fish and other sea foods is usually eaten after being cooked, and therefore, sea food may be a low risk food, even if contaminated with *Aeromonas* species, although the toxin remain in foodstuff. On the other hand, in recent years, the trend of consuming ready-to-eat under cooked seafoods in public places is getting popular, and thus, there is always the possibility of cross-contamination at the processing, food preparation and service steps. Most of modern approaches to control levels of contamination with microorganisms are effective against *A. hydrophila*.

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RESEARCH ARTICLE

Effect of Rapid Chilling and Pelvic Suspension on Meat Quality of *Longissimus dorsi* Muscle of Lamb [1]

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Summary

The objective of this study was to examine the effect of rapid (RC) and conventional (CC) chilling with achilles (AS) and pelvic (PS) suspension on the meat quality of *M. Longissimus dorsi*. Twenty lamb carcasses were randomly allocated immediately prior to slaughter to the two experimental groups which were subjected to four different treatments. In the first group, carcasses were suspended from the Achilles tendon. Right sides (RC/AS; n=10) were rapidly chilled, while the left sides (CC/AS; n=10) were conventionally chilled. In the second group, the carcasses were re-hanged from the pelvic bone. Right sides (RC/PS; n=10) were rapidly chilled whilst the left sides (CC/PS; n=10) were conventionally chilled. Meat quality was evaluated by measuring the water holding capacity (WHC), cooking loss (CL), surface colour and shear force (SF). As a result, CC accelerated the rate of pH decline while RC increased the temperature decline. RC reduced CL and WHC values. PS had no impact on WHC, CL and color of steaks, but decreased the SF values on the 7th days of post-mortem. In conclusion; PS is a useful method for improving tenderness during storage period and the disadvantageous effect of RC on SF could be equalized by using PS.

Keywords: *Pelvic suspension, Meat quality, Rapid chilling, Tenderness, Lamb*

Hızlı Soğutma ve Pelvik Asılmanın Kuzulara Ait *Longissimus dorsi* Kasındaki Et Kalitesi Üzerine Etkisi

Özet

Bu çalışma, aşil tendosundan ve pelvis bölgesinden asma ile birlikte hızlı ve konvansiyonel soğutmanın *M. Longissimus dorsi*'nin et kalitesi üzerine etkisini incelemek için amaçlanmıştır. Yirmi kuzu karkası, kesimden hemen önce rastgele olarak iki deneysel gruba ayrılmış ve kesim sonrası dört faklı muameleye tabi tutulmuştur. İlk gruptaki karkaslar aşil tendosundan asılmıştır. Sol taraflar konvansiyonel olarak soğutulurken (CC/AS; n=10), sağ taraflar hızlı şekilde soğutulmuştur (RC/AS; n=10). Ikinci gruptaki karkaslar pelvik kemikten tekrar asılmıştır. Sol taraflar konvansiyonel soğutulurken (CC/PS; n=10), sağ taraflar hızlı şekilde soğutulmuştur (RC/PS; n=10). Et kalitesi su tutma kapasitesinin (WHC), pişirme kaybı (CL), yüzey rengi ve kesme kuvvetinin (SF) ile ölçülmesi ile değerlendirilmiştir. Sonuç olarak, RC sıcaklık düşüşünü arttırırken, CC pH düşüş oranını hızlandırmıştır. RC reduced pişirme kaybı ve su tutma kapasitesini azaltmıştır. Pelvik asılmanın su tutma kapasitesi, pişirme kaybı ve bifteğin rengi üzerine hiçbir etkisi yokken, SF değerlerini kesim sonrası yedinci günde azaltmıştır. Sonuç olarak, PS saklama periyodu boyunca yumuşaklığı geliştirmek için kullanışlı bir metoddur ve kesme kuvveti üzerinde RC'nin dezavantajlı etkisi pelvik asılma kullanılarak eşitlenebilir.

Anahtar sözcükler: *Pelvik asılma, Et kalitesi, Hızlı soğutma, Yumuşaklık, Kuzu*

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INTRODUCTION

Consumer acceptance of meat depends on quality characteristics such as tenderness, color and palatability attributes, which are influenced by a series of factors, ranging from physical and chemical to histological properties and meat-processing procedures [1-4]. Numerous techniques are currently used to improve meat quality. Chilling and suspension techniques are one of the effective applications worldwide [5,6]. During the first 24 h of post-mortem, the rate of temperature decline affects the bio-chemical and structure changes on the conversion of muscle to meat. The efficacy of temperature and pH on tenderization depend on the carcass chilling rate [7,8]. Nowadays, different applications of chilling processes are used in most parts of the world to reduce the problems associated with temperature/pH relationships. Rapid chilling (RC) appears more applicable system for extending the shelf life and reducing the evaporative loss of meat [6]. However, compared with conventional chilling regimes, application of RC has a risk of producing tough meat with a high shear force [9]. Therefore, it is important to control the meat temperature in order to improve the tenderness of meat concomitant with RC. Pelvic suspension (PS) has been shown to improve the tenderness in beef [10], lamb [11] and pork meat [12].

PS involves hanging carcasses from the *obturator foramen* shortly after slaughter and before the commencement of rigor [13]. The hind leg hangs vertically from the carcass reversing the effects on the muscles involved and the vertebral column is straightened [14]. The present study was aimed to investigate the concominant effects of RC and PS on the meat quality of *M. longissimus dorsi* (LD) of lamb.

MATERIAL and METHODS

The research protocol of the current study was approved by the Ethic Committee of the Istanbul University, Faculty of Veterinary Medicine (Approval number: 2004/078).

Animals and Experimental Design

Twenty lambs, averaging 11 months of age and overal live weight of 45 kg at slaughter were procured from Istanbul University, Faculty of Veterinary Medicine farm. The animals were transported to the slaughterhouse from nearby farm within 15 min for 1 day prior to slaughter. After a rest for 22-24 h, with only water available, animals were electrically stunned at 220-250 V, 1.0-1.3 A for 1-3 second, stunning tongs applied on both sides of the head. Following exsanguinations and evisceration, carcasses were halved by splitting through the vertebral column within approximately 25 min of postmortem.

Carcasses were randomly allocated immediately prior to slaughter to the two experimental groups which were subjected to four different treatments. In the first group, carcasses were suspended from the Achilles tendon. Right sides (RC/AS; n=10) were rapidly chilled (air temperature, -18±1°C; wind velocity, 2 m/s) for 6 h and then placed in a conventional chiller (air temperature, 2±1°C; wind velocity, 1 m/s) for 18 h, while the left sides (CC/AS; n=10) were conventionally chilled (air temperature, 2±1°C; wind velocity, 1 m/s). In the second group, the carcasses were re-hanged from the pelvic bone. Right sides (RC/PS; n=10) were rapidly chilled, whilst the left sides (CC/PS; n=10) were conventionally chilled. At 24 h of post-mortem, the longissimus muscle (LM) was removed from each half carcass after measuring pH and divided into two portions. Portions were vacuum packaged in Cryovac barrier bags (Cryovac Sealed Air Corp., New Jersey, USA) and stored at 2±1°C for up to 7 days post-mortem prior to the evaluating the water holding capacity (WHC), cooking loss (CL), shear force (SF), and instrumental colour (CIE *L**, *a**, *b**).

Meat Quality Measurements

The temperature and pH were monitored in the deep portion of the LM at approximately 1 h, 4 h, 8 h and 24 h post-mortem using a portable thermometer (Hanna HI 145) and pH meter (Hanna HI 8314) [15].

The percentage of free liquid was evaluated as a measure of WHC by the filter press method described by Hertog-Meischke et al.[16]. The outline area of the expressible juice and the meat film traced, and two areas were measured using AUTOCAD 2007 (Apso Ltd, UK). CL was calculated from the weight of samples taken before and after cooking.

SF of steaks was determined by measuring the force required to shear through a cooked sample at 2 and 7 days of post-mortem. Samples were cooked individually in a 100°C water bath (NB20, Nuve, Istanbul, Turkey) until an internal temperature of 75°C was reached. The cooked samples were stored in a refrigerator overnight and the pieces (2.5 cm thick) were removed parallel to the muscle fiber. The pieces were sheared by a Warner-Bratzler shear attachment mounted on an Instron Texture Analyzer (3343 model, Instron, UK) with a 50 kg load transducer and crosshead speed of 200 mm/min. An average of five sub-samples was accepted to be the SF value of the sample [17].

Meat colour was measured using a Color Flex Hunter Lab Colour Measurement System (Hunter Associates Laboratory Inc., Virginia, USA). Colour coordinates values which were refered as *L** for lightness, *a** for redness, and *b** for yellowness, were recorded at each analyzed day. Colour was evaluated using a diffuse illumination (D65 2° observer) with 8 mm viewing aperture and a 25 mm port size with the specular component excluded and readings were averaged. Colour values were obtained considering the average of five readings, performed in different location of the meat surface [18].

Statistical Analysis

Analysis of variance (ANOVA) was conducted for each variable to investigate the effect of chilling regime and suspension type on meat quality of lamb meat. The model used included the fixed effects of storage time, chilling rate and suspension conditions. Means of each characteristic, which were significantly different, were separated using Duncan's multiple range tests and significance of differences was defined as P<0.05 [19].

RESULTS

Changes of pH and temperature values obtained from carcasses are given in *Table 1*. According to the results, it was found that the pH values in RC were higher than CC. Differences among the results of the groups at 4 and 8 h were significant (P<0.001). Carcass temperature at 1 h was 37.44°C (P>0.05) and faster drop on the muscle temperature was remarkable after 4 h in both groups *(Table 1)*. The mean temperatures excepted after 24 h for RC were significantly lower than DC (P<0.05).

The effect of chilling conditions and suspension methods on WHC, CL and SF are presented in *Table 2*. RC reduced significantly CL and WHC (water expelled) values (P<0.001), and SF values were lower in CC carcasses than RC carcasses (P<0.001).

The effect of RC on colour parameters is summerazed in *Table 3*. RC significantly decreased *L** values (P<0.001) only at 2 day of post-mortem. There were no significant differences in *a** and *b** values between RC and CC carcasses. Additionally, colour parameters were not affected by using PS (P>0.05).

DISCUSSION

CC accelerated the rate of carcass pH decline. In the present study, the pH values in RC were higher than CC. Similar results in pH were reported by Li et al.[9] and Hopkins et al.[20]. At the end of the chilling process (24 h), the average pH values in the groups was 5.60 (P>0.05). Likewise, Bayraktaroglu and Kahraman [5] found that the values for ultimate pH were between the range of 5.3 and 5.7 for lamb carcasses.

In this study, carcass mean temperatures excepted after 24 h for RC were significantly lower than DC (P<0.05). Likewise, Janz et al.[21] indicated that RC increased the

Table 1. *Changes in the pH and temperature values of lamb meat*
Tablo 1. *Kuzu etinin pH ve sıcaklık değerlerindeki değişimler*

Attribute	Groups	n	Time (hours)			
			1	4	8	24
pH	RC	10	6.80±0.01	6.45±0.01^{a}	6.12±0.02^{a}	5.60±0.01
	CC	10	6.77±0.01	6.32±0.01^{b}	5.96±0.03^{b}	5.60±0.01
	P	20	NS	***	***	NS
Temperature (°C)	RC	10	37.44±0.06	16.37±0.03^{b}	12.33±0.04^{b}	2.59±0.06
	CC	10	37.44±0.07	20.61±0.04^{a}	17.41±0.03^{a}	2.52±0.07
	P	20	NS	***	***	NS

RC: *Rapid chilling,* ***CC:*** *Conventional chilling; * P<0.05, ** P<0.01, *** P<0.001*

Table 2. *Effect of RC and PS on the water holding capacity (WHC), cooking loss (CL), and shear force (SF) of lamb meat*
Tablo 2. *Kuzu etinin RC ve PS'nin su tutma kapasitesi (WHC), pişirme kaybı (CL) ve kesme kuvveti (SF) üzerine etkisi*

Attribute	Storage (days)	n	Groups				P
			RC/AS	RC/PS	CC/AS	CC/PS	
CL (%)	2	10	25.93±0.28^{bA}	25.87±0.13^{bA}	26.77±0.26^{aA}	26.59±0.30^{aA}	***
	7	10	23.94±0.14^{bB}	23.67±0.09^{bB}	25.52±0.14^{aB}	25.32±0.27^{aB}	***
	P	20	***	***	***	**	
WHC (%)	2	10	13.91±0.16^{bB}	13.90±0.13^{b}	14.10±0.11^{a}	14.09±0.17^{a}	***
	7	10	13.78±0.21^{bA}	13.77±0.21^{b}	13.95±0.17^{a}	13.87±0.21^{a}	*
	P	20	NS	NS	NS	NS	
SF (kgf)	2	10	3.94±0.10^{a}	3.85±0.10^{a}	3.73±0.14^{b}	3.33±0.15^{b}	***
	7	10	3.92±0.13^{a}	3.74±0.10^{b}	3.61±0.15^{b}	3.28±0.08^{c}	***
	P	20	NS	NS	NS	NS	

a, b, c: *Means within a row with different letters are significantly different (P<0.05);* ***A, B:*** *Means within a column with different letters are significantly different (P<0.05); * P<0.05, ** P<0.01, *** P<0.001*

Table 3. *Effect of RC and PS on color values*
Tablo 3. *RC ve PS'nin renk değerleri üzerine etkisi*

Attribute	Storage (days)	n	Groups				P
			RC/AS	RC/PS	CC/AS	CC/PS	
Lightness (*L**)	2	10	31.13±0.53^{b}	31.11±0.44^{b}	33.92±0.49^{a}	33.69±0.22^{a}	***
	7	10	35.94±0.99	36.38±0.73	36.71±0.45	36.65±0.34	NS
	P	20	**	***	**	***	
Redness (*a**)	2	10	12.42±0.89^{A}	13.42±0.52^{A}	11.77±0.91	12.71±0.59^{A}	NS
	7	10	10.50±0.23^{B}	10.61±0.30^{B}	10.38±0.27	10.63±0.37^{B}	NS
	P	20	*	***	NS	**	
Yellowness (*b**)	2	10	12.60±0.38	12.09±0.27^{B}	12.27±0.26^{B}	12.32±0.42	NS
	7	10	13.94±0.29	13.97±0.28^{A}	13.69±0.22^{A}	13.14±0.21	NS
	P	20	NS	**	**	NS	

a,b,c: *Means within a row with different letters are significantly different (P<0.05);* ***A, B:*** *Means within a column with different letters are significantly different (P<0.05) ; * P<0.05, ** P<0.01, *** P<0.001*

rate of temperature decline. Bendall [11] found that muscle temperature less than 10°C are susceptible to cold shortening when muscle pH less than 6.2 is reached and at 16°C cold shortening is less severe. In the present study, the temperature in RC and CC muscles were above 16°C at 4 h and 10°C at 8 h. It was implied that the chilling conditions had decreased the risk of cold shortening. In another study, Bowater [22] reported that beef and lamb carcasses must reach to the internal temperatures of 7°C after 24 h or before moving the carcass to the boning room.

Reduction in CL and WHC values because of RC is validated by earlier studies [9,23]. In another studies, it was concluded that RC had no effect on WHC and CL [24]. These differences may be originated from the different chilling temperature and wind velocity. PS had lower WHC and CL than AS at 2 and 7 days of post-mortem. However, no statistically significant differences were found (P>0.05) between the groups, which indicated that suspension methods had no impact on WHC and CL. Similar results were reported by Fisher et al.[25] and Claus et al.[26]. The results confirmed that WHC and CL depend on the ultimate pH value [27]. In contrary, Ahnstrom et al.[14] stated that PS significantly improved WHC by reducing the losses during storage. The differences could be attributed to the cooking method.

SF values were lower in CC carcasses than RC carcasses in the present study *(Table 2)*. The results showed that the temperature treatment has a powerful effect on tenderness. This is in agreement with previous studies [12,28]. However Li et al.[9] observed no significant difference between the chilling treatments. Differences may be due to variations in animal species and breed. On the other hand, SF values were lower in pelvic suspended carcasses, but significant differences were found only at 7 day post-mortem (P<0.001). Derbyshire et al.[29] stated that SF values were lower in pelvic suspended carcasses at aged for 7 day, but significant differences were found only at 24 h. Ahnstrom et al.[14] reported that PS reduced SF of *M. semimembranosus* from 67.7 to 53.3 N in bull carcasses.

No significant differences were observed in *a** and *b** values between RC and CC carcasses in this study, which showed similar findings with the reports of Bowling et al.[30] and Janz et al.[21]. Pearson and Dutson [31] also reported that a decrease of the free water on the cell surface give the meat darker appearance. On the other hand, no effect on *L** was found by several authors [32.33]. Additionally, colour parameters were not affected by using PS (P>0.05) *(Table 3)* similarly to those were found by Fisher et al.[25] and Claus et al.[26]. The similar colour values belong to PS and AS could be due to the same rate of pH decline at rigor.

The results of this study showed that CC accelerated the rate of pH decline and RC increased the temperature decline. PS had no impact on WHC, CL and colour of lamb meat. PS significantly decreased SF values only at 7 days of post-mortem. In conclusion, PS is a useful method for improving tenderness of LM during storage period and the disadvantageous effect of RC on SF could be equalized by using PS.

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RESEARCH ARTICLE

The Effect of Estrous Cycle on Oxidant and Antioxidant Parameters in Dairy Cows [1]

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Summary

Reactive oxygen species have fundamental roles in reproductive functions. To comprehensively evaluate the relation between reactive oxygen species and infertility, physiological variations across the estrous cycle in healthy cows have to be known. For this purpose 25 healthy multiparous Holstein dairy cows having regular estrous cycles were used. The estrous cycles were synchronized by ovsynch protocol. Oxidant [lipid hydroperoxide (LOOH), total oxidant status (TOS), oxidative stress index (OSI)], antioxidant parameters [total antioxidant status (TAS), total free sulfhydryl groups (SH), ceruloplasmin (CP), paraoxonase-1 (PON1), arylesterase (ARE), uric acid (UA)], lipid profile and progesterone levels were assayed at estrus, metestrus, diestrus and proestrus stages of the estrous cycle in the plasma samples. The plasma levels of oxidant (LOOH, TOS and OSI) and antioxidant (TAS, SH and UA) parameters were significantly decreased during the luteal phase compared to the follicular phase (at proestrus and at estrus) of the estrous cycle. There was also a significant positive correlation between TAS and TOS. The activity of PON1 and ARE significantly increased only at diestrus. Levels of high density lipoprotein, low density lipoprotein and total cholesterol elevated during the follicular phase (estrus) and declined during the luteal phase. In conclusion, oxidant/antioxidant status and lipid profile were affected by cyclic changes. Moreover, antioxidant defense system showed adaptive response to increased oxidative activities by occurring parallel increases and it may indicate that there is a dynamic balance between oxidant and antioxidant status during the estrous cycle in healthy cows.

Keywords: *Cow, Estrous cycle, Lipid peroxide, Lipid profile, Total antioxidant status, Total oxidant status*

Sütçü İneklerde Östrus Siklusunun Oksidan ve Antioksidan Parametreler Üzerine Etkisi

Özet

Reaktif oksijen türlerinin üreme fonksiyonları üzerinde önemli rolleri vardır. Sağlıklı süt sığırlarında reaktif oksijen türleri ve infertilite arasındaki ilişkiyi kapsamlı bir şekilde değerlendirmek için östrus siklusu boyunca reaktif oksijen türü seviyelerindeki değişimlerin bilinmesi gerekir. Bu çalışmada düzenli siklus gösteren 25 adet sağlıklı Holştayn ırkı sütçü inek kullanıldı. Östrus siklusları ovsynch protokolü ile senkronize edildi. Östrus siklusunun proöstrus, östrus, metaöstrus ve diöstrus dönemlerinde kan plazmasında oksidan parametreler [lipit hidroperoksit (LOOH), total oksidan seviye (TOS), oksidatif stres indeksi (OSI)], antioksidan parametreler [total antioksidan kapasite (TAS), total serbest sülfhidril gruplar (SH), seruloplazmin (CP), paraoksonaz-1 (PON1), arilesteraz (ARE), ürik asit (UA)], lipit profili ve progesteron seviyesi ölçüldü. Foliküler dönemle karşılaştırıldığında luteal dönemdeki plazma oksidan (LOOH, TOS, OSI) ve antioksidan (TAS, SH, UA) seviyelerinin önemli derecede düştüğü görüldü. Ayrıca TAS ve TOS değerleri arasında önemli bir pozitif korelasyon vardı. Paraoksonaz-1 ve ARE aktiviteleri sadece diöstrus döneminde anlamlı olarak arttı. Foliküler dönemde (östrus) artan yüksek yoğunluklu lipoprotein, düşük yoğunluklu lipoprotein ve total kolesterol seviyeleri luteal dönemde düştü. Sonuç olarak, oksidan/antioksidan denge ve lipit profili östrus siklusu sırasındaki değişikliklerden etkilenmiştir. Ayrıca, antioksidan savunma sistemi artan oksidatif strese karşı paralel artışlar göstererek uygun savunma cevapları oluşturmuştur. Bu da sağlıklı ineklerde östrus siklusu boyunca oksidan ve antioksidanlar arasında dinamik bir dengenin olabileceğini göstermektedir.

Anahtar sözcükler: *İnek, Östrus siklusu, Lipit peroksit, Lipit profili, Total antioksidan kapasite, Total oksidan seviye*

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INTRODUCTION

Many stress factors including heat stress and high milk yield are impairing the reproductive efficiency and performance of dairy cows. A common denominator of the responses to these stresses is the redox homeostasis. The redox homeostasis is maintained by the balance between the production of reactive oxygen species (ROS) and antioxidant defense system [1]. Oxidative stress occurs when the generation of ROS exceeds the scavenging capacity of antioxidants, either due to excessive ROS production or an inadequate availability of antioxidants. At physiological levels, ROS is required for reproductive functions including oocyte maturation, folliculogenesis, ovarian steroidogenesis, luteolysis, ovulation and cyclical endometrial changes [2]. However ROS, higher than physiological levels, contribute to several pathological conditions, such as retained placenta, udder oedema, mastitis, infertility which in turn may impair reproductive performances [3].

The estrous cycle is associated with several metabolic and hormonal variations. The mechanisms that relate ROS with female fertility are not completely understood. Investigation of the relationships between ROS and female fertility and reproductive outcomes may be masked by normal menstrual cycle variation [4]. In order to comprehensively evaluate the relation between ROS and infertility, physiological variations across the normal estrous cycle in healthy cows should be known. Up to date, few studies concerning to the effect of stages estrous cycle on the oxidative status on plasma in cows have been reported [5,6]. However, oxidant and antioxidant parameters were investigated as single parameters during the estrous cycle in these studies. Furthermore, effects of cyclic changes on plasma cumulative action of antioxidants (Total Antioxidant Status, TAS) and oxidants (Total Oxidant Status, TOS and Oxidative Stress Index, OSI) have not also been determined.

Lipids are one of the most susceptible substrates to ROS damage and biomarkers of lipid peroxidation are considered the best indicators of oxidative stress [7]. Especially, the compositional properties of low density lipoprotein cholesterol (LDL-C; e.g., lipid classes, fatty acids, antioxidants) relevant for its susceptibility to oxidation. In contrast, plasma high density lipoprotein cholesterol (HDL-C) particles exert potent antioxidant activity, which protects LDL-C against oxidative stress [8]. In addition, HDL-C particles transport enzymes exerting antioxidant activity, including paraoxonase (PON), an enzyme produced by the liver [9]. Paraoxonase has three known enzymatic molecules, including PON1, arylesterase (ARE), and dyazoxonase. Paraoxonase-1 hydrolyzes organophosphates such as paraoxon, hydrolyzes aromatic esters such as phenylacetate, and also decreases the accumulation of lipid peroxidation products. Serum PON1 acts in conjunction with ARE to function as a single enzyme having lipophilic antioxidant characteristics [10]. To our best knowledge, PON1 and ARE activities in cow plasma has not been evaluated during the estrous cycle until now.

In light of previous literatures, to be known physiological variations on oxidant/antioxidant parameters and lipids profile during the estrous cycles in healthy cows may help to understanding infertility problems. Therefore, this study was aimed to comprehensively evaluate oxidant/antioxidant status at different stages of the estrous cycle (at follicular phase: proestrus and estrus, and luteal phase: metestrus and diestrus) in cows by measuring cumulative indicator such as TAS, TOS, OSI and single parameters such as lipid hydroperoxide (LOOH), ceruloplasmin (CP), total free sulfhydryl groups (SH), uric acid (UA), PON1 and ARE in plasma. In addition, the relationship between oxidative stress and lipid profile throughout the estrous cycle was also examined.

MATERIAL and METHODS

Animal, Location and Experimental Protocol

All animal experimental procedures were approved by Harran University Animal Experimentation Local Ethics Committee (38/6, 13.01.2012). A total of 25 healthy multiparous Holstein cows having regular estrous cycles, aged 4-5 years, weighing 500±100 kg, in their 2nd to 4th lactation, at least 45 days postpartum were selected from a private dairy farm located in Sanliurfa province (southeastern Turkey) during the May and June. The animals were housed in a free stall barn, milked twice daily, and fed the same diets as total mixed ration.

Ovarian activities were checked by using transrectal ultrasonography (Pie Medical Scanner 100LC, The Netherlands) before estrous synchronization. The estrous cycle of cows were synchronized by Ovsynch protocol [11] consisting of 10 µg Buserelin acetate (Receptal®, Intervet, Turkey; 0. day), 500 µg cloprostenol sodium (Estrumate®, Intervet, Turkey; 7. day) and 10 µg Buserelin acetate (9. day) injections intramuscularly. The second 10 µg Buserelin acetate injection day were accepted as day 0 of the estrous cycle. Blood samples were collected from the jugular vein into evacuated 10 ml tube containing heparin on the 0 day (at estrus), 2 day (at metestrus), 16 day (at late diestrus) and 18 day (at proestrus). The ovarian ultrasonography was also used to confirm the stages of the estrous cycle. Plasma samples were immediately frozen and stored at -80°C, until analyzes.

Assay

Oxidative and antioxidative parameters were measured in the plasma samples by using the Aeroset automated analyzer (Abbott, IL, USA) and spectrophotometer (Cecil 3000, UK). Plasma lipid hydroperoxide level was evaluated

by the fluorimetric method based on the reaction between malondialdehyde and thiobarbituric acid [12]. Briefly, plasma was added to diethylthiobarbituric acid reagent in phosphate buffer and mixed at 95°C. Samples were placed in ice for 5 min and then added 5 ml of butanol. Fluorescence of the butanol extract was measured at excitation wavelength of 539 nm and emission wavelength of 553 nm.

Total oxidant status was measured using an automated colorimetric measurement method developed by Erel [13]. In this method, oxidants in the plasma sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The ferric ion builds a colored complex with xylenol orange in an acidic medium. The color intensity is measured spectrophotometrically.

Total antioxidant status was measured according to method of Erel [14]. In this method, the hydroxyl radical reacts with O-dianisidine to produce the colored dianisyl radical. Upon the addition of a plasma sample, the reactions initiated by hydroxyl radical are suppressed by the antioxidant components of the plasma, preventing the color change. Oxidative stress index, an indicator of the degree of oxidative stress, was calculated by using following formula; OSI = [(TOS, μmol/L)/(TAS, (mmol Trolox Eq./L) x 10]

Total free sulfhydryl groups of plasma was assayed according to the method of Hu et al.[15]. Briefly, plasma and buffer solution was added to a spectrophotometer cuvette followed by the addition of 5.5'-dithio-bis 2-nitrobenzoic acid (DTNB). Samples without DTNB were run for each sample as blanks. Following incubation for 15 min, absorbance was read at 412 nm. Ceruloplasmin activity was measured according to the method of Erel [16] based on the enzymatic oxidation of ferrous ion to ferric ion.

Paraoxonase activity was measured by using paraoxon substrate. The rate of paraoxon hydrolysis was measured by monitoring the increase of absorbance at 412 nm at 25°C [17]. Arylesterase activity was analyzed according to method of Haagen and Brock [18].

Plasma total cholesterol (TC), HDL-C and triglyceride (TG) levels were measured by an autoanalyzer (Aeroset, Abbott, IL, USA) using commercial kits (Abbott, IL, USA). Plasma concentration of LDL-C was calculated using the Friedewald equation [19]. Level of uric acid was measured by using commercial kits (Olympus, AU). Plasma progesterone concentration was determined with a validated electrochemiluminescence method using Roche commercially kits in autoanalyzer (Roche Elecsys E170, IN, USA).

Statistical Analysis

Statistical analysis was carried out with SPSS software 10.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed using General Linear Model for repeated measures and Bonferroni test to determine the differences phases of the estrous cycle. Pearson correlation test was used for determination of correlations among oxidant, antioxidant and lipid parameters. Data were given as means and standard deviation (SD). The differences were considered to be significant when $P<0.05$.

RESULTS

Significant cycle-dependent changes were observed on some parameters related with oxidant/antioxidant status and lipid profile during the estrous cycle in cows *(Fig. 1-4)*. The plasma levels of oxidant parameters such as LOOH and the cumulative indicator of oxidative status including TOS, OSI were significantly decreased ($P<0.05$) during the luteal phase of the estrous cycle (at metestrus and diestrus) *(Fig. 1,2)*. In addition, there were significantly positive correlations between TOS and OSI ($r=0.92$, $P<0.01$) or LOOH ($r=0.68$, $P<0.01$; *Table 1*). On the other hand, antioxidant parameters (SH, UA and TAS) were also decreased during the luteal phases of the estrous cycle *(Fig. 1,2)*. Interestingly, similar fluctuations were observed both oxidant and antioxidant parameters as mentioned above. There was also a significant positive correlation between TAS and TOS ($r=0.55$, $P<0.01$). Activity of CP did not change during the estrous cycle *(Fig. 3)*. The activity of PON1 and ARE significantly increased only at diestrus ($P<0.05$) *(Fig. 3)*. There was also a significant correlation between PON1 and ARE ($r=0.96$, $P<0.01$). On the contrary to our expectation, no significant correlations were observed between HDL-C and PON1-ARE activities ($P>0.05$).

Lipoprotein cholesterol levels were observed to change over the estrous cycle. Levels of TC, HDL-C and LDL-C elevated the highest during the follicular phase of estrous cycle and declined during the luteal phase *(Fig. 4)*. The changes in TC levels were accompanied by a change in the levels of the HDL-C ($r=0.79$, $P<0.01$) and LDL-C ($r=0.96$, $P<0.01$) respectively). TG levels did not vary throughout the estrous cycle. Moreover, the concentration of plasma progesterone showed a normal pattern according to the stage of the estrous cycle increasing until day 16, and then decreasing rapidly during luteal regression.

DISCUSSION

The present study was conducted to evaluate the possible variations in oxidant/antioxidant parameters and the related factors such as lipid profile which affect these parameters during the estrous cycle in lactating dairy cow. To minimize individual animals effects on those parameters, each animal were used for all studied stages of the estrous cycle. Furthermore, the stages of estrous cycle in cow were defined by both their ovarian ultrasonography and plasma progesterone level.

Oxidative stress were evaluated by measuring the levels

Table 1. *The correlation between some oxidant and antioxidant parameters and progesterone levels during the estrous cycle in dairy cows*
Tablo 1. *Sütçü ineklerde östrus siklusu boyunca bazı oksidan ve antioksidan parametreler ile progesterone seviyesi arasındaki korelasyonlar*

Parameters	TAS	TOS	OSI	LOOH	SH	CP	ARE	PON	UA	TG	TC	HDL	LDL
TOS	0.55**												
OSI	0.18	0.92**											
LOOH	0.58**	0.68**	0.53**										
SH	0.30**	0.48**	0.44**	0.33**									
CP	0.14	0.14	0.10	0.09	0.02								
ARE	-0.30**	-0.08	0.04	-0.17	0.37**	0.08							
PON	-0.35**	-0.08	0.07	-0.17	0.40**	0.02	0.96**						
UA	0.79**	0.56**	0.30**	0.59**	0.23*	0.11	-0.21*	-0.23*					
TG	-0.19	0.04	0.15	0.02	0.31*	0.05	0.26*	0.33**	-014				
TC	0.48**	0.07	-0.14	0.23*	-0.15	-0.09	-0.36**	-0.43**	0.42**	-0.46**			
HDL-C	0.57**	0.14	-0.09	0.28*	-0.07	0.05	-0.30**	-0.39**	0.51**	-0.48**	0.79**		
LDL-C	0.38**	0.02	-0.15	0.17	-0.19	-0.15	-0.35**	-0.41**	0.32**	-0.44**	0.96**	0.62**	
P	-0.41**	-0.38**	-0.27**	-0.38**	-0.22*	-0.1	0.11	0.08	-0.48**	0.02	-0.09	-0.18	-0.04

P<0.05, ** P<0.01,* ***TAS:* *Total antioxidant status;* ***TOS:*** *Total oxidant status;* ***OSI:*** *Oxidative stress index;* ***LOOH:*** *Lipid hydroperoxide;* ***SH:*** *Total free sulfhydryl groups;* ***CP:*** *Ceruloplasmin;* ***ARE:*** *Arylesterase activity;* ***PON:*** *Paraoxonase activity;* ***UA:*** *Uric acid;* ***TG:*** *Triglyceride;* ***TC:*** *Total cholesterol;* ***HDL-C:*** *High density lipoprotein cholesterol;* ***LDL-C:*** *Low density lipoprotein cholesterol;* ***P:*** *Progesterone*

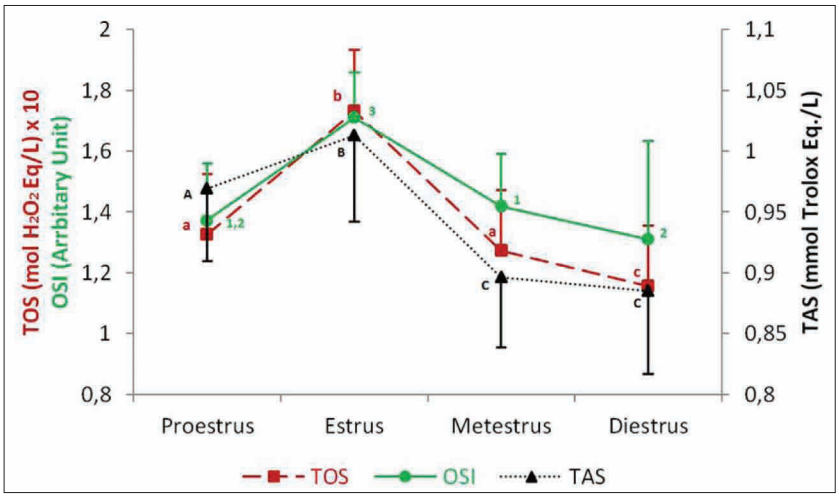
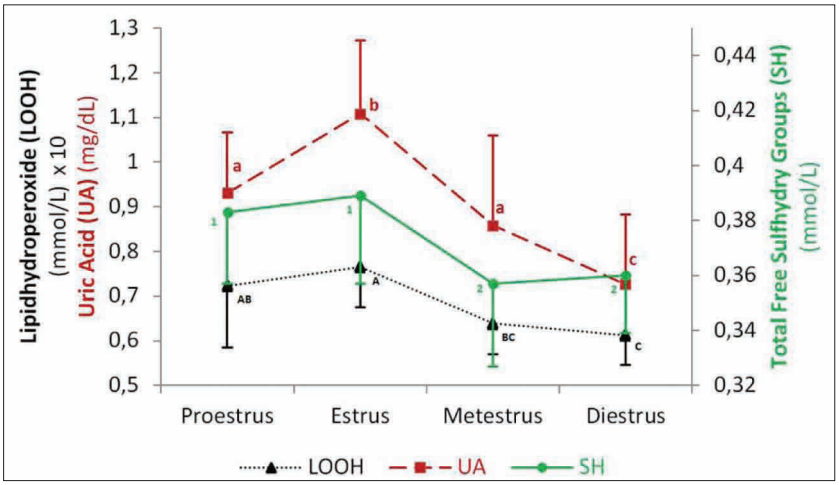


Fig 1. Total Oxidative status (TOS), total antioxidant status (TAS) and oxidative stress index (OSI) parameters during the estrous cycle in dairy cows (mean±SD). a,b: Means with different superscripts on same parameter are statistically different (P<0.05)

Şekil 1. Sütçü ineklerde östrus siklusu boyunca total oksidan stress (TOS), total antioksidan kapasite (TAS) ve oksidatif stres indeksi (OSI) parametreleri (ortalama±SD). a,b: Aynı parametre üzerindeki farklı harfli ortalamalar istatistiksel olarak farklıdır

Fig 2. Lipid hydroperoxide (LOOH), uric acid (UA) and total free sulfhydryl groups (SH) parameters during the estrous cycle in dairy cows (mean±SD). a,b: Means with different superscripts on same parameter are statistically different (P<0.05)

Şekil 2. Sütçü ineklerde östrus siklusu boyunca lipit hidroperoksit (LOOH), ürik asit (UA) ve total serbest sülfhidril grup (SH) parametreleri (ortalama±SD). a,b: Aynı parametre üzerindeki farklı harfli ortalamalar istatistiksel olarak farklıdır



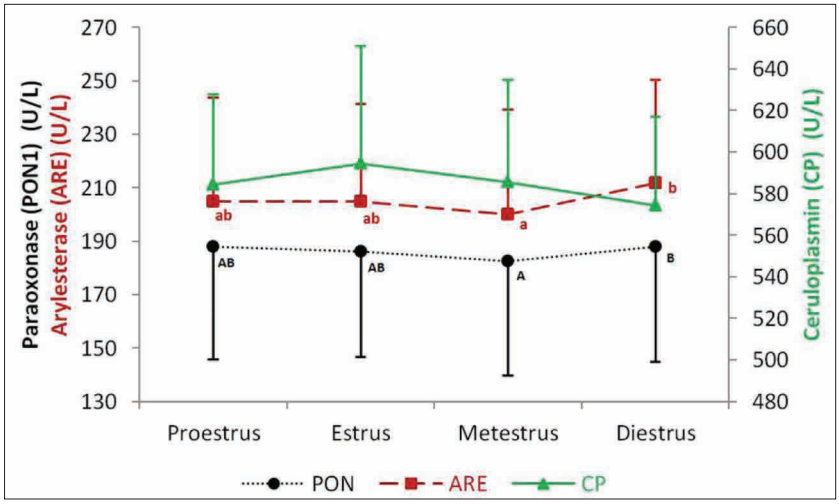
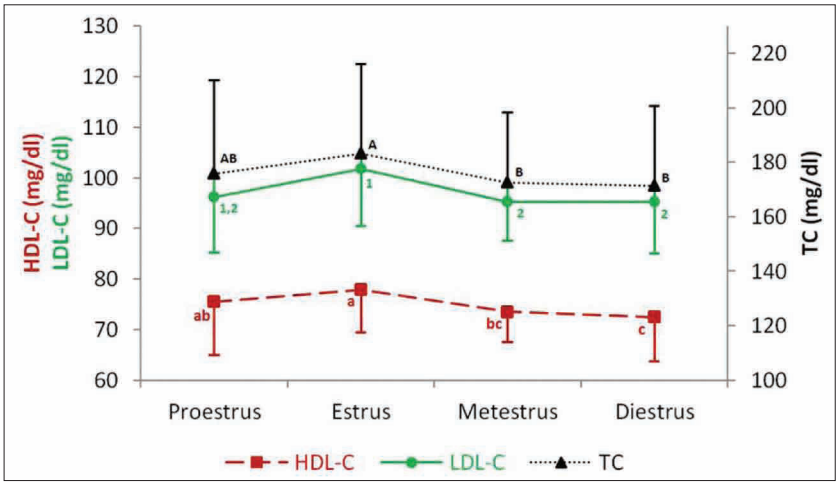


Fig 3. Paraoxonase (PON), arylesterase (TAS) and ceruloplasmin (CP) activities during the estrous cycle in dairy cows (mean±SD). a,b: Means with different superscripts on same parameter are statistically different (P<0.05)

Şekil 3. Sütçü ineklerde östrus siklusu boyunca paraoksonaz (PON), arilesteraz (ARE) ve serulo-plazmin (CP) parametreleri (ortalama±SD). a,b: Aynı parametre üzerindeki farklı harfli ortalamalar istatistiksel olarak farklıdır

Fig 4. Total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) levels during estrous cycle in dairy cows (mean±SD). a,b: Means with different superscripts on same parameter are statistically different (P<0.05)

Şekil 4. Sütçü ineklerde östrus siklusu boyunca total kolesterol (TC), yüksek yoğunluklu lipoprotein kolesterol (HDL-C) ve düşük yoğunluklu lipoprotein kolesterol (LDL-C) parametreleri (ortalama±SD). a,b: Aynı parametre üzerindeki farklı harfli ortalamalar istatistiksel olarak farklıdır



of lipid peroxidation as a single, TOS and OSI as cumulative indicator in the plasma. The measuring of TOS considers the cumulative action of all the oxidants present in plasma and body fluids, thus providing an integrated parameter rather than the simple sum of measurable oxidant [3]. It was observed that both LOOH and TOS levels increased the luteal phase of the estrous cycle (P<0.05). Some studies suggest that dynamic changes in metabolism and energy consumption in many organs can generate byproducts on an extraordinary scale in the estrus phase of the estrous cycle [20]. Among these byproducts, ROS are generated during the physiological process of oxygen consumption [21]. In our study, an increase on oxidative stress in plasma during the follicular phase of the estrous cycle may be closely related to the oxygen demand as a result of cell growth and synthesis activity [20]. In addition, lipids, especially due to compositional properties of LDL-C, are one of the most susceptible substrates to lipid peroxidation [22]. Because of LDL-C susceptibility to oxidation, its high level may be a reason of increased oxidative stress at the follicular phase of the estrous cycle. As for total antioxidant status, it considers the cumulative action of all the antioxidants present in plasma and body fluids, thus providing an integrated parameter rather than the simple sum of measurable antioxidant [23]. We observed that TAS levels in the luteal phase were significantly lower (P<0.05) than the follicular phase *(Fig. 1)*. Among the other antioxidants examined the concentrations of UA and SH showed similar pattern to those of TAS, peaked during the follicular phase and decreased during the luteal phase of the estrous cycle *(Fig. 1)*. Specially, there was a high significant correlation between TAS and UA (r=0.79, P<0.01). Uric acid is a well-known antioxidant that contributes significantly to the plasma antioxidant capacity. These findings are in accordance with previous studies reporting that UA is contributing to approximately 70% of plasma total antioxidant capacity, when measured by FRAP method [24]. In addition, a negative correlation was observed between UA and progesterone level (r= -0.48, P<0.01) in the present study. The lower UA level in the luteal phase of the estrous cycle may be result from that progesterone increases glomerular filtration rate and urinary UA clearance [25]. Total free sulfhydryl groups are mainly responsible for antioxidant response to oxidative stress [26]. In our study, the lower SH level in the follicular phase of the estrous cycle due to elevated oxidative stress compared to the luteal phase of the estrous cycle (P<0.05) seems reasonable and predictable. The activity

of ceruloplasmin, acute phase protein, also shown at tend to be lower profile (P>0.05) during the luteal phase of the estrous cycle and to elevate during the follicular phase of the estrous cycle like other antioxidant parameters *(Fig. 3)*. Similarly, Fox et al.[27] also observed the elevation in plasma CP ferroxidase activity during the follicular phase and they indicated that it may be induced by oxidative stress.

The activity of PON1 and ARE significantly increased only at late diestrus (P<0.05) *(Fig. 3)*. There was also a significant correlation between PON1 and ARE (r=0.96, P<0.01) like the report of Miyamoto et al.[28]. On the other hand, there were no correlations between activities of PON1 or ARE and TAS or HDL-C levels (P>0.05). This result is somewhat surprising because it would be expected that the antioxidant property of PON1 is responsible for the antioxidant action of HDL-C [9] which correlated with TAS (r=0.57, P<0.01) in present study.

As for lipid profile, it undergoes cyclic changes during the estrous cycle in this study *(Fig. 4)*. This pattern is characterized by a marked reduction TC level during the luteal phase of the estrous cycle in plasma as reported earlier by Mumford et al.[29]. The changes in TC levels were accompanied by a change in the levels of the HDL-C and LDL-C (r=0.79, r=0.96; P<0.01 respectively; *Table 1*), whereas TG levels varied no significantly throughout the estrous cycle. Since cholesterol is the precursor of ovarian steroids, lower TC level during luteal phase may be a result of enhanced utilization by steroidogenesis [30].

Interestingly, both oxidant and antioxidant parameters shown similar fluctuations as mentioned above. Moreover, there was a significant positive correlation between TAS and TOS values (r=0.55, P<0.01). The correlation of some antioxidants with oxidative status indicates that anti-oxidative mechanisms are activated to cope with oxidative stress in physiologic conditions. On the other word, an increased antioxidant capacity in plasma may not necessarily be a desirable condition if it reflects a response to increased oxidative stress, or vice versa in physiologic condition such as the estrous cycle [24].

As a result, cyclic changes in oxidant/antioxidant status and lipid profile during the estrous cycle were shown by the present study in lactating cows. Especially antioxidant defense system (TAS, SH and UA) showed adaptive response to increased oxidative activities (TOS and LOOH) during the follicular phase of the estrous cycle and it may indicate that there is a dynamic balance between oxidant and antioxidant status in healthy cows during the estrous cycle. Taken together, these findings suggest the stage of the estrous cycle should always be taken into account when evaluating oxidative status and lipoprotein cholesterol levels in cyclic cows in order to improve interpretation in clinical settings and in future research. It would also indicate a need for studying the nutritional, metabolic and endocrine features that regulate this relationship.

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RESEARCH ARTICLE

The Differentiation of Neuronal Cells from Mouse Embryonic Stem Cells [1][2]

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Özet

With new technologies emerging today, the importance of stem cells in the cell therapy of nervous system diseases is supported by recent studies. Therefore, the development of neuronal cell differentiation protocols from stem cells is of great importance. In our study, the differentiation of neuronal and neuroglial cells from mouse embryonic stem (ES) cell line and their analysis with neuronal cell markers are aimed. Mouse ES cells were differentiated to neurogenic series cells by adding N2 and bFGF to the culture medium on coated Fibronectin dishes. For the identification of differentiated cells, they were evaluated by light microscopy using immunhistochemistry techniques and by electron microscopy. Indirect immunohistochemical staining method was performed with SSEA-1 (mouse embriyonic stem cells marker), Nestin (neural precursor cells marker), βIII-Tubulin (neuronal cells marker), MAP-2 (neuronal cells marker), GFAP (astrocyte marker), and O4 (oligodendrocyte marker). After 1 week of differentiation of cells, immunoreactivities of SSEA-1 and Nestin were detected to be negative and moderate, respectively. After 2 weeks culture time, the differentiation was still continuing and especially positive immunoreactivities of β-III Tubulin and MAP-2 and weak immunoreactivities of O4 and GFAP were supported neuronal differentiation. In conclusion, our results suggest that neuronal cell derived from mouse ES cells were differentiated particularly to neuron using N2+bFGF+fibronectin culture condition. Therefore, these differentiated cells may be used as a treatment method in degenerative diseases of the nervous system.

Anahtar sözcükler: *Mouse embryonic stem cell, Differentiation, Neuron and neuroglia*

Fare Embriyonik Kök Hücrelerden Nöronal Hücrelerin Farklılaşması

Summary

Günümüzde gelişen yeni teknolojiler sayesinde sinir sistemi hastalıklarının hücresel tedavisinde kök hücrelerinin önemi son yıllardaki çalışmalar ile desteklenmektedir. O nedenle kök hücrelerden nöronal hücrelerin farklılaştırılması protokollerinin oluşturulması büyük önem taşımaktadır. Çalışmamızda, fare embriyonik kök (EK) hücre hattından, nöron ve nöroglial hücrelerin farklılaştırılması ve nöronal hücre belirteçleri ile analizi amaçlanmıştır. Fare EK hücreler, fibronektin kaplı petrilerde kültür ortamına N2 ve bFGF ilavesi ile nörojenik seri hücrelerine farklılaştırıldı. Farklılaşmış hücrelerin tanımlanması için hücreler, immünohistokimya tekniği kullanılarak ışık mikroskobu ile ve elektron mikroskobu ile değerlendirildi. İndirek immünohistokimyasal boyama yöntemi SSEA-1 (fare ES hücre belirteci), Nestin (nöron öncülü hücre belirteci), βIII-Tubulin (nöron hücre belirteci), MAP-2 (nöron hücre belirteci), GFAP (astrosit belirteci) ve O4 (oligodendrosit belirteci) için uygulandı. Hücrelerin farklılaşmasının 1. haftasından sonra SSEA-1 ve Nestin immünoreaktivitesi sırasıyla negatif ve orta saptandı. Kültürün 2. haftasından sonra farklılaşmanın hala devam etmesi ve özellikle β-III Tubulin ve MAP-2 immünoreaktivitesinin güçlü pozitif ve O4 ve GFAP immünoreaktivitesinin zayıf olması nöronal farklılaşmayı desteklemiştir. Sonuç olarak, bizim sonuçlarımız göstermiştir ki fare EK hücrelerinden kaynaklanmış nöronal hücreler, N2+bFGF+fibronektin kullanılan kültür koşullarında özellikle nörona farklılaşmıştırlar. Bu nedenle, farklılaştırılmış bu hücreler dejeneratif sinir sistemi hastalıklarının hücresel tedavisinde kullanılabilir.

Keywords: *Fare embriyonik kök hücre, Farklılaşma, Nöron ve nöroglia*

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INTRODUCTION

The differentiation of neuronal cells from stem cells plays an important role in the cell therapy of nervous system diseases, including neurodegenerative disorders, multiple sclerosis, neurotrauma and neurodevelopmental disorders [1]. Recent clinical trials of regenerative therapy for neuronal disease have been performed with transplantation of stem cells [2]. Stem cells using neuronal disease models; somatic (adult derived) stem cells, fetal stem cells and ES cells, induced pluripotent stem (iPS) cells [3]. Numerous studies of transplantation of somatic stem cells using neuronal disease models have been reported and most studies have confirmed its efficiency in repairing these [2]. The transplantation effect of adipose derived mesenchymal stem cells has been reported to be greater than that of bone marrow mesenchymal stem cells [2]. iPS cells have been shown to differentiate towards a neuronal phenotype leading to functional improvement after their application in animal models of Parkinson's disease, stroke and other pathologies [3]. Fetal stem cells demonstrate higher proliferation, more specific differentiation, and better migration after transplantation. However, the use of these cells is limited [3,4]. ES cells are pluripotent stem cells [5]. Studies have shown that they can proliferate undifferentiated *in vitro* [6]. These cells have been shown to proliferate without differentiation in suitable culture medium, but changing the environment may lead these cells to differentiate [7-9]. Furthermore, ES cells have been reported to generate all three neural lineages: neurons, astrocytes and oligodendrocytes [10-12]. Several approaches have been used to achieve in vitro neural differentiation starting from ES cells, aimed at generating specified neural progenitors and/or differentiated neuronal and glial subtypes [10,13]. Differentiation of stem cells to neurons has usually been achieved by protocols that enrich ectoderm derivatives, as a first step in the production of neural progenitors [12,14]. These attempts to influence cell fate decisions in order to obtain cells of the ectodermal layer have utilized neuralizing signals derived from feeder cells, addition of growth factors or growth supplements to the medium [12,15-17].

In this study, neural cell differentiation from mouse embryonic stem cells was indicated by neural and glial cells specific markers. Differentiated neuronal cells were identified with stage specific embryogenic antigen-1 (SSEA-1), nestin, βIII-Tubulin, microtubule associated protein (MAP-2), glial fibrillary acidic protein (GFAP), and oligodendrocyte 4 (O4**)** antigens. SSEA-1 is a carbohydrate antigen that can be identified in mouse and human embryonic stem cells. SSEA-1 is positive in preimplantation stage of mouse embryos while it is lost during differentiation [18]. Nestin is type VI intermediate filament protein and is synthesized in dividing cells during early development, especially in primitive neuroepithelium. Nestin is expressed by neuronal precursor cells. They are down regulated with differentiation of neuronal stem cells [19,20]. MAP-2 is responsible for microtubule formation and shaping dendritic in neurogenesis [21,22]. βIII-Tubulin is widely as an early neuronal cytoskeleton marker in neuronal development studies [23,24]. GFAP is a specific intermediate filament protein that is expressed by numerous cell types of central nervous system. It is associated with many cellular functions, such as cellular structure and movement, in neuronal cells [25-27]. O4 has been commonly used as the earliest recognized marker specific for the oligodendroglial lineage [28].

Recently, in diseases of the nervous system, many studies have been done regarding stem cell therapy. Therefore, the forming nerve cell production protocols from stem cells is essential.This study is important in constituting a study protocol for nerve cells derived from embryonic stem cell in different time periods in medium containing basic fibroblast growth factor (bFGF) and N2 on fibronectin coated plates.

MATERIAL and METHODS

The study protocols and experimental procedures were approved by the Celal Bayar University Scientific Ethics Committee (0259).

Embryonic Stem Cell Culture and Neuronal Differentiation

Mouse embryonic stem cell line (CGR8, 07032901, ECACC, Salisbury, UK) was purchased from the Celal Bayar University Medical Faculty, Histology and Embryology Department. For the feeder layer, mouse fibroblast cells (STO, 86.032.003, ECACC, Salisbury, UK) were used. When the STO cells became confluent after 1 week of culture, they were treated with 20 µg/ml of mitomycin-C (A2190, 0002, Applichem, Darmstadt, Germany) for 1.5h at 37°C with 5% CO_2 in air. Incubation of STO cells with mitomycin-C and embryonic stem cell cultures, was performed simultaneously.

Embryonic stem cells were cultured in a humidified atmosphere at 37°C in 5% CO_2 in a medium [Dulbecco's modified Eagle's medium (DMEM, F0445, Biochrom AG, Berlin, Germany) containing 4500 mg/l glucose and sodium pyruvate,15% fetal bovine serum (FBS, S0113, Biochrom AG, Berlin, Germany), 1% L-glutamine (K0283, Biochrom AG, Berlin, Germany), 1% penicillin/streptomycin (A2213, Biochrom AG, Berlin, Germany), 0.1 mM non-essential amino acid (NEAA, K0293, Biochrom AG, Berlin, Germany), 10^{-6} M β-mercaptoethanol (M7522, Sigma-Aldrich, St. Louis, MO, USA) and 1.000 IU/ml leukemia inhibitory factors (LIF, L5158 Sigma-Aldrich St. Louis, MO, USA)] on mitomycin-C treated STO cells. The medium was changed every other day. When the cells were confluent, they were routinely subcultured using trypsin-EDTA solution (L2143, Biochrom AG, Berlin, Germany). The hanging drop

method was used for forming embryoid bodies (EBs) from embryonic stem cells. On the lid of culture dishes, 20 µl drops containing 5×10^4 undifferentiated cells/ml were plated. The lid was inverted and placed over the culture dish filled with sterile phosphate buffered saline (PBS) to prevent the drops from drying [29]. The culture dishes with hanging drops were incubated in a humidified atmosphere at 37°C in 5% CO_2 for 2 days. Embryoid bodies were collected into media and then were transferred into culture dishes. Cells were cultured in a humidified atmosphere at 37°C in 5% CO_2 for 4 days in culture medium without LIF. 1% N2 solution (R&D System, SC005, USA), containing bovine insulin, human transferrin, sodium selenite, putrescine and progesterone, was added into culture media for neural stem cell expansion. When the cells were confluent, they were transferred to 1% bovine fibronectin (Neural Stem Cell Expansion Kit Monolayer Plus System, SC005, USA) coated plates. Fibronectin used to promote cell attachment and spreading. On the seventh day of the culture, some of the cells were fixed for immunohistochemical analysis, while 0.1% bFGF (Neural Stem Cell Expansion Kit Monolayer Plus System, SC005, USA) was added to the remaining cells to maintain the differentiation. These cells were passed through on the seventh, fourteenth and twenty-first days of the culture, and were fixed for immunohistochemical analysis.

Identification of Neuronal Differentiation Using Transmission Electron Microscopy

Cells were collected after 2 weeks of culture period using trypsinization technique. They were fixed with 2.5% glutaraldehyde (16210, Electron Microscopy Sciences, Hatfield, PA). After washing with buffer solution (Buffer A: 0.06 M KH_2PO_4 + Buffer B: 0.08 M Na_2HPO_4) twice, 10 min for each step, the pellets were fixed with 1% osmium tetroxide (R1015, Agar Scientific, Essex, UK) at +4°C for 1 hour. Cells were embedded in 2% agar (A 2114, AppliChem, Darmstadt, Germany) after washing with buffer solution for 10 min. They were dehydrated with propylene oxide and embedded in epon. Thin sections (120 nm) were contrasted with uranyl acetate and lead citrate. They were evaluated under Zeiss LIBRA 120 electron microscope (Oberkochen, Germany).

Identification of Neuronal Differentiation Using Indirect Immunoperoxidase Staining

For immunohistochemical analysis, cells were stained using the indirect immunohistochemical method. The differentiated cells were fixed with 4% paraformaldehyde in PBS at 4°C for 30 min. Subsequently, they were washed with PBS. Endogenous peroxidase activity was quenched with incubation with 3% hydrogen peroxide (H_2O_2, K31355100, Merck, Darmstadt, Germany) for 10 min at room temperature. Cells were then washed with PBS, and incubated on ice for 15 min with 0.1% Triton-X (A4975,0100, Applichem, Darmstadt, Germany) for permeabilization. Afterwards, cells were incubated with blocking solution (K023, DBS, California, USA) for 1 h. Cells were then washed with PBS, and incubated with primary antibodies: anti-SSEA-1 (1:5 dilution, R&D System, MAB2155, USA), anti-Nestin (1:10 dilution, (R&D System, SC 013, USA), anti-βIII-Tubulin (1:10 dilution, R&D System, SC 013, USA), anti-GFAP (1:100 dilution, R&D System, SC 013), anti-O4 (1:10 dilution, R&D System, SC 013, USA), and anti-MAP-2 (1:100 dilution, Gene-Tex, GTX 48032, USA), all for overnight at room temperature in a humidified chamber. After removing the primary antibodies, the cells were incubated with biotinylated IgG (both anti-mouse and anti-goat supplied ready to use by Zymed, San Francisco, CA, USA) for 30 min, followed by three washes in PBS and then with streptavidin-peroxidase conjugate (supplied ready to use by Zymed) for 30 min (Histostain-Plus Bulk Kits; Zymed) and washed with PBS three times. They were incubated with a solution containing 1:9 concentration of diaminobenzidine (supplied ready to use by Zymed) with 0.3% H_2O_2 (Histostain-Plus Bulk Kits; Zymed), 50 µl for each sample, for 5 min to visualize immunolabeling. Samples were then mounted with mounting medium (AML060, Scytek, Logan, UT, USA). Subsequently, they were viewed using an IX71 inverted microscope (Olympus, Tokyo, Japan). The negative controls received the same treatment as described above, but they were incubated with rabbit IgG instead of the primary antisera [29]. Labelling in all negative control cases was negative. Immunolabelling was evaluated semi-quantitatively using an additive immuno reactive score reflecting signal intensity, as negative (–), mild (+), moderate (++) and strong (+++).

Statistical Analysis

Comparable data groups were evaluated using ANOVA. $P<0.05$ was considered significant [30]. Graph-Pad InStat statistic program (GraphPad Software, USA) was used for analyses.

RESULTS

It was observed that mouse embryonic stem cells were round-shaped with bright nuclei and they formed inexplicit-contoured colonies on the confluent mitomycin treated STO *(Fig. 1A, B)*. Both the outer and the inner layer cells were distinguishable within the embryonic bodies formed from the embryonic stem cells after the hanging drop method. It was observed that the outer layer cells were composed of cubic surface ectoderm-like single layer cells significantly *(Fig. 2A)*. The peripheral cells were seen to have proliferated forward as well *(Fig. 2B)*.

For neuronal differentiation, N2 medium was added into culture condition at fourth day of culture. Some of the cells were observed as fusiform-shaped, while the others had round bodies and axon-dendrite-like extensions just like neurons on the 7th day of differentiation *(Fig. 3A)*.

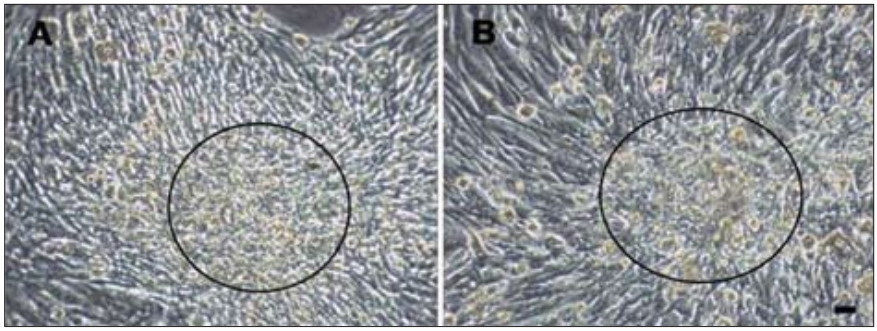
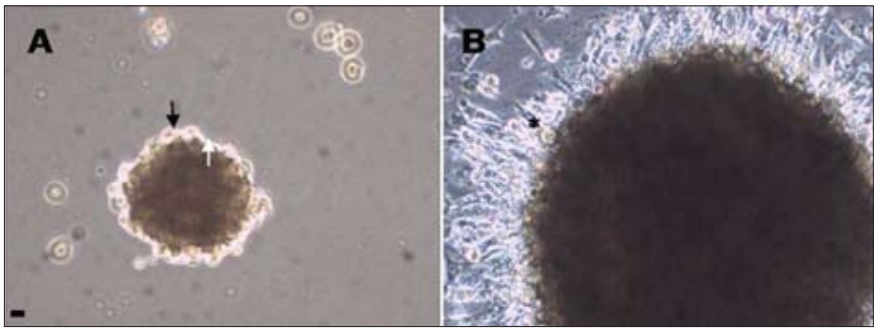


Fig 1. The embryonic stem cell colonies which were marked with the circles on confluent mouse fibroblast cells treated with mitomycin-C **(A, B)**, x200, Scale bar: 25 µm

Şekil 1. Mitomisin-C uygulanmış fare fibro-blast hücreleri üzerindeki daire ile işaretlenmiş embriyonik kök hücre kolonileri **(A, B)**, x200, Ölçek çubuğu: 25 µm

Fig 2. First **(A)** and fourth **(B)** days of culture condition of embryonic bodies. After EB formation, both the outer *(black arrow)* and the inner *(white arrow)* layer cells were distinguishable, and proliferating cells (*) were also observed on the 4th day of the culture time, x100, Scale bar: 25 µm

Şekil 2. Kültürün 1. **(A)** ve 4. **(B)** günündeki embriyonik cisimler. Embriyonik cisim oluşumun-dan sonra hem iç *(siyah ok)* hem dıştaki *(beyaz ok)* hücre tabakası ayırtedilebiliyor, bununla beraber kültürün 4. gününde farklılaşan hücreler (*) de gözleniyordu, x100, Ölçek çubuğu: 25 µm



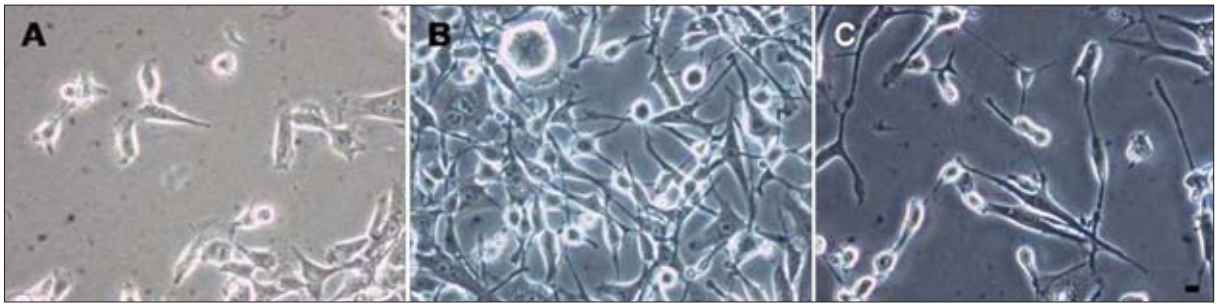


Fig 3. The cells cultured within the N2 added media on the 7th **(A)**, 14th **(B)** and 21st **(C)** days of differentiation, x100 Scale bar: 25 µm

Şekil 3. Farklılaşmanın 7. **(A),** 14. **(B)** ve 21. **(C)** günlerinde kültür ortamına N2 ilavesi ile kültüre edilmiş hücreler, x100, Ölçek çubuğu: 25 µm

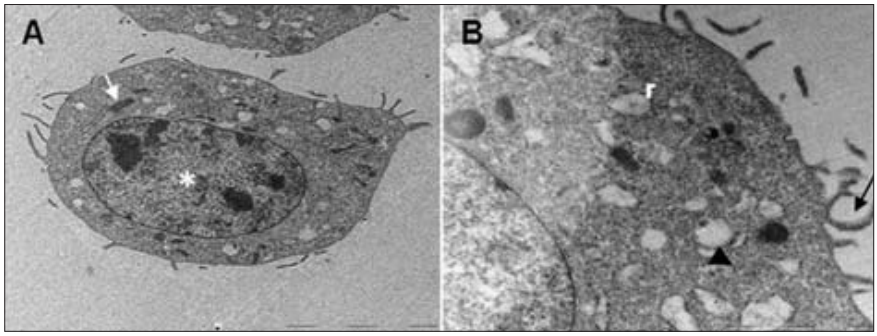


Fig 4. Electron micrograph of differentiated neuron-like cells. **A-** heterochromatin nucleus (*), mitochondria with few cristae *(white arrow)* and polyribosomes (r), Scale bar: 5.000 nm, **B-** Phagocytotic vesicles *(arrow head)* indicative of autophagocytosis, and cross sections of microvilli *(black arrow)*, Scale bar: 5.000 nm

Şekil 4. Farklılaşmış nöron benzeri hücrelerin elektron mikroskobik görüntüsü. **A-** hetero-kromatin çekir-dek (*), bir kaç kristası *(beyaz ok)* olan mitokondri ve poliribozomlar (r). Ölçek çubuğu: 5.000 nm, **B-** Oto-fagositozun göstergesi fagositik kesecikler *(ok başı)* ve mikrovilli kesitleri *(siyah ok)*, Ölçek çubuğu: 5.000 nm

Further culture of differentiated cells including bFGF on fibronectin coated plates showed that most of the cells started to become polarized apparently with extensive neurit like structures. To further promote the maturation, heterogeneous cell populations consisting cells at different stages of maturation were seen at 14th and 21st days of culture condition *(Fig. 3B,C)*. The fusiform shape of cells were accepted as undifferentiated cells, and the neuron-like cells were determined as an early, intermediate and terminal differentiated neurons.

Transmission Electron Microscopy Results: Differentiated cells were morphologically similar to the neuron-like cells, especially after 21st days of culture in N2+bFGF medium *(Fig. 4)*. After TEM analyses, the cells which were cultured in N2+bFGF medium for 9 days consisted lamellipodia, large euchromatic nuclei and sparse endoplasmic reticulum and golgi *(Fig. 4A)*. Neuron-like cells have mitochondria which have more prominent cristae and distinguishable microtubules which is an indicator of mature neurons *(Fig. 4B)*.

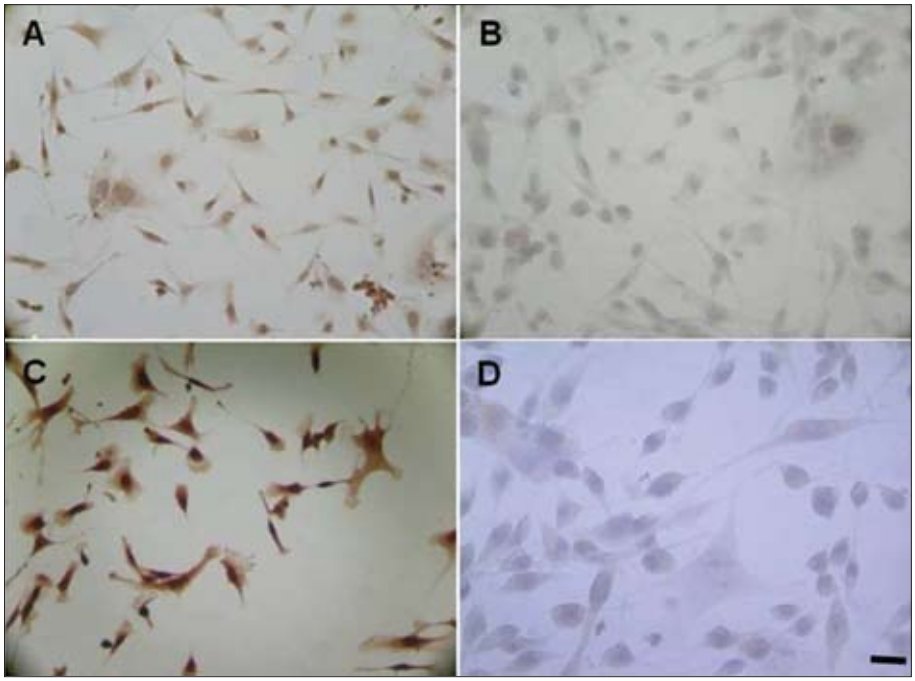


Fig 5. Distributions of SSEA-1 **(A,B)** and Nestin **(C,D)** immunoreactivities of differentiatied cells on the 7th and 14th days of differentiation, x400, Scale bar: 25 µm

Şekil 5. Farklılaşmanın 7. ve 14. günlerinde farklılaşmış hücrelerin SSEA-1 **(A,B)** ve Nestin **(C,D)** immünoreaktivitelerinin dağılımları, x400, Ölçek çubuğu: 25 µm

Immunohistochemical Results: According to the immunohistochemical analyses performed in order to characterize the cells on the 7th and 14th days of differentiation, SSEA-1 immunoreactivity, which is a mouse embryonic stem cell marker, was weak and negative (0.9±0.17), (0.1±0.19) respectively *(Fig. 5A,B)*, whereas the immunoreactivity of Nestin, which is an anti- body specific to the intermediate filament proteins of neural precursor cells, was strongly and weakly positive on the 7th and 14th days of the culture within N2 medium, (2.8±0.18), (1.08±0.10) respectively *(Fig. 5C, D)*. SSEA-1 and Nestin immunoreactivites in both 7th and 14th were statistically different (P<0.001), (P<0.001) respectively. In particularly, statistical significance of nestin immuno-reactivity supported that cells were derived from embryonic cells differentiated to neuron precursor at the 7th day of culture in our differentiated condition.

Table 1. *Immunolabelling intensity of SSEA-1, Nestin antibodies at the 7th and 14th days of differentiation cultures*

Tablo 1. *Farklılaşma kültürünün 7. ve 14. günlerinde SSEA-1, Nestin antikorlarının immünişaretleme yoğunluğu*

Days	Antibody Immunolabelling Intensity[a]	
	SSEA-1	Nestin
7	+	+++
14	-	+

Table 2. *Immunolabelling intensity of MAP_2, βIII-Tubulin, GFAP and O4 antibodies at the 14th and 21st days of differentiation cultures*

Tablo 2. *Farklılaşma kültürünün 14. ve 21. günlerinde MAP_2, βIII-Tubulin, GFAP ve O4 antikorlarının immünişaretleme yoğunluğu*

Days	Antibody Immunolabelling Intensity[a]			
	MAP-2	βIII-Tubulin	GFAP	O4
14	+++	+++	+	+
21	+++	+++	+	+

[a] *Immunolabelling intensity was graded on the following scale: negative (-), mild (+), moderate (++), and strong (+++)*

On the 14th and 21st days of culture, the neuronal cell markers MAP-2 *(Fig. 6A,B)* and ßIII-Tubulin *(Fig. 6C,D)* immuno-reactivites were strongly positive, whereas the neuroglial cell markers GFAP *(Fig. 6E,F)* and O4 *(Fig. 6G,H)* immuno-reactivities were weakly positive. MAP-2 and βIII-Tubulin immunoreactivites were statistically different when compared to the GFAP and O4 immunoreactivites in 14th and 21st days (P<0.001). This supported the fact that cells differentiated into neuronal cells rather than neuroglial cells.

Intensities of SSEA-1 and Nestin immunoreactivity at the 7th and 14th days of differentiation cultures are shown in *Table 1*. Intensities of MAP-2, βIII-Tubulin, GFAP and O4 immunoreactivity at the 14th and 21st days of differentiation cultures are shown in *Table 2*.

DISCUSSION

The nervous system is controlled by a series of epi-genetic and cellular endogeneous signals during embryo-genesis and early postnatal life, and differentiates from the neural tube derived from the configuration of the neuro-ectoderm [31]. All the central nervous system elements (neurons, glial cells, ependymal cells, etc.) are inside this structure. The structural elements of the nervous tissue are completed by the formation of certain cells and structures by the crista neuralis, which is derived from the neuroectoderm. The neurons and neuroglia cells that were derived from their precursors during neurogenic

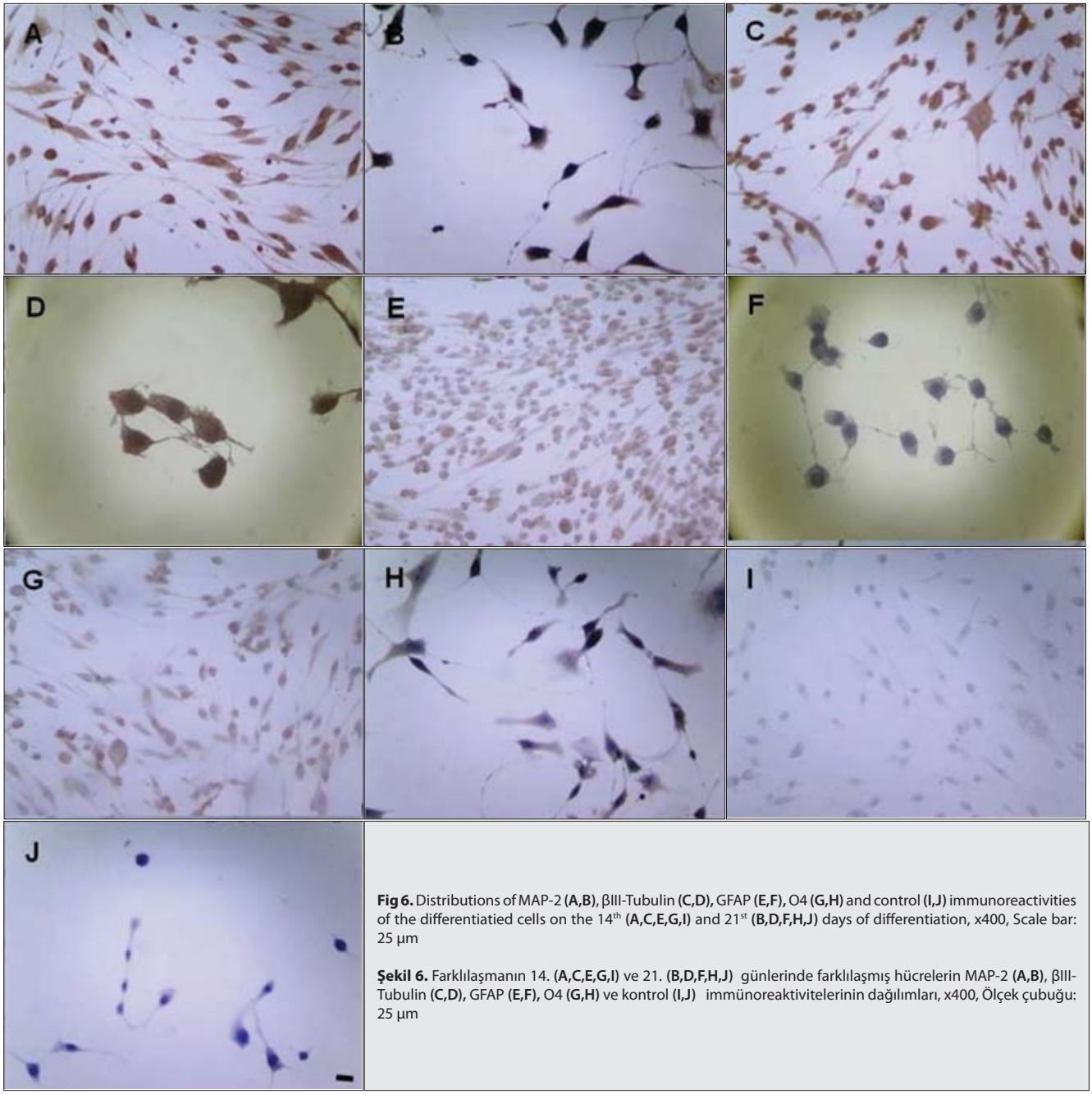


Fig 6. Distributions of MAP-2 **(A,B)**, βIII-Tubulin **(C,D),** GFAP **(E,F),** O4 **(G,H)** and control **(I,J)** immunoreactivities of the differentiatied cells on the 14th **(A,C,E,G,I)** and 21st **(B,D,F,H,J)** days of differentiation, x400, Scale bar: 25 µm

Şekil 6. Farklılaşmanın 14. **(A,C,E,G,I)** ve 21. **(B,D,F,H,J)** günlerinde farklılaşmış hücrelerin MAP-2 **(A,B)**, βIII-Tubulin **(C,D),** GFAP **(E,F),** O4 **(G,H)** ve kontrol **(I,J)** immünoreaktivitelerinin dağılımları, x400, Ölçek çubuğu: 25 µm

development complete their differentiation in the embryonic period. The extreme differentiation of the nerve cells makes them the ultimate controller of all the other cells in the organism. The nerve cells are ultimately developed during the embryogenic development. This situation eliminates their regeneration and proliferation ability in adult nerve cells. This is why the degenerative disorders of the nervous system cannot be amended [32].

Recently, many studies have aimed to clarify the mechanisms that will enable the replacement of the damaged tissues and perform nervous transmission. Thus, nervous cells that may be differentiated both from embryonic or extraembryonic derived stem cells are of great importance.

One of the most important factors determining the development and differentiation of stem cells during the embryonic development is the environment. The cell is differentiated depending on its environment and the signal molecules it is exposed to. The signal molecules build up the micro-environment of the cell [10]. Depending on the settlement within the micro-environment of the cell and certain intrinsic factors, stem cells may enlarge the cellular pool with divisions, turn into different cell lines and progress into programmed cell death [33]. Multiple different protocols exist for achieving neural induction and differentiation of ES cells. For example, the pluripotent cells can differ depending on the altering cell culture condition such as culturing on matrigel, mouse embryonic

fibroblast, and fibronectin coated plates. In addition, neural induction methods from embryonic stem cells also vary which can include embryoid bodies, adherent monolayers, and rosette formation of ES cells. In our condition, we used fibronectin as an extracellular matrix component, bFGF as an inductive factor and N2 as a supportive medium to neuronal lineage differentiation.

Neural differentiation was first experienced in embryonal carsinoma cells. Following the retinoic acid in vitro exposure of P19 (EC cell line) and embryo derived pluripotential stem cell line, they differentiated into neural cells [10,11,34,35]. Different studies have utilized other signal molecules such as βNGF, FGF, Wnt, TGF-β and N2 with different protocols in order to promote neural differentiation [14,36,37].

In our study, a micro-environment was created by mouse fibroblast cells as a feeder layer for mouse embryonic stem cells. The physical conditions of the cells were changed using N2 and bFGF, and their differentiation into neuronal cells and proliferation were promoted. The N2 medium provides the growth of neural stem cells in vitro, and contains insulin, transferrin, sodium selenite, putrescine, and progesterone. bFGF is a mitogen agent that is used in order to promote the proliferation of the neural precursor cells [38-43]. The media supplemented with N2 and bFGF induced embriyonic stem cells to differentiate into more complex neuron-like cells.

At the beginning of the study, the maintenance of the pluripotent properties of the ES cells was provided using nutritive cell layers (STO fibroblast cells) and LIF. After culturing EB cells derived from ES cells in the absence of LIF in culture dishes until the fourth day of the experiment, N2 was added into the ESC differentiation culture medium. The positivity of SSEA-1 and nestin immunoreactivity in the culture at the end of the seventh day showed that these were embryonic stem cells and precursor neuro-genic cells. The lower immunoreactivity of SSEA-1 on the 7th day showed that the embryonic stem cells had begun to differentiate. The positive immunoreactivity of nestin on the7th day indicated that the cells had begun to differentiate into neuron precursor cells in accordance with the decrease in the SSEA-1 immunoreactivity. This result suggested that this culture condition lead to the differentiation of mouse embriyonic stem cells into neuro-genic progenitor cells.

The results of immunohistochemical analyses after 14 days of culture time, the strong positivity of βIII-Tubulin and MAP-2 immunoreactivities, which were neuronal cell indicators, and the weak positivity of O4 and GFAP immuno-reactivities, which were oligodentrocytes and astrocytes markers, were observed respectively. The data suggested that the cells had begun to differentiate into neurogenic (neurons, oligodendrocytes, astrocytes etc.) cells from the precursor cells. The increased immunoreactivities of βIII-Tubulin and MAP-2 compared to those of O4 and GFAP on the 21st day suggested that the differentiation protocols provided a particular differentiation into neuronal cells.

The protocol used in our study induced the differentiation of a higher number of embryonic stem cells into neurons, while providing a lower potential of differentiation into neuroglial cells. Therefore, it may be concluded that the protocol used is appropriate for neuronal differentiation, and that a different mediator should be added into the culture medium in order to promote the activation of astrocyte and/or oligodendrocyte differentiation. Barres et al.[44] have demonstrated that retrieval of FGF from the medium after the 7th day of culture and continuation of the culture with T3 addition resulted in a higher oligo-dendrocyte differentiation from embryonic stem cells.

In the study of Okabe et al.[15], ES cells were increased in number with the addition of N2 and FGF factors into the medium and they differentiated into neuronal precursor cells, which were shown by the detection of nestin, MAP-2, GFAP and O4 positive cells. These results were similar to the results in our study. However, following the retrieval of FGF from the medium after differentiation, the number of nestin-positive cells in the culture decreased, MAP-2 positive cells increased and GFAP and O4-positive cells were visualized.

In the study of Li et al.[45], retinoic acid was used for the neural line specification of the embryonic stem cells. Neural progenitor cells were formed on the 7th day of the culture, neuronal cells were formed on the 10th day and neuronal line specification and functional neuron formation were completed by the 17th day, similar to our study. Sox1, Sox 2 and ß Galactosidase indicators were used as neuroepithelial markers in this study in addition to nestin, Pax3, 6 and Islet expressions which were examined as neural markers. Although there are many studies on the use of embryonic stem cells for the differentiation of neuronal cells, it has been demonstrated that mesenchymal stem cells and multipotent adult progenitor cells are also shown to form neurons, oligodendrocytes and astrocytes in humans, mice and rats *in vitro* [8,46-51].

In conclusion, using the protocols that use the differentiation potentials of embryonic stem cells and considering the cell differentiation criteria of ectodermal serial cells, the cells were demonstrated to start differentiating on the 7th day and this continued until the 21st day. It was also observed that, the neuron-like cell differentiation was to a higher extent in particular. It is necessary to analyze the *in vivo* usability and functionality of the cells that are derived in vitro. This study is important in constituting a study protocol for the analysis of the usability of cells derived from embryonic stem cell differentiation treating nerve cell injuries and degenerative disorders of the nervous system.

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RESEARCH ARTICLE

Measuring the Performance of Cattle Fattening Enterprises with Data Envelopment Analysis: Comparative Analysis of Enterprises in the Northeast Anatolia Region (TRA) Between the Years 2009-2010

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Summary

The present study is the first study which is about measuring the efficiency of cattle fattening enterprises with data envelopment analysis in Turkey. In this study it is aimed to comparatively present the performance score of intensive cattle fattening enterprises in the Northeast Anatolia Region with data envelopment analysis in two fattening periods. The CCR input method model was established with 10 inputs and 3 outputs for analysis. The analysis program was operated with "Super Efficiency" command to see efficiency score differences among DMU. As a result of measurements, performance scores that belong to total 143 DMU were determined. According to that, while the performance score mean of DMU in first fattening period were 112.01 in all DMU, 103.97 in small scale DMU, 113.82 in medium scale DMU, and 124.88 in large scale DMU, DMU's performance score means were 105.89, 108.94, 99.40, and 94.13 respectively in the second fattening period. The inefficiency DMU rate was measured as 22.79% in the first fattening period and 31.25% in second fattening period. When two fattening periods were compared in view of measurements, it was observed that there was a significant difference between medium and large scale DMU's performance scores ($P < 0.05$). In conclusion, it is considered that the increase in the number of inefficiency decision making units in the second fattening period was caused by the excessive increase in the fattening material cost, the low carcass meat price in comparison to the increase in the fattening material cost, and the decrease in the capacity utilization rate.

Keywords: *Data envelopment analysis, Performance measurement, Cattle fattening, Mathematical programming, Efficiency, Productivity*

Sığır Besi İşletmeleri Performansının Veri Zarflama Analizi Yöntemiyle Ölçülmesi: Kuzeydoğu Anadolu Bölgesi'ndeki (TRA) İşletmelerin 2009-2010 Yıllarında Karşılaştırmalı Analizi

Özet

Bu çalışma Türkiye'de sığır besi işletmelerinin veri zarflama yöntemiyle etkinliğinin ölçülmesinde ilk olma özelliği taşımaktadır. Çalışmada veri zarflama analizi yöntemiyle Kuzeydoğu Anadolu Bölgesi'ndeki entansif sığır besi işletmelerinin iki besi dönemi halinde karşılaştırmalı olarak performans skorlarının ortaya konulması amaçlanmıştır. Analiz için 10 girdi ve 3 çıktı ile CCR girdi yönelimli model kurulmuştur. Analiz programı "Karar Verme Birimleri"nin (KVB) kendi aralarındaki etkinlik skor farklarını görmek amacıyla "Super Efficiency" komutuyla çalıştırılmıştır. Hesaplamalar sonucunda toplam 143 KVB'ne ait performans skorları saptanmıştır. Buna göre birinci besi dönemindeki performans skor ortalamaları KVB'nin tamamında 112.01, küçük ölçekli KVB'nde 103.97, orta ölçekli KVB'nde 113.82 ve büyük ölçekli KVB'nde 124.88 iken, ikinci besi döneminde KVB'nin performans skor ortalamaları aynı sırayla 105.89, 108.94, 99.40, 94.13 olarak belirlenmiştir. Etkin olmayan KVB oranı birinci besi döneminde %22.79, ikinci besi döneminde ise %31.25 olarak hesaplanmıştır. İki besi dönemi ölçeklere göre karşılaştırıldığında orta ve büyük ölçekli KVB'nin performans skorları arasındaki fark önemli bulunmuştur ($P<0.05$). Sonuç olarak; etkin olmayan KVB sayısının ikinci besi döneminde artış göstermesine; besi materyali maliyetindeki aşırı yükselmenin, besi materyali fiyatındaki yükselişe göre düşük kalan karkas et fiyatının ve kapasite kullanım oranın düşmesinin neden olduğu düşünülmektedir.

Anahtar sözcükler: *Veri zarflama analizi, Performans ölçümü, Sığır besiciliği, Matematik programlama, Etkinlik, Verimlilik*

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INTRODUCTION

According to the Nomenclature of Units for Territorial Statistics, the provinces of Erzurum, Erzincan, and Bayburt in the Northeast Anatolia Region (TRA) are in TRA-1 and Kars, Ardahan, Ağrı, and Iğdır are in TRA-2 [1]. According to 2012 data of Turkish Statistical Institute Turkey's 14.71% of Turkey's total cattle is in TRA, 3.51% is in Kars, and 4.48% is in Erzurum While considering Turkey's total cattle existence distribution, Kars and Erzurum were among the top four cities as of 2012 [2]. Cattle fattening enterprises in TRA have similar socio-economic features [3].

Cattle fattening, a subsector of the livestock sector, has an important role in transforming male calves, which are an important output of dairy farming, and female breeding stock into high quality and efficiency beef through economic utilization [4]. The live weight increase, feed intake, feed efficiency and their ability and the relationship between profitability are direct factors on cattle enterprises [4,5].

As in all commercial enterprises in cattle fattening enterprises, the main purpose is to make a profit. From this point, evaluating the performances of cattle fattening enterprises gains importance in terms of the continuation of business efficiencies. Performance in general is a concept that quantitatively or qualitatively determines the gains at the end of a purposeful and scheduled activity. In other words, performance is the level of achievement to reach the planned output level. If the business performance is in question, the first concepts that come to mind are efficiency and, productivity [6].

The concepts of effectiveness and efficiency, each a dimension of the performance, are crucial for all units in maintaining their existence, whether or not they seek profit. In order to see their own places, their superiority and inferiority among similar ones, units periodically should measure performance with measured data. Without measurement, it is not possible to decide what is good or bad by whom [6].

The fact that efficiency and productivity are so important gives way to the development of many measurement methods. They can be grouped in three groups: ratio analysis, parametric methods and non-parametric methods [6]. Of the methods used to measure efficiency ratio analysis is the simplest. In this approach, each ratio considers only one of the dimensions of efficiency while others are ignored. Generally regression techniques are used in the efficiency measurement with parametric methods. Mathematical programming has been adopted as the solution technique in performance measurement with non-parametric methods [7]. When ratio analysis and parametric methods cannot over-come situations (particularly multi-input and multi-output conditions) data envelopment analysis (DEA) offers enormous opportunities for solutions [6].

Data envelopment analysis is an activity measurement "without parameters" first developed by Charnes, Cooper and Rhodes (1978-1979) in order to measure "relative" efficiencies of similar commercial decision making units (DMU) in terms of their goods or services [7]. The method is used for performance comparison in multi-input multi-output relations of production, to which classical regression analysis cannot be directly applied [8].

Unlike the single input, single output in the conventional efficiency analysis, DEA acts on the basis of multiple inputs multiple outputs. DEA measuring comparative efficiency originally in nonprofit public institutions, then has been used widely for the measurement of technical efficiency in profit seeking manufacturing and service sectors [9]. In this context, it has been used to measure the performances of many profit businesses including livestock enterprises [10].

In cases which inputs and outputs measured with multiple or different measurement scales make comparison difficult DEA is a linear programming-based technique aiming to measure the relative performances of DMU [11]. In this method it is possible to simultaneously measure various sizes of the DMU with respect to independence of the input and output units from the scale [12].

This study aims to determine the activity in two fattening periods of cattle fattening enterprises located in TRA by DEA based on mathematical programming suitable to measure the efficiencies of DMU using multiple inputs and multiple outputs. The present study is the first study about measuring the efficiency of cattle fattening enterprises with data envelopment analysis in Turkey.

MATERIAL and METHODS

Material

To determine intensive cattle fattening enterprises to be included in the research in 2009 in the provinces of Erzurum and Kars, pre-interviews were conducted with Food, Agriculture and Livestock Departments, Agriculture Credit Cooperatives, Meat and Dairy Institution Erzurum Slaughterhouse employees and some breeders. As a result of pre-interviews it has been identified that in the provinces many intensive cattle fattening enterprises operate through the TAR-ET project conducted by the Meat and Dairy Institution and Agriculture Credit Cooperatives. Therefore, it was decided to include intensive cattle fattening enterprises operating through the TAR-ET project in the provinces of Erzurum and Kars in the scope of the research and a total of 82 enterprises were inter-

viewed in this context.

The first degree material of the study consisted of data obtained through face-to-face interviews conducted with intensive cattle fattening enterprises in the central district of Kars, the districts of Selim and Sarıkamış, the central Erzurum districts (Aziziye, Palandöken, Yakutiye), the distract of Pasinler and affiliated villages. The Meat and Dairy Institution has benefited from data of Erzurum slaughterhouse's TAR-ET project [13]. The research includes the cattle production efficiencies of first fattening period of 2009-2010, while the second fattening period between the years 2010-2011. Cattle fattening in the provinces are usually scheduled between October and April in many enterprises once a year.

First survey application was carried out with a total of 79 voluntary enterprises participating in both provinces aimed at intensive cattle fattening enterprises at the beginning and end of the fattening period. The second fattening period survey continued to operate in provinces with a total of 64 enterprises that were surveyed in the first fattening period. Cattle fattening enterprises with 20 and less cattle are considered small scale, those with 21-40 are considered medium scaled, and those with 41 and more are considered large scaled enterprises.

Methods

Data Evaluation

Data envelopment analysis does not give absolute efficiency values while measuring DMU's activity values, it reveals how effective they are to each other [14]. Therefore, in the study DMU's performance scores were found by analyzing two fattening period's multi-input and multi-output data of cattle fattening enterprises in "Efficiency Measurement System (EMS)" package program which is suitable to measure decision- units' performance [6]. The cause of analyzing a total of 143 DMU's of two fattening periods in the EMS package program as a whole arises from the desire to reveal how effective of two fattening period to each other.

In the study, determining the performance scores' descriptive statistics and controlling the significance of differences between groups were made using One-Way ANOVA to three comparisons and T-Test to pairwise. To perform analyzes, the SPSS 20.0 statistical software package was utilized and to create the scatter diagram of the performance score, Windows Excel 2010 was utilized [15].

Data Envelopment Analysis

DEA is a multi-factor productivity measurement model that measures similar decision making unit's (homogeneous) relative effectiveness. A multi-input and multi-output activity score factor is defined as follows [16]:

$$\text{Efficiency} = \frac{\text{Weighted Output}}{\text{Weighted Input}}$$

Charnes, Cooper and Rhodes expanded Farrell's only input/output technical efficiency measure in 1957 to multiple input/output relative effectiveness measurement, put it to the DEA's literature as the CCR model [17,18].

Following the wide recognition of the data envelopment analysis, the method's basic concepts and principles brought the model variation. A variety of models have been developed as well as CCR (Charnes, Cooper, Rhodes) ratio model, BCC (Banker, Charnes, Cooper) returns to scale model, additive model and multiplicative model [8]. In this study, the input oriented CCR technique has been used; there is no superiority over each other between the CCR and BCC techniques,

It's assumed that each unit has "m" amount of input, "s" amount of output and "n" amount of decision making unit on the problem that will be analyzed. X_{ij} parameter indicates "i" input amount using by "j" DMU and Y_{ij} parameter indicates output amount using by "j" UD. Decision variables for that decision problem are the weight to be given for the, "k" DMU's "i" inputs and "r" outputs. These weights are shown as V_{ik} and U_{rk} respectively. The objective function of fractional linear programming model was defined as maximal ratio of the "k" DMU total weighted output the sum of the weighted [14,16,19].

Objective function:

$$Enb\ h_k = \frac{\sum_{r=1}^{s} u_{rk}\, y_{rk}}{\sum_{i=1}^{m} v_{ik}\, x_{ik}}$$

Subject to:

$$\frac{\sum_{r=1}^{s} u_{rk}\, y_{rj}}{\sum_{i=1}^{m} v_{ik}\, x_{ij}} \le 1 \qquad ;j = 1,2,\ldots,n \quad (2)$$

and:

$$u_{rk} \ge 0;\ r = 1,2,\ldots,s$$

$$v_{ik} \ge 0;\ i = 1,2,\ldots,m$$

CCR data envelopment model can be created with converting the above fractional programming model into a linear programming model [7,17,20].

Objective function:

$$Enb\ h_k = \sum_{k=1}^{s} u_{rk}\, y_{rk} \qquad k = 1,2,\ldots,n$$

Subject to:

$$\sum_{r=1}^{s} u_{rk}\, y_{rj} - \sum_{i=1}^{m} v_{ik}\, x_{ij} \leq 0 \;;\; j = 1,2,\ldots,n$$
$$\sum_{i=1}^{m} v_{ik}\, x_{ik} = 1 \qquad (3)$$

and:

$$u_{rk} \geq 0;\; r = 1,2,\ldots,s$$
$$v_{ik} \geq 0;\; i = 1,2,\ldots,m$$

The above problem has been processed "n times" to determine the effectiveness of all DMU's score. Weighted inputs and outputs are chosen to optimize each decision making unit's efficiency score. In general, if a decision making unit's efficiency score is equal to one, it is efficient, if it's lower than one, it is inefficient [16].

Implementation of Data Envelopment Analysis

The analysis was applied to a total of 143 cattle fattening enterprises; it was applied to 79 of them in the first fattening period and 64 of them in the second period. Cattle fattening enterprises were coded as the "Enterprise (Ent) - Fattening Period (1, 2) - City (Erzurum-E, Kars-K) - Enterprise Number (1, 2, 3, ...)". Accordingly, for example, the first enterprise in Erzurum was "Ent1E1" in first fattening period, while the fifth enterprise in Kars was coded as "Ent2K5" in the second fattening period. The evaluation codes of 15 enterprises that didn't continue to operate in the second fattening period are not available. Cattle fattening enterprises are presented in *Table 1* according to the fattening period and scale

The fattening material costs (FMC {I}), feed expenses (FC {I}), labor costs (LC {I}), veterinary-health expenditure (VHC {I}), care-repair cost (CRC {I}), foreign capital interest (IC {I}), other costs (electricity, water, litter, transport, animals and ranch insurance) (OC {I}), general administrative expenses (GAC {I}), amortization of buildings (ABC {I}) and machinery amortization (MAC {I}) are determined as the input elements for indicating cattle fattening enterprises' performance. The carcass income (CI {O}), incentive bonus income (IBI {O}) and fertilizers income (FI {O}) were included as outputs of enterprises in the analysis. The DMU's incentive bonus income is 1.50 TL per kilogram of male animal carcass that have 60% efficiency and over 190 kg weight in the first fattening period within the TAR-ET project and whereas per men animal is 300 TL in the second fattening period [3].

Thus, by analyzing a total of 143 DMU, 10 inputs and 3 outputs, through the input oriented analysis in two fattening periods, the efficiency scores were calculated. As in all methods of mathematical analyses, in DEA in the presence of a large number of input and output increases the reliability of results. The program has been executed with "Super Efficiency" command for the enterprises on the efficiency border in EMS package program to see differences of the efficiency scores among themselves. Accordingly, the cattle fattening enterprises with the efficiency score 100% and more were evaluated as efficient and the ones with the efficiency score less than 100% as inefficient.

RESULTS

The decision making unit's capacity utilization rates evaluated in the context of research according to the fattening period and scales are given in *Table 2*. In terms of capacity utilization rate, between the first and second fattening period statistical differences are found in their medium and large scale decision making units ($P<0.001$).

The performance scores of the decision making units first and second fattening periods are shown in *Table 3* and in *Fig. 1*. In addition, performance scores according to the evaluation of the decision making unit measures are present in *Table 4*.

Table 1. *Performance measured cattle fattening enterprises*
Tablo 1. *Performans ölçümleri yapılan sığır besi işletmeleri*

Decision Making Units Scale	Fattening Periods	
	First Fattening Period Decision Making Units	Second Fattening Period Decision Making Units
≤20 Head	Ent1E1, Ent1E8, Ent1E9, Ent1E12, Ent1E15, Ent1E17, Ent1E18, Ent1E20, Ent1E22, Ent1E25, Ent1E26, Ent1E28, Ent1E29, Ent1E32, Ent1E34, Ent1E35, Ent1E36, Ent1E43, Ent1E44, Ent1E45, Ent1E49, Ent1E50, Ent1E52, Ent1E57, Ent1E58, Ent1E61, Ent1E63, Ent1E64, Ent1E65, Ent1E66, Ent1E68, Ent1E70, Ent1K2, Ent1K3, Ent1K4, Ent1K5, Ent1K8	Ent2E1, Ent2E8, Ent2E9, Ent2E10, Ent2E12, Ent2E15, Ent2E22, Ent2E25, Ent2E26, Ent2E27, Ent2E28, Ent2E29, Ent2E32, Ent2E33, Ent2E34, Ent2E35, Ent2E36, Ent2E41, Ent2E44, Ent2E49, Ent2E51, Ent2E52, Ent2E54, Ent2E60, Ent2E63, Ent2E64, Ent2E66, Ent2K2, Ent2K3, Ent2K5
21-40 Head	Ent1E2, Ent1E3, Ent1E4, Ent1E10, Ent1E13, Ent1E23, Ent1E24, Ent1E27, Ent1E30, Ent1E31, Ent1E33, Ent1E37, Ent1E41, Ent1E42, Ent1E46, Ent1E51, Ent1E53, Ent1E54, Ent1E59, Ent1E60, Ent1K6, Ent1K7	Ent2E2, Ent2E3, Ent2E4, Ent2E5, Ent2E13, Ent2E21, Ent2E23, Ent2E24, Ent2E31, Ent2E37, Ent2E42, Ent2E46, Ent2E53, Ent2E55, Ent2E58, Ent2E62, Ent2E67, Ent2E68, Ent2K6, Ent2K7
41≥ Head	Ent1E5, Ent1E6, Ent1E7, Ent1E11, Ent1E14, Ent1E16, Ent1E19, Ent1E21, Ent1E38, Ent1E39, Ent1E40, Ent1E47, Ent1E48, Ent1E55, Ent1E56, Ent1E62, Ent1E67, Ent1E69, Ent1K71, Ent1K1	Ent2E6, Ent2E7, Ent2E11, Ent2E16, Ent2E19, Ent2E38, Ent2E39, Ent2E40, Ent2E47, Ent2E48, Ent2E56, Ent2E69, Ent2E71, Ent2K1

Table 2. *Capacity utilization rates according to fattening periods*
Tablo 2. *Besi dönemlerine göre kapasite kullanım oranları*

Parameter	Fattening Periods							
	First Fattening Period Decision Making Units Scale				Second Fattening Period Decision Making Units Scale			
	≤20 Head	21-40 Head	41≥ Head	All	≤20 Head	21-40 Head	41≥ Head	All
DU Amount (Number)	37	22	20	79	30	20	14	64
Total Breeding Animal (Head)	521	684	1455	2660	422	599	863	1884
Total Capacity (Head)	1199	1021	1840	4060	1054	1104	1336	3494
Capacity Utilization Rate (%)	43.45a	66.99a*	79.08a*	65.52a**	40.04a	54.26b*	64.60b*	53.92b**

a, b: *The difference between groups with different letters in the same row and the same scale are significant; * P<0.001; ** P<0.05*

Table 3. *EMS package program performance scores with the super efficiency command for the first and second fattening periods decision making units*
Tablo 3. *Birinci ve ikinci besi döneminde karar verme birimlerinin süper etkinlik komutuyla EMS paket programı performans skorları*

DMU	Performance Score (%)	DMU	Performance Score (%)	DMU	Performance Score (%)	DMU	Performance Score (%)	DMU	Performance Score (%)	DMU	Performance Score (%)
Ent1E1	105.07	Ent1E25	95.85	Ent1E49	102.91	Ent1K2	108.66	Ent2E22	116.60	Ent2E49	92.23
Ent1E2	121.37	Ent1E26	93.80	Ent1E50	100.90	Ent1K3	111.46	Ent2E23	101.81	Ent2E51	100.00
Ent1E3	129.81	Ent1E27	106.86	Ent1E51	111.01	Ent1K4	106.35	Ent2E24	101.68	Ent2E52	124.98
Ent1E4	109.08	Ent1E28	99.50	Ent1E52	112.12	Ent1K5	107.48	Ent2E25	106.25	Ent2E53	98.38
Ent1E5	111.24	Ent1E29	96.52	Ent1E53	105.81	Ent1K6	99.76	Ent2E26	100.99	Ent2E54	106.56
Ent1E6	112.09	Ent1E30	168.24	Ent1E54	86.19	Ent1K7	133.13	Ent2E27	131.39	Ent2E55	104.53
Ent1E7	170.43	Ent1E31	105.82	Ent1E55	125.24	Ent1K8	130.86	Ent2E28	112.48	Ent2E56	87.70
Ent1E8	101.44	Ent1E32	119.14	Ent1E56	115.61	Ent2E1	92.04	Ent2E29	113.35	Ent2E58	91.70
Ent1E9	92.53	Ent1E33	110.75	Ent1E57	107.60	Ent2E2	103.32	Ent2E31	97.22	Ent2E60	93.10
Ent1E10	112.22	Ent1E34	105.35	Ent1E58	114.35	Ent2E3	99.89	Ent2E32	117.25	Ent2E62	107.42
Ent1E11	110.57	Ent1E35	90.54	Ent1E59	113.11	Ent2E4	100.14	Ent2E33	122.43	Ent2E63	154.05
Ent1E12	90.52	Ent1E36	109.16	Ent1E60	100.67	Ent2E5	84.24	Ent2E34	90.64	Ent2E64	100.00
Ent1E13	122.93	Ent1E37	109.25	Ent1E61	115.86	Ent2E6	113.39	Ent2E35	93.88	Ent2E66	94.53
Ent1E14	240.32	Ent1E38	115.95	Ent1E62	110.71	Ent2E7	162.08	Ent2E36	103.36	Ent2E67	110.79
Ent1E15	93.29	Ent1E39	111.86	Ent1E63	135.31	Ent2E8	145.45	Ent2E37	97.15	Ent2E68	107.53
Ent1E16	126.39	Ent1E40	111.33	Ent1E64	96.60	Ent2E9	120.81	Ent2E38	100.59	Ent2E69	91.78
Ent1E17	122.67	Ent1E41	126.00	Ent1E65	126.52	Ent2E10	85.59	Ent2E39	98.74	Ent2E71	113.96
Ent1E18	101.37	Ent1E42	103.79	Ent1E66	84.24	Ent2E11	126.30	Ent2E40	106.70	Ent2K1	107.61
Ent1E19	112.01	Ent1E43	92.38	Ent1E67	116.27	Ent2E12	100.00	Ent2E41	104.79	Ent2K2	125.36
Ent1E20	97.29	Ent1E44	89.57	Ent1E68	102.03	Ent2E13	101.69	Ent2E42	89.22	Ent2K3	103.09
Ent1E21	116.59	Ent1E45	83.12	Ent1E69	93.84	Ent2E15	113.45	Ent2E44	101.55	Ent2K5	101.87
Ent1E22	106.89	Ent1E46	111.41	Ent1E70	97.73	Ent2E16	102.74	Ent2E46	98.38	Ent2K6	85.58
Ent1E23	106.96	Ent1E47	103.93	Ent1E71	123.11	Ent2E19	104.76	Ent2E47	98.31	Ent2K7	104.35
Ent1E24	109.85	Ent1E48	121.69	Ent1K1	148.50	Ent2E21	102.92	Ent2E48	106.26	-	-

In the decision making units first fattening period while the scale grows the performance score rises, it declines in the second fattening period. In addition, performance scores differences while between small scale decision making units and other scale decision making units in the first fattening period, between large scale decision making units and other scale decision making units in the second fattening period and are statistically significant (P<0.05). On the other hand, when the two fattening periods are compared according to the scales

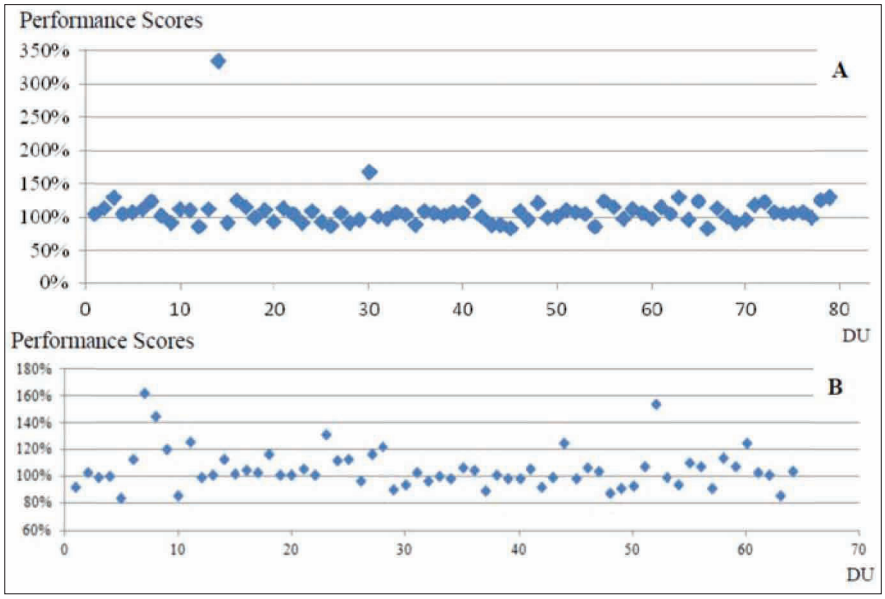

Fig 1. The scatter diagram for the first **(A)** and the second **(B)** fattening period performance scores

Şekil 1. Birinci **(A)** ve ikinci **(B)** besi dönemine ait performans skorları serpilme diyagramı

Table 4. *The evaluation of decision making units performance scores according to in the first and the second fattening periods*
Tablo 4. *Birinci ve ikinci besi döneminde karar verme birimlerinin performans skorlarının ölçeklerine göre değerlendirilmesi*

Decision Making Units Scale	Fattening Periods							
	First Fattening Period				Second Fattening Period			
	n	Performance Score (S±SE)	Inefficiency Decision Making Units		n	Performance Score (S±SE)	Inefficiency Decision Making Units	
			Number	(%)			Number	(%)
≤20 Head	37	103.97±2.02a†	15	40.54	30	108.94±2.94a†	7	23.33
21-40 Head	22	113.82±3.43b†	2	9.09	20	99.40±1.58a‡	9	45.00
41≥ Head	20	124.88±7.06b†	1	5.00	14	94.13±4.84b‡	4	28.57
Total	79	112.01±2.40†	18	22.79	64	105.89±1.86‡	20	31.25

a, b: *The differences between groups in the same column bearing different letters are significant (P<0.05);* ***†‡:*** *The differences between groups with different icons on the same line are significant (P<0.05)*

the differences between medium and large scales' decision making units performance scores are found to be significant (P<0.05).

DISCUSSION

Within the scope of the study, the first survey application done with intensive cattle fattening enterprises for the first fattening period on October 2009 began a rising trend across Turkey with the red meat prices starting with mutton prices. As of the year 2009 in Turkey live animal and carcass meat imports are not in question. However, to balance the rising red meat prices on April 30, 2010 lowering customs tax rates opened the way for live animal and carcass meat imports. On the other hand, on June 2010 the TAR-ET project was repealed [1,3,21,22].

As a result of these developments the operating 15 businesses in the first period, when the survey was applied, withdrew from this branch of production in the second fattening period, and the 64 enterprises with ongoing efficiencies capacity utilization rates on average were reduced by 17.71%.

Intensive cattle fattening enterprises quitting this business or continuing by reducing capacity; fattening material prices demonstrating extreme increases, as of the date of the study the uncertainty of the continuation of live animal and red meat imports, the increase in concentrated feed, and lack of incentives can be shown as reasons.

Aydin at al.[22] and, Aydin and Sakarya [1] reported that due to the excessive rise of costs in red meat in 2010, the general sum of the costs of fattening material shares increased proportionally, in other words the absolute value of fattening material costs according to other input elements increased more. The same study found that the period in which price increases in red meat happened, the prices of live animal that are also fattening material increased by approximately 50%.

According to the performance scores in the study, while in the first fattening period, 22.79% of the decision making units (18 DMU) are not efficient, in the second fattening period the inefficient decision making

units rate rises to 31.25% (20 DMU). Also the first fattening period decision units average performance score was calculated 5.78% more compared to the second fattening period decision making units.

At the end of the first fattening period decision making units who find a possibility to sell carcass meat high priced which is the product of livestock efficiencies, while entering the second fattening period (October 2010) in spite of buying high priced fattening material, at the end of the import the price balancing carcass meat was sold close to the price of the first fattening period [22]. The cost of fattening material in a cattle fattening enterprise makes up about 45%-55% of the total cost [3] when taken into consideration it is possible to say that the second fattening period decreases the profitability of the decision making units. On the other hand, considering the first fattening period in the second fattening period; the average feed costs per animal via current prices, labor and vet health expenditures have increased. Indeed, some of the literature resources report that one of the most important elements that directly affect the profitability of the business is the cost of fattening material [23]. This case also supports the findings of the research.

The research shows that in the first fattening period as the DMU scale grow the performance score average increases, whereas in the second fattening period it decreases remarkably. On the other hand, according to the first fattening period in the second fattening period medium and large scale decision making units differences between; capacity utilization rates ($P<0.001$) and average performance scores ($P<0.05$) are significant.

The capacity of business identified as the ability and opportunity to produce the goods or services of a business being described with a certain measure is large in importance. The capacity utilization rate being low is especially known to have an increasing effect on operating costs [24]. As the capacity utilization rate increases the businesses fixed assets are used more efficiently and fixed costs such as labor, general administrative expenses, building amortization per unit of animal are reduced.

As a result; compared to the first fattening period in the second fattening period from the cost elements the fattening material costs increased by approximately 50%, however, the carcass meat sales revenue did not show the same rate in increase. Compared to the first fattening period in the second fattening period especially medium and large scale decision making units due to the fall in capacity utilization rates fixed costs per unit of animal have increased. The increase in the number of inefficient decision making units in the the second fattening period; excessive rise in the cost of fattening material, according to the rise in the price of fattening material low carcass meat prices and capacity utilization rates are thought to have caused the falling.

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RESEARCH ARTICLE

Effects of Cage and Floor Housing Systems on Fattening Performance, Oxidative Stress and Carcass Defects in Broiler Chicken [1] [2]

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Summary

This study was carried out to compare floor with cage housing systems used for broiler chicken production in terms of performance, some oxidative stress parameters and carcass defects. For this purpose, two cage and two floor housing farms were monitored simultaneously during summer, autumn and winter seasons. Capacities of farms in each housing system were 40.000 and 25.000 chickens. At the end of each summer, autumn and winter season, 15 broilers were selected in both housing systems with capacity of 25.000 chickens for carcass and oxidative stress parameters. Blood samples were taken in slaughtering period from chickens slaughtered with decapitation. Broiler reared in cage housing showed higher live weight at 7 and 14 days (P≤0.05). But this difference disappeared after three weeks, while slaughter weights were found to be similar at both systems. Better feed conversion ratio (FCR) and carcass yield were obtained in floor housing (P≤0.01). Serum malondialdehyde (MDA) level increased in cage housing (P≤0.01). Other carcass traits and antioxidant activity were found to be similar between groups (P>0.05). Cases of wing fractures, wing and breast bruising were found to be higher in cage housing (P≤0.05). Case of shank and drumstick bruising slightly increased in floor housing (P=0.074). The incidence and severity of food pad lesions increased in floor housing (P≤0.01). The results of this study indicated that floor housing had shown better performance and carcass quality at examined production capacities.

Keywords: *Broiler, Floor housing, Cage housing, Performance, Season*

Kafes ve Yer Sistemlerinin Etlik Piliç Üretiminde Besi Performansı, Oksidatif Stres ve Karkas Kusurları Üzerine Etkileri

Özet

Bu araştırma, etlik piliç üretiminde kullanılan yer ve kafes sistemlerini performans, bazı oksidatif stres parametreleri ve karkas kusurları bakımından karşılaştırmak için yürütülmüştür. Bu amaçla, 2 yer ve 2 kafes kümesi yaz, sonbahar ve kış sezonları süresince eş zamanlı olarak takip edilmiştir. Her sistemde kümeslerin kapasitesi 40.000 ve 25.000 piliç şeklindedir. Yaz, sonbahar ve kış sezonları sonunda, 25.000 kapasiteli her iki yetiştirme sisteminden karkas ve oksidatif stres parametreleri için 15 piliç seçilmiştir. Kan numuneleri boyun uçurma yöntemi ile kesimi yapılan piliçlerden kesim esnasında alınmıştır. Kafes sisteminde yetiştirilen piliçler 7 ve 14. günlerde daha fazla canlı ağırlık göstermişlerdir (P≤0.05). Bu farklılık üçüncü haftadan sonra ortadan kaybolmuş ve kesim ağırlıkları her iki sistemde benzer bulunmuştur. Yer sisteminde yemden yararlanma ve karkas randımanı iyileşmiştir (P≤0.01). Serum malondialdehit (MDA) seviyesi kafes sisteminde yükselmiştir (P≤0.01). Diğer karkas özellikleri ve antioksidan aktivite araştırma grupları arasında benzer bulunmuştur (P>0.05). Kanat kırığı, kanat ve göğüs morarması olguları kafes sisteminde yüksek tespit edilmiştir (P≤0.05). İncik ve baget morarması olgusu yer sisteminde önemsiz derecede yükselmiştir (P=0.074). Taban yastığı nekrozlarının görülme oranı ve şiddeti yer sisteminde artmıştır (P≤0.01). Bu araştırma incelenen üretim kapasitelerinde yer sisteminin performans ve karkas kalitesi bakımından daha iyi sonuçlara sahip olduğunu göstermektedir.

Anahtar sözcükler: *Etlik piliç, Yer sistemi, Kafes sitemi, Performans, Mevsim*

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INTRODUCTION

Two housing systems including floor and cage are used in conventional broiler production. Floor housing is widely used, but implementations of cage rearing in broiler production are not recent. Manufacturing companies began to work in this issue in 1960s. The colony cages were developed with different features in accordance with the needs of the broilers [1-3]. From now on, reduced labor costs per square meter, increased uniformity, improved feed efficiency, more production per unit area, unnecessity of the use of litter, disappearance of dust and wet litter problems which are the major problems in floor system, minimum incidence of diseases such as enteritis and coccidiosis because of decreasing contact with manure, increase at annual production because of convenience of disinfection and cleaning operations, easy and stress-free transport to slaughterhouse have been detected as the advantages of use of cages for broiler breeding [4-6]. However, high initial investment cost, difficulty at poultry management and control of environmental factors at large-scale flocks, deteriorating welfare, increased mortality rates related to leg and wing disorders of chickens, softening of bones, leg and wing fractures, perozis, defects of brisket and decline in quality of meat have been obtained as the disadvantages [3,7-10].

Although cages have been banned in broilers and layers in EU, with more intensive production in a small land, the cage production seems quite attractive and has been quickly growing over the World, especially in Russia, the Middle East, several Asian countries, Africa and Eastern European countries in recent years [10]. However, there is the lack of information in cage reared broilers, especially in commercial flocks. This study aimed to compare simultaneously floor housing with cage housing used in broiler production in terms of performance, some stress parameters and carcass traits during three seasons (summer, autumn and winter).

MATERIAL and METHODS

Experimental Design

The study was conducted at cage and floor farms of an integrated commercial company with the approval of Firat University Animal Researches Ethic Committee (FUHADEK, verdict no: 20.01.2012/07). The study was conducted at 4 farms contracted with the same poultry integration company consisting of cage (2) or floor (2) housing systems. Data were collected from each farm during the two consequent production periods in summer, autumn and winter seasons. Therefore, there were 4 replicate flocks of each cage and floor housing systems with the capacities of 40.000 and 25.000 broilers. Any effort has been made to optimize and to have similar environmental conditions in each flock through the seasons. Ross-308 broiler chicks were randomly placed to the farms.

The cage system had 4 storeys, and each cage unit had 165 cm width x 246 cm length x 75.75 cm in height. All storeys base was made from plastic mesh material. First 28 days, 23 h of light and 1 h of dark schedule was applied to the both housing systems. Thereafter, 20 h of light and 4 h of dark schedule was used. All heating, ventilation, lighting, feeding, watering, capture and delivery systems were controlled digitally. Manure was removed from the coops with digital belt system in cage housing. At the end of production, transportation of chickens to slaughterhouse was performed with same belt system *(http://www.kutlusan.com.tr)*. Stocking density was adjusted at cage and floor flocks to be 17-17.5 chicks/m^2. Wood shaving was used as flooring material at floor coops (5 kg/m^2). Feed and fresh water were automatically distributed and ad libitum. Diets were obtained from commercial feed company and were in accordance with NRC [11]. Compositions of the diets were given at *Table 1*.

Weights of chickens were determined on the days 1st, 7th, 14th, 21st, and 28th. On these days, a balance featuring precision of g scale was used for determination of live weights and each time 10 different broilers (5 males and 5 females) were randomly weighed from 5 different points of poultry house. A total of 50 broilers from each flock were weighed each of on these days. Slaughter weight was collectively determined at special scales of slaughterhouse of the company. Broilers were taken from flocks at the evening hours, and were sent to slaughterhouse after 12 h total fasting period. Broilers spent their waiting time in special waiting rooms, in trucks and crates. Trucks were weighed before slaughter process, while it was full and later, while it was empty. Mean live weight were calculated by dividing total live weight to the number of slaughtered birds. Slaughter age was organized according to marketing plan of the company. Digital board was used for feed consumption detection. Food was withdrawn from flocks before 8-10 h of arrival of loading trucks, and within this period remaining food consumption was achieved. Live weight gain and feed consumption per chicken were determined and feed conversion ratio was calculated as feed to gain (kg/kg). Dying chickens during production period were processed to flock board, and at the end of the production period, mortality rates, by percentage were calculated.

Carcass traits and oxidative stress data have been collected only from the flocks of 25.000. Blood samples of birds from each housing system were collected into tubes at slaughter line during the neck cut and were analyzed by the following procedure. To determine carcass traits, enough chickens were individually weighed on slaughter day. 7 females and 8 males having a live weight of ~2.0 and 2.5 kg respectively were picked out and transferred for

Table 1. *Ingredients and chemical composition of diets*
Tablo 1. *Karma yemin bileşimi ve kimyasal kompozisyonu*

Feed ingredients	Days (1-10)	Days (11-27)	Days (28- Slaughter)
Maize	54.10	45.70	54.50
Wheat	-	11.10	6.50
Vegetable oil	1.30	3.50	4.00
Soybean meal (% 48 HP)	30.10	25.10	24.50
Full-fat soy	8.00	8.20	6.17
Meat-bone meal	3.00	3.27	-
Dicalcium phosphate	1.30	1.20	2.00
Ground limestone	0.50	0.30	0.70
Sodium bicarbonate	0.50	0.50	0.50
Salt	0.30	0.30	0.30
DL- Methionine	0.40	0.40	0.40
L- Lysine	0.10	0.05	0.05
L- Threonine	0.10	0.08	0.08
Vitamin mix *	0.20	0.20	0.20
Mineral mix **	0.10	0.10	0.10
Nutritional composition, %			
Dry matter	90.60	90.10	90.89
Crude protein	23.40	22.00	19.70
Crude fibre	3.20	3.50	3.58
Ether extract	5.83	7.75	8.34
Ash	5.50	5.30	3.91
Calcium ***	1.00	0.93	0.85
Available phosphorus ***	0.51	0.51	0.44
Methionine ***	0.69	0.66	0.59
Lysine ***	1.44	1.27	1.11
Threonine ***	0.97	0.88	0.81
ME, Kcal/kg***	3.011	3.176	3.225

** Vitamin premix supplied per 2.5 kg; (ROVIMIX 123-T+CAR 25/5); Vitamin A 12.000.000 IU; vitamin D_3 2.000.000 IU; vitamin E 35.000 mg; vitamin K_3 4.000 mg; vitamin B_1 3.000 mg; vitamin B_2 7.000 mg; Niacine 20.000 mg; Calcium D-pantotenat 10.000 mg; vitamin B_6 5.000 mg; vitamin B_{12} 15 mg; Folik Asit 1.000 mg; D-Biotin 45 mg; vitamin C 50.000 mg; Choline chloride 125.000 mg; Canthaxanthin 2.500 mg; Apo Karotenoik Acid Ester 500 mg; ** Mineral premix supplied per kg; (REMINERAL-S); Mn 80.000 mg; Fe 60.000 mg; Zn 60.000 mg; Cu 5.000 mg; Co 200 mg; I 1.000 mg; Se 150 mg; *** Calculated*

slaughter. Feathers were plucked from selected chickens at slaughter house with wet plucking method, and after feet's cutting, internal organs (except kidneys and lungs) were removed. After removal of internal organs, fat tissue around cloaca, gizzard and duodenum, and covering under surface of peritoneum was removed and determined as abdominal fat weight. Later, carcasses were cut into parts in accordance to TSE [12] shredding technique, and all parts were weighed with skin.

Carcass defects and ammonia burns were performed in company with qualified personnel in slaughterhouse. For this purpose; 300 animals in both flocks were evaluated in each season (150x2). Chicks were selected randomly from cutting lane for each feature. Evaluation of carcasses for lesions was conducted in form of yes/no evaluation, and determination was given as percentage. Evaluation of foot pad ammonia burns was conducted using a 4 scale scoring indicating as 0: No lesion 1: Mild lesion, 2: Moderate lesion, 3: High-intensity lesion, respectively [13].

Chemical Analysis

Chemical composition of food ingredients (dry matter, crude protein, ash and ether extract) were analyzed according to the AOAC [14] procedures and crude fiber was determined by the methods of Crampton and Maynard [15].

Lipid Peroxidation: The levels of malondialdehyde (MDA) were measured in serum with the thiobarbituric acid reaction by the method of Placer et al.[16]. The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption to the standard curve of MDA equivalents generated by acid catalyzed hydrolysis of 1,1,3,3 tetraethoxypropane. Every sample was assayed in duplicate, and the assay coefficients of variation for MDA were less than 3%.

Reduced Glutathione (GSH): The GSH content of the serum was measured at 412 nm using the method of Sedlak and Lindsay [17]. The samples were precipitated with 50% trichloroacetic acid and then centrifuged at 1.000 × g for 5 min. The reaction mixture contained 0.5 ml of supernatant, 2.0 ml of Tris-EDTA buffer (0.2 M; pH 8.9) and 0.1 ml of 0.01 M 5,5′-dithio-bis-2-nitrobenzoic acid. The solution was kept at room temperature for 5 min, and then read at 412 nm on the spectrophotometer.

Catalaz (CAT): The CAT activity of erythrocytes was measured according to the method of Aebi [18]. The degradation rate of H_2O_2 by CAT was spectrophotometrically measured by means of the fact that H_20_2 absorbed light at 240 nm wave length. CAT activity was calculated as k/g Hb.

Glutathione Peroxidase (GSH-PX): The GSH-Px activity was determined according to the method of Lawrence and Burk [19]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM ethylene diamine tetra acetic acid (EDTA), 1 mM sodium azide (NaN_3), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 IU/ml oxidized glutathione (GSSG)-reductase, 1 mM GSH, and 0.25 mM hydrogen peroxide (H_2O_2). Enzyme source (0.1 ml) was added to 0.8 ml of the above mixture and incubated at 25°C for 5 min before initiation of the reaction with the addition of 0.1 ml of peroxide solution. The absorbance at 340 nm was recorded for 5 min on a spectrophotometer. The activity was calculated from the slope of the lines as micromoles of NADPH oxidized per minute. The blank value (the enzyme was replaced with distilled water) was subtracted from each value.

Statistical Analysis

Effects of floor and cage housing systems on fattening performance, oxidative stress and carcass defects in broiler chicken were evaluated by independent-samples t test after test of normality. P-values were given in the tables including each season (summer, autumn and winter) and total effect of the housing systems. All analyses were performed by using SPSS for Windows [20]. The results were considered as significant when P values were lower than 0.05.

RESULTS

Mean values and standard errors of examined parameters were given in the tables. Live weights of 7th and 14th days were given in *Table 2* and found to be higher in cage system (P≤0.05). There were no significant difference between groups at later ages and at slaughter weight (P>0.05). Mortality rate and feed intake were found to be similar between groups (P>0.05). Better feed conversion rate (FCR) was obtained in floor system (P≤0.01).

The data presented in *Table 3* indicate that carcass yield was higher in floor housing (P≤0.01), all carcass parts and internal organ weights were similar between groups (P>0.05).

The data including lipid peroxidation presented in *Table 4* show that serum MDA levels of broiler were higher in cage housing system as compared with floor housing (P≤0.01). Serum CAT and GSH-Px activity and serum GSH level took statistically similar values between groups (P>0.05).

When carcass defects were examined *(Table 5)*, ratios of wings bruising (P≤0.001), wing fractures (P≤0.05) and breast bruising (P≤0.001) increased in cage housing. However, ratio of shank and drumstick bruising slightly increased in floor housing (P=0.074). When food pad lesions were examined *(Table 5)*, incidence of lesions decreased in cage housing (P≤0.001). The lesions of level 1 increased in cage housing (P<0.05), while ratios of degree 2 and 3 found to be higher in floor housing (P≤0.01).

DISCUSSION

Significantly higher live weight at 7 and 14 days were found to be in cage housing. Superior early weight gains at cage reared chickens may be an indication of more uniform control of environmental conditions in early stages at cage housing. In later periods, disappearance of difference in body weights was a sign of deterioration in cage conditions. Due to genetic characteristics of broiler chickens, they tend to be less active with increasing age [21]. This tendency may be increased with decreased possibility of moving in cage systems. Although each cage unit was designed to be large, lack of activity was thought to be an important factor affecting the results of the present research. At last stage of growth, lying chicks are the important factor for other chicks because they prevent them to reach water and food [22].

FCR values of reared chickens on the floor were found to be significantly better than reared chickens in the cage. Decreased activity in cage systems was concluded as an effect for the deterioration of FCR value. Skinner et al.[23] were reported drowsiness as a parameter that adversely affected the broiler FCR. It was reported that due to the lack of activity reducing in the bird feed consumption and increasing in mortality rates deteriorate the feed

Table 2. *Performance parameters of broilers reared in cage and floor housing systems*
Tablo 2. *Kafes ve yer sistemlerinde yetiştirilen etlik piliçlerin performans parametreleri*

Performance parameters	Summer			Autumn			Winter			Total Effect of Housing Systems (P_t)
	CH	FH	P	CH	FH	P	CH	FH	P	
Initial weight of the study, g	42.0±0.29	41.4±0.52	NS	41.2±0.23	42.0±0.29	NS	40.1±0.31	41.4±0.35	NS	NS
Day 7th, g	180±2.96	175±1.56	NS	178±2.18	176±1.54	NS	194±2.86	166±1.60	***	***
Day 14th, g	473±13.39	447±5.34	NS	438±6.34	465±3.33	**	520±7.92	466±6.14	**	*
Day 21st, g	956±6.25	920±6.35	**	969 ±9.40	996±11.14	NS	948±11.58	921±10.90	NS	NS
Day 28th, g	1583±10.93	1499±16.09	NS	1552±12.05	1642±16.23	**	1566±21.09	1561±8.12	NS	NS
Mean of slaughter ages of four production periods, day	36			35			33			-
Mortality rate, %	6.24±1.12	8.98±0.52	NS	9.64±1.01	7.80±1.34	*	5.70±1.39	6.82±1.35	NS	NS
Slaughter weight, kg	2.02±0.06	1.99±0.03	NS	1.93±0.03	2.04±0.05	NS	1.81±0.07	1.77±0.02	NS	NS
Weight gain, kg (1st- slaughter age)	1.97±0.08	1.94±0.05	NS	1.88±0.02	1.99±0.04	NS	1.77±0.09	1.72±0.02	NS	NS
Cumulative feed intake per broiler, kg	3.47±0.13	3.34±0.07	NS	3.27±0.09	3.27±0.09	NS	2.96±0.15	2.82±0.06	NS	NS
Feed conversion, FCR	1.76±0.02	1.72±0.02	NS	1.73±0.02	1.64±0.02	**	1.67±0.03	1.63±0.02	NS	**

CH: *Cage housing;* ***FH:*** *Floor housing;* ***P:*** *Statistical significance; Mean ± SEM.;* ***NS:*** *Not statistically significant; * P≤0.05; ** P≤.0.01; *** P≤0.001*

Table 3. *Carcass traits of broilers reared in cage and floor housing systems*
Tablo 3. *Kafes ve yer sistemlerinde yetiştirilen etlik piliçlerin karkas özellikleri*

Carcass traits	Summer			Autumn			Winter			Total Effect of Housing Systems (P_t)
	CH	FH	P	CH	FH	P	CH	FH	P	
Adjusted slaughter weight, kg	2.30±0.02	2.29±0.03	NS	2.29±0.03	2.29±0.02	NS	2.27±0.02	2.27±0.02	NS	NS
Carcass weight, kg	1.52±0.02	1.49±0.02	NS	1.52±0.02	1.55±0.01	NS	1.47±0.01	1.63±0.01	***	*
Carcass yield, %	66.1±0.68	65.2±0.64	NS	66.3±0.54	67.6±0.43	*	64.8±1.03	71.8±0.68	***	**
Thigh ratio, %	40.0±0.33	40.1±0.33	NS	39.4±0.30	39.9±0.34	NS	41.4±0.41	39.9±0.27	*	NS
Breast ratio, %	37.0±0.37	35.6±0.53	*	37.3±0.50	36.4±0.37	NS	35.7±0.55	37.3±0.56	NS	NS
Wings ratio, %	9.80±0.19	10.2±0.24	NS	9.67±0.15	9.85±0.11	NS	10.1±0.10	9.98±0.21	NS	NS
Back and neck ratio, %	13.2±0.22	13.1±0.34	NS	13.5±0.20	13.8±0.25	NS	12.8±0.25	13.8±0.26	NS	NS
Abdominal fat ratio, %	1.52±0.09	1.62±0.10	NS	1.67±0.08	1.48±0.09	NS	1.57±0.06	1.57±0.09	NS	NS
Liver ratio, %	1.77±0.03	1.84±0.07	NS	1.89±0.02	1.79±0.02	*	1.82±0.06	1.97±0.04	NS	NS
Heart ratio, %	0.433±0.01	0.418±0.01	NS	0.411±0.00	0.460±0.01	*	0.490±0.01	0.492±0.01	NS	NS
Spleen ratio, %	0.092±0.00	0.113±0.00	NS	0.095±0.00	0.098±0.00	NS	0.120±0.01	0.100±0.00	NS	NS

CH: *Cage housing;* ***FH:*** *Floor housing;* ***P:*** *Statistical significance; Mean ± SEM.;* ***NS:*** *Not statistically significant; * P≤0.05; ** P≤.0.01; *** P≤0.001; Weights of hot carcass, liver, heart, spleen and abdominal fat were proportioned to slaughter weight; Weights of thigh, breast, wings, back and neck were proportioned to carcass weight*

Table 4. *Lipid peroxidation and antioxidant activity of broilers reared in cage and floor housing systems*
Tablo 4. *Kafes ve yer sistemlerinde yetiştirilen etlik piliçlerin lipit peroksidasyonu ve antioksidan aktivitesi*

Oxidative stress parameters	Summer			Autumn			Winter			Total Effect of Housing Systems (P_t)
	CH	FH	P	CH	FH	P	CH	FH	P	
Malondialdehyde (MDA), nmol/ml	1.60±0.12	1.32±0.08	*	1.99±0.06	1.74±0.04	NS	2.33±0.16	1.92±0.09	***	**
Glutathione (GSH), mmol/g Hb	2.64±0.19	3.51±0.24	*	4.34±0.16	3.06±0.13	*	3.61±0.23	3.16±0.16	NS	NS
Catalaz (CAT), k/g Hb	5.90±0.66	2.34±0.33	**	7.10±1.38	7.42±1.52	NS	6.57±0.91	5.13±1.28	NS	NS
Glutathione peroxidase (GSH-Px), U/g Hb	26.7±0.69	21.6±0.78	*	43.8±2.98	52.4±3.11	NS	42.0±2.45	41.9±4.61	NS	NS

CH: *Cage housing;* ***FH:*** *Floor housing;* ***P:*** *Statistical significance Mean ± SEM.;* ***NS:*** *Not statistically significant; * P≤0.05; ** P≤.0.01; *** P≤0.001*

Table 5. *Carcass defects and food pad burns of broilers reared in cage and floor housing systems*
Tablo 5. *Kafes ve yer sistemlerinde yetiştirilen etlik piliçlerde karkas kusurları ve taban lezyonları*

Carcass defects	Summer			Autumn			Winter			Total Effect of Housing Systems (P_t)
	CH	FH	P	CH	FH	P	CH	FH	P	
Wings bruising	16.2±1.12	11.0±1.04	*	16.9±1.64	13.2±0.92	NS	15.6±1.08	8.63±0.85	***	***
Wing fractures	10.5±2.06	8.50±1.82	NS	8.68±0.49	4.80±0.69	**	16.4±1.41	10.5±1.13	**	*
Shank and drumstick bruising	3.83±0.75	3.60±0.64	NS	4.20±0.69	5.30±0.84	**	2.33±0.20	4.18±0.52	NS	NS
Breast bruising	2.83±0.52	1.10±0.10	*	4.12±0.35	2.32±0.91	NS	1.20±0.00	1.18±0.14	NS	***
Food pad burns										
No lesion	42.6±6.33	27.3±8.38	NS	41.3±3.00	30.3±3.75	**	46.1±3.64	23.1±3.27	*	***
Level 1	30.2±3.64	22.8±9.07	NS	37.5±2.95	24.9±6.04	NS	38.6±3.19	35.3±3.73	NS	*
Level 2	20.7±1.42	33.9±3.25	NS	19.5±1.36	29.5±4.09	*	13.9±1.29	32.7±10.12	*	**
Level 3	6.56±1.66	16.5±6.05	NS	1.72±0.57	15.3±2.83	*	1.37±0.43	8.83±2.27	*	**

CH: *Cage housing;* ***FH:*** *Floor housing;* ***P:*** *Statistical significance Mean ± SEM.;* ***NS:*** *Not statistically significant; * P≤0.05; ** P≤.0.01; *** P≤0.001;* ***Level 1:*** *Mild lesion;* ***2:*** *Moderate lesion;* ***3****: High-intensity lesion*

efficiency [24,25]. Feed consumption and mortality rates were found to be similar between the groups and this finding suggested another factor for affecting feed efficiency; feed waste. Perforated structure of cage ground leads to spillage of food to manure belt and spilled food cannot be reached by chickens. However, at floor system, spilled food can be consumed again and utilized by chickens. At the same time, rush to food after dark schedule increased the food wastage at cage housing and mortality due to sudden death syndrome. In addition, Santos et al.[26] revealed that broilers reared on litter had a better FCR than those raised in cages (1.71 vs. 1.81 g/g) due to larger the jejunum villus area, mucosal depth and heavier relative gizzard weights, whereas the small intestine was lighter and shorter. In another research, Santos et al.[27] reported that although broiler reared on litter floors showed greater 14 day *Salmonella* colonization than cage reared broiler, their digestion capacity appeared superior than cage reared broiler, and they had fewer undigested feed particles in their distal small intestine which correlates with enhanced growth performance and breast meat yield. Fouad et al.[28] mentioned that floor reared broilers had significantly heavier final body weight, body weight gain, better FCR and lower mortalities throughout the whole rearing period (0-6 weeks). Lacin et al.[9] found higher body weight in floor group than cage without any effect on FCR and carcass traits. Aslam Athar et al.[5] emphasized significant increase in performance of broiler at cage housing systems. However, Bahreiny et al.[29] found no significant difference between cage and floor systems in terms of live weight, feed intake and FCR.

Broiler weights in each system and each season were equalized before slaughter in order to compare results between the groups. Groups were found to be similar in terms of proportional values of parts of carcasses and proportional values of lymphoid organs. The superiority of carcass yield might be associated with better welfare status of broilers reared at floor. Significantly lower serum MDA levels in broiler reared at floor as compared with the caged ones supported this idea. Higher serum MDA levels in caged birds would account for higher stress in these birds as compared with the floor housing. Reactive oxygen species (free radicals) are natural products of cell oxygen metabolism. However, depending on environmental stress, these metabolites increase rapidly in cell. Increased metabolites damage cell structure. This condition is defined as oxidative stress [30,31]. MDA is end product of lipid peroxidation in cell and an important indicator of stress [32]. Due to the increase in the level of MDA values in cage housing, it can be said that chickens were stressed. This parameter can also be associated with worsening feed efficiency and carcass yield in cage reared broiler chickens in the present study. In another study [33], performance and carcass quality of broiler chickens grown under chronic stress were found to be significantly impaired since the increase in synthesis of corticosterone impairing protein synthesis. Sogunle et al.[7] referred that dressing percentage and breast weight were higher in the floor housing than cage housing while Bahreiny et al.[29] and Lacin et al.[9] did not find any difference between groups in carcass yield and parts. Antioxidant metabolism was found to be similar in terms of both groups.

High carcass defects in cage reared broilers could be associated with fall of broiler chickens onto conveyor belts during transport to slaughter and wing flapping on this line during progress. Weak bone structure might be another affecting factor in cases of wing fractures in cage reared broiler [8,34]. Numerical superiority of shank and drumstick bruising in floor housing were associated with capturing of chickens from feet during transport to slaughter (P=0.07). Formation of ammonia burns on foot pad at cage housing was observed to be intensive at level 1. However, deeper and wider lesions were detected at foot pad of chickens in floor housing. Higher level lesions such as level 2 and level 3 in floor housing were related with litter management. The other studies showed that poor litter management caused the higher incidence of foot pad lesions at broiler chickens [35,36]. Incidence and severity of these lesions dropped at cage housing, because litter was not used in this system. However, lack of activity and structural feature of ground and manure that not filtered along cage ground especially in later ages were found to be related with etiology of foot lesions in broiler chickens reared in cage housing.

In conclusion, in spite of increasing automation with subsequently developing technology in cage housing, broiler chickens reared in floor housing showed better performance. Although it is possible to grow more chickens with multi-storey cage housing, cost of cages in this housing system and mending costs are very high. Placing of the chicks from hatcheries to the system and collecting of the dead during production increase the labor cost. Higher mortality rate towards the end of production period leads to early delivery of chicks to slaughter. It might be possible that activity in caged birds was limited by cage conditions and birds were not able to express their natural behavior resulting in increased stress and reduced welfare in birds. However, dust problem in poultry house and litter problems are dissolved in this system. These important considerations should be carefully taken into account in future decisions regarding the expansion of cage system for broiler production.

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RESEARCH ARTICLE

Recovery of Thermophilic *Campylobacter* spp. in Healthy and Diarrhoeic Pets by Three Culture Methods and Identification of the Isolates by Multiplex Polymerase Chain Reaction (mPCR) [1]

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Summary

In this study, the determination of prevalence of thermophilic *Campylobacter* species in dogs and cats with and without diarrhoea using 3 different cultural methods was aimed. For this purpose, rectal swabs were collected from 120 dogs and 15 cats and 14 of them (12 dogs and 2 cats) were taken from diarrhoeic animals. The isolation of thermophilic *Campylobacter* spp. was conducted by direct plating onto modified charcoal cefoperazone deoxycholate agar (mCCDA) supplemented with CCDA (cefoperazone, amphotericin B) or CAT (cefoperazone, amphotericin and teicoplanin) for all samples and membrane filtration method onto Mueller-Hinton Agar supplemented with 5% defibrinated sheep blood for samples from diarrhoeic pets and identification of isolates was performed using multiplex polymerase chain reaction (mPCR). The overall prevalence of *Campylobacter* species was found to be 40.0% and 26.7% in dogs and cats, respectively. *Campylobacter jejuni* was the most frequent bacterium isolated from 36 dogs and 4 cats. *C. upsaliensis, C. coli* and *C. lari* were isolated from 10 dogs and 1 cat, 5 dogs and 2 healthy dogs respectively. For the isolation of thermophilic *Campylobacter* spp., whilst the method using CAT as selective supplement being more sensitive in dogs, the membrane filtration appeared as the most suitable method in diarrhoeic dogs. These results showed the occurrence of a relatively high carriage of *Campylobacter* spp., particularly in healthy dogs that may constitute a non negligible risk for public health.

Keywords: *Campylobacter spp., Membrane filtration method, mPCR, Pet animals, Rectal swab*

Sağlıklı ve İshalli Pet Hayvanlarda Termofilik *Campylobacter* spp.'nin Üç Kültür Metodu ile İzolasyonu ve İzolatların Multipleks Polimeraz Zincir Reaksiyonu m(PZR) İle İdentifikasyonu

Özet

Bu çalışmada, sağlıklı ve ishalli kedi ve köpeklerde 3 farklı kültür metodu kullanılarak termofilik *Campylobacter* türlerinin prevalansının belirlenmesi amaçlandı. Bu amaçla, 120 köpek, 15 kediden rektal svap örneği toplandı ve bunların 14'ü (12 köpek ve 2 kedi) ishalli hayvanlardan alındı. Termofilik *Campylobacter* spp.'nin izolasyonunda, tüm örnekler için CCDA (cefoperazone, amphotericin B) ya da CAT suplement (cefoperazone, amphotericin and teicoplanin) ilave edilmiş modified charcoal cefoperazone deoxycholate agar (mCCDA)'a (sefoperazon, amfoterisin B) direkt ekim, ishalli hayvanlardan alınan örnekler için de %5 defibrine koyun kanı ilave edilmiş Mueller- Hinton Agar üzerine membran filtrasyon yöntemi kullanıldı. İzolatların identifikasyonu multipleks polimeraz zincir reaksiyonu (mPZR) ile gerçekleştirildi. *Campylobacter* türlerinin köpek ve kedilerde genel prevalansı sırasıyla %40.0 ve %26.7 olarak bulundu. *Campylobacter jejuni* en sık rastlanan tür olup 36 köpek ve 4 kediden izole edildi. Ayrıca, 10 köpek ve 1 kediden *C. upsaliensis,* 5 köpekten *C. coli* ve 2 sağlıklı köpekten *C. lari* izole edildi. Termofilik *Campylobacter* spp. izolasyonu için CAT selektif suplementin kullanıldığı metot köpeklerde daha duyarlı iken ishalli köpeklerde membran filtrasyonun en uygun metot olduğu görüldü. Bu sonuçlar, özellikle sağlıklı köpeklerde oldukça yüksek oranlarda bulunan *Campylobacter* spp. taşıyıcılığının halk sağlığı için göz ardı edilemez risk oluşturduğunu göstermektedir.

Anahtar sözcükler: *Campylobacter spp., Membran filtrasyon metodu, mPZR, Pet hayvanları, Rektal svap*

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INTRODUCTION

The genus *Campylobacter* currently contains 18 species with six sub-species and two biovars [1-3]. *Campylobacter* bacteria are the most commonly reported agents causing gastroenteritis in humans in the industrialized countries [4-6]. Campylobacteriosis in humans is a zoonotic disease and the bacteria are frequently found as commensals in the gastrointestinal tract of many domestic and wild animals, especially birds [7-12]. *C. jejuni* is by far the most frequently isolated species from human cases, but other thermophilic *Campylobacter* spp. such as *C. upsaliensis, C. coli and C. lari* have also been associated with diseases in humans [5,6] Consumption of undercooked chickens and handling raw chicken carcasses has been identified as significant risk factors for human infections [2,10,13]. Other known risk factors are consumption of unpasteurised milk or water, travelling abroad and living or working on a farm [2,13-15]. Cat and dogs can harbour *Campylobacter* spp. in their gastrointestinal systems [16-18] and daily contact with pet dogs and cats have been identified as another risk factor for human campylobacteriosis. There are many reports describing presumed or proven associations between *Campylobacter* infections and pet exposure [19,20].

However, there is no detailed report by using different isolation methods and molecular method for *Campylobacter* species from dogs and cats in Turkey. The objective of this study was to evaluate the prevalence of Thermophilic *Campylobacter* spp. in dogs and cats with and without diarrhoea using three different isolation methods and to identify isolates using mPCR (multiplex polymerase chain reaction).

MATERIAL and METHODS

Samples

A total of 135 rectal swabs taken from 120 dogs and 15 cats submitted to Erciyes University, Faculty of Veterinary Medicine, Turkey, was analysed between November 2008 and April 2009. The animals were at different ages and breeds. Fourteen (12 dogs and 2 cats) of the 135 rectal swabs examined were taken from diarrhoeic animals whereas 108 dogs and 13 cats were healthy (being presented for a health check, vaccination or neutering). The majority of the animals was from Kayseri and kept as indoor pets. The samples were immediately transported to the laboratory in a cool box and examined within 15 min after sampling.

Isolation Procedures

Each swab sample taken from animals was homogenized with 500 µL distilled water and 100 µL of this inoculum was plated directly onto mCCDA (modified charcoal cefoperazone deoxycholate agar, Oxoid, CM0739) with CCDA selective supplement (cefoperazone, amphotericin B, Oxoid, SR0155E) (medium 1), and mCCDA with CAT (cefoperazone, amphotericin and teicoplanin, Oxoid, SR174E) selective supplement (medium 2), respectively. Membrane filtration method was used as a third method. In the filtration method, 300 µL of faecal suspension were placed on a 47-mm diameter, 0.45µm-pore-size cellulose acetate membrane filter (Sartorius AG, Goettingen, Germany) placed on Mueller-Hinton Agar supplemented with 5% defibrinated sheep blood without any selective supplement. After incubation at 37°C for 30 min under aerobic conditions, the filter was removed [21]. The plates were then incubated microaerobically for 48-96 h at 42°C. The first and second methods were used both in diarrhoeic and non diarrhoeic animals, but the third method was used in diarrhoeic animals only. After the incubation period, *Campylobacter* spp. were initially identified by observing characteristic morphology and motility using phase contrast microscopy and using morphological features of the colonies (1-3 mm in diameter, white to cream to silver in colour and round in outline), Gram staining, oxidase reaction and catalase production [8,9,22]. *C. jejuni* NCTC 11168 was used as the reference strain. Presumed *Campylobacter* spp. colonies were sub-cultured on mCCDA supplemented with 5% defibrinated sheep blood under the same conditions as described above for purification and the isolates were stored at -80°C until further analysis.

Differentiation of Campylobacter Isolates by Colony mPCR

The primers and PCR assay conditions were used for the simultaneous identification and differentiation of the *Campylobacter* isolates as previously described by Wang et al.[23]. This method was slightly modified and *Campylobacter fetus* primers were not used in the current mPCR. Only 5 pairs of primers were used to identify the genes *hipO* from *C. jejuni*, *glyA* from *C. coli*, *C. lari*, and *C. upsaliensis*; and the internal control 23S rRNA [23]. Chromosomal DNA was prepared by suspending again the cell pellets in 100 µL of sterile distilled water and boiling the suspensions for 10 min. After centrifugation (in 10.000xg, for 10 min, at +4°C), the supernatants were used as DNA templates in mPCR. The primers and expected PCR amplicons are shown in *Table 1*.

The mPCR consisted of 30 cycles (Touchgene Gradient,

Table 1. *Predicted sizes of amplified products of mPCR and primer pairs for thermophilic Campylobacter species used*
Tablo 1. *Termofilik Campylobacter türleri için mPCR amplifikasyon ürünlerinin beklenen band büyüklükleri ve kullanılan primer çiftleri*

Gene	Primer	PCR Amplicon Size (in bp)
C. jejuni hipO	CJF, CJR	323
C. coli glyA	CCF, CCR	126
C. lari glyA	CLF, CLR	251
C. upsaliensis glyA	CUF, CUR	204
C. jejuni 23S rRNA	23SF, 23SR	650

Techne, UK). Amplified products were detected by electrophoresis (EC340 Maxicell, Thermo, USA) on a 1.5% agarose at 100 V for 40 min (EC250-90, Thermo, USA). The gels were stained with ethidium bromide and inspected visually under a UV transilluminator (Vilber Lourmat, Marne La Vallée, France).

RESULTS

All *Campylobacter* spp. isolates were identified at the species level by mPCR *(Fig. 1)*. A total of 48 samples in dogs and 4 samples in cats were positive for *Campylobacter* spp. with at least one of the 3 isolation methods leading to overall prevalences of 40.0% and 26.7%, respectively. As shown in *Table 2* and *Table 3*, *C. jejuni* was the most predominant species identified in sampled dogs (in 32 non diarrhoeic dogs and in 4 diarrhoeic dogs) and cats (in 3 healthy animals and in one with diarrhoea) with at least one isolation method and sometimes found in association with other species such as *C. upsaliensis* (3 times in dogs and once in cats) and *C. coli* (Once in healthy dogs). Three other *Campylobacter* species, *C. upsaliensis*, *C. coli* and *C. lari*, were also isolated in 20.8%, 10.4% and 4.2% respectively dog rectal swab samples.

The 2 isolation methods based on isolation on mCCDA medium with CCDA (method 1) or CAT (method 2) as selective supplements showed different efficiencies. Firstly, 24.1% and 32.4% of the healthy dogs and 25.0% and 41.7% of the diarrhoeic dogs were positive using methods 1 and 2, respectively *(Table 2)* and in healthy cats, the isolation rates were 23.1% and 15.4% for methods 1 and 2, respectively. Contrary to the method 1, no rectal sample from diarrhoeic cats gave positive isolation with the medium 2 *(Table 3)*. As reported in *Table 4*, the overall agreement score (number of identical scores (positive or negative isolation for *Campylobacter* spp.) in dogs and cats) was 81.5% and the agreement score was slightly higher in cats (86.7%) than in dogs (80.8%). Among the 50 samples (46 from dogs and 4 from cats) positive for *Campylobacter* spp. isolation, 25 (23 from dogs and 2 from cats) were positive for the 2 methods (positive agreement score: 50.0%) and these positive scores were similar and have remained as moderate in healthy animals than in diarrhoeic ones (approximately 50.0%). The second method was found to be more appropriate and sensitive in detecting various species of *Campylobacter* spp. in the dog rectal samples (40 positive samples versus 29 with the first method) whereas in cats 4 samples were positive with the first method and only 2 with the second method. In diarrhoeic cases, the membrane filtration method (third method) appeared as the most suitable in dogs *(Table 2)* evidencing *Campylobacter* spp. in 7 samples (versus 5 with the method 2) whereas it failed to detect bacteria in diarrhoeic cats *(Table 3)*.

DISCUSSION

This is the first report using different isolation techniques and mPCR for detecting of thermophilic campylobacters from dogs and cats in Turkey. Several isolation media have been developed and evaluated for the isolation of *Campylobacter* spp. from clinical, food, environmental and animal samples. The most widely used method for the

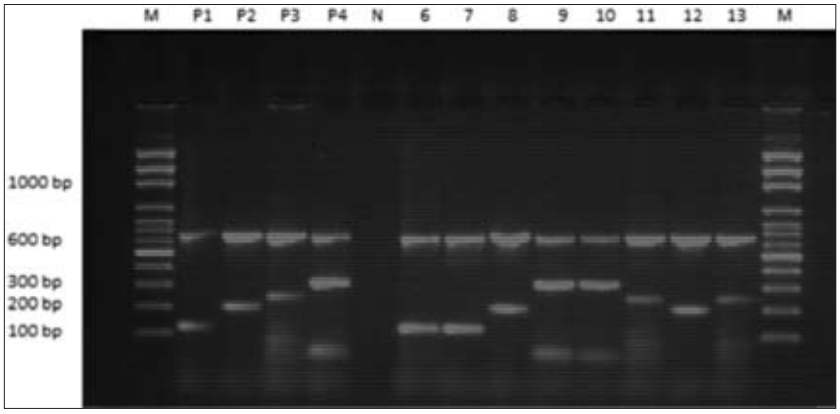

Fig 1. mPCR products from *Campylobacter* isolates by 1.5% agarose gel electrophoresis
M: Marker; **P1:** positive control for *C. coli* DCC2 (126 bp); **P2:** positive control for *C. upsaliensis* DCC3 (204 bp); **P3:** positive control for *C. lari* DCC4 (251 bp); **P4:** positive control for *C. jejuni* NCTC 11168 (323 bp); **N:** negative control; **lanes 6-13:** dog rectal swap isolates (*C. coli* in lanes 6 and 7, *C. upsaliensis* in lanes 8 and 12, *C. jejuni* in lanes 9 and 10, *C. lari* in lane 11and 13); **650 bp:** fragment of 23S rRNA (which occurred from all *Campylobacter* spp.)

Şekil 1. *Campylobacter* türlerine ait mPZR ürünlerinin %1.5 agaroz jel görüntüsü
M: Moleküler marker; **P1:** *C. coli* DCC2 pozitif kontrol (126 bp); **P2:** *C. upsaliensis* DCC3 pozitif control (204 bp); **P3:** *C. lari* DCC4 pozitif control (251 bp); **P4:** *C. jejuni* NCTC 11168 pozitif control (323 bp); **N:** negatif kontrol; **sıra 6-13:** köpek rektal svap izolatları (6,7: *C. coli*, 8,12: *C. upsaliensis,* 9,10: *C. jejuni*, 11,13: *C. lari*); **650 bp:** 23S rRNA (*Campylobacter* spp. için genus pozitif bandlar)

Table 2. *Isolation rates of thermophilic Campylobacter spp. by different methods in diarrhoeic (n = 12) and in healthy (n = 108) dogs*
Tablo 2. *İshalli ve sağlıklı köpeklerden termofilik Campylobacter türlerinin farklı metodlar ile izolasyon oranları*

Media Used	Healthy Dogs (n = 108)	Diarrhoeic Dogs (n = 12)	Total (n = 120)
Positive samples			
Medium 1	26 (24.1%)	3 (25.0%)	29 (24.2%)
Medium 2	35 (32.4%)	5 (41.7%)	40 (33.3%)
Medium 3	ND	7 (58.3%)	ND
Total[1]	**41 (38.0%)**	**7 (58.3%)**	**48 (40.0%)**
***Campylobacter* spp**			
C. jejuni			
Medium 1	22 (84.6%)	3 (100.0%)	25 (86.2%)
Medium 2	28 (80.0%)	4 (80.0%)	32 (80.0%)
Medium 3	ND	4 (57.1%)	ND
Total[1]	**32**	**4**	**36 (75%)**
C. coli			
Medium 1	3 (11.5%)	0 (0.0%)	3 (10.3%)
Medium 2	1 (2.9%)	0 (0.0%)	1 (2.5%)
Medium 3	ND	1 (14.3%)	ND
Total[1]	**4**	**1**	**5 (10.4%)**
C. lari			
Medium 1	1 (3.8%)	0 (0.0%)	1 (3.4%)
Medium 2	1 (2.9%)	0 (0.0%)	1 (2.5%)
Medium 3	ND	0 (0.0%)	ND
Total[1]	**2**	**0**	**2 (4.2%)**
C. upsaliensis			
Medium 1	1 (3.8%)	0 (0.0%)	1 (3.4%)
Medium 2	8 (22.9%)	1 (20.0%)	9 (22.5%)
Medium 3	ND	2 (28.6%)	ND
Total[1]	**8**	**2**	**10 (20.8%)**

Medium 1: *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CCDA (cefoperazone, amphotericin B) selective supplement;* ***Medium 2:*** *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CAT (cefoperazone, amphotericin and teicoplanin) selective supplement;* ***Medium 3:*** *Mueller-Hinton Agar supplemented with 5% defibrinated sheep blood (membrane filtration method);* ***ND:*** *not detected;* [1] *number of positive samples with at least one isolation method*

detection of *Campylobacter* spp. in animals is direct plating of a faecal swab sample onto selective media containing various combinations of antibacterial agents (such as, Preston agar, CAT agar, mCCDA medium, and Karmali medium) [24,25]. In addition, enrichment of campylobacters in a broth medium is used for the isolation of campylobacters when the numbers of bacteria are presumed to be low [26,27]. Membrane filtration method has been extensively used for the isolation of *Campylobacter* spp. (non selective agar base e.g., blood agar base, Mueller Hinton agar, Brucella agar supplemented with 5-7% defibrinated sheep blood) [26-28]. Although faecal-based methods are still the most widely used and considered to be reliable detection methods for *Campylobacter* in animals, their detection ranges are variable with each procedure [9,26-29]. Thermophilic *Campylobacter* spp., *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* are commensally present in the intestinal flora of dogs and cats [30,31]. Hence, dogs and cats also present a risk factor for human campylobacteriosis [19,20]. Isolation rate of *Campylobacter* spp. from these animals have been shown to vary in different studies. Acke et al.[26] isolated *Campylobacter* spp. from both healthy and diarrhoeic animals at a rate of 45.2% in dogs and cats. In another study performed by the same researchers [32], the isolation rate of *Campylobacter* spp. was determined to be 42.9% and 41.5% in cats and dogs, respectively. Sandberg et al.[22] found campylobacters in 18% and 23% of healthy cats and dogs respectively. In the present study, the isolation rates of *Campylobacter* spp. were found to be 25.0%, 41.7% and 58.3% in diarrhoeic dogs by using the method 1, method 2 and method 3, respectively and healthy animals had 24.1% and 32.4% *Campylobacter* isolation rates with methods 1 and 2, respectively. Similar to other studies [22,32], it was found that dogs with diarrhoea were more likely to be carriers of campylobacters than healthy animals *(Table 2)*. In healthy cats, the isolation rates were 23.1% and 15.4% for method 1 and method 2, respectively. The isolation rates of campylobacters in this study were found to be different from the earlier studies, which can be attributed to several factors, such as isolation media and procedures employed, sample size, sampling time.

However, the detection rate of *Campylobacter* spp. in the 135 pets sampled was significantly increased using a combination of the 3 isolation methods in the current study leading to bacteria prevalence of 40.0% (48 positive cases) in dogs and of 26.7% (4 positive cases) in cats (*Table 2* and *3*). As the majority of *Campylobacter* spp. was

Table 3. *Isolation rates of thermophilic Campylobacter spp. by different methods in diarrhoeic (n = 2) or in healthy (n = 13) cats*
Tablo 3. *İshalli ve sağlıklı kedilerden termofilik Campylobacter türlerinin farklı metodlar ile izolasyon oranları*

Media Used	Healthy Cats (n = 13)	Diarrhoeic Cats (n = 2)	Total (n = 15)
Positive samples			
Medium 1	3 (23.1%)	1	4 (26.7%)
Medium 2	2 (15.4%)	0	2 (13.3%)
Medium 3	ND	0	ND
Total[1]	**3 (23.1%)**	**1**	**4 (26.7%)**
***Campylobacter* spp**			
C. jejuni			
Medium 1	3 (100%)	1	4
Medium 2	2 (100%)	0	2
Medium 3	ND	0	ND
Total[1]	**3**	**1**	**4**
C. coli			
Medium 1	0 (0%)	0	0
Medium 2	0 (0%)	0	0
Medium 3	ND	0	ND
Total[1]	**0**	**0**	**0**
C. lari			
Medium 1	0 (0%)	0	0
Medium 2	0 (0%)	0	0
Medium 3	ND	0	ND
Total[1]	**0**	**0**	**0**
C. upsaliensis			
Medium 1	0 (0%)	0	0
Medium 2	1 (50%)	0	1
Medium 3	ND	0	ND
Total[1]	**1**	**0**	**1**

Medium 1: *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CCDA (cefoperazone, amphotericin B) selective supplement;* ***Medium 2:*** *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CAT (cefoperazone, amphotericin and teicoplanin) selective supplement;* ***Medium 3:*** *Mueller-Hinton Agar supplemented with 5% defibrinated sheep blood (membrane filtration method);* ***ND:*** *not detected; [1] number of positive samples with at least one isolation method*

Table 4*. Agreement scores between the methods used for the isolation of Campylobacter spp. from rectal swab samples in healthy (108 dogs and 13 cats) and diarrhoeic (12 dogs and 2 cats) animals*
Tablo 4. *Sağlıklı ve İshalli hayvanların (12 köpek ve 2 kedi) rektal svap örneklerinden Campylobacter spp. izolasyonunda kullanılan metodlar arasındaki uyum değerleri*

Medium 2 and Aggrement Score	Medium 1																	
	Positive (n = 33)									Negative (n = 102)								
	Healthy			Diarrhoeic			Total			Healthy			Diarrhoeic			Total		
	Dogs	Cats	Dogs + Cats	Dogs	Cats	Dogs + Cats	Dogs	Cats	Dogs + Cats	Dogs	Cats	Dogs + Cats	Dogs	Cats	Dogs + Cats	Dogs	Cats	Dogs + Cats
Medium 2																		
Positive (n = 42)	20	2	22	3	0	3	23	2	25	15	0	15	2	0	2	17	0	17
Negative (n=93)	6	1	7	0	1	1	6	2	8	67	10	77	7	1	8	74	11	85
Agreement score																		
in dogs	20			3			23			67			7			74		
in cats		2			0			2			10			1			11	
Total			22			3			25			77			8			85

Medium 1: *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CCDA (cefoperazone, amphotericin B) selective supplement;* ***Medium 2:*** *mCCDA (modified charcoal cefoperazone deoxycholate agar) with CAT (cefoperazone, amphotericin and teicoplanin) selective supplement; Agreement score: number of samples given a same result with the 2 methods*

recovered by direct plating onto mCCD agar medium with CAT supplement (method 2), this would be the method of choice if only a method was selected for detection of the most common *Campylobacter* spp. in pets. The findings of

this method used in this study are in agreement with those of previous studies for the isolation of *Campylobacter* [26,28,32]. Indeed, the overall agreement score between method 1 (mCCD agar medium with CCDA supplement) and method 2 (mCCD agar medium with CAT supplement) was relatively moderate in pets (81.5%) and the positive agreement score (number of samples given positive by the 2 methods) was quite low (50.0%), showing great variations in sensitivity between the 2 methods. As the membrane filtration method was used only in diarrhoeic animals, the number of cases was quite insufficient for determining agreement scores with the 2 other isolation methods.

C. jejuni was the most commonly isolated species from dogs and cats, and *C. upsaliensis* was the second most commonly isolated species in pets with all three method used in the current study. In contrary, it has been reported that *C. upsaliensis* was the predominant species in the recent studies [17,18,33].

Campylobacter spp. can be found as an opportunistic infectious agent in dogs and cats with gastrointestinal signs caused from endoparasites or parvovirus infection and they may act as a primary or secondary pathogen [32]. The link between the gastrointestinal symptoms and the presence of campylobacters in the gastrointestinal system has been studied but it remains obscure [29,32] and in the present study, the sampled animals were found to be negative for endoparasites or parvovirus infections.

Concurrent association between several *Campylobacter* species in dogs and cats has also been reported by other researchers [25,29,34-36]. Koene et al.[25] detected more than one *Campylobacter* species in six samples taken from healthy dogs. Similarly Hald et al.[37] reported that ten dogs were positive for concurrent infection with *Campylobacter* species. Similar findings have been reported by Workman et al.[29] for cat rectal samples. Such *Campylobacter* spp. associations were detected in five samples from pets in the present study, indicating that infections may be simultaneously caused by several bacterial species. In addition, different colony type of *Campylobacter* bacterium should be evaluated in one examined samples. Although *C. lari* was generally found in poultry intestinal system [38] some researchers reported that this bacterium was also encountered in dog intestines [25,32]. Similarly, *C. lari* was found in 2 healthy dog rectal swabs.

As a conclusion, the overall prevalence of *Campylobacter* spp. was 40.0% (48 cases) and 26.7% (4 cases) in dogs and cats, respectively. This study illustrates that dogs and cats carry a potential risk as possible reservoirs for human infections by these bacteria. The mCCD basal agar with CAT supplement (method 2) was found to be more appropriate and sensitive in detecting various species of *Campylobacter* in healthy dog rectal swab samples while in diarrhoeic animals, the membrane filtration was the most effective method for cultural detection.

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RESEARCH ARTICLE

Effects of Stocking Density and Litter Type on Litter Quality and Growth Performance of Broiler Chicken

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Summary

This study was conducted to investigate the effects of stocking density and litter type on growth performance of broiler chickens as well as dressing percentage and some litter quality indicators. 684 male broiler (Ross PM_3) was used for a 6 weeks period in the experiment. The day-old chicks were randomly assigned in two litter groups: Rice hulls and wood shavings. Each litter group was further divided in three stocking density (15, 19 and 23 chicks/m^2). Chicks in the each group were randomized into three replicates at hatch and they were housed in a deep litter pens (1x2 m, each) in an environmentally controlled house. The litter type and stocking density had significant effect on the final body weight of broiler ($P<0.05$, $P<0.001$), lenght of foot pad lesions of the birds ($P<0.001$, $P<0.001$), litter moisture ($P<0.01$, $P<0.05$) and litter pH contents ($P<0.05$, $P<0.05$). The results of this study suggest that greater stocking density more than 15 chicks per square meter and rice hull adversely affects live body weight of broilers and main litter quality indicators. But up to a critical point, profitability increases with increased stocking density because of the reduction of fixed cost and more kilograms production of broiler per area. Depends on the price and availability rice hull can be used as litter material instead of wood shaving.

Keywords: *Broiler, Stocking density, Litter type, Litter quality, Growth performance*

Etlik Piliçlerde Yerleşim Sıklığı ve Altlık Türünün Altlık Kalitesi ve Büyüme Performansı Üzerine Etkileri

Özet

Bu çalışma etlik piliçlerde yerleşim sıklığı ve altlık tipinin büyüme performansı, karkas randımanı ve altlık kalite parametreleri üzerine etkisini araştırmak amacı ile yapılmıştır. Çalışmada Ross PM_3 genotipi 684 adet erkek etlik civciv kullanılmış ve çalışma 6 hafta sürmüştür. Çevre kontrollü bir kümeste yer alan deneme ünitesinde; pirinç kavuzu ve odun talaşının kullanıldığı gruplar ile herbir altlık grubu üzerinde; metrekare alanda 15, 19 ve 23 adet civcivin yer aldığı (2x3=6) altı ana grup oluşturulmuş ve her ana grupta 3 tekrarlı grup yer almıştır. Altlık türü ve barındırma yoğunluğunun canlı ağırlık ($P<0.05$, $P<0.001$), ayak tabanı lezyonu ($P<0.001$, $P<0.001$), altlık rutubet ($P<0.01$, $P<0.05$) ve pH ($P<0.05$, $P<0.05$) üzerine etkisi önemli bulunmuştur. Çalışmada birim metrekare alanda onbeşten fazla hayvan sayısı ve pirinç kavuzunun canlı ağırlık ve altlık kalitesini olumsuz etkilediği sonucuna ulaşılmıştır. Ancak birim alandaki sabit masrafların azalması ve üretim miktarının artmasından dolayı kritik bir noktaya kadar yerleşim sıklığı artırılabilir. Fiyat ve elde edilme imkanlarına bağlı olarak odun talaşı yerine pirinç kabuğu da altlık olarak kullanılabilir.

Anahtar sözcükler: *Etlik piliç, Yerleşim sıklığı, Altlık tipi, Altlık kalitesi, Büyüme performansı*

INTRODUCTION

Great number of research paper about broiler stocking density and litter are focused on growth and economic performance, carcass quality and in recent period, poultry welfare. One of the major welfare concerns in broiler production is detrimental effects of high stocking density, especially in the final weeks of growing period [1,2]. Bokkers et al.[3] showed that a stocking density in large flocks exceeding 16 birds/m^2 leads to compression of birds. But the correlation between broiler welfare and stocking density are not so clear [4]. A large-scale study in Europe on broiler welfare has shown that stocking density did not affect bird behavior [5].

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One of main factor influencing litter quality in broiler production is litter or bedding material and providing high litter quality. An ideal litter material should be dry with high water absorption capacity, but should also be able to release the absorbed moisture quickly [6]. Litter material and therefore the quality of litter directly affects the performance, health, carcass quality, and welfare of poultry [7-9].

There is an important relationship between stocking density and litter quality in broiler production. Especially in high stocking density conditions litter material had been disturbed faster than low density and higher levels of litter moisture may result with some leg problems such as angle-out legs and unblemished hocks [10]. Litter quality also has a direct influence on skin condition of birds and carcass quality. In that reason controlling the environment of the birds, particularly in house humidity and ammonia along with litter moisture is crucial to provide good welfare. Litter materials with high water-holding capacity, such as wood shavings, are believed to result in better litter quality than litter materials with poorer absorption capacity such as straw [11]. Torok et al.[12] showed that litter choice may have an important role in poultry gut health particularly in the absence of in-feed antibiotics. Much attention should be given to create good growing conditions to reducing adverse effect of high stocking density [13]. This could be performed by using the most ideal litter material and more bedding material per unit area in high stocking density conditions. Additionally, the bedding or litter material must be readily available in sufficient quantities and most importantly, it must be economical [14].

Within the last several years, economic and practical conditions in Turkey have led to a shortage of wood shavings conventionally used as poultry litter. Limited supplies, higher cost, and unavailability of suitable material have encouraged the search for alternative litter materials. As a result of this effords the use of rice hull is being becoming very popular as litter material in broiler production in Turkey. But its quality and effects on performance and bird welfare is very questionable. Therefore, the objective of this study was to evaluate effects of rice hull compared to wood shavings on main litter quality indicators and growth performance in broiler production in three different stocking density conditions.

MATERIAL and METHODS

The experimental procedures conducted in this study were in accordance with the principles and guidelines set out by the Committee of the Faculty of Veterinary Medicine. 684 day-old male chicks (Ross PM_3) obtained from a commercial hatchery were reared in a deep litter pens (in total 18 pens, 1x2 m each) in an environmentally controlled house (Faculty farm) in spring season until they were six weeks old. The day-old chicks were randomly assigned in two litter type groups as rice hulls and wood shavings. Each litter groups were further divided into three stocking density (15, 19 and 23 chicks/m^2). Chicks in the each litter type x stocking density treatment groups were randomized into three replicates *(Table 1)*.

Management

All chicks were brooded and reared at 32-33 °C from hatch to 7 d of age, 28-30°C from 8 to 14 d of age, 24-26°C from 15 to 21 d of age, and 21-24°C from 22 day of age to the end of the experiment. Standard commercial broiler feed (220 g protein/kg diet and 3.000 kcal metabolisable energy/kg diet from 1 to 14 d of age, 200 g protein/kg diet and 3.050 kcal metabolisable energy/kg diet from 15 to 35 d of age, 180 g protein/kg diet and 3.100 kcal metabolisable energy /kg diet from 36 to 42 d of age) were used used for each group [15]. Starter feed was provided as crumples, and subsequent feeds were pellets. All birds had ad libitum access to feed and water. Chicks received vaccinations for Newcastle, Infectious Bronchitis and Gumboro diseases ones for each. Each pen was equipped with fresh litter material as 8 kg/m^2 (thickness of litter was 15 cm, approximately), two tube feeders and two bell drinkers. Intermittent lighting program with 2 h Light:2 h Dark was given to both treatment groups during the whole experiment, except first seven days of age. Continuous light was provided for first seven days of the 42-days of study. Feed was withdrawn eight hour before slaughter. All birds were evaluated for carcass weight by neckcutting.

Data

Individual body weight of birds in all groups are measured at the beginning and the end of the experiment. Feed consumption and mortalities were recorded as it required or occurred throughout the experiment and total consumption was measured at the end of experiment. Feed convertion ratio in replicates was calculated by total feed consumed by the birds/total weight gain. Carcass weight (dressing percentage) was determined after chilling and calculated as a percentage of live body weight of all bird in all replicate groups.

Welfare was assessed with the length of foot pad lesions (None:no lesion present; Mild:lesion<7.5 mm;

Table 1. *The bird number in each replicate in the treatment groups*
Tablo 1. *Deneme gruplarında her tekerrürde yer alan hayvan sayıları*

Treatment Groups (Litter type x stocking density, bird number/m^2)	Number of Replicate	Bird Number in Each Replicate
Rice hull x 15	3	30
Rice hull x 19	3	38
Rice hull x 23	3	46
Wood shavings x 15	3	30
Wood shavings x 19	3	38
Wood shavings x 23	3	46

Severe: lesion>7.5 mm), litter moisture and pH which are the main indicator of litter quality at the end of growth period [16,17]. All birds were scored for foot pad lesion.

Litter samples were collected from four locations within each pen (four peripheral, equidistant from each pen corner) and thoroughly mixed to obtain material representative of the entire pen. At least 200 g of litter were placed in a plastic container and a subsample was taken for further analysis at the laboratory. The litter moisture samples were collected by compositing litter from four locations in each pen, mixing, and obtaining a 100 g litter subsample was placed in 15 x 30 cm tray and oven-dried for 48 h at 60°C. The percentage of moisture was calculated by using the weight loss between initial and dried litter [18]. The upper 10 cm of the litter was collected at each sample position and transported back to the laboratory for determination of pH. The pH of each litter type was measured after litter samples of nearly 5 g were suspended for 30 min in 25 mL of distilled water and stirred for 5 min using a pH meter (Mettler Toledo, GmbH, Switzerland) [19].

Statistical Analysis

The live body weight, dressing percentage and food pad lesions were analyzed by ANOVA with three levels of stocking density and two levels litter material [20]. Multiple comparison of means was performed using the Duncan test. Arc sine transformation was performed on dressing percentage data prior to analysis and then analysed. Non-parametric Kruskall-Wallis and Mann-Whitney U tests were used for the rest of the data. All statistical tests were analyzed in SPSS® computer software 13.00 [21].

RESULTS

The effect of litter type and stocking density on the final performance of broilers are summarized in *Table 2*. The litter type and stocking density had significant effect on the final body weight of broiler (P<0.05, P<0.001). The final live weight of broiler in the wood shaving and the lowest stocking density group were found to be 2757 and 2.946 g., respectively. There were no significant differences for the feed conversion ratio (P>0.05), mortality (P>0.05) and dressing percentage (P>0.05) neither in stocking density nor litter type. It was observed no significant stocking density x litter type interactions for the traits related with the growth performance (P>0.05).

The moisture content and pH of the litter at the end of the experiment are given in *Table 3*, along with the foot pad lesions of birds in the main and interactive groups. Both of the main factors investigated in the experiment were significantly affected the foot pad lesions of the birds (P<0.001, P<0.001). Greater stocking density had a negative effect on the foot health of the broiler. Also birds raised in the rice hull litter group had a greater foot pad lesions than birds raised in the wood shaving. Litter moisture

Table 2. *Performance traits in the experimental groups*
Tablo 2. *Denemede yer alan hayvanların performans özellikleri*

Treatments	Body Weight[1] g	Feed Conversion Ratio, g/g	Mortality %	Dressing Percentage[1]*
Litter type				
Wood shaving	2757±42	1.76	2.10	77.3±0.1
Rice hull	2589±43	1.65	2.31	78.8±0.1
Stocking density (chicks/m²)				
15	2946±56[a]	1.59	1.94	77.1±0.2
19	2638±49[b]	1.71	2.01	79.2±0.1
23	2436±45[c]	1.82	2.71	77.9±0.2
Litter type x Stocking density				
W x 15	3000±79	1.59	1.66	77.1±0.3
W x 19	2748±70	1.76	1.75	77.1±0.2
W x 23	2524±64	1.92	2.89	79.4±0.3
R x 15	2891±80	1.58	2.22	79.0±0.3
R x 19	2528±68	1.65	2.19	75.5±0.2
R x 23	2347±62	1.71	2.53	80.3±0.3
ANOVA				
Litter type	0.05	n.s.	n.s.	n.s.
Stocking density	0.001	n.s.	n.s.	n.s.
Litter type x Stocking density	n.s.	n.s.	n.s.	n.s.

a-c: *within rows, values with different superscript letters differ significantly (P<0.05, P<0.001), n.s.;no significant; * As a percentage of live weight; [1] Mean ± SE*

Table 3. *Some litter quality traits and foot-pad lesions in the groups*
Tablo 3. *Gruplarda bazı altlık kalite özellikleri ve ayak tabanı lezyonları*

Treatments	Length of Foot Pad Lesion (mm)	Litter pH	Litter Moisture %
Litter type			
Wood shaving	2.19	8.52	58.16
Rice hull	2.99	8.86	57.46
Stocking density (chicks/m²)			
15	0.89[a]	8.58[a]	57.42[a]
19	2.80[b]	8.70[b]	57.89[b]
23	4.08[c]	8.76[b]	58.13[b]
Litter type x Stocking density			
W x 15	0.77	8.35	54.04
W x 19	2.18	8.55	59.75
W x 23	3.65	8.58	60.70
R x 15	1.03	8.81	55.10
R x 19	3.43	8.84	55.55
R x 23	4.52	8.93	61.75
ANOVA			
Litter type	0.001	0.01	0.05
Stocking density	0.001	0.05	0.05
Litter type x stocking density	n.s.	n.s.	n.s.

None: *no lesion present;* ***Mild:*** *lesion<7.5 mm; Severe:lesion>7.5 mm;* ***a-c:*** *within rows, values with different superscript letters differ significantly (P<0.05, P<0.01, P<0.001);* ***n.s.:*** *no significant*

content of the groups were significanly affected by litter type and stocking density (P<0.05, P<0.05). It was 58.16 and 57.42% in litter type group, 57.42, 57.89 and 58.43% in three different stocking density groups, respectively. There were significant differences for the pH value of the litter in both main groups (P<0.01, P<0.05). The pH value of wood shaving and rice hull were 8.52 and 8.86, while it were 8.58, 8.70 and 8.76 in stocking density group of 15, 19 and 23 chicks per square meter, respectively. No significant stocking density x litter type interactions were calculated for the litter quality indicators investigated in this study (P>0.05).

DISCUSSION

In comparison to rice hull group, broiler raised in wood shaving group had significantly better live body weight at the end of this experiment (P<0.05). There were no significant differences for the feed conversion ratio, mortality and dressing percentage between the main groups. In terms of performance data, our findings agreed with those of earlier studies, except the live body weight [22,23]. Numerous studies in which alternative materials have reported that the type of litter material used does not affect the broiler performance [8,24,25]. The observed differences in body weight in our study may be attributed to depression of feed intake associated with litter consumption in birds reared on the rice hull. As birds can eat some litter particle, the base material must be free of any harmful contaminants. Litter materials should also be free of other substances-including chemicals, disease organisms and moulds-that may damage the birds' health. A good litter material should be dust free not cake or compact into layers. Take into account of the growing conditions of rice hull, these adverse effects may be observed more in rice hulls than in wood shavings.

As reported by Petek et al.[13], Uzum and Oral [23], Skomorucha et al.[26] the final body weight of broiler raised in this experiment significantly worsened with increasing stocking density (P<0.001). But the total live weight in per square meter area was found to be highest in greater stocking density group. Similar with some findings [27] there were no significant differences for the feed conversion ratio and survival rates in stocking density groups at the slaughter age. As previously showed by Skrbic et al.[28] rearing of broilers in lower stocking density provides better body development, more intensive growth and higher absolute yield of processed carcass parts which contain more meat, especially in breast.

We found that foot pad lesions in broilers were increased when stocking density increased from 15 to 19 and 23 birds/m². Similar with the findings of Ferrante et al.[29] and Petek et al.[13] stocking density could have negatively affected foot pad lesions and wood shaving resulted to be the better solution for foot condition compared to

rice hull. The incidence of foot pad lesions was positively correlated with increasing humidity and pH as previously identified as main factor for foot pad dermatitis by Ekstrand et al.[30]. Moisture content of litter was significantly greater in high stocking density ($P<0.05$). As a result of this high moisture content resulted in poor litter quality. The decrease of litter quality with increasing stocking density can be explained by different effects. Petek et al.[13] and Ravindran et al.[31] reported that litter quality was poorer at high population densities, but there were no welfare implications as indicated by the lack of effect of density on gait scores and the incidence of hock and foot pad burns. The more birds in the same area mean more manure production. Also, the quality of the in-house environment is highly dependent upon the litter quality. One of the management factors affecting broiler welfare is those relating to good ventilation and air quality such as the type of ventilation, type of drinker and litter type. The type of the watering system we used (bell drinker) may be showed a negative effect on the poor litter quality and greater foot pad lesions. The wetter the litter, the more likely it will promote the proliferation of pathogenic bacteria and moulds. The litter is ideal for bacterial proliferation and wet litter is also the primary cause of high level of ammonia, one of the most serious performance and environmental factors affecting broiler production today. Controlling litter moisture is the most important step in avoiding ammonia problems [32]. To limit ammonia production, the litter pH should be below 7.0; litter moisture below 30%; and temperature at the level of the broiler's comfort demands. In this study, both of these parameters were greater than this ideal condition. In a well-managed broiler house, litter moisture averages between 25 to 35 percent [14]. Interestingly, as reported by Asaniyan et al.[33] thickness of the litter layer might be increased the foot pad lesions score in the groups as thin layers of litter resulted in lower levels of foot pad dermatitis than thicker layers probably because the chickens are less prone to peck, scratch and turn the litter particles over, and thereby help to ventilate the litter, if the layer of litter is thick and compact. Litter that is too dry and dusty can also lead to problems such as dehydration of new chicks and respiratory disease.

The water absorption capacity of wood sawing better than the rice hulls and it is required more effort to stable the litter moisture content of litter rice hull. Similar with our findings Sreehari and Sharma [34] reported that the most suitable litter type and stocking density according to net profit per kilogram body weight was wheat straw litter and 0.18 sq m./bird. Stocking density is important for better welfare quality, but it is not sufficient. The real improvements in broiler welfare will come from establish production standards that combine stocking density and good environment. As reported by Feddes et al.[35] and Jones et al.[8] the control of environment particularly good ventilation, to control temperature and humidity is a key factor in improving broiler welfare. A significant improvement in performance can be obtained by significantly lower litter moisture [36]. Because chickens spend all their life in contact with litter, therefore if litter conditions are not optimal there is a considerable risk that the birds will develop contact dermatitis on their feet and breast. In several experiments the prevalence of contact dermatitis in broilers is related to litter quality [30,37] when the litter is wet, sticky and compact, dermatitis are commonly present.

In this study, greater stocking density and litter material of rice hull were adversely affected live body weight of broilers and main litter quality indicators. But high stocking densities contribute to reduce the fixed costs of production and produce more kilograms of broiler per area. Therefore, up to a critical point, profitability increases with increased stocking density. Depends on the price and availability rice hull can be used as litter material instead of wood shaving. Also, better litter management is very crucial to provide good litter quality and broiler welfare in high stocking densities and rice hull litter condition.

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RESEARCH ARTICLE

The Evaluation of Important Biomarkers in Healthy Cattle [1]

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Summary

In this study the aim is to determine the blood serum levels of biological markers as procalcitonin, neopterin, TNF-α, MDA, PGE2, IL-8, IFN-γ which are considered as highly beneficial on diagnosing the infections in the veterinary medicine and evaluating the prognosis in the healthy cattle at different ages and in different gender. The materials of this study are 48 (25 female and 23 male) cattle and calf bred (neonatal <1 month, young 12-24 month and mature >24 month) in operations in Sivas region and which are determined to be healthy via the biochemical and hematological findings. Serum procalcitonin level was found lower in neonatal group than the young and adult group (P<0.05). It was realized that neopterin level is reasonably higher in neonatal group than both young and adult group (P<0.05). MDA level in the young and adult group was measured as higher than of the neonatal group (P<0.05). There was found between the adult group and neonatal group a statistically reasonable difference for the serum PGE2 levels (P<0.05). In IL-8 level, there was found a statistically important difference only between young group and neonatal group (P<0.05). There was found no statistic difference among the levels of procalcitonin, neopterin, TNF-α, IFN-γ, MDA, PGE2, IL-8 between the sexes. As a result, it is concluded that determining the levels of markers used in defining the prognosis of the infection in healthy cattle at different ages would be a base data for further studies.

Keywords: *Procalcitonin, Cattle, TNF-α, MDA, Neopterin, PGE2, IFN-γ, IL-8*

Sağlıklı Sığırlarda Önemli Biyomarkerların Değerlendirilmesi

Özet

Bu çalışmada veteriner hekimlikte enfeksiyonların teşhisinde ve prognozunun değerlendirilmesinde önemli faydalar sağlayacağı düşünülen prokalsitonin, neopterin, TNF α, IFN-γ, MDA, PGE2, IL-8 gibi biyolojik markerlerin farklı yaş gruplarında ve cinsiyetlerdeki sağlıklı sığırlardaki kan serumu seviyelerinin belirlenmesi amaçlanmıştır. Çalışmanın materyalini Sivas yöresinde bulunan işletmelerde yetiştirilen biyokimyasal ve hematolojik bulgularıyla sağlıklı olduğu belirlenen neonatal (<1 ay), genç (12-24 ay) ve ergin (>24 ay) 48 adet (25 dişi 23 erkek) sığır ve buzağı oluşturmuştur. Serum prokalsitonin seviyesi, neonatal grupta genç ve ergin gruba göre düşük bulundu (P<0.05). Neopterin seviyesi neonatal grupta hem genç hem de ergin gruba göre anlamlı düzeyde yüksek olduğu görüldü (P<0.05). Genç ve ergin gruptaki MDA seviyesi neonatal gruba göre daha yüksek ölçüldü (P<0.05). Ergin grup ile neonatal grup arasında serum PGE2 seviyeleri açısından istatistiki olarak anlamlı fark belirlendi (P<0.05). IL-8 seviyesinde ise sadece genç grup ile neonatal grup arasındaki fark istatiksel olarak önemli bulundu (P<0.05). Cinsiyetler arasında neopterin, prokalsitonin, TNF-α, IFN-γ, MDA, PGE2, IL-8 seviyelerinde istatistikî olarak fark belirlenemedi (P>0.05). Sonuç olarak farklı yaş aralığındaki sağlıklı sığırlarda enfeksiyonun prognozu belirlenmesinde kullanılan markerlerin seviyeleri belirlenerek sonraki yapılacak farklı çalışmalara temel veri niteliği taşıyacağı düşünülmüştür.

Anahtar sözcükler: *Prokalsitonin, Sığır, TNF-α, MDA, Neopterin, PGE2, IFN-γ, IL-8*

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INTRODUCTION

Despite to all the advantages as too many diagnose methods' defining the inflammatory reactions entering into applications and the increase in the diversity of medicines used against the infections practically, it is still deemed that there have been difficulties in observing the prognosis of infections in domestic animal and there still occurs deaths resulted from sepsis, multiple organ failure and shock developed in parallel with the infections. It is known that a considerable amount of deaths occur because of septic shock both in economically important livestock and both in the pets [1].

Many parameters are used for diagnosis of illnesses caused by infection. C-reactive protein (CRP) as an acute phase reactants and cytokines such as neopterin, interleukins (IL-8, IL-6), tumor necrosis factor (TNF) are major ones used for diagnosis. Procalcitonin, can be used both for of infections despite being sensitive to bacterial inflammations and protein based during the illness it is a long half-life important marker. Among these markers for diagnostic and prognostic the illnesses, procalcitonin stands out for reasons such as its specificity to bacterial infections and unlike cytokines being stabil at room temperature during analyze at plasma concentrations [2].

TNF-α and interferon gamma (IFN-γ) are one of those cytokines activating macrophage. When they are activated, they could phagocytized pathogens since they have turned into the cells which are capable of producing reactive oxygen products and lysosomal enzymes. It is also known that after IFN-γ's stimulating IL-2 receptors in macrophage, IL-2 released from T lymphocyte is increasing the microbicidal effects of macrophages [3-5].

TNF-α stimulates acute inflammation in order to initiate the extrinsic coagulation mechanism and activate the clotting mechanism through stimulating the tissue factor formation in endothelium cells [6]. Moreover, it increases the platelet activating factor (PAF) release by stimulating the endothelium cells and also increases the synthesis of phase proteins by effecting the hepatocyte and it eases chemotaxis and plays an effective role in stimulating the neutrophils for formation of superoxide radicals in phagocytose. There has been various studies conducted on both people and animals demonstrating that there are a positive correlation between TNF-α level and mortality rates and that TNF-α could be used in observing the inflammation [3,7,8].

The release of TNF together with IL-1 both stimulates hypothalamus cells so prostaglandinE_2 (PGE_2) synthesis increases [5] and PGE_2 which are the product of arachidonic acid metabolism are released from mast cells, capillary endothelial cells and macrophages [9]. If the amounts of prostaglandins are low in tissues, then it stimulates the inflammation, however; if high, they behave as if anti-inflammatory. Prostaglandins have some effects such as pain and fever [6].

TNF-α stimulates the formation and release of other cytokines (IL-1, IL-6, IL-8) such cytotoxic factors like free oxygen radicals and nitric oxide (NO) [5].

Reactive oxygen species and free radicals produced by lipid peroxidation in tissue damage has been implicated in the pathogenesis of many diseases. Unsaturated phospholipids and cholesterol appearing in the structure of membranes and free radicals can easily react and so result in lipid peroxidation. During that process a range of reactions occur and as a result of them MDA, an important biological marker used in marking the membrane damage occurs. Through the measuring of the amount of malondialdehyde an indirect data on the level of lipid peroxidation could be gained [10].

IL-8 is produced by inflammatory cells. For leucocytes, potential chemotaxis is an effective cytokine [11]. Cytokines are important proinflammatory mediators during early phase in the sepsis released from macrophages and endothelial cells and monocytes with the stimulation of infections. In numerous studies, in human neonatal and adult sepsis, TNF-α, IL-6 and IL-8 levels are found high [12,13]. IL-8 plays an important role in the organ dysfunction developed in sepsis and lung damage. In studies conducted on determining the diagnostic value of IL-8, Gram has stated that in negative bacteremia the positive predictive value is 73% and the negative predictive value is 94% [8]. In the study done by Martin et al.[12], it was assigned that in newborns serum IL-6, IL-8 and TNF-α levels have increased.

Neopterin is low molecular weight 2-amino-4-hydroxy-(1'2'3'trihydroxypropyl)-pteridin. Neopterin cell is accepted as a marker of cell-mediated immunity [2]. It is produced by active monocyte/macrophage. IFN-γ is a potential neopterin producer and it demonstrates the increase in neopterin concentrations and the existence of IFN-γ in body fluids [2,14].

Procalcitonin is precursor of calcitonin hormone which is produced in thyroid C cells and which is responsible for the calcium homeostasis. Apart from the neuroendocrine in the thyroid gland, procalcitonin which could be released from lung and bowel, as well, in cases of sepsis, is found low in healthy persons. According to studies, procalcitonin in bacterial diseases is reported to increase rapidly in a short time after TNF- α, IL-6 and IL-8. It is known by various researchers that in septicemias, procalcitonin could increase as one hundred times more than normal serum levels [2,15-18].

It is stated that for humans; procalcitonin to be under 0.1 ng/ml in healthy persons, >0.5 ng/m1 in viral infections and 1.5 ng/ml as highest, moreover, in serious bacterial infections this rate might increase to five more times at least, that it could go beyond 10ng/ml and that this rate could even exceed 1.000 ng/ml [2,8].

Biological markers used mostly in determining the type of the ongoing inflammation and observing the respond to the treatment in human medicine. However, in veterinary medicine there known very little parameter used routinely and could be useful in determining the inflammation or observing the respond to the treatment.

In this study the aim is to determine the blood serum levels of the biological markers such as; procalcitonin, neopterin, TNF-α, IFN-γ, MDA, PGE_2, IL-8 in healthy cattle at different ages and of different genders in order to enlighten the studies to be done in the field of veterinary medicine related to the biological markers known for their important benefits for diagnosing the infections and evaluating the prognosis in human medicine.

MATERIAL and METHODS

The materials of this study are heparin containing blood and blood serums taken from 48 (25 female and 23 male) cattle and calf which were separated into three groups as neonatal (<1 month), young (12-24 month) and adult (>24 month) determined as healthy in systematic clinical examinations, whose serum biochemical values and hematological parameters were found among the normal bounds and which were raised in the operations in Sivas region.

In the blood serums taken, glucose, total bilirubin, direct bilirubin, total protein, albumin, globulin and AST levels were determined via auto analyzer device (Mindray BS 200, PRC).

Hematological examinations were given through the heparin containing blood samples taken through the methods acclaimed by literature [19].

Among the blood samples, IFN-γ, IL-8 and procalcitonin levels were determined through the sandwich enzyme immunoassay method, TNF-α, MDA, neopterin and PGE_2 levels were determined through competitive inhibition enzyme immunoassay method and through commercial kits (Cusabio, PRC) and in accordance with the kit procedures and by using the ELISA device (Thermo Multiskan).

In analyzing the facts reached, Student-T Test and ANOVA tests were used. While Levene was evaluating the homogeneity of variance according to the test results, intergroup comparisons were made by using Duncan and Tamhane tests [20] and through the SPSS 14.00 packet program (SPSS Inc, Chicago).

This study was carried out with permission dated 03.04.2013 No. 373 with local Ethics Committee for Animal Experiments of the University of Cumhuriyet.

RESULTS

Serum biochemistry values reached in the study are shown in *Table 1* and the results of clinical examination and hematological values are shown in *Table 2*.

In the statistical analysis of serum biochemistry values determined in the studies, there could not be found any difference among age and gender groups (P>0.05).

The comparison on the age base, of the levels of procalcitonin, neopterin, MDA, TNF-α, IL-8, PGE_2, IFN-γ determined in the serums is shown in *Table 3*. The comparison of determined values to the gender is given in *Table 4*.

The level of serum procalcitonin was found lower in neonatal group than the young and mature groups (P<0.05). The comparison of procalcitonin levels according to age group is shown in *Fig. 1*. Neopterin level was regarded as reasonably high comparing to both young and mature group (P<0.05). The comparison of neopterin levels according to age group is shown in *Fig. 2*. MDA level in young and mature group was measured higher than the

Table 1. *Serum biochemistry evaluations determined in the study*
Tablo 1. *Çalışma gruplarında belirlenen serum biyokimya değerleri*

Parameters	Age Groups						P Value
	Neonatal		Young		Mature		
	n	Mean±SE	n	Mean±SE	N	Mean±SE	
Glucose (mg/dL)	15	61.40±2.85	17	63.88±1.83	16	62.56±1.89	0.729
Creatine (mg/dL)	15	1.13±0.06	17	1.11±0.05	16	1.27±0.07	0.182
T. Bilirubin (mg/dL)	15	0.14±0.03	17	0.12±0.02	16	0.22±0.05	0.234
D. Bilirubin (mg/dL)	15	0.07±0.02	17	0.10±0.02	16	0.14±0.05	0.377
Total Protein (g/dL)	15	7.08±0.11	17	7.01±0.11	16	7.05±0.11	0.898
Albumin (g/dL)	15	3.32±0.12	17	3.42±0.07	16	3.23±0.10	0.394
Globulin (g/dL)	15	3.76±0.14	17	3.59±0.15	16	3.75±0.13	0.611
AST (IU/L)	15	82.73±3.51	17	80.47±3.13	16	83.75±3.27	0.766

Tablo 2. *The clinical examination findings and hematological values determined in the study*
Tablo 2. *Çalışmada belirlenen klinik muayene bulguları ve hematolojik değerler*

Measurements	Neonatal	Young	Mature	Normal Values
Body temperature °C	38.78±0.05	38.73±0.06	38.65±0.02	36.7-39.1
Respiratory rate	49.00±2.15	42.13±1.11	37.75±1.28	26-50
Heart rate	95.00±1.92	74.00±2.07	72.25±1.62	48-84
Packed cell volume %	32.13±0.90	30.75±0.75	30.00±0.93	24-46
Leukocyte (10^3/µl)	5.43±0.27	5.25±0.23	5.13±0.23	4-12
Erythrocyte (10^6/µl)	6.15±0.19	6.34±0.26	6.24±0.28	5-10

Table 3. *The comparison of procalcitonin, neopterin, TNF-α, MDA, PGE_2 IL- 8 and IFN-γ levels according to the age groups*
Tablo 3. *Çalışmada belirlenen prokalsitonin, neopterin, TNF-α, MDA, PGE_2, IL- 8 ve IFN-γ seviyelerinin yaş gruplarına göre karşılaştırılması*

Parameters	Age Groups						
	Neonatal		Young		Mature		P*
	n	Mean±SE	n	Mean±SE	n	Mean±SE	
Procalcitonin (pg/ml)	15	43.257±0.780^{b}	15	52.929±3.71^{a}	15	53.349±3.166^{a}	0.021
Neopterin (ng/ml)	15	4.181±0.266^{a}	17	3.204±0.495^{b}	16	2.493±0.101^{b}	0.004
TNF-α (ng/ml)	13	0.667±0.033	17	0.861±0.123	14	0.707±0.050	0.259
MDA (ng/ml)	13	267.430±20.956^{b}	16	454.379±64.052^{a}	14	473.088±45.813^{a}	0.037
PGE2 (pg/ml)	13	113.343±4.607^{b}	15	138.080±12.968^{ab}	15	150.120±11.887^{a}	0.029
IL-8 (pg/ml)	15	199.187±8.433^{b}	17	293.294±47.031^{a}	16	238.929±9.406^{ab}	0.011
INF-γ (pg/ml)	13	-	15	-	13	-	-

*^{a,b,c} In the same row with different letters are statistically significant differences between the values (P<0.05); -: specified value was not significant for healthy cattle; * Biggest P value of differences*

Table 4. *The comparison of procalcitonin, neopterin, MDA, TNF-α, IL- 8, PGE_2 and IFN-γ levels according to the gender*
Tablo 4. *Çalışmada belirlenen belirlenen prokalsitonin, neopterin, MDA, TNF-α, IL- 8, PGE_2 ve IFN-γ seviyelerinin cinsiyete göre karşılaştırılması*

Parameters	Sex Groups						
	Male		Female		P Value	Total	
	n	Mean±SE	n	Mean±SE		N	Mean±SE
Procalcitonin(pg/ml)	23	50.066±2.800	22	49.614±2.147	0.89	45	49.845±1.755
Neopterin (ng/ml)	24	3.460±0.373	24	3.084±0.224	0.39	48	3.272±0.217
MDA (ng/ml)	22	402.061±53.994	21	405.931±32.292	0.95	43	403.951±31.439
TNF-α (ng/ml)	24	0.758±0.086	20	0.752±0.051	0.95	44	0.755±0.052
IL-8 (pg/ml)	24	217.435±6.735	24	274.093±34.208	0.11	48	245.764±17.734
PGE_2 (pg/ml)	24	132.422±7.755	19	137.806±11.402	0.69	43	134.801±6.571
INF-γ (pg/ml)	21	-	20	-		41	-

neonatal group's. There determined a reasonable difference between the mature group and neonatal group on the basis of PGE_2 levels (P<0.05). In IL-8 level, only the difference between young group and neonatal group was found statistically important (P<0.05). The comparison of PGE_2, MDA and IL-8 levels according to age group are shown in *Fig. 3*. The difference of TNF-α levels between groups was not statistically meaningful (P>0.05). IFN- γ levels were not taken in to account owing to the fact that they were under the sensitivity of the ELISA kit used.

There could not be determined a statistic difference among the levels of procalcitonin, neopterin, MDA, TNF-α, IL-8, PGE_2, IFN-γ between the sexes (P>0.05).

DISCUSSION

It has been reported for many times by various researchers that markers such as IFN-γ, TNF-α, MDA, IL-8, PGE_2, procalcitonin and neopterin which are revealing the

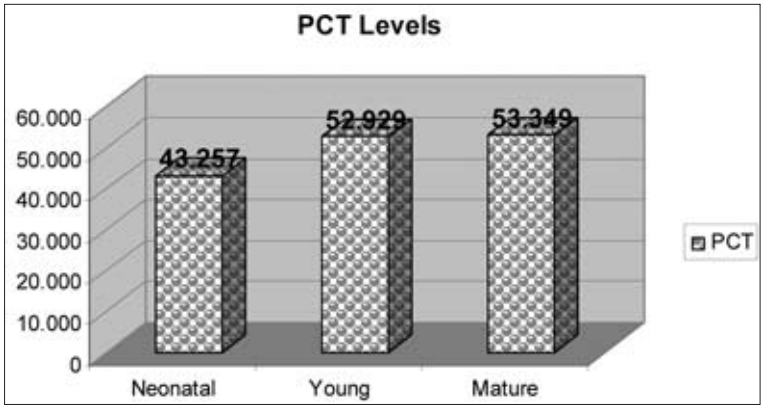
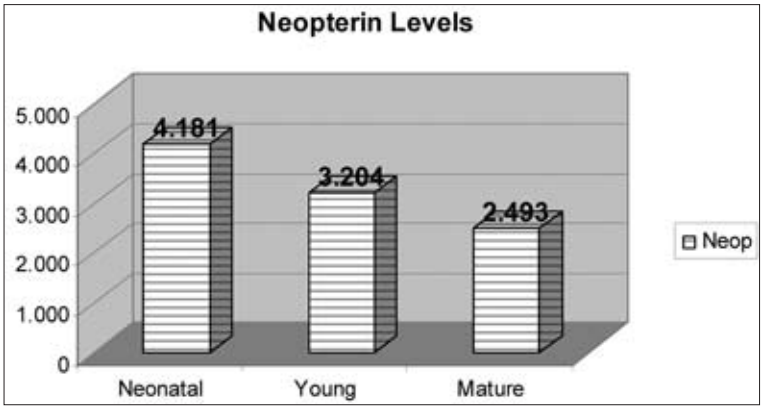


Fig 1. Procalcitonin levels according to age groups

Şekil 1. Yaş gruplarına göre prokalsitonin düzeyleri

Fig 2. Neopterin levels according to age groups

Şekil 2. Yaş gruplarına göre neopterin düzeyleri



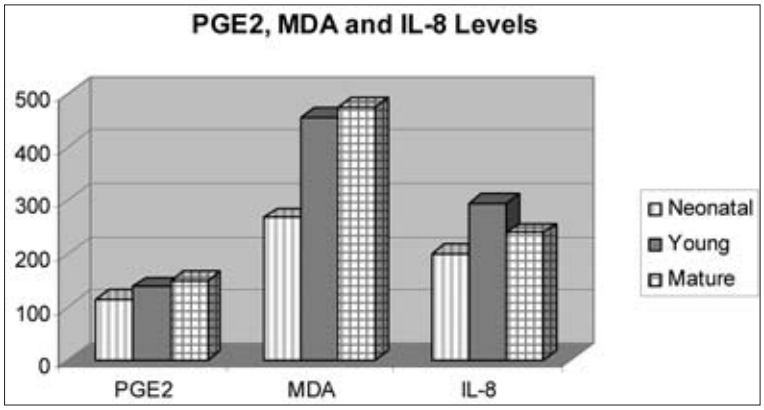


Fig 3. PGE_2, MDA and IL-8 levels according to age groups

Şekil 3. Yaş gruplarına göre PGE_2, MDA ve IL-8 düzeyleri

results of growing immune during the infections could be used in observing the prognosis in infections [3,8,9,14,21,22].

The reported clinical examination results of the healthy cattle and hematologic value ranges [19,23,24] are in parallel with the values determined in the cattle which were the materials of the study as well, and shown in *Table 2*.

It has been stated by numerous researchers that in healthy cattle glucose is 45-75 mg/dl, creatine is 1-2 mg/dl, total bilirubin is 0.01-0.47 mg/dl, direct bilirubin is 0.04-0.44 mg/dl, total protein is 6.7-7.5 g/dl, albumin 3-3.6 g/dl, AST 43-127 IU/L, A/G (albumin/globulin) and its rate is on the range of 0.8 and 0.9 [23,24]. Biochemical values of the serums which are the materials of this study are determined as the in the value ranges accepted for healthy cattle.

IFN-γ, is synthesized by lymphocyte and is one of cytokines that activating macrophage [4]. Hisaeda et al.[3], who claim that IFN-γ increases in cows with mastitis reported that IFN-γ levels in the serums are of the amounts in that could not be reported in the cows of the control group of the study they were doing. This fact coincides with those healthy cows' serum INF-γ levels are not found reasonable.

In the studies conducted by Hisaeda et al.[3] and in the study searching the utility of serum levels in revealing the prognosis of natural coliform mastitis those are stated that; serum TNF-α levels are high in cattle with mastitis however, even though there confirmed no statistical difference of the TNF-α levels between the cattle responding to the treatment and the ones euthanized, TNF-α levels of milk serums have increased statistically reasonably. Moreover,

in the same study they determined that TNF-α level in mature healthy cattle as a control group is lower than 10 ng/ml [3]. In this study, TNF-α levels of neonatal, young and mature are determined respectively as 0.66, 0.86 and 0.70 ng/ml and the average of all groups are calculated as 0.75 ng/ml. These values are in accordance with the values of healthy ones in Hisaeda et al.'s study [3]. In this study, the measured TNF-α levels difference between healthy male and female cattle are not found statistically reasonable (P>0.05).

It is proved by too many studies that procalcitonin could be used in human medicine newborn units as an inflammation mark in pneumonia, septicemia, meningitis, fungal and parasitic infection and that inflammation could be used safely in determining the prognosis. It is demonstrated with various studies that the determination of serum procalcitonin amount caused by bacterial inflammations compared to other cytokines is more specific and sensitive [2,15-18,25-27].

In literature search, it has been found out that that studies on veterinary medicine are limited and that the studies on animals are mostly conducted on experimental animals in order to provide data with the human medicine. In this study the statistical lowness of the serum procalcitonin level in neonatal period relatively to the young and mature animals displays that the age has an effect upon the procalcitonin level (P<0.05) and in statistical analysis of serum procalcitonin measured after grouping the blood serums as male and female it displays that the gender has not got an effect upon procalcitonin serums (P>0.05). In the light of these indications, procalcitonin levels can be detected in the blood of healthy cattle has shown that this parameter can be used in latter studies in the field of veterinary medicine.

INF-γ and other cytokines are effective stimulants in the formation of neopterin by monocytes. In the study done by Stang et al.[14] determined that they were searching the neopterin levels of cattle, horse, lama, dog and cat and it is also determined that the serum neopterin level in both sexes in cattle was 2.85±0.65 nmol L^{-1}. However the serum neopterin levels' not differing between the sex groups are in accordance with the facts of Stang et al.[14], in this study neopterin level is found statistically reasonably higher in neonatal group than young and mature group, apart from Stang et al.[14] (P<0.05). The acceptance as a marker of neopterin immune activation can be associated with its being produced by active monocytes at neonatal period.

Baker et al.[9] have reported that in the study they have arranged to determine PGE_2 levels and histamine and PGD_2 levels in cows with ostertagyiosis, in the healthy cattle assigned as the control group PGE_2 levels are between 178±74 - 266±135 $pgml^{-1}$. In the studies of Fraccaro et al.[28], PGE_2 levels were first measured by immunoenzymatic method for 5 min, 360 min, 720 min and 24 h. While the levels at control were between 500-1.000 pg/ml until min 720, it is indicated that the levels were close to 1.500 pg/ml levels at 24 h measurement. In the study there found no difference according to the sex and though there found reasonable differences according to the age groups, calculated values are lower than of reported in these studies. Higher levels of PGE_2 seen at adults compared to the young and neonatal group was interpreted as antiinflammatory response rised accordingly to the age.

The existence and level of oxidative stress could be set forth by determining the amount of malondialdehyde formed as end product in the process known as lipid peroxidation. The increasing amount of MDA in the serum is an important marker of oxidative stress [10]. In a study researching the markers of oxidative stress and immune system in cattle with anaplasmosis, malondialdehyde level is found as 15.23±2.33 umol/L in 15 healthy cattle whose ages are ranging from 1 to 3 [29]. In cattle infected with *Brucella abortus* as a control group in the study received a mean serum MDA levels in 10 healthy cattle was 1.74±0.25 nmol/mL have been reported [30]. In this study the average MDA level of newborn, young and mature cattle are determined respectively as 264.4, 454.3, 473 ng/ml. It is determined that sex difference hasn't got an effect upon MDA level and that MDA level is reasonably and statistically higher in young and mature cattle than neonatal group (P<0.05).

Due to the fact that IL-8 stimulates neutrophil chemotaxis, its measurement is important at some liquids and serum during the course of certain diseases. The fact that the level of IL-8's normal serum levels show increase in 24 h are reported by many researchers [12,13]. The study comparing viral and bacterial pneumonia IL-8 in cattle have supported the fact that in bronchoalveolar lavage fluid compared to viral IL-8 levels are higher especially in bacterial infections [31]. Serum IL-8 levels are determined in neonatal, young and mature groups respectively as 199.187 pg/ml, 293.294 pg/ml and 238.929 pg/ml. It is also determined that statistically all groups are different from each other and that IL-8 level is higher in young group than other groups (P<0.05). Even though TNF-α levels between groups were not statistically meaningful, when related with IL-8 levels it is seen that these two parameters seem parallel at neonatal, young and adult levels.

As a conclusion, in this study the scope is to determine the blood levels of biological markers such as procalcitonin, neopterin, TNF-α, IL-8, MDA, PGE_2, IFN-γ in healthy cattle at different age and of different sex groups and to put forward the effects of age and sex differences upon serum levels in order to provide base data for further studies in the field of veterinary medicine on biological markers used routinely in diagnosing infectious diseases in human medicine and evaluating prognosis. The results

achieved show that, in the field of veterinary medicine in determining inflammation and prognoses, procalcitonin, neopterin, TNF-α, IL-8, MDA, PGE_2 serum levels can be indicated by ELISA method.

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RESEARCH ARTICLE

Effects of Dietary Yeast Cell Wall Supplementation on Performance, Carcass Characteristics, Antibody Production and Histopathological Changes in Broilers

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Summary

This study was carried out to determine the effects of dietary yeast cell wall supplementation on growth performance, carcass traits, antibody production to sheep red blood cells (SRBC) and histopathological changes in broilers. A total of 272 Ross 308 male broiler chicks aged one day were allocated into one control group and three treatment groups each containing 68 chicks. A basal diet was supplemented with 0, 1, 2 and 3 g/kg yeast cell wall (InteMos) to obtain dietary treatments. The experimental period lasted 6 weeks. Dietary yeast cell wall increased body weight gain during the first three weeks ($P<0.001$). Feed conversion during the first three weeks ($P<0.001$) and during the overall experimental period ($P<0.01$) were improved with the dietary supplementation of yeast cell wall. No differences were observed in feed intake, carcass yield and the relative weights of gizzard, liver and heart. Yeast cell wall supplementation decreased the relative weight of abdominal fat ($P<0.05$) and increased antibody titres to SRBC ($P<0.01$) in broilers. Control and treatment groups had similar histological grade; hepatic lobular inflammation, steatosis and cell ballooning scores and, cardiac steatosis. It is concluded that yeast cell wall was an effective feed additive in broiler feeding due to the increased growth performance, increased humoral immune response and the reduction in abdominal fat.

Keywords: *Broiler, Carcass traits, Histopathological changes, Performance, Yeast cell wall*

Broyler Karma Yemlerine Maya Hücre Duvarı İlavesinin Performans, Karkas Özellikleri, Antikor Üretimi ve Histopatolojik Değişiklikler Üzerine Etkileri

Özet

Bu araştırma broyler karma yemlerine maya hücre duvarı ilavesinin büyüme performansı, karkas özellikleri, koyun eritrositine karşı antikor üretimi (SRBC) ve histopatolojik değişiklikler üzerine etkilerini belirlemek amacıyla yapılmıştır. Toplam 272 adet günlük Ross 308 erkek broyler civciv her biri 68 adet içeren bir kontrol ve üç deneme grubuna ayrılmıştır. Bazal karma yeme 0, 1, 2 ve 3 g/kg düzeyinde maya hücre duvarı (InteMos) ilave edilerek deneme karma yemleri oluşturulmuştur. Deneme 6 hafta sürdürülmüştür. Karma yeme maya hücre duvarı ilavesi ilk üç haftada canlı ağırlık kazancını artırmıştır ($P<0.001$). Denemenin ilk üç haftası ($P<0.001$) ve deneme süresince ($P<0.01$) yem dönüşüm oranı maya hücre duvarı ilavesi ile olumlu yönde etkilenmiştir. Yem tüketimi, karkas randımanı ile relatif taşlık, karaciğer ve kalp ağırlıkları bakımından gruplar arasında farklılık gözlenmemiştir. Maya hücre duvarı ilavesi broylerlerde relatif abdominal yağ ağırlığını azaltmış ($P<0.05$) ve SRBC'ye karşı antikor titresini ($P<0.01$) artırmıştır. Kontrol ve deneme gruplarında hepatik lobüler inflamasyon, yağlanma ve hücre balonlaşma skorları ile kalp yağlanma oranlarını içeren histolojik skorlamalar benzer bulunmuştur. Sonuç olarak, büyüme performansındaki artış, humoral immun cevaptaki artış ve abdominal yağdaki azalmadan dolayı maya hücre duvarı etkili bir yem katkı maddesidir.

Anahtar sözcükler: *Broyler, Histopatolojik değişiklikler, Karkas özellikleri, Maya hücre duvarı, Performans*

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INTRODUCTION

Yeast cell walls have been used increasingly in poultry diets as a feed additive after the ban on the use of antibiotic growth promoters in the EU [1]. Yeast cell walls contain prebiotic oligosaccharides such as fructooligosaccharides, mannanoligosaccharides and β-glucans that beneficially affect gut health [2,3] and modulate immunity [4,5]. Dietary β-1,3/1,6-glucan, derived from yeast *(Saccharomyces cerevisiae)* cell walls, increases performance by improving average daily gain, reducing the feed/weight gain ratio and enhancing immunological response [6].

Reisinger et al.[7] observed that the yeast derivative (contains 0.017% mannan and 0.025% glucan) when fed at 0.1% of the diet positively influenced the final body weight, the daily body weight gain, feed conversion ratio and jejunum goblet cell density and reduced the number of apoptotic enterocytes. They [7] concluded that increased goblet cell density might have protected the broilers against primary infections and this could have been a reason for the improved performance. Chae et al.[8] reported that dietary levels of β-glucan (derived from *Saccharomyces cerevisiae*) at 0.02% and 0.04% improved weight gain, nutrient retention and immunity in broilers. However the feed conversion ratio was not improved with β-glucan supplementation [8]. Reports on the effects of yeast cell wall as prebiotics on the gut pH, antibody titer to SRBC and histopathological changes in broilers are lacking. Therefore the present study was designed to determine the effects of dietary yeast *(Saccharomyces cerevisiae)* cell wall on performance, carcass characteristics, humoral immune response and histopathological changes in broilers.

MATERIAL and METHODS

Animals and Diets

A total of 272 Ross 308 male broiler chicks aged one day were randomly assigned to one control group and three treatment groups each containing four replicate groups of 17 chicks. Chicks of each replicate groups were placed in separate floor pen measured as 170 x 94 x 90 cm, width x length x height, respectively. Each pen had wood shavings litter, two nipples and one hanging suspended feeder. Feed in mash form and water were provided *ad libitum* during 42 days. Continuous lighting was applied during the whole experiment. Average room temperature was 32±2°C on the first week and then gradually lowered to average 24-26°C and this temperature was maintained up to slaughter age. All animal use protocols were in accordance with the Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010 on the protection of animals used for scientific purposes [9].

Basal diets were supplemented with the yeast cell wall derived from bakers yeast, *Saccharomyces cerevisiae* (InteMos, NCYC R 625, Integro Food and Feed Manufacturing Company, İstanbul, Turkey) at the level of 1, 2 and 3 g/kg for the diets of the first, second and third treatment groups, respectively. Yeast cell wall had 227.2 g/kg crude protein, 37.4 g/kg ether extract, 4.3 g/kg crude fibre, 64 g/kg crude ash, 7.39 g/kg calcium and 5.91 g/kg phosphorus. The basal diet was formulated according to the commercial management guide (Ross 308 Broiler). The ingredients and chemical composition of the basal diet are presented in *Table 1*.

Measurements, Sample Collection and Laboratory Analysis

Moisture, crude ash, crude fibre, ether extract and crude protein contents of basal diet was determined according to the AOAC [10]. The samples were ashed in a muffle furnace prior to the analysis of calcium and total phosphorus [11,12]. Metabolizable energy levels of samples were estimated using the Carpenter and Clegg's equation [13].

Chicks were weighed individually at the beginning of the experimental period and weekly for calculating body weight gains. The birds were observed daily for evaluating mortality. Feed consumption was recorded weekly and

Table 1. *Ingredients and chemical composition of the basal diets*
Tablo 1. *Bazal karma yemlerin yapısı ve kimyasal bileşimi*

Ingredients (g/kg)	Starter Diet 1-14 days	Grower Diet 15-28 days	Finisher Diet 29-42 days
Corn	490.5	544.5	544.5
Soybean meal	240.0	190.0	160.0
Full fat soya	165.0	165.0	195.0
Meat and bone meal	40.0	40.0	40.0
Sunflower oil	30.0	26.0	26.0
Limestone	15.0	15.0	15.0
Dicalcium phosphate	12.0	12.0	12.0
Salt	2.5	2.5	2.5
DL-Methionine	2.0	2.0	2.0
Lysine	0.5	0.5	0.5
Vitamin mineral premix[1]	2.5	2.5	2.5
Chemical composition (Analyzed)			
Metabolizable energy[2] (MJ/kg)	13.15	13.28	13.46
Crude protein (g/kg)	223.0	205.0	202.8
Calcium (g/kg)	14.2	14.1	14.1
Total phosphorus (g/kg)	8.2	8.0	8.0

[1] Supplied the following per kilogram of diet: 12.000 IU vitamin A, 2.400 IU vitamin D_3, 30 mg vitamin E, 2.5 mg vitamin K_3, 2.5 mg vitamin B_1, 6 mg vitamin B_2, 4 mg vitamin B_6, 20 µg vitamin B_{12}, 25 mg niacin, 8 mg calcium-D-panthotenate, 1 mg folic acid, 50 mg vitamin C, 50 µg D-biotin, 80 mg Mn, 60 mg Zn, 60 mg Fe, 5 mg Cu, 1 mg I, 0.5 mg Co, 0.15 mg Se;
[2] Metabolizable energy content of diets was estimated according to the equation of Carpenter and Clegg [11]

expressed as g per bird per week and the feed conversion ratio was calculated as kg feed per kg body weight gain.

To collect excreta, broilers in each replicate were put on cleaned plastic sheet in a separate pen during 5-10 min at day 40. Then excreta samples of each replicate pen were collected and mixed. The samples were dried in an air-forced oven at 60°C until reaching constant weight, and then the moisture content of samples was determined according to the AOAC [10].

At day 36, 20 broilers from each diet group (5 from each replicate) were randomly selected from each pen and injected with 0.1 ml of 0.25% suspension of sheep erythrocytes (SRBC, provided from a healthy male sheep) in phosphate buffer saline. Circulating anti-SRBC antibody titers were determined by the microhemagglutination technique from samples taken at 5 days after the immunization. All titers were expressed as the $\log_2$ of the reciprocal of the serum dilution [14].

Blood samples were collected from vena brachialis under the wing from 20 fed broilers randomly chosen from each group (five from each replicate) at day 41 and centrifuged at 3.220 x g for 8 min. Serum was collected and stored at -20°C for determination of total protein, albumin, uric acid, triglyceride, cholesterol, and levels of aspartate amino transferase (AST) and alanine amino transferase (ALT) by an autoanalyser (Product code 680-2153, Vitros 350; Johnson-Johnson Company, New York, USA) using their accompanying commercial kits (Vitros Chemistry Products, Ortho-Clinical Diagnostics; Johnson-Johnson Company).

At the end of the experiment (on day 42) 16 broilers from each group (4 from each replicate) were weighed and slaughtered by severing the jugular vein. Their gastrointestinal tracts were excised. Hot carcasses were weighed to determine the carcass yield. Absolute and relative weights of abdominal fat, liver, heart, gizzard, spleen and bursa of Fabricius were determined. Duodenal, jejunal, ileal and caecal digesta contents were pooled and homogenized. pH values of digesta contents were measured immediately by pH meter (Selecta pH meter, pH 2004, J.P. Selecta, Barcelona).

For histopathological analysis, half of the liver and heart samples were frozen in -80°C in liquid nitrogen and cut at 5 µm and stained with haematoxylin and eosin and oil-red (-O) stain. The other halves were fixed in 10% neutral buffered formalin solution and embedded in paraffin wax and cut 5 µm and stained with haematoxylin and eosin and trichrome stains. Histopathological examination was carried out by independent investigators blinded to treatment groups using light microscopy in 30 high-power fields per sample with a magnification of 200x. Histopathological features of hepatic steatosis were evaluated using a semiquantitative, histopathology scoring adapted from the recently accepted AASLD criteria for steatosis staging, and then scored for steatosis, lobular inflammation, and hepatocyte ballooning using the NAFLD activity score [15].

Statistical Analysis

Statistical analysis were done using SPSS program (SPSS Inc., Chicago, IL, USA). The experimental unit was the cage (n = 4). The normality of data distribution was checked using the Kolmogorov-Smirnov test. Values were reported as means ± SEM. The significance of mean differences among groups was tested by Duncan [16]. Level of significance of $P<0.05$ was used.

RESULTS

The effects of dietary yeast cell wall on growth performance and excreta moisture are shown in *Table 2*. Supplementing diets with yeast cell wall increased the weight gains during the starter period ($P<0.001$) in broilers. Dietary treatments did not significantly affect feed intake. Feed conversion during the starter period ($P<0.001$) and during the whole period ($P<0.05$) was improved by yeast cell wall supplementation. During the experimental period, 2 (2.9%), 1 (1.5%), 2 (2.9%) and 1 (1.5%) broilers died in the control group and groups fed with diets containing yeast cell wall at the level of 1, 2 and 3 g/kg, respectively. Dietary yeast cell wall supplementation had no effect on excreta moisture.

The effects of dietary yeast cell wall on anti-SRBC titer and blood serum parameters in broilers are shown in *Table 3*. Dietary yeast cell wall supplementation increased antibody titers to SRBC ($P<0.01$), increased serum protein concentration ($P<0.05$) and decreased total cholesterol and triglyceride concentrations ($P<0.001$). No differences were observed in serum albumin, uric acid, ALT and AST among groups.

Carcass yield and the relative weights of gizzard, liver, heart, spleen and bursa of Fabricius were not affected by the yeast cell wall supplementation *(Table 4)*. However the relative weight of abdominal fat was decreased with dietary yeast cell wall. Intestinal pH *(Table 4)* tended to be decreased in broilers supplemented with yeast cell wall and the jejunal pH and ileal pH was significantly lower than that of the control group ($P<0.05$).

No significant differences were seen in histological grade; hepatic lobular inflammation, steatosis and cell ballooning scores and cardiac steatosis among groups *(Table 5)*.

DISCUSSION

Dietary yeast cell wall supplementation increased the weight gains during the starter period ($P<0.001$) in broilers. Total live weight gain and weight gain during the grower

Table 2. *The effects of dietary supplementation of yeast cell wall on growth performance and excreta moisture in broilers*
Tablo 2. *Karma yemlere maya hücre duvarı ilavesinin broylerlerde büyüme performansı ve dışkı nemi üzerine etkileri*

Items	Yeast Cell Wall (g/kg)				SEM	P-value
	0	1	2	3		
Live weight gain (g)						
1-21 days	838b	879a	880a	893a	6	<0.001
22-42 days	1831	1895	1896	1900	16	0.375
1-42 days	2669	2775	2776	2793	19	0.059
Feed intake (g)						
1-21 days	1080	1074	1071	1079	2	0.253
22-42 days	3436	3428	3400	3414	8	0.428
1-42 days	4516	4501	4471	4492	8	0.268
Feed conversion ratio (g/g)						
1-21 days	1.29a	1.22b	1.22b	1.21b	0.01	<0.001
22-42 days	1.88	1.81	1.79	1.80	0.02	0.219
1-42 days	1.69a	1.62b	1.61b	1.61b	0.01	0.030
Excreta moisture (g/kg)	797.0	803.6	804.2	803.8	1.3	0.149

[a-b] Means with different superscript in the same row are different at P<0.05 in instances with significant interaction; n = 4

Table 3. *The effects of dietary supplementation of yeast cell wall on anti-SRBC titers and blood serum parameters in broiler*
Tablo 3. *Karma yemlere maya hücre duvarı ilavesinin broylerlerde SRBC'ye karşı antikor düzeyi ve kan serum parametreleri üzerine etkileri*

Items	Yeast Cell Wall (g/kg)				SEM	P-value
	0	1	2	3		
Anti SRBC titer (log_2)	5.20b	5.95a	6.10a	6.10a	0.11	0.004
Total protein (g/L)	30.3b	32.6ab	34.0a	33.3a	0.5	0.026
Albumin (g/L)	13.5	13.7	13.8	14.0	0.1	0.585
Uric acid (mg/L)	56.7	56.9	57.3	56.6	0.8	0.995
Total cholesterol (g/L)	1.26a	1.16b	1.08c	1.10c	0.01	<0.001
Triglyceride (g/L)	1.29a	1.14b	0.96c	0.94c	0.02	<0.001
ALT (U/L)	14.6	14.1	14.8	13.9	0.3	0.637
AST (U/L)	226	221	225	235	3	0.344

[a-b] Means with different superscript in the same row are different at P<0.05 in instances with significant interaction; n = 20

period tended to be increased in broilers supplemented with yeast cell wall but differences with not supplemented group were not statistically significant. Dietary yeast cell wall supplementation improved feed conversion during the starter period (P<0.001) and during the whole period (P<0.05) however had no effect on feed intake. This improvement in yeast cell wall supplemented groups might be due to the improvement of the intestinal lumen health and thereby increasing the absorption and utilization of the dietary nutrients [17,18]. Zhang et al.[19] reported that the live weight gains by yeast cell wall fed broilers were greater than those of the control broilers from 4 to 5 weeks of age and from 0 to 5 weeks of age. Live weight gain [20,21], feed intake [19,20] and feed conversion [21] were not affected by using yeast cell wall in some studies. The differences in animal response may be related to the differences in the type and dose of yeast cell wall and diet composition. In the present study 2 (2.9%), 1 (1.5%), 2 (2.9%) and 1 (1.5%) broilers died in the control group and groups fed with diets containing yeast cell wall at the level of 1, 2 and 3 g/kg, respectively during 42 days. Similarly some researchers [7,20,22] reported that dietary supplementation of yeast cell wall had no effect on mortality. Dietary yeast cell wall supplementation did not significantly affect excreta moisture, as previously reported with yeast in broilers [23].

Antibody responses have been used as measures of the humoral immune status of poultry [24]. As shown in

Table 4. *The effects of dietary supplementation of yeast cell wall on carcass yield, relative organ weights and intestinal pH in broilers*
Tablo 4. *Karma yemlere maya hücre duvarı ilavesinin broylerlerde karkas randımanı, relatif organ ağırlıkları ve bağırsak pH'sı üzerine etkileri*

Items	Yeast Cell Wall (g/kg)				SEM	P-value
	0	1	2	3		
Carcass yield (%)	72.7	72.8	72.8	72.9	0.2	0.983
Gizzard (%)	1.33	1.33	1.34	1.32	0.02	0.960
Heart (%)	0.52	0.52	0.53	0.51	0.01	0.743
Liver (%)	1.91	1.89	1.92	1.88	0.02	0.792
Spleen (%)	0.11	0.10	0.11	0.11	0.01	0.261
Bursa Fabricius (%)	0.17	0.18	0.18	0.19	0.01	0.796
Abdominal Fat (%)	1.49a	1.33b	1.33b	1.27b	0.03	0.024
Duodenum pH	5.64	5.58	5.52	5.55	0.04	0.723
Jejunum pH	5.63a	5.54ab	5.46b	5.43b	0.03	0.048
İleum pH	6.52a	6.30b	6.32b	6.29b	0.03	0.041
Caecum pH	6.50	6.37	6.40	6.44	0.04	0.672

[a-b] Means with different superscript in the same row are different at P<0.05 in instances with significant interaction; n = 16

Table 5. *The effects of dietary supplementation of yeast cell wall on cardiac and hepatic histopathology in broilers*
Tablo 5. *Karma yemlere maya hücre duvarı ilavesinin broylerlerde kalp ve karaciğer histopatolojisi üzerine etkileri*

Items	Grade	Yeast Cell Wall (g/kg)				P-value
		0	1	2	3	
Cardiac steatosis(%)	0	75.0	87.5	100.0	93.8	0.218
	1	18.8	12.5	0	0	
	2	6.3	0	0	6.3	
Hepatic histopathology (%)						
Histological grade (%)	0	81.3	81.3	68.8	62.5	0.599
	1	18.8	18.8	31.3	31.3	
	2	0	0	0	6.3	
Hepatic steatosis (%)	0	81.3	93.8	100.0	87.5	0.500
	1	12.5	6.3	0	6.3	
	2	0	0	0	6.3	
	3	6.3	0	0	0	
Cell ballooning (%)	0	81.3	93.8	87.5	87.5	0.767
	1	18.8	6.3	12.5	12.5	
Lobular inflammation (%)	0	0	18.8	25.0	25.0	0.444
	1	81.3	62.5	50.0	62.5	
	2	18.8	18.8	25.0	12.5	

Table 3, dietary yeast cell wall supplementation increased antibody titers to SRBC (P<0.01). This might be due to the glucans and the mannans present in the yeast cell wall on the immune system [4,5,25]. It can be assumed that prebiotics would bind to macrophage reception sites by recognizing spesific sugars found in glucoproteins of the epithelial surface, triggering a cascading reaction that would eventually activate macrophages and release cytokines, thereby activating the acquired immune response [26,27]. Higher antibody responses in broiler breeders fed MOS were observed in the study of Shashidhara and Devegowda [5]. Chae et al.[8] showed that CD8 and TCR I cells were higher in the 0.04% β-glucan supplemented diet as compared with non-added diets. Similarly, Yalçın et al.[23] also reported greater antibody titre in broilers fed diets containing 1, 2, 3 or 4 g/kg of yeast autolysate.

Blood serum biochemical parameters may provide useful information about the evaluation of the health status of broilers. Dietary yeast cell wall supplementation increased serum protein concentration (P<0.05) and decreased total cholesterol and triglyceride concentrations (P<0.001). Increased serum total protein in broilers fed diets supplemented with yeast cell wall may reflect a more intensive metabolism of the proteins and effective protein utilization in the broiler metabolism. This situation may also be explained by increasing weight gain. Similarly some researchers observed that serum total cholesterol and triglyceride concentrations were reduced by dietary MOS [28] or yeast autolysate [29]. Krasowska et al.[30] reported that *Saccharomyces* strains are able to remove cholesterol from the growth medium and that baker's yeast *Saccharomyces cerevisiae* seems to be the perfect organism for lowering cholesterol in the gastrointestinal tract. Nicolosi et al.[31] also indicated that yeast derived β-glucan lowered total cholesterol concentrations in hypercholesterolaemic men. However these results contradict the findings of Konca et al.[32] who reported that dietary MOS supplementation did not affect serum cholesterol and total protein concentration in turkeys. In the present study dietary supplementation of yeast cell wall had no effect on serum albumin, uric acid, ALT and AST. Yalçın et al.[23] also showed that serum uric acid, ALT and AST levels were not affected from dietary yeast autolysate.

No significant differences in the carcass yield and the relative weights of gizzard, liver, heart, spleen and bursa of Fabricius were observed among groups *(Table 4)*. However the relative weight of abdominal fat was significantly lower (P<0.05) in broilers fed with diets containing yeast cell wall than in birds fed with the control diet. This result shows a change in energy partitioning. It might be that the extra energy that was not being stored by the broilers fed yeast cell wall supplemented diets was being used to up regulate the immune system and increase the titer response to SRBC. In agreement with previous reports yeast cell wall or yeast cell wall ingredients had no significant effect on gizzard weight [21], relative weight of spleen and bursa of Fabricius [20]. The results of present study contradict the findings of previous work by Guo et al.[33] in which dietary supplementation of yeast β-glucan resulted in increased relative spleen and bursa weights. Corduk et al.[34] reported that MOS supplementation did not significantly affect carcass yield and the relative weights of abdominal fat and gizzard.

Intestinal pH tended to be decreased in broilers supplemented with yeast cell wall and the jejunal pH and ileal pH was significantly lower than that of the control group (P<0.05) as shown in *Table 4*. Low pH of the digesta of broilers fed yeast cell wall could improve utilization of the diets as reported in the study of Afsharmanesh et al.[35]. In contrast to the present study, some researchers observed that MOS supplementation did not affect [36] or increased [37] ileal pH. According to the findings of some studies, pH of duodenal contents [32,38] and pH of ileal and caecal contents [38-40] were unaffected by dietary MOS supplementation. The difference among the studies may be due to the diet composition, diet type, age of birds and method of pH measurement.

During the growth of broiler chickens by intensive feeding, some health problems occur, mainly limb defects, sudden death syndrome, or excessive fat deposition [41]. Maxwell et al.[42] reported that feed overconsumption in chicken results in fat deposition throughout the body, which leads to coronary and hepatic steatosis in chickens. In the present study control and treatment groups had similar histological grade; hepatic lobular inflammation, steatosis and cell ballooning scores and cardiac steatosis as shown in *Table 5*. However, these results do not exclude the possibility that yeast cell wall supplementation may be beneficial in other circumstances. In the previous study of Yalçın et al.[43] the dietary probiotic (Primalac 454; *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium* and *Bifidobacterium thermophilus*) supplementation (at the dose of 0.05%) have significantly alleviated the development of a non-alcoholic fatty liver disease induced by dietary protein restriction.

The differences between the results of present study and those of previous reports may be the species, age, and sex of the birds, dietary nutrient composition, type, dose and composition of yeast cell wall or environmental conditions.

The results of this study indicate that dietary yeast cell wall at the level of 1, 2 and 3 g/kg improved body weight and feed efficiency, decreased abdominal fat and increased humoral immune response. Therefore it is concluded that yeast cell wall derived from bakers yeast (InteMos) was an effective and beneficial feed additive in broiler feeding.

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RESEARCH ARTICLE

The Effect of Heat Processing and pH on PCR Detection of Genetically Modified (GM) Soy in Meat Products [1]

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Summary

Soybean is the most cultivated GM crop worldwide, being planted on 47% of the global GM crop area. However, GM crops have not been widely publicly accepted. Thus, countries have established regulations for the labeling of GM foods to inform consumer decision making. In 2010, regulations on GM foods began to be enforced in Turkey. To meet these legislation requirements, the development of reliable detection methods is an important priority in this research area. PCR-based methods are most commonly used for this purpose. However, processing factors (low pH, heat etc.) affect DNA quality and thus the sensitivity of PCR. The aim of this study was to evaluate the combined effects of heat and pH on the detection of GM soy in meat products. We found that the combined effects of heat and low pH affect the detection limit, but low levels of GM soy can still be detected after processing.

Keywords: *GMO, PCR, Meat products, GM soy, DNA degradation, Process factor*

Et Ürünlerinde Isıl İşlem Uygulamaları ve pH'nın Genetiği Değiştirilmiş (GD) Soyanın PCR ile Tespiti Üzerine Etkisi

Özet

Soya fasulyesi dünya genelinde en yaygın ekilen GD tahıl ürünü olup toplam GD tarım üretim alanının %47'sini teşkil etmektedir. Buna karşın, GD tarım ürünleri tüketiciler tarafından yaygın olarak kabul görmemektedir. Bu yüzden, çeşitli ülkeler tüketicilere karar verme olanağı tanımak üzere GD gıdaların etiketlemesine yönelik yönetmelikler hazırlamıştır. Nihayetinde, 2010 yılında ülkemizde de GD gıdalarla ilgili mevzuat yürürlüğe girmiştir. Yönetmelik şartlarını karşılamak için, güvenilir tespit metotlarının geliştirilmesi bu bilim alanının önceliklerinden biri haline gelmiştir. Bu amaçla PCR-temelli metotlar en yaygın kullanılan teşhis yöntemlerindendir. Ancak, çeşitli gıda üretim yöntemleri (düşük pH, sıcaklık v.b.) DNA kalitesini ve böylece PCR hassasiyetini etkilemektedir. Çalışmamızın amacı, ısı ve pH'ın et ürünlerinde GD soyanın PCR tespitine olan kombine etkisini değerlendirmektir. Sonuçlarımıza göre, düşük pH ve ısının kombine etkisi tespit limitini etkilemekle beraber, proses sonrası dahi oldukça düşük seviyelerde GD soyanın tespiti mümkün olabilmektedir.

Anahtar sözcükler: *GMO, PCR, Et ürünleri, GD soya, DNA yıkımlanması, Proses etkisi*

INTRODUCTION

Soy is an important crop for the food industry because it is widely used in many foods [1-3]. In the case of meat products, soy protein is a common ingredient because of its unique functional properties, such as water and fat binding capabilities and the ability to improve organoleptic features. Furthermore, soy protein is also an economic protein replacement that can reduce production costs [2-5]. However, soy is also important for being the first commercial GM crop (Roundup Ready (RR) soy). It was developed by the Monsanto Company and is still the most cultivated GM crop; presently, it accounts for 47% of the global GM crop area [6,7].

Similar to several other countries, Turkish food regulation also enforces the labeling of foods that contain approved GM material above a threshold level [7-14]. To meet these regulation requirements, various studies have been performed to develop reliable and sensitive detection methods [1,15,16]. PCR is the most common method used for this aim [3,17,18]. By using PCR, general GMO screening

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and event-specific identification can be performed. Screening methods that are based on the detection of common DNA elements, such as the cauliflower mosaic virus (CaMV) 35S promoter and/or the nopaline synthase (*nos*) terminator, are generally the initial step in GMO detection (before event identification or GMO quantification); therefore, their reliability is important for most users [1,11,19-21]. Thus, screening assays based on the detection of CaMV 35S and *nos* sequences have been interlaboratory validated, and the method has been accepted as an official standard method [22-25].

Although DNA is more stable than protein in processed foods, it can still be degraded under processing conditions [14,25-29]. Because PCR-based detection of GMOs depends on the quality, purity and quantity of DNA, degradation reduces the sensitivity of analysis and has a negative impact on the detection limit of the method [30,31]. Temperature and pH are known to be the most contributory factors to DNA fragmentation [26,30]. In several studies, the degradation effect of various heating processes (drying, cooking, baking, autoclaving and spray-drying) commonly used for food production were evaluated. The results of all these studies proved that heat processing of foods caused mild to strong fragmentation of DNA and thus limited the ability to perform PCR screening [16,20,31-34]. Additionally, Bauer *et al.*[26], found that the highest DNA degradation occurred due to a combined exposure to acidic conditions and heat. In that study, researchers lowered the pH of soy flour with acetic acid and extracted DNA from these samples. The researchers also informed that the stability of DNA in different food matrices would also be different because each food processing technique and matrix would led to a unique environment. Gryson [30], also explained the importance of the effects of the type of food matrix on the performance of DNA extraction and PCR testing.

Therefore, in this study, we aimed to evaluate the combined effects of pH and heat, which are the most common processing factors used in meat production, on the PCR screening of GMOs.

MATERIAL and METHODS

Certified Reference Materials and Food Samples

Certified reference materials (CRMs) consisting of soybean powder (0, 0.1, 0.5 and 1% of RR soybean powder) produced by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and commercial soy containing meat sausage, soy flour and soy-free bread samples were used for quality control and verification purposes in the study.

Model Processed Sausage Production

Model processed sausages were produced from a formula of a commercial meat product producer in Turkey. The sausage mixtures were prepared from 390 g of beef, 160 g of fat emulsion, 340 g of ice, 40 g of oil, 0.05 g of paprika, 0.15 g of nitrate, 15 g of salt, 15 g of mixed spices (ginger, white pepper), 40 g of potato starch, and 0.20 g of carmine. Then, the appropriate amount of 1.25% RR soy (SDI diagnostics, USA) was added to the sausage mixtures to give final concentrations of 0.1, 0.5 and 1%. Each of the model sausage mixtures were divided into two groups, and the pH of these groups were adjusted to either 5.2 or 6.2. Following pH adjustment, each group was further divided into three subgroups, two of which were heated for 15 min at either 65°C or 85°C, while the third subgroup was left untreated (control group) *(Fig. 1)*.

DNA Extraction and Purification

DNA was extracted and purified in duplex from raw and heated model sausages, CRMs and food samples, using a Promega Wizard™ DNA isolation kit (Promega, Madison,

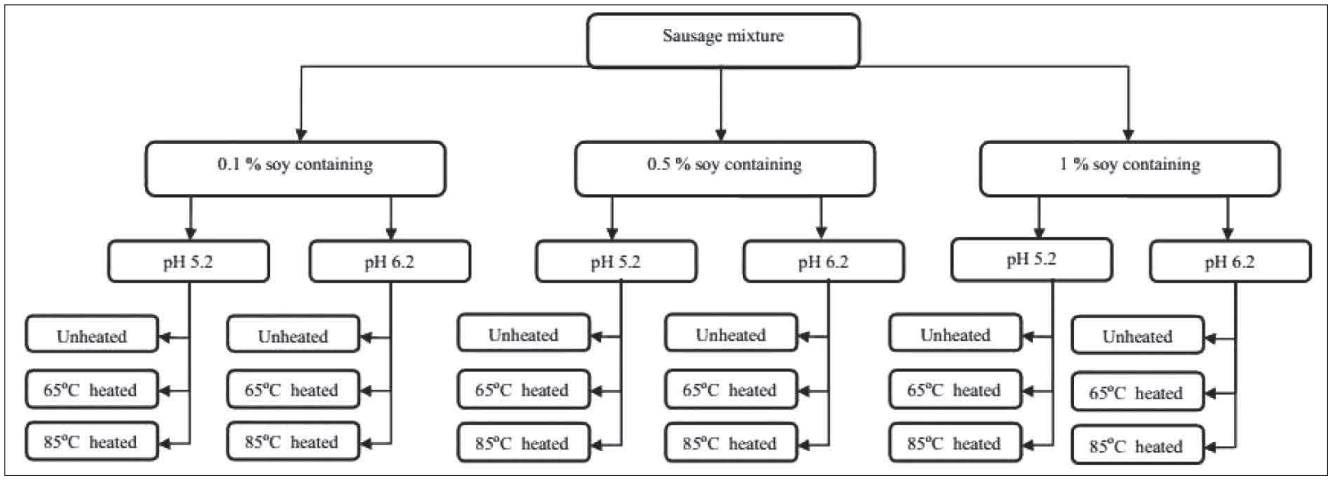

Fig 1. Model processed sausage production
Şekil 1. Model sosislerin üretimi

USA) according to the manufacturer's instructions and as described in other studies [17,23]. Briefly, between two hundred to three hundred milligrams of food material from a previously homogenized sample was mixed with 860 µl of extraction buffer (10 mM Tris-OH, 150 mM NaCl, 2 mM EDTA and 1% w/v sodium dodecyl sulfate), 100 µl of guanidine hydrochloride (5 M) and 40 µl of proteinase K (20 mg/ml) and then incubated at 65°C overnight. The samples were then centrifuged at 13.500 g for 10 min. After centrifugation, 500 µl of supernatant was mixed with 1 ml of Wizard™ resin (Promega, Madison, USA) and pushed through a Wizard™ minicolumn (Promega, Madison, USA). The column was further washed with 2 ml of isopropanol. Following centrifugation of the column at 12.000 g for 5 min, DNA was eluted with 50 µl of pre-warmed (65°C) elution buffer (10 mM Tris-OH). The columns were incubated at room temperature for 1 min and centrifuged at 10.000 g for 2 min. The collected DNA was stored at -20°C until it was used.

The quantity and purity of the DNA were monitored by measuring the UV absorption at 260 nm and 280 nm using a T80 UV/VIS spectrometer (PG Ins. Ltd., UK). The integrity of the DNA was verified by loading the DNA onto a 2% agarose gel containing ethidium bromide.

PCR Primers

The primers p35S-cf3 (5'-CCA CGT CTT CAA AGC AAG TGG-3') and p35S-cr4 (5'-TTC TCT CCA AAT GAA ATG AAC TTC C3') that amplify a PCR fragment of 123 bp were used for screening PCR of the CaMV 35S sequence [22]. The primers Lectin 1 (5'GAC GCT ATT GTG ACC TCC TC-3') and Lectin 6 (5'- GAA AGT GTC AAG CTT AAC AGC GAC G-3') were used for amplification of soy specific lectin sequence and yielded a longer PCR product of 318 bp [28].

PCR Conditions

All PCR reactions were performed with a CG Palm-Cycler (CG 1-96 Genetix Biotech, Australia & Asia). The amplification reactions contained 5 µl of genomic DNA (10 ng/µl) and 20 µl of the appropriate PCR reaction mixture. The PCR reaction mixture was varied: for the CaMV 35S amplifications, it consisted of 1X buffer (Fermentas), 1.5 mM $MgCl_2$ (Fermentas), 0.6 µM primers for 35S, 0.16 mM aliquots of each dNTP (Fermentas) and 0.8 U of Maxima™ Hot Start *Taq* polymerase (Fermentas); for soy-specific lectin amplifications, it consisted of 1X buffer (Fermentas), 2 mM $MgCl_2$ (Fermentas), 0.5 µM primers for lectin, 0.2 mM aliquots of each dNTP (Fermentas) and 2 U of Maxima™ Hot Start *Taq* polymerase (Fermentas) [22,28].

The amplification profiles used for these mixtures were as follows:

- For CaMV 35 S: denaturation for 10 min at 95°C; amplification for 25 s at 95°C, for 30 s at 62°C and for 45 s at 72°C; number of cycles 50; final extension for 7 min at 72°C.

- For lectin: denaturation for 3 min at 94°C; amplification for 45 s at 94°C, for 45 s at 60°C and for 25 s at 72°C; number of cycles 50; final extension for 7 min at 72°C.

Agarose Gel Electrophoresis

The PCR products were electrophoresed through a 2% agarose gel containing ethidium bromide. As a size reference, a 50 bp DNA ladder (Fermentas) was used. Visualization of the gels was performed with a UV trans-illuminator, and the gels were captured with a Dolphin-DOC system and Dolphin 1D Gel analyzing software (Wealtec, Nevada, USA).

RESULTS

Verification and Quality Control Testing

The PCR conditions and primers were verified in the beginning of the study. For this, a lectin PCR with soy containing sausage samples, soy-free bread sample, soy flour and RR soy CRMs was performed. The results are given in *Fig. 2*. According to these results the primers used in the assay generated PCR product only with the food samples containing soy and did not generate any amplification with non-soy containing bread sample.

The results of verification of CaMV 35S assay with CRMs are given in *Fig. 3*. These results also showed that the primers generate amplification with RR soy CRMs even when the GM soy content is as low as 0.1% and did not generate any PCR products with non GM soy CRM (0% RR soy CRM).

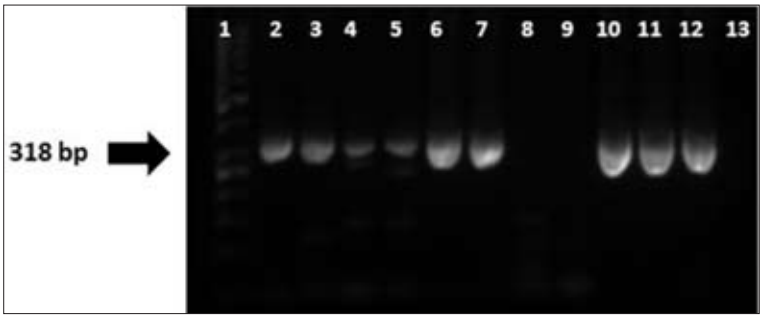

Fig 2. Lectin PCR: Lane-1:50 bp DNA ladder, Lane 2-5: Soy containing sausages, Lane 6-7: Soy flour, Lane 8-9: Soy free bread sample, Lane 10-12: RR soy CRMs Lane-13: PCR milli q water

Şekil 2. Lektin PCR: 1. Sıra: 50 bp DNA marker, 2-5. Sıra: Soya içeren sosis örnekleri, 6-7. Sıra: Soya unu, 8-9. Sıra: Soya unu içermeyen ekmek örneği, 10-12. Sıra: RR soya SRM'leri, 13. Sıra: PCR milli q su

Quality and Quantity of Extracted DNA

The results of agarose gel electrophoresis performed with DNA extracts of the model samples are given in *Fig. 4*. Although both groups showed a band above 1.000 bp (The highest band of the DNA ladder is 1.000 bp), the band intensities of DNA extracted from pH 5.2 sausages were significantly lower compared to the intensity of the DNA bands from the pH 6.2 samples. The DNA concentration and purity ratios which were calculated from the 260 nm absorbance readings and 260/280 absorbance reading ratios of the extracts respectively are detailed in *Table 1*. According to the results of our study, the overall average DNA concentrations were 116 ng/µl and 119 ng/µl for pH 5.2 and pH 6.2 sausages, respectively. The overall mean purity ratio of DNA extracts of pH 5.2 sausages was 1.51, while it was 1.39 for pH 6.2 sausages *(Table 1)*.

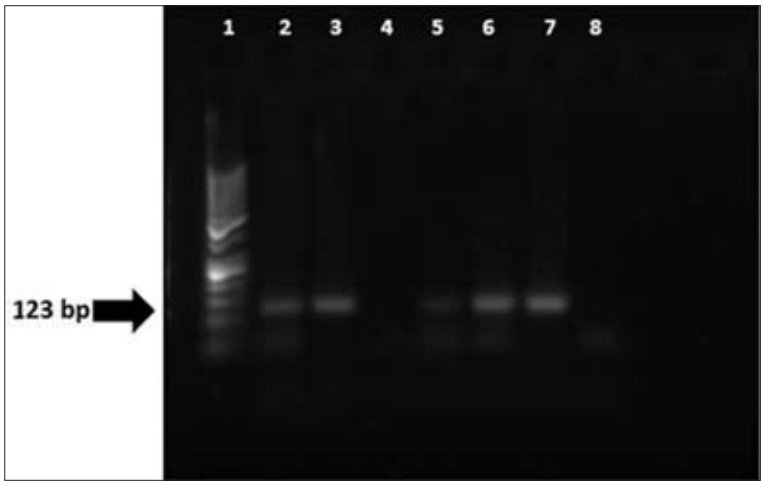

Fig 3. CaMV 35S PCR: Lane-1:50 bp DNA ladder, Lane 2-3: Heated model sausage (0.5%, pH 6.2, 85ºC), Lane 4-7: RR soy CRMs 0%, 0.1%, 0.5% and 1%, Lane-8: PCR milli q water

Şekil 3. CaMV 35S PCR: 1. Sıra: 50 bp DNA marker, 2-3. Sıra: Isıl işlem görmüş model sosis örnekleri (%0.5, pH 6.2, 85ºC), 4-7. Sıra: RR soya SRM'leri %0, %0.1, %0.5 ve %1, 8. Sıra: PCR milli q su

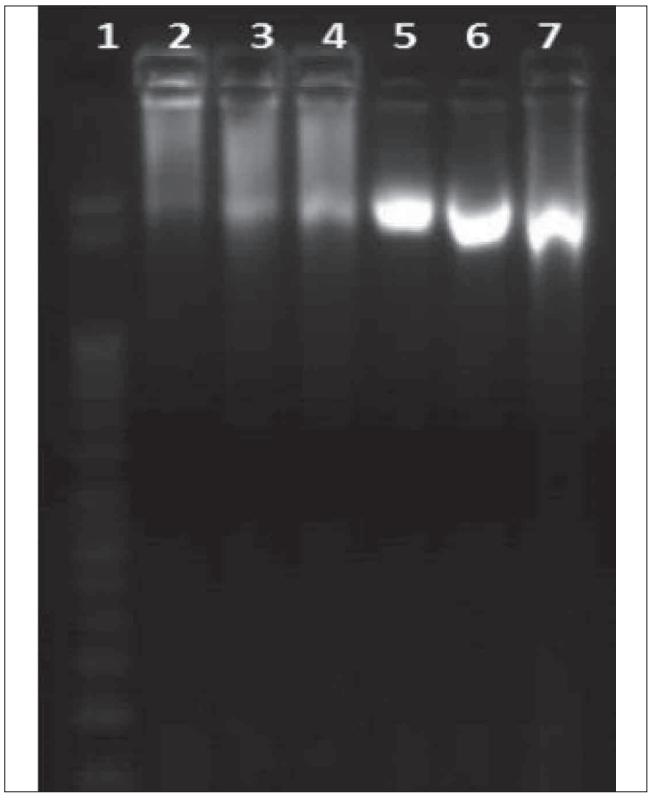

Fig 4. Agarose gel electrophoresis of the DNA extracts of model sausages; Lane-1:50 bp DNA ladder, Lane 2: pH 5.2 raw, Lane 3: pH 5.2 65ºC, Lane 4: pH 5.2 85ºC, Lane 5: pH 6.2 raw, Lane 6: pH 6.2 65ºC, Lane 7: pH 6.2 85ºC

Şekil 4. Model sosis örneklerine ait DNA ekstarktlarının Agar Jel Elektroforezi; 1. Sıra: 50 bp DNA marker, 2. Sıra: pH 5.2 çiğ, 3. Sıra: pH 5.2 65ºC, 4. Sıra: pH 5.2 85ºC, 5. Sıra: pH 6.2 çiğ, 6. Sıra: pH 6.2 65ºC, 7. Sıra: pH 6.2 85ºC

PCR Testing

Duplex extractions were made from the model sausage samples, and PCR tests were then repeated to obtain four amplification results for each sample for both the lectin and the CaMV 35S sequences. The results of the replicated amplifications are summarized in *Table 2*. The results of CaMV 35S PCR detection showed that when the ratio of 1.25% RR soy in the sausage sample was 0.5% or higher, 100% (4/4) positive amplification of GM-specific sequences was observed in raw, 65°C- and 85°C-heated samples from both pH 5.2 and 6.2 sausages. However, when the content of 1.25% RR soy was lowered to 0.1%, positive amplification could only be obtained from 50% (2/4) of the pH 6.2 samples. In the pH 5.2 sausages, detection was not possible when the sausage was heated at 85°C, while 50% detection was achieved for samples heated at 65°C.

Parallel to the PCR screening of RR soy, PCR testing for amplification of the lectin sequence was also performed *(Table 2)*.

According to these results, 100% amplification could be performed from all the samples, irrespective of the soy flour ratio and processing conditions.

DISCUSSION

The effects of processing factors on the quality and quantity of extracted DNA have also been studied by

Table 1. *The concentration and purity of the DNA extracts*
Tablo 1. *DNA ekstraktlarının konsantrasyon ve saflığı*

Sample Type		DNA Concentration (ng/µl)	Mean DNA Concentration	Purity	Mean Purity
5.2	Unheated*	108	116	1.69	1.51
	65°C*	93		1.38	
	85°C*	146		1.47	
6.2	Unheated*	112	119	1.43	1.39
	65°C*	137		1.44	
	85°C*	108		1.30	

** The concentration and purity are mean of 0.1, 0.5 and 1% samples*

Table 2. *PCR screening results of model samples determined with primer pairs for CaMV 35S and lectin sequences*
Tablo 2. *Model sosis örneklerinin CaMV 35S ve lektin dizilimleri için primer çiftleri ile gerçekleştirilen PCR tarama test sonuçları*

1.25% RR Soy/mixture	pH	Heat	CaMV 35S*	Lectin*
0.1%	5.2	Unheated	2/4	4/4
		65°C	2/4	4/4
		85°C	0/4	4/4
	6.2	Unheated	2/4	4/4
		65°C	2/4	4/4
		85°C	2/4	4/4
0.5%	5.2	Unheated	4/4	4/4
		65°C	4/4	4/4
		85°C	4/4	4/4
	6.2	Unheated	4/4	4/4
		65°C	4/4	4/4
		85°C	4/4	4/4
1%	5.2	Unheated	4/4	4/4
		65°C	4/4	4/4
		85°C	4/4	4/4
	6.2	Unheated	4/4	4/4
		65°C	4/4	4/4
		85°C	4/4	4/4

** The number of positive results in 4 repeated PCR*

other researchers; in these studies, heating was evaluated for certain food types [20,32-34]. However, because the food matrix would also affect the extractability and amplifiability of the DNA, studies should also be performed on various food types. In this study, the possibility of detecting low levels of GM soy in processed meat products by PCR is evaluated. For this purpose, model processed sausages containing various levels (0.1, 0.5 and 1%) of 1.25% RR soy were prepared. The pH of the products was adjusted to 6.2 or 5.2, and both groups were further divided into subgroups that were heated at 65°C or 85°C for 15 min or left unheated as a control.

The results of the verification PCRs proved that primer pairs used in the study are specific to target DNA and do not generate any amplification with non-target DNA (*Fig. 2* and *3*). The sensitivity of the CaMV 35S screening assay was determined by testing 0.1, 0.5 and 1% RR soy CRMs in parallel with the samples in each PCR. Positive detection of 0.1% RR soy CRM proved that the detection limit of the CaMV 35S assay is below 0.1% *(Fig. 3)*. For elimination of any false positive results, a no template control (sterile MILLI Q water) was run in each lectin and CaMV 35S specific PCR and 0% RR soy CRM was run in each CaMV 35S PCR [35].

Many other researchers have shown that a sufficient quality and quantity of DNA is necessary for successful PCR testing [19,28,30]. Additionally, processing technologies and extraction methods are crucial for maintaining the integrity of extracted DNA [16,20,26,30]. For this purpose, the effects of temperature and pH on the integrity of DNA extracted from raw and processed model sausages were examined by loading some of the DNA extracts onto a 2% agarose gel *(Fig. 4)*. These results proved that, although

DNA of low pH samples had a lower intensity, DNA fragments of sufficient length are still present, indicating that PCR should be possible. The DNA concentration in the extracted DNA which were determined by measuring the absorbance at 260 nm showed that the DNA concentrations did not indicate a significant difference between low and high pH sausages and different heating temperatures *(Table 1)*. The purity of the DNA extract is reported as another important variable that has an effect on PCR detection. The purity of the extracted DNA can be determined by measurement of A260/280 absorbance ratios with a spectrophotometer. DNA extracts are considered "suitable for PCR" when the ratio is between 1.5 and 2.0 [30]. Although the mean ratio of the DNA extracts from some of the model sausages were out of the purity ratio recommended for PCR in our study, lectin and CaMV 35S sequences could still be amplified from these extracts. Similarly, the results of Kakihara *et al.*[16], showed that although the 260/280 nm absorbance ratios of DNA solutions extracted using an alkaline lysis method were as low as 1.2, they could detect PCR products of 100 to 150 bp fragments from these solutions.

Screening PCR is reported to be the most sensitive PCR in GMO analysis, and it is generally used as the first step in GMO detection before both event identification and GMO quantification in routine monitoring [20,23]. Therefore, we used the CaMV 35S screening method to determine the effects of temperature and pH on the ability of GM DNA to serve as a template for amplification. Because most testing laboratories prefer internationally validated standard methods for routine testing, as recommended in the ISO 17025 laboratory accreditation standard, the primers used in the standard method for amplifying the CaMV 35S promoter were also used in this study [22,36].

According to the results of CaMV 35S PCR, detection was possible, when the ratio of 1.25% RR soy in the sausage sample was 0.5% or higher, after all types of processing conditions. The decrease in the ratio of the (2/4) positive results obtained from pH 6.2, 0.1% RR soy containing sausage samples was most likely related to the low RR soy content. The lack of amplification in 85°C heated pH 5.2 samples while it was present in 85°C heated pH 6.2 samples proved that heat processing more strongly effects the detection in lower pH matrixes. The amplification of the lectin sequence in the same extracts proved that negative results were not related to the absence of amplifiable quality soy DNA but, rather, to the practical detection limit of the method. Similarly, the results of Gryson *et al.*[20], showed that the practical detection limit of GM soy screening in cooked model cookies was higher compared to raw dough. The results obtained for sausages at pH 5.2 and heated to 85°C agreed with the results of Bauer *et al.*[26], who also showed that the combined effects of pH and heating were stronger than either effect alone.

Because lectin is present in both GM and non GM soy, PCR testing for amplification of the lectin sequence was also performed for evaluating homogeneity of the sample and amplifiability of the DNA extract. Additionally, information about the effects of processing on endogenous and exogenous DNA is also valuable for quantitative PCR testing. Even though the target fragment length necessary for the lectin assay is significantly longer than the CaMV 35S assay used in this study, detection could be possible from all samples. Similarly, other results showed that processing conditions have different effects on endogenous and exogenous genes of Roundup Ready soy [33]. However, the relatively lower ratio of GM soy (1.25% RR soy) in the final product might also be a reason.

In conclusion, the efficiency of a PCR detection method strongly depends on the quality, quantity and amplifiability of the DNA extract, which is affected by processing techniques. Our results proved that the effect of low and high temperature pasteurization processing used for meat products does not have a very strong effect on GMO screening and can be used to a certain extent. However, it was also determined that the combined effects of pH and heating are stronger than either individually, and the method should be further evaluated for low pH meat products heated at higher temperatures. The effect of processing endogenous and exogenous genes seemed to be different, and further studies into this concept should be performed because of its important effect on the accuracy of quantitative methods.

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RESEARCH ARTICLE

To Determine the Occurence of Aflatoxin M1 (AFM1) in Samples of Cyprus Traditional Cheese (Halloumi): A Cross-Sectional Study

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Summary

This Cross-Sectional present study was conducted to determine the occurence of aflatoxin M1 (AFM1) in samples of Turkish Republic of North Cyprus (TRNC) traditional cheese (halloumi). In the current study a total of 128 halloumi cheese samples including 36 industrial made and 92 home made were selected by using cluster sampling method and analyzed for AFM1 with the competitive ELISA. The percent of AFM1 contamination in halloumi cheese was found to be low, since 28.8% (0-16.66 ng/kg) of the samples were positive in industrial made and 21.7% (0-4.63 ng/kg) in home made. The mean, standard error of mean (SEM), 95% Confidence Interval (95% CI) values of aflatoxin M1 in halloumi cheese with industrial and home made samples were 0.84±0.24, (95% CI :0.35-1.35) and 1.28±0.32, (95% CI: 0.63-1.93) respectively. Both means were not significantly difference (P=0.422) and found very low from the limits of European Commission (EC) (250 ng/kg) and Turkish Food Codex (TFC) (500 ng/kg) (P<0.001). In order to prevent from introduction of aflatoxin M1 into cheese industry cycle, hygienic conditions, appropriate storage and control of livestock feed at all stages of planting and requires system that makes aflatoxin control are necessary.

Keywords: *Halloumi cheese, Aflatoxin M1, Cheese, Enzyme-linked immunosorbent assay, ELISA*

Kıbrıs Geleneksel Peynir (Hellim) Örneklerinde Aflatoksin M1 (AFM1) Oluşumunun Belirlenmesi: Kesitsel Bir Çalışma

Özet

Bu kesitsel çalışma ile Kıbrıs geleneksel peynir (Hellim) örneklerinde aflatoksin M1 (AFM1) oluşumunu belirlemek için yapılmıştır. Çalışmada küme örnekleme yöntemiyle 36 endüstriyel yapımı ve 92 ev yapımı olmak üzere toplam 128 Hellim peyniri örneğe seçildi. Peynir örneklerindeki AFM1 varlığı ELISA yöntemi ile analiz edilerek belirlendi. Endüstri yapımı Hellim peynirinde AFM1 pozitif bulunma oranı %28.8 (0.00-16.66 ng/kg), ev yapımı Hellim peynirinde ise bu oran %21.7 (0.00-4.63 ng/kg) şeklinde düşük bir oran olduğu belirlendi. Endüstri ve ev yapımı Hellim peynirindeki AFM1 değerinin ortalama, standart hata (SEM) ve %95 güven aralığında sırasıyla 0.84±0.24, (%95 CI: 0.35-1.35) ve 1.28±0.32, (%95 CI: 0.63-1.93) şeklinde bulundu. Her iki ortalama arasındaki farklılık önemli bulunmadı (P=0.422) ve European Commission (EC) (250 ng/kg) and Turkish Food Codex (TFC) (500 ng/kg) değerlerinden önemli derecede düşük olduğu bulundu (p<0.001). Aflatoksin M1 'in peynirin endüstri döngüsündeki girişini önlemek için, hijyenik koşullar, uygun depolama ve hayvan yemlerinin tüm aşamalarda kontrolünün sağlanması ve aflatoxin düzeyini denetleyen bir sistemin olması gerektiği bilinmelidir.

Anahtar sözcükler: *Hellim peyniri, Aflatoxin M1, Peynir, Enzyme-linked immunosorbent assay, ELISA*

INTRODUCTION

Halloumi is a firm pickled cheese with its origins in TRNC where it is made from sheep or goat milk or a mixture of both. It can also be made from cow milk. Starter is not used. The cheese may be eaten fresh or after storage in a cool store. If it is stored at below 12°C it will keep for several months. After salting the cheese pieces may also be stored in plastic bags without brining; if stored at about 10°C the cheese has a shelf-life of two to three months.

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About one kilogram of cheese will be obtained from nine liters of milk. The production and energy-nutritional values (100 g) of Halloumi cheese are presented as follows; the production of hallumi cheese was Milk, Coagulation, Processing of the Curd, Taking the Nor Cheese, Cooking, Salting and Folding, Packaging. Energy and nutritional values (100 g) of halloumi cheese were Energy: 352.6 kcal, Protein: 26 g, Calcium: 700 mg, Phosphorus: 590 mg, Carbohydrates: 1.4 g, Fat: 27 g [1].

Aflatoxins are carcinogenic compounds produced predominantly by certain strains of the Aspergillus genus. They are both acutely and chronically toxic, mutagenic, teratogenic and carcinogenic compounds for animal and human Contamination of milk and dairy products to aflatoxin M_1 is a risk for human health. Afloatoxin M_1 (AFM_1) is relatively stable during milk pasteurization and storage as well as during the preparation of various dairy products. Aflatoxin M_1 is the principle hydroxylated metabolite of aflatoxin B_1 which is transformed at the hepatic level by means of cytochrome p450 enzymes and excreted into the milk in the mammary glands of both human and lactating animal after ingestion by the animal of pellets and forage contaminated with aflatoxin B_1 [2-5].

Of the 550.000-600.000 new hepatocellular carcinoma (HCC) cases worldwide each year, about 25.200-155.000 may be attributable to aflatoxin exposure. Most cases occur in sub-Saharan Africa, Southeast Asia, and China where populations suffer from both high HBV prevalence and largely uncontrolled aflatoxin exposure in food. Liver cancer, is the third leading cause of cancer deaths worldwide, with prevalence 16-32 times higher in developing countries than in developed countries. Aflatoxin may play a causative role in 4.6-28.2% of all global HCC cases [6-8].

When cheese-making is carried out using AFM_1 contaminated milk, this toxin is likely to have become enriched in the final curd compared to that found in milk. This could be explained by both the capacity of AFM_1 to somehow bind caseins and increased dry matter content. The affinity of AFM_1 has been tested not only with these proteins, but also with other different ones present in whey as a larger amount of this toxin has been demonstrated to be present in the retentive where the protein-rich fraction appears. Therefore it is necessary to note whether AFM_1 is present in final products like cheese because its concentration in them has been reported to be around 2.1-4.5 times higher than in the original milk used, depending on the cheese type [9,10].

The measured AFM_1 concentration was correlated to four factors which were presumed to influence the contamination level: manufacturing, production season, milking animal, and maturation. Statistical analyses demonstrated that milking animals and manufacturing affect AFM_1 concentrations, as cheeses obtained from cows' milk and from artisanal production are more contaminated than cheeses produced with milk belonging to other animals and in industrial contexts [11].

Markets in developing countries generally do not reward reduced aflatoxins in crops because it is difficult to discern aflatoxin contamination or its risks. The presence of mold is a potential, but highly imperfect, indicator of aflatoxin contamination. Surveys in a few African countries show that farmer knowledge and awareness are far from perfect, as are storage and drying practices. While some moldy grain is diverted to uses that somewhat reduce direct human exposure (such as for brewing and animal feeds), quality differentiation based on either market rewards or public standards is still unusual in most developing countries [12].

Vacuum packaging is sometimes used to inhibit fungal growth on cheese, but some fungal species are able to grow under these condition. It has been isolated that several fungal species from vacuum packaged cheeses, the most commonly occurring being species of Cladosporium, Penicillium and Phoma [13,14].

The European Commission (EC) has approved a maximum admissible level of 250 ng/kg for AFM_1 in cheese [15]. However, the Turkish Food Codex (TFC), has accepted 500 ng/kg as the action level for AFM_1 [16].

Although there are some literature published about the occurrence of AFM_1 in various cheese like feta, Parmesan, Manchego, Kahramanmaraş, white, kashar, cream, civil and cheeses produced by dairy ewe's milk [17-24], there is not any information about the occurrence of AFM_1 in Halloumi is a firm pickled cheese with its origins in Cyprus.

For this purpose, the current study was designed to determine the presence and levels of AFM_1 in halloumi cheese that especially consumed fresh or after storage in a cool store in Cyprus province and to suggestion how could it protect from aflatoxin. Also the levels of AFM_1 found for halloumi cheese will compare the results with the legal regulations for AFM_1 legislated by EC and Turkish Food Codex (TFC).

MATERIAL and METHODS

Collection of Samples

A total of 128 samples of TRNC traditional cheese (halloumi) were collected during November and December 2013 from main districts of TRNC. The samples of halloumi were collected from dairy farmhouses and retail markets.

Cluster Sampling Method was used in selection the dairy farmhouses and retail markets. Cyprus was divided into four main clusters. Each cluster was selected to be heterogeneous as possible. Farmhouses and retail markets were selected from each cluster by using Random

Number Tables with Random Sampling Method. Thus, the randomization for sampling was completed carefully.

The size of halloumi samples were at least 200 g. The samples were preserved in their original packages or plastic bags, and during collection and transportation, samples were kept in an icebox. The samples were immediately transported to the laboratory in a cooler with ice packs and stored at -20°C until analysis. All samples were analyzed before their expiry dates. Laboratory studies of research were completed in Nutrition and Dietetics Laboratories in Eastern Mediterranean University (EMU).

Method of Analysis

The quantitative analysis of AFM_1 was performed using enzyme immunoassay: Ridascreen aflatoxin M kit (R-Bipharm AG, Germany).

The test is based on the antigen-antibody reaction. The assay was performed according to the manufacturer's recommendation and as described elsewhere [25]. The mean lower detection limit of the assay was 5 ng/l.

Extraction and ELISA Analysis

The analysis of AFM_1 in halloumi samples were performed according to the R-Biopharm Aflatoxin M_1 test kit's instructions. Determination of AFM_1 in the cyprus traditional cheese was determined using Aflatoxin M_1 Test Kit (R-Biopharm AG, Darmstadt, Germany), which is competitive enzyme immunoassay kit. One hundred micro liter of standart solutions and prepared samples were added into separate microtitre wells and incubated for 60 min at room temperature in the dark. The liquid was then poured out and the wells washed with washing buffer (250 µl) twice. In the following stage, 100 µl of the diluted enzyme conjugate was added to the wells and incubated for 60 min at room temperature in the dark. Again, the wells were washed 3 times with washing buffer. Afterwards, 50 µl of substrate and 50 µl chromogen was added, mixed gently and incubated in the dark at room temperature for 30 min. Finally, 100 µl of the stop reagent was added into the wells and absorbance was measured at 450 nm in ELISA plate reader.

Statistics

All continuous variables were presented as mean ± standard error of mean (SEM), 95% Confidence Interval (95% CI) and all categorical variables were presented as number of patients and percentages. Significant differencies between the mean values for two kind of Halloumi cheese (industrial and home made) were analyzed by using Student's t test for two independent groups. Student's t test for one population mean was also used to test the both means of AFM_1 found for industrial and home made with the mean values of the European Commission (EC) and Turkish Food Codex (TFC).

A power analysis using "Proportion Difference Power/ Sample Size Calculation" software was conducted to calculate sample size. Using previously published data for AFM_1, proportion difference of 27%, with the power of the test set to 0.85, and significance level at 0.05 resulted in sample size 128.

For all statistical analyses a P value ≤ 0.05 was considered statistically significant. The SPSS 15.0 statistical package was used to perform all statistical analyses (SPSS Inc., Chicago, IL, USA).

RESULTS

In the current study a total of 128 industrial made halloumi cheese samples including 36 industrial made and 92 home made were analysed for AFM_1 with the competitive ELISA.

The occurrence and the distribution of AFM_1 concentration in various ranges in cheese samples for industrial and home made are presented in *Table 1* and *Table 2* respectively.

The mean, standart error of mean (SEM), 95% Confidence Interval (95%CI) values of aflatoxin M_1 in halloumi cheese with industrial and home made samples were presented by *Table 3*. *Table 3* also contents the results of Student's t test and significant value.

Table 1. *Distribution of aflatoxin M_1 contents in various range in halloumi cheese with industrial made samples*
Tablo 1. *Endüstri yapımı hellim peyniri örneklerinde aflatoksin M_1 dağılımı*

Samples	AFM1 (ng/kg)	Percent (%)	Total Percent (%)	Min-Max (ng/kg)
26	0.00 (None)	72.2(None)	72.2(None)	0.00-4.63
8	1.00-3.90	22.2	28.8	
2	4.00-6.90	6.6		
36 (Total)		100.0	100.0	

Table 2. *Distribution of aflatoxin M_1 contents in various range in halloumi cheese with home made samples*
Tablo 2. *Ev yapımı hellim peyniri örneklerinde aflatoksin M_1 dağılımı*

Samples	AFM_1 (ng/kg)	Percent (%)	Total Percent (%)	Min-Max (ng/kg)
72	0.00 (None)	78.3(None)	78.3(None)	0.00-16.66
8	1.00-3.90	8.7	21.7	
6	4.00-6.90	6.4		
3	7.00-9.90	3.3		
2	10.00-12.90	2.2		
0	13.00-15.90	0.0		
1	16.00-18.90	1.1		
92 (Total)		100.0		

Table 3. *Descriptive statistics and test results for industrial and home made of halloumi cheese*
Tablo 3. *Endüstriyel ve ev yapımı hellim peyniri için tanımlayıcı istatistikler ve test sonuçları*

Halloumi	n	X AFM_1 (ng/kg)	SEM	95%CI	t	p
Industrial made	36	0.84	0.24	0.35-1.35	0.81	0.422
Home made	92	1.28	0.32	0.63-1.93		

x: *Mean,* ***SEM:*** *Standard Error of Mean; 95%CI: 95% Confidence Interval,* ***t:*** *Student's t test was analyzed two different means*

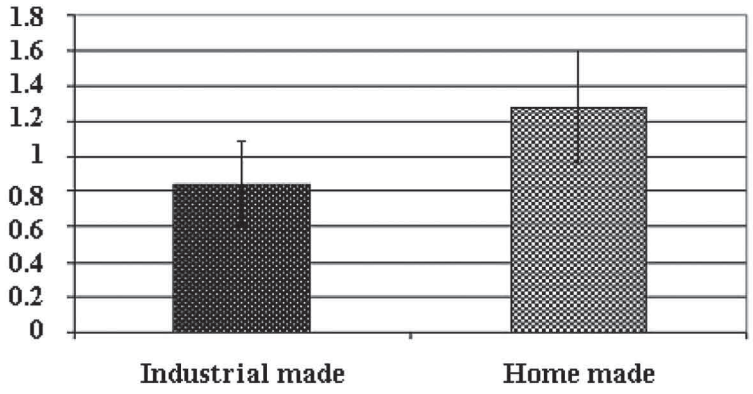

Fig 1. The distribution of mean and SEM values of industrial and home made halloumi cheese

Şekil 1. Endüstriyel ve ev yapımı hellim peynirinin ortalama ve standart hata dağılımı

According to the results of *Table 3*, there was not found significant differencies between the mean values for two kind of Halloumi (P=0.422).

Both means of AFM_1 found for industrial (0.84 ng/kg) and home made (1.28 ng/kg) were analyzed with the mean values of the European Commission (EC) (250 ng/kg) and Turkish Food Codex (TFC) (500 ng/kg) by using Student's t test for one population mean. The results were found significantly difference (P<0.001).

Fig. 1 represents the distribution of mean and SEM values in two kind of Halloumi cheese.

DISCUSSION

The percent of AFM contamination in halloumi cheese was found to be low, since 28.8% (0-4.63 ng/kg) of the samples were positive in industrial made and 21.7% (0-4.63 ng/kg) in home made. Both persentages were not significantly difference and found very low from the limits of European Commission (EC) (250 ng/kg) [15] and Turkish Food Codex (TFC) (500 ng/kg) [16].

Filazi et al.[24] reported that AFM_1 in cheeses may be hazardous to human, particularly children. For this reason, there are many studies concerning the presence of AFM_1 in dairy products [26-28]. The presence of AFM_1 was detected in concentrations between 20-2000 ng/kg in 14 of 50 samples (28%). Altogether, 5 cheese samples (10%) were found to have levels that exceed the legal limits of 250 ng/kg established by the Turkish Food Codex. It was therefore concluded that, widespread occurrence of AFM_1 in ewe's milk cheese samples produced in Urfa city were considered to be possible hazards for human health.

Atasever et al.[22] have examined in terms of AFM_1 in 304 cheese samples 85 white cheese, 75 kashar cheese, 62 civil cheese, 82 cream cheese) put up for sale in various places in Erzurum. The AFM_1 ontent and concentrations of the samples were researched by competitive ELISA method. Determinable limit was 50 ng/kg and it was determined that white cheese samples included 82.4% AFM_1, kashar cheese samples 80%, civil cheese samples 19.4% and cream cheese samples 84.2%. According to European Commission limit (250 ng/kg), the sample incidence exceeding the acceptable limits were 27.1%, 34.7%, 17.1% in white cheese, kashar cheese and cream cheese samples, respectively. The sample ratio exceeding the limits regulated by Turkish Food Codex (500 ng/kg) was determined in white cheese, kashar cheese and cream cheese samples as 16.5% (14/85), 14.7% (11/75) and 6.1% (5/82) respectively, any sample exceeding these limits was not met in civil cheese samples. As understood from these results, high AFM_1 level determined in some cheese types is an important problem threatening the public health in Turkey.

In the study of Turgay et al.[20] have also selected 46 cheese samples that were obtained from various markets located in Kahramanmaraş. In all, 22 of the 46 samples were made from bovine milk, 6 were made from ovine milk, and 18 were made from goat milk. None of the ovine milk cheese samples contained AFM_1. AFM_1 was present in 32 samples (69.6%) of bovine and goat cheese. Bovine milk cheese and goat milk cheese samples contained 0.069-1.2 ng g^{-1} and 0.06-0.22 ng g^{-1} of AFM_1, respectively. With the exception of 2 bovine milk cheese samples (one contained 1.2 ng g-1 of AFM_1, the other contained 0.25 ng g of AFM_1), the other samples (96%) had levels of AFM_1 below the acceptable limit for cheese (0.25 ppb)

set forth by the Turkish Alimentarus Codex.

Tekinsen and Tekinsen [29] reported in 60 samples of Van otlu (herb) and 50 white pickle cheese samples obtained from retail outlets in Van and Hakkari, Turkey. The rate of AFM_1 in Van otlu and white pickle cheese samples ranged from 0.16 to 7.26 µg kg and from 0.10 to 5.20 µg kg^{-1} respectively. In all, 80% of Van otlu cheese and 40% of white pickle cheese samples exceeded the maximum acceptable level. However in the study of Kivanc [30] the absence of AFM_1 was found at detectable levels in Van otlu and white cheese samples in Van, Turkey.

The result of studies for AFM_1 were examined for the cheeses produced outside of Turkey were presented as follows. In the results of Rubio et al.[10], Aflatoxin M_1 distribution in curd, whey, Manchego cheese, the traditional Spanish whey cheese Requesón and Requesón whey, and its stability during two different cold treatments, have been studied. At the end of study the contamination AFM_1 in Manchego cheese was found at the EU limit level (50 ng/kg).

In the study of Oliveira et al.[31], 24 samples of Minas Frescal cheese and 24 samples of Minas Padrao cheese produced in the North-east region of the state of Sao Paulo, Brazil, were analyzed for aflatoxin M_1. AFM_1 was detected in 13 (27.1%) samples at concentrations ranging from 0.037 to 0.313 ng g^{-1}. The mean concentrations of high incidence of AFM_1 in positive samples of Minas Frescal and Minas Padrao cheese were 0.142±0.118 and 0.118±0.054 ng g^{-1}, respectively. In another study, fresh cheese produced in Argentina from artificially contaminated milk with AFM_1 at levels of 1.7-2.0 ng mL^{-1} had 60% of AFM_1 in the whey and 40% in cheese [32].

The mean values of AFM_1 found in the present study in halloumi were found lower than the results of all cheeses reported above. This result is noteworthy for human exposure to this toxin.

Aflatoxicosis is primarily a hepatic disease. The susceptibility of individual animals to aflatoxins varies considerably depending on species, age, sex and nutrition. In fact, aflatoxins cause liver damage, decreased milk and egg production, recurrent infection as a result of immunity suppression, in addition to embryo toxicity in animals consuming low dietary concentrations. While the young of a species are most susceptible, all ages are affected but in different degrees for different species. Clinical signs of aflatoxicosis in animals include gastrointestinal dysfunction, reduced reproductivity, reduced feed utilization and efficiency, anemia and jaundice. Nursing animals may be affected as a result of the conversion of aflatoxin B_1 to the metabolite aflatoxin M excreted in milk of dairy cattle. Aflatoxin B_1, M_1, and G_1 have been shown to cause various types of cancer in different animal species. Aflatoxin develops in the field when grains are exposed to severe environmental conditions. Management practices that improve plant health strongly discourage aflatoxin development which timely planting, adequate fertility, good weed and insect control, supplemental irrigation, suitable plant population, and hybrid selection should help reduce aflatoxin potential [33].

Naturally, the diet of the cow has a major impact on its health. The type of grass, greatly affects the nutrition profile of the cow. The reasons of low level AFM_1 in Halloumi cheese, sheep and cows grazing on fields of luscious green grass in Cyprus. This is the main reason reduses the AFM_1 level in cheese.

In order to prevent and reduce the negative implications of these mycotoxins in cheese production, it is necessary to create both global and national strategies to reduce the amount of mycotoxins in grain. In order to prevent from introduction of aflatoxin M_1 into cheese industry cycle, hygienic conditions, appropriate storage and control of livestock feed at all stages of planting and requires system that makes aflatoxin control are necessary.

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RESEARCH ARTICLE

Contractile Effects of *Eryngium kotschyi* Boiss. on Rat Isolated Ileum and Detrussor Muscle [1]

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Summary

The pharmacological activity of the aerial (EKA) and root (EKR) parts of the endemic plant, *Eryngium kotschyi* Boiss., on rat isolated ileum and detrusor muscle was investigated. Plant extracts alone and with the presence of agonist (acethylcholine) and antagonist (atropin, verapamile, oxybutinine-detrusor muscle, and papaverine-ileum) drugs, along with Ca^{2+} applications on calcium-free medium, were applied. Plant extracts induced contraction in ileum and detrusor muscle where the contractions were concentration dependant for EKA and EKR single dose applications in detrusor muscle and concentration-free contractions were observed in cumulative applications for both tissues. Aerial and root parts of Eryngium extracts induced contractions in dose, tissue and protocol dependent manner where the contractions were affected by the tested antagonists, which could be attributed to non-specific pathways including calcium ions and calcium channel stimulations.

Keywords: *Eryngium kotschyi, Detrusor muscle, Bladder, Ileum, Motility, Rat*

Eryngium kotschyi Boiss.'in İzole Rat İleum ve İdrar Kesesi Düz Kasında Kastırıcı Etkisi

Özet

Bu çalışmada, ülkemizdeki endemik bitkilerden *Eryngium kotschyi* Boiss.'in toprak altı (EKTA) ve toprak üstü (EKTU) kısımlarının izole sıçan ileum ve idrar kesesi kasında farmakolojik etkinliği araştırıldı. Bitki ekstrelerinin dokulardaki etkinliği tek, agonist (asetilkolin) ve antagonist (atropin, verapamil, oksibutinin-idrar kesesi, papaverin-ileum) varlığında ve kalsiyumsuz ortamda Ca^{2+} uygulamaları ile birlikte değerlendirildi. Bitkinin her iki kısmı doku türü, ekstre dozu ve uygulama protokolüne bağlı değişiklik gösterecek şekilde kontraksiyon oluştururken; bu kasılmaların EKTU ve EKTA tek uygulamalarında doza bağımlı, kümülatif uygulamalarında ise dozdan bağımsız olduğu görüldü. Oluşan kasılmaların test edilen antagonistler ile değiştiği; dolayısıyla kontraktil etkinliğin kalsiyum iyonu ve kalsiyum kanallarının uyarılması gibi nonspesifik yolaklara özellikle bağlı olabileceği görüşüne varıldı.

Anahtar sözcükler: *Eryngium kotschyi, İdrar kesesi, İleum, Düz kas, Motilite, Sıçan*

INTRODUCTION

Medicinal plants and products thereof are doubtlessly of great medicinal and economic importance [1]. Since herbal medicines are considered to be safe and effective and regarded as free from undesirable side effects; many people turn to use instead of conventional drug therapy, globally [2].

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The genus *Eryngium*, belonging to the subfamily Saniculoidea of Apiaceae, are represented by 317 accepted taxa worldwide [3]. These species are named as "Boğadikeni" in Turkish folk medicine and are widely distributed around the country. *Eryngium kotschyi* named as "Çakır dikeni" is recorded as one of the ten endemic *Eryngium* taxa in Turkey [4]; however, ethnopharmacological use of *E. kotschyi* have not been reported yet. Detailed phytochemical investigation on *E. kotschyi,* resulted in isolation of four triterpenoid saponins [5]. Some Eryngium subspecies are used as vegetables or for sweetmeats in the Eastern Anatolia and assumed as cultural ornaments. On the other hand, infusions of the aerial and the root parts of some are used in folk medicine for its antitussive, diuretic, antiedema, appetizing, spasmolytic, stimulant, carminative and aphrodisiac effects and particularly used for urinary system disorders such as uremia and nephritis [6-10]. Related to the ethnopharmacological use of *Eryngium* species, especially as diuretic and spasmolytic [11,12], this study was based on investigating the potential effects of these plants on ileum and vesica urinaria *in vitro*. Therefore, the aim of this study was to investigate the pharmacodynamic effects of aerial and root parts of the promising endemic plant, *E. kotschyi,* on rat isolated ileal and detrusor muscle to gather *in vitro* data for further *in vivo* follow-up studies.

MATERIAL and METHODS

Preparation of Plant Extracts

E. kotschyi was collected during flowering time from Konya, Hadim-Beysehir Lake (1.500 m) in August 2009; authenticated by Prof. Dr. Hayri Duman (Gazi University Faculty of Arts and Sciences); given a voucher specimen and stored in Hayri Duman Herbarium (H. Duman - 9137). The root and the aerial parts of the plant were seperated (*E. kotschyi* aerial - EKA, *E. kotschyi* root - EKR) and dried in a cool-dark place and powdered using pulverizing mill. The powdered material was then extracted under reflux. The extract filtered and stored at -80°C [13]. The frozen extract was then lyophilized. The desired concentrations (w/v) were prepared from this extract for the analysis.

Animals

Thirty six male Wistar albino rats of 5 months weighing 250-350 g, were obtained from Ankara University, Animal Experimentation Unit (Ethical Approval: 2009-51-261). The animals were fasted overnight (had free access to water) and anesthetized with ether and sacrificed by decapitation.

Tissue Preparation

Detrusor smooth muscle (vesica urinaria) was removed and immersed in Krebs (in mM: NaCl 118, KCl 4.6, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 10, EDTA 0.025, pH 7.4) physiological solution; where as ileum was mounted in Tyrode (in mM: NaCl 136.89, KCl 2.68, $MgCl_2$ 1.05, $CaCl_2$ 1.80, NaH_2PO_4 0.42, $NaHCO_3$ 11.90, glucose 5.5, pH 7.4) physiological solution. Both tissues were cleaned from the surrounding connective tissue and cut into the strips of 1.5 cm length and then, gently suspended and isomerically connected to an isomeric force transducer (MAY-COM FDT 10-A, Commat Iletisim Ltd. Ankara, Turkey) on 10 ml organ baths filled with physiological solution. The baths were aerated with 95% O_2, 5% CO_2 at 37°C under a resting tension of 1.000 mg and allowed to equilibrate for 60 min in physiological solution [14]. The measurement of isometric force was continuously displayed and recorded on-line on a personal computer via a data acquisition system (TDA 94, Commat Iletisim Ltd.) using a software (Polywin 95, Ver 1.0; Commat Iletisim Ltd., Turkey) which also had the capacity to analyze the data.

Drugs

Lyophilized aqueous extracts of both the aerial and root parts of *E. kotschyi* were dissolved in water at the concentration of 150 mg/ml, and further dilutions were done accordingly and added to 10 ml Krebs-Tyrode containing organ bath as 100 µl where the concentrations were calculated as 100 folds. Papaverine hydrochloride, oxybutynin chloride, atropine sulfate, verapamil hydrochloride, and acetylcholine (ACh) (all chemicals were purchased from Sigma, St. Louis, MO, USA) were dissolved in water (Milli-Q double distilled) as 100 mM stock solutions and further diluted as required. Calcium chloride (Sigma-Aldrich, USA) were dissolved and diluted in Tyrode solution for ileal and Krebs solution for detrusor muscle applications.

Experimental Design

After a stabilization period of 60 min, contractile responses were recorded by stimulating the tissues with the EC_{50} value of ACh (10^{-6} M). All tissues were then given a 30 min equilibration period during which they were washed and the resting tension was adjusted in every 15 min.

***Single and cumulative dose administration of EKA and EKR to detrusor muscle and ileum*:** EKA was studied at 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 75, 100, 150 mg/ml single doses in detrusor muscle. The dose response curve was then assessed by 12.5, 25, 50, 75, 100, 150 mg/ml single doses where the response was found to be more accurate. Cumulative applications of EKA were carried out by 3.125, 6.25, 12.5, 25, 50, 75, 100, 150 mg/ml. EKR were studied at 25, 50, 75, 100 mg/ml for single doses and 12.5, 18.75, 25, 37.5 mg/ml for cumulative doses. EKA was studied at 0.078, 0.156, 0.3125, 0.78, 1.56 mg/ml single doses in ileum. The dose response curve was then assessed by 0.156, 0.3125, 0.78, 1.56 mg/ml single doses where the response was found to be more accurate. Cumulative applications of EKA were carried out by 0.078, 0.156, 0.3125, 0.78, 1.56, 3.125, 6.25 mg/ml. EKR were studied at 25, 50, 75, 100 mg/ml for single doses and 12.5, 18.75, 25, 37.5 mg/ml for cumulative doses.

***Cumulative and single dose of ACh administration to detrusor muscle and ileum after single dose incubation of EKA and EKR*:** After 10 min incubation of the muscle strips by the working doses close to EC_{50} for EKA (75 mg/ml) and EKR (50 mg/ml) ACh in cumulative doses (0.5 log folds of 10^{-8}-10^{-3} M) were applied. Same protocol was repeated by single dose application of ACh (10^{-6} M). After 10 min incubation of the muscle strips by the working doses close to EC_{50} for EKA (2.343 mg/ml) and the working dose close to E_{max} value (50 mg/ml) for EKR, ACh in cumulative doses (0.5 log folds of 10^{-8}-10^{-3} M) were applied. Same protocol was repeated by single dose application of ACh (10^{-6} M).

***Single dose of EKA and ACh administration to detrusor muscle and ileum followed by single dose of atropine, verapamil, oxybutynin, and papaverine incubation*:** EKA single dose (75 mg/ml) and ACh single dose (10^{-6} M) were administred to detrusor muscle and the results were recorded. After washing and equilibration, EKA single dose (75 mg/ml) and ACh single dose (10^{-6} M) were administered followed by 10 min incubation of antagonist drugs atropine (10^{-6} M) [15], verapamil (10^{-7} M) [15], and oxybutynin (10^{-8} M) [16]. EKA single dose (2.343 mg/ml) and ACh single dose (10^{-6} M) were administred to ileum and the results were recorded. After washing and equilibration, EKA single dose (2.343 mg/ml) and ACh single dose (10^{-6} M) were administered followed by 10 min incubation of antagonist drugs atropine (10^{-6} M) (7), verapamil (10^{-7} M) and papaverine (10^{-6} M) [17].

***Single dose of EKR and ACh administration to detrusor muscle and ileum followed by single dose of atropine, verapamil, oxybutynin, and papaverine incubation*:** EKR working dose (50 mg/ml) and ACh single dose (10^{-6} M) were administred to detrusor muscle and the results were recorded. After washing and equilibration, EKR single dose (50 mg/ml) and ACh single dose (10^{-6} M) were administered followed by 10 min incubation of antagonist drugs atropine, verapamil, and oxybutynin. EKR working dose (50 mg/ml) and ACh single dose (10^{-6} M) were administred to ileum and the results were recorded. After washing and equilibration, EKR single dose (50 mg/ml) and ACh single dose (10^{-6} M) were administered followed by 10 min incubation of antagonist drugs atropine, verapamil and papaverine.

***$CaCl_2$ administration, followed by the single dose incubation of EKA and EKR with or without the presence of verapamil to the detrusor muscle and ileum*:** Responses of the detrusor muscle to 1 mM $CaCl_2$ were recorded in calcium-free Krebs solution. After washing and equilibration, working doses of EKA (75 mg/ml), EKR (50 mg/ml) with and without the presence of verapamil (10^{-7} M) were administered, results were recorded. Following washing and equilibration, 1 mM $CaCl_2$ were applied after incubation with the plant extracts for 10 min and the responses were recorded. Responses of the ileum to 1 mM $CaCl_2$ were recorded in calcium-free Tyrode solution. After washing and equilibration, working doses of EKA (2.343 mg/ml), EKR (50 mg/ml) with and without the presence of verapamil (10^{-7} M) were administered, results were recorded. Following washing and equilibration, 1 mM $CaCl_2$ were applied after incubation with the plant extracts for 10 min and the responses were recorded.

Statistical Analysis

The results were presented as the mean±SEM of *n* observations. The contraction responses were expressed as apparent affinity constant (pD_2) and percentage of the corresponding maximal responses to the plant extract were calculated as a percentage of the maximal response to Ach (E_{max}). pD2 value was given by the negative logarithm of the molar agonist concentration that produces 50% of the maximal response produced by ACh (pD_2 = –logEC50). Values were analysed using Student's *t*-test or Mann Whitney U test, as appropriate after checking normality with Shapiro-Wilk and homogeneity of variances with Levene test as parametric test assumptions. Minimum of 5% of significance were considered to differ significantly. pD_2, EC_{50}, E_{max} values were calculated by interpolation from semilogarithmic plots by GraphPad Prism® and all statistical analysis were calculated using SPSS® 14.01 for Windows.

RESULTS

***Single and cumulative dose administration of EKA and EKR to detrusor muscle*:** pD_2 values for single dose and cumulative dose applications of EKA were calculated as 1.283±0.42 and 1.276±0.26, with the EC_{50} values of 52.13 and 53.02. E_{max} values for single dose applications were found at the highest dose (150 mg/ml) as 91.35±8.50 and for cumulative applications as 55.38±10.89 since downregulation were recorded at the highest dose for EKA *(Fig. 1)*. 75 mg/ml for EKA was decided as the working dose for detrusor muscle tissue giving precise results for both single and cumulative dose applications in accordance with the calculated EC_{50}. Dose dependant contractile response was observed by single dose administration of EKR in 25, 50, 75, 100 mg/ml single doses where EC_{50}, E_{max} and pD_2 were calculated as 48.81, 89.45±5.86 and 1.31±0.68, respectively. Highest tension was recorded at 150 mg/ml dose application as 1748±133.03 mg. Further protocols were carried out by 50 mg/ml close to the calculated EC_{50} (48.81) *(Fig. 2)*.

Single and cumulative dose administration of EKA and EKR to ileum: Single dose applications of EKA to ileum induced slight contractility in the muscle; however this contractility was not found dose-dependant. Highest tension was recorded at 0.078 mg/ml dose application as 1591±79.94 mg. In cumulative dose applications, EKA induced tension at lower doses; however this response was not dose-dependant where the responses decreased after 6.25 mg/ml. Single dose applications of EKR to ileum induced contractility in the muscle free from the

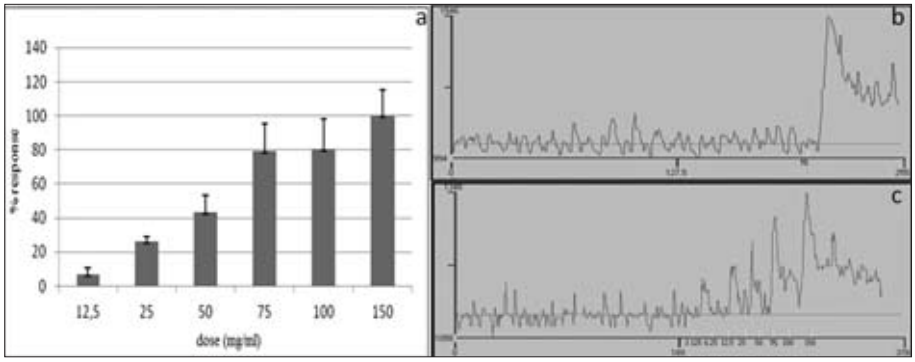

Fig 1. Single **(a,b)** and cumulative **(c)** dose responses of *Eryngium kotschyi* aerial in detrusor muscle

Şekil 1. İdrar kesesinde *Eryngium kotschyi* toprak üstü tek **(a,b)** ve kümülatif doz **(c)** cevapları

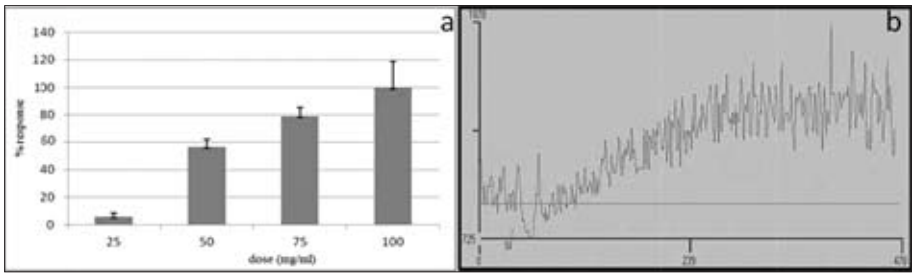

Fig 2. Single dose response of *Eryngium kotschyi* root in detrusor muscle

Şekil 2. İdrar kesesinde *Eryngium kotschyi* toprak altı tek doz cevapları

dose (except 25 and 50 mg/ml) giving long duration of contractility (≈7 min). Highest tension was recorded at 50 mg/ml dose application as 2315±110.93 mg where the working dose was chosen accordingly. Noncumulative contractility was observed until 75 mg/ml with the down-regulation at further doses.

Cumulative and single dose of ACh administration to detrusor muscle and ileum after single dose incubation of EKA and EKR: Isolated detrusor showed 30.14% contractile activity with the incubation of EKA (75 mg/ml) alone compared to single dose ACh (10^{-6} M) contraction alone. By the addition of ACh (10^{-6} M) over the EKA incubated bath, contractile responses increased to 144.78% (P<0.05). Similar results were obtained by EKR incubation as 29.85% EKR alone and 154.12% by ACh addition *(Table 1)*. Dose-response relation was not observed in cumulative applications. Isolated ileum showed 14.29% contractile activity with the incubation of EKA (2.343 mg/ml) alone compared to single dose ACh (10^{-6} M) contraction alone. By the addition of ACh (10^{-6} M) over the EKA incubated bath, contractile responses increased to 105.24% (P<0.05). EKR incubation (50 mg/ml) induced as 20.95% contraction alone and 77.14% by ACh addition *(Table 1)*. Dose-response relation was not observed in cumulative applications.

Single dose of EKA and ACh administration to detrusor muscle and ileum followed by single dose atropine, verapamil, oxybutynin, and papaverine incubation: Induced contractility in detrusor tissue by the incubation of EKA (75 mg/ml) alone according to ACh (10^{-6} M) contraction alone as 24.07% was decreased by the presence of atropine, verapamil, oxybutynin as 9.10%, 7.01%, 10.63%, respectively. No significant difference between different antagonists by the presence of the plant extract. ACh

Table 1. *Single dose responses of acethylcholine (ACh; 10^{-6} M) over Eryngium kotschyi aerial (EKA; 75 mg/ml for detrusor muscle and 2.343 mg/ml for ileum) and Eryngium kotschyi root (EKR; 50 mg/ml) incubation*

Tablo 1. *İdrar kesesinin ve ileumun Eryngium kotschyi toprak üstü (2.343 mg/ml) ve Eryngium kotschyi toprak altı (50 mg/ml) tek doz uygulaması üzerine tek doz asetilkolin (ACh; 10^{-6} M) cevapları*

Application	Response (%)			
	Detrusor muscle	P	Ileum	P
ACh (n: 4)	100±24.08		100±27.34	
EKA (n: 4) EKA + ACh (n: 4)	30.14±3.86[a] 144.78±23.52[b]	*	14.29±4.27[a] 105.24±32.86[b]	*
EKR (n: 3) EKR + ACh (n: 4)	29.85±3.16[a] 154.12±25.81[b]	*	20.95±5.44[a] 77.14±24.85[b]	*

*Different superscript letters ([a,b]) in a column for EKA and EKR groups, shows statistically significant difference represented by * for P<0.05*

(10^{-6} M) response to the same antagonists were found as 6.42%, 37.45%, 17.83%, respectively *(Table 2)*. EKA was found to be affected by the antagonists as ACh. Contractile responses (9.01%) of EKA in ileum were decreased by the presence of atropine, verapamil and papaverine (2.96, 3.38, and 3.18%, respectively). Whereas ACh administration with the presence of the antagonist drugs are found as 5.14%, 51.52%, and 77.39%, respectively where significant difference was observed compared to the EKA applications (P<0.05) *(Table 2)*.

Single dose of EKR and ACh administration to detrusor muscle and ileum followed by single dose atropine, verapamil, oxybutynin, and papaverine incubation: Contractile responses of EKR (29.37%) in detrusor muscle was decreased by the presence of atropine, verapamil, and oxybutynine (13.79%, 2.86%, and 6.57%, respectively). Verapamil response was found significantly different than

the other antagonist drugs for EKR applications (P<0.05). ACh responses with the presence of the antagonist drugs are found as 6.48%, 30.84%, and 12.22%, respectively where significant difference was observed compared to the EKR applications (P<0.05) *(Table 3)*. Contractile responses of EKR (33.54%) in ileum was decreased by the presence of atropine, verapamil and papaverine (19.27%, 14.11%, and 10.18%, respectively) without significant difference in between (P>0.05). ACh responses for the same antagonists were recorded as follows, 6.55%, 52.44%, and 83.97%, respectively with a significant difference between the EKR extracts by the contraction (P<0.05). Atropine, decreased the contractile responses of both ACh and EKR in ileum, whereas the results were found significant in ACh (P<0.05). On the other hand, EKR was more effected by papaverine and verapamil compared to ACh group *(Table 3)*.

$CaCl_2$ administration, followed by the single dose incubation of EKA and EKR with or without the presence of verapamil to the detrusor muscle and ileum: 1 mM $CaCl_2$ induced 24.29 mg tension difference in calcium-free Krebs solution; whereas EKA (75 mg/ml) induced 5.00±1.58 and EKR (50 mg/ml) induced 9.17±3.96 tension difference in detrusor muscle. After single dose incubation of EKA (75 mg/ml), EKR (50 mg/ml) and verapamil (10^{-7} M) 1 mM $CaCl_2$ found to increse the responses of the plant extract as potentialization whereas it was not able to break the antagonist effect of verapamile *(Table 4)*. 1 mM $CaCl_2$ induced 50.22 mg tension difference in calcium-free Tyrode solution. After single dose incubation of EKA (2.343 mg/ml), EKR (50 mg/ml) and verapamil (10^{-7} M); 1 mM $CaCl_2$ found to increse the responses of the plant extract as potentialization whereas it was not able to break the antagonist effect of verapamile *(Table 4)*.

DISCUSSION

For many of the medicinal plants of current interest, a primary focus of research to date has been in the areas

Table 2. *Eryngium kotschyi aerial (EKA; 75 mg/ml for detrusor muscle and 2.343 mg/ml for ileum) and acethylcholine (ACh; 10^{-6} M) responses with atropine (10^{-6} M), verapamil (10^{-7} M), oxybutynin (10^{-8} M), and papaverine (10^{-6} M)*

Tablo 2. *İdrar kesesinin ve ileumun Eryngium kotschyi toprak üstü (idrar kesesi için 75 mg/ml, ileum için 2.343 mg/ml) tek doz uygulaması üzerine tek doz asetilkolin (ACh; 10^{-6} M), atropin (10^{-6} M), verapamil (10^{-7} M), oksibutinin (10^{-8} M) ve papaverin (10^{-6} M) cevapları*

Application	Response (%)			
	Detrusor muscle	P	Ileum	P
ACh (n: 5)	100±15.70		100±5.64	
EKA (n: 5)	24.07±3.93		9.01±1.59	
Atropine + EKA (n: 6) Atropine + ACh (n: 5)	9.10±4.46 [a] 6.42±2.51 [b]	*	2.96±0.88 [a] 5.14±1.98 [b]	*
Verapamil + EKA (n: 5) Verapamil+ ACh (n: 5)	7.01±2.01 [a] 37.4±6.74 [b]	*	3.38±2.18 [a] 51.52±5.99 [b]	*
Oxybutynin + EKA (n: 4) Oxybutynin + ACh (n: 4)	10.63±1.04 [a] 17.83±6.97 [b]	*	- -	
Papaverine + EKA (n: 5) Papaverine + ACh (n: 7)	- -		3.18±1.99 [a] 77.39±6.63 [b]	**

*Different superscript letters ([a,b]) in a column, shows statistically significant difference represented by * for P<0.05 and ** for P<0.01*

Table 3. *Eryngium kotschyi root (EKR; 50 mg/ml) and acethylcholine (ACh; 10^{-6} M) responses with atropine (10^{-6} M), verapamil (10^{-7} M), oxybutynin (10^{-8} M), and papaverine (10^{-6} M)*

Tablo 3. *İdrar kesesinin ve ileumun Eryngium kotschyi toprak altı (idrar kesesi ve ileum için 50 mg/ml) tek doz uygulaması üzerine tek doz asetilkolin (ACh; 10^{-6} M), atropin (10^{-6} M), verapamil (10^{-7} M), oksibutinin (10^{-8} M) ve papaverin (10^{-6} M) cevapları*

Application	Response (%)			
	Detrusor muscle	P	Ileum	P
Ach (n: 6)	100±7.87		100±7.76	
EKR (n: 4)	29.37±2.89		33.54±6.73	
Atropine + EKR (n: 4) Atropine + ACh (n: 6)	13.79±4.91 [a] 6.48±3.12 [b]	*	19.27±3.97 [a] 6.55±3.69 [b]	*
Verapamil + EKR (n: 6) Verapamil+ ACh (n: 5)	2.86±1.43 [a] 30.84±3.99 [b]	*	14.11±3.11 [a] 52.44±7.18 [b]	*
Oxybutynin + EKR (n: 6) Oxybutynin + ACh (n: 4)	6.57±2.08 [a] 12.22±4.78 [b]	*	-	
Papaverine + EKR (n: 4) Papaverine + ACh (n: 5)	-		10.18±2.04 [a] 83.97±10.50 [b]	*

*Different superscript letters ([a,b]) in a column, shows statistically significant difference represented by * for P<0.05*

Table 4. *Responses of Eryngium kotschyi aerial (EKA; 75 mg/ml for detrusor muscle and 2.343 mg/ml for ileum), Eryngium kotschyi root (EKR; 50 mg/ml) and verapamil (10^{-7} M) incubation and coapplications with 1 mM $CaCl_2$*

Tablo 4. *Eryngium kotschyi toprak üstü (idrar kesesi için 75 mg/ml ve ileum için 2.343 mg/ml) ve Eryngium kotschyi toprak altı (idrar kesesi ve ileum için 50 mg/ml) ve veya verapamil (10^{-7} M) ile inkübasyonu takiben 1mM $CaCl_2$ cevapları*

Application	Tension Difference (mg)			
	Detrusor muscle	P	Ileum	P
EKA (n: 7)	5.00±1.58		-77.83±43.49	
EKR (n: 7)	9.17±3.96		171.67±33.30	
$CaCl_2$ (n: 7) EKA + verapamil + $CaCl_2$ (n: 6) EKR + verapamil + $CaCl_2$ (n: 7)	24.29±5.17 [a] 32.50±13.15 [a] 135.71±26.89 [b]	* * *	50.22±11.27 [a] 165.00±28.36 [b] 285.00±71.20 [c]	* * *

*Different superscript letters ([a,b,c]) in a column, shows statistically significant difference represented by * for P<0.05*

of phytochemistry, pharmacognosy, and horticulture. In the area of phytochemistry, medicinal plants have been characterized for their possible bioactive compounds, which have been separated and subjected to detailed structural analysis. Since phytomedicines exert their beneficial effects through the interaction of multiple chemical compounds at the same time through single or multiple target sites; the pharmacological effect could not be attributed to the main active component but the synergistic action of several compounds [18]. Phytochemical studies on *Eryngium* species revealed that this genus contains mainly the phenolic compounds and terpenoids including triterpenoid saponins, monoterpene, sesquiterpenes, triterpenoids, flavonoids, coumarins, steroids, acetylenes and other compounds [11]. Antispasmodic activity of triterpenoid saponin "zygophylosides" from *Zygophylum gaetulum* [19] and "ginseng saponins" from *Panax ginseng* [20] on isolated guinea pig ileum were reported previously. On the contrary, for the current study, since contraction responses were recorded in ileal and detrusor muscle strips; it could be suggested that, other compounds in the extract might have masked the triterpenoid effect where antispasmodic activity was expected or the extract acts through a cholinergic mechanism with the role of calcium ions which was confirmed by the responses by the antagonists in both tissues. In several *in vitro* studies, the importance of the muscarinic receptor systems on the mechanism of action of plant extracts with the influence of Ca^{2+} were discussed in details. For instance, Hu et al.[21] studied M3 muscarinic receptor- and Ca^{2+} influx-mediated muscle contractions induced by croton oil in isolated rabbit jejenum. In the study of Elorriaga et al.[22] muscarinic receptor-induced phasic contractions in the rat ileum were depended on the release of internal Ca^{2+} entry from the extracellular space through voltage-dependent Ca^{2+} channels. Parry et al.[23] investigated papaverine-like relaxant effects of the aqueous extract of the root bark of *Heteromorpha trifoliata* on gastrointestinal smooth muscle strips where the mechanism of action were related to the prevention of the Ca^{2+} influx into the smooth muscle cells, inhibiton of the calcium-induced Ca^{2+} release mechanism, prevention of the release of calcium from the sarcoplasmic reticulum, or prevention of the binding of calcium to calmodulin [24]. Therefore, Ca^{2+} has a critical role on the effects of the muscarinic receptor antagonists such as papaverine and oxybutynine. In the current study, atropine, papaverine and verapamil induced inhibitory effect over plant extract contraction. Among these antagonists, verapamil induced inhibition by both extract coadministration contractions were found to be more preponderant compared to ACh induced contractions. To sum up, the contractile responses of EKR and EKA could be related to receptor mediation and by voltage dependant (L type) calcium channels where the contractility were found to be affected at various levels by tested antagonists revealing the importance of the Ca^{2+} for the mechanism of action.

Inhibitory responses of 1 mM $CaCl_2$ administration, followed by the single dose incubation of the plant extracts and verapamil to both tissues in calcium-free media with respect to ACh, showed that Ca^{2+} had a nonspecific role in the contractility by *Eryngium* extracts. The decrease of contractility by plant extracts in calcium-free media compared to calcium included media; increase of the contractility by addition of 1 mM $CaCl_2$ in calcium-free media and the responses of the plant extracts by ACh compared to verapamil incubation; strengthens the theory of Ca^{2+} mediated pathways for the contractile responses of the extracts [25].

In conclusion, aerial and root parts of *E. kotschyi* induced contractility on tissue-dose dependent manner where the contractions were affected by the tested antagonists. The mechanism of action could be related to non-specific pathways including calcium ions and calcium channel mediated pathways. The present study revealed the contractile responses of *E. kotschyi* on detrusor and ileal tissues which should be further investigated by *in vivo* studies for its promising pharmacological effects on motility along with complementary toxicity tests and more research studies should be encouraged on the pharmacological activity of the endemic plants in Turkey [26] to reveal a basis for their ethnomedicinal use.

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RESEARCH ARTICLE

The Combined Effect of Prostaglandin Administration and Ram Introduction in Multiparous and Nulliparous Sheep in Anestrous Period on Prolificacy [1]

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Summary

In the study it was aimed to investigate and compare the combined effectiveness of ram introduction (ram effect) and prostaglandin F2 alpha (PGF2α) administration in multiparous and nulliparous Kangal White Karaman ewes during the out of breeding season. The ewes were first divided into two main groups: non-lactating multiparous (Group M, n=104) and nulliparous (Group N, n=101). The multiparous and nulliparous animals were further divided in to two subgroups. Group MRP (n=50 multiparous) and NRP (n=51 nulliparous) were injected with a single dose of PGF2α on the first day of ram introduction. And the Group MR (n= 54) and NR (n= 50) served as controls with ram introduction but no PGF2α injection. In all of the groups, adult, purebred and fertile rams stayed with the ewes for 45 days. The blood samples were collected at 3-day intervals for 18 days after ram introduction from subsets of ewes (n=17 per group) to monitor the serum progesterone concentration. The total lambing ratios in multiparous and nulliparous animals were 72.1% (75/104) and 44.6% (45/101), respectively (P<0.001). Among the PGF2α and non-PGF2α subgroups of multiparous and nulliparous ewes, the lowest lambing rate was observed in Group NR (36.0%). In multiparous ewes (Groups MR and MRP), the mean progesterone level varied significantly among the days (P<0.001). In contrast, in Group NRP, the progesterone levels varied significantly over the tested time course (P<0.001), but no differences were detected in Group NR (P>0.05). We concluded that being multiparous contributes to the success of PGF2α administration in combination with ram introduction in the anestrous period in ewes. Furthermore, PGF2α administration together with ram introduction positively affects the lambing rate in nulliparous ewes.

Keywords: *Ram effect, Prostaglandin F2 alpha, Multiparous, Nulliparous Kangal White Karaman, Ewe, Out of breeding season*

Anöstrus Dönemindeki Multipar ve Nullipar Koyunlarda Koç Etkisi ve Prostaglandin Uygulamalarının Dölverimi Üzerine Kombine Etkisi

Özet

Bu çalışmada, üreme sezonu dışında multipar ve nullipar Kangal Akkaraman ırkı koyunlarda koç katımı (koç etkisi) ve prostaglandin F2α (PGF2α) uygulaması kombinasyonunun etkinliğinin karşılaştırılması ve araştırılması amaçlandı. Koyunlar öncelikle, laktasyonda olmayan multipar (n=104, Grup M) ve nullipar (n=101, Grup N) koyunlar olmak üzere iki ana gruba ayrıldı. Daha sonra multipar ve nullipar koyunlar iki alt gruba ayrıldı. Grup MRP (n=50 multipar) ve Grup NRP'ye (n=51 nullipar) koç katımının ilk günü, tek doz PGF2α enjekte edildi. Grup MR (n= 54) and NR (n= 50) ise PGF2α enjekte edilmeden sadece koç katımı yapılarak kontrol grubu olarak seçildi. Tüm gruplarda fertil, ergin ve safkan koçlar 45 gün süre ile sürüde kaldı. Kan progesteron düzeylerinin izlenmesi amacıyla her bir grup için oluşturulan subsetgruplardan (n= 17) 18 gün boyunca, üç gün aralıklarla kan alındı. Multipar ve nullipar hayvanlarda total kuzulama oranları sırasıyla %72.1 (75/104) ve %44.6 (45/101) olarak belirlendi (P<0.001). Multipar ve nullipar koyunlarda PGF2α uygulanan ve uygulanmayan alt gruplarda %36.0 kuzulama oranı ile en düşük sonuç Grup NR'de belirlendi (P<0.001). Multipar koyunlarda (Grup MR ve Grup MRP) günler arasındaki ortalama progesteron düzeyleri arası fark önemli bulundu (P<0.001). Ancak nullipar koyunlarda günler arası progesteron düzeyi Grup NRP'de önemli iken (P<0.001), Grup NR'de önemsiz bulundu (P>0.05). Sonuç olarak; anöstrus döneminde koç katımı ve PG F2α kombinasyonu uygulamalarında multipar olmanın etkili olduğu, bununla birlikte nullipar koyunlarda koç katımı ile birlikte PGF2α uygulamasının kuzulama oranını artırdığı tespit edildi.

Anahtar sözcükler: *Koç etkisi, Prostaglandin F2 alfa, Multipar, Nullipar Kangal Akkaraman, Koyun, Üreme mevsimi dışı*

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INTRODUCTION

Kangal White Karaman sheep represent a local and national breed that was registered in 2012 and is characterized by adaptation to the hard environmental conditions found in Turkey [1,2]. In northern hemisphere countries such as Turkey, sheep usually show a seasonal sexual cycle. The breeding season usually begins at the end of August and continues to the end of November [3,4].

Since sheep breeding is naturally seasonal, prolificacy is restricted temporally. Therefore, expanding the application of reproductive biotechnologies such as estrous and ovulation synchronization that can be performed in and/or out of breeding season is essential to increase prolificacy and market availability and provide economically significant and controlled breeding and genetic advancement [5]. In addition, these types of applications should not result in additional costs for the breeder or harm the environment, animal welfare or public health.

When rams are introduced into a herd of ewes during the anestrous period, the sexual cycle is stimulated and copulation, ovulation and pregnancy occur; this is defined as the 'ram effect' [6-8]. The ram effect has been well established [5,9]. Introducing rams into a sheep herd out of breeding season to stimulate estrous is one of the easiest, most economical and environmentally friendly synchronization methods that does not compromise animal welfare or public health. Therefore, its use has become widespread [10-13]. However, new applications are needed to increase the effectiveness of the ram effect.

Sudden introduction of rams during the anestrous period stimulates the release of luteinizing hormone (LH) [14-16]. The time between ram introduction and LH release varies between 20 and 30 hours [7,17]. Applications that arrange the luteal phase would be helpful in increasing the effectiveness of estrous. Ungerfeld and Rubianes [18] reported that estrous signs were more prominent in sheep that received medroxyprogesterone prior to ram introduction than in untreated sheep; in the early period of the breeding season, increased follicular growth rates and rising estrogen concentrations led to increased LH release. However, steroids have negative effects on the environment and human and animal health; thus, the administration of prostaglandin F2 alpha (PGF2α) instead of steroids would be beneficial [13,19]. Therefore, studies have been conducted to find effective methods of using PGF2α administration together with the ram effect, particularly in sheep herds in out of breeding season [20].

Varying results have been reported regarding the effects of PGF2α administration on estrous synchronization in and out of breeding season [15,21-23].

The aim of this study was to test the following hypotheses: 1) The combination of ram introduction (ram effect) and PGF2α administration stimulates cycle activities better then only ram effect in out of breeding season, 2) Nulliparous and multiparous animals could have different responses to these applications.

MATERIAL and METHODS

In the study, 205 Kangal White Karaman ewes in the anestrous period (101 nulliparous and 104 non-lactating multiparous) and 20 fertile rams of the same breed bred by a family company in the Sivas province were used. The animals grazed on native pasture during the study and were kept in the same yard at night. The study was conducted during May and June, in out of breeding season [3].

The ewes included in the study were initially divided into two main groups: Group M (multiparous, n=104) and Group N (nulliparous, n=101). Subsequently, each group was divided into two subgroups (Group MR, MRP and Group NR, NRP). The animals in Group MRP (n=50 multiparous) and Group NRP (n=51 nulliparous) were injected with a single dose of 125 µg cloprostenol (PGS®, Alke, Turkey) via the intramuscular (IM) route on the first day of ram introduction. Groups MR (n=54 multiparous) and NR (n=50 nulliparous) served as controls and were not administered cloprostenol. After being separated for 2 months, all of the ewes were exposed to 20 rams for 45 days.

Seventeen animals were randomly selected from each group, and subsets were created to measure the progesterone concentrations. Blood was obtained from the vena jugularis on days 0, 3, 6, 9, 12, 15 and 18 of ram introduction. The serum samples were separated and stored at -20°C until all the samples had been collected. The progesterone levels were then determined using a commercial ELISA kit (Catalogue No: EIA 1561, DRG International Inc., Marburg, Germany) and an ELISA reader (Bio Tek Instruments, USA) [24].

SPSS 14.01 software was used for the statistical analyses. Variance analysis was used to compare the mean progesterone levels on different days. Student's t-test was used to compare the progesterone levels between groups on a given day. A chi-square test was used to compare the lambing ratios between groups and cyclic conditions at the beginning of the study. Differences were considered to be significant when $P<0.05$.

Ethics committee approval was obtained from the Animal Tests Ethics Committee, Firat University (FÜHADEK, 2011/11-138).

RESULTS

The total lambing ratio was 72.1% (75/104) for the multiparous animals and 44.6% (45/101) for the nulliparous animals; the difference found significant ($P<0.001$). When

the lambing ratios were compared among the four groups (NRP, NR, MRP and MR), the lowest lambing ratio was detected in Group NR (18/50, 36%) (P<0.05). There was no significant difference between the lambing ratio in subgroups of multiparous and nulliparous ewes (MRP and MR/NRP and NR) *(Table 1)*.

The mean progesterone concentrations were greater than 1 ng/ml in both the nulliparous and multiparous animals on the first day of ram introduction *(Table 2, Table 3)*. Also the luteal and follicular phase ratios were found similar in all groups (P>0.05).

Comparisons of the progesterone concentrations on different days in inter-groups without being multiparous and nulliparous, significant differences were detected between the PGF2α and non- PGF2α groups (P<0.001). While the differences in the mean progesterone concentrations on different days were significant in the Groups MRP, MR and NRP (P<0.001), no such differences were found in the nulliparous control ewes (NR) (P>0.05).

DISCUSSION

The Kangal White Karaman breed of sheep is a local breed, registered in 2012. It is considered a seasonal polyestrous breed. Although the breeding season is primarily in October and November, it is defined as autumn and the beginning of winter [1,4]. This study was conducted in May and June, the out of breeding season [3].

In recent years, methods such as the introduction of rams have been used to induce sheep breeding because they have fewer pharmacologic side effects, are more environmentally friendly, and pose less risk to animal welfare, public and human health. Increasing the effectiveness of these methods outside of the breeding season would be economically beneficial to the sheep industry [25,26]. Although long-term use of progesterone to increase breeding efficiency raises concerns due to the harmful effects on the environment and human and animal health, PGF2α is more environmentally friendly and poses less

Table 1. *The lambing ratios in nulliparous and multiparous ewes*
Tablo 1. *Nullipar ve multipar koyunlarda kuzulama oranı*

Animal	**Multiparous (n=104)**		**Nulliparous (n=101)**		**P**
Application	**PGF (+) (Group MRP)**	**PGF (-) (Group MR)**	**PGF (+) (Group NRP)**	**PGF (-) (Group NR)**	
n	54	50	51	50	
Lambing ratios (%)	37/54 (74.0)^{a}	38/50 (70.4)^{ac}	27/51 (52.9)^{bc}	18/50 (36.0)^{b}	*
Total (%)	75/104 (72.1)		45/101 (44.6)		*

PGF (+): *cloprostenol injection,* ***PGF (-):*** *no cloprostenol;* ^{a,b,c} *Numbers/percentages with different superscripts within a row differ significantly; * P<0.001*

Table 2. *Comparison of the mean progesterone concentrations (ng/ml) (± standard deviation) in multiparous ewes at 3-day intervals*
Tablo 2. *Multipar koyunlarda 3 gün aralıklarla ortalama progesteron (ng/ml) (± standard hata) konsantrasyonlarının karşılaştırılması*

Groups	**Days (X±Sx)**							
	0	**3**	**6**	**9**	**12**	**15**	**18**	**P**
Group MRP	1.02±0.17^{a}	0.91±0.25^{a}	2.42±0.32^{b}	3.46±0.86^{b}	4.25±0.77^{b}	5.36±1.04^{b}	4.15±1.16^{b}	*
Group MR	1.41±0.44^{a}	0.71±0.15^{a}	2.95±0.57^{b}	4.21±0.70^{b}	4.66±0.63^{b}	4.34±0.66^{b}	3.58±1.12^{b}	*
P	-	-	-	-	-	-	-	

^{a,b} *Values with different superscripts within a row differ significantly; - P>0.05; * P<0.001*

Table 3. *Comparison of the mean progesterone concentrations (ng/ml) (± standard deviation) in nulliparous ewes at 3-day intervals*
Tablo 3. *Nullipar koyunlarda 3 gün aralıklarla ortalama progesteron (ng/ml) (± standard hata) konsantrasyonlarının karşılaştırılması*

Groups	**Days (X±Sx)**							
	0	**3**	**6**	**9**	**12**	**15**	**18**	**P**
Group NRP	1.70±0.41^{a}	1.28±0.29^{a}	2.66±0.45^{b}	2.69±0.54^{b}	3.72±0.58^{b}	4.03±0.80^{b}	2.17±0.57^{b}	*
Group NR	1.85±0.39	1.59±0.45	3.05±0.58	2.58±0.42	3.30±0.64	3.14±0.80	3.36±0.97	-
P	-	-	-	-	-	-	-	

^{a,b} *Values with different superscripts within a row differ significantly; - P>0.05; * P<0.001*

health risks than progesterone. However, the low efficiency obtained with PGF2α use in and/or out of breeding season has compelled researchers to improve such methods. In the present study, a single dose of PGF2α was used as an alternative to a double dose. The aim of the administration of a single dose of PGF2α combined with ram introduction was to increase the ram effect-induced ovulation ratios [22,26]. The results obtained from the study demonstrate that this goal was achieved, particularly in nulliparous sheep.

The inadequate results obtained from estrous synchronization with synthetic prostaglandin analogues and artificial insemination may be explained by corpus luteum dysfunctions due to insufficient hormones, early embryonic death, implantation disorders, late embryonic death and/or fetal losses [23]. In contrast, it has been reported that estrogen receptor alpha and progesterone receptor protein expression levels are decreased in oviductal and uterine cells in response to progesterone compared with prostaglandin in synchronization studies [27]. However, the variability in ovulation time after PGF2α application may be minimized by the ram effect. The ram effect is commonly used for LH pulsation and ovulation induction during seasonal anestrous in ewes [16]. This effect is also observed in sheep that are administered progesterone [28] and PGF2α [5]. Thus, the administration of PGF2α combined with the ram effect may be of benefit during the early luteal phase to provide fixed-time artificial insemination without estrous detection in ewes [29]. There is little or no knowledge regarding the use of PGF2α administration to stimulate cycles on the first day of ram introduction in ewes in the anestrous period.

The corpus luteum formed after male-induced ovulation has either a normal life span (luteal phase lasting 18-19 days) or regresses early (5-6 days). An ovulatory subestrous period may follow this period. The progesterone levels detected in the present study are consistent with those reported in the literature [7,30,31]. When the reduction in progesterone concentration is compared with natural luteolysis, it is more prominent after PGF2α-induced luteolysis. Although luteal regression after natural PGF2α release lasts for 72 h, after exogenous PGF2α administration, it lasts for only 6-24 h [32]. In the present study, for multiparous and nulliparous anestrous ewes, on the first day of ram introduction, no difference in the progesterone levels was observed between the PGF2α-treated sheep and controls *(Table 2, Table 3)*.

Based on the progesterone levels measured at the beginning of the study (day 0), 19/34 (55%) nulliparous animals and 17/34 (50%) multiparous animals were found to be in the luteal phase (P>0.05). In a similar study, Smith et al.[20] found that 14% of progesterone-treated ewes were in the luteal phase at the first day of application. The difference may be attributed to the breed of sheep and the location.

In the present study, serum progesterone levels in multiparous and nulliparous ewes were greater than 1 ng/ml on the first day of ram introduction, demonstrating that at least partial cyclic activities are present during the anestrous period in Kangal White Karaman ewes. Consistent with the results presented in this study, Kaulfuss et al.[33] detected 100% cyclic luteal structures in ewes in breeding season with no differences noted between breeds. However, depending on the breed, they observed 10-60% cyclic luteal structures during the rest of the year. Similarly, in a study by Maatoug-Ouzini et al.[34] using Barbarina sheep, the different cycle types formed after ram introduction were analyzed. They found that 30% of the Barbarina sheep continued cyclic activities out of breeding season, and this rate reached 60% with the ram effect.

In the present study, both nulliparous and multiparous sheep were found to respond to the ram effect. Nevertheless, the lambing ratios were significantly higher in the PGF2α-administered groups on the first day of ram introduction *(Table 1)*. In addition, it was found that PGF2α administration on the first day of ram introduction was more effective in the nulliparous animals than multiparous animals. Also when we compared the progesterone levels between days, multiparous animals were more affected by the ram introduction instead of PGF2α application. Nonetheless, in nulliparous animals PGF2α administration with ram introduction found more effective.

In our study, the lambing rates of the multiparous and nulliparous ewes, 72.1% and 44.6%, respectively, were significantly different (P<0.001). The difference is most likely due to ovulation insufficiency and high pre-implantation losses in the nulliparous animals. Supporting this assumption, Khan et al.[35] reported the embryonic loss rate as 20-40% in adults and 50-63% in young ewes. In a review by Rosa and Bryant [4], it was reported that ovulation in multiparous sheep was approximately three-fold greater than in 14- to 15-month-old nulliparous sheep when using the ram effect.

In a study conducted by Reyna et al.[36] in Australia, the pregnancy rates achieved with fixed-time insemination were 11-32% in different groups of multiparous and nulliparous animals out of breeding season. These rates are lower than those observed in the present study. The low pregnancy rates detected in similar studies conducted both in and out of breeding season may be attributed to factors such as artificial estrous or ovulation induction, different concentration or type of sperm, fixed-time inseminations and different techniques [23,37,38]. The higher lambing ratios obtained in the present study may due to the continuous presence of rams with the ewes until mating [39].

It is also important to emphasize that the progesterone values measured in this study demonstrate that nulliparous ewes respond more strongly to ram introduction when treated with PGF2α than multiparous ewes. Thus, being

nulliparous must be considered a negative factor for breeding in normal situations.

The results of the current study suggest that cyclic activities continue to some degree throughout the year in Kangal White Karaman sheep. Multiparous Kangal White Karaman sheep in anestrous period responded better to the combined PGF2α administration and ram effect than nulliparous ewes. PGF2α administration at the time of ram introduction increased the lambing ratio in nulliparous sheep. Further studies are required to determine the cycle-induction mechanism of PGF2α administration and ram introduction and to assess their impact on ovulation.

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RESEARCH ARTICLE

Investigation of Relationships between DNA Integrity and Fresh Semen Parameters in Rams [1]

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Summary

The aim of the present study was to evaluate the correlation between the routine semen analyses and sperm DNA integrity, as assesed by TUNEL in fresh semen of rams in Kivircik and Awassi breeds in breeding season. Semen was collected by electro-ejaculation five times every other day. For that purpose, 50 fresh ejaculates from 6 Kivircik and 4 Awassi rams were evaluated. The mean percentages of semen volume, concentration, mass activity, motility, acrosomal defects, HOST and DNA fragmentation of Kivircik and Awassi rams were 1.3 ml, 1.9 x109, 3.3 (0-5 scale), 72.6%, 13.2%, 73.6%, 16.2% and 1.0 ml, 1.9 x109, 3.3 (0-5 scale), 72.5%, 11.1%, 77.9%, 15.9%; respectively (P>0.05). Sperm DNA fragmentation was correlated adversely with semen volume (r = -0.329, P<0.05), concentration (r = -0.188, P>0.05), mass activity (r = -0.349, P<0.05), motility (r = -0.448, P<0.01), acrosomal defects (r = -0.103, P>0.05) and HOS test (r = -0.513, P<0.01). This study provided clear evidence that most of the parameters evaluated herein related to DNA fragmentation at one point or the other. Hence, DNA structure evaluation of sperm might be a useful tool for accurate prediction of the male fertility in individual rams.

Keywords: *Ram, Fresh semen, DNA fragmentation*

Koçlarda Taze Sperma Parametreleri ve DNA Bütünlüğü Arasındaki İlişkilerin Araştırılması

Özet

Bu çalışmanın amacı, Kıvırcık ve İvesi ırkı koçlardan alınan spermaların, sezon içerisinde rutin sperma analizleri ve TUNEL ile yapılan sperm DNA bütünlüğü arasındaki korelasyonu değerlendirmektir. Sperma elektro-ejakülasyon yöntemiyle 5 kez ve birer gün aralıklarla alındı. Bu amaçla; 6 baş Kıvırcık ve 4 baş İvesi ırkı koçtan alınan 50 ejakülat değerlendirildi. Kıvırcık ve İvesi ırkı koçların ortalama sperma hacmi, konsantrasyonu, mass aktivitesi, motilitesi, akrozomal bozukluğu, HOST ve DNA fragmantasyonu yüzdeleri sırasıyla 1.3 ml, 1.9 x109, 3.3 (0-5 skala), %72.6, %13.2, %73.6, %16.2 ve 1.0 ml, 1.9 x109, 3.3 (0-5 skala), %72.5, %11.1, %77.9 ve %15.9 olarak bulundu. Sperm DNA fragmantasyonu; sperma hacmi (r = -0.329, P<0.05), sperma konsantrasyonu (r = -0.188, P>0.05), mass aktivite (r = -0.349, P<0.05), motilite (r = -0.448, P<0.01), akrozomal bozukluk (r = -0.103, P>0.05) ve HOS testi (r= -0.513, P<0.01) ile negatif korelasyon gösterdi. Bu çalışma, değerlendirilen çoğu parametrenin DNA fragmantasyonu ile bağlantılı olduğunu açıkça gösterdi. Dolayısıyla, DNA yapısının incelenmesi her bir koç ejakülatının fertilitesinin doğru olarak tespit edilmesi konusunda faydalı olabilir.

Anahtar sözcükler: *Koç, Taze sperma, DNA fragmantasyonu*

INTRODUCTION

In livestock breeding, the impact of male infertility upon the reproductive efficiency of farms is high, since a male animal can serve a large number of females, either by artificial insemination or by mating. Therefore, male infertility is observed commonly as increased return to oestrus rate or decreased lambing rate in ewes [1]. The widespread use of artificial insemination in domestic animals has encouraged the development of laboratory tests that accurately predict the individual fertility of rams.

In order to assess the potential fertility of rams, semen evaluation, complementary to the clinical examination, is useful [2,3]. Semen quality and its relationship to fertility are of major concern in animal production. The fertilization

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process involves complex biochemical and physiological events that cannot be measured solely by routine semen evaluation. The general methods for evaluation of semen quality have been primarily based on routine semen analyses (i.e., motility, morphology and acrosomal integrity), however such routine examinations have a narrow capacity for predicting the potential fertility of a given ejaculate [4-7]. Numerous studies were conducted on semen freezing [8-11], and rutine ram semen evaluation [7,12,13]. Also most of the studies explain the routine semen parameters and DNA integrty in man [14-16], especially in patients with miscarriage history. However, there appears no study available on the relation between rutine semen parameter and DNA integrity of fresh semen in rams.

Evaluation of sperm DNA damage can satisfy the expectations on the prediction of the outcome of assisted reproductive techniques (ART) than conventional sperm parameters. The most commonly used tests to measure sperm DNA damage are the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and the sperm chromatin structure assay (SCSA). The TUNEL assay measures both single- and double-strand DNA integrity, measures a definitive end point (presence of free 3 = hydroxyl groups), and can provide more meaningful information on the implantation potential of an embryo.

Therefore, the aim of this study was to evaluate the correlation between routine semen analyses and sperm DNA integrity as assesed by TUNEL in fresh semen of rams in Kivircik and Awassi breeds in breeding season.

MATERIAL and METHODS

Chemicals

PBS tablets and poly-$_L$-lysine were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Triton X-100 (10% stock solution) (11332481001) and an *In Situ* Cell Death Detection Kit were purchased from Roche (Roche Diagnostics GmbH, Mannheim, Germany). Proteinase K (003011) and antibody diluents were purchased from Zymed (Zymed, San Francisco, California, USA). Bovine anti-rabbit fluorescein (FITC) (Sc: 2365) and mounting medium (Sc: 24941) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Semen Collection and Preparation

A total of ten rams (6 Kivircik and 4 Awassi breeds) aged 3-5 years kept at Uludag University, Faculty of Veterinary Medicine in Bursa, Turkey, were used during the breeding season. Semen was collected five times every other day by an electro-ejaculation with 12 cm probe length, 2.5 cm in diameter and 12 V (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand) [17]. To collect semen, rams were restrained physically and a lubricated probe was inserted into the rectum with downward pressure upon the front of the probe, so the electrodes rested on the upper portion of the ampullary region. An electrical stimulation was applied for 4-8 sec. The electrostimulation was stopped briefly (3-4 sec) while further massage was applied with the probe. This cycle was repeated until a 1-2 ml of semen sample was collected (usually 3-4 electro-stimulations). After the collection, each ejaculate was placed in a warm water bath (30°C) and immediately assesed for the volume, concentration, mass activity (0-5 scale), motile spermatozoa (zero to 100%), acrosomal defects, plasma membrane integrity, and DNA fragmentation rates (%).

Semen Evaluation

All semen parameters were measured by the same person throughout the study. Sperm motility was evaluated subjectively using a phase-contrast microscope (Olympus BX 51) (400x) on a warm slide (38°C) [17].

Fluorescein Lectin Staining Assay (Florescein Isocyanate-conjugated Pisum Sativum Agglutinin [FITC-PSA]): Acrosomal integrity was assessed by using FITC-conjugated *PSA* [9]. Briefly, 20 µl of diluted semen was re-suspended in 500 µl PBS and centrifuged at 2.000 rpm for 20 min; the supernatant was then discarded. The spermatozoa pellet was re-suspended in 250 µl PBS. One drop of resuspended spermatozoa was smeared on a glass microscope slide and dried in the air. Air-dried slides were fixed with acetone at 4°C for 10 min, and the slides were covered with FITC PSA solution (50 µg/ml in PBS solution) in the dark for 30 min. Stained slides were rinsed with PBS solution, covered with glycerol, and examined under a fluorescence microscope. At least 100 spermatozoa per smear were evaluated for acrosomal integrity.

Hypo-osmotic Swelling Test (HOST): Sperm membrane integrity was evaluated using a method as described by Nur et al.[18] with minor modifications. The semen was submitted to HOS test. A volume of 20.0 µl of semen was added into 1 ml of warmed hypo-osmotic swelling solution (100 mOsm/l) containing sodium citrate (25 mmol/l) and fructose (75 mmol/l) and incubated at 37°C for 60 min. Immediately after the incubation, one drop of semen was placed on a clean glass slide, covered with onother slide and assessed within 5 min under phase-contrast microscopy (400 x). For each sample, a total of 100 spermatozoa were counted per slide and the percentages of swollen and curled tailed spermatozoa were recorded.

Sperm DNA Integrity by Terminal Deoxynucleotidyl Transferase-Mediated Fluorescein-TUNEL Assay: For the TUNEL technique, we used the *In Situ* Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with slight modifications. In brief, one drop of re-suspended spermatozoa was smeared on a glass slide and fixed with 10% formaldehyde for 20 min at room temperature. The slides were washed in PBS and stored at 4°C. Upon the

removal from storage, the samples were washed again in PBS (for three times, 5 min each). They were then treated in a humidified chamber with proteinase K for 10 min at room temperature, washed with PBS, treated with 3% H_2O_2 in distilled water for 10 min at room temperature and washed again with PBS. The slides were permeabilized with 0.1% Triton X-100 for 5 min on ice.

The permeabilized slides were incubated in the dark at 37°C for 1 h with the TUNEL reaction mixture, that contained terminal deoxynucleotidyl transferase (TdT) plus dUTP label. After the labeling, the samples were washed with PBS and analyzed immediately via fluorescence microscopy. Negative (omitting TdT from the reaction mixture) and positive (using DNase I, 1 mg/ml, for 10 min at room temperature) controls were included in each trial. At least 100 spermatozoa were evaluated for determining the percentage of TUNEL-positive sperm. Each microscopic field was evaluated first under fluorescence microscopy (40x) for determining the number of reactive sperm and then under phase-contrast microscopy) for determining the total number of sperm per field.

Statistical Analyses

Data were analyzed by independent samples T test. Spearman's correlation coefficient was used to assess the relationship between sperm volume, concentration, mass activity, motility, acrosomal defects, plasma membrane integrity and DNA fragmentation (TUNEL-positive) rate. All data were analyzed using the SPSS statistical package (SPSS 20.0 for Windows; SPSS, Chicago, IL, USA). Differences were considered significant when P<0.05.

RESULTS

The general means of the spermatological parameters in Kivircik and Awassi rams were summarized in *Table 1*. There were no significant differences in sperm volume, concentration, mass activity, motility, HOST, acrosomal defects and DNA fragmentation between the two breeds (P>0.05).

The correlation analyses between the semen parameters studied were presented in *Table 2* and *Fig. 1*. TUNEL assay demonstrated that spermatozoa with DNA fragmentation exhibited a green fluorescence *(Fig. 2)*. Sperm DNA fragmentation was correlated adversely with sperm volume (P<0.05), concentration (P>0.05), mass activity (P<0.05), motility (P<0.01), acrosomal defects (P>0.05) and swollen tailed spermatozoa obtained by HOS test (P<0.01).

Also, sperm functional plasma membrane integrity (HOS test) was correlated favourably with mass activity (P<0.01) and motility (P<0.01). Furthermore, the acrosomal defects were correlated with volume (P<0.01), while mass activity was correlated favourably with volume (P<0.05) and sperm concentration (P<0.05). Besides, there was a positive relationship between sperm concentration and volume (P<0.01).

DISCUSSION

Semen quality and its relation to fertility are of major concern in animal production. Quality tests are routinely used for determining the acceptability of processed semen

Table 1. *General means of the spermatological parameters in Kivircik and Awassi rams*
Tablo 1. *Kıvırcık ve İvesi ırkı koçların ortalama spermatolojik parametre değerleri*

Rams	Volume (ml) X±Sx	Sperm Concentration ($x10^9$) X±Sx	Mass Activity (0-5) X±Sx	Motility (%) X±Sx	Acrosomal Defects (%) X±Sx	HOST (%) X±Sx	DNA Fragmentation (%) X±Sx
Kivircik	1.34±0.16	1.90±0.17	3.31±0.19	72.59±2.24	13.16±0.93	73.59±1.75	16.19±1.87
Awassi	0.96±0.16	1.87±0.09	3.25±0.19	72.50±1.77	11.11±0.82	77.94±2.36	15.86±2.63

The values are the mean ± standard error of mean (SEM); There was no significant difference (P>0.05)

Table 2. *Correlation coefficients (r) between the results of semen characteristics and TUNEL*
Tablo 2. *Spermatolojik değerler ve TUNEL arasındaki korelasyon katsayıları (r)*

Spermatological Parameters	Sperm Concentration ($x10^9$)	Mass Activity (0-5) Scale	Motility (%)	Acrosomal Defects (%)	HOST (%)	DNA Fragmentation (%)
Volume (ml)	0.415**	0.301*	0.059	0.455**	0.073	-0.329*
Sperm concentration ($x10^9$)		0.536*	0.222	0.098	0.183	-0.188
Mass activity (0-5)			0.702**	-0.251	0.557**	-0.349*
Motility (%)				-0.086	0.601**	-0.448**
Acrosomal defects (%)					0.004	-0.103
HOST (%)						-0.513**

*Correlations are significant when * P<0.05, ** P<0.01*

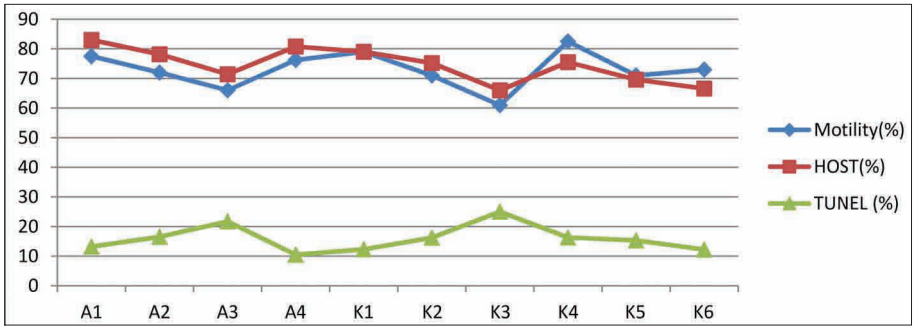
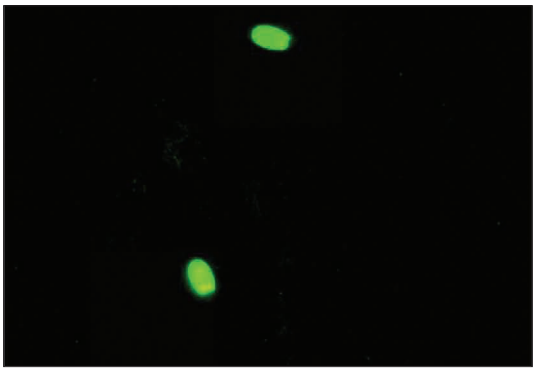


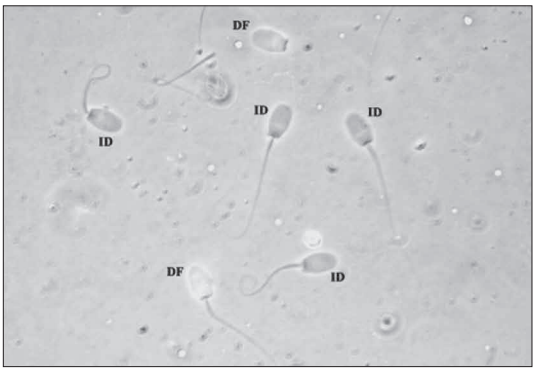
Fig 1. Correlation between the motility, HOST and DNA fragmentation rates (TUNEL)

Şekil 1. Motilite, HOST ve DNA fragmantasyon (TUNEL) oranları arasındaki korelasyon

Fig 2. DNA fragmentation (DF) and intact DNA (ID) detected by TUNEL assay

Şekil 2. DNA fragmantasyonu (DF) ve DNA bütünlüğünün (ID) TUNEL ile belirlenmesi





for breeding purposes. Thus, the accurate measurement of the quality is a major importance. The widespread semen evaluation generally includes the measurement of semen volume, sperm concentration, mass activity and the percentage of motile and morphologically normal spermatozoa [19]. Although some of these parameters are correlated with fertility in rams [7], several authors suggest that this information does not accurately predict whether a male is truly fertile [20,21].

The process of fertilization involves complex biochemical and physiological events that are not completely reflected in the conventional measures of concentration, motility, and morphology. Since the functional activity of the nuclear structure is crucial for the viability and fertilizing ability of spermatozoa, it is important to assess the DNA integrity of sperm [14]. A number of tests are currently available for the measurement of sperm DNA fragmentation. These include the TUNEL assay [7], the comet assay [14] and the SCSA test [7]. To the best of our knowledge, only very limited information is available for the correlation between semen parameters and DNA integrity in rams. In the present study, we evaluated the relationship between the routine semen analyses and sperm DNA integrity, as assesed by TUNEL in fresh Kivircik and Awassi ram semen during breeding season.

Semen collection methods, season, age and breed of rams may all affect the ejaculate volume. The mean semen volume varies between 0.6-2 ml in fertile ejaculates in different breeds [22]. Present study demonstrated that the general mean ejaculate volumes were 1.34 and 0.96 ml in Kivircik and Awassi rams, respectively (P>0.05).

Hafez [23] reported that the sperm motility is a prerequisite for sperm transportation to the fertilization area, but it is not indicative of the fertilizing ability. Also, the sperm acrosome has an effect on the fertilizing ability. Fresh ram sperm motility may vary between 70% [9,11] to 90% [22] and fresh acrosome defect may vary between 3.8% [11] to 6.7% [24] after different fixation and staining assays. The general means of motility and acrosomal defects were 72.59% *vs.* 13.16% and 72.50% *vs.* 11.11% in Kivircik and Awassi rams, respectively. The increasing rates of acrosomal defects may be related to the staining of assay. There are no studies documented on fresh ram semen about the acrosomal defects evaluated by FITC-PSA staining assay.

Sperm membrane integrity is a crucial parameter for the evaluation of sperm quality, because the intact plasma membrane is an essential borderline for survival of sperm cell [25]. Hypo-osmotic swelling test has recently been shown to be useful in detecting subtle changes in the functional integrity of ram sperm membranes [26]. Ollero et al.[27] reported that fresh semen exhibited 72% HOS test response. Similar results were also obtained for both breeds herein.

DNA damage may originate from improper packaging and ligation during spermatogenesis and epididymal sperm maturation [28]. The ram sperm DNA has the most degredation response under the similar experimental conditions when compared to other mammalian species [29]. Therefore, the evaluation of individual fresh semen DNA integrity could give important information related to the fertilizing ability. Therefore, we used TUNEL assay to

determine the DNA integrity. Nur at al.[7] reported that the mean percentage of spermatozoa with damaged DNA were 1.8% in Tris diluted ram semen. In men, the fertile percentage of fresh spermatozoa with damaged DNA was 12.9 % while in infertil men it was 48.8% obtained with commet assay [14]. Semen collection time, breeding season, ejaculation frequency, sexual arrest, age, breed, body condition and nutritional regime may all have an effect on semen quality and fertility [12,30]. The mean percentages of TUNEL positive spermatozoa were 16.19% and 15.86% in Kivircik and Awassi breeds, respectively.

Comparation of the semen parameters studied revealed that most of the parameters correlated to other paramaters at one point or the other. In this study, swollen tailed spermermatozoa corelated to motility (r: 0.601) and mass activity (r: 0.557). These findings are not suprising because the motility partly depends on transport of compounds across the membrane of spermatozoa [13]. Similar findings have been reported for sperm motility and HOST values earlier [13,31].

There exist remarkable numbers of motile spermatozoa from fertile donors containing fragmented DNA [32,33]. The degree of DNA damage in sperm cells leads to impairment of fertilization, embryo development [34-36], and reduced chance of producing live offspring [37-39]. The energy source of motilty that plays critical roles for sperm to reach to the fertilization site is provided by mitochondria, as controlled by sperm nucleus [40]. The failure of nuclear integrity also affects the sperm motility. In our study, the relationship between motilty and DNA integrity supports our theory. These relationships also present for the sperm plasma membrane functional integrity and the mass activity. There is an increasing interest in the use of DNA integrity related assays as a predictor of fertility potential [7]. A previous study showed that there is a positive relationship between sperm motility and DNA damage [41]. However, a markedly inverse correlation has been found between sperm motility and DNA integrity (r: -0.448, P<0.01). These findings were in agreement with results reported by Piasecka et al.[42] and Sheikh et al.[14]. Undoubtedly, poor-quality semen has a greater percentage of spermatozoa with DNA fragmentation than that of superior quality semen [32,33].

Functional integrity of sperm plasma membrane is a sign of healthy substance exchange needed for the viability of cells. The percentages of spermatozoa with damaged plasma membrane were higher than those sperm with DNA defects in both breeeds. Balasuriya et al.[15] reported that the sperm plasma membrane damage was higher than that of nuclear damage. It is expected that, the higher motility rates can be found in those spermatozoa that have solid membranes [43]. This study showed that the functional membrane integrity of sperm has a strict relationship with the motility. This condition was also proved in the earlier studies [18]. The increase in failure of the plasma membrane integrity results in a decrease in DNA integrity [44]. Sperm DNA fragmentation rate was correlated adversely with functionally active sperm population (P<0.01). However, according to Fatehi et al.[45], the cells with DNA damage did not show signs of functionally affected integrity of membranes and motility.

In summary, we have demostrated that sperm DNA fragmentation correlated unfavourably with the ejaculate volume (P<0.05), mass activity (P<0.05), motility (P<0.01), and HOST values (P<0.01). Also, there were positive correlations among HOST values, motility and mass activity (P<0.01). According to the results, we can conclude that testing DNA damage, in addition to standard methods may be a useful tool for the accurate prediction of the fertility in ram semen.

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RESEARCH ARTICLE

Treatment of Orthopaedic Problems with Manuflex® Disposable External Fixator in 15 Dogs and 7 Cats

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Summary

The aim of this study was to use Manuflex® disposible external fixator (MDEF), designed by two Hungarian orthopaedists, one of which is MD and the other is DVM, for treatment of orthopaedic problems, and to present the outcomes in dogs and cats. Cases included fractures of humerus (n=1), tibia (n=4) and radius-ulna (n=4), an angular deformity (with radius-ulna fracture), tibio-tarsal luxations (n=3), a tarso-metatarsal fracture (n=1), mandibular fractures (n=2) in dogs; and fractures of tibia (n=2), radius (n=1) and humerus (n=2), a radio-carpal luxation (n=1), bilateral tibio-tarsal luxation and malleolar tibial fracture (n=1) in cats. Three types of fixators (large, medium and small) has been used according to animal sizes and bone fracture types. All cases had tolerated their apparatus well and did not have any postoperative reactions and they started using the limbs immediately after surgery. Pin tract infections were seen in 2 dogs. Functional outcomes were very good in 10 cases, good in 4 cases and satisfactory in 1 case, in dogs; and very good in 6 cases and satisfactory in 1 case in cats. It was concluded that MDEF can be an alternative system compared to other external fixators, because of its easier application, forming rigid fixation and stability, applying without any complicated equipments and being more economic.

Keywords: *External fixator, Dog, Cat, Manuflex®*

Ortopedik Problemli 15 Köpek ve 7 Kedinin Manuflex® Eksternal Fikzatör İle Sağaltımı

Özet

Bu çalışma, biri beşeri, diğeri veteriner hekim olan iki Macar ortopedist tarafından geliştirilen Manuflex® eksternal fikzatörün kedi ve köpeklerde karşılaşılan ortopedik problemlerin sağaltımında kullanılması ve sonuçların değerlendirilmesini amaçlamıştır. Çalışma materyalini, köpeklerde humerus (n=1), tibia (n=4) ve radius-ulna (n=4) kırığı, angular deformitesi (radius-ulna kırığıyla birlikte), tibio-tarsal lukzasyon (n=3), tarso-metatarsal kırık (n=1) ve çene kırığı (n=2), kedilerde tibia (n=2), radius (n=1) ve humerus (n=2) kırığı, radio-karpal lukzasyon (n=1), çift taraflı tibio-tarsal lukzasyon ve malleolar tibia kırığı (n=1) oluşturmuştur. Hayvanların büyüklüğüne ve kırık kemik tiplerine göre üç tip fikzatör (büyük, orta ve küçük boy) kullanılmıştır. Operasyon sonrası tüm olgular apareyi iyi tolere etmiş, herhangi bir postoperatif reaksiyon göstermemiş ve hayvanlar uzuvlarını oldukça hızlı kullanmaya başlamışlardır. Yalnız iki köpekte pin dibinde enfeksiyon şekillenmiştir. Fonksiyonel sonuçlar 10 köpekte çok iyi, 4 köpekte iyi ve 1 köpekte yeterli; 6 kedide çok iyi ve 1 kedide yeterli görülmüştür. MDEF'nin, karmaşık ekipmana gereksinim duyulmadan kolay uygulanabilmesi, yeterli fikzasyon ve stabilite oluşturması ve daha ekonomik olması yönlerinden diğer eksternal fikzatörlerle karşılaştırıldığında alternatif bir sistem olarak kullanılabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Eksternal fikzatör, Köpek, Kedi, Manuflex®*

INTRODUCTION

External fixators are used in animals, for emergency or temporary stabilisation of bones, for treatment of fractures, correction of limb deformities, stabilisation of arthrodeses, treatment of non-unions and osteomyelitis, treatment of

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quadriceps muscle contracture, and in modern trauma management [1-3].

An external fixator is composed of inserting multiple percutaneous transcortical stainless steel pins or wires placed proximal and distal to the fracture or joint. The external frame can be linear (rods or columns) or circular (rings). It is applied using either closed or open reduction. The pins are connected by clamps, acrylic or epoxy putty compounds to an external connecting bar [4,5]. This fixation system is modular and thus can be assembled in numerous construct configurations. External fixation is also used in combination with internal fixation (especially intramedullary Steinmann pins and/or cerclage wires) to provide adjunctive fracture stabilization [6].

Three types of external fixator systems are classified: Type I (a and b) utilizes half pin splintage. Pins pass through both cortices of the bone, clamps and bars are placed on one side of the limb and pins only penetrate one skin surface. Type II utilizes full-pin splintage. Pins pass through both cortices of the bone, clamps and bars are placed on both sides of the limb and pins penetrate opposing skin surfaces. Type III utilizes combination of half-pin and full-pin splintage, type I and II systems are placed at 90 degrees to each other and the frames are enterconnected. External fixators can also be classified either uniplanar (pins and bars occupy a single plane) or biplanar (two half-pin splintage fixators are placed at 60 degrees to 90 degrees of axial rotation to each other and the frames are interconnected) [6].

Both in human and veterinary surgery, the bone-pin interface (pin diameter and interference), the components of the fixator and the fixator configuration (how it is assembled on the inserted bone pins) directly influence the contribution to stability by the external fixator. All these forms external fixator biomechanics and directly affect the animal comfort, limb using, fracture and tissue healing [6,7].

The aim of this study was to describe the technique and surgical outcomes of the use of Manuflex©, the unique disposable external fixator (MDEF) which is not connected by clamps, for treatment of different orthopaedic problems (fractures of the long bones, fragmented fractures of condylar areas, luxations, stabilization of bones, angular deformities and mandible fractures) in cats and dogs.

MATERIAL and METHODS

Animals

This study had been performed in Szent Istvan University, Faculty of Veterinary Medicine, Department of Surgery, Ankara University, Faculty of Veterinary Medicine, Department of Surgery and Veterinary Surgery and Orthopaedic Center. Seven cats and 15 dogs of different age, sex and breeds, with different orthopaedic problems were assessed for the study. Considering the anamnesis and clinical examinations, plain radiograms were taken in all cases. All radiograms were examined by the same orthopaedists. Before surgery, MDEF configuration was determined from assessment of radiograms of orthopaedic problems. Manuflex type and Kirschner wires in suitable diameters (1.0, 1.2, 1.5 and 2.0 mm) were chosen.

Operations

Surgery was performed under general anesthesia by the same operators. Xylazine 2% (Alfazyne, 2 mg/kg, IM, Egevet, Türkiye) and Ketamine HCl 10% (Alfamine, 10 mg/kg, IM, Egevet, Türkiye) were used for dogs and cats. Also, cefazolin (Sefazol, 20 mg/kg, IV, Mustafa Nevzat, Türkiye) was administered 1 h before the operation. Preoperative analgesia included SC administration of 0.2 mg/kg meloxicam. All animals were treated with MDEF (Trade-Coop, Hungary) which was made of unalloyed aluminium material. MDEF was used also with polymethyl methacrylate (PMMA) in 7 animals (2 cats and 5 dogs). Closed reduction was performed on 7 cases (4 cats and 3 dogs), limited open reduction was performed on 2 cats and open reduction was performed on 13 cases (1 cat and 12 dogs) *(Table 1)*.

Manuflex Apparatus

Three types of device have been used in animals of different sizes and weights, and different bone fractures.

- Large Size Manuflex® (40.0 cm lengthening of rods, 9.0 mm diameter of rod, 10.0 cm distance without any hole between lower and upper hole, 5.0 mm inner diameter of a hole, 3.0 mm distance between holes)
- Medium Size Manuflex® (50.0 cm lengthening of rods, 8.0 mm diameter of rods, 3.0 mm inner diameter of a hole, 3.0 mm distance between holes)
- Small Size Manuflex® (50.0 cm lengthening of rods, 5.0 mm diameter of rods, 2.0 mm inner diameter of a hole, 1.0 mm distance between holes) *(Fig. 1)*

In open reduction technique, the fracture fragments were exposed surgically by dissecting the tissues and the fragments were reduced. After fracture reduction, pins (K-wires) were placed unicortically or bicortically (according to fracture type and animal weight) with a high torque drill. In this technique, tension band wires and intramedullary pins were also used depending on the animal's weight and fracture type. In close reduction technique, small longitudinal stab incisions were made in the skin over the insertion sites for each pin. Pins were placed through as little soft tissue as possible *(Fig. 2)*. K-wires with different sizes and numbers were placed on the proximal and distal fragments unilaterally or bilaterally. MDEF position was determined by suitable pin insertion points. MDEF has not got a clamp apparatus.

Table 1. *Summary of data recorded for 22 cases (15 dogs and 7 cats) managed with MDEF*
Tablo 1. *MDEF uygulanan 22 olguya (15 köpek ve 7 kedi) ait bilgiler*

Case No	Signalment	Fracture Type, Localization, and History	Procedure and Apparatus Configuration	Radiologic Consolidation (Days after operation)	Fixator Removal (Days after operation)	Complications	Functional and Cosmetic Results
1	Dog, German Shepherd, 2 years-old, M, 33 kg (AUFVM)	Left tibia; Grade II-Open fragmented fracture on distal 1/3 of diaphyseal region; Vehicular trauma	Closed reduction; Large size Manuflex®; Four K-wires 2.0 mm Ø on the proximal fragment (bilateral); Three K-wires 2.0 mm Ø on the distal fragment (Two of them unilateral, the other bilateral); Two K-wires 2.0 mm Æ on the metacarpus (bilateral); With PMMA	43	55	No complication	Very good
2	Cat, Mix, 11 years-old, M, 5 kg (AUFVM)	Right tibia; Fragmented fracture on distal 1/3 of diaphyseal region; Fall from height	Limited open approach and reduction; Small size Manuflex®; One K-wire 1.5 mm Ø intramedullary pinning; Three K-wires 1.5 mm Ø on the proximal fragment (bilateral); Two K-wires 1.5 mm Ø on the proximal fragment (unilateral); Two K-wires 1.5 mm Æ on the distal fragment (bilateral); With PMMA	38	45	No complication	Very good
3	Dog, Bull Terrier, 2 months-old, F, 5 kg (SIU)	Left radius and ulna; Fragmented fracture on mid 1/3 of diaphyseal region; Compression injury	Closed reduction; Small size Manuflex®; Two K-wires 1.5 and 1.0 mm Ø on the proximal fragment (bilateral, near to fracture line 1.0 mm Ø); Two K-wires 1.5 mm and 1.0 mm Ø on the distal fragment (bilateral, near to fracture line 1.0 mm Ø); Non-PMMA	27	35	No complication	Very good
4	Cat, Siamese, 11 years-old, MN, 4.5 kg (VSOC)	Right radiocarpal luxation; Fall from height	Closed reduction; Small size Manuflex®; Three K-wires 1.5 mm Ø on the radius and ulna (bilateral); One K-wire 1.5 mm Ø on the carpal bones (bilateral); Two K-wires 1.5 mm Ø on the carpal bones (unilateral); With PMMA	25	35	No complication	Very good
5	Dog, Peckingese, 5 years-old, M, 13 kg (SIU)	Right tibio-tarsal stabilization and fracture on Mt IV; Vehicular trauma	Open approaches and arthrodesis; X pinning for tarsal arhtrodesis; Medium size Manuflex®; One K-wires 1.5 mm Ø on the tibia (bilateral); One K-wires 1.5 mm Ø on the calcaneus (bilateral); Two K-wires 1.5 mm on the metatarsus (bilateral); With PMMA	45	55	Pin tract infection on the postoperative 6th day	Good
6	Cat, Mix, 4 months-old, F, 2.8 kg (AUFVM)	Left radius; Simple fracture on mid 1/3 of diaphyseal region; Vehicular trauma	Closed reduction; Small size Manuflex®; Two K-wires 1.2 mm and 1.0 mm Ø on the proximal fragment (unilateral, near to fracture line 1.0 mm Ø); Two K-wires 1.2 mm and 1.0 mm Ø on the distal fragment (unilateral, near to fracture line 1.0 mm Ø); Non-PMMA	32	45	No complication	Very good
7	Dog, Mix, 3 years-old, M, 17 kg (VSOC)	Right humerus; Oblique fracture on midline diaphyseal region; Unknown trauma	Open approaches and reduction; Medium size Manuflex®; Two K-wires 2.0 mm on the proximal fragment (unilateral); Two K-wires 2.0 mm on the distal fragment (unilateral); Two K-wires 2.0 mm Ø for intramedullary fixation; Non- PMMA	35	45	No complication	Very good
8	Cat, Mix, 2.5 years-old, FM, 4.5 kg (VSOC)	Right humerus; Fragmented oblique fracture on mid 1/3 of diaphyseal region; Unknown trauma	Open approach and reduction; Small size Manuflex®; Three K-wires 1.5 mm Ø for intramedullary fixation; Collaps of fracture line on postoperative 3rd week; Second operation; Removal all K-wires, Closed reduction; Three K-wires 1.5 mm Ø on the proximal fragment (unilateral, far to fracture line 2.0 mm Ø threaded pin); Two K-wires 1.5 mm Ø on the distal fragment (unilateral, far to fracture line 2.0 mm Ø threaded pin); With PMMA	30	45	No complication	Very good
9	Cat, Mix, 4 months-old, M, 2.0 kg (SIU)	Left humerus; Fragmented oblique fracture on mid 1/3 of diaphyseal region; Unknown trauma	Limited open approach and reduction; Small size Manuflex®; Two K-wires 1.0 mm Ø for intramedullary fixation; Two K-wires 1.0 mm Ø on the proximal fragment (unilateral); Two K-wires 1.0 mm Ø on the distal fragment (unilateral); Non-PMMA	30	45	No complication	Very good
10	Dog, German Shepherd, 2.5 years-old, M, 42 kg (SIU)	Left tibia; Tibio-tarsal stabilization and shearing injury; Vehicular trauma	Closed reduction; Large size Manuflex®; Four K-wires 2.0 mm Ø on the tibia (bilateral); One K-wire 2.0 mm Ø on the tarsal bones (bilateral); Three K-wires 2.0 mm Ø on the metatarsus (bilateral); With PMMA	21	35	No complication	Good
11	Dog, Jack Russell Terrier, 1 years-old, M, 8.5 kg (AUFVM)	Left radius and ulna; Fracture on distal 1/3 of diaphyseal region Vehicular trauma	Open approaches and reduction; Medium size Manuflex®; Plate fixation (DCP 3.5 mm Ø, 10 holes, 8 cortical screws 3.5 mm Ø); Plate removal on postoperative 3rd months; Fracture line not enough (protection for refracture); Two K-wires 1.5 mm Ø on the proximal fragment (bilateral); Two K-wires 1.5 mm Ø on the distal fragment (bilateral); Non-PMMA	30	45	No complication	Very good

M: male, F: female, MN: male neutered ; AUFVM: Ankara University Faculty of Veterinary Medicine, SIU: Szent Istvan University, VSOC: Veterinary Surgery and Orthopaedic Center

Table 1. *Summary of data recorded for 22 cases (15 dogs and 7 cats) managed with MDEF (continued)*
Tablo 1. *MDEF uygulanan 22 olguya (15 köpek ve 7 kedi) ait bilgiler (devam)*

Case No	Signalment	Fracture Type, Localization, and History	Procedure and Apparatus Configuration	Radiologic Consolidation (Days after operation)	Fixator Removal (Days after operation)	Complications	Functional and Cosmetic Results
12	Dog, German shepherd; 1 years-old, M, 31.5 kg; (AUFVM)	Left radius and ulna; Angular deformity; Early closure to distal epiphyseal growth plate of radius; Unknown trauma	Open approaches and wedge osteotomy for radius, simple osteotomy for ulna; Cross pinning for osteotomy line; Large size; Manuflex®; Two K-wires 2.0 mm Ø on the proximal fragment of radius (one of them bilateral, other unilateral); One K-wire 2.0 mm Ø on the distal fragment of radius (bilateral); Two K-wires 2.0 mm Ø on the metacarpus (bilateral); With PMMA	40	55	No complication	Very good
13	Dog, German shepherd, 3 years-old, M, 38 kg (AUFVM)	Left tarso-metatarsal stabilization; Fall from height	First operation; Open approaches and arthrodesis; X pinning and tension band wire for tarso-metatarsal arhtrodesis; Large size Manuflex®; Second operation after 3 weeks; One K-wire 2.3 mm Ø on the tibia (bilateral); Two K-wires 2.0 mm Ø on the tarsal bones (bilateral); Three K-wires 1.8 mm on the metatarsus (bilateral); Three angular technique; With PMMA	50	60	No complication	Good
14	Cat, Mix, 2 years-old, M, 3 kg (SIU)	Left tibia; Shearing injury distal 1/3 of diaphyseal region of tibia; Vehicular trauma	Closed reduction; Small size Manuflex®; Two K-wires 1.2 mm Ø on the proximal tibia (bilateral); One K-wire 1.2 mm Ø on the tarsal bones (bilateral); Two K-wires 1.2 mm Ø on the metatarsus (bilateral); With PMMA	21	35	No complication	Very good
15	Cat, Mix, 4 years-old, F, 3 kg (AUFVM)	Left and right tibia; Tibio-tarsal luxation and fracture of malleolar region of tibia (right side is open fracture; Grade II and left side is closed); Fall from height	Closed reduction; Left and right side; Small size Manuflex®; Four K-wires 1.5 mm Ø on the tibia (bilateral); One K-wire 1.0 mm Ø on the tarsal bones (bilateral); Three K-wires 1.0 mm Ø on the metatarsus (bilateral); With PMMA	35	45	No complication	Satisfactory
16	Dog, Mix, 5 months-old, M, 30 kg (SIU)	Left tibia and fibula fracture; Fragmented oblique fracture on mid 1/3 of diaphyseal region; Vehicular trauma	Open reduction; Large size Manuflex®; Two K-wires 2.0 mm Ø on the proximal fragment of tibia (unilateral); Three K-wires 2.0 mm Ø on the distal fragment of tibia (unilateral); Tension band wire on tibial fracture line; With PMMA	30	45	No complication	Very good
17	Dog, Mix, 6 months-old, F, 16 kg (VSOC)	Left tibia; Fragmented oblique fracture on proximal 1/3 of diaphyseal region; Vehicular trauma	Open reduction; Large size Manuflex®; Two K-wires 2.0 mm Ø on the proximal fragment (unilateral); Three K-wires 2.0 mm Ø on the distal fragment (unilateral); Non-PMMA	35	45	No complication	Very good
18	Dog, Mix, 9 months-old, F, 18 kg (AUFVM)	Right radius and ulna + right femur; Fragmented simple fracture on radius; Vehicular trauma	Open reduction; Intramedullary pinning for femur osteosenthesis; Medium size Manuflex®; For tibial fracture; Two K-wires 2.0 mm Ø on the proximal fragment (unilateral); Three K-wires 2.0 mm Ø on the distal fragment (unilateral); Non-PMMA	30	38	No complication	Very good
19	Dog, Mix, 3 months-old, F, 10 kg (SIU)	Right tibio-tarsal stabilization and m. quadriceps contracture; Vehicular trauma	Open reduction; Medium size Manuflex®; Two K-wires 1.5 mm Ø on the femur (unilateral); Four K-wires 1.5 mm Ø on the tibia (unilateral); Two K-wires 1.5 mm on the tarsal bones (unilateral); Non-PMMA	-	21	Pin tract infection on the postoperative 4th day	Satisfactory
20	Dog, Mix, 8 months-old, F, 20 kg (SIU)	Right tibio-tarsal stabilization and shearing injury; Vehicular trauma	Open reduction; Large size Manuflex®; One K-wire 1.5 mm Ø on the metatarsus (unilateral); Four K-wires 2.0 mm Ø on the tibia (unilateral); One K-wire 2.0 mm on the tarsal bones (unilateral); The second Manuflex® apparatus at the level of the third and fourth wire of tibia; Non-PMMA	-	21	No complication	Good
21	Dog, Mix, 2 years-old, M, 20 kg (AUFVM)	Right mandible multiple fractures; Vehicular trauma	Open reduction; Small size Manuflex®; Two K-wires 1.0 mm Ø on the body of mandible, between the fourth premolar and third molar teeths; Three K-wires 1.0 mm Ø between the third molar tooth and ramus mandible; With PMMA	27	35	No complication	Very good
22	Dog, Labrador retriever, 6 years-old, F, 25 kg (VSOC)	Right mandible simple fracture; Vehicular trauma	Open reduction; Small size Manuflex® Four K-wires 1.5 mm Ø on the body of mandible and ramus mandible; With PMMA	30	40	No complication	Very good

M: male, F: female, MN: male neutered ; AUFVM: Ankara University Faculty of Veterinary Medicine, SIU: Szent Istvan University, VSOC: Veterinary Surgery and Orthopaedic Center

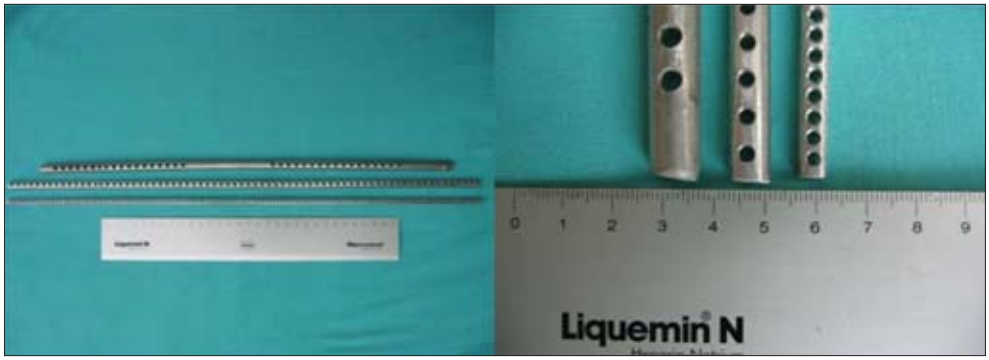

Fig 1. Manuflex Disposible External Fixator types (large, medium and small size)

Şekil 1. Manuflex Eksternal Fiksatör çeşitleri (büyük, orta ve küçük boy)

Fig 2. Close reduction technique in a cat with left radius simple fracture (case 6)

Şekil 2. Sol radiusta basit kırık bulunan bir kedide kapalı redüksiyon tekniği (olgu 6)

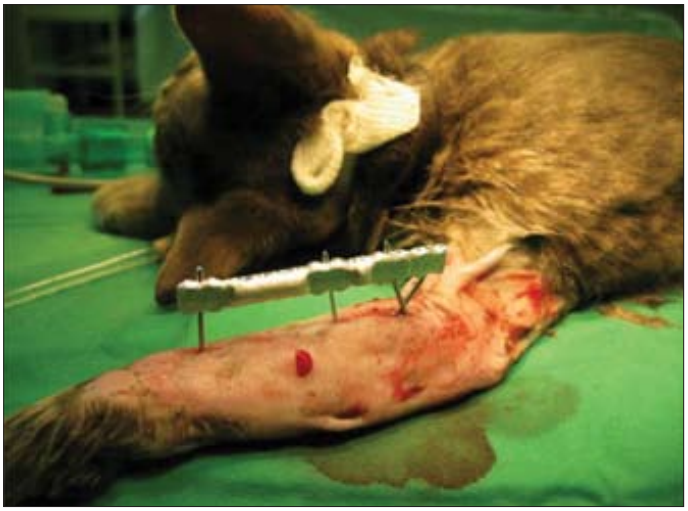

Fig 3. The application of crimping forceps

Şekil 3. Sıkıştırma forsepsinin uygulanışı

Then pin insertion site within the fixator was compressed by a special crimping forceps *(Fig. 3)*. MDEF is a unique system in all external fixators with clamp system due to crimping issue. In some animals, PMMA was covered on the Manuflex apparatus completely *(Fig. 4,b)*. In 2 dogs with mandibular fractures, Kirschner wires were placed in the mandible and the fragments were transfixed with them. The ends of the pins were incorporated in apparatus laterally. PMMA did not use for both dogs.

Postoperative Follow-up

Radiograms were taken after surgery. In the postoperative period, the owners were warned that cases should be taken to avoid stairs and should not play with other animals for at least 21 days. The skin of the area where the pins exit was cleaned daily with antiseptic solutions and tetracyclin spray was applied to the area. Carprofen (Rimadyl, 2 mg/kg, bid, orally, Pfizer, USA) was administered for 5 days, amoxicillin-clavulanic acid (Amoklavin forte, 20 mg/kg, bid, orally, Deva, Türkiye) was used as a broad spectrum antibiotic for 7 days. Owners were called for clinical and radiological assessments weekly until fracture healings were complete and the devices were removed. Fixator was removed under sedation using a wire twisting forceps at the suitable time of fixator removal. The area was cleaned with warm povidon iodine antiseptic solutions daily for 7 days.

RESULTS

In the study, mean age of dogs was 2.4 years (range, 2 months-5 years) and cats was 5 years (range, 4 months-11 years). Mean body weight was 21.8 kg for dogs, 3.5 kg for cats. There were 6 female and 9 male dogs, 3 female and

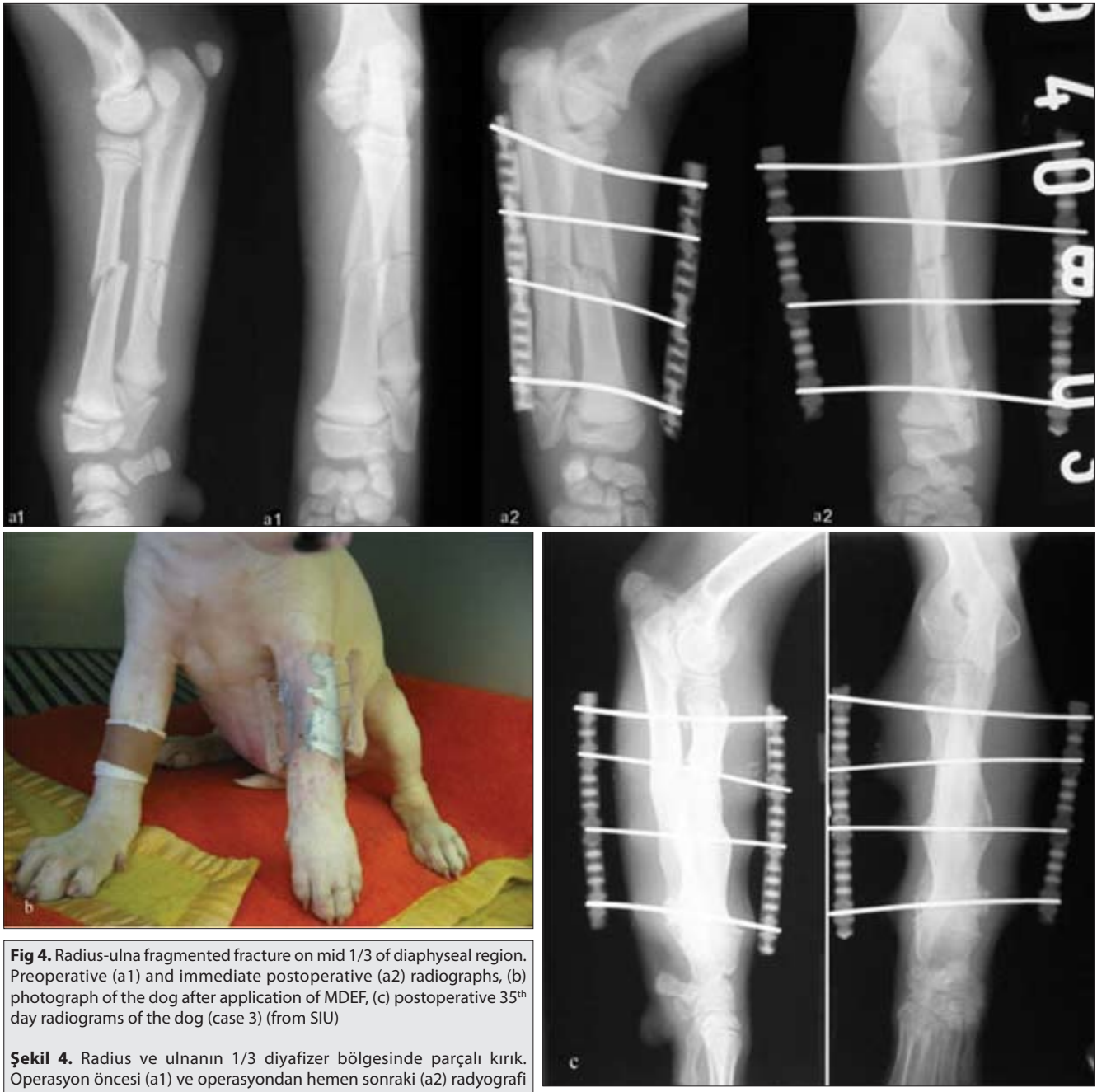

Fig 4. Radius-ulna fragmented fracture on mid 1/3 of diaphyseal region. Preoperative (a1) and immediate postoperative (a2) radiographs, (b) photograph of the dog after application of MDEF, (c) postoperative 35th day radiograms of the dog (case 3) (from SIU)

Şekil 4. Radius ve ulnanın 1/3 diyafizer bölgesinde parçalı kırık. Operasyon öncesi (a1) ve operasyondan hemen sonraki (a2) radyografi görüntüsü, (b) MDEF yerleştirilen köpeğin fotoğraf görüntüsü, (c) köpeğin operasyon sonrası 35. gün radiografisi (olgu 3) (SIU'dan)

4 male cats. Orthopaedic problems included fractures of humerus (n=1) *(Fig. 5)*, tibia (n=4) *(Fig. 6)* and radius-ulna (n=4), angular deformity (with radius-ulna fracture), tibio-tarsal luxations (n=3), tarso-metatarsal fracture (n=1), mandibular fractures (n=2) in dogs; and fractures of tibia (n=2), radius (n=1) and humerus (n=2), radio-carpal luxation (n=1), bilateral tibio-tarsal luxation with malleolar tibial fracture (n=1) *(Fig. 7)* in cats. Fractures were caused by vehicular trauma (11 dogs, 2 cats), falling from height (1 dog and 3 cats), compression injury (1 dog) and unknown trauma (2 dogs and 2 cats) *(Table 1)*.

Mean time to fixator removal was 39 days (range, 21-60 days) in dogs, 42 days (range 35-45 days) in cats. Two dogs (case 5 and 19) had pin tract infection as complication on the postoperative period. There were no other complications such as vein or nerve damage, malunion, nonunion or osteomyelitis. In postoperative period, it was observed that all cases had tolerated their apparatus well and could use their limbs immediately, and they did not form any reaction.

DISCUSSION

External skeletal fixation (ESF) has become a well established treatment technique for many traumatic and degenerative orthopaedic problems in dogs and cats.

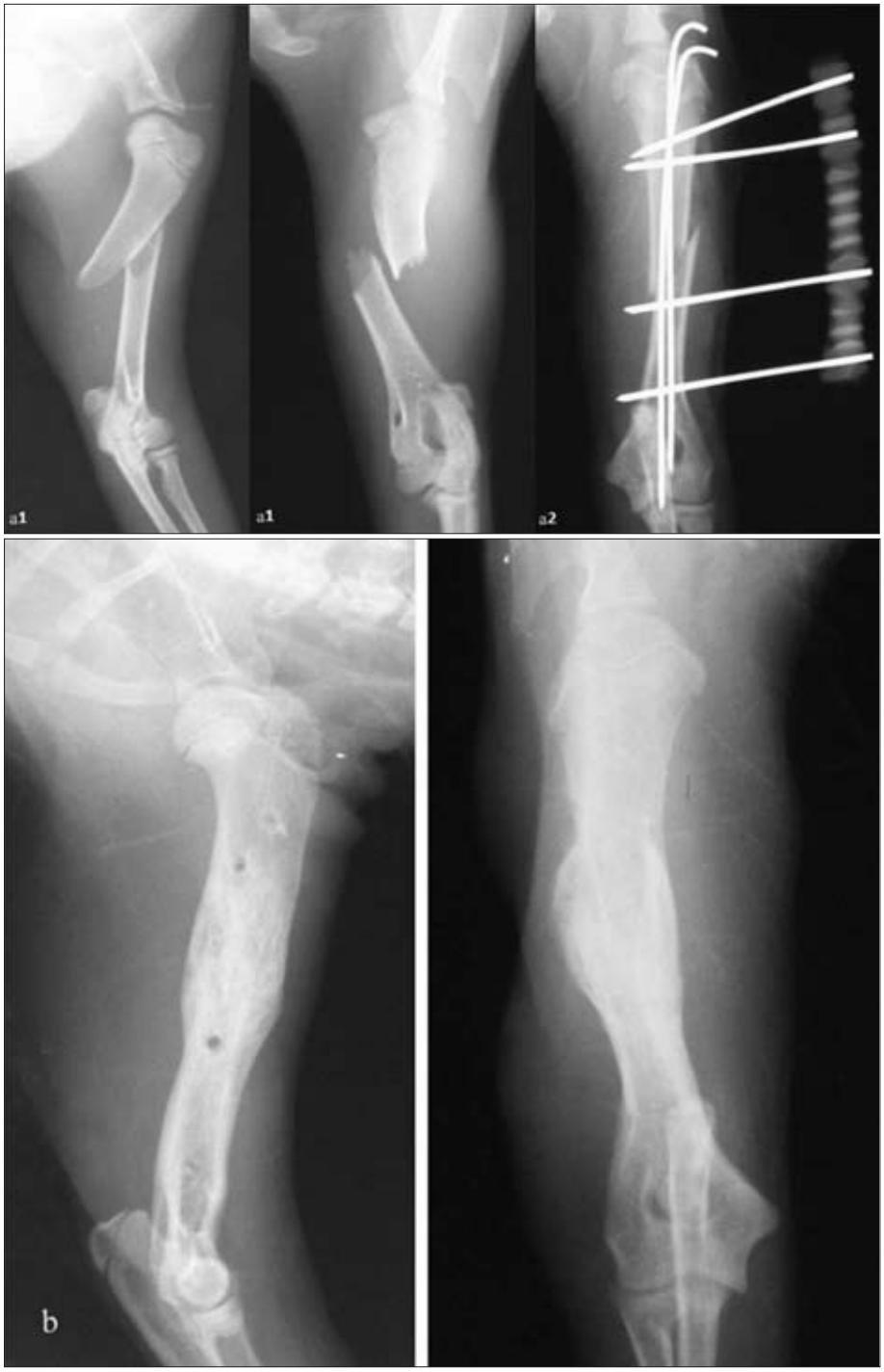

Fig 5. Humerus oblique fracture. Preoperative (a1) and immediate postoperative (a2) radiographs. (b) radiographs of the humerus after MDEF removal on the postoperative 45th day (case 7) (from VSOC).

Şekil 5. Humerusta oblik kırık. Operasyon öncesi (a1) ve operasyon sonrası (a2) radyografileri, (b) humerusun operasyon sonrası 45. gün MDEF uzaklaştırıldıktan sonraki radyografisi (olgu 7) (VSOC'dan)

The frame type, configuration, implant size, pin types and numbers are important for the apparatus accomodation to the bone. They can be applied in either open or closed reduction, used alone or combined with internal fixation. Especially the past decades, clinical and experimental studies have led to technological advances and modifications in external fixator design and techniques [4,6]. Considering all these circumstances, as a new external fixator, MDEF has been designed. In this study, in different orthopaedic problems, MDEF was evaluated with its surgical outcomes in dogs and cats.

ESF is commonly used for the long bone fracture repair. All kinds of frames can easily be applied to tibia and radius. Type II frames may be applied for providing rigid stabilization in tibial and radial fractures. Type III frames may be used for fractures in large dogs and for comminuted fractures in which limited exists between the fixator and fragments [5,8,9]. In the study, type I and type II frames were used for tibia and radius fractures both in dogs and cats. All animals either with simple or fragmented fractures could tolerate their MDEF well and they could use their limbs without any problems.

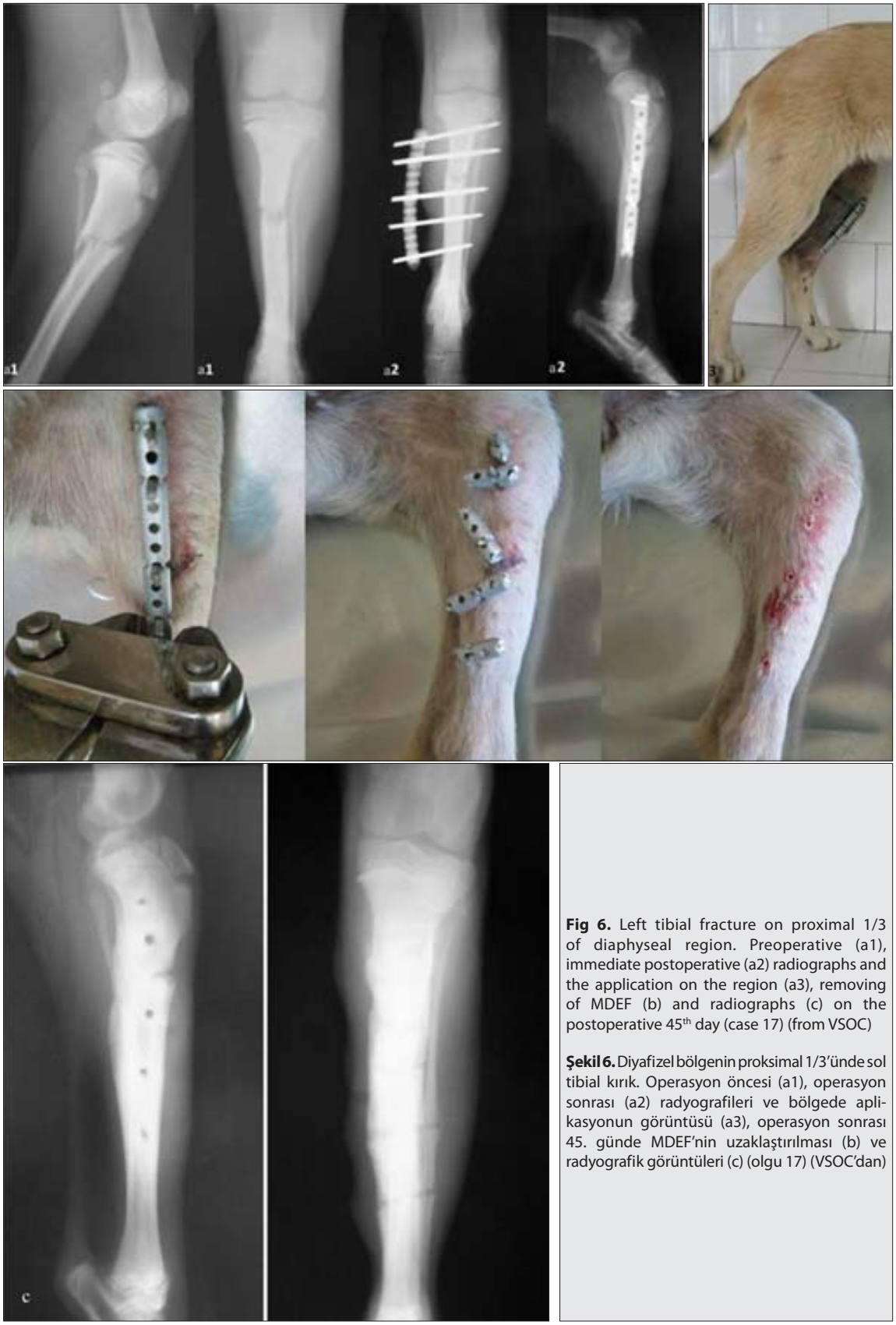

Fig 6. Left tibial fracture on proximal 1/3 of diaphyseal region. Preoperative (a1), immediate postoperative (a2) radiographs and the application on the region (a3), removing of MDEF (b) and radiographs (c) on the postoperative 45th day (case 17) (from VSOC)

Şekil 6. Diyafizel bölgenin proksimal 1/3'ünde sol tibial kırık. Operasyon öncesi (a1), operasyon sonrası (a2) radyografileri ve bölgede aplikasyonun görüntüsü (a3), operasyon sonrası 45. günde MDEF'nin uzaklaştırılması (b) ve radyografik görüntüleri (c) (olgu 17) (VSOC'dan)

Mandibular fractures are seen less frequently in dogs than in cats. They generally involve the premolar or the molar regions in dogs. Methods of its fixation include plate fixation, external fixators, transverse pinning and wire sutures. In comminuted fractures of the ramus, the use of an external fixator may be more applicable

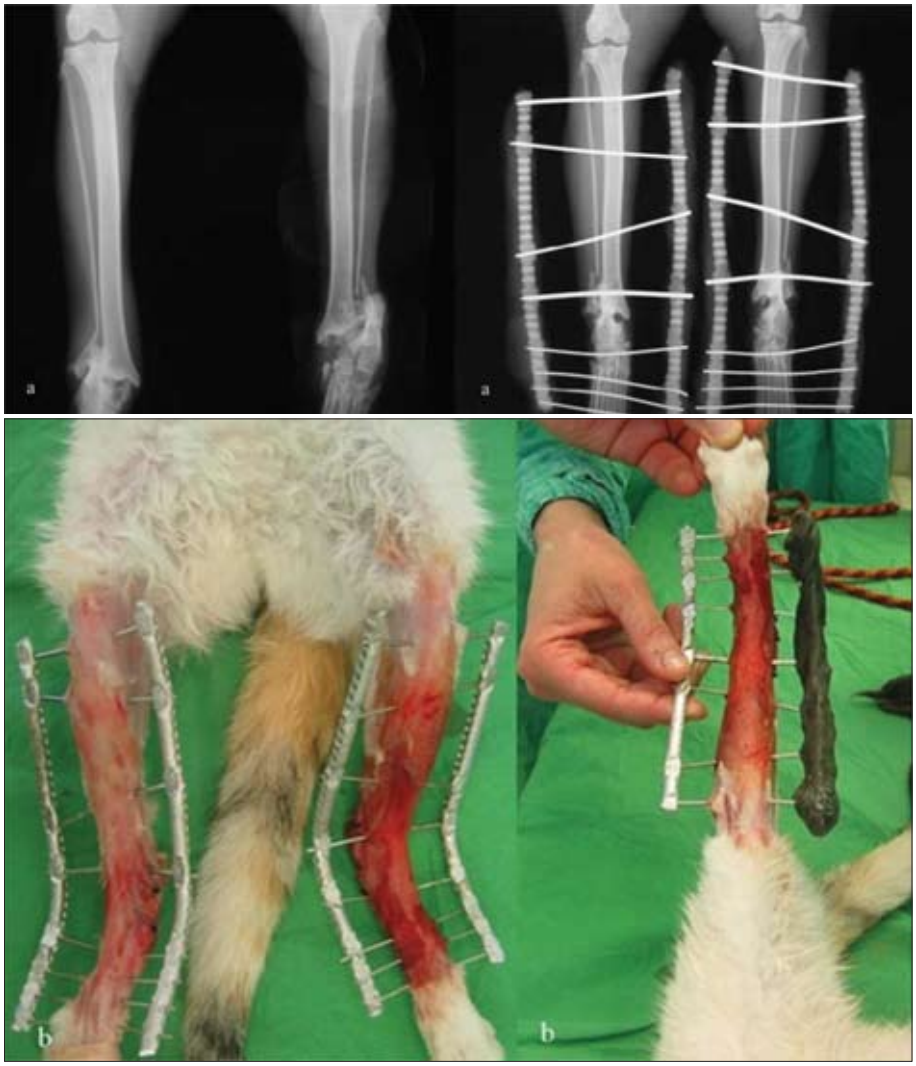

Fig 7. (a) Radiograms of bilateral tibio-tarsal luxation and MDEF application in a cat. (b) Photograph of the cat after application of MDEF with PMMA (case 15) (AUFVM)

Şekil 7. (a) Bir kedide bilateral tibio-tarsal luksasyon ve uygulanan MDEF'nin radyografik görünümü. (b) Aynı kedinin PMMA ile birlikte MDEF uygulandıktan sonraki fotoğraf görüntüsü (olgu 15) (AUFVM'dan)

than a plate. Also the shape of the mandible in breeds like brachicephalics may make use of a traditional connecting bar on the fixator difficult. In this situation, the pins or Kirschner wires are driven into the bone fragments and joined on the lateral aspect of the mandible with cement or dental acrylic [10,11]. In this study, MDEF applied to the bone fragments easily and satisfactorily. Because of its lightness, dogs might use their mandibles immediately after the operation. Although there were only two dogs, fixator availability and the absence of any complications after surgery were assessed as the advantage of the apparatus might be in mandibular fractures in dogs.

Close reduction technique is less traumatic to the soft tissues, but can not yield an adequate alignment as open reduction [6]. In this study, reduction technique was chosen according to the bone fractures and also fragments positions.

For external fixation systems, acrylics (methacrylates, epoxy putties) can be used instead of Kirschner clamp. Especially PMMA are available as bone cements and can be used either as a liquid poured into tubing placed over the fixation pins, or at the dough stage rolled into a cylinder then pressed around the pins [12]. It is lighter, less bulky, can be customized for any size and shape of bone, allows flexibility for pin placement and cheaper than using Kirschner external skeletal fixators. According to studies, when acrylic columns compared to Kirschner external fixators, it is improved more stiff [12-14] especially in cats and small breed or low weighted dogs [15]. MDEF is similar to PMMA in some of its advantages. It is light and can be in any shape and size. It can be adapted for any number and size of pins. Its holes can be pressed and pins are stabilized to the fixator system. In the study, MDEF was used alone or also used with PMMA. Using fixator with or without PMMA was not a standard procedure. But in general PMMA was preferred in operations for increasing frame stiffness, in which large size MDEF was used.

Acrylic external fixator systems include toxic fumes that are produced during polymerization and acrylic can reach high temperatures (range between 50 to 100°C) that can cause bone necrosis. Because of the risk the skin-acrylic column distance must be adjusted [14,15]. PMMA-

MDEF combination did not cause a risk and results were satisfactory.

ESF postoperative complications are generally soft tissue healing problems, pin tract infections and fixator failures [16]. In the study, pin tract infections were seen as a complication in 2 cases (case no. 5 and 19) because of the poor wound hygiene and inconsistent use of antibiotic. It was treated with oral antibiotics and daily pin tract care successfully in these two cases.

In previous studies, premature loosening of the pins is the other most frequent complication due to the stress on the pins both in cats and dogs, especially in heavy animals [17]. In the present study, the appropriate pin size and numbers were selected that would ensure adequate stiffness at the fracture site and prevent fragment motion, to prevent this complication. In general, with large size MDEF, 3-4 pins were applied on each fragment; with small and medium size MDEF, 2-3 pins were applied on each fragment. Secondly, special design fixator Manuflex® allows latitude in fixation pin placement and pins do not need to be aligned in the same longitudinal plane. Manuflex® shape, size and diameter of pin holes could be designed perfectly according to the bone, fracture type and localization.

In the study, high torque drill was used for inserting fixation pins into the bone. This was preferred, because previous studies show that high speed drills (or with low speed and excessive pressure) may cause thermal necrosis in the bones and because of the holes excessive enlargement for the pins resulting in premature loosening [18].

Soft tissue swelling is frequently seen between connecting bar and skin, postoperatively. This is because of the tissue inflammation around the pin-skin contact area. However, increasing the distance between the bone and the connecting bar decreases the strength of the system [7]. In the present study, sufficient distance was left between the bar and skin, so that it was not encountered any consequences.

In conclusion, MDEF can be an alternative system compared to other external fixators, because of its easier and less complicated application, lighter frame weight, benefit of rigid fixation and stability, and being more economic. It can be easily customized to accommodate all fracture types.

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RESEARCH ARTICLE

Effect of Knockdown TAGLN on the Migration Capacity of Wuzhishan Pig's Bone Marrow Mesenchymal Stem Cells [1]

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Summary

Mesenchymal stem cells (MSCs) are multipotent adult stem cells, can easily be isolated from the bone marrow and subsequently expand *in vitro*. Bone marrow MSCs (BMMSCs) appear to be poorly immunogenic, and are the most widely used MSCs in tissue regenerative medicine, and the migration capacity of BMMSCs is the key determinant for the efficiency of their regenerative therapy. The Wuzhishan pig (WZSP) is characterized by its physiological and general biochemical indices very similar to humans, and may be potential organ donors for human being owing to its size. In this study, the BMMSCs from WZSP were used to study their migration capacity which regulated by TAGLN *in vitro*. The specific short hairpin RNA (shRNA) for TAGLN was designed to knock down TAGLN gene, and the scratch assay and transwell migration assay was employed to estimate effect of knockdown TAGLN on the migration capacity of BMMSCs from WZSP. The results showed that the specific shRNA for knocking down TAGLN efficiently was found for the BMMSCs, and there was a significant effect on the migration capacity of the BMMSCs from WZSP with knockdown TAGLN *in vitro*. In conclusion, TAGLN was an important factor in maintaining the migration capacity of the BMMSCs, which may be benefit for the BMMSCs from WZSP to be used in regenerative therapy for human being.

Keywords: *Bone marrow mesenchymal stem cells, Migration capacity, TAGLN, Wuzhishan pig*

TAGLN Geninin Bloke Edilmesinin Wuzhishan Domuz Kemik İliği Mezenkimal Kök Hücrelerinde Göç Etme Kapasitesi Üzerine Etkisi

Özet

Mezenkimal kök hücreleri (MSCs) kemik iliğinden kolayca izole edilebilen ve takibinde *in vitro* olarak çoğalabilen mültipotent olgun kök hücreleridir. Kemik iliği MSC (BMMSC)'lerinin zayıf immunojenik özellikte olduğu görülmekte olup doku yenileme tedavisinde en sıklıkla kullanılan MSC'lerdir ve BMMSC'lerin göç etme kapasiteleri yenileme tedavisindeki başarının anahtarıdır. Wuzhishan domuzu (WZSP) fizyolojik ve genel biyokimyasal belirtileri açısından insana oldukça benzerdir ve boyutu düşünüldüğünde insana organ nakli bakımından oldukça yüksek potansiyele sahiptir. Bu çalışmada WZSP'den elde edilen BMMSC'lerin TAGLN tarafından düzenlenen göç kapasiteleri *in vitro* olarak çalışıldı. TAGLN için spesifik kısa hairpin RNA (shRNA) TAGLN genini bloke etmek için dizayn edildi. Scratch metodu ve transwell göç metodu bloke TAGLN genin WZSP'den elde edilen BMMSC'lerin göç kapasiteleri üzerine etkisini araştırmak amacıyla uygulandı. Sonuçlar TAGLN genini bloke etmede kullanılan spesifik shRNA'nın BMMSC için etkili olduğunu ve *in vitro* ortamda TAGLN blokajının WZSP'den elde edilen BMMSC'lerin göç kapasiteleri üzerine anlamlı bir etkisinin var olduğunu gösterdi. Sonuç olarak TAGLN BMMSC'lerde göç kapasitesini sağlamada önemli bir faktördür. Bu durum; WZSP'den elde edilen BMMSC'lerin insanlarda yenileme tedavilerinde kullanılmasına olanak sağlayabilir.

Anahtar sözcükler: *Kemik iliği mezenkimal kök hücreleri, Göç kapasitesi, TAGLN, Wuzhishan domuzu*

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INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent adult stem cells, and can easily be isolated from the bone marrow (BM) and subsequently expand *in vitro*. MSCs can differentiate into mesodermal lineages (osteogenic, adipogenic, and chondrogenic lineages), and also can differentiate towards endodermal or ectodermal derivatives. In addition, MSCs can secrete many bioactive molecules which affect local cellular environment with strong anti-inflammatory and immunosuppressive properties [1]. The bone marrow MSCs (BMMSCs) are the best characterized adult stem cells, which has a great therapeutic potential in tissue engineering, regenerative medicine and autoimmune diseases [2]. BMMSCs constitutively express low levels of major histocompatibility complex (MHC) class I molecules and no MHC class II molecules, thus appear to be poorly immunogenic. There is no expression of co-stimulatory molecules in BMMSCs, including CD40, CD80, or CD86 which are involved in the activation of T cell for transplant rejection. It is due to the easy isolation of BMMSCs and the reports of differentiation into extra-mesodermal cell types, which has made BMMSCs the most widely used MSCs in cell therapy for pre-clinical and clinical trials of a variety of diseases [3].

TAGLN is a gene that encodes the Transgelin protein in humans, is also known as SM22 and WS3-10. Transgelin is ubiquitous in vascular and visceral smooth muscle, and is an early marker of smooth muscle differentiation. Transgelin is also an actin cross-linking/gelling protein which is involved in calcium interactions and regulates contractile properties. Transgelin contains a C-terminal calponin-like module and an upstream positively charged amino acid region which is required for actin binding [4]. It is by stabilizing the cytoskeleton through binding to actin that Transgelin plays a role in cell differentiation, cell migration, cell invasion and matrix remodeling [5,6]. Transgelin is upregulated in repopulating mesangial cells, and promotes their migratory and proliferative repair response after injury [7].

The Wuzhishan pig (WZSP) is characterized by its small adult size, and has a mature body weight of only approximately 30 kg with physiological and general biochemical indices very similar to human being. The heart, small intestine and guts of WZSP are similar to those of human body, and the pig also is a unique pharmacology and toxicology model for the investigation of human health [8]. However, it is not clear whether knockdown TAGLN has an effect on the migration capacity of BMMSCs from WZSP. In this study, the specific short hairpin RNA (shRNA) for TAGLN gene was designed to explore the influence for expression of TAGLN in BMMSCs through the quantitative real time PCR (qRT-PCR) and Western blot analysis. In addition, the effect of Transgelin on the migration capacity of BMMSCs from WZSP was evaluated through scratch assay and transwell migration assay.

MATERIAL and METHODS

Culture of Porcine BMMSCs

The BMMSCs were isolated from femur and tibia of WZSP, and flow cytometric analysis, adipogenic and osteogenic differentiation had been used to evaluate the character as described previously [9]. The BMMSCs were cultured in D-MEM/F-12 (Gibco) medium with 10% (v/v) fetal bovine serum (FBS, Gibco) containing penicillin/streptomycin (50 IU/ml, 50 μg/ml), and incubated at an atmosphere of 5% CO_2 in air at 37°C. Media were changed every other day. All animals were handled according to the animal protocols approved by the Chinese Academy of Agricultural Sciences Institutional Animal Care and Use Committee.

Design and Assessment of Specific Short Hairpin RNA for TAGLN Gene

Specific shRNA sequences for TAGLN gene were designed based on TAGLN gene order from NCBI Gene database (Gene ID 6876) and design principle for shRNA [10], and synthesized by Shanghai GenePharma Co, Ltd. *(Table 1)*. The negative control was also designed and synthesized, which had no homology to TAGLN gene sequences with the same composition of nucleic acids to the specific shRNA sequences. The expression vectors encoding different shRNA included TAGLN-sus-246, TAGLN-sus-473, TAGLN-sus-496 and TAGLN-sus-626. The above four expression vectors and non-specific transfection vector of TAGLN-NC (Control) were utilized to transfect the BMMSCs using X-tremeGENE HP DNA transfection reagent following the manufacturer's protocol (Liposomal transfection reagent kit, Roche). The expression of TAGLN mRNA was detected by qRT-PCR assay after post-transfection for 24 h, and the expression vector with best interference effect was selected and used in the following experiments. The best vector (TAGLN-sus-473) was recovered after digested with the restriction endonuclease ApaLI (New England Biolabs).

The linearized shRNA vector with no specific transfection to the BMMSCs was named as shRNA-NC (Control), and the linearized shRNA vector with best interference effect to the BMMSCs was shRNA-473. The experimental group was the BMMSC treated with the transfection vector of shRNA-473, and the control group was the BMMSC treated with shRNA-NC. The G418 (200 μg/ml) was added to the culture medium for the BMMSCs after the cells had been transfected with shRNA-NC and shRNA-473 for 36 h, and then the culture media were changed once every two days. After elimination of non-transfected cells and selection for 14 days, the stable cell lines transfected with shRNA-NC and shRNA-473 were obtained.

Table 1. *The sequence of specific short hairpin RNA for TAGLN*
Tablo 1. *TAGLN için spesifik kısa hairpin RNA'nın sekansı*

Vector	Sequence Name	Sequences
TAGLN-sus-246	S	5′-CACCGCTGGTGGAGTGGATCATAGTTTCAAGAGAACTATGATCCACTCCACCAGCTTTTTTG-3′
	A	5′-GATCCAAAAAAGCTGGTGGAGTGGATCATAGTTCTCTTGAAACTATGATCCACTCCACCAGC-3′
	Transcript	GCTGGTGGAGTGGATCATAGTTTCAAGAGAACTATGATCCACTCCACCAGCTT
TAGLN-sus-473	S	5-CACCGCGTCACCAAGACTGACATGTTTCAAGAGAACATGTCAGTCTTGGTGACGCTTTTTTG-3′
	A	5′-GATCCAAAAAAGCGTCACCAAGACTGACATGTTCTCTTGAAACATGTCAGTCTTGGTGACGC-3′
	Transcript	GCGTCACCAAGACTGACATGTTTCAAGAGAACATGTCAGTCTTGGTGACGCTT
TAGLN-sus-496	S	5′-CACCGCAGACTGTTGACCTCTTCGAATTCAAGAGATTCGAAGAGGTCAACAGTCTGTTTTTTG-3′
	A	5′-GATCCAAAAAACAGACTGTTGACCTCTTCGAATCTCTTGAATTCGAAGAGGTCAACAGTCTGC-3′
	Transcript	GCAGACTGTTGACCTCTTCGAATTCAAGAGATTCGAAGAGGTCAACAGTCTGTT
TAGLN-sus-626	S	5′-CACCGCCAGGAGCATAAGAGGGAATTTCAAGAGAATTCCCTCTTATGCTCCTGGTTTTTTG-3′
	A	5′-GATCCAAAAAACCAGGAGCATAAGAGGGAATTCTCTTGAAATTCCCTCTTATGCTCCTGGC-3′
	Transcript	GCCAGGAGCATAAGAGGGAATTTCAAGAGAATTCCCTCTTATGCTCCTGGTT

RNA Extraction and qRT-PCR Assay

The MicroElute Total RNA Kit (OMEGA bio-tek, USA) was employed to extract the total RNA, and the RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas, International Inc., Ontario, Canada) was used to synthesize the cDNA. The qRT-PCR was performed using TaKaRa RNA PCR Kit (AMV), and primer sequences of TAGLN (forward: 5′-CCCATCCTGTCTGTCCAAGT-3′, reverse: 5′-CCAGCTCCTC GTCGTACTTC-3′) and GAPDH (forward: 5′-GTGAAGGTCG GAGTGAACG-3′, reverse: 5′-CTCGCTCCTGGAAGATGGTG -3′) were designed and synthesized by Shanghai Sangon Biotech. GAPDH was used as an internal control gene to calculate the relative mRNA expression levels.

Western Blot

The BCA Protein Assay Kit (Thermo Scientific) was used to measure the protein concentration with bovine serum albumin as the standard after the BMMSCs were washed and lysed. Lysates (10 µl/lane) were separated using 12% SDS-PAGE followed by transferring proteins to 0.22 µm PVDF membranes (Millipore Corp, Bedford, MA, USA). The PVDF membranes were blocked in 5% non-fat milk (w/v) at 4°C overnight. Transgelin was detected by Western blot using a Transgelin primary antibody (Abcam, Anti-SM22 alpha antibody, ab14106) to probe the membrane. After washed with TBS-T, the membrane was incubated with second antibody. The immunoreactive bands were detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce) and recorded on x-ray films (Fuji Medical, Tokyo, Japan). The GAPDH antibody (MBL International Corp) was used to monitor variation in loading of samples.

BMMSCs Migration Analyzed by Scratch Assay and Transwell Migration Assay

The migration capacity of BMMSCs was evaluate using scratch assay and transwell migration assay. The BMMSCs were cultured in 60 mm culture plate to confluence and treated with 10 µg/ml mitomycin-C for two hours. The growth arrested BMMSCs were transferred into 6 well-plate in a density of 3×10^6 per well, and the 'scratches' were made using a 10 µl pipette tip along the bottom of the plate after cultured for six hours, and then the BMMSCs were cultured for another 36 h. At the selected time points (0 h, 6 h, 12 h, 24 h and 36 h), the images of the BMMSCs were acquired from the culture dishes under phase contrast microscope.

The BMMSCs at a density of 4×10^6 cells/ml were placed in the apical well of the transwell assembly (6.5 mm diameter inserts, 8.0 µm pore size, Corning Costar, NY) with 100 µl serum-free medium, and 800 µl culture medium containing 10% FBS was filled into lower chambers as a source of chemoattractants. The nucleus of BMMSCs was stained with Hochest 33342 after incubation at 37°C for 12 h, and the number of migrating cells was ascertained through calculating nine fields per well at random under the fluorescence microscope.

Statistical Analysis

The experiment was repeated at least three times and the results were expressed as the mean ± SD. Statistical analyses were performed by Student *t* test. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Assessment of Specific Short Hairpin RNA for TAGLN

After the BMMSCs were transfected by the vectors of TAGLN-NC (Control), TAGLN-sus-246, TAGLN-sus-473, TAGLN-sus-496 and TAGLN-sus-626, the expression of TAGLN mRNA by the BMMSCs was evaluated through qRT-PCR assay. The result showed that the expression of TAGLN

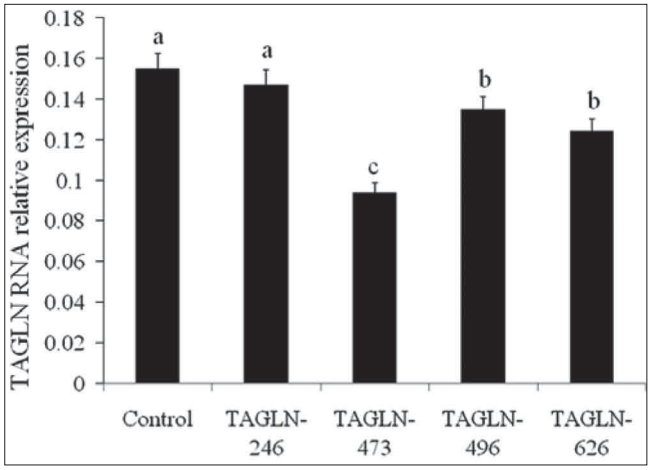

Fig 1. The expression level of TAGLN in BMMSCs through qRT-PCR analysis. The groups of TAGLN-246, TAGLN-473, TAGLN-496, TAGLN-626 and Control were the BMMSCs which were transfected with the vectors of TAGLN-sus-246, TAGLN-sus-473, TAGLN-sus-496, TAGLN-sus-626 and TAGLN-NC. Significant differences (P<0.05) are indicated by different letters

Şekil 1. qRT-PCR analisi ile elde edilen BMMSC'lerin TAGLN'lerinin ekspresyon seviyesi. TAGLN-246, TAGLN-473, TAGLN-496, TAGLN-626 ve kontrol grupları TAGLN-sus-246, TAGLN-sus-473, TAGLN-sus-496, TAGLN-sus-626 ve TAGLN-NC ile transfekte edilen BMMSC'leridir. Anlamlı farklılıklar (P<0.05) farklı harfler ile belirtilmiştir

in the BMMSCs transfected with the vectors of TAGLN-sus-473 and TAGLN-sus-496 TAGLN-sus-626 were lower than that transfected with the vector of TAGLN-NC (Control) (P<0.05), and the specific shRNA for TAGLN induced TAGLN knockdown in the BMMSCs efficiently *(Fig. 1)*. The vector of TAGLN-sus-473 was the most efficient one among the four experimental groups in knockdown TAGLN.

It was found in *Fig. 2A* that the linearized shRNA vectors for shRNA-NC (Control) and TAGLN-sus-473 (shRNA-473) were reclaimed efficiently. As illustrated in *Fig. 2B* that all BMMSCs were observed with fluorescence, and the stable cell lines were obtained after transfected with the vectors of shRNA-NC and shRNA-473 through selection for 14 days, and non-transfected cells were removed. It was showed in *Fig. 2C* that there was a significant difference between the group of shRNA-NC (Control) and shRNA-473 by qRT-PCR analysis (P<0.05), and TAGLN was knocked down effectually in the BMMSCs which were transfected with the shRNA vector of shRNA-473. There was an obviously decreasing expression of Transgelin in the group of shRNA-473 comparing with that in the group of shRNA-NC (Control) through Western blot analysis, but the GAPDH protein was expressed equally in both the groups of shRNA-NC (Control) and shRNA-473 *(Fig. 2D)*.

The Role of Transgelin on BMMSCs Migration

The scratched BMMSCs transfected with the vector of shRNA-NC (Control) confluenced after cultured for about 36 h, but the scratched BMMSCs transfected with the vector of shRNA-473 did not confluence after cultured for about 36 h *(Fig. 3)*. The scratched BMMSCs transfected with the vector of shRNA-473 migrated more slowly than that transfected with the vector of shRNA-NC. Therefore the scratch assay analysis suggested that TAGLN knockdown degraded the migration capacity of BMMSCs significantly.

It was demonstrated through the transwell migration assay that the number of BMMSCs that migrated across the filters with TAGLN knockdown was 85.30±7.13 (shRNA-473), but the number that migrated across the filters with the normal TAGLN expression was 120.53±16.32 (Control), so the BMMSCs with TAGLN knockdown had inferior ability of transwell migration comparing with the normal BMMSCs *(Fig. 4)*. It was obvious that Transgelin may play a key role in BMMSCs migration.

DISCUSSION

MSCs are multipotent stromal cells which can easily be isolated from the bone marrow. BMMSCs can differentiate into osteoblasts, adipocytes, chondrocytes, myocyates and many other tissues, and characteristically lack hematopoietic antigens, MHC class II, and endothelial antigens, which enable BMMSCs to be used as cell-based regenerative therapy for large bone defects, maxillofacial skeletal reconstruction, cardiovascular and spinal cord injury and other defects without immune rejection [11]. *In vitro* studies manifest that MSCs can inhibit a variety of immune cell functions and cell proliferation of T cells, B cells, natural killer cells and dendritic cells [12]. BMMSCs may play a key role in cell-based regenerative therapy for bone marrow, skin, heart, and corneal transplantation, graft versus host disease, hepatic and renal failure, lung injury, multiple sclerosis, rheumatoid arthritis, diabetes and lupus diseases.

In this study, the BMMSCs from WZSP were used to explore their migration capacity. WZSP is considered useful for medical and veterinary research due to its physiological and general biochemical indices similar to human being and its small size. It was reported that there was a small amount of porcine endogenous retrovirus and a lack of type C retroviruses in the WZSP genome, and pigs strongly resemble human being through investigating gene evolution in the pig and the pig counterparts of human druggable domain and disease related genes, which indicated that this pig may be used as potential organ donors for human being with low risk of pig-to-human infection during xenotransplantation [8].

RNA interference (RNAi) is a general method to silence gene expression in many organisms. It is possible that creation of continuous cell lines and transgenic animals through shRNAs expression method in which suppression of a target gene is stably maintained [13]. In this study, the specific shRNAs for TAGLN was found to knock down

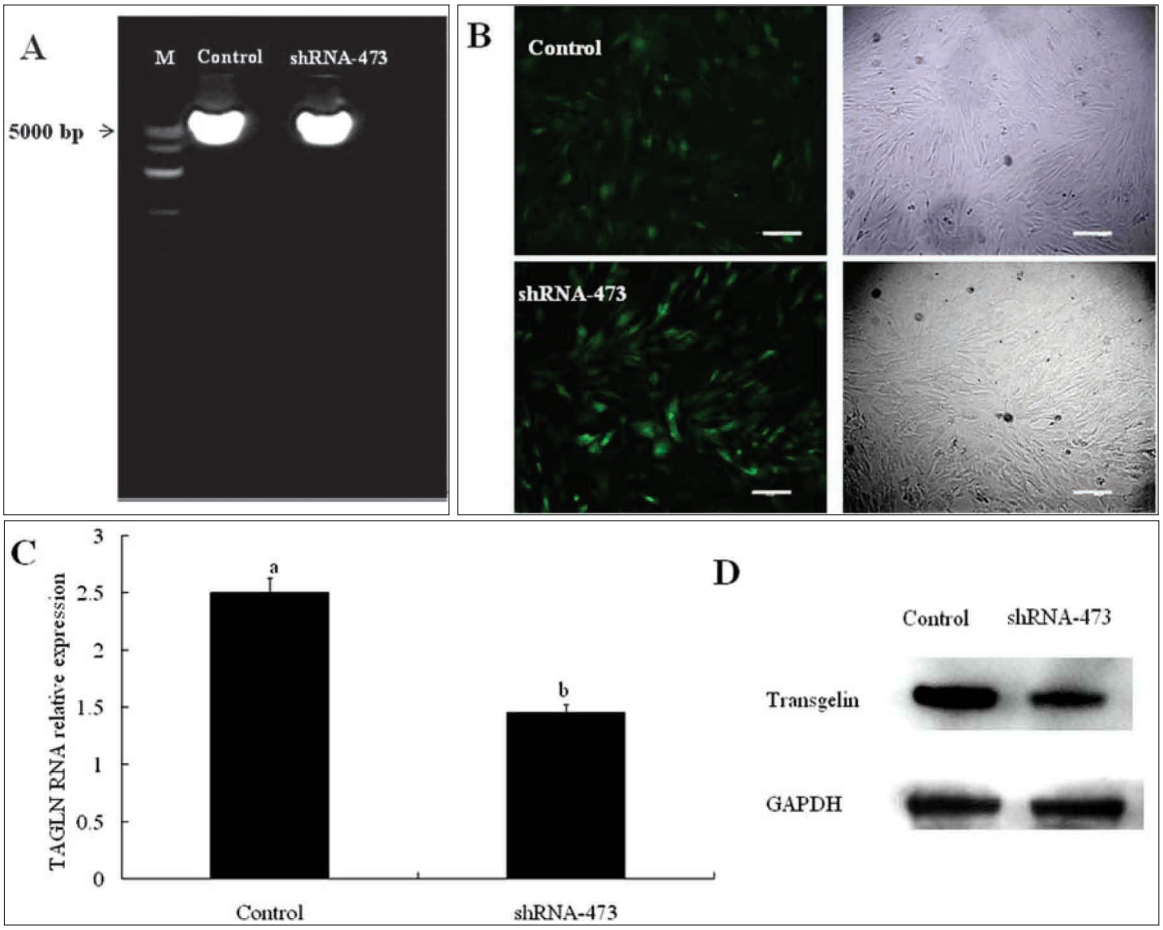

Fig 2. Effect of specific shRNA for TAGLN on knockdown TAGLN. **A-** The linearized shRNA vectors for shRNA-NC (Control) and shRNA-473 were reclaimed. *M:* marker, **B-** All BMMSCs were observed with fluorescence, and the stable cell lines transfected with the vectors of shRNA-NC (Control) and shRNA-473 were obtained. *Bar:* 100 μm, **C-** The expression level of TAGLN mRNA in the BMMSCs through qRT-PCR analysis. Control and shRNA-473 were the BMMSCs which were transfected by the vectors of shRNA-NC and shRNA-473, **D-** The expression of Transgelin in the BMMSCs through Western blot analysis using a specific Transgelin antibody. GAPDH antibody was used to monitor variation in loading of samples. Significant difference ($P<0.05$) is indicated by different letters

Şekil 2. Spesifik shRNA için TAGLN'nin TAGLN blokajındaki etkisi. **A-** shRNA-NC (Kontrol) ve shRNA-473 için linear shRNA vektörler dizayn edildi. *M:* markır, **B-** Tüm BMMSC'ler florasans ile gözlemlendi ve stabil hücre kültürleri shRNA-NC (Kontrol) vektörleri ile transfekte edildi ve shRNA-473 elde edildi. *Bar:* 100 μm, **C-** qRT-PCR analisi ile BMMSC'lerdeki TAGLN mRNA ekspresyon seviyesi. Kontrol ve shRNA-473 shRNA-NC ve shRNA-473 vektörleri ile transfekte edilen BMMSC'lerdir, **D-** spesifik Transgelin antikor kullanılarak yapılan Western Blot analizinde BMMSC'lerdeki Transgelin ekspresyonu. GAPDH antikoru örnekleri yüklemedeki varyasyonu takip etmek için kullanıldı. Anlamlı farklılık ($P<0.05$) farklı harflerle belirtildi

TAGLN expression in the BMMSCs from WZSP. The results showed that specific shRNAs for TAGLN can reduce the expression of TAGLN gene effectively *(Fig. 2)*. The TAGLN expression by the BMMSCS transfected with shRNA-473 was significant lower than that transfected with shRNA-NC (Control) through the qRT-PCR assay and Western blot analysis. Therefore the design of specific shRNAs for TAGLN was suitable for knockdown TAGLN gene in the BMMSCs, and a BMMSCs line with stable TAGLN gene silencing was established. However, the TAGLN gene was not knocked down completely, which may be owing to the liposomal transfection method.

Transgelin is an actin cross-linking protein, which plays a key role in cell differentiation, cell migration cell invasion and matrix remodeling through stabilizing the cytoskeleton [5,6]. Daniel et al.[7] reported that Transgelin is upregulated in repopulating mesangial cells and promotes their migratory. However, it was reported that Apigenin induced up-regulation of Transgelin and inhibited invasion and migration in colorectal cell lines [14]. In this study, the results showed that knockdown TAGLN in the BMMSCs lead to lower migration capacity of the BMMSCs (*Fig. 3* and *Fig. 4*), and it was essential for the BMMSCs to maintain high level expression of Transgelin so as to keep their migration capacity.

It has been shown that BMSCs are able to migrate to the sites and start the differentiation process in the injury and recovery process, suggesting that BMSCs possess migratory capacity. The efficiency and efficacy of BMSCs therapy is limited by the fact that very few transplanted

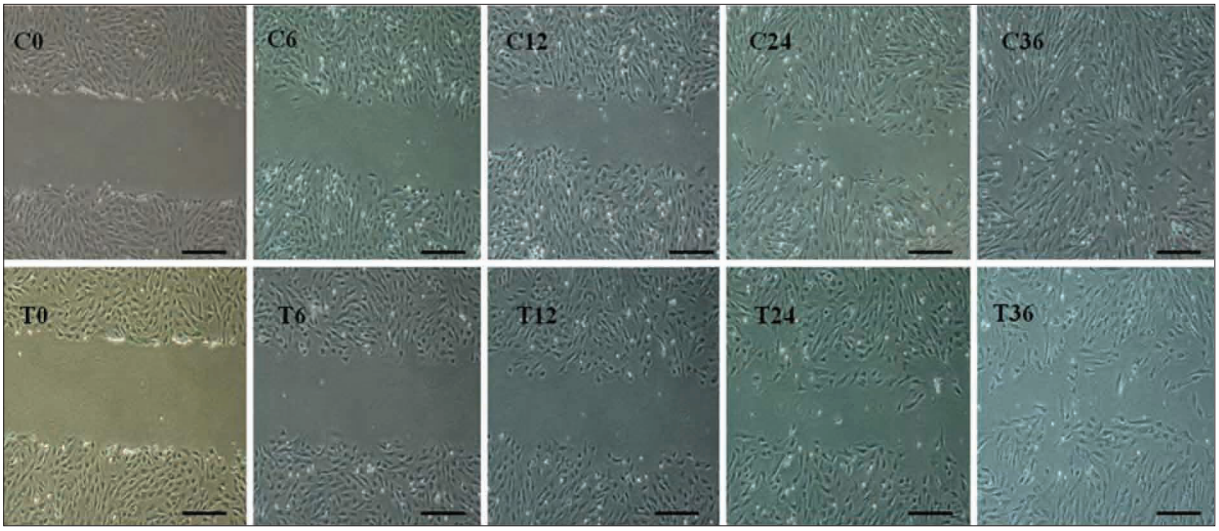

Fig 3. BMMSCs migration capacity analyzed by scratch assay. C0, C6, C12, C24 and C36 were the scratched BMMSCs transfected with the vector of shRNA-NC (Control) after 0 h, 6 h, 12 h, 24 h and 36 h respectively. T0, T6, T12, T24 and T36 were the scratched BMMSCs transfected with the vector of shRNA-473 after 0 h, 6 h, 12 h, 24 h and 36 h respectively. *Bar:* 50 μm

Şekil 3. Scratch analizi ile yürütülen BMMSC göç kapasite analizi. C0, C6, C12, C24 ve C36, sırasıyla 0, 6, 12, 24 ve 36. saatlerde shRNA-NC (Kontrol)'nin vektörü ile transfekte edilen çizilmiş BMMSC'lerdir. T0, T6, T12, T24 ve T36 sırasıyla 0, 6, 12, 24 ve 36. saatlerde shRNA-473 vektörü ile transfekte edilen çizilmiş BMMSC'lerdir. *Bar:* 50 μm

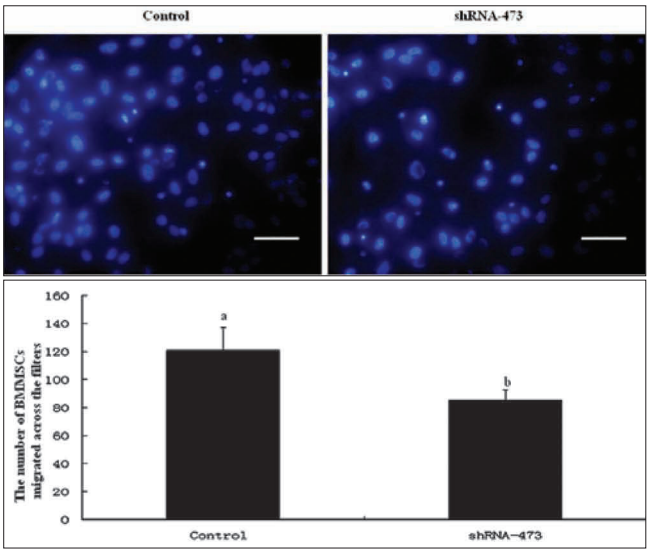

Fig 4. The transwell migration results of the BMMSCs. The shRNA-473 was the BMMSCs with TAGLN knockdown. *Bar:* 100 μm. Significant difference (P<0.05) is indicated by different letters

Şekil 4. BMMSC'lerin transvel göç sonuçları. shRNA-473 TAGLN boklanmış BMMSC'lerdir. *Bar:* 100 μm. Anlamlı farklılık (P<0.05) farklı harflerle belirtildi

cells home to the injured tissues, and functional recovery is often inadequate [15]. The migration potential of BMSCs is one of the determinants of the efficiency in tissue repair *in vivo* [16]. Our results indicated that it needed more time for the BMMSCs with TAGLN knockdown to confluence by scratch assay, and less number of the BMMSCs with TAGLN knockdown migrated across the filters through transwell migration assay (*Fig. 3* and *Fig. 4*). Therefore, TAGLN was an important gene for BMMSCs based transplant therapy.

In conclusion, WZSP may be used as potential organ donors for human being due to the low risk of pig-to-human infection during xenotransplantation. Our results showed that the specific shRNA for knocking down TAGLN efficiently was found for the BMMSCs from WZSP. The results also demonstrated that as an actin cross-linking/gelling protein, Transgelin is involved in maintaining the migration capacity of BMMSCs, which may be benefit for the BMMSCs from WZSP to be used in regenerative therapy for human being.

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SHORT COMMUNICATION

Prevalence of *Argulus foliaceus* and Fungal Infections in Some Ornamental Fishes [Discus *(Symphysodon discus)*, Dwarf Gourami *(Trichogaster lalius)* and Guppy *(Poecilia reticulata)*] in Isfahan City of Iran

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Summary

This study was aimed to determine the prevalence *Argulus foliaceus* and some fungal diseases of ornamental fishes (discus, dwarf gourami and guppy) in Isfahan, Iran. The 90 ornamental fish from 2 pet fish supply store in Isfahan city of Iran were randomly selected. The study demonstrated that only 2 out of those 90 samples were infested with *A. foliaceus*. The highest infestation rate was in Dwarf gourami *(Trichogaster lalius)* (23.31%), and the lowest infestation rate was in Guppy *(Poecilia reticulata)* (6.66%). A total of 90 fishes of 3 species were examined. The contaminant fungal species were identified as *Acremonium* sp., *Aspergillus* sp., *Alternaria* sp., *Penicillium* sp., and *Saprolegnia* sp.. Fungal infection was observed in discus, dwarf gourami and guppy. According to the presented study, it is clear that *A. foliaceus* can act as a potential risk factor for natural ecosystems and native fish population of Iran and other countries.

Keywords: *Prevalence, Argulus foliaceus, Fungal infections, Ornamental fishes, Isfahan, Iran*

İran'ın İsfahan Kentinde *Argulus foliaseus* ve Mantar Enfeksiyonların Bazı Süs Balıklarında [Diskus *(Symphysodon discus)*, Cüce Gurami *(Trichogaster lalius)* ve Lepistes *(Poecilia reticulata)*] Yaygınlığı

Özet

Bu çalışmanın amacı İran'ın İsfahan kentinde *Argulus foliaseus* ve mantar enfeksiyonlarin bazi süs baliklarinda (Diskus, Cüce Gurami ve Lepistes) yaygınlığının belirlenmesidir. İran'ın İsfahan kentinde 2 pet balığı tedarik deposundan 90 süs balığı rastgele seçildi. Çalışma sadece 90 öreneğin iksinin *A. foliaceus* ile enfeste olduğunu göstermiştir. En yüksek enfestasyon oranı, Cüce Gurami *(Trichogaster lalius)* (23.31%) ve en düşük enfestasyon oranı Lepistes *(Poecilia reticulata)* (%6.66) olarak belirlendi. Üç türden toplam 90 balık incelenmiştir. Kontaminan mantar türleri *Acremonium* sp., *Aspergillus* sp., *Alternaria* sp., *Penicillium* sp. ve *Saprolegnia* sp. olarak belirlendi. Mantar enfeksiyonu Disk, Cüce Gurami ve Lepistes balıklarında gözlemlendi. Mevcut çalışmaya gore *A. foliaseus*'un İran ve diğer ülkelerde doğal ekosistemler ve yerli balık nüfusu için potansiyel bir risk faktörü olarak hareket edebileceği oldukça açıktır.

Anahtar sözcükler: *Prevalans, Argulus foliaseus, Mantar enfeksiyonları, Süs balıkları, İsfahan, İran*

INTRODUCTION

Ornamental fish keeping has become an ever more common hobby global. The trade of ornamental fish is a multi-million dollar industry currently. Several species of ornamental fishes are imported from Southeast Asian countries into other countries such as Iran [1,2]. Fungal diseases of fish are considered to be a chief problem for both aquaculture and fisheries and happen in brood stock and totally life stages of fish and eggs [1,3]. Among many aquatic fungi, *Achlya, Penicillium* sp., *Alternaria* sp., *Aphanomyces, Aspergillus* sp., *Dictyuchus, Fusarium solani, Protoachlya, Pythium, Saprolegnia,* and *Thraustotheca* were reported [1,3]. Fungus has been reported to cause serious diseases in estuarine and freshwater fishes in Australia, Japan and throughout South Asia [4].

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The genus *Argulus* (Crustacea: Branchiura), or fish louse, are common parasites of freshwater fish [2,5]. Nearly 15 spp. are found on freshwater fishes and several of the species are parasitic on marine fishes [2,6]. *Argulus foliaceus (A. foliaceus)* have a direct life cycles that because of not need to the intermediate hosts for whole its life cycle and transports rapidly among the fishes [7]. Adults may live free from the host for up to fifteen days. *A. foliaceus* is an obligatory blood sucker and can survive for only a little day without the host fish, depending on size and ambient temperature [8]. A lot of researches have been done on prevalence of *A. foliaceus*, nonetheless the researches on prevalence of this ectoparasite in Iran are limited [2,5-7,9].

Consequently, this study was designed to find the prevalence *A. foliaceus* and some fungal diseases of ornamental fishes (discus, dwarf gourami and guppy) in Isfahan, Iran. This is the first study on prevalence of *A. foliaceus* in Isfahan, Iran. There is a lack of data on the characterisation and identification of fungal diseases of ornamental fishes in Iran. Such data are significant for fisheries management.

MATERIAL and METHODS

The 90 ornamental fish [30 pieces discus *(Symphysodon discus)*, 30 pieces dwarf gourami *(Trichogaster lalius)* and 30 pieces guppy *(Poecilia reticulata)*] from 2 pet fish supply store in Isfahan city of Iran (which are imported from Southeast Asian countries such as: Thailand, Malaysia and Singapore into Iran), in the summer 2013 were randomly selected. Then, the fish caught via hand tour and were transported to laboratory of mycology and parasitology in the School of Veterinary Medicine, Islamic Azad University Shahrekord Branch of Shahrekord, in sterile polyethylene bags in aerated aquarium/pond water. The fishes were kept separate in glass aquariums by continuous air supply at ambient temperature. Samples were examined macroscopically, microscopically, clinically for presence of *A. foliaceus* and fungal diseases.

Identification of Fungi

For culturing of fungal specimens, three different types of media counting Malt extract agar (MEA), Sabouraud dextrose agar (SDA) and Potato dextrose agar (PDA) were prepared and streptomycin sulphate was supplemented to each preparation of media to avoid bacterial infection. The body surfaces of everything the fishes using in study were disinfected via dipping each fish in 1% formaldehyde for 1 to 5 min followed via 70% alcohol and lastly in sterile water in which it was thoroughly rinsed. The fungal isolates were collected from infected organs (skin, fins, gills, eyes) of fish with sterile needle and inoculated on MEA (Oxoid, UK), SDA (Oxoid, UK) and PDA (M096-India) agars. The agar plates were incubated at 28-30°C and fungal growth was observed after 4-7 days. The fungal colonies of many colors were observed in the agar plates. For microscopic examination, slides were ready from each colony and stained with 0.05% trypan blue in lactophenol. The slides were observed under Olympus microscope and photographed. The fungi were identified by the help of available fungal identification keys and literature [10].

Identification of A. foliaceus

In the macroscopic technique, contaminated fishes were carried out and their parasites were removed by forceps from different parts of the body. Via microscopic technique (using a compound light microscope at ×10 and ×40 magnification.), the fish were examined via dissecting microscope. The *Argulus* parasites were fixed in 70% ethanol and transferred to laboratory. Then, parasites were identified by the diagnostic key [11].

Statistical Analysis

Prevalence of infection was calculated by using the following formula:

$$\text{Prevalence of infection (\%)} = \frac{\text{No. of fungal affected fish}}{\text{Total no. of examined fish}} \times 100$$

RESULTS

The study demonstrated that only 2 out of those 90 samples were *infested* with *A. foliaceus,* meaning 2.22% prevalence. The highest infestation rate was in guppy (6.66%) and no detected in discus and dwarf gourami. Under the light microscope, these parasites were identified at *A. foliaceus* according to the rounded lobes of abdomen and the posterior emargination not reaching the mid-line and posterior lobes cephalothoracic carapace not extended beyond the beginning of abdomen *(Fig. 1)*. In this study, all fish had generalized symptoms including lack of appetite and abnormal swimming. Bloody spots were also observed on skin and fins. In the present study, the distribution of *A. foliaceus* infection on different body regions was examined.

Results of fungal infection by any details showed in *Table 1, 2,* and *3*. The fungal isolates were collected from infected organs (skin, fins, gills, eyes) of fish and the contaminant fungal species were identified as *Acremonium* sp., *Aspergillus* sp., *Alternaria, Penicillium* sp., *and Saprolegnia* sp.. The highest infestation rate was in Dwarf gourami *(Trichogaster lalius)* (23.31%), and the lowest infestation rate was in Guppy *(Poecilia reticulata)* (6.66%). A total of 90 fishes of *Acremonium* sp., *Aspergillus* sp., *Alternaria*, *Penicillium* sp., and *Saprolegnia* sp.. were examined *(Table 1)*. Fungal infection was observed in discus, dwarf gourami and guppy. However, no *Saprolegnia* sp. infection was no observed in discus and dwarf gourami; no *Acremonium* sp. infection was no observed in guppy; no *Aspergillus* sp.

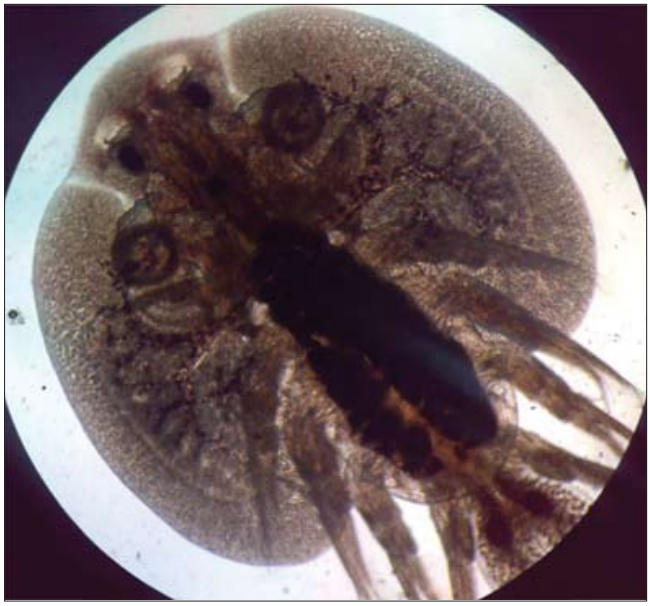

Fig 1. *Argulus foliaceus* magnification (×4)
Şekil 1. *Argulus foliaceus* büyütme (×4)

Table 1. *The number and percentage of healthy and fungi infected fishes*
Tablo 1. *Sağlıklı ve mantarlarla enfekte balıkların sayı ve yüzdesi*

Fish Species		Number	Ratio (%)
Discus (*Symphysodon discus*)	Infected	6	20
	No Infected	24	80
Dwarf gourami (*Trichogaster lalius*)	Infected	7	23.31
	No Infected	23	76.69
Guppy (*Poecilia reticulata*)	Infected	2	6.66
	No Infected	28	93.34

infection was no observed in dwarf gourami and guppy; no *Alternaria* infection was no observed in guppy *(Table 3)*.

DISCUSSION

In the current study, some ornamental fishes were infected by fungus and *A. foliaceus*. The propagating and rearing of ornamental fish have a remarkable situation in the globe and plays a valuable role in exchange income of several nations and in Occupation. The world trade share of these fish which was estimated about 900 million dollar, has given a vital insight into it [12]. Since of the economic importance of these aquatics, several researchers have paid helpfulness the different factors threatening their health. *Argulus* sp. have been reported from various fish species global [2,6,7,9,13-15].

Does research carried out on Koi and goldfish checks the *Argulus* as the greatest prevalent parasite [14]. A small number of parasites species like protozoa are apparently non-host specific species and have been found in a large-scale of brackish water or fresh fish species all over the

Table 2. *Frequency percentages of the identified fungi species*
Tablo 2. *Tespit edilen mantar türlerinin sıklık oranları*

Isolated Fungi	Ratio (%)
Penicillium sp.	40.0
Acremonium sp.	26.6
Aspergillus sp.	20.0
Alternaria	6.7
Saprolegnia sp.	6.7

Table 3. *The percentage of different fungal species isolated from fishes*
Tablo 3. *Balıklardan izole edilen farklı mantar türlerinin oranları*

Identified Fungi Species	Fish Species			Total n (%)
	Discus n (%)	Dwarf gourami n (%)	Guppy n (%)	
Penicillium sp.	2 (33.3)	3 (42.8)	1 (50)	6 (40)
Acremonium sp.	1 (16.7)	2 (28.6)	0 (0)	3 (20)
Aspergillus sp.	1 (16.7)	0 (0)	0 (0)	1 (6.7)
Alternaria	2 (33.3)	2 (28.6)	0 (0)	4 (26.6)
Saprolegnia sp.	0 (0)	0 (0)	1 (50)	1 (6.7)
Total	6 (100)	7 (100)	2 (100)	15 (100)

world. *A. foliaceus* were reported from different fish species global and reported by some of the goldfish in Turkey [8,13,16]. In the present study, *A. foliaceus* was reported on guppy *(Poecilia reticulata)* which this was first recorded in Isfahan, Iran and findings of the present study are comparable to the findings of Mirzaei and Khovand [2].

Freshwater ornamental fish in Sri Lanka examined. Three species of copepod arthropods *(Lernaea cyprinacea, Ergasilus ceylonensis, Argulus foliaceus)*, were identified [5]. The lice fish is a risky parasite lacking specificity, therefore that it can perhaps infested all freshwater fishes and happening in coastal or estuarine and marine water habitats [16]. In the other study, tiger Oscar *(Astronotus ocellatus)* species taken from a local pet shop with symptoms were examined for bacterial, fungal and parasitical infections. The parasites observed on the skin and fins of fish were identified as *A. foliaceus* [13].

Another study in Iran, lionhead goldfish *(Carassius auratus)*, taken from a goldfish aquarium with symptoms, were examined for ectoparasites. The parasites collected from the skin and fins of fish were identified as *A. foliaceus*. This is the first report of infection with *A. foliaceus* of lionhead goldfish *(Carassius auratus)* in Iran [6]. Al-Dulaimi [7] in Babylon province of Iraq reported cases of the infection with *A. foliaceus* lice in various goldfish species in the earthen ponds. Notash [15] studied on the goldfishes in east Azerbaijan province of Iran and reported that they were infested by at least one species of crustacean. Results showed that of 300 samples, 85 (28.33%) samples were positive and 215 (71.67%) samples were negative from existence of *Argulus*. Too, Ebrahimzadeh Mousavi *et*

al.[9] studied on the 10 various ornamental fishes farm in Iran and reported that 230 pieces of the goldfishes were infested by 3 species of this ectoparasite. Other study, the concurrent fungal and parasitic infection of *Argulus foliaceus,* was observed in 3 fishes (12.5%). The parasitic infection of *A. foliaceus* is discussed elsewhere. Five fishes (20.83%) did not show any clinical signs [4]. In the new study in Iran, *A. foliaceus* was reported on goldfish and Koi which this was first recorded in Kerman, southeast of Iran [2].

There is a lack of information on the identification and characterization of fungal diseases of ornamental fishes in Iran. Such information is very important for fisheries management. Five fungi, *Aspergillus* spp., *Fusarium* spp., *Mucor* spp., *Penicillum* spp., and *Rhizopus* spp., were reported from 8 edible smoked-dried freshwater fishes via Fayioye *et al.*[17]. Junaid *et al.*[18] isolated seven fungal species from stock fish in Nigeria and these included *A. flavus, A. fumigatus, A. niger, Trichophyton verrucosum, Rhizopus* spp., *Mucor* spp., and *Penicillum* spp. and between these *Mucor* spp., displayed the highest occurrence. In another study, fungi of 8 various genera; *Saprolegnia, Aspergillus, Fusarium, Mucor, Penicillium, Rhizopus, Scopulariopsis* and *Curvularia* were isolated from 2 fish species, *Oreochromis* spp., and *Claris gariepinus* [19]. Shahbazain *et al.*[20] isolated *Penicillium expansum, Penicillium citrinium; Aspergillus terruse, Aspergillus clivatus; Alternaria* spp.*; Saprolegnia parasitic, Saprolegnia lapponica, Saprolegnia ferax* and *Saprolegnia hypogyna* and seven other species of fungi from infected eggs of rainbow trout, *Oncorhynchus mykiss* in Iran. Fadaeifard *et al.*[21] isolated eight species of fungi from eggs and brood stock of rainbow trout *O. mykiss*. These isolates were *Penicillium* spp., *Acremonium* spp., *Alternaria* spp.*, Fusarium solani, Aspergillus* spp.*, Mucor* spp.*, Saprolegnia* spp., and *Cladosporium* spp. In another study, 5 fungal species viz. *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp., *Blastomyces* sp., and *Rhizopus* sp., were isolated from 4 species of carps, *C. auratus* L.; *Hypophthalmichthys molitrix* Richardsons; *Labeo rohita* Hamilton and *C. idella* [1]. The fungal genera like *Penicillium* spp.*, Fusarium* spp.*, Mucor* spp., and *Saprolegnia* sp., were isolated from *Acipenser persicus* eggs, where they caused 22% mortality of these eggs [3]. Too, fungal species like *Branchiomyces* sp.*, Saprolegnia* spp., and *Aphanomyces* spp., have also been reported to be pathogenic to fish [3].

Absence of good aquarium keeping in pet shops and fish farms increases the chances of fungal infection in fishes. The basic health management practices may be easily over looked by reason of lack of trained personal or resources. In the current study, *A. foliaceus* was reported on guppy *(Poecilia reticulata)* which this was first recorded in Isfahan, Iran. According to the presented study, it is clear that *A. foliaceus* can act as a potential risk factor for natural ecosystems and native fish population of Iran and other nations, that should be mentioned to prevent the burst of new parasitic fauna to Iran and various nations as well as stop direct economic losses caused via mortality derived from infestation with this ecto-parasite. Too, lack of good aquarium keeping in pet shops and fish farms increases the chances of fungal infection in fishes. The basic health management practices may be easily over looked due to dearth of trained personal or resources.

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YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup, derleme ve çeviri türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.
2- Dergide yayımlanması istenen yazılar Times New Roman yazı tipi ve 12 punto ile A4 formatında, 1.5 satır aralıklı ve sayfa kenar boşlukları 2.5 cm olacak şekilde hazırlanmalı ve resim, tablo, grafik gibi şekillerin metin içindeki yerlerine Türkçe ve yabancı dilde adları ve gerekli açıklamaları mutlaka yazılmalıdır.
Dergiye gönderilecek makale ve ekleri (şekil vs) *http://vetdergi.kafkas.edu.tr* adresindeki online makale gönderme sistemi kullanılarak yapılmalıdır.
Başvuru sırasında yazarlar yazıda yer alacak şekilleri (13 X 18 cm boyutlarından büyük olmamalı) online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış *Telif Hakkı Devir Sözleşmesi* editörlüğe gönderilmelidir.
3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.
4- ***Makale Türleri***
Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır.
Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, giriş, materyal ve metot, bulgular, tartışma ve sonuç ile kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 10 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.
Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 4 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.
Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 2 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.
Gözlem, uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri giriş, olgunun tanımı, tartışma ve sonuç ile kaynaklardan oluşmalı ve 4 sayfayı geçmemelidir.
Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 1 sayfayı geçmemelidir.
Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri giriş, metin ve kaynaklardan oluşmalı ve 10 sayfayı geçmemelidir.
Çeviri, makalenin orijinal formatı dikkate alınarak hazırlanmalıdır.
Yazarla ilgili kişisel ve kuruma ait bilgiler ana metin dosyasına değil, on-line başvuru sırasında sistemdeki ilgili yerlere unvan belirtilmeksizin eklenmelidir.
5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.
6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır.
Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.
Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.
Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.
Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.
Örnek: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.
Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi kaynak bilgilerinin sonuna eklenmelidir.
Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.
Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.
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