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Research Article

The Effects of Increase in Threonine to Lysine Ratio on Performance, Blood Parameters and Humoral Immune Responses of Male Broiler Chickens Challenged with *Salmonella*

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Abstract

The aim of this study was to assess the effect of threonine to lysine (Thr/Lys) ideal ratio for optimum performance, blood parameters and immunity of broiler chicks. Supplemental threonine (equal or 25% more than breed's threonine/lysine ratio requirement) was added to a control, threonine deficient and high crude fiber diet, then fed to 288 one-day-old male Ross 308 broiler chicks (three treatments and eight replications per treatment). On day 11 and 21 of age, infectious bursal disease virus and infectious bronchitis virus vaccines were orally administered individually, respectively. Then, 21 days after each administration, blood antibody titers against viruses were measured. On day 32 of age, four birds in each treatment group were infected orally with equal numbers of *Salmonella* Paratyphi A (5×10^4 cfu/bird). As a result of this study, challenging with *Salmonella* led to increase in mortality rate and increase in Thr/Lys ratio could not decrease it. Increase in Thr/Lys ratio decreased feed intake and weight gain of ensemble challenged and non-challenged groups, however improved feed conversion ratio of challenged group in finisher period. Feed consumption cost increased by salmonellosis and increase in Thr/Lys ratio could not improve salmonellosis based economic loss. Salmonellosis increased serum urea, uric acid and AST and decreased serum glucose and cholesterol and increase in Thr/Lys ratio did not alleviate triglyceride. Increase in Thr/Lys ratio improved non-significantly humoral immune response in the challenged and non-challenged groups. These findings indicate that higher Thr/Lys ratio in infected birds improved production performance, however could not be an economical lucrative medicative agent.

Keywords: *Broiler, Humoral immune responses, Lysine, Salmonella, Threonine*

Salmonella'ya Maruz Kalan Erkek Broyler Piliçlerde Threonine-Lysine Oranındaki Artışın Performans, Kan Parametreleri ve Humoral İmmun Tepki Üzerine Etkileri

Özet

Bu çalışmanın amacı, etlik piliçlerde ideal lisin (Thr/Lys) oranının optimum performans, kan parametreleri ve immunite üzerine etkisini değerlendirmekti. 288 adet bir günlük erkek Ross 308 etlik civcivlere kontrol, treonin yönünden yetersiz ve yüksek ham selüloz diyetine treonin (türün treonin/lizin oranı gereksinimine eşit veya %25 daha fazla) eklendikten sonra verildi (üç uygulama ve her bir uygulama için sekiz tekrarlı tarzda). Bireylere 11. ve 21. günlükken sırasıyla bulaşıcı bursal hastalık virüsü ve bulaşıcı bronşit virüsü aşıları oral yolla ayrı ayrı uygulandı. Her uygulamadan 21 gün sonra, virüslere karşı kan antikor titreleri ölçüldü. 32 günlükken, her bir tedavi grubundan dört eşit sayıda piliç ağızdan *Salmonella* Paratyphi A (5 x 104 cfu/piliç) ile enfekte edildi. Bu çalışmanın sonucunda, *Salmonella* maruziyeti ölüm oranında artışa yol açarken, Thr/Lys oranındaki artış bu oranı azaltamadı. Thr/Lys oranındaki artış maruziyet olan ve olmayan grupların toplam yem alımı ve ağırlık kazançlarını azaltırken, maruziyet grubunun son periyottaki yem dönüşüm oranını artırdı. Yem tüketim maliyeti salmonellosis ile artarken, Thr/Lys oranındaki artış salmonellosis-kökenli ekonomik kaybı azaltamadı. Salmonellosis serum üre, ürik asit ve AST'yi artırıp serum glukoz ve kolesterolü azaltırken, Thr/Lys oranındaki artış ise trigliseridi baskılamadı. Thr/Lys oranındaki artış maruziyet olan ve olmayan gruplardaki humoral immun tepkiyi nispeten yükseltti. Bu bulgular, yüksek Thr/Lys oranının enfekte kuşlardaki üretim performansını artırdığını, ancak ekonomik yönden kazançlı bir tıbbi ajan olamadığını göstermektedir.

Anahtar sözcükler: *Broyler, Humoral immun tepkiler, Lisin, Salmonella, Treonin*

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INTRODUCTION

Threonine is the third most limiting amino acid in most plant-based broiler diets behind the total sulphur-containing amino acids and lysine [1]. Among the essential amino acids, threonine is particularly important for maintenance of gut barrier integrity and has an important role in the structure and function of gastrointestinal tract [2-4]. A higher threonine to lysine (Thr/Lys) ratio in intestinal infected broilers by coccidiosis or subclinical *Clostridium* infection improved production performance [5,6].

Threonine is found in high concentrations in chicken gamma globulins [7]. Gamma globulins represent the fraction of serum containing the highest concentration of immuno-globulins (antibodies) as determined by electrophoresis [8]. Because immunoglobulins depend on amino acid sequences to form the variable regions for antigen binding and provide structural support [8], threonine deficiency may suppress antibody activity [1].

More threonine requirement was reported for optimal responses in the cellular and humoral immune systems of rats than requirement for optimum growth [9]. Because of threonine participation in immune system functions and influence of nutrition and vaccination programs on diseases prevention, as substitutes for antibiotics, Kidd [1] recommended such researches should evaluate cellular and humoral immune system functions as they are affected by threonine to understand more completely the birds' needs for this essential amino acid [10].

Low Thr/Lys ratio was reported by Ross broiler nutrition specificatio™n [11] compared with NRC [12]; but, Aviagen [11] reported more threonine to metabolizable energy, lysine to metabolizable energy, threonine to crude protein and lysine to crude protein for broilers. This means more amino acid intake per daily feed intake [11]. Kidd [1] deduces that NRC [12] overestimated the threonine requirement of broilers (Thr/Lys ratio admittedly) for optimum performance. Likewise, NRC [12] reported that findings about broilers Thr requirement was insufficient. Lysine and protein requirement of modern broiler strains is more than that reported by NRC [12] as a result of their fast growth rates. Novel researches with new points of view should be done because more Lysine and protein in the diet changes the physiological characteristics and quantity of amino acids requirement [13-19].

Salmonella infects poultry and humans by the oral route through contaminated food or water [20]. *Salmonella* especially Paratyphoid serovar that are motile makes colony on gut epithelium then transmit across from gut mucosal and immune barrier to blood flow and can cause septicemia or tissues infection and damage [20]. Today complementary controls via gut health promotion by non antibiotic therapy method are used to prevent *Salmonella* from entering the food chain; consequently, to improve poultry products consumer's health [21-23].

The objective of this study was to determine variations in performance, blood parameters and humoral immune responses of broiler chickens challenged with *Salmonella* Paratyphi A, fed diet supplemented with L-Threonine to meet various Thr/Lys ratios.

MATERIAL and METHODS

The study was approved by the Ethics Committee of Islamic Azad University, Science and Research Branch (approval date: 16.11.2013; no: 3740, AEC 11).

Experimental Design and Diets

In a completely randomized design 288 one-day old *Salmonella* negative male (Ross 308) broiler chicks were randomized in 3 treatments with 8 repetitions, 12 chicks per repeat. Average body weight (means ± standard error) of chicks at the beginning of the experiment was 40.3±1.6 g. Chicks were housed in floor pen (10 chicks/1 m^2 pen) and had free access to feed and water during the experimental period and 24 h light daily in a temperature-controlled room. The relative humidity was controlled at 65% and temperature was set at 32°C on day 1 and lowered gradually to 24°C for the rest of the experiment period. After 24 h eating similar prestarter pellet, chicks were fed experimental starter (2-10 d), grower (11-24 d) and finisher (25-42 d) rations based on breed's nutritional catalog [11]. Performance assay was accorded with these periods.

Near Infrared Reflectance Spectroscopy (NIRS) (AMINONIR®, 43076) was used to determine amino acids profiles of all ingredients (by: Paya Amin Mehr Co. Ltd., Evonik Animal Nutrition Service, Tehran, Iran).

Treatment 1 had no threonine supplement, treatment 2 had threonine supplement to meet Thr/Lys ratio requirement as pointed in Ross broiler nutrition specification [11] and treatment 3 had more threonine supplement to meet Thr/Lys ratio 25% more. Basal diets ingredients and nutrient analysis and L-Threonine supplement quantity of experimental diets showed in *Table 1*. The difference between threonine supplement content of treatments 1 and 2 with treatment 3, correct with adding grind fine sand as filler to treatments 1 and 2.

Salmonella Challenge and Recovery

Salmonella enterica subsp. *enterica* serovar Paratyphi A (ATCC® 9150™) was used for infection induction. This bacterium was obtained from microorganism's bank of Iranian Biological Resource Centre (IBRC), ACECR, Tehran, Iran (IBRC No.: IBRC-M 10668). The challenge organism for this experiment was grown in tryptic soy broth (Sigma-Aldrich, UK) at 37°C then diluted to 5×10^4 cfu/ml [24].

Table 1. *Composition of experimental diets*
Tablo 1. *Deneysel diyetlerin içeriği*

Items	Starter 2-10 d	Grower 11-24 d	Finisher 25-42 d
Ingredients (g/kg)			
Corn	432.91	472.21	503.00
Wheat	100.00	105.00	105.00
Barley	50.00	60.00	60.00
Wheat bran	25.00	30.00	30.00
Rice bran	25.00	30.00	30.00
Soybean meal (46% CP)	311.20	247.00	209.67
Soybean oil	6.02	11.45	20.60
Choline chloride 60%	1.45	1.40	1.30
L-Threonine suppl. ^{T}-11	-	-	-
L-Threonine suppl. T-2[1]	0.59	0.48	0.36
L-Threonine suppl. T-3[1]	2.81	2.44	2.13
L-Lysine monohydrochloride	2.45	2.20	1.93
DL- Methionine	2.97	2.40	2.10
Limestone	11.84	9.60	9.57
Dicalcium phosphate	18.15	15.90	14.90
Sodium bicarbonate	4.10	3.90	3.70
Salt	0.60	1.00	1.10
Vitamin premix[2]	2.50	2.50	2.50
Mineral premix[2]	2.50	2.50	2.50
Maduramycin 1%	0.50	0.50	-
Analysis results of nutrients			
AME_n[3] (kcal/kg)	2720	2820	2920
CP (%)[4]	21.07[4]	18.78	17.34
Thr T. 1 (%)	0.790	0.697	0.641
Thr T. 2 (%)	0.846	0.743	0.675
Thr T. 3 (%)	1.057	0.929	0.843
Lys (%)	1.287	1.110	0.994
Thr /Lys T.1 (%)	61.38	62.82	64.47
Thr /Lys T.2 (%)	65.73	66.93	67.91
Thr /Lys T.3 (%)	82.16	83.68	84.83
Met + Cys (%)	0.965	0.852	0.785
Val (%)	0.990	0.882	0.815
Ile (%)	0.871	0.761	0.693
Arg (%)	1.379	1.201	1.090
Trp (%)	0.258	0.225	0.204
Crude fiber (%)	4.108	3.89	3.70
Ca (%)	0.96	0.80	0.77
Available Phosphorus (%)	0.45	0.40	0.38
Na (%)[5]	0.15	0.17	0.16
Cl (%)[5]	0.15	0.17	0.17
K (%)[5]	0.87	0.78	0.71
DCAD (meq/kg)[5,6]	249	224	207

*[1] L-Threonine supplement of treatment 1&2&3. Feed grade and 98.5% purity; [2] Breed's special supplement made as Ross nutrition catalog suggested (Anonymous, 2009), contain: 4.400.000 IU/kg of Vit. A, 2.000.000 IU/kg of Vit. D_3, 30.000 IU/kg of Vit. E, 1.200 mg/kg of Vit. K (Menadione), 1.200 mg/kg of B_1, 3.200 mg/kg of B_2, 24.000 mg/kg of Nicotinic Acid, 6.000 mg/kg of Pantothenic Acid, 1.600 mg/kg of B_6, 60 mg/kg of Biotin, 800 mg/kg of Folic Acid, 6 mg/kg of B_{12}; 6.400 mg/kg of Copper, 500 mg/kg of Iodine, 16.000 mg/kg of Iron, 48.000 mg/kg of Manganese, 120 mg/kg of Selenium, 40.000 mg/kg of Zinc; [3] AME_n: apparent metabolizable energy corrected for nitrogen excretion; [4] CP: crude protein; All limiting essential amino acids were supplied in basal diet by increase in ration crude protein content; [5] by calculation; [6] **DCAD:** dietary cation anion difference*

All chickens of four replications of each treatment individually infected by oral gavage using an animal feeding needle with equal numbers of *Salmonella* Paratyphi A (5×10^4 cfu/ml per bird) at the age of 32-d of old [25]. In a 2×3 factorial arrangement, other four replications of each treatment received normal saline. Challenge pens were separated by 2 meter distance and separated instrument from unchallenged pens.

Three cloacae swab samples from each pen of challenged and non-challenged chickens were cultured on day 39 to confirm that no *Salmonella* was present in the unchallenged group and success of *Salmonella* present in challenged group. A resistance again tylosin observed in an antibiogram test that initially done on this *Salmonella* serovar. Cloacae samples were streaked for isolation onto xylose lysine deoxycholate (XLD, Sigma-Aldrich, UK) agar plates containing tylosin (20 µg/mL) and incubated for 24 and 48 h at 37°C. Plates were evaluated for the presence or absence of *Salmonella*, which grow as red colonies on this selective medium [25-27].

Blood Sampling and Measurements

On days 10, 17, 24, 33 and 42 of age growth efficiency (all birds/pen) and on day 42, blood parameters (2 birds/pen) measured. Blood samples (without fasting) had been caught from wing vein after washing skin with distilled water and then drying. Serum glucose, urea, uric acid, cholesterol, triglyceride and aspartate aminotransferase (AST) measured by human Roche diagnostics kits with automatic analyzer COBAS INTEGRA 400 plus, (Roche Diagnostics Ltd. CH-6343 Rotkreuz, Switzerland). Principle of these test's methods was enzymatic-spectrophotometric of hexokinase, urease/glutamate dehydrogenase, uricase/peroxidase, cholesterol esterase/cholesterol esterase/peroxidase, Lipoprotein lipase/Glycerol kinase/GPO/Peroxidase respectively and AST method was according to the IFCC but without P-5'-P [28].

Humoral Immune Assay

On day 11 of age, infectious bronchitis H 120 strain live vaccine product of MERIAL, 17 rue Bourgelat 69002 (Lyon, France) (Batch no.: L395281) and on day 21 of age, Gumboro D78 live vaccine product of Intervet International B.V. (Boxmeer, Holland) (Batch no: 12648BM01) were diluted by disinfectant-free drinking water then orally administered individually (1.1 dose per chicken). These vaccines were the only vaccine throughout the study. Blood antibody titer against each vaccine measured with ELISA method on 21 days after each administration in serum. Antibody test kit used for Infectious Bronchitis Virus (IBV; code: CK119; Lot no: FS5674) and Infectious Bursal Disease (IBD; code: CK113; Lot no: FS5709) was product of BioChek veterinary diagnostics, (BioChek (UK) Ltd., 11 Mill farm business park, Millfield Road, Hounslow, London TW4 5PY).

Statistical Analysis

The statistical normality of all data were tested in MINITAB software, confidence level=95% [29]. Statistical normal data of each variable with normal distribution (P>0.05) used for ANOVA procedure and statistical un-normal data (P<0.05) normalized by especial equations according to each variable properties [29,30]. Then treatments analyzed by ANOVA procedure using the GLM procedure of SAS software [31]. When significant differences among means were found, means were separated using Duncan's Multiple Comparison test (α=5%) for post hoc multiple comparisons.

RESULTS

Salmonella Recovery

Salmonella culture of cloacae samples showed that only one chicken of challenged group in treatment 1 was not infected and no *Salmonella* were present in unchallenged group.

Performance

Salmonellosis led to increase (P<0.01) in mortality and addition of Thr level did not alleviate it significantly *(Table 2)*. Feed intake (FI) was not affected by salmonellosis significantly; but, the increase in Thr/Lys ratio over catalog recommendation decreased (P<0.05) feed intake in whole period *(Table 3)*. Salmonellosis and addition of Thr/Lys ratio over catalog recommendation decreased (P<0.05) weight gain *(Table 2, Table 3)*. In *Salmonella* positive group, recommended Thr/Lys ratio increased (P<0.05) weight gain than other Thr/Lys ratio level *(Table 3)*; but, increase in Thr/Lys ratio in *Salmonella* positive group at finisher period did not improve weight gain significantly *(Table 2)*. Feed conversion ratio (FCR) and protein efficiency ratio

Table 2. *Response to different Thr/Lys ratio and Salmonella challenge for performance*
Tablo 2. *Farklı Thr/Lys oranı ve Salmonella maruziyetine karşı performans tepkisi*

Traits[1]	Starter (2-10 d)				Grower (11-24 d)				Finisher (25-42 d)			
	Mortality %	FI (kg)	WG (kg)	FCR	Mortality %	FI (kg)	WG (kg)	FCR	Mortality %	FI (kg)	WG (kg)	FCR
Treatments[2]												
T.1	2.59	0.272	0.218	1.255	3.89	1.532^{ab}	0.943^{ab}	1.624	4.77^{b}	2.546	1.327^{a}	1.918^{c}
T.2	0.00	0.271	0.224	1.212	5.19	1.588^{a}	0.997^{a}	1.593	0.00^{b}	2.553	1.311^{a}	1.932^{c}
T.3	0.00	0.269	0.216	1.255	4.02	1.463^{b}	0.901^{b}	1.626	0.00^{b}	2.453	1.340^{a}	1.831^{c}
T.4	-	-	-	-	-	-	-	-	19.09^{a}	2.526	1.005^{b}	2.509^{a}
T.5	-	-	-	-	-	-	-	-	19.39^{a}	2.590	1.076^{b}	2.405^{ab}
T.6	-	-	-	-	-	-	-	-	19.09^{a}	2.397	1.034^{b}	2.320^{b}
Factors[3]												
Sal. -	-	-	-	-	-	-	-	-	1.59^{b}	2.511	1.326^{a}	1.894^{b}
Sal. +	-	-	-	-	-	-	-	-	19.19^{a}	2.504	1.038^{b}	2.412^{a}
Thr.1	-	-	-	-	-	-	-	-	10.90	2.537	1.210	2.171^{a}
Thr.2	-	-	-	-	-	-	-	-	8.31	2.557	1.209	2.135^{a}
Thr.3	-	-	-	-	-	-	-	-	8.18	2.429	1.189	2.040^{b}
SEM[4]												
Treatments	0.522	0.002	0.003	0.016	1.003	0.015	0.011	0.006	3.496	0.073	0.022	0.041
Sal.	-	-	-	-	-	-	-	-	2.018	0.042	0.013	0.023
Thr.	-	-	-	-	-	-	-	-	2.472	0.051	0.16	0.029
P-value[5]												
Treatments	0.119	0.906	0.657	0.516	0.863	0.017	0.021	0.150	0.001	0.614	0.0001	0.0001
Sal.	-	-	-	-	-	-	-	-	0.0001	0.925	0.0001	0.0001
Thr.	-	-	-	-	-	-	-	-	0.781	0.221	0.523	0.022
Sal.×Thr.	-	-	-	-	-	-	-	-	0.756	0.769	0.204	0.388
CV[6]	295.8	4.48	8.53	6.33	112.4	4.81	6.14	2.06	76.55	5.85	3.78	3.90

*[1] **FI:** Feed Intake (kg/41d), **WG:** Weight Gain (kg/41d), **FCR:** Feed Conversion Ratio (feed intake/weight gain); [2] Comparison of treatments effects: **T.1** = Low Thr/Lys ratio+ no Salmonella challenge, **T.2** = Standard Thr/Lys ratio + no Salmonella challenge, **T.3** = High Thr/Lys ratio+ no Salmonella challenge, **T.4** = Low Thr/Lys ratio + Salmonella challenge, **T.5** = Standard Thr/Lys ratio + Salmonella challenge, **T.6**= High Thr/Lys ratio + Salmonella challenge; [3] Comparison of factors effects: **Sal.** - = Salmonella negative group, **Sal.** + = Salmonella positive group, **Thr.1** = Low Thr/Lys ratio group, **Thr.2** = Standard Thr/Lys ratio group, **Thr.3**= High Thr/Lys ratio group; [4] Standard error of mean for treatments or factors (Sal. = Salmonella grouping; Thr. = Threonine grouping); [5] Significance level of calculated F in analysis of variance; [6] Coefficient of variation (%); [abc] Means without a common superscript letter differ within each part of a column (P<0.05)*

Table 3. *Response to different Thr/Lys ratio and Salmonella challenge (whole period)*
Tablo 3. *Farklı Thr/Lys oranı ve Salmonella maruziyetine karşı tepki (tüm periyot)*

Traits[1]	Mortality %	FI (kg)	WG (kg)	FCR	FCC (USD $)	PER	IBV titer	IBD titer	Urea (mg/dL)	Uric acid (mg/dL)	AST (IU/L)	Glucose (mg/dL)	Chol. (mg/dL)	Trig. (mg/dL)
Treatments[2]														
T.1	9.3^{ab}	4.393	2.519^{a}	1.743^{b}	0.691^{b}	3.125^{a}	1412.5	4475.3	2.83^{b}	2.97^{c}	292.5^{bc}	236.4^{ab}	132.8	114.1
T.2	9.0^{ab}	4.412	2.553^{a}	1.727^{b}	0.686^{b}	3.154^{a}	1368.8	4093.7	2.95^{b}	3.53^{bc}	282.2^{c}	228.1^{ab}	136.7	145.2
T.3	4.7^{b}	4.250	2.513^{a}	1.691^{b}	0.680^{b}	3.222^{a}	1505.0	4780.6	3.12^{b}	3.37^{c}	309.3^{bc}	264.0^{a}	148.2	138.3
T.4	21.2^{a}	4.276	2.126^{c}	2.009^{a}	0.796^{a}	2.717^{b}	1235.6	3879.6	4.17^{a}	3.89^{abc}	370.1^{ab}	198.8^{b}	118.9	104.9
T.5	24.2^{a}	4.425	2.271^{b}	1.946^{a}	0.773^{a}	2.805^{b}	1203.9	3561.6	4.34^{a}	4.61^{a}	358.8^{abc}	192.4^{b}	122.7	136.4
T.6	21.2^{a}	4.046	2.077^{c}	1.951^{a}	0.785^{a}	2.799^{b}	1320.9	4162.9	4.58^{a}	4.37^{ab}	393.4^{a}	222.5^{ab}	132.7	127.0
Factors[3]														
Sal. -	7.7^{b}	4.351	2.528^{a}	1.720^{b}	0.686^{b}	3.167^{a}	1428.8	4449.9	2.97^{b}	3.29^{b}	294.6^{b}	242.8^{a}	139.2^{a}	132.6
Sal. +	22.2^{a}	4.249	2.158^{b}	1.969^{a}	0.785^{a}	2.773^{b}	1253.5	3868.0	4.36^{a}	4.29^{a}	374.1^{a}	204.6^{b}	124.8^{b}	122.7
Thr.1	14.4	4.343^{ab}	2.351^{ab}	1.857	0.736	2.950	1336.7	4220.0	3.40	3.37	325.7	220.2	126.8	110.2^{b}
Thr.2	15.5	4.418^{a}	2.432^{a}	1.821	0.724	3.004	1298.1	3865.6	3.55	4.00	315.0	212.8	130.7	141.5^{a}
Thr.3	11.8	4.162^{b}	2.326^{b}	1.802	0.725	3.040	1426.1	4515.9	3.74	3.80	345.3	246.2	141.5	133.5^{a}
SEM[4]														
Treatments	4.25	0.088	0.039	0.024	0.009	0.033	238.0	348.3	0.24	0.28	24.8	14.7	6.8	9.4
Sal.	2.45	0.051	0.022	0.014	0.005	0.019	137.4	201.1	0.13	0.16	14.3	8.5	3.9	5.4
Thr.	3.00	0.062	0.027	0.017	0.006	0.023	168.2	246.3	0.17	0.20	17.5	10.4	4.8	6.7
P-value[5]														
Treatments	0.037	0.117	0.0001	0.0001	0.0001	0.0001	0.963	0.273	0.0001	0.004	0.026	0.034	0.101	0.061
Sal.	0.001	0.210	0.0001	0.0001	0.0001	0.0001	0.409	0.066	0.0001	0.0003	0.0009	0.005	0.022	0.248
Thr.	0.726	0.036	0.037	0.139	0.398	0.069	0.880	0.243	0.411	0.117	0.511	0.107	0.139	0.011
Sal.×Thr.	0.878	0.536	0.209	0.641	0.611	0.562	0.999	0.993	0.973	0.964	0.988	0.983	0.993	0.991
CV[6]	60.9	4.11	3.33	2.70	2.66	2.20	49.73	23.45	19.10	21.65	21.35	18.42	14.59	20.88

*[1] **FI:** Feed Intake (kg/41d), **WG:** Weight Gain (kg/41d), **FCR:** Feed Conversion Ratio (feed intake/weight gain), **FCC:** Feed Consumption Cost (cost of feed intake in Rials/kg of weight gain), **PER:** Protein Efficiency Ratio (kg of weight gain/kg of consumed crude protein), **IBV titer:** ELISA titer of Infectious Bronchitis Virus, **IBD titer:** ELISA titer of Infectious Bursal Disease, **Chol.:** Cholesterol, **Trig.:** Triglycerides; [2] Comparison of treatments effects: **T.1** = Low Thr/Lys ratio + no Salmonella challenge, **T.2** = Standard Thr/Lys ratio + no Salmonella challenge, **T.3** = High Thr/Lys ratio + no Salmonella challenge, **T.4**= Low Thr/Lys ratio + Salmonella challenge, **T.5** = Standard Thr/Lys ratio + Salmonella challenge, **T.6**= High Thr/Lys ratio + Salmonella challenge; [3] Comparison of factors effects: **Sal. -** = Salmonella negative group, **Sal. +** = Salmonella positive group, **Thr.1** = Low Thr/Lys ratio group, **Thr.2** = Standard Thr/Lys ratio group, **Thr.3** = High Thr/Lys ratio group; [4] Standard error of mean for treatments or factors (Sal. = Salmonella grouping; Thr. = Threonine grouping); [5] Significance level of calculated F in analysis of variance; [6] Coefficient of variation (%); [abc] Means without a common superscript letter differ within each part of a column (P< 0.05)*

(PER) negatively affected (P<0.01) by salmonellosis *(Table 3)*. Improved FCR and PER by adding Thr to basal diet was inconsiderable in whole period but Thr/Lys ratio over catalog recommendation in *Salmonella* positive group at finisher improved (P<0.01) FCR than lowest Thr/Lys ratio *(Table 2)*. Salmonellosis led to increase (P<0.01) in feed consumption cost per 1 kg weight gain *(Table 3)*.

Improved feed consumption cost (FCC) by increasing Thr/Lys ratio was inconsiderable. Results on mortality, feed intake, weight gain and FCR at starter and grower are given in *Table 2*. A trend (P<0.05) was observed between increases in Thr/Lys ratio over catalog recommendation and decrease in feed intake and weight gain at grower *(Table 2)*. At grower negative effect of maximum Thr/Lys ratio than catalog recommendation on FCR was inconsiderable and recommended Thr/Lys ratio by catalog has the best FCR non-significantly *(Table 2)*.

Blood Parameters

Salmonellosis increased serum urea, uric acid and AST and decreased serum glucose and cholesterol (P<0.01). Salmonellosis did not decrease serum triglycerides significantly. A non-significant trend in challenged and non-challenged groups was observed between increasing Thr/Lys ratio and increased serum urea and uric acid. Increasing Thr/Lys ratio had no significant incremental effect on serum AST and glucose but ratio 25% over catalog recommendation led to a small increasing in challenged and non-challenged groups. Increasing Thr/Lys ratio led to increase (P<0.05) in serum triglyceride but the similar effect on cholesterol was not significant.

Humoral Immune Response

Decreased humoral immune response by salmonellosis was not significant. Increasing Thr/Lys ratio over catalog recommendation did not improve humoral immune response in challenged and non-challenged groups.

DISCUSSION

Salmonellosis inducted by *Salmonella* Paratyphi A, negatively affected production performance (similar to *Clostridium* infection) and mortality rate and increasing Thr/Lys ratio over catalog recommendation could not improve them significantly [5]. However, a trend (P<0.05) was observed between increase in Thr/Lys ratio and improvement of FCR in finisher period that may be because of its small negative effect on feed intake and obtained similar weight gain.

In whole period, recommended Thr/Lys ratio by catalog showed increase in feed intake and weight gain than level 25% more, that was also significant in weight gain of infected chickens *(Table 3)*. Weight gain of infected chickens fed recommended Thr/Lys ratio by catalog was more than other levels, similar to *Clostridium* infected chicken [5,32]. This is may be because low Thr and high Thr led to amino acid imbalance and amino acid imbalance led to decreased feed intake and weigh gain and impairment of FCR [33-35]. Therefore, recommended Thr/Lys ratio by catalog seems to be adequate for optimum performance [11].

Increase in diet's crude fiber can affect intestinal mucosa and consequently digestive tract health that may lead to gut susceptibility to infections [36,37]. Therefore, with the aim of increasing diet's crude fiber and decreasing threonine content of treatment 1 as far as possible, the basal diet was formulated in total amino acids system on base of corn, wheat, barley, wheat bran, rice bran and soybean meal. It is known that a threonine deficiency will affect mucin secretion and, thereby, gut barrier integrity [38]. Mucus contains relatively high threonine levels, suggesting that the threonine requirement in birds with intestinal problems may increase [3]. European Centre for Disease Prevention and Control [39] reported *Salmonella Paratyphi* A was the most commonly identified serotype in human cases of paratyphoid fever in EU/EEA countries. Because of epidemiological importance of this serovar around the world, this serovar was selected for study. In the present study, FCR and PER generally not improved by increasing Thr/Lys ratio. In *Salmonella* challenge group, that may be because of severity of inducted infection by this *Salmonella* serovar similar to *Clostridium* infected chicken or relatively few differences between Thr/Lys ratios of basal diets and recommended Thr/Lys ratio [5,6]. Nevertheless this general resulting, FCR of challenged group in finisher period tended to improve with increasing Thr/Lys ratio; however, no significant effects on feed intake or weight gain of these chickens were observed. This may be because of other beneficial metabolic effect of threonine, like on immune response to *Salmonella* infection as coccidiosis challenge [6].

Some animals, like rats and pigs, have a specific requirement for threonine to optimize immunity that is much higher than that of growth [9,40,41]. In the present investigation no differences in humoral immunity or mortality were observed in challenged and non challenged groups in response to different Thr/Lys ratios. Similar investigations reported that high Thr/Lys ratio over (well-nigh) Ross nutrition specification recommendation had no significant effect on improvement of cellular or humoral immunity [2,42,43]. Nevertheless, improved cellular or humoral immunity in response to increased Thr/Lys ratio were reported in diets with great Thr deficiency and more threonine supplementing than recommended Thr/Lys ratio by Ross nutrition specification had no significant effect [4,5,44].

Identification of the arginine pathway that produces nitric oxide has led to research demonstrating that Arg is a potent immunological modulator [45]. Animal and human studies suggest outcome benefit to the use of supplemental dietary arginine [46]. Complimentary effects of arginine on immune function and health of broiler chickens showed high levels of Arginine accelerated antibody production [47]. Depressed kidney arginase activity by feeding high level of threonine was reported that may leading to more bio-availability of arginine for immune responses and less urea excretion in chickens [48,49]. In the present study no decrease in serum urea in response to increased diets Thr level were observed, whereas serum urea showed a non-significant incremental trend. More investigation about relationship between arginase activity and threonine levels on immune response alteration mechanism is recommendable.

No significant negative effect of highest examined Thr level on AST was observed. Correlation with no significant alteration of serum glucose and cholesterol, showed highest examined Thr level might have had no adverse effect on liver functions. Significant increased of serum triglycerides in response to increased Thr/Lys ratio on ensemble challenged and non-challenged groups might indicate on better absorption of triglycerides from healthier absorptive surface.

Increase in Thr/Lys ratio 25% over catalog recommendation could slightly be economically lucrative; however, Thr/Lys ratio 25% over catalog recommendation significantly improved FCR compare with Thr deficient diet in *Salmonella* challenged group. In severe gastric infection that leading to mortality, increasing Thr/Lys ratio over catalog recommendation seemed to have inconsiderable effect on subjugating the infection and seems cannot be a choice for replacing the antibiotics.

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Research Article

Evaluation of Lead, Cadmium, Arsenic and Mercury Heavy Metal Residues in Fish, Shrimp and Lobster Samples from Persian Gulf [1]

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Abstract

Severe discharge of sewage and industrial effluents into the Persian Gulf causes the deposition of various types of heavy metals and especially lead, cadmium, arsenic and mercury in the muscles of marine animals. The present study was carried out to evaluate the concentration of lead, cadmium, arsenic and mercury in the fish *(Scomberomorus commerson)*, shrimp *(Fenneropenaeus indicus)* and lobster (Panulirus homarus) samples from Persian Gulf. All of the samples were collected from the shopping centers in the Boushehr city. Weight and length of samples were measured and recorded. Concentrations of lead, cadmium, arsenic and mercury in fish, shrimp and lobster samples were analyzed using atomic absorption spectrophotometer. Average length and weight of collected samples were 46.9±2.68 cm and 642.237±52 g for fish, 18.88±1.67 cm and 45.779±4.51 g for shrimp and 22.3±2.13 cm and 203.098±20 g for lobster. Heavy metals concentrations in fish samples were 91.67±9.21 for lead, 49.00±4.77 for mercury, 60.37±7.07 for cadmium and 101.33±9.85 µg/g for arsenic. Lead, mercury, cadmium and arsenic concentration ranges were 64-93, 104-135, 18-34 and 211-265 µg/g in shrimp and 260-390, 71-130, 114-348 and 118-318 µg/g in lobster, respectively. ANOVA test showed significant statistically differences (P<0.05) between the type of seafoods and concentration of heavy metals. However, the levels of toxic elements were less than allowable concentrations but consumption of the contaminated seafoods with low levels of heavy metals may be harmful for human health.

Keywords: *Arsenic, Lead, Mercury, Cadmium, Persian Gulf, Seafood*

Basra Körfezindeki Balık, Karides ve Istakoz Örneklerinde Kurşun, Kadmiyum, Arsenik ve Civa Ağır Metal Seviyelerinin Değerlendirilmesi

Özet

İran Körfezine aşırı miktarda lağım ve endüstriyel atığın salınması çeşitli ağır metallerin ve özellikle de kurşun, kadmiyum, arsenik ve cıvanın sucul canlıların kaslarında birikmesine neden olmaktadır. Mevcut çalışma, İran Körfezindeki balık *(Scomberomorus commerson)*, karides *(Fenneropenaeus indicus)* ve ıstakoz (Panulirus homarus) örneklerinde kurşun, kadmiyum, arsenik ve cıva ağır metal konsantrasyonlarının belirlenmesi amacıyla gerçekleştirildi. Tüm örnekler Boushehr şehrindeki alışveriş merkezlerinden toplandı. Örneklerin ağırlık ve uzunlukları ölçülerek kaydedildi. Balık, karides ve ıstakoz örneklerinde kurşun, kadmiyum, arsenik ve cıva ağır metal konsantrasyonları atomik absorpsiyon spektrofotometre ile belirlendi. Ortalama uzunluk ve ağırlıklar sırasıyla balıkta 46.9±2.68 cm ve 642.237±52 g, karideste 18.88±1.67 cm ve 45.779±4.51 g ve ıstakozda 22.3±2.13 cm ve 203.098±20 g olarak kaydedildi. Balık örneklerindeki ağır metal seviyeleri kurşun için 91.67±9.21, cıva için 49.00±4.77, kadmiyum için 60.37±7.07 ve arsenik için 101.33±9.85 µg/g olarak tespit edildi. Kurşun, cıva, kadmiyum ve arsenik konsantrasyonları karideste sırasıyla 64-93, 104-135, 18-34 ve 211-265 µg/g ıstakozda ise 260-390, 71-130, 114-348 ve 118-318 µg/g değerleri arasında belirlendi. ANOVA testi deniz ürünleri ve ağır metal konsantrasyonları yönünden istatistiksel olarak anlamlı farlılıkların olduğunu gösterdi (P<0.05). Toksik maddelerin seviyeleri izin verilen seviyenin altında olmasına rağmen düşük düzeyde ağır metal ile kontamine deniz ürünlerinin tüketilmesi insan sağlığı açısından tehlikeli olabilir.

Anahtar sözcükler: *Arsenik, Kurşun, Cıva, Kadmiyum, İran Körfezi, Deniz ürünü*

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INTRODUCTION

Seafoods such as fish, shrimp and lobster provide essential nutrients for the human nutrition. They contain complete proteins, vitamins and minerals which are considered to be major dietary compounds for the health, in particular, including high levels of polyunsaturated fatty acids such as omega-3 and omega-6. Tens of millions of people consume these products daily. This situation makes the quality of the seafoods much more important for the public health. Otherwise, the seafoods may be potential reservoirs for the pathogens and harmful chemical pollutants, leading to the seafood-related diseases [1-3].

One of the most important issues in the present century is the discharge of industrial waste water into the rivers and seas. Chemical waste waters are usually the sources of heavy metals. Unfortunately, heavy metals can be accumulated in different organs of fish and marine animals and may ultimately affect the human food chain [4-6]. Consumption of seafoods contain consider levels of heavy metals may lead to several disorders. Heavy metals can easily be accumulate in organs such as the liver and the kidney in long time periods. Nervous system and kidneys are the most common targets of lead and cadmium [7,8]. Cardiovascular collapse, acute paralytic syndrome and loss of brain function are the main disorders caused due to the consumption of foods containing high levels of arsenic [9]. Long-term ingestion of arsenic increases the risk of skin, bladder, and lung cancers [9].

Mercury is an element of special concern because its inorganic form is biologically transformed in aquatic environments into methylmercury (MeHg), which is a lipophilic organic compound that bioaccumulates and biomagnifies as it moves up the aquatic food chain [10]. Methylmercury may mimic biologicals and be transported by amino acid or organic anion transporters. Further, the generation of reactive oxidative species is often induced by metals in their ionic form, resulting in oxidative modification of DNA or proteins, including aberrant gene expression and carcinogenesis [11,12].

According to the uncertain status of contamination of seafoods of Persian Gulf with dangerous heavy metals, the present study was carried out to evaluate the concentration of lead, cadmium, arsenic and mercury heavy metals in the fish, shrimp and lobster samples of Persian Gulf.

MATERIAL and METHODS

Samples Collection

During the period of October 2013 to May 2014, a total of 200 samples (80 fish, 60 lobster, and 60 shrimp) from the supermarkets were randomly collected in the Boushehr province of the Persian Gulf, Iran. All samples were caught recently from the Persian Gulf. All samples were maintained in cold box and transferred to the laboratory at 4°C. Total length (cm) and body weight (g) of the samples were measured and recorded before dissection. Average length and weight of collected samples were 46.9±2.68 cm and 642.237±52 g for fish *(Scomberomorus commerson)*, 18.88±1.67 cm and 45.779±4.51 g for shrimp *(Fenneropenaeus indicus)* and 22.3±2.13 cm and 203.098±20 g for lobster *(Panulirus homarus)*. The muscle samples were maintained in −20°C freezer prior to analysis.

Devices, Reagents and Materials

All glassware was soaked overnight in 10% (v/v) nitric acid (Merck, Germany), followed by washing with 10% (v/v) hydrochloric acid (Merck, Germany) and rinsed with double distilled water and dried before using.

A Varian Model 220 atomic absorption spectrophotometer (Varian AA 220FS Atomic Absorption Spectrometer System, United States) equipped with a deuterium background corrector was used for the determination of heavy metals. Lead and cadmium concentrations were determined by a graphite furnace atomic absorption spectrophotometer (GFAAS, Analytik Jena AG, Germany, AAS ZEEnit 650) 110 employing pyrolytic platform graphite tubes (Agilent Tech, Santa Clara, California) [13]. Hydride generation was with a Varian model 77 with quartz tubes.

All reagents used were of analytical reagent grade (Merck, Germany). Standard stock solutions of mercury, arsenic, cadmium and lead were prepared from Titrasol (1000 mg/l) (Merck, Germany) and were diluted to the corresponding metal solution. The working solution were freshly prepared by diluting an appropriate aliquot of the stock solutions using 10% HNO_3 (Merck, Germany) for diluting lead and cadmium solutions, 1 M HCl (Merck, Germany) and 5% H_2SO_4 (Merck, Germany) for diluting mercury solution, 7 M HCl for diluting arsenic solution and 5% HCl for diluting tin solution. Stannous chloride, for mercury analysis, was freshly prepared by dissolving 10 g in 100 ml of 6 M HCl. The solution was boiled for about 5 min, cooled, and nitrogen bubbled through it to expel any mercury impurities.

Sample Preparation and Digestion

Each sample was homogenized thoroughly in a food blender with stainless steel cutters. A sample were then taken and digested promptly as follows: 2 g of the homogenized sample was weighed into a 0.5 l glass digestion tube, and for mercury, 10 ml of concentration of HNO_3 (Merck, Germany) and 5 ml of concentration of H_2SO_4 (Merck, Germany) were slowly added. The tube was then placed on top of a steam bath unit to complete dissolution. It was then removed from the steam bath, cooled and the solution transferred carefully into a 50 ml volumetric flask; for the reduction of mercury 5 ml $SnCl_2$ (Merck, Germany)

were used. For arsenic determination 2 g of homogenized sample was weighed after pre-digestion. Then, HNO_3 mixed with 4 ml of $MgNO_3$ 20% (Merck, Germany) as ashing aid, dried on a hot plate and ashed in a 450°C furnace. The ashes were dissolved in 7 ml of HCl and diluted to 50 ml. For the determination of lead and cadmium, about 2 g of homogenized sample were weighed into a 200 ml beaker and 10 ml of concentration of HNO_3 were added. The beaker was covered with a watch glass and, after most of the sample had dissolved by standing overnight, heated on a hot plate with boiling until any vigorous reaction had subsided. The solution was allowed to cool in room temperature, transferred into a 50 ml volumetric flask and diluted to the mark with distilled water.

Aquatic samples were spiked with various concentrations of heavy metals for the recovery repeatability tests and for verifying the analytical methodology. For each run, triplicate samples, spiked samples and blanks were carried through the digestion reaction. The results are shown in *Table 1*.

Chemical Analysis

Mercury and arsenic were determined by the hydride generation system. The manufacturer operation procedure involves continuous addition of reductant, consisting of 0.3% $NaBH_4$ (Merck, Germany), 0.5% NaOH (Merck, Germany) for mercury and 0.6% $NaBH_4$ (Merck, Germany), 0.5% NaOH, 10% KI for arsenic. The manufacturer's operating procedure consists of adding sample, reductant and acid, with the aid of argon gas, to a reaction coil; then any vapour generated is swept into the absorption quartz cell, and heated for arsenic detection. Cells were aligned in the light path of the hollow cathode lamp where the absorption was measured. Cadmium and lead concentrations were determined by graphite furnace atomic absorption spectrophotometry, employing pyrolytic platform graphite tubes (Agilent Tech, Santa Clara, California), ascorbic acid and palladium for matrix modification and using the method of additions for quantification. Graphite Tube Atomizer (GTA) was equipped with an auto sampler and the analysis was done according to the manual instruction, optimized conditions and the method of peak area [13].

Table 1. *Evaluation of the recovery rates of lead, cadmium, mercury and arsenic in fish, shrimp and lobster*
Tablo 1. *Balık, karides ve ıstakozlarda kurşun, kadmiyum, cıva ve arsenik gerikazanım oranları*

Heavy Metals	Added Concentration (µg g^{-1})	Achieved Concentration (µg g^{-1}) (Average±SD)	% Recovery
Lead	50	47.66±1.15	95.32
Cadmium	50	48.33±2.00	96.66
Arsenic	50	51.00±1.00	102.00
Mercury	50	45.33±1.52	90.60

Statistical Analysis

The results of the mercury, arsenic, cadmium and lead concentration in fish, lobster and shrimp were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), Analysis of Variance (ANOVA) test were used for analysis of the variances. Differences were considered significant at values of $P<0.05$.

RESULTS

The results of the present investigation showed that the aquatic food samples of Persian Gulf were contaminated with lead, mercury, cadmium and arsenic heavy metals. Concentration of heavy metals in each studied samples is shown in *Table 2*. Heavy metals concentrations in fish samples were 91.67±9.21 for lead, 49.00±4.77 for mercury, 60.37±7.07 for cadmium and 101.33±9.85 µg/g for arsenic. Lead, mercury, cadmium and arsenic concentration ranges were 64-93, 104-135, 18-34 and 211-265 µg/g in shrimp and 260-390, 71-130, 114-348 and 118-318 µg/g in lobster, respectively. Significant statistically differences were seen for the concentration of lead between shrimp and lobster ($P=0.015$), concentration of mercury between fish and shrimp ($P=0.029$), concentration of cadmium between shrimp and lobster ($P=0.024$) and finally concentration of arsenic between fish and shrimp ($P=0.023$) and lobster and shrimp ($P=0.027$).

DISCUSSION

The results of the present study showed that the fish, shrimp and lobster samples of Persian Gulf have been

Table 2. *Average and standard deviation of the concentration of the lead, mercury, cadmium and arsenic heavy metals in fish, shrimp and lobster*
Tablo 2. *Balık, karides ve ıstakozlarda kurşun, cıva, kadmiyum ve arsenik konsantrasyonlarının ortalama ve standart sapmaları*

Samples	Concentration of Heavy Metals (µg g^{-1})							
	Lead		Mercury		Cadmium		Arsenic	
	Average±SD	Range of Contamination	Average±SD	Range of Contamination	Average±SD	Range of Contamination	Average±SD	Range of Contamination
Fish	91.67±9.21	63-121	49.00±4.77	19-98	60.37±7.07	18-87	101.33±9.85	62-148
Shrimp	75.67±8.33	64-93	115.67±10.86	104-135	26.00±2.00	18-34	237.67±22.01	211-265
Lobster	316.67±29.58	260-390	98.33±7.74	71-130	131.46±12.57	114-348	105.28±10.21	118-318

contaminated with considerable levels of lead, cadmium, arsenic and mercury heavy metals. However, the levels of these toxic elements were entirely less than their allowable concentrations but consumption of the contaminated seafoods even lower than their permissible levels may be harmful for human health. One of the most important point to evaluate the levels of lead, cadmium, arsenic and mercury heavy metals in seafood is comparison of the permitted extent and acceptance daily intake of heavy metals with the amount obtained. The acceptance limits recommended of mercury, lead, cadmium and arsenic is 0.5 mg/kg [14-16], 0.5 mg/kg [17], 0.5 mg/kg [14-17] and 6 mg/kg [18], respectively. The levels of detected lead, cadmium, arsenic and mercury in our study were 47.66, 48.33, 51.00 and 45.33 µg/g, respectively. The levels of detected elements were lower than the acceptable limits recommended.

Average daily lead intake through diet was about 114 microg/day for adults and 50 microg/day in children and tolerable limit is 250 microg/day for adults and 90 µg/day for children. Acceptance daily intake of cadmium is 3.0 µg/kg body weight per day (2-7 µg/kg body weight per day) and the tolerable weekly intake of this element 15 µg/kg body weight. Tolerable weekly intake of mercury is 1.6 µg/kg body weight. Average daily arsenic intake is 3.0 µg/kg and its tolerable weekly intake is 15 µg/kg body weight [19-21].

Similar studies have been done of the determination of heavy metals such as copper, lead, cadmium, zinc, mercury and arsenic in the seafood samples of the Persian Gulf [6,22-24]. Previous study [6] reported that the obtained range of heavy metals in the fish species of Persian Gulf were 0.024-0.111 µg/g for cadmium and 0.057-0.471 µg/g for lead which was entirely lower than our results. Agah et al.[22] reported that the concentration range of lead was 0.2-25 ng/g in various species of fish caught from the Persian Gulf. Reissy et al.[23] showed that the heavy metals concentrations in lobster samples of Persian Gulf were 32-73 µg/kg for mercury, 118-275 µg/kg for arsenic, 379-1120 µg/kg for lead and 101-401 µg/kg for cadmium which was entirely lower than our results except lead concentration. In a study which was conducted on Vietnam [24], the lead, cadmium, and mercury concentration ranges in shellfish were 0.008-0.083, 0.013-0.056, and 0.028-0.056 mg/kg, respectively. Islam et al.[25] reported that the concentrations of mercury, arsenic, cadmium and lead varied between 0.24±0.007 - 0.01±0.001, 44.54±5.69 - 1.23±0.20, 0.13±0.05 - ND (not detected), 1.32±0.47 - 0.09±0.02 and 0.74±0.28 - 0.05±0.03 mg/kg, respectively.

One possible explanation for the higher presence of heavy metals in our results is the fact that the fish, shrimp and lobster samples of our study are in close contact with contaminant sources like oil tankers and industrial wastewaters. Besides, differences in the races of studied seafoods of our investigation with those of other studies is another reason for the higher prevalence of heavy metals in our research. Considerable differences in the concentration of heavy metal between various races of seafoods have been reported previously [6,22,26,27]. The season which the seafood samples were collected and analyzed for presence of heavy metals is another determinative factor in the concentration of toxic elements. It seems that different seasonal dependent conditions such as water temperature, dietary factors and growth and reproductive cycles are effective on heavy metal fluctuations [28-31]. The higher metal content in winter might be a result from considerable rainfall which washed down the wastes [28-31]. Therefore, variation in the seasons of sampling may has the direct effect of the concentration of heavy metals. Large differences in the levels of heavy metals in the seafood samples of our study may be related to the variation in the weight and length of samples. There were no significant differences between the concentrations of heavy metals and average length and weight of seafood samples ($P>0.05$). Average length and weight of collected samples were 46.9±2.68 cm and 642.237±52 g for fish, 18.88±1.67 cm and 45.779±4.51 g for shrimp and 22.3±2.13 cm and 203.098±20 g for lobster. Fish samples had the highest length and weight but had the lowest concentrations of mercury and arsenic. Petroody et al.[32] reported the significant relationships between lengths and lead concentrations ($P<0.01$), but no significance correlation between length and cadmium concentrations were observed. In a study which was conducted by Jafarzadeh Haghighi et al.[33] concentrations of cadmium in the fish samples was positively correlated with length and weight.

Level of arsenic in the seafood samples in the current study was entirely higher than previous studies that reported arsenic concentration in crabs, shrimps, lobster, fishes and bivalves of Persian gulf [23,34-36]. In a study which was conducted by Heidarieh et al.[34] the concentrations of heavy metals was evaluated in crab and shrimp samples of Persian Gulf, Iran. Their results showed that arsenic concentrations in crab and shrimp samples were 21.38±3.31 and 8.28±2.82 µg/g, respectively. In another study which was conducted by Javaheri Babooli and Velayatzadeh [37] the mean concentrations of mercury, arsenic, cadmium and lead in shrimp samples of Persian Gulf was 0.032±0.002, 0.117±0.07, 0.175±0.006, 0.414±0.012 µg/g which was lower than our results. The main anthropogenic sources of arsenic in Persian Gulf are emissions from coal burning electrical generating facilities, mining and smelting operations, herbicide or algicide applications especially for algae bloom and leaching from hazardous waste facilities and from insecticide [38].

High concentration of mercury was seen in the seafood samples of our investigation. Another Iranian survey showed the mean levels of mercury in fish samples of Persian Gulf was 0.05 µg/g [39]. Agah et al.[40] reported that total mercury concentrations in fish ranged from 0.0123 to 0.0867 mg/kg. Documented data reported that in several coastal areas of the Persian Gulf such as United Arab

Emirates, Iran, Kuwait and Qatar, mercury levels exceeded 0.1 mg/kg in various fish species. Only in the coastal area of Bahrain, the average concentrations of mercury were below 0.1 mg/kg [41]. Mercury toxic effects have been emphasized by some cases of collective poisoning in people who consumed a large amount of fish [42]. It is generally accepted that seafood represents one of the major sources of mercury in the human food chain.

Other important heavy metals studied in our study were cadmium and lead. Our results showed that the mean concentration of lead and cadmium in fish, shrimp and lobster samples of Persian Gulf had the range of 75.67±8.33 to 316.67±29.58 µg/g and 26.00±2.00 to 131.46±12.57 µg/g, respectively. In a study which was conducted by Dadolahi and Nazarizadeh Dehkordi [43] on Sediments from the North of the Strait of Hormuz, Persian Gulf, Iran the mean concentrations of lead and cadmium were 3.00 to 28.00 and 2.96 to 10.11 mg/g, respectively which was entirely lower than our findings. Haghighi et al.[33] reported the lower range of cadmium in two different fish samples of Persian Gulf (0.1±.05 to 0.31±0.09 mg/g). The concentrations of cadmium and lead in the fish samples of Persian Gulf have been studied previously by Mohammadnabizadeh et al.[44]. They showed that the total concentration of cadmium and lead in the fish samples had the ranges of 0.58±0.03 to 1.7±0.2 and 0.22±0.06 to 1.12±0.17 mg/g, respectively.

The main factors which may lead to the pollution of water sources with heavy metals mainly are leakage of industrial sewage from seaside, industrial effluents, agricultural runoff, domestic and municipal sewage and pedogenic background contributions.

In conclusion, the results of the present study indicated that the seafoods of the Persian Gulf were contaminated with low range of lead, cadmium, arsenic and mercury heavy metals. Our results indicated the improvement of local environmental conditions with respect to mercury, lead, arsenic and cadmium concentrations, which is explained by reduced industrial activity in the region (Boushehr seaside), the use of more efficient dust traps and filters in recent years, and, hence, the decreased discharge of these metals into the sea. However, the contents of toxic metals in Persian Gulf were below the permitted levels but more studies are needed to properly assess other sources and even other sites of the Persian Gulf to monitoring the levels of heavy metals.

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Research Article

Determination of Potential Biomarker among Plasma Sphingosine-1-Phosphate, Total Sialic Acid and Adenosine Deaminase in Cattle with Naturally Infected Liver Cystic Echinococcosis

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Abstract

The purpose of this trial was to assay the alterations of some blood parameters such as sphingosine 1 phosphate (S1P), total sialic acid (TSA) and adenosine deaminase (ADA) and clarification of possible potential biomarker among them in naturally infected liver cystic echinococcosis in cattle. After observation of severe liver parasitic infestation (as cystic form) and blood sampling from parasitized and healthy ones, all selected biochemical analytes were clarified and results determined significant increase ($P \leq 0.01$) of aforementioned parameters in parasitized group rather than healthy ones. In conclusion, based on high sensitivity of TSA and ADA than S1P, they could be considered as potential biomarker in CE.

Keywords: *Biochemical parameters, Cattle, Echinococcosis*

Doğal Enfekte Karaciğer Kistik Ekinokokozisli Sığırlarda Plazma Sfingosin-1-fosfat, Total Sialik asit ve Adenozindeaminaz Arasından Potansiyel Biomarkerın Belirlenmesi

Özet

Bu çalışmanın amacı, doğal enfekte karaciğer kistik ekinokokozisli sığırlarda sfingozin-1-fosfat (S1P), total siyalik asit (TSA) ve adenozin deaminaz (ADA) için kan değerlerinin belirlenmesi ve böylece aralarından hangisinin muhtemel potansiyel bir biyomarker olabileceğinin belirlenmesidir. Parazitli olan (şiddetli kistik formu) ve sağlıklı sığırlardan kan örnekleri alındıktan sonra, belirtilen tüm biyokimyasal analizler yapıldı ve sonuçlar karşılaştırıldığında parazitli olan grupta anlamlı bir artış ($P \leq 0.01$) olduğu belirlendi. Sonuç olarak, TSA ve ADA S1P`e göre daha yüksek hassasiyetlik göstermesi sebebiyle, kistik ekinokokkozisde potansiyel biyomarker olarak değerlendirilebilir.

Anahtar sözcükler: *Biyokimyasal parametreler, Sığır, Ekinokokozis*

INTRODUCTION

Echinococcus granulosus contributes in the occurrence of cystic echinococcosis (CE) in animals (domestic and wild herbivores) and man. Echinococcosis is known as one of the most essential zoonotic diseases in the world and possesses important effect on human and animal health with remarkable economic detriments [1]. Carnivores play substantial effect as definitive hosts, while domestic ungulates and human are involved as the intermediate hosts. Similarly, sheep participate in disease transmission and the strain (G1) of *Echinococcus granulosus* causes CE. It is usually asymptomatic in livestock, however, during inspection of carcass at the slautherhouse can lead to detection of disease [2,3].

Sphingosine 1-phosphate (S1P) is known as novel bioactive lipid mediator which belongs to sphingolipids group and it participates in both cellular physiological and pathophysiological pathways [4]. Subsequent studies determined that S1P is abundantly existed in plasma and other body fluids, where it acts as autocrine or paracrine effects onto fundamental cell functions [5]. Erythrocytes and platelets store and liberate S1P into blood and they are considered as fundamental sources of S1P [6]. In addition, S1P involve as major bioactive molecule in exhausting

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of lymphocyte from the secondary lymphoid tissues into the lymph. Thus, these findings forcefully suggest that S1P has fundamental effects *in vivo* as well as potentially pathophysiological roles as a circulating paracrine mediator [5]. It should be noted that ability of S1P in the modulation of fibroblasts migration has been determined and plays substantial role in fibrosis in different tissues [7].

Sialic acid (SA) is acetylated derivative of neuraminic acid which has been broadly distributed in mammal tissues and body fluids. SA is classified in three forms namely protein-bounded sialic acid (PBSA), lipid-bounded sialic acid (LBSA) and free form and it is involved at the end chain of many acute phase proteins [8]. Thus, the detection of SA might be a momentous marker for diagnosis and prognosis of inflammatory diseases [8,9]. In many infectious diseases, SA are determined in cattle, such as keratoconjunctivitis, leptospirosis, pneumonia, theileriosis, anaplasmosis and traumatic reticulo-peritonitis [8]. It is linked to residues of the carbohydrate chains of glycoproteins and glycolipids (especially non-reducing sections) [10]. This linkage becomes it susceptible to get involved in cellular and molecular interrelationships and also participates in lipoproteins and lipid metabolism [11].

Adenosine deaminase (ADA) is involved in degradation of adenosine and deoxyadenosine into inosine and deoxyinosine. ADA, as an essential enzyme, contributes to the maturation and differentiation of T lymphocytes and its activity is higher in T cells than B cells [12]. The ADA regulates the cellular mechanisms associated with blood flow, vasodilatation, angiogenesis and proliferation [13]. It has been indicated that serum ADA activity is higher in diseases associated with immune response stimulation such as liver cirrhosis, chronic hepatitis and hepatocellular carcinoma. Generally, serum ADA level has been referred in human and animals as an important indicator for detection of liver diseases [14].

To our knowledge, no research has been conducted to determine potential biomarker among TSA, ADA and S1P in cattle echinococcosis. Thus, the present study aimed to investigate the above-mentioned issue in cattle echinococcosis.

MATERIAL and METHODS

This study was conducted in Urmia city (West Azerbaijan province), Iran. Ten milliliters of blood were collected via the jugular vein of cattle (20-22 months) that had been admitted for slaughtering at the abattoir of Urmia and blood samples transferred equally to EDTA-contained and non-EDTA contained tubes. After slaughtering, animals were surveyed based on observation of severe hepatic CE in liver. Eighty animals were infected to hepatic CE and same number were also selected as control group (healthy animals). Microscopic examination of blood smears staining in the immersion objective (X100) revealed no parasite in infected and healthy sheep.

All samples were centrifuged 6.000 g for 10 min at room temperature for plasma and serum preparation and were kept frozen (–25°C) until the analysis. The plasma S1P level was determined using a RA1000 in accordance with the ELISA method by (East Biopharm Co, Hangzhou, China). TSA was measured by Sydow method [15] in serum (spectrophotometer, model Spekol 1500, Germany). Total bilirubin and unconjugated bilirubin were measured colorimetrically by (Pars Azmoon Co. kits Tehran, Iran) in serum. Finally, ADA activity was determined by the electrochemiluminescence method (Roche Co. Elecsys 2010).

Statistical analysis was accomplished in all analyses. The Mean ± SD and the determination of variation between the data results were carried out with Student's *t*-test through SAS v9.1 (SAS Institute Inc., Cary, NC, USA). The significance level was specified at ($P<0.01$). Moreover, determination of cut-off point among with ROC analysis were carried out in all parameters for sensitivity and specificity detection.

RESULTS

All of the altered parameters are shown in *Table 1*. Significant increase ($P<0.01$) in S1P, TSA, ADA, total bilirubin and unconjugated bilirubin levels were revealed in the patient group compared to the healthy ones. In the respect of *Table 2*, based on cut-off point, AUC and ROC curve statistical analysis, all parameters possesses different sensitivity and specificity percentage and among them TSA and ADA sensitivity are better than S1P sensitivity. In the *Fig. 1*, significant increase of TSA (42.84 mg/dl) in the patient group has been noted compared with control ones (14.07 mg/dl). The *Fig. 2* illustrate ADA alterations between two groups. Considerable elevation (42.17 U/L) in patient group versus (15.92 U/L) in control ones. Regarding S1P as bioactive mediator *(Fig. 3)*, considerable increase (271.77ng/L) was demonstrated compared control group (89.33 ng/L). In connection with bilirubin metabolism *(Fig. 4)*, total and unconjugated form have been severely increased

Table 1. *Alterations of plasma S1P, ADA and TSA levels in the control and patient groups*
Tablo 1. *Kontrol ve hasta gruplarında plasmadaki S1P, ADA ve TSA seviyelerindeki değişiklikler*

Parameters	Control Group	Patient Group
TSA (mg/dl)	14.07±2.85	42.84±8.56†
ADA (U/L)	15.92±3.15	42.17±10.02†
S1P (ng/l)	89.33±20.48	271.77±82.57†
Total bilirubin (mg/dl)	0.34±0.012	1.49±0.02†
Unconjugated bilirubin (mg/dl)	0.07±0.001	0.82±0.06†

Data are expressed as mean ± standard deviation. † Significantly different from the control group (P<0.01)

Table 2. *According to table, as area under the curve for each parameter is equal to 1. Hence all parameters have good specificity, but TSA and ADA sensitivity are higher than S1P*

Tablo 2. *Tablo ya göre, her bir parametre için eğrinin altında kalan alan olarak 1'e eşittir. Dolayısıyla tüm parametrelerin iyi özgüllüğü vardır, fakat TSA ve ADA hassasiyeti S1P göre daha yüksektir*

Parameter	Cut-off Point	AUC	P Value	Sensitivity (%)	Specificity (%)
S1P	55/180	1	0001/0	5/87-85	100
TSA	69/28	1	0001/0	100- 5/97	100
ADA	81/28	1	0001/0	95	100

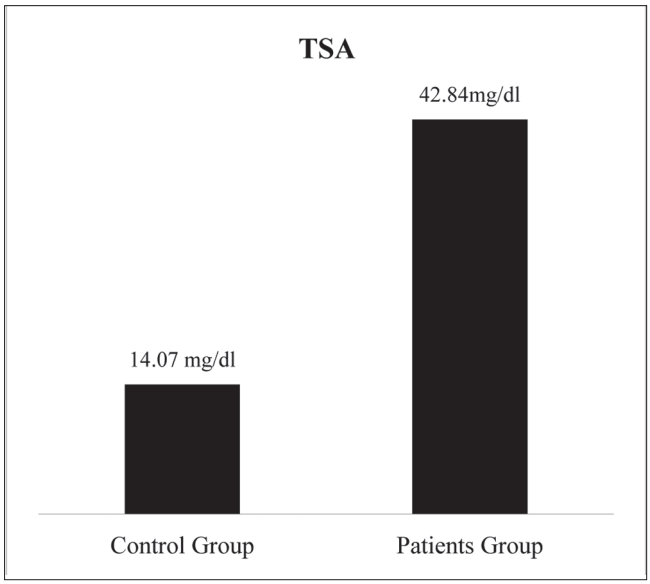

Fig 1. Alterations of TSA levels in infected group compared with control ones

Şekil 1. Kontrol grubuyla karşılaştırıldığında enfekte grupta TSA düzeylerindeki değişimler

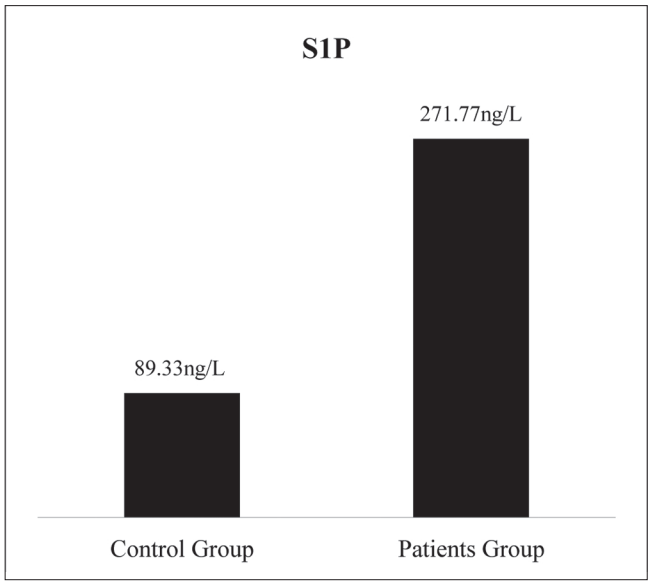

Fig 3. Alterations of S1P levels in infected group compared with control ones

Şekil 3. Kontrol grubuyla karşılaştırıldığında enfekte grupta S1P düzeylerindeki değişimler

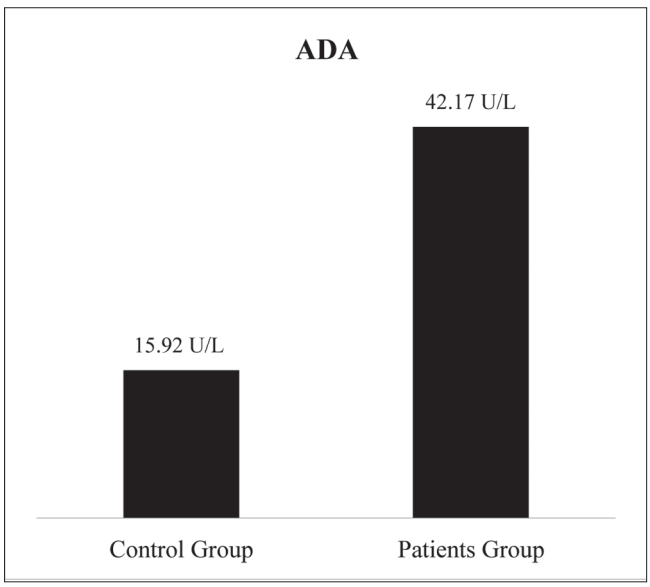

Fig 2. Alterations of ADA levels in infected group compared with control ones

Şekil 2. Kontrol grubuyla karşılaştırıldığında enfekte grupta ADA düzeylerindeki değişimler

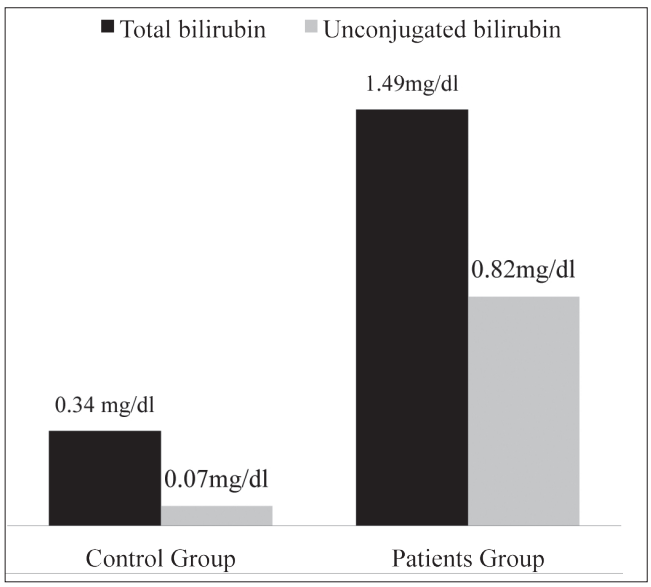

Fig 4. Alterations of total bilirubin and unconjugated bilirubin levels in infected group compared with control ones

Şekil 4. Kontrol grubuyla karşılaştırıldığında enfekte grupta total bilirubin and konjuge olmayan bilirubin düzeylerindeki değişimler

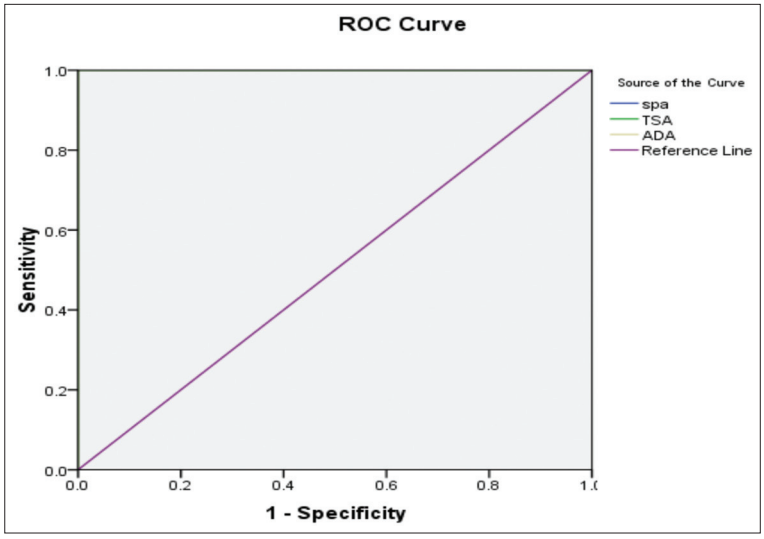

Fig 5. ROC Curve for three parameters

Şekil 5. Üç parametre için ROC eğrisi

in patient group (1.49 and 0.82 mg/dl) rather than healthy group (0.34 and 0.07 mg/dl) respectively. *Fig. 5*, illustrates ROC (Receiver Operating Characteristic) among three parameters along with AUC (Area under Curve) for determination of sensitivity and specificity.

DISCUSSION

Sialic acid, plasma proteins, glycoproteins and lipids are mostly synthesized in the liver [11] and sialic acid is linked to non-reducing residues of the carbohydrate chains of glycoproteins and glycolipids [16]. High levels of TSA were determined in echinococcosis group compared with the healthy ones. We could not clarify any information about sialic acid alterations in cattle CE. Stefenelli et al.[17] reported a significant decrease of TSA in chronic liver diseases such as cirrhosis than control ones. Yurtseven et al.[10] revealed low concentration of TSA in cattle with theileriosis. Chrostek et al.[11] demonstrated decrease of lipid-bound sialic acid in non-alcoholic cirrhosis and attributed it to liver diseases that impressed serum level of lipids and lipoproteins and also the level of sialic acid bounded with these compounds which are not in accordance with our study. It worth mentioning that many studies have revealed significant increase of sialic acid in various diseases such as cancer, inflammatory disorders, cardiovascular diseases and diabetes mellitus and even sialic acid has been demonstrated as one of the inflammatory markers [9]. Since, sialic acid is considered to be inflammatory marker. It is likely, significant increase in plasma TSA in cattle with hepatic CE can be attributed to disease-mediated TSA synthesis and production followed by infection in liver.

The substantial effects of S1P has been determined on the function of T and B lymphocytes which is included in the maturation and migration of them. It plays especially as an substantial effect onto activation of T cell [18,19]. It has been demonstrated that during the T and B lymphocytes activation and stimulation, subsequently, S1P synthesis is reduced [20,21] which is not in accordance with our evidence. One of the reasons of S1P increase may be attributed to high concentration of HDL. The HDL is known to be the major bio-molecule which possesses S1P. The HDL participates in remove of some blood parasites and may play a substantial role in the increase of S1P in CE. The significant increase of S1P revealed in this study. We could not find any evidence which related to S1P alterations in hepatic CE. Li et al.[22] showed that S1P concentration to be increased in CCL_4-mediated hepatic fibrosis in rat which is in accordance with our study. Ikeda et al.[23] showed S1P reduction in liver fibrosis patients that is not consistent with our evidence. Platelets are known to be one of the main sources of S1P in plasma during sphingosine phosphorylation by sphingosine kinase [24,25]. In line with this, platelets are recognized to reserve S1P plentifully and after activation, release it into the plasma [26]. In the respect of platelets, Togill et al.[26] suggested arising of platelets activation followed by chronic hepatic diseases. Moreover, CE is generally associated with long time hepatic damage in cattle. Consequently, platelets might be activated in cattle with liver CE which could cause S1P elevation. Furthermore, it is possible that plasma S1P may be raised due to unknown source(s) which need further clarified. The ADA activity was significantly raised in the hepatic CE group than healthy ones. Isik et al.[27] showed low levels of ADA activity in surgically treated hydatid patients and ascribed it to amelioration of damaged tissue or cease of lymphocyte proliferation during parasite elimination. Moreover, in another study, the ADA activity decrease during experimental infection of mice with a secondary hydatid disease was attributed to immunosuppression associated with the disease [28] that are not consistent with our finding. Umaramani et al.[29] reported significant increase of ADA activity followed by viral hepatitis which is in accordance with our study. This may have been the case in our study as liver echinococcosis causes cellular-immunity stimulation related to ADA activity increase.

In conclusion, on the basis of our findings, the results denote increase of all plasma parameters. Moreover, due to high sensitivity of TSA and ADA than S1P, they could be utilized as potential biomarker in cattle CE.

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Research Article

Semen Characteristics and Cardiac Enzymes in Healthy Male Cats Fed with Commercial Cat Food Containing *Yucca schidigera* [1] [2]

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Abstract

This study has been designed to examine the effect on cardiovascular enzymes, testosterone and semen characteristics of the Yucca schidigera extract (YSE), used as a feed additive in commercial cat food for the purpose of decreasing faecal odour in male cats. In eighteen healthy male cats, while the biomarker cardiac troponin I (cTnI), known as the "gold standard" for cardiovascular assessment in cats was used, creatin kinase-MB (CK-MB), total cholesterol and triglyceride levels were also investigated as aiding parameters. The level of cTnI was 0.16±0.03 ng/ml before feeding without YSE. It was found 0.20±0.04 ng/ml after feeding with YSE. Similarly, results of the study demonstrated that there was no difference statistically in cardiovascular or lipidic parameters after feeding on this type of cat food for 12 weeks. The level of testosterone was 0.29±0.01 ng/ml before feeding without YSE. It was found 0.21±0.02 ng/ml after feeding with YSE. (P>0.05). Therefore, semen analysis revealed a statistically significant increase in sperm motility alongside a significant decrease in sperm abnormalities. The mean volume, concentration, motility and total sperm defect rates were 118.61±8.55 µl, 197.78±12.83x10^6/ml, 83.33±1.06% and 27.22±1.33% before feeding without YSE, respectively. These values were 213.06±21.29 µL, 300.56±16.59x10^6/ml, 90.00±1.07% and 18.67±0.59% after feeding with YSE, respectively. The differences between these values were statistically significant (P<0.001). It was concluded at the end of the study that the commercial cat food containing Yucca schidigera extract could be beneficial effects of the sperm volume, concentration, motility and total morphological defect rates in healthy male cats.

Keywords: *Male cat, Yucca schidigera, Semen, Cardiac enzymes*

Yucca schidigera İçeren Ticari Kedi Mamaları ile Beslenen Sağlıklı Erkek Kedilerde Sperma Özellikleri ve Kardiyak Enzim Analizleri

Özet

Bu çalışma, erkek kedilerde fekal kokuyu azaltmak amacıyla ticari kedi mamalarına bir yem katkı maddesi olarak kullanılan Yucca schidigera ekstraktının (YSE) kardiyovasküler enzimler, testosteron ve spermatolojik özellikler üzerindeki etkisini incelemek için dizayn edilmiştir. Onsekiz adet sağlıklı erkek kedide, kedilerde kardiyovasküler değerlendirme için "altın standart" olarak bilinen biyobelirtec kardiyak troponin I (cTnI), kreatin kinaz-MB (CK-MB), total kolesterol ve trigliserid düzeyleri incelendi. cTnI düzeyi YSE içermeyen beslenmeden önce 0.16±0.03 ng/ml bulundu. Bu değer YSE ilavesi ile beslendikten sonra 0.20±0.04 ng/ml olarak tespit edildi. Benzer şekilde, 12 hafta boyunca beslenme sonrası kardiyovasküler veya lipidik parametrelerde istatistiksel fark olmadığı gözlendi. Testosteron düzeyi YSE içermeyen beslemeden önce 0.29±0.01 ng/ml olarak saptandı. YSE ile beslendikten sonra ise 0.21±0.02 ng/ml olarak bulundu. (P>0.05). Buna karşın, YSE ile beslenme sonrası spermatolojik incelemelerde spermatozoon anomalilerinde önemli oranda bir azalma ile birlikte sperm motilitesinde istatistiksel olarak anlamlı bir artış saptandı. Ortalama sperma hacmi, konsantrasyon, motilite ve toplam spermatozoon anomalileri YSE içermeyen beslenmeden önce sırasıyla; 118.61±8.55 µL, 197.78±12.83x10^6/ml, 83.33±1.06% ve 27.22±1.33% olarak saptandı. Bu değerler YSE ile beslendikten sonra sırasıyla; 213.06±21.29 ul, 300.56±16.59x10^6/ml, %90.00±1.07 ve %18.67±0.59 olarak bulundu. Bu değerler arasındaki fark istatistiksel olarak anlamlı bulundu (P<0.001). Bu çalışmanın sonunda, Yucca schidigera ekstraktı içeren ticari kedi maması ile beslenen sağlıklı erkek kedilerde sperm hacmi, konsantrasyon, motilite ve toplam morfolojik bozukluk oranları üzerine yararlı etkileri olabileceği kanısına varıldı.

Anahtar sözcükler: *Erkek kedi, Yucca schidigera, Sperma, Kardiyak enzimler*

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INTRODUCTION

Use of natural herbal extracts known as "Phytomedicines" is growing on the basis of both their aid in the treatment of various diseases as well as their health benefits. Addition of herbal extracts to animal food products is preferred due to their low cytotoxicity and residual properties. Across the globe, and particularly in the US, the herbal market is on the rise in the fields of human and animal health. Negativities caused by development of resistance and residue in relation to feed additive antibiotics has led the animal nutrition industry to focus on herbal extracts and oils. There are over 80.000 plants known for their bioactive characteristics [1]. Worldwide use of these plants in the veterinary field has been documented. In Italy [2], reported 280 ethno veterinary plants, while [3] listed ~ 200 plants with ethno veterinary use in Southern Africa. For instance, it is known that aromatic herbs such as; fennel, thyme, sage, eucalyptus and cinnamon are used in egg poultry and pet food [4]. Echinacea, flaxseed, garlic, ginger, ginseng and yucca are also among herbal supplements given to horses [5]. In recent years, to prevent significant economic losses due to mould spoilage in pet food products and human salmonellosis contamination related to these foods, natural plant oils and extracts such as rosemary, oregano and lemongrass have been used [6]. *Yucca schidigera* extract has been used as a feed additive for a long time in human, equine, livestock and pet diets. The Federal Drug Administration of the USA has described the extract of this plant as safe for human consumption [7,8]. According to the European Commission [9] (EC 1831, 2003), regarding feed additives in animal nutrition, phytogenic compounds have been categorized as "sensory additives" and, in particular, as flavouring compounds, i.e. substances the inclusion of which in feeding stuffs increases feed smell and palatability. In present day pet industry, *Yucca schidigera* is also used as an alternative diet formulation, particularly to reduce spreading of faecal odour [10].

Scientists have claimed that the Yucca plant is a phytogenic feed additive (PFA) containing steroids, saponins and glycocomponents. Essential oils and spices have distinct biological functions, such as; antiprotozoal, antimicrobial, or antioxidant properties [8]. Numerous previous studies have demonstrated the antibacterial effects of *Yucca schidigera* extract on the inhibition of gram-positive bacteria [11]. Yucca extract supplements have proved to have beneficial effects on the quality of carcass and meat in storage, as well as improving resistance of the immune system against diseases and reducing mortality. Yucca products have also been reported to stimulate the laying rate, egg weight and decrease serum glucose, cholesterol and triglyceride levels in laying quails [12]. Yucca contains resveratrol, polyphenolics and other stilbenes (yuccaols A, B, C, D and E). Therefore, yucca is used as an antioxidant and free-radical scavenger [13]. The positive effects of antioxidants on stress and production of free radicals have been among the most important research topics in recent years regarding human and animal reproduction. Furthermore, since it was established that it decreased faecal odour in pet animals, *Yucca schidigera* has become very popular as an alternative diet formulation. In other studies aimed at the use of yucca extracts as an additive, weight gain, growth and reproduction in animals were investigated. However, despite its widespread use in pet nutrition, studies on male animal reproduction, in particular, are scarce. Research directed at the cardiovascular system is also insufficient. The most recent long-term large-scale epidemiological study has revealed a strong relationship between low testosterone levels and deaths related to cardiovascular diseases [14]. The purpose of this study is to contribute to recent studies aiming at drawing attention to the relationship between the reproductive and cardiovascular systems. In this study, effects on testosterone, cardiac enzymes and semen characteristics in healthy male cats feeding with commercial cat food containing *Yucca schidigera* extract has been assessed.

MATERIAL and METHODS

Animals

Eighteen healthy shorthair male cats from, approximately 24-36 months old and 3.5-4.5 kg bodyweight, were used. The cats were initially fed a commercial cat food for 12 weeks, which was produced by the Eagle Pack Natural Pet Food Company (Indoor adult dry cat food, Tewksbury, MA, USA) and did not contain the Yucca schidigera plant. Following this, a feeding programme was undertaken with the same cat food containing 150 ppm Yucca schidigera extract (DK Powder-35, produced by Desert King Int. USA) for 12 weeks and exposed to a 12-h light/dark cycle, at room temperature. Cats consumed average 60-65 g commercial dry cat food daily and were given drinking water *ad libitum*. The study was approved by the Animal Experimentation Ethics Committee of Istanbul University (Protocol 76/2009). All cat owners were given detailed information and signed a research consent form. Food ingredients was shown in *Table 1*.

Table 1. *Chemical and basic composition of commercial cat food**
Tablo 1. *Ticari kedi mamasının temel ve kimyasal içeriği**

Nutritional Level	Analysis Values
Moisture	10.0%
Crude protein	30.0%
Crude fat	14.0%
Crude fiber	7.3%
Crude ash	4.9%
Mineral matter	2.0%
Metabolizable energy	967 kcal/kg

** taurine, magnesium, omega 3 and omega 6 fatty acids, vitamin A, D_3 and E*

Semen Analysis

All cats produced ejaculates with 80% progressively motile sperm; sperm counts were within normal limits. Semen samples were collected from the animals before eating (control) and after a period of 12 weeks feeding with commercial cat food containing YSE. Semen collection was performed from January and April using an electro-ejaculator (P-T Electronics, Boring, OR, USA) under general anesthesia. The volume of the semen was measured using an adjustable automatic micropipette (10-1.000 µL) and the value was recorded in microliters. Sperm motility was assessed subjectively (3 µL sample) using phase-contrast microscope at x200 magnification by viewing at least 3 fields. An aliquot (5 µL) of semen was diluted with 10% formol saline and evaluated for total number of sperm using a hemocytometer chamber (Neubauer, Boeco, Hamburg, Germany). A spermac® stain kit (Stain enterprises, Onderstepoort, Repuclic of South Africa) was employed in sperm morphologic observations. The preparation was examined by light microscope at x1.000 magnification by counting 200 cells (acrosome, head, mid-piece, tail and total abnormal spermatozoa rate) as described by Baran et al.[15].

Blood Serum Analysis

Blood samples were collected from the animals before eating (control) and after a period of 12 weeks feeding with commercial cat food containing YSE. In serum samples; CK-MB, Total cholesterol and Triglyceride levels were measured with the enzymatic method using the Abbott C8000 autoanalyser and identical commercial kits.

Cardiac troponin-I (cTnI) and total testosterone levels were determined using the IMMULITE® commercial kit (solid-phase, competitive chemiluminescent enzyme immunoassay) with an immulite one immunoassay analyzer (DPC, USA). Due to the commercial kit properties, readings below 0.20 ng/ml could not be measured quantitatively and were considered to be negative. For cTn-I, values of 0.20 mg/ml and above were considered to be positive and measured quantitatively.

Statistical Analysis

Statistical analysis was performed using the SPSS for Window version 21.0 programme (SPSS Inc., Chicago, IL, USA). Analysis was performed using Student's t-test. $P<0.05$ was considered statistically significant.

RESULTS

No difference was observed among the male cats with regard to testosterone levels and mid-piece sperm morphology after with YSE feeding ($P>0.05$). The motility rate, volume and concentration after with YSE feeding were observed higher than before with YSE feeding ($P<0.001$).

Results revealed that there was a statistically significant positive effect on motility, volume and morphological properties following feeding on pet food containing *Yucca schidigera* extract *(Table 2)*.

No statistically significant difference was found between the enzymes CK-MB, cTn-I, total cholesterol and triglyceride examined to assess the cardiovascular system, before and after feeding *(Table 3)*.

DISCUSSION

It is necessary to investigate the health effects of adding natural herbal extracts to animal diets. Haematologic parameters examined during research can provide information on clinical and nutritional status. These parameters also aid in diagnosing the metabolic condition of tissues and organ function disorders. In the present study, the haematologic parameters used were; total cholesterol, triglyceride, troponin and ck-mb levels. The cholesterol-lowering action mechanism of herbal extracts containing

Table 2. *Means of spermatological traits and testosterone levels of male cats in before (control) and after with Yucca schidigera extract feeding*
Tablo 2. *Yucca schidigera ekstraktlı beslenme öncesi (kontrol) ve sonrasında erkek kedilerin testosterone ve spermatolojik özelliklerinin ortalamaları*

Parameters	Before	After	Significance
Testosterone (ng/ml)	0.29±0.01	0.21±0.02	NS
Volume (microliter)	118.61±8.55^{a}	213.06±21.29^{b}	P<0.001
Concentration (x10^{6}/ml)	197.78±12.83^{a}	300.56±16.59^{b}	P<0.001
Motility (%)	83.33±1.06^{a}	90.00±1.07^{b}	P<0.001
Acrosome (%)	15.11±0.90^{b}	10.83±0.83^{a}	P<0.01
Head (%)	6.22±0.68^{b}	3.83±0.33^{a}	P<0.01
Mid-piece (%)	2.11±0.47	1.33±0.34	NS
Tail (%)	3.78±0.41^{b}	2.67±0.38^{a}	P<0.05
Total (%)	27.22±1.33^{b}	18.67±0.59^{a}	P<0.001

^{a,b} Different superscripts within the same line demonstrate significant differences (n=18); Means±SE, ***NS:*** *Not significant*

Table 3. *Means of creatine kinase-MB, troponin, total cholesterol and triglyceride levels in before (control) and after with Yucca schidigera extract feeding*
Tablo 3. *Yucca schidigera ekstraktlı beslenme öncesi (kontrol) ve sonrasında kreatin kinaz-MB, troponin, toplam kolesterol ve trigliserit düzeylerinin ortalamaları*

Stage	Creatine Kinase-MB (ng/ml)	Troponin (ng/ml)	Total Cholesterol (mg/dl)	Triglyceride (mg/dl)
Before control feeding	197.89±7.56	0.16±0.03	109.44±5.81	28.33±3.25
After YSE feeding	194.94±7.58	0.20±0.04	116.56±6.73	24.56±2.76
Significance	NS	NS	NS	NS

Means±SE, P>0.05 ***NS:*** *Not significant, (n=18)*

sterol is based on decreasing and preventing cholesterol absorption. Some present views state that, plant sterols cause cholesterol to drop in broilers by limiting blood cholesterol increase and allowing its secretion via bile [4]. Similarly, Aazami et al.[16] pointed out the cholesterol-lowering effect of saponins present in yucca, either directly by way of inhibiting small intestinal absorption of cholesterol or indirectly by mucosal cell desquamation or preventing reabsorption of bile acids. There are studies demonstrating the presence of a cholesterol-lowering effect in goats receiving *Yucca schidigera* powder compared to the control group [17]. Mardalena et al.[18] reported that *Yucca schidigera* powder possesses an anti-oxidant function and this lowered cholesterol by preventing lipid oxidation through its ability to capture free radicals and radical peroxy salts. In this study, while the commercial food containing yucca extract was not seen to have a triglyceride- and cholesterol-lowering effect in cats, other studies suggest that the use of the herb may originate from differences in dosage.

Plant compounds cause diverse effects in the cardio-vascular system. There are those that cause cardiac arrhythmias, haemorrhagic syndrome and cardiac necrosis and this effect is often associated with plant secondary compounds such as glycosides [19,20]. In this study, in order to assess cardiac function, the authors utilized cardiac markers used as biomarkers. In veterinary medicine, cardiac troponins are used in the diagnosis and prognosis of myocardial injury originating from the damage in myocardial cells. Circulating cardiac troponin I, in particular, is known to be the most important biomarker - the "gold standard" - in the diagnosis of myocardial injury in cats. It has been reported that, it is significantly raised compared to the control group in hypertrophic cardiomyopathy, the most important cardiac disease in cats. Also, while a relationship could not be found between histopathological investigation and diagnosis, it was established that the significantly raised cTnI level was useful and this was confirmed by other veterinary practices participating in the study [21,22].

While cTnI levels are low to non-detectable in healthy mammals, raised cTnI levels have been reported to be useful myocardial injury biomarkers in calves, cattle, horses, dogs and lambs [23-28]. In the present study, the cTnI level was seen to be normal in healthy cats and no change was observed in parameters when the cats were fed food containing *Yucca schidigera* extract.

Creatin Kinase myocardial band (CK-MB) is another cardiac biomarker and it has been reported that it is raised in chronic coronary artery occlusion in animals, as well as in cases of post-surgical tissue damage and renal failure. However, due to its higher sensitivity between cardiac parameters, cTnI is favoured over CK-MB and troponin C and T [22]. Based on this information, while the authors preferred cTnI since it is the most sensitive parameter in response to cardiac disorders in cats, in this study, in view of the fact that it may aid in cardiac function assessment, CK-MB values were also examined and found to be within normal limits.

Consistent with other studies [14] in which testosterone level and cardiac function are related, hormone and cardiac parameters were seen to be within normal levels in the present study. Today, the *Yucca schidigera* plant is used as an additive in pet food products. However, there are few studies directed at its reproductive activity. In this study, semen characteristics examined in the andrological assessment of male cats were; volume (ml), concentration (sperm count/ml), motility (%) and abnormal spermatozoa count (%). In healthy male cats fed food containing yucca extract, while sperm volume, concentration and motility showed a statistically significant increase; acrosome, head, tail and total morphological abnormalities were found to be statistically lower than pre-administration values. In a study [13] determining reproductive activity in rabbits, the powder extract of the whole plant was used at doses of 5 g/100 kg and 20 g/100 kg. Both doses were found to be high compared to semen and progressive motility in the control group. The results of the study are similar to the sperm characteristics recorded in the present study. On this subject, there is data regarding the anti-oxidants and free radical scavengers present in yucca improving spermatological responses by aiding in the suppression of reactive oxygen species. Also, in a study carried out by Baláži et al.[29] it has been demonstrated that yucca intake in female rabbits aids in achieving the best birth rate by increasing progressive motility of spermatozoa in genitalia.

Detectability of natural herbal extracts after addition to food is a priority when determining their effects on health. In a study [30] carried out in Japan, it was reported that only 5-6% of yucca extract used as a food additive contained saponin and over 60% was constituted of

unknown substances. In this study it was also reported that, the extract of this plant is recorded in the food additive list in Japan and is included in food as a shelf life extender. However, no specification has been stated of this widely used plant as a food additive. In Turkey, it is used without any standardization or toxicological assessments. The present study, on the other hand, confirmed that examined parameters showed there to be no negative effects on the cardiovascular system, that the normal levels were preserved in analyses related to testosterone and heart, and concluded that it has a beneficial effect on reproduction.

Addition of crude plant extracts or plant mixtures to food is based mostly on traditional knowledge. It is necessary for this information to be supported by scientific research. There is a need for scientific research directed towards the health effects of plant active components. In research, dosage, toxicological risk assessment and standardization must be considered and inclusion of synthetic drugs and feed additives into feeding must be in this direction.

In keeping with present day animal production processes, the main scope in pet food industry is to promote better quality of life through an eco-friendly clean and natural mission. The current study shows that the used herbal extract has no long-term negative effects. Moreover, it has positive effects on semen characteristics in male cats. Nevertheless, there are deficiencies regarding suitable dosage, determining phytochemical composition and mechanism of action in the use of feed additives.

It was concluded at the end of the study that the commercial cat food containing *Yucca schidigera* extract (150 ppm) could be beneficial effects of the sperm volume, concentration, motility and total morphological defect rates in healthy male cats.

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Research Article

Critical Thresholds of Nonesterified Fatty Acids and β-hydroxybutyrate in Transition Dairy Cows for Prediction of First Service Conception Rate

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Abstract

The objective of this study was to establish cow level critical thresholds for β-hydroxybutyrate (BHBA) and nonesterified fatty acids (NEFA) to predict conception to first service. The data were generated from 97 Holstein cows (2 to 5 parities) on a large commercial farm. Serum concentrations of BHBA and NEFA were measured in all cows on day 10 prepartum and, weeks 1, 2, 4 and 6 postpartum. NEFA and BHBA analyzed with receiver operator characteristic (ROC) analysis to determine critical thresholds for predicting pregnancy to first service. NEFA in weeks 4 and 6 postpartum were the only significant predictors identified in the ROC analysis. Optimum critical thresholds for NEFA in weeks 4 and 6 were 201.15 µmol/L and 203.4 µmol/L, respectively (P<0.05). The critical threshold for serum BHBA in the prepartum cohort was 600 µmol/L (P=0.1), which predicted conception to first service. Logistic regression analysis indicated that the risk of conceiving was 82.4% and 88.6% lower for cows with NEFA ≥201.15 in week 4 (OR=0.176; P=0.001) and NEFA ≥203.4 µmol/L in week 6 (OR=0.114; P=0), respectively. In conclusion, NEFA concentrations within 4 and 6 weeks after calving were associated with lower probability of pregnancy at the first AI.

Keywords: *Dairy cow, Nonesterified fatty acids, β-hydroxybutyrate, First service conception*

Geçiş Dönemi Sütçü İneklerde İlk Tohumlamada Gebe Kalma Oranını Tahmin Etmede Esterlenmemiş Yağ asitleri ve β-hidroksibütirat Kritik Eşik Değerleri

Özet

Bu çalışmanın amacı ineklerde ilk tohumlamada gebe kalma oranını tahmin etmede β-hidroksibütrat (BHBA) ve esterlenmemiş yağ asitleri (NEFA) kritik eşik değerlerini belirlemektir. Çalışmanın verileri ticari büyük bir çiftlikte 97 adet Holstein ırkı inekten (2 ile 5 parite) elde edildi. Tüm ineklerde BHBA ve NEFA serum konsantrasyonları prepartum 10. günde ve postpartum 1, 2, 4 ve 6. haftalarda ölçüldü. BHBA ve NEFA ilk tohumlamada gebe kalma eşik değerini belirlemek amacıyla Receiver Operator Characteristic (ROC) ile analiz edildi. Postpartum 4. ve 6. haftalardaki NEFA ROC analizinde belirlenen tek anlamlı değerlerdi. 4. ve 6. haflarda NEFA için optimal kritik eşik değerleri sırası ile 201.15 µmol/L ve 203.4 µmol/L olarak tespit edildi (P<0.05). Prepartum grubunda serum BHBA için kritik eşik 600 µmol/L (P=0.1) olarak belirlendi. Lojistik regresyon analizi ile gebe kalma riski 4. haftada NEFA ≥201.15 ineklerde (OR=0.176; P=0.001) %82.4 ve 6. haftada NEFA ≥203.4 µmol/L ineklerde (OR=0.114; P=0) %88.6 daha düşük olarak belirledi. Sonuç olarak, doğumdan sonra 4 ve 6. haftalarda NEFA konsantrasyonları ilk suni tohumlamada gebe kalma olasılığı ile düşük derecede ilişkilidir.

Anahtar sözcükler: *Süt ineği, Esterlenmemiş yağ asitleri, β-hidroksibütrat, İlk tohumlamada gebelik*

INTRODUCTION

Most transition dairy cows enter a state of negative energy balance (NEB) for three important reasons: 1) increased energy demands at parturition, 2) decreased DMI shortly before parturition, and 3) lagging DMI compared with energy demands due to milk production [1,2].

Body fat stores are mobilized into the bloodstream in the form of nonesterified fatty acids (NEFAs) because of increased energy demand and decreased DMI and contribute

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to overall energy requirements during early lactation, some of which is taken up by the liver. In the liver, some NEFA are oxidized or re-esterified into triglycerides that are either exported as very low density lipoproteins or stored in the liver. During the periparturient period, high rates of NEFA enter the liver and sometimes exceed the liver's capacity to secrete triglycerides as very low density lipoproteins, leading to an accumulation of triglycerides [3]. Increased amounts of NEFA that are removed by the liver control ketogenesis and thus, β-hydroxybutyrate (BHBA) production [4].

At the cow level, increased BHBA and NEFA concentrations have been used as markers of excessive NEB. Previous studies have indicated that increased concentrations of these metabolites are related to increased risk of developing detrimental health [5,6], reproductive [7,8], and production outcomes [9].

The aims of this research are the followings: 1) to specify whether concentrations of BHBA and NEFA measured at 10 days prepartum and in each of the first, second, fourth and sixth weeks postpartum could be used at herd level to predict success of conception to first service and in which times relative to calving were most effective in predicting fertility; 2) to determine the cutoff point of NEFA and BHB concentrations for diagnosis of conception using receiver operating characteristic (ROC) analysis.

MATERIAL and METHODS

Study Population and Design

The study was conducted on 97 lactating Holstein cows of parities two to five in a large commercial dairy herd, in Chaharmahal and Bakhtiari province of Iran. In this study, cows have fed a TMR-based diet (All diets were based on alfalfa, corn silage, and a combination of concentrate including corn, soya meal and bone meal).

Seasonal effects were minimized as most of the cows on farm calved during a one-hundred-day period from August until November in 2014. Blood samples were collected at 5 to 6 a.m. (before feeding) on day 10 prepartum and weeks one, two, four and six postpartum, via the tail vein into a glass tube.

Blood samples were left to clot at room temperature for about thirty minutes and then centrifuged at 2.000×g. The obtained serum samples were kept at -20°C until analyzed for BHBA and NEFA concentrations. These metabolites were determined by a D-3-hydroxybutyrate kit and a NEFA Kit (Randox Laboratories Ltd, Ardmore, UK). The cows were inseminated by an expert inseminator when standing heat was observed. Pregnancy diagnosis was performed by ultrasonography 30 to 40 days after service and the second palpation was done two weeks later to validate the pregnancy.

Statistical Analysis

Receiver Operator Characteristic (ROC) Analysis for Critical Thresholds: In this study, BHB and NEFA in different times were evaluated with receiver operator characteristic (ROC) analysis in order to determine critical thresholds for predicting conception.

The ROC curves analyze sensitivity versus 100 - specificity. Sensitivity was the proportion of animals conceived at first service that were below a given metabolite threshold, and specificity was the proportion of animals that did not conceive that was above a given threshold [10].

The point on the ROC curve that had the highest combined sensitivity and specificity was considered the critical threshold. In this analysis, there is an area under the curve (AUC) and a P-value for each parameter in different times. The value of P indicates if this parameter is an appropriate indicator for prediction of conception or not? Interpretation of this critical threshold was based on the area under the curve (AUC) such that if the AUC=0.5, it was noninformative; if $0.5 < AUC \leq 0.7$, it was accurate; if $0.7 < AUC \leq 0.9$, it was very accurate; if $0.9 < AUC < 1$, it was highly accurate; and if AUC = 1, then it was considered perfect [11].

Logistic regression: The odds ratios (OR) of conception to first service outcome given NEFA or BHBA concentrations were modeled with multivariable regression techniques, accounting for clustering of cows within herds.

Univariable analyses were first performed to assess the association between pregnancy at the first AI and categorical cow-level covariates (calf sex, calf weight, parity, BCS, BCS loss from calving to first service, postcalving clinical disease, and occurrence of dystocia) with t-test as a random effect. Parity was categorized into 2 and more than 2. Body condition score loss was categorized as less than one unit and one or more than one unite. A binary disease variable was created and coded 1 if a cow was diagnosed with dystocia, retained placenta, metritis, endometritis, ovarian cyst or at least one of these disease before 30 DIM. Variables that were not associated with pregnancy at the first AI in the univariable analysis (P>0.0.05) were not considered further.

For each significant metabolite and week of sampling in ROC analysis, dichotomized metabolites concentrations based on determined thresholds and significant covariates in the univariable analysis were submitted to multivariable logistic regression, using a binary distribution. The predicted probabilities of pregnancy were estimated from the model.

RESULTS

Conception rate to first service was 29% (28/97). The present study revealed several relationship estimates

between traits and fertility that were statistically significant (P<0.05) or tended to be significant (P=0.1).

Parity and body condition score loss from calving to first service were associated with the odds of pregnancy at first AI in univariable analyses whereas sex, weight, the mean of BCS, postcalving clinical disease and dystocia were not significantly different between pregnant and not pregnant cows after first service. First service conception rate progressively decreased from 33.7% for cows losing <1 unit of BCS to 10% for cows losing ≥1 unit of BCS from calving to first service (P=0.03). Also when cows were divided in to parity 2 (n=54) and ≥3 (n=43), cows with second parity had significantly higher conception rate compared with older cows (37% vs. 18%; P<0.05).

Critical Thresholds

NEFA and BHBA analyzed with ROC curves to determine the cow-level critical thresholds (combined highest sensitivity and specificity) to predict conception to first service. Non-esterified fatty acids in weeks 4 and 6 postpartum, was the only significant predictor identified in the ROC analysis. Tabular results of ROC curve determination of critical BHB and NEFA thresholds (μmol/L) for the prediction of conception are in *Table 1* and *2*. In summary, optimum critical threshold that had the highest combined sensitivity and specificity, for prepartum BHB was 600 μmol /L (P=0.1) and for NEFA in weeks 4 and 6 were 201.15 μmol/L (P=0.04) and 203.4 μmol/L (P=0.01), respectively, with 16.5, 51.5 and 49.5% of the animals at or above the threshold. All metabolite concentrations below these thresholds were associated with higher reproduction performance (i.e., cows with NEFA concentrations below related thresholds most likely conceived than cows with concentrations at or above the same thresholds).

Measures of Association

Odds ratios (OR) were calculated based on critical thresholds determined by ROC analysis. When NEFA was evaluated as the only main predictor (i.e., without BHB in the model) and after controlling for parity and BCS loss, the odds of conceiving were 82.4% and 88.6% lower for cows with NEFA ≥201.15 (OR=0.176; P=0.001) and NEFA ≥203.4 μmol/L (OR=0.114; P=0) in weeks 4 and 6 postpartum, respectively.

DISCUSSION

This study was an analysis of the association of serum metabolites in the transition period with early lactation reproductive performance on commercial dairies. This study was done in a commercial dairy farm with about 1450 lactating dairy cows in a mountainous area in Iran. Conception rate to first service on our farm was 29% which was the same as that of (27%) reported recently for 87 Iranian dairy cows by Kadivar et al.[12].

The results showed no significant association between BHBA concentrations and the odds of pregnancy at first AI. However, as far as we know, this is the first study which has reported an association (P=0.1) between precalving BHBA and reproductive performance. The optimal threshold of ≥600 μmol/L for predicting a reduction in reproductive performance was the same as that associated with a decreased milk yield in Chapinal et al.[13]. This finding is

Table 1. *Receiver operator characteristic curve determination of critical BHB thresholds as predictors of conception in transition dairy cows*
Tablo 1. *Geçiş dönemi ineklerde gebe kalma tahmini amacıyla kritik BHB eşik değerleri Receiver operator characteristic eğrisinin belirlenmesi*

Pregnancy Status	Critical Threshold[1]	Se[2]	Sp[3]	AUC[4]	P-value
Before parturition	421.55	53.6	30.4	0.605	0.1
Week 1	621.05	53.6	47.8	0.497	0.9
Week 2	480.7	53.6	42	0.502	0.9
Week 4	453.35	64.3	47.8	0.452	0.4
Week 6	468	60.7	47.8	0.487	0.8

*[1] Highest combined specificity (Sp) and sensitivity (Se), μmol/L; [2] **Se** = epidemiologic sensitivity; [3] **Sp** = epidemiologic specificity; [4] **AUC** = area under the curve*

Table 2. *Receiver operator characteristic curve determination of critical NEFA thresholds as predictors of conception in transition dairy cows*
Tablo 2. *Geçiş dönemi ineklerde gebe kalma tahmini amacıyla kritik NEFA eşik değerleri Receiver operator characteristic eğrisinin belirlenmesi*

Pregnancy Status	Ccritical Threshold[1]	Se[2]	Sp[3]	AUC[4]	P-value
Before parturition	133.5	57.1	47.8	0.486	0.8
Week 1	673.95	57.1	53.6	0.495	0.9
Week 2	466.65	60.7	46.4	0.490	0.8
Week 4	201.15	78.6	63.8	0.628	0.04
Week 6	203.4	85.7	63.8	0.654	0.01

*[1] Highest combined specificity (Sp) and sensitivity (Se), μmol/L; [2] **Se** = epidemiologic sensitivity; [3] **Sp** = epidemiologic specificity; [4] **AUC** = area under the curve*

very interesting because, in contrast to NEFA, ketones can be simply measured in the field [14].

The optimal threshold of ≥ 600 µmol/L was somewhat lower than the threshold of 800 µmol/L associated with an increased risk of displaced abomasum in Chapinal et al.[15].

Walsh et al.[7] determined BHBA concentration thresholds for the prediction of probability of pregnancy after the first insemination early in lactation. They showed that cows with serum BHBA 1000 µmol/L in the first week or 1400 µmol/L in the second week were significantly less probably to be diagnosed pregnant after first insemination.

The same association between fertility and BHBA concentration has been reported by Ospina et al.[8]. In their study, in animals which were sampled postpartum, the risk of pregnancy within 70 days post-voluntary waiting period (VWP) was reduced by 13% when BHBA concentrations were 970 µmol/L.

In our study, mean BHBA concentration in all sampling times were lower than threshold values reported by Walsh et al.[7] and Ospina et al.[6] that above this, risk of pregnancy after first service is reduced. This fact can point out why BHBA did not affect the probability of pregnancy in this study. In agreement with our results, Fahey et al.[16], Waters et al.[17] and Falkenberg et al.[18] did not report any significant relationship between peripartum BHBA concentrations and reproductive parameters. Moreover, detrimental effects of ketonebodies on reproductive success rely on the longevity of their increased levels [19]. In this study, the duration of increasing BHBA concentration may be too short to have a negative effect on fertility. Therefore, under the conditions of the present study, this variate is not likely to be useful predictors of reproductive performance.

In the current study, higher NEFA concentrations in weeks 4 and 6 postcalving were associated with decreased odds of pregnancy which showed that a moderate degree of fat mobilization in these times of lactation may be critical to get low performance. This shows that postpartum NEFA concentration is a dependable indicator for defining conception status after the first service.

The optimal thresholds in these two times were 201.15 µmol/L and 203.4 µmol/L, respectively. Excessive magnitude or rate of mobilization of fat supplies will cause suboptimal metabolic performance and is likely an indicator of a reduced adaptive response to NEB.

A number of studies have focused on the relationship between NEFA concentrations and reproductive performance [20-22]. The negative impact of NEFA concentration on commencement of luteal activity postpartum was reported during the 4 and 7 week of lactation [21]. A delay in the resumption of ovulation limits the number of oestrous cycles before service, which may lead to the reduced conception rates [23]. However, Reist et al.[24] and Fahey et al.[16] did not report any significant relationships between NEFA concentration and fertility.

After considering the stage of sampling in which significant relationships were observed, we note that these relationships were found in the last weeks of sampling, when the most animals were already cyclic. This is completely reasonable because this is the time that the most of the cows were inseminated for the first time and energy balance indicators are expected to be more informative in this period.

An association between reproductive efficiency in early lactation and elevated peripartum NEFA was reported by Ospina [8]. In all animals sampled prepartum, the risk of pregnancy within 70 d post-VWP was reduced by 19% when NEFA concentrations were ≥270 µmol/L. In all animals sampled postpartum, those with NEFA concentrations ≥720 µmol/L had a 16% decrease in risk of pregnancy.

On the other hand, these thresholds are related to the assessment of conception within 70 days post-voluntary waiting period (VWP). The level at which elevated NEFA is associated with conception at first service was not evaluated.

Although elevated concentrations of both NEFA and BHBA decline the risk of conception, through direct toxic effect on the follicles with induction of cumulus cells apoptosis, necrosis and follicular development arrest [25,26], however, in our study, NEFA concentration was found to have the stronger relationship with reproductive performance than BHBA. This situation is likely because of the more direct physiological relationship between NEFA concentrations and negative energy balance [27].

The current analysis allowed the opportunity to examine the effect of elevated concentrations of pre- and postpartum NEFA and BHBA on reproduction at the cow level. In summary, elevated serum concentrations of BHB within 1 week before calving and NEFA in weeks 4 and 6 after calving were associated with lower probability of pregnancy at the first AI in the present study.

Establish cow level critical thresholds for serum concentrations of NEFA and BHBA to predict conception at first service was a notable feature of the current study. The following cow-level critical thresholds should be considered general guidelines for monitoring cattle: NEFA concentrations ≥201.15 µmol/L for cattle in week 4 postpartum; and NEFA concentrations ≥203.4 µmol/L for cattle in week 6 postpartum. Both postpartum NEFA concentrations above these critical thresholds were associated with decreased occurrence for conception at first service.

This information allows the identification of individual cows at risk for this downstream outcomes based on their NEB status during the transition period. Recognizing cows at risk for decreased PR based on the effects of increased NEFA or BHBA concentrations during the transition period

may help farmers focus on improving herd energy balance. Thus, it is necessary to improvement in management of transition cows so as to minimize the effect of NEB.

Efforts to improve NEB status should be implemented at the herd level, where decisions about nutritional management, diet, comfort, social adaptation, and access to feed, which may be the best methods of minimizing the lagging DMI during the transition period, which is one of the major factors associated with NEB.

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Research Article

The Distribution and Heterogeneity of Mast Cells in the Cecum of Quail *(Coturnix Coturnix Japonica)* [1]

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[1] *A part of the study was presented as a thematic presentation at the 10th National Congress of Histology and Embryology, May 17-20, 2010, Çeşme /İzmir, Turkey*

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Abstract

The aim of the present study is to investigate the location and heterogeneity of mast cells in quail cecum. Cecum samples were fixed in basic lead acetate (BLA), Carnoy's, isotonic formaldehyde acetic acid (IFAA) and 10% neutral buffered formalin (NBF) solutions. The sections were stained with an alcian blue-critical electrolyte concentration (AB-CEC) (pH 5.8, 0.3 M $MgCl_2$) and safranin O (SO) (pH 1.0) used in a combined method and with toluidine blue (TB) (pH 0.5). Mast-cell population was shown in highest ratios in all layers of Carnoy fixed cecum parts within TB-stained sections. Metachromatic mast cell density was determined the most in tunica mucosa layers of the middle and distal cecum. It was seen that AB-CEC (+)/SO (-) mast-cell density was greater in tunica mucosa layers of the proximal and middle cecum in IFAA and BLA fixed tissues campare to Carnoy fixation. At the end of the study; it can be said that Carnoy solution fixed the connective tissue mast cells, as well as IFAA and BLA solutions fixed the mucosal mast cells better.

Keywords: *Cecum, Fixative solution, Mast cell, Quail*

Bıldırcın *(Coturnix Coturnix Japonica)* Sekumunda Mast Hücrelerinin Dağılımı ve Heterojenitesi

Özet

Sunulan çalışmanın amacı, bıldırcın sekumunda mast hücrelerinin yerleşimini ve heterojenitesini araştırmaktır. Sekum örnekleri bazik kurşun asetat (BLA), Carnoy, izotonik formaldehit asetik asit (IFAA) ve %10'luk nötr tamponlu formalin (NBF) solüsyonlarında tespit edildi. Kesitler alsiyan mavisi-kritik elektrolit konsantrasyonu (AB-CEC) (pH 5.8, 0.3 M $MgCl_2$)/safranin O (SO) (pH 1.0) kombine metodu ve toluidin mavisi (TB) (pH 0.5) ile boyandı. Mast hücresi populasyonu, TB ile boyanmış kesitlerde Carnoy solüsyonu ile tespit edilen sekum bölümlerinin bütün katmanlarında en yüksek oranlarda gösterildi. Metakromatik mast hücresi yoğunluğu en fazla orta ve distal sekumun tunika mukoza katmanlarında belirlendi. AB-CEC (+)/SO (-) mast hücresi yoğunluğunun ise, Carnoy tespitine göre IFAA ve BLA ile tespit edilen dokularda, proksimal ve orta sekumun tunika mukoza katmanlarında daha fazla olduğu görüldü. Araştırma sonunda; bıldırcın sekumunda Carnoy tespit solüsyonunun bağ doku mast hücrelerini, IFAA ve BLA tespit solüsyonlarının ise mukozal mast hücrelerini daha iyi tespit ettiği söylenebilir.

Anahtar sözcükler: *Sekum, Tespit solüsyonu, Mast hücresi, Bıldırcın*

INTRODUCTION

The avian cecum consists of two blind-ended sacs located on the right and left of the juncture between the small and large intestines [1,2]. It comprises three parts, the proximal, middle and distal [3], and plays a role in various functions, including the digestion and fermentation of cellulose and the absorption of water, sodium, carbon dioxide [4] and nutrients [2]. Additionally, it contains diffuse and nodular lymphatic tissues in its submucosa and lamina propria. It therefore acts as a defensive organ [5]. These lymphatic tissues are called as cecal tonsils, and they are located in the proximal region of the cecum [6].

In particular, mast cells are frequently found around blood vessels and nerves [7]. They contain basophilic, cyto-

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plasmic granules, which have histamine, heparin and eosinophil chemotactic factors [8], and they can also secrete numerous cytokines that induce lymphocyte functions [9]. Moreover, mast cells play an important role in regulating the sensitivity and permeability of the gastro-intestinal system [10]. Consequently, the number of mast cells increases with the presence of some diseases, such as irritable bowel syndrome [11,12].

Mast cells are classified as either mucosal mast cells (MMCs) or connective-tissue mast cells (CTMCs), depending on their locations, responses to applied fixative solutions and mediator compositions in rats [13,14]. Whereas MMCs are most commonly found in the lamina propria and epithelial layers of mucosal surfaces [15,16], CTMCs are located in the skin, tongue, intestinal serosa and lung parenchyma [17]. Mucosal mast-cell granules contain chondroitin sulphate [18] and rat mast-cell protease-II (RMCP-II) [17]. They are sensitive to formalin [13] and are stained with AB (+) [14]. CTMC granules, in contrast, are rich in heparin [18], contain rat mast-cell protease-I (RMCP-I) [17], are resistant to formalin [13] and are stained with SO (+) [14].

The present study sought to determine the distribution of mast cells and subtype cells using several fixative solutions and staining methods to better understand their functions in the cecum of quails.

MATERIAL and METHODS

In the present study, a sample of 21 six- to nine-week-old adult quails (*Coturnix coturnix japonica*) was used. The quails were obtained from Adnan Menderes University's Department of Laboratory Animals at Aydin, Turkey. They were kept in a storeyed cage system and fed and watered *ad libitum* under conventional conditions. All procedures were approved by the Adnan Menderes University Ethics Committee (Decision No: B.30.2.A DÜ.0.00.00.00/050.04/2010/40).

Examination with Light Microscopy

The quails were euthanized by decapitation, and samples of their cecum were removed. The right and left portions of cecum were separated. Then, right portions of first and second seven animals' cecum were divided into three parts, the proximal, middle and distal cecum, Next, they were fixed in basic lead acetate (BLA) (n=7) and 10% neutral buffered formalin (NBF) (n=7). Additionally, both right and left portions of third seven animals' cecum were divided into three parts, the proximal, middle and distal cecum. Then, the parts of the right portion in Carnoy's and the parts of left portion in isotonic formaldehyde acetic acid (IFAA) solutions were fixed (n=7). After routine histologic processing, the tissues were embedded in paraffin, and six serial sections, each 5 µm thick, were taken at 70 µm intervals. The histologic sections were then stained with toluidine blue (TB, pH 0.5) [19] or with alcian blue-critical electrolyte concentration (AB-CEC) (pH 5.8, 0.3 M $MgCl_2$) and safranin O (SO, pH 1.0) in a combined method [20,21]. Finally, the sections were examined under a light microscope (Leica DMLB) equipped with an image-analysis system (Leica Q Win Standard), and appropriate locations were photographed.

Cell Count and Statistical Analyses

A subjective scoring system was used to determine mast-cell distribution in the TB- and AB-CEC/SO-stained tissues. Six sections of each animal's cecum were scored with a value from zero to four [22,23]. Mast-cell populations in the cecum's tunica mucosa (lamina propria+submucosa), tunica muscularis and tunica serosa were determined. Scores indicating the cecum's mast-cell densities [23] were assigned as follows: 0 = absent (-); 1 = weak (+); 2 = moderate (++); 3 = strong (+++); 4 = very strong (++++). Differences in mast cell-density values based on the various fixative solutions used were calculated using the SPSS 17.0 program package. Data were analysed using one-way analyses of variance (ANOVAs), and the source of the group's differences was determined *post hoc* with a Duncan's test [24]. Furthermore, mast-cell densities between the tunica mucosa folds of cecum parts were compared using paired t-tests [25]. Values are presented herein as mean ± standard error. Values for which $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) were considered statistically significant.

RESULTS

Mast-cell distributions and densities for the proximal, middle and distal parts of the studied cecum were stained with TB or AB-CEC/SO and examined using various fixative solutions. Mast cells were observed in the cecum's lamina propria, submucosa, tunica muscularis and serosa *(Fig. 1)*. They were especially noticeable in the connective tissue of the villi surrounding the blood vessels and between the glands in the tunica mucosa, as well as in the peripheral nerves; they were observed less frequently overall in the cecal tonsils *(Fig. 2)*, and notably, they were most evident when BLA, Carnoy's and IFAA fixative solutions were used. But they were very few when the NBF solution was used. Therefore, the data could not be obtained from NBF fixed sections and they could not be shown in tables.

Mast cells were stained metachromatically with TB, and orthochromatically stained TB (+) cells were also detected *(Fig. 1)*. Metachromatic mast-cell densities were greater in the tunica mucosa than in the cecum's other tissue folds, regardless of the fixative solution used. Otherwise, they were most evident in the tunica muscularis and serosa when Carnoy's fluid was used in conjunction with TB stain. Of course, it was evident that metachromatic mast cell density was determined the most in tunica mucosa layers of the middle and distal cecum. Mast cell population

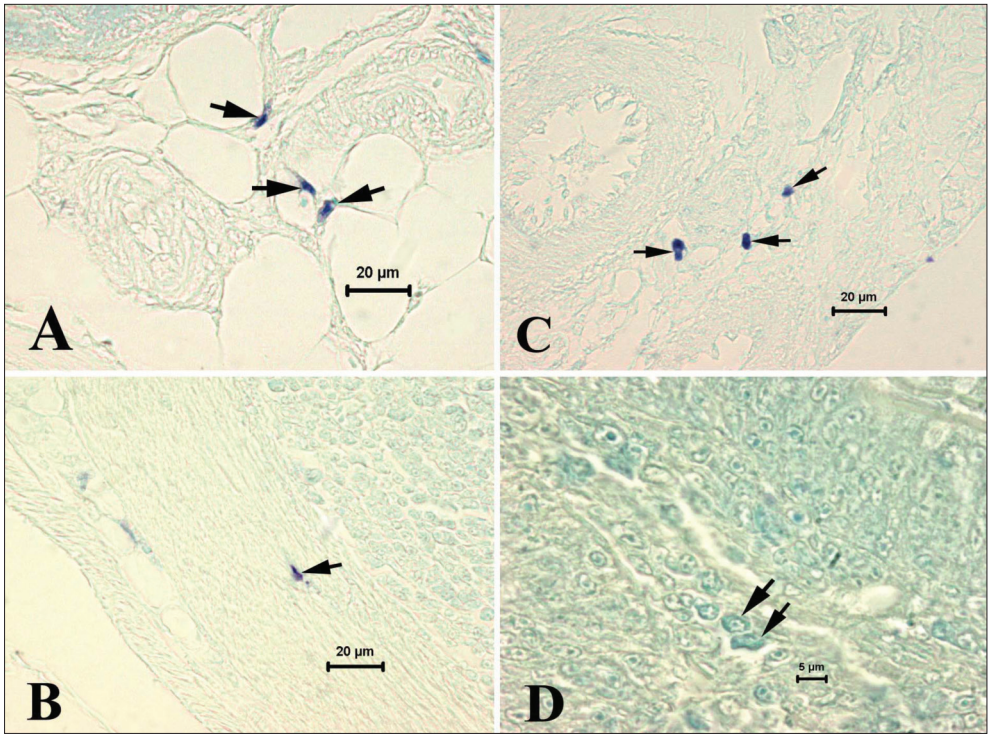

Fig 1. The metachromatic mast cells are seen in submucosa with IFAA (A), in tunica muscularis with BLA (B), in tunica serosa with Carnoy (C) and the orthochromatic TB (+) cells in villus with Carnoy fixed cecum (D). TB staining method. Bar: 20 µm (A,B,C), 5 µm (D)

Şekil 1. Submukozada IFAA (A), tunika muskulariste BLA (B), tunika serozada Carnoy (C) ile tespit edilmiş sekumda metakromatik ve villusta Carnoy (D) ile tespit edilmiş sekumda ortokromatik TB (+) mast hücreleri görülmektedir. TB boyama metodu. Bar: 20 µm (A,B,C), 5 µm (D)

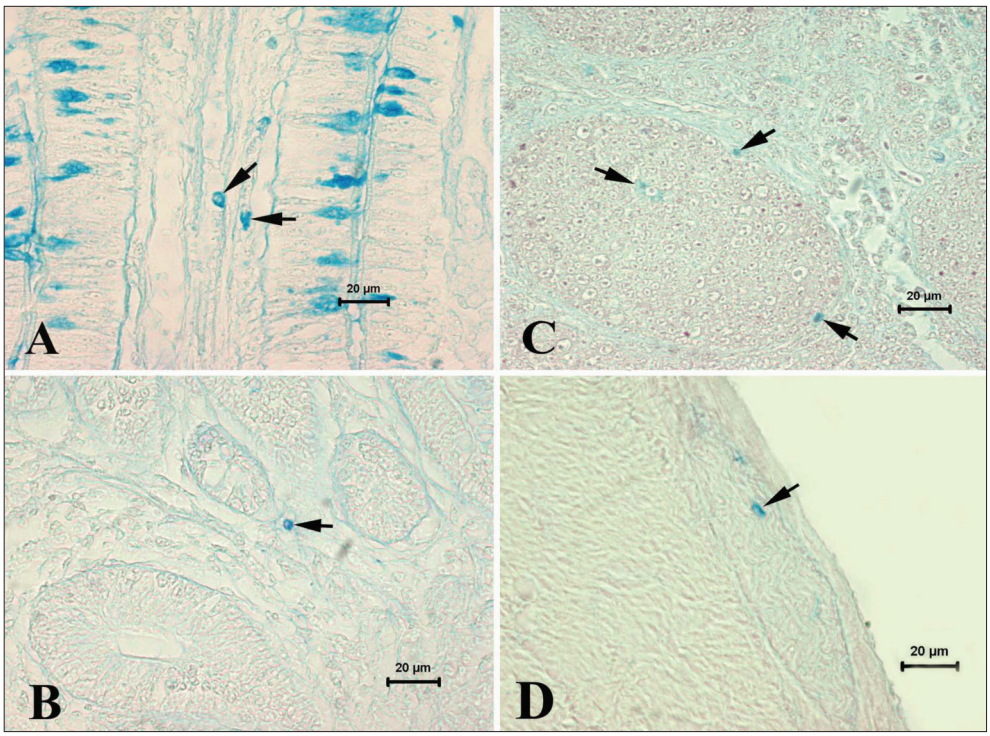

Fig 2. The AB-CEC (+) / SO (-) mast cells are seen in villus with IFAA (A), between glands with BLA (B), in lymphatic nodule of cecal tonsil with Carnoy (C) and in peripheral nerve with NBF fixed cecum (D). AB-CEC/SO staining method. Bar: 20 µm

Şekil 2. Villus IFAA (A), bezler arasında BLA (B), sekal tonsilin lenfatik nodülünde Carnoy (C) ve perifer sinirde NBF (D) ile tespit edilmiş sekumda AB-CEC (+) / SO (-) mast hücreleri görülmektedir. AB-CEC/SO boyama metodu. Bar: 20 µm

was shown in highest ratios in all layers of Carnoy fixed cecum parts within TB-stained sections ($P<0.05$, $P<0.01$ and $P<0.001$). Yet, while the mast-cell densities were significantly lower in the tunica mucosa of the proximal regions of cecum fixed with Carnoy's and IFAA fixatives than they were at the most rates in the middle and distal regions of TB-stained cecum sections. All mast cell-density values from the presently described experiment are presented in *Table 1*.

The cecum's mast cells were AB-CEC (+) and SO (-) reactive when the AB-CEC/SO staining method was used *(Fig. 2)*. That said, mast-cell densities were greater in the tunica mucosa than they were in other folds fixed with IFAA and BLA solutions. Additionally, when Carnoy's fluid was used AB-CEC (+)/SO (-) mast cells were more evident in the tunica muscularis and serosa of the tested cecum's proximal and distal sections; thus, it was concluded that mast-cell populations increased in the presence of IFAA

Table 1. *The density values of metachromatic mast cells in proximal, middle and distal parts of cecum*
Tablo 1. *Sekumun proksimal, orta ve distal bölümlerinde metakromatik mast hücrelerinin yoğunluk değerleri*

TB	n	Proximal			Middle			Distal		
Fixatives		TM	TMs	TS	TM	TMs	TS	TM	TMs	TS
IFAA	7	$0.45\pm0.07^{B,b}$	0.23 ± 0.05^{c}	0.29 ± 0.05^{b}	$1.52\pm0.10^{A,a}$	0.54 ± 0.08^{b}	0.09 ± 0.04^{b}	$1.52\pm0.14^{A,a}$	0.45 ± 0.07^{b}	0.14 ± 0.05^{b}
BLA	7	1.02 ± 0.15^{a}	0.54 ± 0.09^{b}	0.26 ± 0.06^{b}	1.11 ± 0.09^{b}	0.50 ± 0.08^{b}	0.16 ± 0.05^{b}	1.16 ± 0.07^{b}	0.57 ± 0.09^{b}	0.21 ± 0.06^{b}
CARNOY	7	$1.12\pm0.12^{B,a}$	0.87 ± 0.11^{a}	0.91 ± 0.10^{a}	$1.70\pm0.10^{A,a}$	0.81 ± 0.07^{a}	0.65 ± 0.09^{a}	$1.76\pm0.12^{A,a}$	1.00 ± 0.09^{a}	1.07 ± 0.09^{a}
P		***	***	***	***	*	***	**	***	***

^{a,b,c} Different superscripts in the same column indicate the significant difference. ^{A,B} Different superscripts in the same row indicate the significant difference. ***NS:*** *Non-significant, * P<0,05, ** P<0,01, *** P<0,001,* ***TB:*** *Toluidine blue,* ***IFAA:*** *Isotonic formaldehyde acetic acid,* ***BLA:*** *Basic lead acetate,* ***TM:*** *Tunica mucosa,* ***TMs:*** *Tunica muscularis,* ***TS:*** *Tunica serosa*

Table 2. *The density values of AB-CEC (+) / SO (-) mast cells in proximal, middle and distal parts of cecum*
Tablo 2. *Sekumun proksimal, orta ve distal bölümlerinde AB-CEC (+) / SO (-) mast hücrelerinin yoğunluk değerleri*

AB-CEC/SO	n	Proximal			Middle			Distal		
Fixatives		TM	TMs	TS	TM	TMs	TS	TM	TMs	TS
IFAA	7	$2.01\pm0.07^{A,C,a}$	0.44 ± 0.06^{b}	0.51 ± 0.06^{b}	$2.16\pm0.10^{A,a}$	0.83 ± 0.10	0.40 ± 0.09^{b}	1.85 ± 0.11^{C}	$0.88\pm0.08^{a,b}$	0.45 ± 0.08^{b}
BLA	7	2.09 ± 0.11^{a}	0.97 ± 0.09^{a}	0.57 ± 0.08^{b}	$1.95\pm0.09^{a,b}$	0.79 ± 0.08	$0.62\pm0.08^{a,b}$	1.78 ± 0.08	0.71 ± 0.07^{b}	0.54 ± 0.08^{b}
CARNOY	7	$0.85\pm0.11^{B,b}$	1.08 ± 0.09^{a}	1.10 ± 0.10^{a}	$1.77\pm0.12^{A,b}$	0.93 ± 0.06	0.77 ± 0.11^{a}	1.68 ± 0.12^{A}	1.08 ± 0.10^{a}	1.10 ± 0.08^{a}
P		***	***	***	*	NS	*	NS	*	***

^{a,b} Different superscripts in the same column indicate the significant difference. ^{A,B,C} Different superscripts in the same row indicate the significant difference, ***NS:*** *Non-significant, * P<0.05, *** P<0.001,* ***AB-CEC/SO:*** *Alcian blue-critical electrolyte concentration/safranin O,* ***IFAA:*** *Isotonic formaldehyde acetic acid,* ***BLA:*** *Basic lead acetate,* ***TM:*** *Tunica mucosa,* ***TMs:*** *Tunica muscularis,* ***TS:*** *Tunica serosa*

and BLA solutions to the tunica mucosas of a cecum's proximal and middle portions when compared with the introduction of Carnoy's solution to the same (P<0.05, P<0.001). It was also concluded that mast-cell density is significantly lower for the tunica mucosa of a cecum proximal region to which Carnoy's fixative has been introduced than it is for middle and distal cecum regions stained with AB-CEC/SO. All values associate with mast-cell population are presented in *Table 2*.

DISCUSSION

Rodent mast cells are classified into two types, MMCs and CTMCs [13,14]. MMCs are stained with AB (+), and CTMCs are stained with SO (+) [14]. Uslu and Yoruk [19] state that MMCs stain blue and CTMCs stain red with a combined AB/SO staining method. And yet, the aforementioned researchers also report that three kinds of mast cells were found to be AB (+), SO (+) and AB/SO (+) when AB/SO staining was introduced to some digestive-tract organs in chickens *(gallus gallus domestica)* [23], quails [26] and rats [27]. Moreover, Harem and Liman [21] have demonstrated that rat and quail mast cells have varied glycosaminoglycan types with regards to both combined AB-CEC (pH 5.8, 0.3 M $MgCl_2$) and aldehyde fuchsin (AF) tissue-staining techniques. In the present study, the tested cecum mast cells were AB-CEC (+) and SO (-) reactive when the AB-CEC/SO staining method was used. For this reason, it was concluded that quail-cecum mast cells were MMCs. Furthermore, in the present study, orthochromatic TB (+) cells were observed in the TB-stained sections. Mendonca et al.[28] report that immature mast cells contain orthochromatic granules. They also state that the granules become metachromatic when fully developed. Based on this information, it was concluded that the orthochromatically stained TB (+) cells observed in the present study may be immature quail-cecum mast cells.

The effects of fixative solutions on various species' mast-cell counts and distributions have been previously reported. For example, it was found that BLA solution was better than Carnoy's and IFAA solutions in fixing the mast cells in turkey digestive systems [19]. Karaca and Yoruk [26] have reported as well that the mast-cell distributions in chicken and quail digestive tracts are best determined with BLA and Carnoy's solutions. Similarly, Carnoy's fixative solution is noted as the most effective fixative for identifying mast cells in chicken intestinal mucosa [29], and IFAA solution is seen as the most suitable fixative for determining mast-cell density in the same. BLA solution, meanwhile, has been shown comparatively superior to other fixatives in helping to identify the granule structures in the lower respiratory tracts and lungs of local ducks and geese [30]; as for identifying human MMCs, Strobel et al.[31] recommend using either Carnoy's or BLA solution. Similarly, both Asti et al.[32] and Eren [33] have showed that more mast cells are identified in dog skin when the tissue is fixed with IFAA solution than when it is fixed with 10% formaldehyde solution; it has been stated as well that

a greater number of mast cells is found in Bouin-fixed esophagus [34] and mast-cell density is greater in IFAA-fixed rat duodenum [27] and cow uterus [22]. On the other hand, while MMCs are commonly found in the lamina propria and epithelial layers of mucosal surfaces [15,16], CTMCs are found in skin, muscle and peritoneal cavity [35]. For its part, the present study showed that mast-cell population was in highest ratios in all layers of Carnoy fixed cecum parts within TB-stained sections. Furthermore, it showed that AB-CEC (+)/SO (-) mast-cell density was greater when IFAA and BLA solutions were introduced into the tunica mucosa of the tested proximal and middle cecum. In the study, it showed that metachromatic mast cells are more evident in the tunica muscularis and serosa folds of all parts of the cecum when Carnoy's fluid is used. Based on these results, the present study's authors have concluded that different fixative solutions and staining methods affect the distribution of mast cells in quail cecum. Also, it can be said that Carnoy solution fixed the CTMCs, as well as IFAA and BLA solutions fixed the MMCs better. However, the density values of mast cell were determined using a semi-quantitative scoring method in the study. For this reason, it is needed the studies that count of mast cells will be revealed with stereological methods in order to improve the reliability of the study data.

The cecum contains diffuse and nodular lymphatic tissues [5], and these are located in the cecum's proximal region [6]. Kiernan [36] has stated that mast-cell granules containing heparin are stained with TB metachromatically and AB 8GX. The present study showed that mast-cell density was significantly lower in the tunica mucosa of the cecum's proximal region when Carnoy's and IFAA fixatives were used than it was in other cecum regions stained with TB, and these same circumstances were observed when Carnoy's solution was used to fix cecum sections stained with AB-CEC/SO. It is therefore apparent that fewer mast cells containing heparin are located in the tunica mucosa of the cecum's proximal region if Carnoy's and IFAA fixatives are applied to it. Also, lymphatic tissues that are called as cecal tonsils are located in the proximal part of the cecum. That's why, fewer mast cells may be found in proximal part of the cecum. Furthermore, in the present study mast cells were rarely observed with two-stain methods when NBF solution was used as a fixative. This is consistent with other studies showing that mast-cell counts are lower in NBF-fixed tissue than in tissues fixed with other solutions [29,31,34]. As with previous studies, the present study shows that MMCs are sensitive to formalin. It may therefore be concluded that they are not sufficiently preserved in NBF solution.

Mast-cell densities vary in different poultry digestive-tract folds. Uslu and Yoruk [19] report that mast-cell numbers are highest in the submucosa, and they were similar in lamina propria and tunica muscularis + tunica serosa of turkey digestive tract. Additionally, Karaca and Yoruk [26] state that lamina propria and submucosa have greater numbers of mast cells than do tunica muscularis and serosa in quail cecum. In the present study, metachromatic mast cell density was greater in the tunica mucosa than it was in other folds of the cecum. Based on these results, it can be asserted that mast cells are more often located in the mucosal folds of quail cecum than they are in their tunica muscularis and serosa.

In conclusion, the distribution of mast cells for quail cecum was determined in the present study by applying TB and AB-CEC/SO staining methods to all regions and folds of said cecum. Moreover, it was revealed that the type of fixative solution used when testing quail cecum tissue affects mast-cell distribution.

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Research Article

Thymoquinone, the Main Constituent of *Nigella sativa*, Could Impact on Adenosine A_2 Receptors in Ovalbumin-sensitized Guinea Pigs

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Abstract

Thymoquinone has demonstrated anti-asthmatic effects in many studies but its exact mechanism is not yet fully known. This investigation aims to demonstrate its prophylactic effect in the presence of selective A_{2A} and A_{2B} adenosine receptors (AR) antagonist; MRS1706 and ZM241365, in sensitized guinea pigs. The gene expression of A_2 AR in blood lymphocytes and lung tissue, lung pathological changes and blood cytokines were evaluated in seven groups. The experiments in blood lymphocytes and lung tissue showed that thymoquinone could increase A_{2A}AR mRNA expression and decrease A_{2B} AR mRNA expression significantly ($P<0.001$ to $P<0.05$); however sensitization had opposite effects. Administration of A_{2A} receptor antagonist attenuated inflammation and A_{2B} receptor antagonist could prevent asthma-induced inflammatory changes. Moreover, the administration of thymoquinone and A_{2A}receptor antagonist together relieved inflammation. Gene expression of A_{2B} receptor showed that thymoquinone administration has more influence on blood lymphocytes while administration of the selective A_{2B} receptor antagonist was more effective in lung tissue. The results showed some of the therapeutic effects of thymoquinone in reducing asthma symptoms might be partially mediated through A_2 adenosine receptors.

Keywords: *Asthma, Adenosine, MRS1706, ZM241365, Gene expression*

Nigella Sativa'nın Biyoaktif Komponenti Olan Timokinon Ovalbuminle Uyarılmış Ginedomuzlarında Adenozin A_2 Reseptörlerini Etkileyebilir

Özet

Timokinonun birçok çalışmada anti-astmatik etkileri olduğu gösterilmesine rağmen tam mekanizması tamamıyla anlaşılamamıştır. Bu çalışma A_{2A} ve A_{2B} adenozin reseptör (AR) antagonistlerinin (MRS1706 ve ZM241365) mevcudiyetinde timokinonun uyarılmış ginedomuzlarındaki proflaktik etkisini araştırmak amacıyla yapılmıştır. Çalışmada toplam yedi grupta kan lenfositleri ve akciğer dokusunda A_2 AR gen ekspresyonları, akciğerdeki patolojik değişiklikler ve kan sitokinleri değerlendirildi. Kan lenfositleri ve akciğer dokusunda yapılan incelemeler timokinonun A_2AAR mRNA ekspresyonunu arttırdığını ve A_{2B} AR mRNA ekspresyonunu ise anlamlı oranda azalttığını, ancak uyarılmanın ters etki yaptığını ortaya koydu ($P<0.001$ to $P<0.05$). A_{2A} reseptör antagonisti yangıyı azaltırken ve A_{2B} reseptör antagonisti astma tarafından oluşturulan yangısal değişikliklere karşı koruyucu etki gösterebilir. Ayrıca, timokinon ve A_{2A} reseptör antagonistinin birlikte uygulanması yangıyı hafifletti. A_{2B} reseptör gen ekspresyonu timokinon uygulamasının kan lenfositleri üzerinde daha etkili olduğunu A_{2B} reseptör antagonistinin ise akciğer dokusunda daha etkili olduğunu gösterdi. Elde edilen sonuçlar astma semptomlarının azaltılmasında timokinon uygulamasının terapötik etkilerinin kısmen A_2 adenozin reseptörleri yoluyla oluştuğunu göstermiştir.

Anahtar sözcükler: *Astma, Adenozin, MRS1706, ZM241365, Gen ekspresyonu*

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INTRODUCTION

Asthma is a chronic and acute respiratory disease of the airways [1] which has been identified by various properties such as the increased responsiveness of airways to physical and chemical stimuli [2]. In asthma, the responses of the immune system and related cells including T lymphocytes are influenced. Asthma is accompanied by the increase in the immune system of Th_2 cells and a decrease in the immune response of Th_1 cells. Th_2 cells are the regulators of pre-inflammatory responses and Th_1 cells directly or indirectly inhibit Th_2 cells [3,4]. Therefore, the increased level of IL-4 (cell markers of Th_2) and decreased level of IFN-γ (cell markers of Th_1) can be seen in this disease [5,6].

Other regulating factors which are propounded in the inflammation are adenosine and adenosine receptors. Adenosine is a purine nucleoside of adenine that mediates the various physiological functions through four transmembrane receptors; A_1, A_{2A}, A_{2B} and A_3 [7]. In asthmatic patients, the level of adenosine in liquid bronchoalveolar lavage is noticeably more than the healthy people [8]. Moreover, inhaled adenosine causes bronchial contraction in asthmatic patients but in healthy people it does not cause any contraction [9]. In fact, adenosine receptors act as sensor and extracellular adenosine acts as a reporter [10]. The stimulation of adenosine A_{2A} receptor inhibits the release of inflammatory mediators [11] and also prevents the activation of T cells [12] and inhibits the mobilization of inflammatory cells in the airways. The stimulation of A_{2B} receptor in human beings activates the mast cells [13], and as a result, increases the release of IL-8 [14]. The inhibition of A_{2B} receptors can have helpful therapeutic effects on asthma [15] and lead to the decrease of bronchospasm in response to such stimuli as AMP [16].

Nowadays, a lot of medicines are used for treating asthma, but the long-term use of them results in therapeutic resistance and will have some undesirable side effects. Therefore, in recent years, the researchers have tried to find new medicines with high effectiveness and fewer side effects. One of these approaches is using herbs. Many studies showed that *Nigella sativa* had anti-inflammatory and therapeutic effects [17-20]. Moreover, our previous studies demonstrated that the extract of *Nigella sativa* and its main constituent, thymoquinone, had preventive effects on asthma and precluded its inflammatory and pathological changes [5,6,21,22].

The exact mechanism of anti-asthmatic effect of thymoquinone is not known yet and the adjustment of Th_1/Th_2 balance has been suggested as one of the possible mechanisms for thymoquinone. In light of this, and in order to find out the intracellular mechanisms of thymoquinone, this investigation was proposed to demonstrate its prophylactic effect on gene expression of A_2 adenosine receptors in blood lymphocytes and lung tissue in the presence of selective A_{2A} and A_{2B} adenosine receptor antagonists; ZM241365 and MRS1706, in asthmatic guinea pigs. In addition, blood IL-4 and IFN-γ level and lung pathological changes were assessed in different groups.

MATERIAL and METHODS

Animal Sensitization and Animal Groups: Seventy male adult Dunkin-Hartley guinea pigs (400-700 g) were used throughout the study. After transportation, they were allowed to acclimatize to the new situation for ten days. They were kept in individual cages at 22±2°C on a 12-h light/dark cycle and allowed free access to standard chow and water. Then the animals were randomly divided into seven groups; control group (C), sensitized group with ovalbumin (S), sensitized group pretreated with thymoquinone (3 mg/kg i.p.; S+TQ) [23], sensitized group pretreated with selective A_{2A} adenosine receptor antagonist (ZM241385, 3 mg/kg i.p.; S+Anta A_{2A}) [24], sensitized group pretreated with selective A_{2B} adenosine receptor antagonist (MRS1706, 3 mg/kg i.p.; S+Anta A_{2B}) [9], sensitized group pretreated with thymoquinone and A_{2A} adenosine receptor antagonist (S+TQ+Anta A_{2A}) and sensitized group pretreated with thymoquinone and A_{2B} adenosine receptor antagonist (S+TQ+Anta A_{2B}). Thymoquinone, ZM241385 and MRS1706 with 3 mg/kg dose were injected intraperitoneally on day 10 of induction protocol. All groups were housed in climate-controlled animal quarters and were given water and food *ad libitum,* while a12-h on/12-h off light cycle was maintained.

Sensitization of animals to ovalbumin (OA) was performed using the method used in our previous study [25]. Briefly, guinea pigs were sensitized to ovalbumin (Grade II Sigma Chemical Ltd., UK) dissolved in saline by injecting 100 mg i.p. and 100 mg s.c. on the first day and a further 10 mg i.p. on the 8th day. From day 14, sensitized animals were exposed to an aerosol of 4% ovalbumin for 18±1 days, 4 min daily. The aerosol was administered in a closed chamber, dimensions 30 × 20 × 20 cm. Control animals were treated similarly but saline was used instead of ovalbumin solution. The study was approved by the ethical committee of the Tabriz University of Medical Sciences (No: TBZMED.REC.1394.862).

Evaluation of the Gene Expression of A_2 Receptors in Blood Lymphocytes and Lung Tissue: One day after the induction period, the animals were killed by cervical dislocation and 5 ml blood sample and approximately, 100 µg of left lung tissue were obtained immediately. At first, blood RBCs were eliminated by RBC lysis buffer containing 9 g NH_4Cl, 1g $KHCO_3$ and 0.2 ml of 0.5M EDTA per liter. The pH was adjusted to 7.3. Then TRIzol reagent was used for RNA extraction. Lung specimens were also grinded and subjected to total RNA extraction by 1 ml of TRIzol reagent (life technologies Co) according to the manufacturer's recommendations. After that, cDNA was

synthesized by Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific Co) using 5 µg of total RNA. The effect of thymoquinone on the gene expression of A_2 adenosine receptors was investigated with RT-PCR. Specific primers were designed for control and receptor genes. A_{2B} primers were designed by OLIGO (version 5.0). The sequences were A_{2A} F: 5′-GCA GAA CGT CAC CAA CTA CTT-3′, A_{2A} R: 5′-CAG GTC ACC AAG CCA TTG TA-3′, A_{2B} F: 5′-CTT TGG CAT TGG ATT GAC TC-3′ and A_{2B} R: 5′-CCA GCA TGA TGA GCA GTG G-3′. β-actin was employed as a housekeeping gene to normalize expression levels of target genes. Primer sequences for β-actin were: β-actin F: 5′- TCC CTG GAG AAG AGC TAC G-3′ and β-actin R: 5′- GTA GTT TCG TGG ATG CCA CA-3′.

The cycling included denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 58°C for 40 s and 72°C for 30 s and a final extension at 72°C for 10 min. PCR products were electrophorized utilizing 1.5% agarose gel and appropriate amount of safe stain (Cinnagen Co,Islamic republic of Iran) and were visualized by gel documentation apparatus. 100 bp DNA ladder (SM0311, Fermentas Co.) was used as size marker. Finally the electrophoresed gene was photographed by document gene device and the intensity of bands was investigated through Image J software (National Institutes of Health, Bethesda, Maryland, USA) and normalized to its actin loading control.

Evaluations of Blood IL-4 and IFN-γ Levels: A total of 5 ml peripheral blood was obtained immediately after sacrificing the animals and placed at room temperature for 1 h. The samples were then centrifuged at 3500×*g* at 4°C for 10 min. The supernatant was collected and immediately put at -70°C until analyzed. Finally, blood IL-4 and IFN-γ were measured using the enzyme-linked immunosorbent assay (ELISA) Sandwich method [6].

Pathological Evaluation: Lungs and trachea were kept in 10% (v/v) buffered formalin. A week later tissues were dried by a range of ethanol concentrations (70%-100%) through Passage method. The samples were saturated in paraffin and put into blocks after being cleared by xylol. Then 4 micron-width slices were prepared using microtome. Finally, the specimens were stained by hematoxylin-eosin (H&E) and evaluated under a light microscope. The pathological changes in the lungs of all groups included vascular and airways smooth muscle hypertrophy and hyperplasia, the presence of mucosal plug, respiratory epithelial denudation, inflammatory infiltration and emphysema [5,21]. The pathological changes were scored according to previous studies as follows: 0: no pathologic changes, 1: patchy changes, 2: local changes, 3: scattered changes and 4: severe changes.

Statistical Analysis: All the results were considered as mean ± SEM. The data of six sensitized groups were compared with controls using one-way analysis of variance (ANOVA) with Tukey-Kramer post-test. Furthermore, the data of five pretreated groups were compared with sensitized guinea pigs using one-way analysis of variance (ANOVA) with Tukey-Kramer. The data of four pretreated groups were compared with S+TQ group using one-way analysis of variance (ANOVA) with Tukey-Kramer.

RESULTS

Analysis of A_{2A} Adenosine Receptor Gene Expression in Blood Lymphocytes: Compared to the control group, the gene expression of A_{2A} adenosine receptor in S and S+Anta A_{2A} groups decreased significantly ($P<0.05$ and percentage changes = -43% for S, $P<0.01$ and percentage changes = -62% for S+Anta A_{2A}) and in S+TQ group increased significantly ($P<0.001$ and percentage changes = 120%, *Fig. 1*). There was a significant increase in A_{2A} adenosine receptor gene expression in S+TQ and S+TQ+Anta A_{2A} groups compared to the sensitized group ($P<0.001$ to $P<0.01$). Percentage changes in these groups are as follows: S+TQ=288% and S+TQ+AntaA_{2A}=91% *(Fig. 2)*.

The gene expression of A_{2A} adenosine receptor in S+

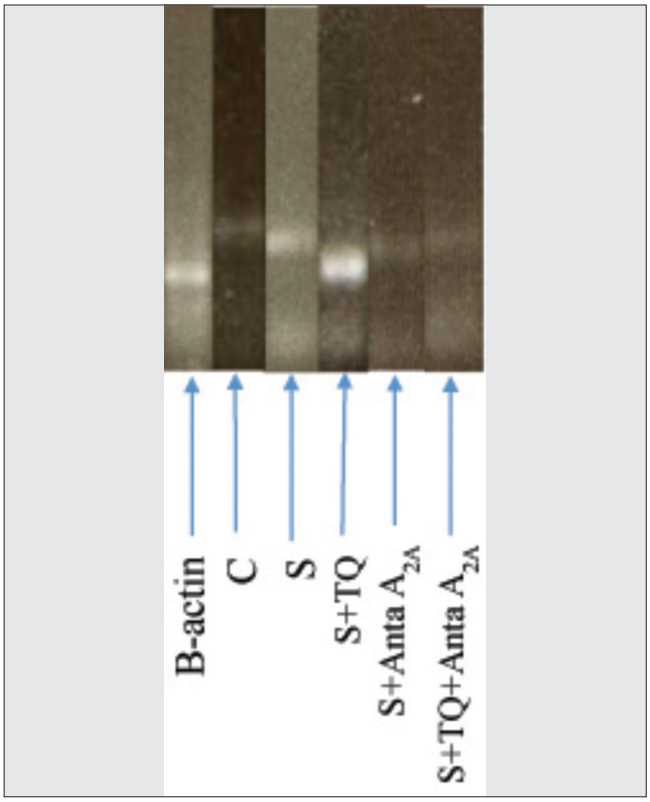

Fig 1. A_{2A} adenosine receptor gene expression analysis using RT-PCR in blood lymphocytes in the control (C), sensitized (S), S pretreated with thymoquinone (S+TQ), S pretreated with A_{2A} adenosine receptor antagonist (S+Anta A_{2A}) and S pretreated with thymoquinone and A_{2A} adenosine receptor antagonist (S+TQ+Anta A_{2A}) guinea pigs

Şekil 1. RT-PCR ile kan lenfositlerinde A_{2A} adenozin reseptör gen ekspresyonu analizi; Kontrol (C), uyarılmış (S), timokinon verilmiş S (S+TQ), A_{2A} adenozin reseptör antagonisti verilmiş S (S+Anta A_{2A}) ve timokinon ile birlikte A_{2A} adenozin reseptör antagonisti verilmiş (S+TQ+Anta A_{2A}) ginedonumuzu

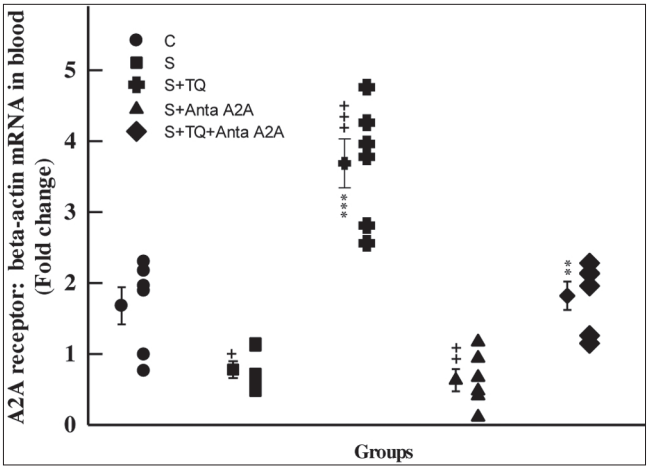

Fig 2. Individual values and mean±SEM (big symbols with bars) of the gene expression of A_{2A} adenosine receptor in blood lymphocytes of control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2A} antagonist (S+Anta A_{2A}), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}), sensitized pretreated with selective A_{2A} antagonist and thymoquinone (S+Anta A_{2A}+TQ) and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups (for each group, n=6). Statistical differences between control and different groups: + P<0.05, ++ P<0.01, +++ P<0.001. Statistical differences between pretreated groups vs sensitized group: * P<0.05, ** P<0.01, *** P<0.001

Şekil 2. Kan lenfositlerinde A_{2A} adenozin reseptör gen ekspresyonun bireysel değerleri ve ortalama ± SEM (Barlı büyük semboller). Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), timokinon ile birlikte A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}+TQ), timokinon ile birlikte A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) ginedonumuzu (Her grupta n=6). Kontrol ve diğer gruplar arasındaki istatistiksel farklar: + P<0.05, ++ P<0.01, +++ P<0.001. Madde uygulanmış ve uyarılmış gruplar arasındaki istatistiksel farklar: * P<0.05, ** P<0.01, *** P<0.001

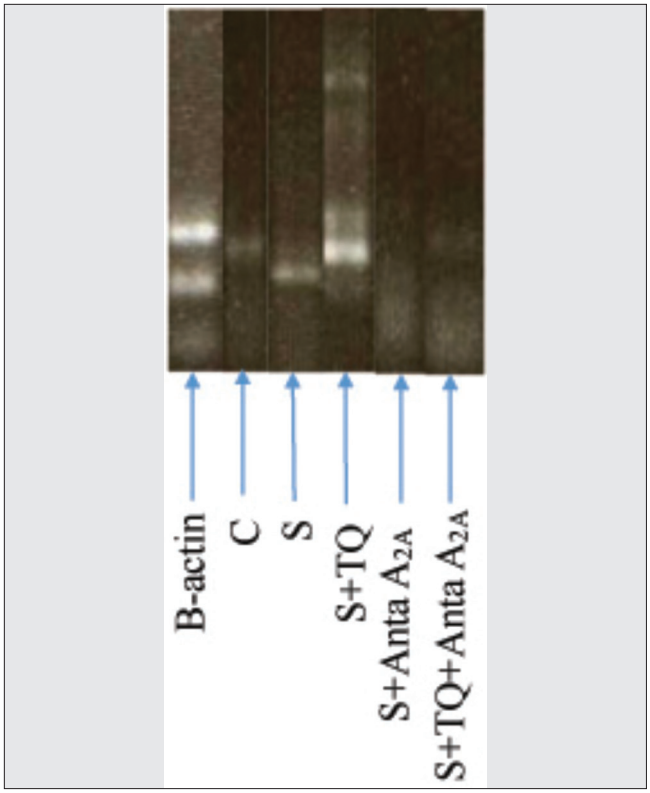

Fig 3. A_{2A} adenosine receptor gene expression analysis using RT-PCR in lung tissue in the control (C), sensitized (S), S pretreated with thymoquinone (S+TQ), S pretreated with A_{2A} adenosine receptor antagonist (S+Anta A_{2A}) and S pretreated with thymoquinone and A_{2A} adenosine receptor antagonist (S+TQ+Anta A_{2A}) guinea pigs

Şekil 3. RT-PCR ile akciğer dokusunda A_{2A} adenozin reseptör gen ekspresyonu analizi; Kontrol (C), uyarılmış (S), timokinon verilmiş S (S+TQ), A_{2A} adenozin reseptör antagonisti verilmiş S (S+Anta A_{2A}) ve timokinon ile birlikte A_{2A} adenozin reseptör antagonisti verilmiş (S+TQ+Anta A_{2A}) ginedonumuzu

Anta A_{2A} and S+TQ+Anta A_{2A} groups decreased significantly compared to thymoquinone pretreated group (P<0.001 and percentage changes = -82% for S+Anta A_{2A}, P<0.001, percentage changes = -50% for S+TQ+Anta A_{2A}). The gene expression of A_{2A} adenosine receptor in S+TQ+Anta A_{2A} group was significantly higher than in S+Anta A_{2A} group (P<0.01, percentage changes= 188%).

Analysis of A_{2A} Adenosine Receptor Gene Expression in Lung Tissue: In comparison to the control group, the gene expression of A_{2A} adenosine receptor in S and S+Anta A_{2A} groups decreased significantly (P<0.05 and percentage changes = -36% for S, P<0.01 and percentage changes = -61%, *Fig. 3*). There was significant increase in A_{2A} adenosine receptor gene expression in S+TQ and S+TQ+Anta A_{2A} groups compared to group S (P<0.01 and percentage changes = 129% for S+TQ, P<0.05 and percentage changes = 76% for S+TQ+Anta A_{2A}, *Fig. 4*).

A_{2A} adenosine receptor gene expression in S+Anta A_{2A} group was significantly lower than the S group (P<0.05, percentage changes= -39%). The gene expression of A_{2A} adenosine receptor in lung tissue of S+Anta A_{2A} group decreased compared to thymoquinone pretreated group (P<0.001). The gene expression of A_{2A} adenosine receptor in S+TQ+Anta A_{2A} group was significantly more than S+Anta A_{2A} group (P<0.01, percentage changes = 188%).

Analysis of A_{2B} Adenosine Receptor Gene Expression in Blood Lymphocytes: Compared to the control group, the gene expression of A_{2B} adenosine receptor in S and S+TQ groups increased significantly (P<0.001 and percentage changes = 632% for S, P<0.01 and percentage changes = 256% for S+TQ, *Fig. 5*). All pretreated groups showed a significant decrease in A_{2B} adenosine receptor gene expression compared with sensitized group (P<0.001 to P<0.01). Percentage changes in these groups are as follows: S+TQ = -51%, S+Anta A_{2B} = -63% and S+TQ+Anta A_{2B} = -191%, *Fig. 6*).

The gene expression of A_{2B} adenosine receptor in blood of S+TQ+Anta A_{2B} group decreased significantly compared to thymoquinone pretreated group and S+Anta A_{2B} group (P<0.001, *Table 1*).

Analysis of A_{2B} Adenosine Receptor Gene Expression in Lung Tissue: As compared with control group, the

gene expression of A_{2B} adenosine receptor in S and S+TQ groups increased significantly (P<0.001 and percentage changes = 291% for S, P<0.01 and percentage changes = 171% for S+TQ, *Fig. 7*). With regard to the sensitized group, the gene expression of A_{2B} adenosine receptor in lung tissue of all pretreated groups decreased significantly (P<0.001 to P<0.05, *Fig. 8*).

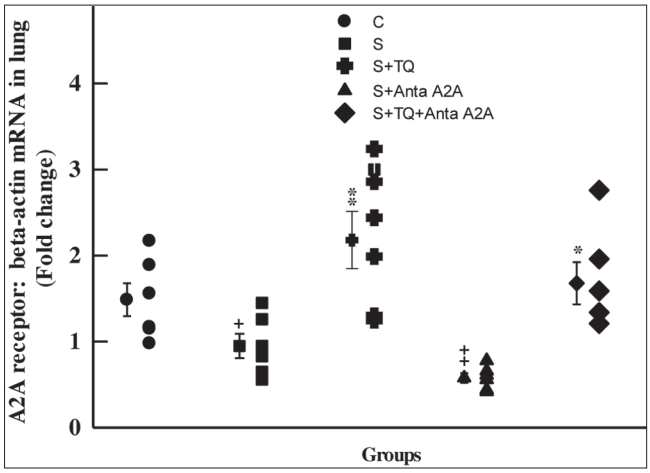

Fig 4. Individual values and mean±SEM (big symbols with bars) of the gene expression of A_{2A} adenosine receptor in lung tissue of control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2A} antagonist (S+Anta A_{2A}) and sensitized pretreated with selective A_{2A} antagonist and thymoquinone (S+Anta A_{2A}+TQ) groups (for each group, n=6). Statistical differences between control and different groups: + P<0.05, ++ P<0.01. Statistical differences between pretreated groups vs sensitized group: * P<0.05, ** P<0.01

Şekil 4. Akciğer dokusunda A_{2A} adenozin reseptör gen ekspresyonun bireysel değerleri ve ortalama ± SEM (Barlı büyük semboller). Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), timokinon ile birlikte A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}+TQ), timokinon ile birlikte A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) ginedonumuzu (Her grupta n=6). Kontrol ve diğer gruplar arasındaki istatistiksel farklar: + P<0.05, ++ P<0.01. Madde uygulanmış ve uyarılmış gruplar arasındaki istatistiksel farklar: * P<0.05, ** P<0.01

The gene expression of A_{2B} adenosine receptor in the lung tissue of S+Anta A_{2B} and S+TQ+Anta A_{2B} groups decreased compared to thymoquinone pretreated group (P<0.001, percentage changes = -68% for S+Anta A_{2B} and percentage changes = -70% for S+TQ+Anta A_{2B}). In the lung tissue of S+TQ+Anta A_{2B} group, the gene expression of A_{2B} adenosine receptor was significantly lower than S+Anta A_{2B} group (P<0.01, percentage changes = -5.50%, *Table 1*).

Pathology: With regard to this scoring, all pathological changes in the S, S+Anta A_{2A} and S+TQ+Anta A_{2A} groups, including the vascular membrane hyperplasia, airway membrane hyperplasia, the presence of mucosal plug, respiratory epithelial denudation, cellular infiltration and emphysema were significantly higher than those in the C group (P<0.05 for all cases). Moreover, the presence of mucosal plug, respiratory epithelial denudation and emphysema in S+TQ and S+Anta A_{2B} groups were significantly higher than those in the C group (P<0.05, *Fig. 9a-g*).

All pathological changes in S+TQ, S+Anta A_{2B} and S+TQ+ Anta A_{2B} groups (except inflammatory infiltration in S+Anta A_{2B}), and the presence of mucosal plug in S+TQ+Anta A_{2A} group were significantly decreased in comparison with the sensitized group (P<0.05). However, there were still significant variations in presence of mucosal plug, respiratory epithelial denudation and emphysema between the C group and S+TQ group (P< 0.05, *Table 2*).

All criteria in S+Anta A_{2A} group, and airway membrane hyperplasia, the presence of mucosal plug and cellular infiltration in S+Anta A_{2B} group were significantly higher than S+TQ group (P<0.001 to P<0.05). There were no differences in the pathological changes between S+TQ+ Anta A_{2A} and S+TQ+Anta A_{2B} groups and the S+TQ group. There was not any significant differences between S+Anta A_{2A} and S+TQ+Anta A_{2A} groups and between S+Anta A_{2B} and the S+TQ+Anta A_{2B} group *(Table 2)*.

Table 1. *The mean value of the gene expression of A_{2B} adenosine receptor in blood lymphocyte and lung tissue and blood cytokines (IL-4 and IFN-g) in control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2A} antagonist (S+Anta A_{2A}), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}), sensitized pretreated with selective A_{2A} antagonist and thymoquinone (S+Anta A_{2A}+TQ) and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups(for each group, n = 8)*

Tablo 1. *Kan lenfosit ve akciğer dokusu A_{2B} adenozin reseptör gen ekspresyonu ile kan sitokinlerinin ortalama değerleri. Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), A_{2A} antagonisti ve timokinon verilmiş ve uyarılmış (S+Anta A_{2A}+TQ) ve A_{2B} antagonisti ve timokinon verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) (Her grupta n=8)*

Parameter	C	S	S+TQ	S+Anta A_{2A}	S+Anta A_{2B}	S+TQ+Anta A_{2A}	S+TQ+Anta A_{2B}
The gene expression of A_{2B} adenosine receptor in blood lymphocyte	0.54±0.23	3.93±0.51	1.91±0.16	0.63±0.15 +++	1.41±0.34	1.82±0.20 **	0.34±0.13 +++ ###
The gene expression of A_{2B} adenosine receptor in lung tissue	0.59±0.18	2.32±0.27	1.61±0.13	0.58±0.05 +++	0.51±0.08 +++	1.68±0.24 **	0.48±0.14 +++ ##
Blood IL-4 level	39.78±2.01	47.41±1.98	43.88±1.70	49.48±2.74	45.20±2.15	46.24±1.38	39.98±0.82 #
Blood IFN-gamma level	104.97±5.93	117.37±2.71	124.93±2.31	101.14±3.24 +++	120.03±2.95	107.87±2.04 +++	126.04±2.81

*Statistical differences between pretreated groups vs S+TQ group: +++; P<0.001; Statistical differences between S+TQ+Anta A_{2A} group vsS+Anta A_{2A} group: ** P<0.01; Statistical differences between S+TQ+Anta A_{2B} group vsS+Anta A_{2B} group: #; P<0.05; ##; P<0.01, ###; P<0.001*

Table 2. *The mean value of different pathological changes in control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2A} antagonist (S+Anta A_{2A}), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}), sensitized pretreated with selective A_{2A} antagonist and thymoquinone (S+Anta A_{2A}+TQ) and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups (for each group, n = 8*

Tablo 2. *Değişik patolojik değişimlerim ortalama değeri. Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), A_{2A} antagonisti ve timokinon verilmiş ve uyarılmış (S+Anta A_{2A}+TQ) ve A_{2B} antagonisti ve timokinon verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) (Her grupta n=8)*

Parameter	C	S	S+TQ	S+Anta A_{2A}	S+Anta A_{2B}	S+TQ+Anta A_{2A}	S+TQ+Anta A_{2B}
Vascular smooth muscle hypertrophy and hyperplasia	0.33±0.21	3±0.3 +++	1.33±0.30 **	2.90±0.28 +++ ##	1.42±0.48 *	2.29±0.42 +	0.86±0.34 **
Airways smooth muscle hypertrophy and hyperplasia	0.17±0.16	2.54±0.28 +++	0.83±0.24 ***	2.54±0.31 +++ ###	0.86±0.34 **	2.29±0.42 ++ #	0.71±0.29 **
Presence of mucosal plug	0±0	2.72±0.14 +++	0.67±0.19 ***	2.27±0.23 +++ ###	1.14±0.34 + ***	1.71±0.29 +++ * #	0.29±0.18 ***
Respiratory epithelial denudation	0.17±0.16	2.18±0.23 +++	0.83±0.20 ***	2.45±0.21 +++ ###	0.86±0.26 **	1.71±0.29 ++	0.29±0.18 ***
Inflammatory infiltration	0±0	1.55±0.20 ++	0.41±0.19 **	2.18±0.22 +++ ###	0.71±0.35	1.85±0.34 +++ ##	0.57±0.30
Emphysema	0.17±0.16	2±0.27 +++	0.92±0.19 *	2.18±0.23 +++ ##	1.43±0.30	1.77±0.29 ++	0.71±0.29 *

*Statistical differences between groups vs C group: ++; P<0.01, +++; P<0.001; Statistical differences between pretreated groups vs S group: * P<0.05, ** P<0.01, *** P<0.001; Statistical differences between pretreated groups vs S+TQ group: #; P<0.05, ##; P<0.01, ###; P<0.001*

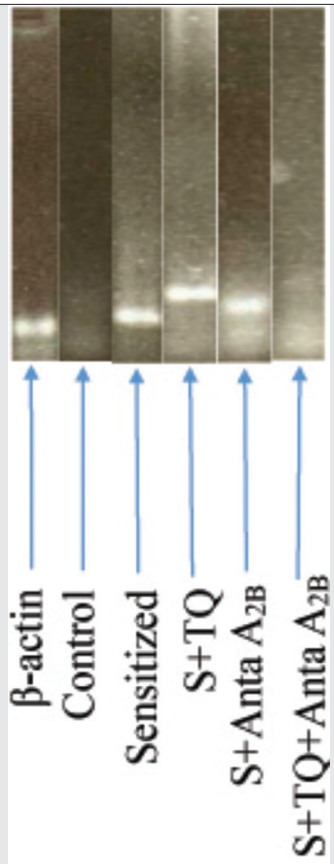

Fig 5. A_{2B} adenosine receptor gene expression analysis using RT-PCR in blood lymphocytes in the control (C), sensitized (S), S pretreated with thymoquinone (S+TQ), S pretreated with A_{2B} adenosine receptor antagonist (S+Anta A_{2B}) and S pretreated with thymoquinone and A_{2B} adenosine receptor antagonist (S+TQ+Anta A_{2B}) guinea pigs

Şekil 5. RT-PCR ile kan lenfositlerinde A_{2B} adenozin reseptör gen ekspresyonu analizi; Kontrol (C), uyarılmış (S), timokinon verilmiş S (S+TQ), A_{2B} adenozin reseptör antagonisti verilmiş S (S+Anta A_{2B}) ve timokinon ile birlikte A_{2B} adenozin reseptör antagonisti verilmiş (S+TQ+Anta A_{2B}) ginedonumuzu

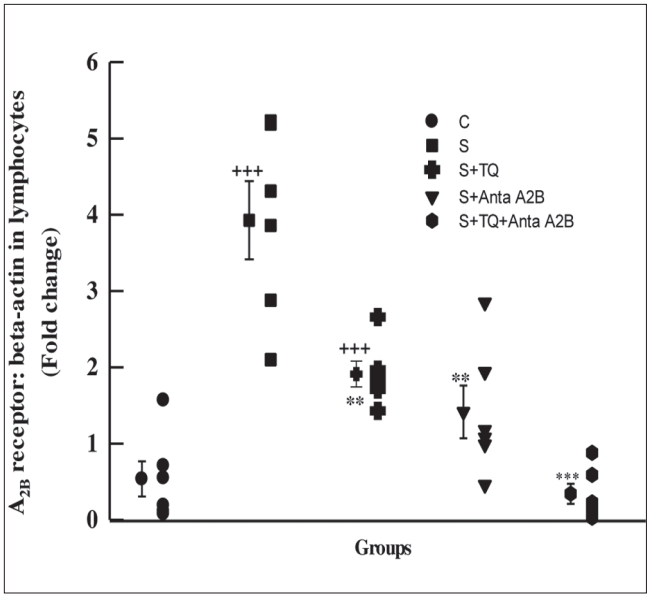

Fig 6. Individual values and mean±SEM (big symbols with bars) of the gene expression of A_{2B} adenosine receptor in blood lymphocytes of control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}) and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups (for each group, n=6). Statistical differences between control and different groups: ++ P<0.01, +++ P<0.001, Statistical differences between pretreated groups vs sensitized group: * P<0.05, ** P<0.01, *** P<0.001

Şekil 6. Kan lenfositlerinde A_{2B} adenozin reseptör gen ekspresyonun bireysel değerleri ve ortalama ± SEM (Barlı büyük semboller). Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), timokinon ile birlikte A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) ginedonumuzu (Her grupta n=6). Kontrol ve diğer gruplar arasındaki istatistiksel farklar: + P<0.05, ++ P<0.01, +++ P<0.001. Madde uygulanmış ve uyarılmış gruplar arasındaki istatistiksel farklar: * P<0.05, ** P<0.01, *** P<0.001

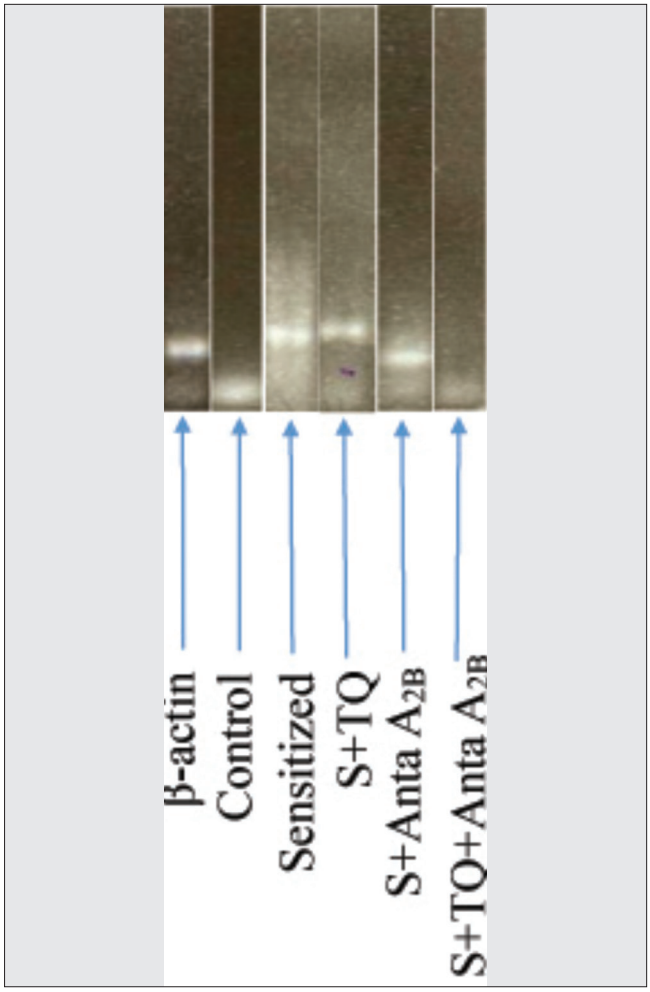

Fig 7. A_{2B} adenosine receptor gene expression analysis using RT-PCR in lung tissue in the control (C), sensitized (S), S pretreated with thymoquinone (S+TQ), S pretreated with A_{2B} adenosine receptor antagonist (S+Anta A_{2B}) and S pretreated with thymoquinone and A_{2B} adenosine receptor antagonist (S+TQ+Anta A_{2B}) guinea pigs

Şekil 7. RT-PCR ile akciğer dokusunda A_{2B} adenozin reseptör gen ekspresyonu analizi; Kontrol (C), uyarılmış (S), timokinon verilmiş S (S+TQ), A_{2B} adenozin reseptör antagonisti verilmiş S (S+Anta A_{2B}) ve timokinon ile birlikte A_{2B} adenozin reseptör antagonisti verilmiş (S+TQ+Anta A_{2B}) ginedonumuzu

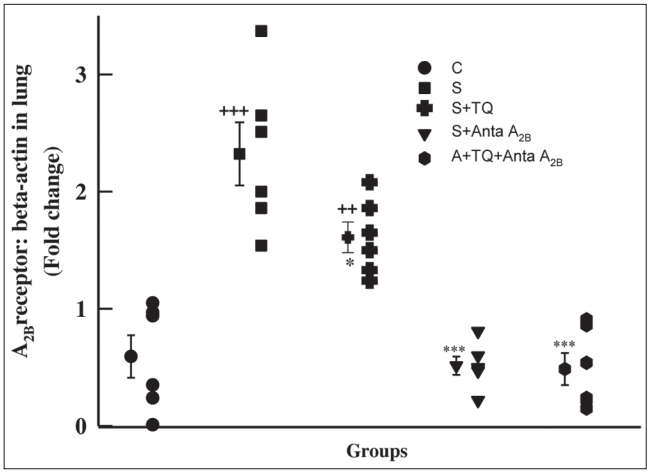

Fig 8. Individual values and mean±SEM (big symbols with bars) of the gene expression of A_{2B} adenosine receptor in lung tissue of control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}) and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups (for each group, n=7). Statistical differences between control and different groups: ++ P<0.01, +++ P<0.001, Statistical differences between pretreated groups vs sensitized group: * P<0.05, *** P<0.001

Şekil 8. Akciğer dokusunda A_{2B} adenozin reseptör gen ekspresyonun bireysel değerleri ve ortalama ± SEM (Barlı büyük semboller). Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), timokinon ile birlikte A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}+TQ), timokinon ile birlikte A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) ginedonumuzu (Her grupta n=6). Kontrol ve diğer gruplar arasındaki istatistiksel farklar: + P<0.05, ++ P<0.01. Madde uygulanmış ve uyarılmış gruplar arasındaki istatistiksel farklar: * P<0.05, ** P<0.01

Blood IL-4 and IFN-γ Levels: The mean value of blood IL-4 level in S, S+Anta A_{2A}, S+Anta A_{2B} and S+TQ+Anta A_{2A} groups was significantly higher than that in the C group (P<0.05). In S+TQ+Anta A_{2B} group, this cytokine is significantly lower than S group (P<0.01). The blood IL-4 level in S+TQ group showed non-significant decrease compared to that of the S group. However, the mean value of the IL-4 in this group was not significantly higher than that in the C group. There were not significant differences between pretreated groups *(Fig. 10a)*.

The mean value of the blood IFN-γ level of S, S+TQ, S+Anta A_{2B} and S+TQ+Anta A_{2B} groups was significantly higher than that of the C group (P<0.01 to P<0.05). There was significant increase in blood IFN-γ of S+TQ and S+TQ+Anta A_{2B} groups compared to that in the S group (P<0.05); however the mean value of IFN-γ in S+Anta A_{2A} and S+TQ+Anta A_{2A} groups was significantly lower than that in the S group (P<0.01 to P<0.05). The mean value of IFN-γ in S+Anta A_{2A} and S+TQ+Anta A_{2A} groups was significantly lower than that in S+TQ group (P<0.001, *Fig. 10b*).

DISCUSSION

The present study attempted to assess the effect of thymoquinone, alone and in the presence of A_{2A} adenosine receptor antagonist, on the A_{2A} and A_{2B} adenosine receptor mRNA gene expression in blood lymphocytes and lung tissue of ovalbumin-sensitized guinea pigs by the use of Real Time PCR method. In addition, blood IL-4 and IFN-γ level and lung pathological changes were analyzed.

The Effect of Thymoquinone on A_{2A} Adenosine Receptor: Data from these experiments in lymphocytes and lung tissue showed that thymoquinone could increase A_{2A} adenosine receptor mRNA expression significantly; however, administration of selective antagonist of A_{2A} adenosine receptor decreased mRNA expression of A_{2A} receptor. The concurrent administration of these two exogenous factors has a slight effect on the expression of A_{2A} adenosine receptors.

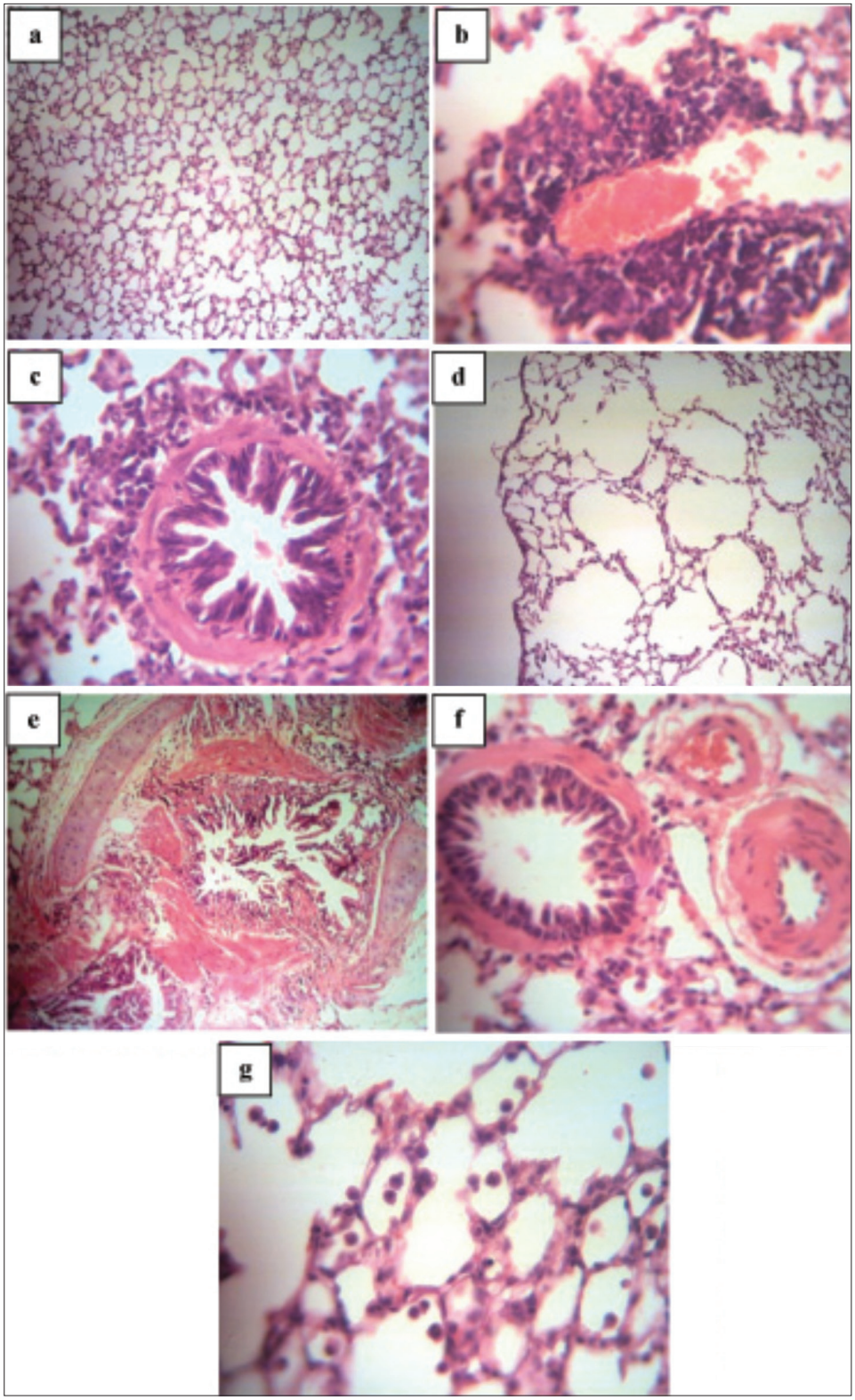

Fig 9. Photographs of lung specimen in guinea pigs: **a-** control normal lung tissues (C, 10x10), **b-** sensitized group (S, 10x40) with perivascular inflammatory infiltration, **c-** sensitized group pretreated with thymoquinone (S+TQ, 10x40) with airway smooth muscle hyperplasia and mild inflammatory infiltration, **d-** sensitized group pretreated with selective A_{2A} antagonist (S+Anta A_{2A},10x10) with emphysema and atelectasis, **e-** sensitized group pretreated with selective A_{2B} antagonist (S+Anta A_{2B}, 10x10) with respiratory epithelial denudation, **f-** sensitized group pretreated with selective A_{2A} antagonist and thymoquinone (S+TQ+Anta A_{2A}, 10x40) with vascular and airway smooth muscle hyperplasia and hypertrophy and **g-** sensitized group pretreated with selective A_{2B} antagonist and thymoquinone (S+TQ+Anta A_{2B}, 10x100) with inflammatory infiltration

Şekil 9. Gine domuzlarında akciğer örneklerinin fotoğrafları. **a-** Kontrol normal akciğer dokusu (C, 10x10), **b-**uyarılmış grup (S, 10x40), perivasküler yangısal infiltrasyon, **c-** timokinon verilmiş ve uyarılmış grup (S+TQ, 10x40), havayollarında düz kas hiperplazisi ve orta şiddette yangısal infiltrasyon, **d-** A_{2A} antagonisti uygulanmış ve uyarılmış grup (S+Anta A_{2A},10x10), amfizem ve atelektazi, **e-** A_{2B} antagonisti uygulanmış ve uyarılmış grup (S+Anta A_{2B},10x10), respiratorik epitel dökülmesi, **f-** A_{2A} antagonisti ile birlikte timokinon uygulanmış ve uyarılmış grup (S+TQ+Anta A_{2A}, 10x40), vasküler ve havayolu düz kas hiperplazi ve hipertrofisi ve **g-** A_{2B} antagonisti ile birlikte timokinon uygulanmış ve uyarılmış grup (S+TQ+Anta A_{2B}, 10x100), yangısal infiltrasyon

A_{2A} adenosine receptors are known as the anti-inflammatory mediators that are highly expressed on lymphocytes [26]. Extracellular adenosine increases during asthma and inflammation and this elevated plasma adenosine level could activate A_{2A} receptors.

As extracellular adenosine has anti-inflammatory effects by means of A_{2A} receptors, the activation of these receptors can change the level of blood cytokines [27]. The expression of A_{2A} receptors on lymphocytes correlates with adenosine levels in plasma and occupation of these receptors and does not have any relationship with the response of A_{2A} receptors [28]. In addition, the expression of A_{2A} receptors is slightly sensitive to changes in concentration of exogenous

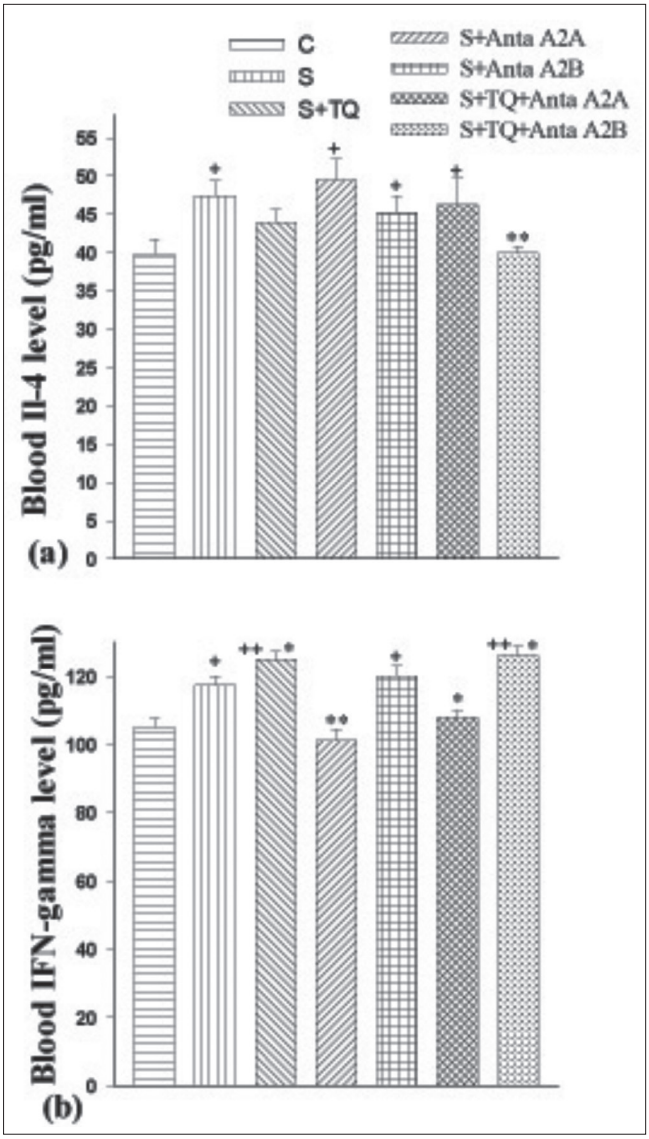

Fig 10. The blood IL-4 (a) and IFN-γ (b) levels (pg/ml) of control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2A} antagonist (S+Anta A_{2A}), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}), sensitized pretreated with selective A_{2A} antagonist and thymoquinone (S+Anta A_{2A}+TQ)and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups (for each group, n=6). Statistical differences between control and different groups: + P<0.05, ++ P<0.01, Statistical differences between pretreated groups vs sensitized group: * P<0.05, ** P<0.01

Şekil 10. Kan IL-4 (a) ve IFN-γ (b) seviyeleri (pg/ml). Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), timokinon ile birlikte A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}+TQ), timokinon ile birlikte A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) ginedonumuzu (Her grupta n=6). Kontrol ve diğer gruplar arasındaki istatistiksel farklar: + P<0.05, ++ P<0.01. Madde uygulanmış ve uyarılmış gruplar arasındaki istatistiksel farklar: * P<0.05, ** P<0.01

and endogenous factors involved in inflammation [29]. On the other hand, thymoquinone as an exogenous factor could change blood cytokine secretion (IL-4 & INF-γ) in the process of asthma; these changes were in accordance with previous studies [5,21]. So thymoquinone probably influences the secretion of cytokines of different types of lymphocytes (Th_2/Th_1) and changes the activity of A_{2A} receptors on the surface of lymphocytes and extracellular adenosine levels. Subsequently, these changes affect intracellular cAMP level and A_{2A} receptors mRNA expression.

A_{2A} adenosine receptors have intermediate expression in lung tissue [29] and are most relevant to lung disease [30]. Administration of thymoquinone caused enhanced A_{2A} receptor gene expression in lung tissue similar to the results of blood lymphocytes. A study in 2006 demonstrated that thymoquinone could affect IFN-γ level in bronchoalveolar lavage fluid [31]. So it is possible that thymoquinone might have affected lung cytokines secretion and induced intracellular mechanism of A_{2A} adenosine receptor mRNA expression in lung tissue; however, this effect was weaker than blood lymphocytes. This elevated A_{2A} receptor gene expression in lung tissue leads to wound healing and improving tissue destruction in group S + TQ during sensitization. Brown and his colleagues in 2008 showed that stimulation of wound healing in bronchial epithelial cells is the distinguished function of A_{2A} adenosine receptor expression [2].

The level of IL-4 increased significantly in S+Anta A_{2A} group although the level of IFN-γ decreased significantly in this group compared to the sensitized group. Administration of selective A_{2A} adenosine receptor antagonist, ZM241385, caused the elimination of A_{2A} adenosine receptor or the reduction of their numbers by decreasing cAMP level [32] and influencing secreted cytokines [33]. The previous study indicated that administration of this antagonist caused an increase in serum level of IL-4 and a decrease in IFN-γ [34].

As expression of pro-inflammatory cytokines can stimulate the gene expression of A_{2A} adenosine receptor [35], it has been suggested that A_{2A} adenosine receptor antagonist and increased serum IL- 4 probably cause improvement of A_{2A} receptors expression in lymphocytes. Although the administration of this antagonist suppresses the anti-inflammatory effect of A_{2A} receptors on the cell surface, but results of A_{2A} receptor mRNA expression were controversial. Nowadays it is accepted that the level of mRNA expression does not correlate exactly with proteins expressed on the cell surface because of some epigenetic factors [36].

The results of the present study indicate that all pathological criteria significantly increased in S+Anta A_{2A} group compared to controls. Spicuzza in 2006 demonstrated that ZM241385, selective A_{2A} adenosine receptor antagonist, could affect respiratory smooth muscle and blocking of these receptors exacerbated asthmatic symptoms [29].

The results of this investigation showed a significant reduction in gene expression of A_{2A} adenosine receptor in S+TQ+Anta A_{2A} group compared to the S + TQ and S

+ Anta A_{2A} groups. It is suggested that thymoquinone prevented the secretion of blood cytokines during the sensitization process [5,21] and the elevated level of IL-4 did not occur to stimulate A_{2A} adenosine receptor expression during administration of selective A_{2A} adenosine receptor antagonist.

There were not significant differences between A_{2A} adenosine receptor expression in lung tissue of group S+TQ+Anta A_{2A} and S+TQ group. According to the results of the present study, it is concluded that thymoquinone had a significant effect on A_{2A} receptor expression in respiratory epithelial cells since respiratory epithelial cells express A_{2A} adenosine receptors, although the A_{2A} receptors expression and its regulation under inflammatory conditions in epithelial cells have not been documented and the biological importance of increased expression on epithelial cells is unknown [35]. Our results suggest that the A_{2A} adenosine receptors probably play a role in the pathogenesis of inflammatory diseases. A previous study of ours showed that concurrent administration of thymoquinone and selective adenosine A_{2A} receptor antagonist improved tracheal smooth muscle contraction and WBC count in bronchoalveolar lavage fluid in comparison with the S+Anta A_{2A} group [37]. Polosa's study also showed that ZM241385, selective A_{2A} adenosine antagonist, affected bronchial smooth muscle [30].

The Effect of Thymoquinone on A_{2B} Adenosine Receptor: The second part of this study evaluated the effect of thymoquinone on blood cytokines, lung pathology and the gene expression of A_{2B} adenosine receptors, in the presence of MRS1706; selective A_{2B}receptor antagonist, in asthmatic guinea pigs.

The gene expression of A_{2B} receptors in sensitized animals (S group) and pretreated group with thymoquinone (S+TQ group) significantly increased compared to controls. This increase of expression in S group and S+TQ group was 86% and 72% respectively. In these groups, the blood IL-4 and IFN-γ levels and pathological changes were significantly raised in comparison to the control group. These findings were in accordance with results of our previous studies [19,34,38]. The increment in A_{2B} receptors gene expression, cytokines and pathological changes in S+TQ group compared with C group was due to inflammation process but there was statistically significant reduction in comparison with the S group. This supports the notion that thymoquinone can improve inflammation and relieve its symptoms.

This study showed that single dose administration of MRS1706, selective A_{2B} adenosine receptor antagonist, could improve lung pathological changes; however, it could not decrease to the control level. These results are in line with Mustafa's study [16]. In addition, the percentage of increase in A_{2B} receptor gene expression in S+Anta A_{2B} group significantly plummeted compared to the S group. There was not a statistically significant difference between A_{2B} receptors gene expression and pathological changes in this group and the control group. This indicated that administration of A_{2B} receptor antagonist could prevent asthma induced inflammatory changes.

It is demonstrated that the anti-inflammatory and pro-inflammatory effects of adenosine depend on their level in lung tissue. Lung inflammation causes a hypoxic environment in which adenosine is produced. At first, the low level of adenosine stimulates high-affinity receptors such as A_{2A} adenosine receptors which shows the protective effect of adenosine. However, in severe lung inflammatory conditions, the high level of produced adenosine affects low-affinity receptors such as A_{2B} receptors and exacerbates the inflammation [39].

The gene expression of A_{2B} receptor, pathological changes and blood IL-4 level in the sensitized group pretreated by thymoquinone and Anta A_{2B} (S+TQ+Anta A_{2B} group) were not statistically different from the control group. Also, the blood level of IFN-γ in S+TQ+Anta A_{2B} group rose significantly compared to the control and sensitized groups. Moreover, in the S+TQ+Anta A_{2B} group, the administration of thymoquinone and selective antagonist together caused more improvement than S+TQ and S+AntaA_{2B} groups. These results are in accordance with results of our current study [37] which is related to the effect of administration of thymoquinone and A_{2B} receptor antagonist together to relieve inflammation.

Cytokines can regulate the number of adenosine receptors in inflammatory environment [40] and our previous study demonstrated that IFN-γ level in sensitized group pretreated with A_{2B} receptor antagonist group has increased significantly compared to the S group and this change could influence the expression of adenosine receptors or T-cells [34]. These observations, therefore, support the hypothesis that blocking of A_{2B} receptor with selective antagonist probably causes changes in levels of secreted cytokines related to inflammation. These findings are in accordance with the results of Anvari's study which showed that in A_{2B} receptor-knockout mice, the expression and secretion of T-cell decreases significantly [41].

The results of gene expression of A_{2B} adenosine receptors in lung tissue are already similar to the findings in blood lymphocytes. The comparison of percentage of gene expression of A_{2B} receptor in blood lymphocytes and lung tissue in the analyzed groups showed that thymoquinone administration has more influence on blood lymphocytes while administration of the selective A_{2B} receptor antagonist was more effective in lung tissue. It was probably due to high expression of A_{2B} receptor in pulmonary resident cells; epithelial, bronchial smooth muscle and endothelial cells, because the pre-inflammatory effect of A_{2B} receptor stimulation was demonstrated in these cells [41]. On the other hand, this receptor could increase the expression of

pre-inflammatory mediators in different cells of chronic pulmonary diseases and over-expression of Th_2 cytokine in lung was associated with increasing levels of A_{2B} receptor [42]. While local cytokines played an important role in regulating pre/anti-inflammatory activities of adenosine, and while these cytokines could also regulate the levels of adenosine metabolic enzymes and adenosine receptors in inflammatory environment [40], MRS1706, selective A_{2B} receptor antagonist, probably inhibits their influence on the expression of pre-inflammatory mediators by blocking receptors. However, it is suggested that the effects of agonists of adenosine receptors are evaluated in future researches to determine the exact mechanism(s) of thymoquinone.

In conclusion, the results showed that thymoquinone could affect A_{2A} and A_{2B} adenosine receptors gene expression and some of the therapeutic effects of thymoquinone in reducing asthma symptoms might be partially mediated through A_2 adenosine receptors.

Acknowledgement

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Conflict of Interest: None declared.

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Research Article

The Effects of Erythropoietin on the Penicillin Induced Epileptiform Activity in Rats [1]

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Abstract

Erythropoietin (Epo), a cytokine hormone produced in the kidney, promotes the formation of red blood cells in the bone marrow. The penicillin-induced epilepsy model is a commonly used experimental model for epilepsy research. The present study was conducted to elucidate the effect of Epo on penicillin-G (500 IU/2.5 µl dose, intracortically (i.c)) -induced epileptiform activity in anesthetized adult Wistar-Albino rats (n=39). The animals were randomly divided into four groups as three treatment groups (groups 1-3) and a control group (no drug application). Rats in groups 1, 2 and 3 were intraperitoneally administered 2.000, 4.000 and 6.000 IU Epo/kg, respectively. The effects on penicillin G induced epilepsy were compared across groups using electrocorticography. Epo at 2.000 IU/kg did not cause a significant change ($P>0.05$) in epileptiform spike-wave activity (number/min) and/or amplitude (µV) values, whereas the average number of spike-waves per minute and seizure severity decreased significantly in the 4.000 and 6.000 IU/kg Epo groups compared with the control ($P<0.05$). Consequently, the results of the present study show that administration of Epo has a dose-dependent antiepileptic effect in penicillin induced model of epilepsy in rats.

Keywords: *Erythropoietin, Electrocorticography, Epilepsy, Penicillin, Rat*

Sıçanlarda Penisilin ile Oluşturulan Epileptiform Aktivitesi Üzerine Eritropoietinin Etkileri

Özet

Eritropoietin (Epo), böbreklerde sentezlenen ve kemik iliğinde eritrosit üretimini sağlayan bir sitokin hormonudur. Deneysel epilepsi araştırmalarında, genel olarak penisilin ile oluşturulan epilepsi modeli kullanılmaktadır. Çalışmamızda, anestezi altındaki yetişkin Wistar-Albino türü sıçanlarda (n=39), Penisilin-G (intrakortikal (i.c) olarak, 500 I.U/2.5 µl dozda) ile oluşturulmuş epileptik aktivite üzerine Epo'nun etkileri araştırıldı. Sıçanlar, üç tedavi grubu (grup 1-3) ve bir kontrol grubu (ilaç uygulanmadı) olarak rastgele dört farklı gruba ayrıldı. Grup 1, 2 ve 3'de bulunan sıçanlara, intraperitonal olarak sırayla 2.000, 4.000 and 6.000 IU Epo/kg'lık dozlarda Epo uygulandı. Gruplar arasında, penisilin G ile oluşturulan epilepsi üzerine Epo'nun etkisi elektrokortikografi kullanılarak karşılaştırıldı. Kontrol grubu ile Epo grubu karşılaştırıldığında, 4.000 ve 6.000 IU/kg Epo uygulaması, dakika başına diken dalgaların ve dikenlerin ortalama sayısı ve nöbet şiddetini anlamlı ($P<0.05$) derecede azaltır iken, 2.000 IU/kg Epo uygulamasında epileptiform diken dalga akitivitesi (sayı/dk) ve/veya genlik (µV) değerlerinde anlamlı bir değişime neden olmadı ($P>0.05$). Sonuç olarak yapılan çalışma, Epo'nun sıçanlarda penisilin ile oluşturulmuş deneysel epilepsi modeli üzerine uygulanmasının, doz bağımlı antiepileptik etkiye neden olduğu ortaya çıkarmıştır.

Anahtar sözcükler: *Eritropoietin, Elektrokortikografi, Epilepsi, Penisilin, Sıçan*

INTRODUCTION

Epilepsy is a clinical condition characterized by spontaneous recurrent seizures of cerebral origin [1]. As a common chronic neurological disorder, it affects 1-3% of the population, and approximately 10% of the general population has one or more seizures during their lifetime [2]. Experimental investigations of epilepsy in animal models have contributed important information regarding epilepsy pathogenesis [3,4]. Experimental epilepsy is induced by

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penicillin, topically or intracortically (i.c.) administered at the surface of the cortex. The penicillin-induced epilepsy model has been used in numerous studies. Penicillin causes acute focal epileptic activity similar to that which decreases the activity of the GABA inhibitory system in the brain and increases glutamate, which becomes the main excitatory neurotransmitter in the brain [5-9]. Researchers [6-9] continue to study the antiepileptic effects of agents in animal models of experimental epilepsy, but the therapeutic effectiveness of these agents may not be the same in humans [10,11].

Erythropoietin (Epo), a hematopoietic glycoprotein cytokine hormone produced in the kidney, promotes red blood cell formation in bone marrow and is expressed in other tissues, including the nervous system. Epo mediates a number of biological actions in the central nervous system (CNS), where it is also neuroprotective [12-15]. Epo can cross the blood–brain barrier (BBB) via a receptor-mediated mechanism [16]. Uzum et al.[17] have shown that Epo pretreatment confines BBB leakage to the cerebellum and cortical areas and lessens the intensity of tonic-clonic seizures during pentylentetrazol-induced seizures.

In recent years, many studies have investigated the presence and protective effect of Epo and the erythropoietin receptor (EpoR) on neurons, demonstrating both epileptic and antiepileptic effects of Epo in different experimental animal models [17-23]. However, no study has shown the effects of Epo in a penicillin-induced experimental epilepsy model. Here, we report the effect of Epo at various doses on epilepsy after seizure

MATERIAL and METHODS

Experimental Procedures

A total number of thirty-nine adult male Wistar-Albino rats (200-250 g; 12-14 weeks) were used in this study. These rats were taken by Duzce University Medical and Surgical Research Center, Duzce-Turkey before experiment and they were housed in groups of 4-5 per cage (42x26x15 cm) in a room with controlled temeparature (21±2°C) and relative humudity (60±5%) with lights on from 8:00-20:00. This study was approved by the Duzce Animal Care and Usage University Ethics Committee (Approval Number: 2009-24). Animal handling during all experiments was consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23).

Rats were randomly assigned to the following groups: (1) 500 IU penicillin (2.5 µl, i.c.) control group (n=10); (2) 500 IU penicillin (2.5 µl, i.c.) + 2.000 IU/kg Epo (n=10); (3) 500 IU penicillin (2.5 µl, i.c.) + 4.000 IU/kg Epo (n=9); and (4) 500 IU penicillin (2.5 µl, i.c.) + 6.000 IU/kg Epo (n=10) groups. All rats were anesthetized with 1.25 g/kg intraperitoneal urethane (Sigma Aldrich Co., St. Louis, MO, USA) and placed in a stereotaxic frame (Harvard Apparatus, Holliston, MA, USA). The left cerebral cortex was exposed by craniotomy. Two Ag-AgCl ball electrodes were placed over the left somatomotor cortex (first electrode: 2 mm lateral to sagittal suture, 1 mm anterior to bregma; second electrode: 2 mm lateral to sagittal suture, 5 mm posterior to bregma). The common reference electrode was fixed on the right pinna. Electrocorticography (ECoG) recordings were continuously monitored. The signals from the electrodes were amplified and filtered (0.1-50 Hz bandpass) using bio-amplifiers (BioAmp; AD Instruments, Bella Vista NSW, Australia). Then the ECoG signal was digitized at a sampling rate of 1024 using a four-channel data acquisition system (PowerLab 8/SP; AD Instruments). Baseline activity was recorded for 10 min in each group.

An epileptic focus was produced by intracortical injection of penicillin G (500 IU/2.5 µl) in all animals. Using a Hamilton microsyringe (type 701 N; Hamilton Co., Reno, NV, USA), penicillin was injected into the left sensorimotor cortex (2 mm posterior to bregma, 3 mm lateral to the sagittal suture, and 1 mm beneath the brain surface) at an infusion rate of 0.5 µl/min. Epileptiform activity was observed by ECoG for 5-6 min. Activity reached a constant level within 30 min following the administration of penicillin G and lasted for 3-5 h. After about 30 min when the spike-waves become stable, the rats were given intraperitoneally Epo at a dose of 2.000, 4.000, or 6.000 IU/kg. All recordings were displayed and stored using a computer. Spike frequencies and amplitudes for each animal were automatically calculated and measured using the data-acquisition Chart v.5.1.1 system (PowerLab software; AD Instruments). The frequency and amplitude of epileptic activity were analyzed offline.

Statistics

The frequency and amplitude values acquired from animals in all groups were converted to a scaling percentage in a time-dependent manner. The percentage changes were used for statistical analyses and graphics. All statistical procedures were performed using SPSS statistical software package version 12.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as means±SD. The data were analyzed by one-way analysis of variance followed by Tukey's post hoc test to correct for multiple comparisons of treatments. Statistical significance was accepted at $P<0.05$.

RESULTS

The penicillin-induced epileptiform discharges were characterized by bilateral spikes and spike-wave complexes on a background of ECoG activity *(Fig. 1)*. Data comprising mean spike frequency and mean spike amplitude, and latencies to onset of epileptiform activity in all experimental groups during 120 min record following penicillin injection. Epo was administered 30 min after

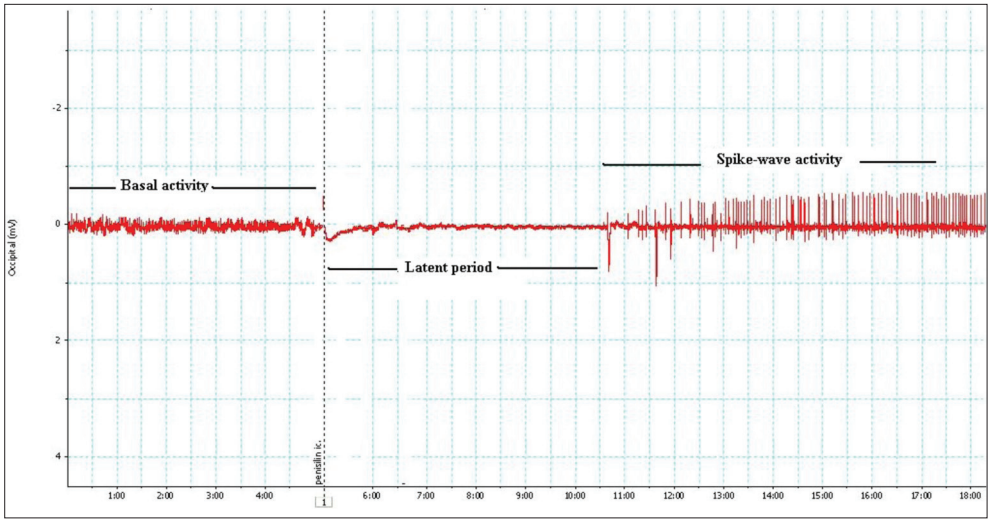

Fig 1. Changes in ECoG activity after administration of penicillin G
Şekil 1. Penisilin G verilmesinden sonra ECoG aktivitesinde değişiklikler

Table 1. *Number of spike or spike-wave discharges per minute (number/minute) at each dose during each period (mean±SD and P values)*
Tablo 1. *Farklı dozlarda uygulanan EPO nun, her bir periyot aralığındaki dakikadaki diken dalga deşarjı sayıları (sayı/dk) (ortalama±SD ve P değerleri)*

Treatment Period (min)	Erythropoietin Treatment (dose)[1]				P Value[2]
	Control group (n:10)	2.000 IU/kg (n:10)	4.000 IU/kg (n:9)	6.000 IU/kg (n:10)	
Baseline	31.78±10.3	27.70±11.0	34.64±13.2	34.80±11.2	-
1-10	32.09±13.2	27.34±12.0	29.92±9.2	32.61±17.5	0.229
11-20	33.58±12.9	24.38±13.5	26.11±8.0[a]	29.33±15.1	0.018[a]
21-30	30.44±11.1	22.35±12.3	23.35±6.4[a]	24.64±15.1[b]	0.034[a,b]
31-40	30.44±12.0	20.81±11.5	22.16±5.5[a]	23.25±13.2[b]	0.027[a,b]
41-50	29.45±12.6	16.88±13.5	18.95±5.9[a]	19.73±14.7[b]	0.034[a,b]
51-60	27.72±13.0	16.01±13.6	15.11±9.0[a]	15.94±15.3[b]	0.037[a,b]
61-70	26.47±13.5	14.93±13.4	14.27±9.1	17.31±16.9	0.099
71-80	24.71±13.2	13.31±12.5	13.70±9.3	18.29±20.0	0.224
81-90	22.71±11.4	12.07±11.4	13.07±9.3	17.97±22.0	0.310
91-100	20.42±8.8	11.99±12.6	12.23±9.6	13.08±17.1	0.196
101-110	19.25±7.4	12.21±13.3	12.09±10.0	11.80±14.2	0.191
111-120	16.87±5.2	10.86±12.7	11.76±10.1	8.18±11.2	0.133

[1] Values are the mean±SD for rats in each group, [2] Statistical significance; P<0.05; [a,b] P<0.05: Compared with control

the penicillin injection. The mean spike-wave frequencies of each group are shown in *Table 1*. The mean number of spike-waves per minute wave frequencies in 4.000 IU and 6.000 IU Epo doses between 21-30, 41-50, 51-60 minutes time period were significantly (P<0.05) decreased than control groups. Also, the spike wave frequencies in 4.000 IU Epo dose between 11-20 min time period were significantly (P<0.05) decreased than control groups. However, at 2.000 IU, Epo produced no significant change in the spike-wave frequency of epileptiform activity *(Table 1)*. Additionally, there were no significant differences between all groups of Epo in terms of spike wave amplitude (μV) of penicillin-induced epileptiform activity (P>0.05) *(Table 2)*.

DISCUSSION

In the present study, we investigated the antiepileptic effects of Epo in penicillin induced epilepsy in rats. This is the first study to demonstrate that Epo has an antiepileptic effect in the penicillin G-induced experimental epilepsy model. Epo at doses of 4.000-6.000 IU/kg inhibited the rate of spikes and spike-waves.

Many researchers [18,19,23] have investigated the effects of Epo in different experimental models of epilepsy. In a kainic acid (KA)-induced seizure model in rats, Kondo et al.[18] used intraventricular infusion of anti-Epo antibody

Table 2. *Spike-wave amplitudes (μV) at each dose during each period (mean±SD and P values)*
Tablo 2. *Farklı dozlarda uygulanan EPO nun, her bir periyot aralığındaki diken dalga amplitütleri (μV) (ortalama±SD ve P değerleri)*

Treatment Period (min)	Erythropoietin Treatment (dose)[1]				P Value[2]
	Control Group (n:10)	2.000 IU/kg (n:10)	4.000 IU/kg (n:9)	6.000 IU/kg (n:10)	
Baseline	0.66±0.2	0.96±0.4	0.85±0.2	0.85±0.4	-
1-10	0.71±0.3	0.99±0.4	0.89±0.3	0.86±0.4	0.979
11-20	0.71±0.3	0.88±0.3	0.87±0.3	0.82±0.3	0.713
21-30	0.75±0.3	0.84±0.2	0.88±0.4	0.78±0.4	0.598
31-40	0.75±0.3	0.77±0.3	0.85±0.4	0.78±0.4	0.533
41-50	0.74±0.3	0.65±0.4	0.80±0.4	0.69±0.2	0.319
51-60	0.76±0.3	0.60±0.4	0.61±0.6	0.49±0.1	0.266
61-70	0.76±0.2	0.60±0.5	0.55±0.6	0.46±0.2	0.226
71-80	0.72±0.2	0.58±0.4	0.50±0.5	0.39±0.2	0.190
81-90	0.67±0,2	0.56±0.4	0.45±0.4	0.30±0.2	0.182
91-100	0.65±0.2	0.50±0.5	0.43±0.4	0.26±0.2	0.168
101-110	0.62±0.2	0.49±0.5	0.41±0.4	0,32±0,2	0.231
111-120	0.69±0.2	0.48±0.5	0.41±0.4	0,28±0,2	0.110

[1]Values are the mean± SD for rats in each group, [2] Statistical significance; P<0.05

to reveal the antiepileptic effect of endogenous Epo and intraventricular infusion of anti-neuropeptide Y antagonist to eliminate the neuroprotective effect of exogenous Epo. Chu et al.[19] studied the effects of Epo (5.000 IU/kg) in a lithium-pilocarpine-induced status epilepticus (SE) model and reported that Epo administration during the latent period following SE prevented BBB leakage, neuronal death, and microglia activation in the dentate hilus, CA1, and CA3; inhibited the generation of ectopic granule cells in the hilus and new glia in CA1; and reduced the risk for developing spontaneous recurrent seizures. Another study [23] also demonstrated that Epo administration reduced seizure activity in the lithium-pilocarpine-induced SE model. Sozmen et al.[22] showed that Epo significantly decreased neuronal cell death in CA1, CA2, CA3, and the dentate gyrus of the hippocampus.

In literature, it has been shown that Epo/erythropoietin receptors (EpoR) has anti-toxic, anti-oxidant, anti-inflammatory and anti-apoptatic effects in different tissues [24,25] *in vivo* and *in vitro* studies. The Epo/ EpoR plays an important role in neurodevelopment and neuroprotection. Also, it is thought that Epo increases the choline acetyltransferase enzyme activity and reduce the epileptic activity with cholinergic effects in neurons [26]. Also, Epo has neurotrophic properties for neuronal stem cell mobilization in damaged regions [27]. In the epilepsy process, cellular events underlying the neuroprotective effects of Epo are dependent on an increase in the total number of (EpoR) and anti-apoptatic (Bcl-2, Bcl-w) molecules, and the total number of pro-apoptatic (Bim, Bid) molecules in hippocampal neurons [28]. Furthermore, Sargin et al.[29] have determined that early intervention with Epo prevents microgliosis caused by neurodegenerative changes. Won et al.[30] demonstrated that Epo protects spinal GABAergic neurons against KA-excitotoxic damage in rat spinal cord cell cultures. They [30] found that post-treatment with Epo for 48 h after KA-induced injury remarkably enhanced the expression of EpoR and glutamate decarboxylase 67, which is an isoform of a GABA-producing enzyme diminished by KA. They suggested that the neuroprotective effect of post-treatment Epo on the GABAergic neurons is mediated by signal transduction involving the EpoR-dependent Janus kinase 2 pathway. We showed an anticonvulsant effect of Epo in a penicillin-induced epilepsy model by antagonizing suppressed GABA inhibition. In addition, Morishita et al.[31] reported that Epo protected cultured neurons from glutamate neurotoxicity mediated by N-methyl-D-aspartate receptors, in a dose and time dependent manner; glutamate-dependent neuronal cell death was reduced by low Epo doses administered 24 h before glutamate exposure, whereas high Epo doses were not effective. In a more recent study, pretreatment with Epo 24 h before the experiment antagonized glutamate-mediated astrocyte water permeability in mice, thereby reducing neurological symptoms [32].

Attempts have been made to explain the mechanism of action of Epo in experimental models of epilepsy. Our observations provide direct evidence that Epo has dose-dependent antiepileptic effects in a penicillin G induced epilepsy model. We revealed that Epo at doses of 4.000-6.000 IU/kg was effective in reducing the frequency, without changing the amplitude, in this model of epilepsy. However, Epo may produce different results in different experimental epilepsy models, in different brain areas, with

different routes of administration, or at different treatment doses. Our findings represent a first attempt to study the antiepileptic role of Epo in penicillin-induced epilepsy in rats. Epo clearly decreased the frequency of penicillin-induced epileptiform activity in a dose-dependent manner, without changing the amplitude of epileptiform activity.

Further studies are needed to clarify the exact mechanisms of Epo at the cellular and molecular levels in various experimental animal models. Epo may be the most promising agent identified thus far for neuroprotection and neuroregeneration in many neurological and psychiatric conditions.

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Conflict of Interest

The authors declare that there are no conflicts of interest. All authors approved the final manuscript.

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Research Article

The Isolation of *Dichelobacter nodosus* and Identification by PCR from Ovine Footrot in Kars District, Turkey [1]

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Abstract

In this study it was aimed to isolation and identification by PCR of specific agent from ovine footrot in Kars district and thus determination of prevalence of disease. To this end, 8,970 sheep belong to 10 different flocks were examined clinically, and in 1532 of these (17.07%) were found lameness for various reasons. Out of 247 (2.75%) of these cases were evaluated to be footrot suspect clinically. Bacteria were isolated in 205 (82.99%) of the 247 samples that were cultured in an anaerobic environment due to the suspicion of footrot. When Gram stains and microscopic investigation was carried out on these isolates, 195 of them (95.12%) were found to be Gram negative rod-type bacteria. These isolates were subjected by polymerase chain reaction (PCR) using *Dichelobacter nodosus* specific primer and amplicons (440bp) of expected weight in 153 (78.46%) of isolates were found. Considering of these findings, it was concluded that prevalence of disease is high in sheep in Kars district.

Keywords: *Sheep, Footrot, Dichelobacter nodosus, Isolation, PCR*

Kars Yöresi Koyunlarında Piyeten Olgularından *Dichelobacter nodosus* İzolasyonu ve PCR İle İdentifikasyonu

Özet

Bu araştırmada, Kars yöresinde footrotlu koyunlardan hastalığın spesifik etkenin izolasyonu, PCR ile identifikasyonu ve böylece hastalığın prevalansının saptanması amaçlanmıştır. Bu amaçla 10 farklı sürüye ait 8970 koyun klinik olarak incelenmiş 1532'sinde (%17.07) çeşitli nedenlere bağlı topallık görülmüştür. Bunlardan 247'si (%2.75) klinik açıdan footrot şüpheli olarak belirlendi. Footrot şüphesiyle anaerobik ortamda kültürel olarak değerlendirilen 247 örneğin 205 inde (%82.99) bakteriyel izolasyon gerçekleştirilmiş, izolatların yapılan Gram boyama ve mikroskobik incelenmeleri sonucu bunların 195'i (%95.12) Gram negative çomak morfolojisinde bakteriler olarak görülmüştür. Bu izolatlar *Dichelobacter nodosus*'a spesifik primer kullanılarak polymerase chain reaction (PCR) a tabi tutulmuş ve 153'ünde (%78.46) beklenen ağırlıkta (440 bp) amplikonlar saptanmıştır. Bu bulgular dikkate alındığında, Kars yöresindeki koyunlarda hastalığın prevalansının yüksek olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Koyun, Piyeten, Dichelobacter nodosus, İzolasyon, PCR*

INTRODUCTION

Footrot is a specific contagious disease of sheep and goats, although it has been reported in cattle, horses, pigs, deer and mouflon. It is an infectious syndrome caused by synergistic action, where *Dichelobacter nodosus* is the main transmitting agent. Ovine footrot is characterized by the separation of keratinous hoof from the underlying tissue resulting in severe lameness, degraded body condition and reduced wool production [1,2]. *D. nodosus* is a rod shaped, Gram-negative, obligate anaerobic bacterium that has proteases and keratinases that are able to dissolve sheep hooves [3,4]. The primary predisposing factors for disease include environmental conditions as well as the

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host's genetics, immunity, diet and stocking rates [5,6].

Although footrot is widespread in many areas of the world where sheep are raised, it is particularly prevalent in temperate, rainy regions such as the UK, Australia and New Zealand [7]. Reports have been made in many countries regarding the disease's aetiology, pathogenesis, epidemiology, treatment, control and eradication. Turkey ranks seventh in the world with regard to sheep population. There are approximately 31 million sheep in Turkey, and 1.49% of them are raised in the region of Kars [8]. Approximately 70% of the people in Kars province work in the area of farming and animal husbandry. More than 90% of the sheep raised in the region are from the Morkaraman and Akkaraman breeds. Outside of the winter months, the sheep spend a bit more than half the year (from April to November) grazing in pastures. The animal owners and shepherds have very little knowledge or interest in foot diseases, so they do not conduct immunization, foot baths or hoof care. Furthermore, the government does not carry out any program to inform farmers about the disease or control and eradicate it. The goal of this study was to establish the status of footrot in sheep in the region of Kars, isolate the agent and identify using PCR. The study did not take into consideration the breed, age and diet of the sheep or environmental factors such as rainfall, moisture and type of terrain.

MATERIALS and METHODS

Reference Bacterial Strain

The reference strain of *D. nodosus* (ATCC 25549) was obtained from the Leibniz-Institut DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Study Region, Period and Sampling

This study was conducted on sheep raised in Kars province, which is in the Northeast Anatolia region of Turkey. The study was conducted on 8.970 sheep from 10 herds (herd size ranged from 800 to 1.100) grazing at various locations in the region from April 2013 to October 2014 (the pasture grazing period).

Clinical Investigation and Sampling Method

Of the 8.970 sheep that were evaluated from 10 different herds, 1.532 of them were found to have lameness for various reasons. These sheep were analysed with the scoring system recommended by Egerton and Roberts [9] for footrot lesions. Those sheep with a lesion score of 2 (interdigital dermatitis) to 4 (severe interdigital dermatitis and under-running of the hard horn of the hoof) were suspected to have footrot, and samples were collected from these 247 sheep using sterile cotton swab. These samples were transferred to a Stuart Transport Medium (Oxoid CM0111) [10], rapidly transported to the laboratory with an unbroken cold chain, and then immediately evaluated.

Isolation

All of the samples were cultured by streaking them in Eugon agar (BD Bacto, Sparks, MD, USA) and trypticase-arginine-serine agar (TAS) [11]. To assist in the growth of *Dichelobacter* spp. colonies, 5% defibrinated sheep's blood was added to the mediums. After the plates were streaked, they were incubated at 37°C for 4-5 days in a 2.5 liter jar (Merck) using an Anaerogen kit (Oxoid) to ensure anaerobiosis. Afterward, if the Gram stain and microscopic characteristics of the colonies that grew were similar to *D. nodosus* [12], passage was performed onto Eugon agar.

DNA Extraction of Bacterial Cultures

Standard methods were used to extract DNA from the bacteria colonies. Using a sterile toothpick, selected colonies of *D. nodosus* cells were prepared in 1.5 ml microcentrifuge tubes in 100 µl of sterile phosphate buffered saline (PBS). The tubes were placed in a boiling water bath for 10 min, cooled on ice for 5 min and centrifuged at 13.000 x g for 10 min. One microliter of the supernatant was used for PCR.

Detecting the fimA Gene of D. nodosus Using PCR

The primers *(Table 1)* and PCR settings used to identify the *fimA* gene of *Dichelobacter nodosus* were chosen according to Cagatay and Hickford [13]. A standard strain obtained from DSMZ was used for a positive control,

Table 1. *Primers used to amplify the fimA gene region of Dichelobacter nodosus*
Tablo 1. *Dichelobacter nodosus'un fimA gen bölgesinin amplifikasyonunda kullanılan primerler*

Primer	Sequence (5'→3')	Serogroup Specificity
Forward		
U1	ATCCCTGCATACAACGACTACAT	A, B, C, E, F, G, I and M (class I)
U2	GC TATTC CACAATAC CAAAACTACAT	D and H (classII)
Reverse		
D1	AC TCAAGAGAGAGGC TTTTAAGTAAG	B, C, G, E and M
D2	AGAGAGGCTTTCACATTTAAGAGC	A, F, I, E and M
D3	GTAC CGAAGTA CAC C TTTGATTG	D and H

while sterile distilled water was used for a negative control.

Analysis of the PCR Products

The PCR products were electrophoresed in 1% agarose gels, stained with nucleic acid stain (Safeview, NBS Biologicals Ltd) and visualized under UV illumination (UVP LMS-20E, Upland, CA, USA) and then photographed.

RESULTS

Clinical Examination

An evaluation was performed on 8.970 sheep from 10 flocks, and 1.532 of the sheep (17.07%) were found to have lameness for various reasons. The cause of lameness in the majority of the cases was hoof horn deformities, and clinical footrot was found to be the cause in 247 of these cases (16.12%). When compared to all of the sheep that were clinically evaluated in the study, the prevalence of footrot was found to be 2.75%.

Isolation Results

Bacteria were isolated in 205 (82.99%) of the 247 samples that were subjected to a bacterial culture in an anaerobic condition because footrot was suspected. When Gram stains and microscopic investigation was carried out on these isolates, 195 of them (95.12%) were found to be Gram negative rod-type bacteria. These isolates were subjected to PCR because *D. nodosus* was suspected.

PCR Detection of the fimA Gene

Of the 195 potential isolates that were subjected to a PCR, a 440-bp amplicon that was of the expected size for the *D. nodosus fimA* gene was identified in 153 of them (78.46%) *(Fig. 1)*.

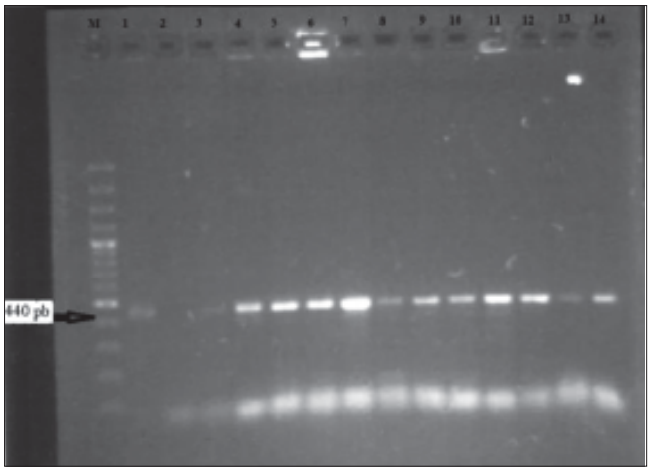

Fig 1. Specific PCR products of *Dichelobacter nodosus* from clinical samples of sheep with footrot. Lane M: 100 bp DNA marker, lane 1: positive control, lane 2: negative control, lane 3-14: *D. nodosus* positive samples

Şekil 1. Piyetenli koyunlara ait klinik örneklerden izole edilen *Dichelobacter nodosus* spesifik PCR ürünleri. M: 100 bp DNA marker, 1: pozitif kontrol, 2: negative kontrol, 3-14: *D. nodosus* pozitif örnekler

DISCUSSION

The disease of footrot in sheep is economically significant because it causes lameness, weight loss and wool production loss to various degrees in many countries around the world where sheep are raised. The prevalence of the disease has been reported at 12.54% [2] and 16.41% in Kashmir, India [14], 10% in the United Kingdom [15] and 3.1% in Bhutan [16].

Turkey is divided into seven different geographical regions based on climactic conditions and altitude. In spite of this variety of geography, there are very few studies that isolate and identify the agent for ovine footrot due to the difficulty of culturing many of the microbes that cause foot diseases in cloven hoof animals (anaerobiosis, fragility, mixed infection etc.) [17]. Previous studies have been largely focused on the healing effects of different diagnosis and treatment options using clinical radiological imaging. In a study that conducted a clinical and radiological investigation of 9.052 sheep in the Burdur region (an area in Turkey's Mediterranean region) during the sheep pen and pasture seasons in order to analyse the distribution and environmental factors of foot diseases in sheep, Avki et al.[18] found that 1.576 animals (16.30%) had foot disease, that 13.46% of these diseases were hoof deformities, and that 2.55% of them were ovine footrot. In another study that conducted a clinical and radiological evaluation of foot diseases observed in sheep raised in the regions of Kars and Iğdır [19]; 4,230 sheep were examined in the pasture season and 3.770 were examined in the pen/stall period for a total of 3.770 sheep. Foot disease was found in 1.080 (25.51%) and 520 (13.76%) sheep, respectively. Hoof horn deformities were the most prevalent in both seasons, while the prevalence of footrot was found to be 2.83% in the pasturing period and 0.82% in the pen/stall period.

This study evaluated 8.970 sheep from 10 flocks being raised in various parts of Kars province, and 1.532 of them (17.07%) were found to have lameness for various reasons. The cause of lameness in the majority of the cases was hoof horn deformities, while clinical footrot was found to be the cause of lameness in 247 of these cases (16.12%). When compared to all of the sheep that were clinically evaluated in the study, the prevalence of footrot was found to be 2.75%. This percentage was lower than some of the aforementioned studies [2,14,15] but quite close to the results of other studies [16,19]. It is known that some factors related to the host animal (such as breed and immunity) as well as certain environmental factors (such as rain, temperature and moisture) have an effect on the natural progression of the disease. For example, the average annual rainfall in the UK and India is much higher than that of Kars province, while Bhutan's average annual rainfall is quite similar to

this area. The prevalence figures obtained in this study prove that atmospheric conditions such as rainfall, temperature and moisture have a definite effect on the progression of the disease.

Isolating *D. nodosus* is an extremely difficult and time consuming process, partly because of the fastidious nature of this strict anaerobe, but also because of the large number of different bacteria in the microflora of the footrot lesion [20,21]. Furthermore, taking samples correctly and quickly transporting them to the laboratory under suitable conditions is absolutely crucial to obtain an accurate isolation rate. Bacteria were isolated in 205 (82.99%) of the 247 samples that were cultured under anaerobic conditions due to suspicion of footrot. This isolation rate can be affected by how the samples are taken and transported to the laboratory, the streaking stages and the incubation conditions. When Gram stains and microscopic investigation were carried out on the isolates, 195 of them (95.12%) were found to be Gram negative rod-type bacteria. These isolates were subjected to PCR because *D. nodosus* was suspected. Of the 195 isolates subjected to PCR, 153 (78.46%) of them were found to be *D. nodosus*. It is known that cases with ovine footrot feature bacterial complexity [6] and that *Fusobacterium necrophorum* has a synergistic effect in the formation of the lesions [3,22]. This leads to the conclusion that the isolates that could not be identified as *D. nodosus* may be other agents that are part of the aetiology of the disease.

This was the first study to be conducted to establish the status of footrot in sheep from the region of Kars, isolation the agent and identify it with PCR. The results have shown that the disease is significantly prevalent in the sheep of the region and *D. nodosus* was isolated and identified in a high percentage of the subjects. The goal of subsequent studies should be to identify the serogroup and serotypes of the strains of *D. nodosus* that are isolated. This is necessary in order to develop a vaccination against the disease. In view of the disease's infectiousness and economic impact, it is critically important that a range of programs and activities be carried out, including vaccination, hoof care, foot baths, evaluating the effects of environmental factors on the disease, separating sick animals from healthy ones, treating sick animals as soon as possible, and training sheep ranchers about these topics.

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Effect of Piggery Microclimate on Ejaculate Performance of Artificial Insemination Boars

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Abstract

The study was carried out on 1913 ejaculates collected from 51 Landrace boars. The ejaculates were collected manually. The study included all ejaculates collected by one artificial insemination station over a span of 12 consecutive months. The ejaculates were subjected to the standard analysis which involved: ejaculate volume, sperm concentration, percentage of progressive motility spermatozoa, total number of spermatozoa, and number of artificial insemination doses per ejaculate. Temperature, relative air humidity, and atmospheric pressure in the piggery were monitored during the semen collections. The resulting data were grouped by air temperature, humidity and pressure, and analyzed. It has been found that the microclimate in the place of semen collection may affect the quality of the collected semen. This effect is varied; temperature, humidity and atmospheric pressure act differently in relation to the semen traits. Temperature and atmospheric pressure have an effect on sperm concentration. Ejaculates with the highest sperm concentrations were collected at the lowest measured temperatures (10°C and lower) as well as with the lowest relative air humidity. Sperm motility revealed a strict and oriented relationship with both temperature and humidity, but also with atmospheric pressure. Semen collected at the lowest air humidity (50% and lower) and at the lowest observed atmospheric pressure (below 900 hPa) contained the most sperm. The highest number of artificial insemination doses are produced from ejaculates collected in such conditions.

Keywords: *Microclimate, Semen quality, Artificial insemination boars*

Mikro Klima İklim Şartlarının Erkek Damızlık Domuzlardaki Ejekulasyon Kalitesine Etkileri

Özet

Araştırma Landrace cinsi 51 erkek damızlık domuzdan alınan 1913 ejekulasyonda gerçekleştirilmiştir. Numuneler el yöntemiyle alınmıştır. Ejekulasyonlar sonraki 12 ay süresince uygulanacak yapay döllenme için tek bir domuz yetiştirme istasyonundan elde edilmiştir. Standart tahliller olan, semen hacmi, spermlerin konsantrasyonu, hareketli sperm sayısının oranı, toplam sperm sayısı ve yapay döllenmedeki doz sayısı analizleri yapılmıştır. Semen örneği alımı sırasında damızlık hayvanların bulundupu yerdeki atmosfer basıncı, havadaki nem ve sıcaklık durumu izlenmiştir. Elde edilen veriler, sıcaklık, nem ve hava basıncına göre gruplandırılmış vede tekrar analiz edilmiştir. Bu çalışmalar neticesinde damızlık hayvanların tutulduğu yerdeki mikro klima şartlarının semen kalitesine etkisi olabileceği görülmüştür. Sıcaklık, nem ve atmosfer basıncı sperm özelliklerine farklı biçimde etki etmektedir. Sıcaklık ve atmosfer basıncı spermlerin konsantrasyonunu doğrudan etkilemektedir. En yüksek sperm konsantrasyonu bulunan ejekulasyonlar, havaya göre en düşük nem ortamı vede en düşük sıcaklıkta (10°C ve aşağısı) elde edilmiştir. Sperm motilitesinin hava basıncı, nem ve sıcaklığa doğrudan bağlı olduğu ortaya çıkmıştır. En fazla sayıda sperm miktarı, en düşük nem oranlarında yani %50 ve daha azı ile 900 hPa hava basıncı şartlarında sağlanmıştır. Bu şartların hakim olduğu ortamlarda hayvanlardan alınan ejakulasyonlardan yapay döllenmeler için gerekli en fazla sayıda elde edilmiştir.

Anahtar sözcükler: *Mikroklima, Sperm kalitesi, Damızlık erkek domuz*

INTRODUCTION

Artificial insemination (AI) plays an important role in swine production and brings numerous advantages; it enables an efficient use of sires, facilitates the organization of breeding in the herd, and reduces the risk of spreading infectious diseases [1]. The main criterion of boars selection for AI centers is the breeding value of the boar, which

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is usually much higher than the average of a given population of young boars classified for evaluation [2]. The economic viability of an AI boar does not depend much on its breeding value though. The decisive factor is the ejaculatory performance, which is the number of AI doses obtained per ejaculate. It is important that the boar shows the ability to produce ejaculates of rather equal quality throughout its lifetime [3]. Therefore, the aim is to use the boars that demonstrate outstanding ejaculate traits, i.e. a large volume of discharged semen with a high concentration and good motility of sperm [4].

Numerous studies demonstrate that the quality and quantity of semen depend on a complexity of factors. These include genetics, primarily the breed of the boar [5,6]. Crossbred boars, which attain sexual maturation sooner, grow faster and have a better breeding performance, as compared with pure-bred pigs, have found a wide application in artificial insemination [7]. Other than genetic factors also affect boar performance, and these include boar's age [8], year season [9,10], changing photoperiod [11], feeding [12], as well as the time interval between ejaculations [13]. It has been found that ejaculation performance in boars is also related with the size of the testes [14]. Such a variety of genetic and environmental factors, as well as interactions between them, result in extremely varied ejaculates of AI boars, which complicates their efficient management. This study is an attempt to evaluate the effect of air temperature, relative humidity, and atmospheric pressure during semen collection on the physical traits of ejaculates of artificial insemination boars.

MATERIAL and METHODS

All boars were fed commercial food for AI boars and were housed in individual pens equipped with nipple drinkers. The boars were maintained in accordance with the principles of animal welfare [15]. Ethics committee approval for this study was given by the Decision of the District Veterinary Officer (14262001). In the study air temperature, relative humidity and the atmospheric pressure on the moment of semen collection were measured. Temperature and humidity was recorded using a thermo-hygrometer TERMIK PLUS (1000209, Termo-produkt, PL). Temperature was measured with a precision of one degree Celsius. Humidity, expressed as a percentage, was measured with a precision of one percent point. Atmospheric pressure was measured using an barometer ADLER (Bar 003, Demus, PL) with a resolution one hPa.

The temperature and humidity sensors, as well as the barometer, were placed in the central part of the piggery, halfway in the aisle dividing the facility into two equal parts. The readings were taken during the semen collection from each boar. The analysis included 1913 ejaculates obtained from 51 Landrace boars. The semen was collected using the gloved-hand technique, using a clean semen collecting flask that filters out gel, dust, and bristles [16]. The evaluation included all ejaculates collected over the period of 12 consecutive months. In order to evaluate the effect of the facility microclimate on the semen quality, each ejaculate was subjected to standard analysis of the following physical traits: ejaculate volume, sperm concentration, percentage of spermatozoa showing progressive motility, the total number of spermatozoa, and the number of insemination doses per ejaculate. Ejaculate volumes were determined by weight, without the gelatinous fraction, using electronic scales. Sperm concentration in the ejaculates was determined with a photometric method, using a spectrophotometer (IMV Technologies, France). Sperm motility was evaluated with a Nikon Eclipse 50i light microscope equipped with a heated stage. A sample of 5 µl of sperm suspension was placed on a pre-warmed slide and sealed with a coverslip at 37ºC. Under 200x magnification, the percentage of normally motile spermatozoa was determined in the overall number of sperms present in the field of vision of the microscope. The total number of motile spermatozoa and the number of insemination doses per ejaculate were calculated using SYSTEM SUL (v. 6.35; Gogosystem, Poland) software package.

In order to evaluate the effect of air temperature, ejaculates were assigned to the following four groups:

Group I : Ejaculates collected at a temperature up to 10ºC
Group II : Ejaculates collected at a temperature 11-15ºC
Group III : Ejaculates collected at a temperature 16-20ºC
Group IV : Ejaculates collected at a temperature above 20ºC

In order to evaluate the effect of air relative humidity, the ejaculates were assigned to the following six groups:

Group I : Ejaculates collected at an air humidity up to 50%
Group II : Ejaculates collected at an air humidity 51-55%
Group III : Ejaculates collected at an air humidity 56-60%
Group IV : Ejaculates collected at an air humidity 61-65%
Group V : Ejaculates collected at an air humidity 66-70%
Group VI: Ejaculates collected at an air humidity above 70%

In order to evaluate the effect of atmospheric pressure, the ejaculates were assigned to the following six groups:

Group I: Ejaculates collected at an atmospheric pressure up to 990 hPa
Group II: Ejaculates collected at an atmospheric pressure 991-995 hPa
Group III: Ejaculates collected at an atmospheric pressure 996-1000 hPa
Group IV: Ejaculates collected at an atmospheric pressure 1001-1005 hPa
Group V: Ejaculates collected at an atmospheric pressure 1006-1010 hPa

Group VI: Ejaculates collected at an atmospheric pressure above 1010 hPa

The resulting data were processed statistically according to the following model:

$Y_{ij} = \mu + a_i + e_{ij}$

where:

Y_{ij} - factor level,

μ - population mean,

a_i - effect of air temperature, humidity or atmospheric pressure,

e_{ij} - error.

The significance of differences between the groups was tested using the Tukey test.

RESULTS

Table 1 presents the data on physical characteristics of the ejaculates in relation to air temperature measured at semen collection. The data reveal that nearly 70% of ejaculates were collected at the range 11-20°C.

The data of *Table 1* reveal that the ejaculates of Group II had the lowest ejaculate volume (P<0.01). Ejaculates collected at temperature up to 10°C (Group I) showed a higher sperm concentration by 19 x10^3/mm^3 compared with those collected at 11°C or higher (Groups II-IV, P<0.01). The data shown in *Table 1* indicate a distinct relationship between motility and air temperature at semen collection. Sperm motility in ejaculates increased with air temperature they were collected at. The highest sperm motility was found in ejaculates collected at temperature above 16°C (Groups III and IV). It exceeded 72.75% and was by more than 1.5% higher than in the semen collected at 11-15°C (P<0.01) and by more than 2.7% higher compared to those collected below 10°C (P<0.01).

No clear relationship has also been found between the number of insemination doses and air temperature at semen collection *(Fig. 1)*. The average was approx. 24.61-25.05 insemination doses obtained from an ejaculate (Groups I, III and IV), but Group II (11-15ºC) was an exception; these ejaculates gave by more than 1.5 fewer doses (P<0.05).

Table 2 presents data on the physical characteristics of ejaculate, depending on the relative humidity recorded

Table 1. *Relationship between ejaculate physical characteristics and air temperature in the piggery during ejaculate collection*
Tablo 1. *Ejakulat toplanması esnasında domuz ahırındaki hava sıcaklığı ile ejakulatın fiziksel özellikleri arasındaki ilişki*

Specification		Air Temperature in the Piggery (°C) (Groups)			
		I (≤10°C)	II (11-15°C)	III (16-20°C)	IV (>20°C)
Average air temperature during ejaculates collection (°C)		9.05	12.89	18.17	22.54
Number of ejaculates	n	409	747	564	193
Ejaculate volume (ml)	X±Sx	241.93±86.78^B	234.71±75.36^A	243.63±84.72^B	244.00±84.34^B
Sperm concentration (x 10^3/mm^3)	X±Sx	398.56±103.98^B	375.33±94.14^A	379.49±90.55^A	378.86±95.26^A
Percentage of spermatozoa with progressive motility (%)	X±Sx	70.00±0.00^A	71.23±3.29^{AB}	72.77±4.48^B	72.75±4.47^B
Total sperm count per ejaculate (x 10^9)	X±Sx	64.09±20.23^b	60.82±19.79^a	64.16±18.97^b	64.4420.41^b
Number of insemination doses	X±Sx	24.61±7.97^b	23.97±7.58^a	25.05±7.15^b	24.82±7.58^b

^{a,b} Differences between average values, represented by different letters in the same row, is important (P<0.05); ^{A,B} Differences between average values, represented by different letters in the same row, is important (P<0.01)

Fig 1. Monthly distribution of temperatures during semen collection (number of days in each month when semen was collected at a specific temperature)

Şekil 1. Semen toplanması boyunca sıcaklığın aylara göre dağılımı (belli bir sıcaklıkta semen toplandığında her aydaki günlerin sayısı)

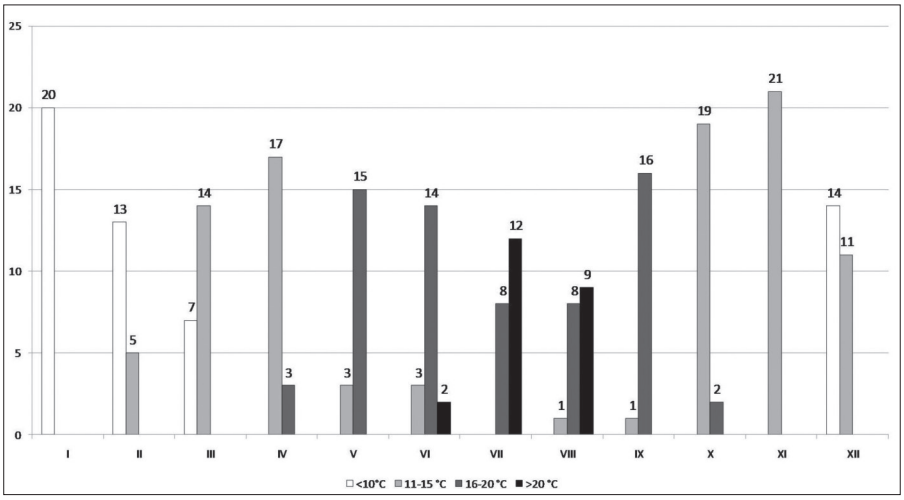

Table 2. *Relationship between ejaculate physical characteristics and air relative humidity in the piggery during ejaculate collection*
Tablo 2. *Ejakulat toplanması esnasında domuz ahırındaki nem ile ejakulatın fiziksel özellikleri arasındaki ilişki*

Specification		Air Relative Humidity in the Piggery (%) (Groups)					
		I (≤50%)	II (51-55%)	III (56-60%)	IV (61-65%)	V (66-70%)	VI (>70%)
Average air relative humidity during ejaculates collection (%)		44.52	53.32	58.44	63.58	68.62	172
Number of ejaculates	n	446	395	437	325	138	172
Ejaculate volume (ml)	X±Sx	230.90±79.43^{A}	233.93±83.35^{AB}	250.17±80.34^{C}	238.73±82.30^{B}	251.96±82.23^{C}	242.52±82.04^{BC}
Sperm concentration (x 10^3/mm^3)	X±Sx	412.91±96.94^{C}	382.48±95.63^{B}	367.73±89.39^{AB}	378.09±95.15^{B}	351.23±87.44^{A}	367.67±96.60^{AB}
Percentage of spermatozoa with progressive motility (%)	X±Sx	71.14±3.19^{A}	71.65±3.71^{B}	71.17±3.21^{A}	71.85±3.89^{B}	72.32±4.24^{B}	72.44±4.31^{B}
Total sperm count per ejaculate (x 10^9)	X±Sx	65.22±20.72^{c}	61.51±20.82^{ab}	62.66±18.33^{ab}	62.52±19.66^{ab}	60.59±16.98^{ab}	62.95±20.21^{ab}
Number of insemination doses	X±Sx	25.06±8.15	24.15±7.68	24.46±7.05	24.55±7.62	24.23±6.42	24.22±7.58

^{a,b} Differences between average values, represented by different letters in the same row, is important (P<0.05); ^{A,B} Differences between average values, represented by different letters in the same row, is important (P<0.01)

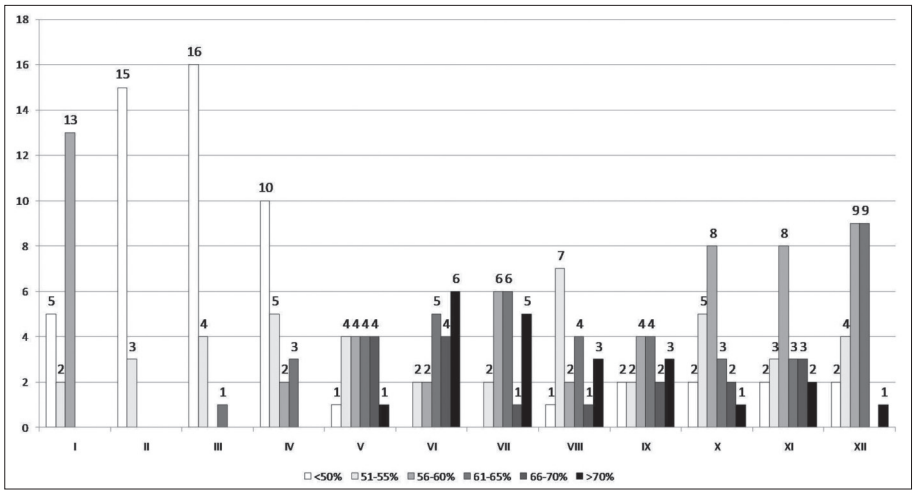

Fig 2. Monthly distribution of relative humidities during semen collection (number of days in each month when semen was collected at a specific humidity)

Şekil 2. Semen toplanması boyunca nemin aylara göre dağılımı (belli bir nemde semen toplandığında her aydaki günlerin sayısı)

during the collection. These data show that ejaculates of the greatest volume were collected at a relative humidity of 66-70%. The volume exceeded 251 ml and was higher by 21.06 ml than in those collected at a humidity not exceeding 50% (P<0.01) and by more than 18 ml higher than in those collected at a relative humidity of 51-55% (P<0.05). Ejaculates collected at a relative humidity equal or lower than 50% (Group I) had a higher concentration of sperm, by 30 x10^3/mm^3, as compared with ejaculates collected at a relative humidity of 51% or higher (Groups II, III, IV, V and VI; P<0.01).

The highest sperm motility was found in ejaculates collected at a relative humidity above 70%. It exceeded 72.4% and was higher by more than 1.27% compared to those collected at 56-60% (P<0.01) and by 1.3% higher than in those collected at a relative humidity not exceeding 50% (P<0.01). Ejaculates collected at very low relative humidities (below 50%) contained the most sperm. Sperm counts in these ejaculates averaged 65.22 billion, by 2.27-4.63 billion more than in those collected at higher humidity (Group II, III, IV, V and VI). No relationship was found between the number of insemination doses and air relative humidity. The average number of insemination doses obtained from the ejaculate was about 24.15-25.06 *(Fig. 2)*.

Table 3 presents the data on physical traits of the semen in relation to the atmospheric pressure measured at the moment of ejaculation. Group VI, with the highest atmospheric pressure, was found to have the lowest ejaculate volume. It exceeded 232 ml and was by about 10.3 ml less than those collected at 991-995 hPa (P<0.01) and by about 11.5 ml less than in ejaculates collected at 1001-1005 hPa (P<0.01).

Ejaculates collected at the lowest atmospheric pressure (not more than 990 hPa, Group I) had the highest sperm concentration. The concentration of sperm decreased as atmospheric pressure increased to 996-1000 hPa (Group III). Ejaculates collected in this group had the lowest sperm concentration, by about 21 x10^3/mm^3 less than those collected at a pressure below 990 hPa (P<0.05). A further increase in atmospheric pressure resulted in an increase

Table 3. *Relationship between ejaculate physical characteristics and atmospheric pressure in the piggery during ejaculate collection*
Tablo 3. *Ejakulat toplanması esnasında domuz ahırındaki atmosferik basınç ile ejakulatın fiziksel özellikleri arasındaki ilişki*

Specification		Atmospheric Pressure in the Piggery (hPa) (Groups)					
		I ≤990 hPa	II 991-995 hPa	III 996-1000 hPa	IV 1001-1005 hPa	V 1006-1010 hPa	VI >1010 hPa
Average atmospheric pressure during ejaculates collection (hPa)		981.56	993.26	998.61	1004.10	1007.87	1015.35
Number of ejaculates	n	137	153	316	632	474	201
Ejaculate volume (ml)	X±Sx	239.90±84.21^{B}	242.63±84.22^{BC}	234.67±77.32^{AB}	243.83±83.77^{C}	240.16±81.52^{BC}	232.32±78.13^{A}
Sperm concentration (x 10^3/mm^3)	X±Sx	395.69±95.09^{c}	380.78±101.25^{ab}	374.87±97.78^{a}	376.82±94.90^{a}	385.34±94.00^{b}	392.04±94.52^{bc}
Percentage of spermatozoa with progressive motility (%)	X±Sx	70.00±0.00^{A}	71.05±3.07^{B}	71.52±3.59^{B}	71.99±4.00^{B}	71.48±3.55^{B}	72.04±4.04^{B}
Total sperm count per ejaculate (x 10^9)	X±Sx	64.07±19.87^{b}	62.65±19.86^{ab}	59.94±19.11^{a}	63.46±20.20^{b}	63.45±19.10^{b}	63.60±20.62^{b}
Number of insemination doses	X±Sx	25.23±8.56^{b}	24.94±7.90^{a}	23.83±6.79^{a}	24.41±7.54^{a}	24.83±7.30^{a}	24.97±8.17^{a}

^{a,b} Differences between average values, represented by different letters in the same row, is important (P<0.05); ^{A,B} Differences between average values, represented by different letters in the same row, is important (P<0.01)

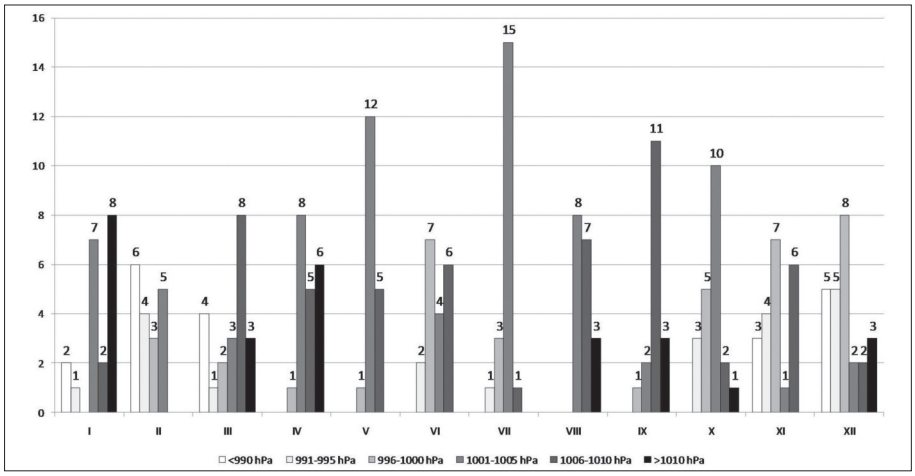

Fig 3. Monthly distribution of air pressure during semen collection (number of days in each month when semen was collected at a specific pressure)

Şekil 3. Semen toplanması boyunca hava basıncının aylara göre dağılımı (belli bir hava basıncında semen toplandığında her aydaki günlerin sayısı)

in the concentration of sperm in the ejaculate. The data in *Table 3* show there is a dependence of sperm motility on atmospheric pressure at ejaculation. As the atmospheric pressure increased, motility in the ejaculates increased too. The highest level of sperm motility was found in ejaculates collected at atmospheric pressure above 1010 hPa. Motile sperm rate exceeded 72% and was higher by 0.99% than in ejaculates collected at 991-995 hPa (P<0.01) and by 2.04% higher than in those collected at atmospheric pressure not exceeding 990 hPa (P<0.01).

Sperm count was highest in ejaculates collected at the lowest atmospheric pressure range, not more than 990 hPa. *(Fig. 3)* It exceeded 64 billion (10^9) and was higher by more than 4.1 billion than in ejaculates collected at 996-1000 hPa (P<0.05). The greatest number of insemination doses per ejaculate, exceeding 25, was found in low atmospheric pressures not exceeding 990 hPa (Group I) and at high pressures above 1010 hPa (Group VI). The average number of insemination doses obtained from ejaculates qualified for the Groups II, III, IV and V was lower by more than 1.5 insemination doses (P<0.05).

DISCUSSION

The resulting data suggest that the characteristics of ejaculates depend on the piggery microclimate parameters present during semen collection. The highest sperm concentration was found in ejaculates collected at low air temperatures, below 10°C. Moreover, ejaculates collected at lower temperatures had a good volume. It seems that boars do not have a particular susceptibility to low air temperatures during ejaculation. Low temperatures then have no negative effects on ejaculation performance of boars. Ejaculates collected at higher temperatures had a significantly lower sperm concentration. Spermatogenesis runs best in air temperature ranging from 15 to 20°C in bulls [17]. Most ejaculates in this study were collected within this thermal range (groups II and III). Air temperature at ejaculation is not, however, the only factor that may affect the ejaculate traits. Air temperature during the entire period of spermatogenesis is also vital, and this period may begin as early as 70 days before ejaculation [18].

Motility and morphology of sperm are sensitive indicators of heat stress [19]. The extent of morphological changes in spermatozoa depends on the duration of heat impact and on the adaptability of males to the conditions of heat stress [20]. Adaptation is less easy with higher diurnal temperature variations [21]. Heat stress directly affects reproductive performance of boars, but also has an indirect impact, by provoking changes in the energy balance [22]. Wettemann et al.[23] observed that increased temperature of the air is accompanied by a reduced quality of porcine semen without distinct changes in the ejaculate volume or libido. Increased thermal conditions resulted in sperm concentration reduced by 50% and in decreased average sperm motility, from 79.5% to 46.4% between the 3rd and 6th week of observations [23]. Stone [24], on the other hand, observed that sperm motility - as a result of temperature increase from 20°C to 40°C - dropped from about 93% to 19% after 3 weeks of such treatment. Our results reveal that sperm motility increases with an increase in temperature at ejaculation. Semen was collected at temperatures rather remaining within the thermal comfort zone for swine. In this study, the temperature never exceeded 29°C, at which - according to Sonderman and Luebbe [25] - spermatogenesis undergoes serious disturbances. Our study revealed that the highest proportion of progressively motile sperm was in ejaculates collected within the range 16-20°C and above 20°C (respectively 72.77% and 72.75%).

Season may affect the air temperature during the collection of ejaculates, which is particularly important in a temperate climate zone [2]. Therefore, in this study we recorded the number of days per each month when ejaculates were collected at a specific temperature *(Fig. 1)*.

The data presented in *Table 1* show that the best semen traits (ejaculate volume, motility, total sperm count) were found in semen collected at temperatures above 16°C. These ejaculates allowed preparation of the most AI doses. Monthly temperature distribution depicted in *Fig. 1* shows that the most days of semen collection at such temperatures are located between May and September. The period from May to September is not a good time in swine reproduction cycle [26]. At this time, boars generally produce ejaculates of lower quality. In summer the sexual activity of boars is often less intensive, which manifests in smaller ejaculate volumes and lower sperm counts, as reflected in the number of insemination doses made. Lower sperm counts in ejaculates collected in the summer months, as compared with the rest of the year, were found in the studies by Flowers [27]. During this period also more morphologically abnormal sperm are found [28]. The data of the presented study indicate, however, that this might not always be the case.

Air temperature in swine farming facilities strongly depend on the year season, which is depicted in *Fig. 1*. The most days of semen collection in which temperature did not exceed 10°C were from December to February. Ejaculates collected at 10°C and lower had the highest sperm concentrations. These results in conformity with data published by Ciereszko et al.[9] and Trudeau and Sanford [29], who analyzed ejaculates in relation to the season of semen collection. According to Knecht et al.[6], a high sperm concentration in ejaculates collected in winter results from lower temperatures, which has a positive effect on spermatogenesis. Auvigne [30] believes that this effect is due to the close kinship between the modern breeds of pigs and the European wild boar, for which the period of breeding activity is in late autumn and early winter, a period of a shortening length of daylight. This was confirmed by Knecht et al.[31], who obtained ejaculates of larger volume and with higher motility sperm from boars of various breeds during the period July - December, which was reflected in more insemination doses obtained from one ejaculate.

The results obtained in this study indicate that air relative humidity during the collection of ejaculates varied and depended largely on the season *(Fig. 2)*.

Air relative humidity in the piggery was 56-60% over most of the days of semen collection during autumn and winter (September - January). Ejaculates collected during this period were characterized by a large volume (250.17 ml on average) and a very low sperm concentration ($367.73 \times 10^3/mm^3$). On the other hand, ejaculates collected at a relative humidity of 50% and lower had the smallest volume (230.90 ml) and the highest sperm concentration ($412.91 \times 10^3/mm^3$). Such an air humidity level was most frequent from February to April *(Fig. 2)*, when ejaculates also had the highest sperm count (65.22 billion), enabling preparation of the highest number of doses (25.06 per ejaculate). With an increase in air humidity in the piggery, a higher rate of sperm showing progressive motility was observed too. Ejaculates collected at humidity lower or equal 60% demonstrated a lower percentage of progressively motile sperm as compared with the semen collected at an air humidity above 66%.

Relative air humidity is closely related to air temperature. There is the view that a combined effect of high temperature and high humidity is more detrimental to the functioning of testes than the effects of these two factors acting separately [32]. McNitt and First [33] found that placing boars for 72 h in a climatic chamber at 33°C and a relative humidity of 50% resulted in a decrease in the total spermatozoa count in the ejaculate and an increase in the percentage of spermatozoa with morphological abnormalities within about two weeks of the treatment. Similar conclusions were reached by Larsson and Einarsson [34], who placed boars for 100 h at 35°C and a relative humidity of 40%. The results were reduced ejaculate volume and a higher percentage of abnormal spermatozoa; total sperm counts in the ejaculates, however, remained unchanged. Suriyasomboon et al.[35] demonstrate that temperature and relative humidity have a significant effect on the volume of

ejaculate, and the total sperm count in ejaculates obtained from boars managed in various systems in Thailand. The authors found that a temperature increase in facilities for boars above 29°C at a relative humidity higher than 70% may have a negative effect on spermatogenesis. The number of articles on the impact of humidity on ejaculation performance is low and their results do not lead to clear conclusions. The data in this study prove that humidity is important for the quality of boar ejaculates and that in practice there are large fluctuations in air humidity during the collection of ejaculates. Therefore, there is a need for research and analysis of the impact of relative humidity on the basic quantitative and qualitative characteristics of ejaculate.

The impact of atmospheric pressure during the collection of ejaculates on their physical characteristics proved to be minor [36]. The highest sperm concentrations were obtained at an atmospheric pressure of 990 hPa and lower. Ejaculates collected at the lowest atmospheric pressure were found to have the lowest sperm motility. Differences between the other groups were insignificant and were not confirmed statistically. Atmospheric pressure is also partly related to the season of the year. Monthly distribution of atmospheric pressure at the time of collecting ejaculates is shown in *Fig. 3*.

The data presented in *Fig. 3* demonstrate that during spring and summer (April - August), air pressure in the piggery during semen collection was usually 1001-1005 hPa, and in autumn (November, December) 996-1000 hPa. Winter was characteristics for its highly varied pressure distribution in each month of the season. The data presented in this study, however, are hard to compare with other studies, since reports on these are lacking.

In conclusion, the studied microclimate parameters in the place of ejaculate collection may affect the quality of ejaculates. This effect, however, is varied. Each of the tested microclimate factors (temperature, humidity and atmospheric pressure) affects the characteristics of collected ejaculates differently and to a varying degree. Air temperature and atmospheric pressure at the time of semen collection affect the volume of the ejaculate and concentration of sperm in the semen. Ejaculates with the highest sperm concentration are obtained at extremely low temperatures (10°C or less), and at extremely low relative humidity. Sperm motility exhibits a distinct and oriented dependence in relation to air temperature, relative humidity of the air and atmospheric pressure. In ejaculates collected at the lowest air temperature, relative humidity of the air and atmospheric pressure were found least sperm with progressive motility. The highest numbers of sperm are found in ejaculates obtained at extremely low relative humidity (50% and lower) and at extremely low atmospheric pressure (not exceeding 900 hPa). Ejaculates collected in these conditions yielded the highest number of artificial insemination doses. Air temperature during semen collection has little influence on the total number of sperm per ejaculate and the number of artificial insemination doses obtainable from a single ejaculate.

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Research Article

Presence of *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157 and Nitrate-Nitrite Residue Levels in Turkish Traditional Fermented Meat Products (Sucuk and Pastırma) [1] [2]

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Abstract

Turkish sucuk and pastırma are traditional meat products commonly consumed in Turkey. These products are generally known as dry fermented meat products (FMP), fermented and ripened naturally. Curing is a preparation method for FMPs used for prolonging shelf life. As well as additives such as nitrate and nitrite are used to obtain the desired colour and flavour, also inhibit the mentioned bacteria. Despite the advantages of the curing agents, FMPs may pose a risk for human health via uncontrolled (out of limits) usage. The present study was conducted to investigate the incidence of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 and nitrate-nitrite contents in 132 sucuk and 66 pastırma samples collected from producers and retailers in Istanbul, Adapazari, Afyon and Kayseri. *Salmonella* spp. and *L. monocytogenes* were detected 2.52% and 2.02% in all samples, respectively. All samples were negative for *E. coli* O157. The nitrate level of sucuk and pastırma samples were found was in the acceptable range. Only, 5 of sucuk samples exceeded the nitrite limit value. The results indicate that meat products may be contaminated with pathogens and nitrosamines can be present in meat products. Furthermore, the essential precautions should be taken to apply sanitation procedure and improve the quality of production technology.

Keywords: *Sucuk, Pastırma, Listeria monocytogenes, Salmonella spp., Nitrate-nitrite*

Geleneksel Türk Fermente Et Ürünlerinde (Sucuk ve Pastırma) *Salmonella* spp., *Listeria monocytogenes, Escherichia coli* O157 ve Nitrat-Nitrit Varlığı

Özet

Türk tipi sucuk ve pastırma Türkiye'de yaygın olarak tüketilen geleneksel et ürünleridir. Doğal olarak fermente olup olgunlaşan bu ürünler genellikle fermente edilerek kurutulmuş et ürünleri olarak bilinirler. Kürleme fermente edilerek kurutulmuş et ürünlerinde kullanılan bir hazırlama metodu olup raf ömrünü uzatmak amacıyla kullanılır. Nitrat ve nitrit gibi katkı maddeleri de arzu edilen renk ve aromanın şekillenmesini sağlarken aynı zamanda bazı bakterilerin üremesinin inhibe edilmesi için de kullanılır. Kürleme ajanlarının avantajlarına rağmen, fermente edilerek kurutulmuş et ürünlerinde kontrolsüzce (limit değerlerin üzerinde) kullanımları sağlık risklerine sebep olabilir. Bu çalışma İstanbul, Adapazarı, Afyon ve Kayseri'deki perakende satış noktaları ve üreticilerden toplanan 132 sucuk ve 66 pastırma örneğinde *Salmonella* spp., *Listeria monocytogenes* ve *Escherichia coli* O157 varlığını ve nitrat-nitrit içeriğini tespit etmek için yürütüldü. *Salmonella* spp. ve *L. monocytogenes* sırasıyla %2.52 ve %2.02 olarak tespit edildi. Hiçbir örnekte *E. coli* O157 tespit edilemedi. Sucuk ve pastırma örneklerinin nitrat düzeyi kabul edilebilir düzeyde bulundu. Sadece sucuk örneklerinin beşinde nitrit limit düzeyinin aşıldığı tespit edildi. Sonuçlar et ürünlerinin patojenlerle kontamine olabileceğini ve et ürünlerinde nitrozaminlerin bulunabileceğini göstermiştir. Buna ek olarak, uygulanan sanitasyon prosedürlerinde zorunlu tedbirler alınmalı ve üretim teknolojisinde kalite iyileştirilmelidir.

Anahtar sözcükler: *Sucuk, Pastırma, Listeria monocytogenes, Salmonella spp., Nitrat-nitrit*

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INTRODUCTION

Turkish type dry fermented sausage (sucuk) and pastırma are traditional meat products widely consumed in Turkey. These products are known as dry fermented meat products (FMP) manufactured by natural fermentation and generally consumed without cooking [1-3]. Despite the fermentation periods the foodborne pathogens that can be present in the gastrointestinal tract of food-producing animals are potential sources of risk for human health [4]. These organisms are subsequently transferred to meat products and people due to poor hygiene, sanitation conditions and handling procedures during slaughtering and production [5].

Curing is a traditional culinary technique is used for prolonging the shelf life of meat products [6]. The curing agents nitrite and nitrate not only help producers obtain the desired flavour and colour; but also have inhibitor effect on several pathogen microorganisms in fermented meat products [7]. Despite their technological and safety advantages, high intake of nitrate-nitrite constitutes a risk to human health, in rare occasions causing allergenic effects and carcinogenic nitrosamines [8,9].

The present study was undertaken to determine the prevalence of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 and the contents of nitrate-nitrite in sucuk and pastırma obtained from retail markets and producers in Istanbul, Adapazari, Afyon and Kayseri, the major sucuk and pastırma producing cities in Turkey.

MATERIAL and METHODS

Sample Collection: Sucuk and pastırma samples were collected at intervals between March 2012 and February 2013. A total of 132 sucuk and 66 pastırma samples were examined for the presence of *Salmonella* spp., *L. monocytogenes, E. coli* O157 and nitrate-nitrite contents. Samples were obtained from producers and retailers in Istanbul, Adapazari, Afyon and Kayseri. All samples were collected in their original packages and transferred to the laboratory at 4ºC.

Microbiological Analysis: For isolation of *Salmonella* spp., pre-enrichment was done by suspending 25 g of sample in 225 ml buffered peptone water (BPW - Oxoid CM0509), followed by incubation at 37ºC for 16 to 20 h. A 0.1 ml sample of the mixture was transferred to Rappaport-Vassiliadis (RV - Oxoid CM0866) and Muller Kaufmann Tetrathionate Broth (MKTTn - Oxoid CM0343) and they were incubated for 24 h at 42ºC. Samples were streaked on Hectoen Enteric Agar (Oxoid CM0419) and XLD Agar (Oxoid CM0469) after incubation and incubated an additional 24 h at 35ºC. The typical colonies were identified by biochemical tests and confirmed with Salmonella antiserum (O and H-Vi polyvalent antiserum) [10].

For detection of *L. monocytogenes*, 25 g food samples were mixed with 225 ml of Listeria Enrichment Broth (Oxoid CM0862) containing Listeria Selective Supplement (Oxoid SR 141). Samples were homogenized in a stomacher bag for 60 sec and incubated at 32ºC for 24 h. 0.1 ml portion of the enrichment broth was streaked onto Chromogenic Listeria Agar (Oxoid CM1080) supplemented with Listeria Selective Supplement (Oxoid SR0227) and Listeria Differential Supplement (Oxoid SR0228). After incubation, typical colonies were transferred to Tryptic Soy-Yeast Extract Agar (Oxoid CM0131) and incubated for 24 to 48 h at 30ºC. These colonies were verified by Gram's staining, catalyses reaction, tumbling motility at 25ºC, Methyl Red-Vogues Proskauer (MR-VP) reactions, CAMP test, nitrate reduction and fermentation of sugars [11].

For detection of *E. coli* O157, each sample was examined by combining 25 g with 225 ml of modified Tryptone Soya Broth (Oxoid CM0989) into a stomacher bag, homogenized for at least 2 min and incubated at 37ºC for 24 h. Enriched cultures were streaked onto Sorbitol MacConkey Agar (Oxoid CM0813) supplemented with Cefixime Tellurite Selective Supplement (Oxoid SR172) and incubated at 37ºC for 18 to 24 h. Following the incubation period, the colourless colonies were tested by *E. coli* O157 latex kit (Oxoid DR0620) [12].

Determination of Nitrate and Nitrite Content: Nitrate and nitrite concentrations in the samples were determined by the HPLC method based on the Nordic Committee on Food Analysis Method No. 165. The solution was injected onto the Shimadzu LC10 chromatograph. Nitrate and nitrite were separated by an Alltech C18 column and measured with an ultraviolet light detector at a wavelength of 205 nm. The limit of quantification for both ions was 5mg/kg^{-1}; the measurement uncertainty (U) at a concentration of 100 mg/kg was 12 mg/kg (k=2, normal) [13].

RESULTS

Salmonella spp. and *L. monocytogenes* were detected at 1.52% and 1.52% in sucuk and 4.55% and 3.03% in pastırma samples, respectively *(Table 1)*. All samples were negative for *E. coli* O157. According to Turkish Food Codex [14], the presence of *Salmonella* spp. and *E. coli* O157 in 25 g of raw beef or ground beef as well as the presence of *Salmonella* spp. and *L. monocytogenes* in 25 g of sucuk is unacceptable. The results of nitrate and nitrite concentrations are shown in *Table 2*.

DISCUSSION

The presence of *Salmonella* spp. in fermented meat products have been examined in several studies. Oksuztepe et al.[15] demonstrated that *Salmonella* spp. was isolated from 3.0% of products. Erdogrul and Ergun [16] reported that 1.66% was found to be the positive for *Salmonella*

Table 1. *Prevalence of Salmonella spp., L. monocytogenes and E. coli O157 in sucuk and pastırma samples*
Tablo 1. *Sucuk ve pastırma örneklerinde Salmonella spp., L. monocytogenes ve E. coli O157 prevalansı*

Products	*Salmonella* spp.		*L. monocytogenes*		*E. coli* O157	
	+	%	+	%	+	%
Sucuk (n=132)	2	1.52%	2	1.52%	-	-
Pastırma (n=66)	3	4.55%	2	3.03%	-	-
Total (n=198)	5	2.53%	4	2.02%	-	-

+: number of positive samples

Table 2. *The results of nitrate and nitrite concentrations in sucuk and pastırma samples*
Tablo 2. *Sucuk ve pastırma örneklerinde nitrat ve nitrit konsantrasyon sonuçları*

Parameters	Sucuk (n=132)			Pastırma (n=66)			Satisfactory Limit by TFC	Unacceptable Samples (Sucuk/Pastırma)
	Min	Max	Mean	Min	Max	Mean		
Nitrate (mg/kg)	28.10	174.62	87.28	64.12	187.66	108.83	250	(0/0)
Nitrite (mg/kg)	6.41	90.02	24.83	4.26	46.28	17.33	50	(5/0) (3.79%)

TFC: *Turkish Food Codex*

spp. The findings were highly consistent with these results (1.52% in sucuk and 4.55% in pastırma). In other studies, no *Salmonella* spp. was isolated [17,18]. Contrarily, two studies which reported higher results (5.0% and 7.0%) than this study were those of Kok et al.[19] and Siriken et al.[20]. They explained the high *Salmonella* spp. prevalence with varying hygiene applications at slaughterhouses and meat markets. Different detection rates may originate from detection methods, sampling procedures and the sanitation applications.

The prevalence of *L. monocytogenes* in the fermented samples tested in this study was lower than those detected by Farber et al.[21] in Canada (20.0%), Cantoni et al.[22] in Italy (13.0%), Jemmi et al.[23] in Switzerland (15.0%) and Colak et al.[24] in Turkey (11.6%). On the other hand, Mena et al.[25] reported the prevalence rate of *L. monocytogenes* was 3.7% in 27 Spanish fermented sausages while Ciftcioglu and Ugur [26] detected only 2.0% in their Turkish samples. The findings of this study showed similarity with the mentioned results. Differences between the findings from these studies can be related to production techniques, contaminations after production process, preservation conditions and inadequate personal hygiene. In this study *Listeria* species were also detected in 9 samples as *L. innocua* (in 5 samples), *L. seeligeri* (in 2 samples) and 2 *L. welshimeri* (in 2 samples).

According to the results from this study, *E. coli* O157 was not detected. Similar results were reported by Ferreira et al.[27] in Portugal and Siriken et al.[20] in Turkey. In Argentina, 4.8% of 83 fresh sausages and 3.3% of 30 dry sausages were contaminated with *E. coli* O157 [28]. Tilden et al.[29] reported that *E. coli* O157:H7 was linked to consumption of fermented salami in USA. *E. coli* O157 presents sporadically at very low levels together with very high levels of competitor organisms which is making it difficult to detect.

Regarding the contamination rate of sausages, the results in this study were low. The reason for the low contamination rate is likely due to the fermentation process which reduces the number of pathogens during curing or storing time. Lactobacilli play an important role in the protection against the pathogens and in the development of flavour by producing lactic acid [30]. The presence of lactic acid accelerates pH decline and water activity, improving the safety and stability of these products [31].

In the present study, the nitrate concentrations of samples were found a mean of 87.28 mg/kg in sucuk and 108.83 mg/kg in pastırma. The mean value of nitrite concentrations was 24.83 and 17.33 with a range of 6.41 to 90.02 mg/kg and 4.26 to 46.48 mg/kg in sucuk and pastırma samples, respectively. Today, many countries enforce production limits of nitrate and nitrite. In general, 250 mg/kg of nitrate and 50 mg/kg of nitrite are to be permitted for all meat products [32].

In the present study, the highest rates of *Salmonella* spp. (4/5) and *L. monocytogenes* (3/4) were detected in June, July and August which have the highest temperatures in Turkey. Temperature is considered the most critical factor for the microbial quality of meat at the stage of manufacture, distribution and consumption. Microbial growth is seen corresponds directly with temperature increase [33].

Nitrate is necessary in a long curing process to act as a source of nitrite [34]. In the present study, the nitrate level of sucuk and pastırma samples were found all to be in the acceptable range. The findings corroborated studies of Sanlı and Kaya [35] and Servi [36]. On the contrary, higher values were found by Sezer et al.[37] and Soyutemiz and Ozenir [38]. Five (3.79%) of the sucuk samples exceeded the nitrite limit value, but the nitrite level in pastırma was within the satisfactory limit. Similar results were reported by Aksu ve Kaya [39], El-Khateib et al.[40] and Sancak et al.[41].

In conclusion, the result of this study confirmed that meat products can become contaminated pathogens such as *Salmonella* spp. and *L. monocytogenes*, which can cause serious public health problems. The quality of raw meat, heat treatment of the meat, the activity of the starter culture, the salting process and the storage conditions are

the most important points of control for the prevention of growth/survival of undesirable microorganisms during the manufacturing process [42]. Therefore, it is essential and beneficial to apply good hygienic practice, good manufacturing practice, HACCP and to develop standard production procedures for controlling foodborne pathogens and enhancing the safety of food. Further studies to identify the risk factors for the presence of pathogens in the production of sucuk and pastırma should be carried out.

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Research Article

Isolation and Characterization of Olfactory Stem Cells from Canine Olfactory Mucosa [1]

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Abstract

Olfactory stem cells have great potential in the treatment of neurodegenerative diseases and they are good candidates for cell therapy due to the easy accessibility of olfactory mucosa. The main objectives of this study were isolation, proliferation and characterization of olfactory mucosa stem cells that were further differentiated into olfactory neurospheres derived cells. When grown on poly-D-lysine with a serum-free culture medium supplemented with EGF (50 ng/ml) and FGF2 (50 ng/ml), olfactory stem cells gave rise to neurospheres. When grown in serum-containing culture medium newly plated spheres gave rise to olfactory neurosphere derived cells. Gene expression analysis revealed that, OCT4, SOX2, Nanog, Nestin, β-tubulin and NCAM were expressed in olfactory stem cells. While the mRNA expressions of Nanog, Nestin, Oct4, βIII-tubulin and NCAM were downregulated in neurospheres, the mRNA expression of SOX2 upregulated in neurospheres. According to the gene levels of neurospheres generated from olfactory stem cells, beta tubulin and NCAM gene expressions were upregulated, whereas OCT4, Nanog, Sox2 and Nestin mRNA expressions were downregulated in Olfactory neurospheres derived cells. Olfactory mucosa of canine is a suitable alternative source of stem cells and can be applied to cell therapy in neurodegenerative diseases.

Keywords: *Olfactory stem cell, Canine, Neurosphere, Pluripotent*

Köpek Olfaktorik Mukozasindan Olfaktorik Kök Hücrelerin Izolasyonu ve Karakterizasyonu

Özet

Olfaktorik kök hücreler nörodejeneratif hastalıkların tedavisinde büyük bir potansiyele sahiptir ve olfaktorik mukozaya kolay erişilebilirliği sayesinde hücre tedavisi için uygun bir adaydır. Bu çalışmanın amacı olfaktorik nörosfer kaynaklı hücrelere kadar farklılaştırılabilen olfaktorik kök hücrelerin izolasyonu, proliferasyonu ve karakterizasyonudur. Olfaktorik kök hücreler EGF (50 ng/ml) ve FGF (50 ng/ml) içeren serumsuz kültür vasatı içerisinde kültüre edildiğinde nörosferleri oluşturdular. Serum içeren kültür vasatında tekrar kültüre edildiklerinde olfaktorik nörosfer kaynaklı hücreleri şekillendirdiler. Gen ekspresyon analizleri OCT4, SOX2, Nanog, Nestin, β-tubulin ve NCAM genlerinin olfaktorik kök hücrelerinde eksprese olduğunu ortaya çıkardı. Nanog, Nestin, Oct4, β tubulin ve NCAM gen ekspresyonları nörosferlerde downregüle olurken, SOX2 geni upregüle oldu. Olfaktorik kök hücrelerin oluşturduğu nörosferlerin gen seviyeleri ile karşılaştırıldığında olfaktorik nörosfer kaynaklı hücrelerde β tubulin ve NCAM gen ekspresyonları upregüle olurken OCT4, Nanog, Sox2 and Nestin mRNA ekspresyonları downregüle oldu. Köpeğin olfaktorik mukozası uygun alternatif bir kök hücre kaynağıdır ve köpek nörodejeneratif hastalıklarında hücre tedavisi için uygulanabilir.

Anahtar sözcükler: *Olfaktorik kök hücre, Köpek, Nörosfer, Pluripotent*

INTRODUCTION

Neurogenesis takes place in three primary areas in the nervous system. These areas include: the subgranular zone, which supplies new granule cells to the dentate gyrus of the hippocampus; the subventricular zone, which supplies new interneurons to the olfactory bulb; and the olfactory neuroepithelia, which generates new excitatory sensory

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neurons that extend their axons to the olfactory bulb [1]. NSCs (Neural stem cells) that are derived directly from CNS (Central nervous system) tissue are considered to be safe and non-tumorigenic [2]. Furthermore, NSCs are excellent candidates for cellular transplantation therapy because they have been shown to replace the dead or dying neural tissues, elaborate trophic factors to rescue dysfunctional endogenous neurons, inhibit inflammation, and deliver therapeutic proteins in a widely disseminated manner [3-7]. However, harvesting such cells directly from the CNS is an invasive procedure with ethical considerations [8]. OM (Olfactory mucosa) of human and dog can easily be obtained from cribriform plate of ethmoidal bone with a non-invasive nasal biopsy [9-12]. Thus, stem cells derived from canine OM stand as a promising candidate for a source of autologous graft, due to their accessibility [10,12,13].

It is well known that new neurons are continuously generated from the stem cells in OM throughout life [14,15]. Neurogenesis within the OM is substantiated by niches of stem cells, located both in the OE (olfactory epithelium) and in the underlying olfactory lamina propria. Within the OE, two distinct populations of stem cells contribute to the neurogenic process, namely the HBCs (horizontal basal cells) and the GBCs (globose basal cells) [16]. A new stem cell population from mesenchymal stem cell family has been recently discovered in lamina propria of OM [17-19]. Lamina propria derived stem cells named as ecto-mesenchymal stem cells have attracted the interest of the researchers, having advantages of easily accessible location, a high proliferation rate, an ability to proliferate in long-term cultures and a tendency to differentiate into neural cells [20]. Therefore, OM has been considered to be an essential source for adult neural stem cells. Neural stem cells in OM are multipotent and can be grown into NSs (neurospheres), as well as further differentiate into neurons, astrocytes, and oligodendrocytes *in vitro* [21]. Thus the generation of NSs is often considered as a sufficient evidence for the existence of a stem cell.

The importance of the dog, being as a large animal model of human neurodegenerative diseases, has led to interest in the isolation and characterization of dog stem cells derived from various tissues such as adipose, bone marrow and amnion [22-24].

In this study, our aim was to isolate and characterize a stem cell population from canine OM as an alternative source of adult stem cells rather than bone marrow and adipose tissue for the treatment of neurodegenerative diseases in canine.

MATERIAL and METHODS

Cell Culture

OM was obtained from each dog according to previous description [25]. Primary cell culture was performed according to a previous report [10]. Briefly the mucosal biopsies were dissected under a stereomicroscope to remove cartilage fragments, blood vessels, connective tissues and non-olfactory mucosa. The remaining OM was rinsed three times with Hank balance salt solution (HBSS) with 1% PS (Peniciline and Streptomycine; Invitrogen; 15140122), and transferred (with minimal dissection) into a 35 mm petri dish containing HBSS with 1% PS. OM was cut into pieces of about 1 mm^3 with a scalpel blade for 1 min *(Fig. 1a,b)*, and by applying explants culture, the tissue was kept in culture flasks of 25 cm^2 at 37°C in High Glucose DMEM/F-12 medium (Biochrom, Cat. #FG-0445) supplemented with 10% fetal bovine serum (FBS, Biochrom Cat. #S0113), and 1% PS. After 7 days of incubation of the explants in culture medium, medium was begun to change every two days. These cells were confluents after 8 day of culture. At this time, the culture medium was aspirated and cells were washed with HBSS. Then, the cells were incubated with 1 ml of trypsin-EDTA solution (Gibco Cat #25200-056) for 5 min at 37°C. The separated cells were collected, centrifuged and re-plated at the rate of 1:2 for subculture. The medium was changed each two days up to the confluence of the cells in the flask.

Olfactory Neurosphere Formation and Growing

To form NSs, trypsinized cells were re-plated into culture T25 flask pretreated with poly-D-lysine and fed with DMEM/HAM F12 (Serum-free culture medium, Invitrogen #31331-028) supplemented with ITS-X 1%, (insulin, transferrin, selenium invitrogen #51500056); EGF (epidermal growth factor, 50ng/ml, R&D Systems Cat. #236-EG); FGF2(basic fibroblast growth factor, 50 ng/ml, Cat. #233-FB) and 1% PS as previously described [9]. In order to collect olfactory neurospheres lysates, the culture medium with the floating NSs was transferred to 15 ml tubes. Then, 2 ml of DMEM/HAM F12 was added to the flask, and with a micropipette, fluxes and refluxes were performed to release the NSs that were still adherents. These NSs in suspension were added to the same 15 ml tubes.

The tubes were centrifuged at 1.100 rpm for 3 min (Nuve NF 800R) and the supernatants were removed.

These were dissociated with trypsin, replated into T25 flasks and cultured in serum containing culture medium. These ONS (olfactory neurospheres derived) cells were then expanded by passage and these cells were stored in -80ºC for Real Time PCR analysis.

Total RNA Isolation and mRNA Expression Levels of Genes by Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated by RNAeasy kit (QIAGEN) and cDNA was generated with a First Strand cDNA Synthesis kit (Thermo Scientific) at a total volume of 20 µl according to the manufacturer's instructions. Real-time quantitative

PCR was performed in a Strategene Mx3005P QPCR system (USA). Expression levels of target genes were normalized to the housekeeping gene β-actin (ΔCt). Gene expression values were then calculated based on the ΔΔCt method using the equation: RQ = 2−ΔΔCt. PCR amplification was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). The primer sequences used in PCR reactions and PCR conditions are described in *Table 1*. Each assay was performed in triplicate and repeated three times.

RESULTS

Each slice of OM was plated in T25 flask and fed with a serum- containing High Glucose DMEM/F-12 medium. After 5 to 7 days, OM derived cells started to grow out of the explant and proliferate *(Fig. 1c)*. The culture medium was totally renewed every 2 days. When the culture had reached confluency after 8 days, the cells were passaged and grown in T75 flasks to obtain large quantities of cells. In the new flasks culture, proliferation, expansion and cell clusters formations were also observed after passage. Primary cultures were mainly composed of elongated adherent cells *(Fig. 1d)*.

To assay the potential of OS (olfactory stem) cells for generatation of NSs, OS cells were plated onto poly-D-lysine coated T25 flask culture petri dishes filled with DMEM/ HAM F12 supplemented with ITS-X (1%), EGF and FGF2. OS cell generated NSs by the next day. As shown in *(Fig. 1e,f)*, the NSs had a spherical structure.

In order to assay their ability to differentiate into ONS cells, olfactory NSs were collected and re-plated in serum containing culture medium. The medium was totally renewed once every 2 days. ONScells rapidly proliferated as an adherent monolayer *(Fig. 1g,h)*.

mRNA Expression Levels of Genes in Olfactory Stem Cells, Neurosphere, Olfactory Neurospheres-Derived Cells

Real time PCR analysis showed that OS cells expressed pluripotent stem cell genes such as OCT4, Nanog, Sox2

Table 1.*Oligonucleotide primer sequences and PCR programs*
Tablo 1. *Oligonükleotid primer sekanslarıve PCR programları*

Transcripts	Primer Sequences	PCR Programs	Cycles
Nestin	F: 5′-5′-GAGAACCAGGAGCAAGTGAA-3′ R: 3′-TTTCCAGAGGCTTCAGTGTC-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	35
βIII-tubulin	F: 5′-GAGGGCGAGATGTACGAAGA-3′ R: 3′-CCTATGGTGGGAAAACAGGA-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	35
GFAP	F: 5′-CGAGTTACCAGGAGGCACTA-3′ R: 3′-TCCACGGTCTTTACCACAAT-5′	In.95˚C 5′/95˚C 10′-56˚C 30″-72˚C 1′	35
NCAM	F: 5′-AGGCAGAGCATAGTGAATGC-3′ R: 3′-AGGCTTCACAGGTCAGAGTG-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	35
NANOG	F: 5′-GAATAACCCGAATTGGAGCAG-3′ R: 3′-AGCGATTCCTCTTCACAGTTG-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	45
OCT4	F: 5′-GCAGTGACTATTCGCAACGA-3′ R: 3′-ATTTGAATGCATGGGAGAGC-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	35
SOX2	F: 5′-AGTACAACTCCATGACCAGC-3′ R: 3′-ATCATGTCCCGGAGGTC-5′	In.95˚C 5′/95˚C 10′-58˚C 30″-72˚C 1′	35
GAPDH	F: 5′-TGACACCCACTCTTCCACCTTC-3′ R: 3′-CGGTTGCTGTAGCCAAATTCA-5′	In.94˚C 2′/94˚C 20″-55˚C 15″-72˚C 1′	35

Table 2. *Comparison of mRNA expression levels of genes*
Tablo 2. *Genlerin mRNA ekspresyon düzeylerinin karşılaştırılması*

Groups	mRNA Expression Levels of Genes (fold increase +/decrease -)						
	Oct4	Nanog	Sox2	Nestin	β Tubulin	NCAM	GFAP
1	1.0	1.0	1.0	1.0	1.0	1.0	-
2[a]	(-) 1.8	(-) 1.8	(+) 1.3	(-) 1.6	(-) 2.8	(-) 5.2	-
3	1.0	1.0	1.0	1.0	1.0	1.0	-
4[b]	(-) 2.1	(-) 1.1	(-) 3.4	(-) 1.1	(+) 1.4	(+) 3.3	-

a: *Compare to the group 1. Group 1: OS cell, Group 2: neurospheres generated from OS cells;* ***b:*** *Compare to the group 3. Group 3: neurospheres generated from OS cells, Group 4: olfactory neurospheres-derived cells*

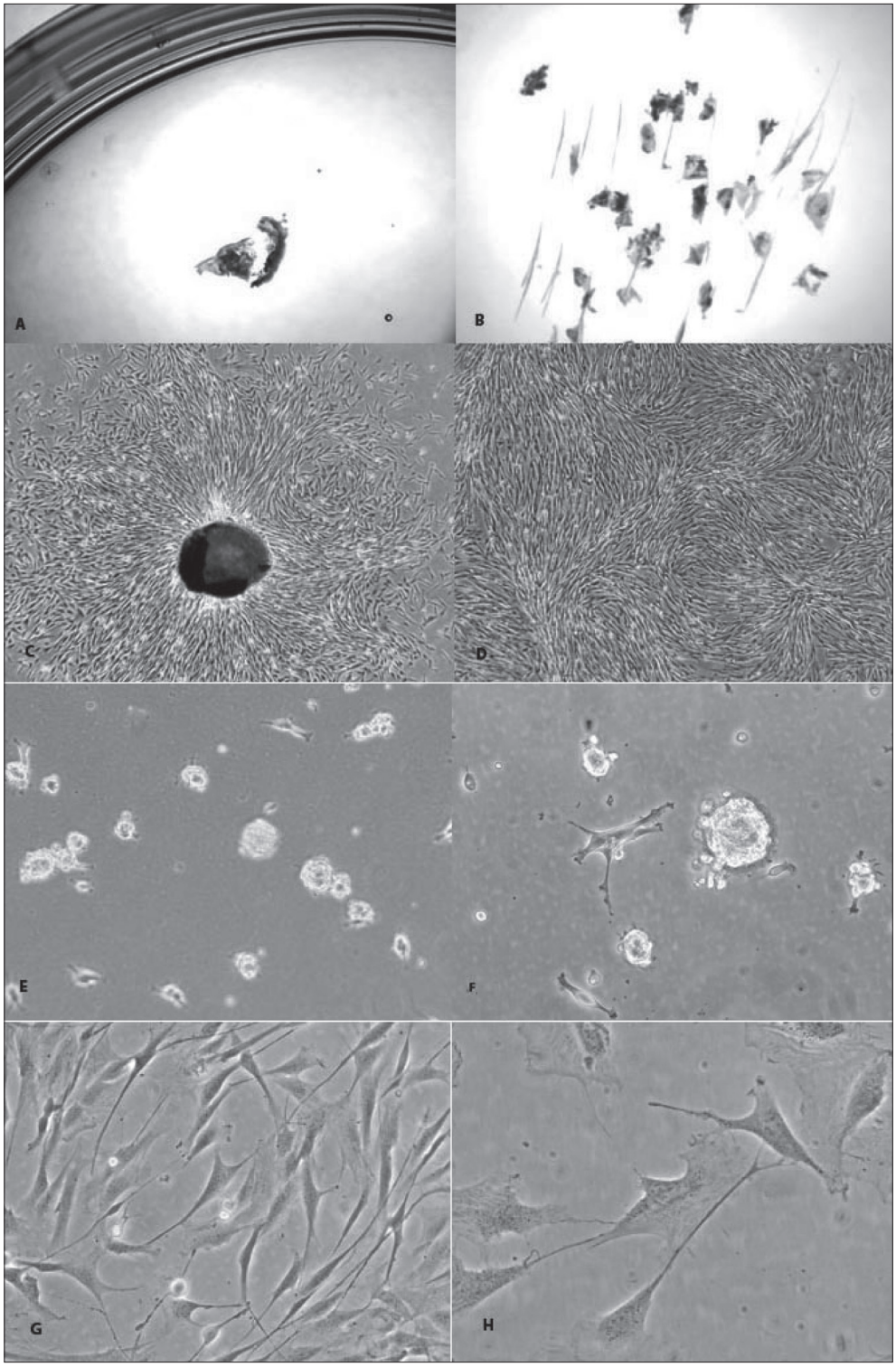

Fig 1. A. OM collection and B. OM tissue pieces of about 1 mm^3; C. cells adhesions close to the fragments; D. spindle shaped adherent cells; E and F. neurosphere formation; G and H. ONS cells

Şekil1. A. OM toplanması ve B. Yak-laşık 1 mm^3 büyüklükte OM doku parçaları; C. doku parçacıkların yakınında hücre tutunmaları; D. mekik şeklinde tutunan hücreler; E ve F. nörosfer şekillenmesi; G ve H. ONS hücreleri

genes, neural stem cell gene Nestin and neural specific genes NCAM, beta tubulin III, but the expression of astrocyte-specific gene that was GFAP was not present *(Table 2)*.

The expression of the neural stem cell marker Nestin was downregulated in the NSs derived from OS cells. Also, beta tubulin and NCAM expressions were determined to be downregulated in the NSs. GFAP was detected in

neither NS nor ONS cells as in OS cells. In particular, the expression of pluripotent stem cell markers OCT4 and Nanog was downregulated in NSs when compared with the mRNA levels in OS cells. But the expression of another pluripotent stem cell marker Sox2 was found high in the NSs *(Table 2)*. According to the gene levels of NSs generated from OS cells, beta tubulin and NCAM gene expressions were upregulated, whereas OCT4, Nanog, Sox2 and Nestin mRNA expressions were downregulated in ONS cells *(Table 2)*.

DISCUSSION

OM generates 2 distinct types of NSs as follows: mesenchymal-like olfactory stem cells from lamina propria and epithelial-like OS cells from OE [9,20]. OE stem cells are similiar to olfactory propria stem cells with their identical primary cultures morphological appearance, expression of the stemness marker nestin or ability to form NSs which can subsequently proliferate as ONS cells or terminally differentiate into neuron-like cells [20]. Thus, we did not need to separate OE from lamina propria to isolate stem cells from each other. We collected canine OM according to a previous report [25] and to obtain OS cells, we chosed the non-enzymatic method in which the tissue slices left undisturbed for five days caused adherence of the explants to the plastic surface and subsequent emergence of the cell population [26]. Some researchers reaffirmed our approach [17,27] that nonenzymatic methods conserved the quality of the both OS cells and olfactory ensheating cells. Explantation causes minimal trauma to the tissue which is critical for cell quality. Within 5-7 days of explant culture, the spindle-shaped cells were observed migrating out of tissue explant onto the culture dish. Similar observations were observed by Alves et al.[10]. The migrated cells possessed typical mesenchymal morphology and continued to proliferate till 7 days. The cells were maintained in culture for a total of 15 days to attain confluence. When the culture reached confluence, the cells were passaged and transfered into the new culture flask. We observed OS cells in the form of elongated- spindle shaped morphology in the culture flask.

Various culture conditions generate NSs [19,28-32] that differ varying by species (human, rat, mouse), developmental stage (embryo, neonatal, adult), presence of serum, chemicals, and trophic factors. Here we used a serum-free culture method (supplemented with ITS, EGF and FGF2) to generate NSs from adult canine OS cells, because stem cells differentiated in the presence of growth factors and serum-free medium are safer for clinical use [24]. At the same time EGF has been demonstrated to be a mitogen for neural stem cells as has FGF2, and both factors in combination have been used to expand neural stem cells [16]. Also FGF2 causes growth in neural tube formation [33]. Thus, EGF and FGF2 together resulted in the most neurospheres forming [16]. Once the OS cell reach confluent in serum containing culture medium, they were plated on poly-D-lysine coated T25 flask that was filled with serum free culture medium supplemented with ITS, EGF and FGF2 at a 1:2 split ratio. The following day, OS cells had given rise to NSs under neurospheres forming conditions. Optimal cell plating density for NS formation is important. Optimal plating cell density should be 16.000 cell/cm^2 according to Girard et al.[9] but it should be 50.000 cell/cm^2 according to Carvalho et al.[20]. We got the best results for NS formation when we cultured the stem cells at a 1:2 split ratio. In this way, we successfully made OS cells turned into neurospheres in one day. In the present study, the expressions of the neural stem cell marker Nestin as well as the neural precursor markers βIII-tubulin and NCAM were detected in canine OS cells. In previous studies [23,24,34], these markers were also found in mesenchymal stem cells which were consistent with our findings. On the otherhand, we could not determine the GFAP mRNA expression in stem cells consisted with results of the study of Chung et al.[24]. Furthermore, ecto-mesenchymal stem cells (also named "mesenchymal-neural" precursors) have the capacity to differentiate into ectoderm and mesoderm cell types. Accordingly, ecto-mesenhcymal stem cells can also be expressing these neural markers in olfactory mucosa. At the same time, co-expression of neural markers such as Nestin, βIII-tubulin and NCAM except for GFAP shows high potential of canine OS cells to differentiate into multiple neural lineage in vitro. Nestin is expressed not only in nervous system organs such as olfactory mucosa but also in other organs and tissues. This may be the evidence that nestin-containing cells are pluripotent and may not be exclusively of neuroepithelial origin, such that nestin cannot be unambiguously interpreted as a marker of neural stem/progenitor cells [35]. Additionally, olfactory stem cells differ from bone marrow stem cells by over-expressing CD9 and under-expressing CD146 and CD200 [17]. CD9 belongs to the tetraspanin family and is considered as a pluripotency marker [36]. Afterwards Chaker N. Adra discovered and patented [37] that pluripotent stem cell populations could be obtained from olfactory mucosa and reported that some cells of the pluripotent stem cell population could differentiate into cells of one or more various lineages such as mesenchymal lineages or neuronal lineages or both. Thus, in this study, we also investigated the expression of pluripotent genes such as OCT4, Nanog and Sox2 because canine embryonic stem cells were demonstrated to express Oct4, Nanog, and Sox2 at high levels [38]. Sox2 governs ESC specification to neuroectoderm while Oct4 and Nanog promote their differentiation to mesendoderm, a common precursor of mesoderm and definitive endoderm [39], and Sox2 is a critical factor for directing the differentiation of pluripotent stem cells to neural progenitors and for maintaining the properties of neural progenitor stem cells [40]. In this study, we observed expressions of these pluripotent genes in OS cells and detected the down-regulation of Nanog, Oct4 and the upregulation of Sox2

in the NSs derived from OS cells. Furthermore, Nestin expression was downregulated in neurospheres and ONS cells consisted with the results of Carvalho et al.[41]. NSs contain a mixed population consisting of neural stem cells, proliferating neural progenitors and postmitotic neurons and glia [41]. Thus, in this study, the downrgulation of Oct4, Nanong and Nestin and the upregulation of Sox2 in NSs derived from OS cells indicated that there was a decrease in pluripotent characteristics of NSs and NSs highly expressing Sox2 gene have a high potential for differentiating towards functional neurons. Olfactory NSs were collected, dissociated and rapidly grown in the presence of serum as an adherent monolayer of ONS cells. These ONS cells had a flattened and undifferentiated appearance with a marker phenotype similar to the ectomesenchymal cells derived from primary cultures of OM [17,35]. That is, they expressed markers of mesenchymal stem cells (CD105 and CD73) as well as other stem and progenitor cell proteins (Oct4, Nestin, and beta tubulin) [35]. In this study, we demonstrated that ONS cell expressed a lower level of pluriptency genes and a higher level of neuronal genes compared with that of NS. Our findings highlight that pluripotent stem cells could be isolated from OM and they could be differentiated into NS and ONS cells, and therefore, these cells are suitable candidates for cell transplantation in neurodegenerative diseases of canine.

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Research Article

RelA/p65-mediated Innate Immune Response Affecting NDV Replication in CEF

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Abstract

Newcastle disease virus (NDV) is a non-segmented and single-stranded negative-sense RNA (-)ssRNA virus. Previous studies indicate that NDV elicit hosts to induce strongly innate immune responses. The virus replication can cause hosts to express more type I interferon (IFN) via nuclear kappa B (NF-kB) pathways and further activate IFN-stimulated genes (ISGs) to retard virus growth and induce cells for developing specific protection. This study investigated the role of RelA/p65 in innate immune responses against NDV replication by using the virus infection chicken embryo fibroblast cells (CEF). The result showed that the mRNA and protein levels of RelA/p65 increased markedly in CEF infected with strains of NDV which differed in their virulence, and RelA/p65 translocated from the cytoplasm to the nucleus. Further research indicated that specific siRNA could inhibit expression of RelA/p65, accordingly, mRNA levels of IFN-α, IFN-β and STAT1 decreased significantly and the replication kinetics of 2 NDVs were enhanced after inhibition of RelA/p65. In conclusion, CEF can synthesize more type I IFN via RelA/p65 pathways after NDV infection, which retards NDV replication and spreads in the early phase of viral infection.

Keywords: *NDV, RelA/p65, Interferon, Virus replication, CEF*

CEF'teki NDV Replikasyonunu Etkileyen RelA/p65-güdümlü Doğal İmmun Tepki

Özet

Newcastle hastalığı virüsü (NDV) tek-parçalı ve tek-sarmallı negatif-yönlü RNA (-) SsRNA virüsüdür. Önceki çalışmalar, NDV'nin konakçıların güçlü doğal bağışıklık tepkilerini indüklediğini göstermektedir. Virüs replikasyonu nükleer kappa B (NF-kB) yolakları üzerinden daha fazla tip I (IFN) interferon ekspresyonuna, ve ayrıca IFN-uyarımlı genlerin (ISGs) aktive ederek daha fazla virüs büyümesini geciktirmek ve spesifik korumanın geliştirilmesi için hücrelerin uyarılmasına neden olabilir. Bu çalışmada, tavuk embriyo fibroblast hücreleri (CEF) virüs enfeksiyonu kullanılarak NDV replikasyonuna karşı doğal bağışıklık yanıtlarında RelA/p65'in rolü araştırıldı. Sonuç, RelA/p65'in mRNA ve protein düzeylerinin virülansları farklı NDV suşlarıyla enfekte CEF'de belirgin oranda arttığını ve RelA/p65'in sitoplazmadan çekirdeğe transloke olduğunu gösterdi. Ayrıca, araştırma spesifik siRNA'nın RelA/p65 ekspresyonunu inhibe edebildiğini, buna uygun olarak, IFN-α, IFN-β ve STAT1 mRNA düzeylerinin önemli ölçüde azaldığını ve 2 NDV'nin replikasyon kinetiğinin RelA/p65 inhibisyonu sonrası geliştirildiğini gösterdi. Sonuç olarak; CEF, NDV enfeksiyonu sonrasında RelA/p65 yolakları aracılığıyla daha fazla IFN türü sentezleyebilir ki, bu NDV replikasyonunu geciktirir ve viral enfeksiyonun erken döneminde yayılma gösterir.

Anahtar sözcükler: *NDV, RelA/p65, İnterferon, Virüs replikasyonu, CEF*

INTRODUCTION

Newcastle disease (ND) is recognized as a significant poultry disease; it is caused by Newcastle disease virus (NDV), a member of the Paramyxoviridae family [1]. NDV is a non-segmented and single-stranded negative-sense RNA virus. NDV isolates are usually classified into three groups on the basis of their virulence: non-virulent (lentogenic), mildly virulent (mesogenic) and highly virulent (velogenic). The NDV genome is approximately 15200 nucleotides long, and it composed of six units that encode their corresponding structural proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and a

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RNA-dependent RNA polymerase (L) [1,2]. RNA editing of the P protein produces additional non-structural proteins V and W [3]. NDV V protein can block various components of interferon (IFN) pathways to help the virus replication and spread in host [3-6].

The host's innate immune response to virus infection is an immediate reaction designed to retard virus growth and help the host develop specific protection [7-9]. One key protein that regulates the response is the nuclear transcription factor, NF-kB, which is held quiescent in the cytoplasm when incomplex with IkBa [10,11]. Once in response to virus via transmembrane toll-like receptors (TLRs) or the cytoplasmic sensors (e.g., RIG-I, MDA5, PKR and NOD proteins), IkBa is phosphorylated by IKK and subject to ubiquitin-dependent proteasomal degradation, allowing NF-kB to translocate to the nucleus from the cytoplasm and activate the transcription of a cascade of proinflammatory cytokines and chemokines to induce inflammatory and antiviral responses [12-15]. Of particular interest is RelA/p65, a subunit of the heterodimeric NF-kB protein, which had gained attention for its diverse roles. It plays a central role in initiating the host's antiviral responses by inducing IFNs and ISGs [16,17].

Past research showed that NDV not only could induce IFNα and IFNβ mRNA in macrophages, IFNγ mRNA in peripheral blood mononuclear cells *in vitro* [18-20], but also IFNα and IFNγ mRNA in splenocytes and peripheral blood *in vivo* [21]. Yet this existing knowledge of NDV does not provide a full understanding role of RelA/p65 in the IFN pathway, and whether the pathway affects NDV replication. Here, using an in vitro model of NDV infection in CEF, we examined the global expression of RelA/p65, IFN-α/β and STAT-1, analysed the sub-cellular localisation of RelA/p65 protein in NDV-infected CEF, and determined NDV replication kinetics after inhibition of RelA/p65. The experimental results indicated that two strains of NDV, which differed in their virulence could upregulate the RelA/p65-mediated pathway, increased the expression of IFN-α and IFN-β, and limited the NDVs replication.

MATERIAL and METHODS

Cell Culture and Viruses

CEF and Vero cells were cultured under an atmosphere of 5% CO_2 at 37°C in D-Minimum Essential Medium (D-MEM, GIBCO®) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Ontario, Canada). The velogenic NDV strain GM (of VII genotype) was stored in our laboratory and the lentogenic NDV strain LaSota was a kind gift from the National Reference Laboratory for Newcastle Disease. The GenBank accession numbers of the GM and LaSota strains are DQ486859 and AF077761, respectively. The virus titers were determined on CEF by standard endpoint dilution and expressed as 50% tissue culture infection doses ($TCID_{50}$) per ml. UV-inactivation of viruses were performed by using a UV Stratalinker 2400 (Stratagene, USA) to prevent the expression of NDV hemagglutinin-neuraminadase (HN) viral gene, and evaluated by immuno-fluorescence analysis.

Real-time Quantitative Polymerase Chain Reaction (qPCR)

CEF were infected with NDV strain LaSota or GM at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$/cell when they were grown to 70-80% confluence in 12-well plates. The cells were harvested at 3, 6, 12 and 24 h post infection (h p.i). Total RNA was extracted by using RNeasy® Mini kit (Promega, U.S.) and was used as a template for synthesizing cDNA strands. QPCR was prepared by using the following primers: 5'-AAGCCGACAAAACCACCC-3' (IFN-α-sense), 5'-CAGGAACCAGGCACGAGC-3' (IFN-α-anti-sense), 5'-CCTCAACCAGATC -CAGCATT-3'(IFN-β-sense) and 5'-GGATGAGGCTGTGAGAGGAG-3'(IFN-β-antisense), 5'-TG AACGGATCCGCACCAATA-3' (RelA/p65-sense), 5'-CGTTCAC CCACACCTGGAAG -3 '(RelA/p65-antisense), 5'-GAACGGCTA CATTAGGACTG-3' (STAT1-sense), 5'-GATCCGAGA-TACCT CATCAAAC-3' (STAT1-antisense), 5'-CCTCTCTGGCAAAGTCC AAG -3' (GAPDH-sense), 5'-CATCTGCCCATTTGATGTTG -3' (GAPDH-antisense).

Real time PCR (SYBR green) was performed by the ABI 7500 real-time PCR detection system (Roche Applied Bio Systems, Germany). The Tm and Cp values were calculated using the analysis software provided by Roche Applied Science. The experimental data on the mRNA levels of RelA/p65, IFN-α, IFN-β and STAT1 genes were normalized against internal controls, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene. Fold changes in the expression of each gene were compared with expression level in non-infected CEF, and analyzed by a comparative threshold cycle (Ct) method using the formula $2^{-(\Delta\Delta Ct)}$.

Preparation of Cell Extract and Western Blot Analysis

Cells were washed three times with phosphate buffered saline (PBS), and then were lysed by a RIPA buffer containing protease inhibitor PMSF. Lysates were incubated on ice for 10 min and then spun at 12.000xg for 10 min; clarified supernatants were stored at -80°C. The supernatants were diluted 1:1 in a 2×Tris-glycine SDS sample buffer (Invitrogen, U.S.). Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% Tris-glycine gels and then transferred onto nitrocellulose (NC) membranes. The membranes were blocked with 5% skimmed milk and then incubated with anti-RelA p65 antibody which could detect RelA/p65 for chicken (Abcam, U.K.). Following primary incubation, a western blot analysis was performed with a FITC-conjugated goat anti-rabbit antibody (Millipore, Billerica, U.S.) according to the manufacturer's protocol. Protein bands were imaged and quantified with the

Odyssey infrared imaging system (Li-Cor, Odyssey). The GAPDH expression levels, measured in parallel, served as a control.

Immunofluorescence Staining

CEF were infected by NDV (LaSota or GM) with MOI of 0.1 $TCID_{50}$/cell or were treated by NDV (LaSota or GM)-UV with same titer for 24 h. The cells were fixed with 4% paraformaldehyde for 15 min and incubated with anti-RelA/p65 antibody (Abcam, U.K.) in PBS containing 0.1% BSA at 4°C overnight. Following incubation with FITC-conjugated goat anti-rabbit antibody (Millipore, Billerica, U.S.) for 1 h, the cells were stained with the nuclei, 4′, 6-diamidino-2-phenylindole (DAPI; Sigma) for 10 min. After the cells were washed three times, they were photographed with a laser copolymerization microscope (Olympus, Japan).

RelA/p65 siRNA Treatment

CEF were seeded to 30% confluence into 12-well dishes the day before transfection with small interfering RNA (siRNA). The spectific siRNA (GenePharma, China) for chicken RelA/p65 (the sense strand is 5′- CGUGCACAGU UUCCAGAAUTT-3′ and the antisense strand is 5′-AUUCUGG AAACUGUGCACGTT-3′) and non-targeting siRNA (the sense strand is 5′- UUCUCCGAACGUGUCACGUTT-3′ and the antisense strand is 5′-ACGUGACACGUUCGG AGAATT-3′) was transfected into cells at a concentration of 40 pM using the Lipofectamine RNAi Max reagent (Invitrogen, U.S.), Knockdown efficiency was monitored using qPCR analysis for RelA/p65. The mRNA levels of IFN-α, IFN-β and STAT1 were also measured at 24 h following RelA/p65-siRNA transfection into CEF. Fold changes in the expression of each gene were compared with expression level in normal cells.

NDV Growth Kinetics

RelA/p65-siRNA (40 pM) was transfected into CEF when the cells were grown to 70-80% confluence in 6-well plates. Twenty-four hours later, the cells were infected with NDV strain LaSota with MOI of 1 $TCID_{50}$/cell and GM with MOI of 0.1 $TCID_{50}$/cell, respectively. One h after virus adsorption, the cells were incubated in virus-production medium (basal D-MEM with 2 percent FBS). Supernatants were harvested every 12 h and virus titers were determined by $TCID_{50}$ on Vero cells. Briefly, Vero cells were seeded in 96-well plates at a density of 10^4 cell per well. The following day, Vero cells were infected with 100 uL virus samples of serial tenfold dilutions. One hour after virus adsorption, the cells were incubated in D-MEM with 2% FBS for 7 days, and then analysed for cytopathic effect to determine $TCID_{50}$, as described previously [22].

Statistical Analysis

The statistical significance of experiment data was determined by Student's *t*-test.

RESULTS

NDV up-regulate mRNA Levels of IFN-α, IFN-β, STAT1 and RelA/p65

To decipher how NDV enhanced the transcriptional activity of IFN-α, IFN-β and STAT1, we first examined whether NDV altered their mRNA levels along with that of RelA/p65 in NDV-infected CEF. Our results indicate that two strains of NDV could differentially modulate mRNA levels of the four molecules which are central to the innate antiviral response. As shown in *Fig. 1*, after 3 h of NDV LaSota infection CEF, the mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65 were strongly increased by 92, 8, 19 and 23 fold, respectively, in comparison with normal control cells. As time went by, the mRNA levels of the four molecules followed an obvious downward trend. The highly virulent strain GM also induced CEF up-regulated mRNA levels of the four molecules compared to the control group, after 3 h of GM infection CEF, the mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65 were increased by 2.8, 1.4, 1.5 and 12 fold, respectively, in comparison with normal control cells. Compared to strain LaSota, the strain GM induced a lower mRNA level of the four molecules in infected CEF. Taken together, we know that two strains of NDV, which differ in their virulence, upregulated mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65 in CEF, but there were obvious differences between the two NDVs induction mRNA levels of the four molecules.

NDV up-regulate Protein Level of RelA/p65

Western blot analysis was carried out to demonstrate the protein level of RelA/p65 in NDV-infected CEF. The results showed that NDV could induce CEF to synthesize more RelA/p65 protein. We also found differences in the relative amount of RelA/p65 protein induced by two stains of NDV. In NDV strain LaSota infection CEF, the RelA/p65 protein was increased markedly at 3 h p.i., and the amount reached to peak at 12 h p.i. Compared to the control group, the RelA/p65 protein was expressed at a higher level, which was stably maintained after NDV GM infection *(Fig. 2)*.

NDV Promote RelA/p65 Localization in the Nucleus

It has been reported that RelA/p65 is held quiescent in the cytoplasm in cells. In this study, CEF were infected with the NDV of lentogenic strain LaSota or the velogenic strain GM to reveal the subcellular distribution of RelA/p65 (green) and the nuclei (blue). RelA/p65 showed a predominantly nuclear localization pattern in CEF (*Fig. 3,* up two panels). However, in the mock-infected CEF cells, RelA/p65 was localized predominantly in the cytoplasm (*Fig. 3,* middle panel). In order to further gain insight into the translocation mechanism of RelA/p65, the laser confocal analysis was conducted after CEF cells were treated by UV-inactivated NDV strain GM or LaSota for 24 h and the

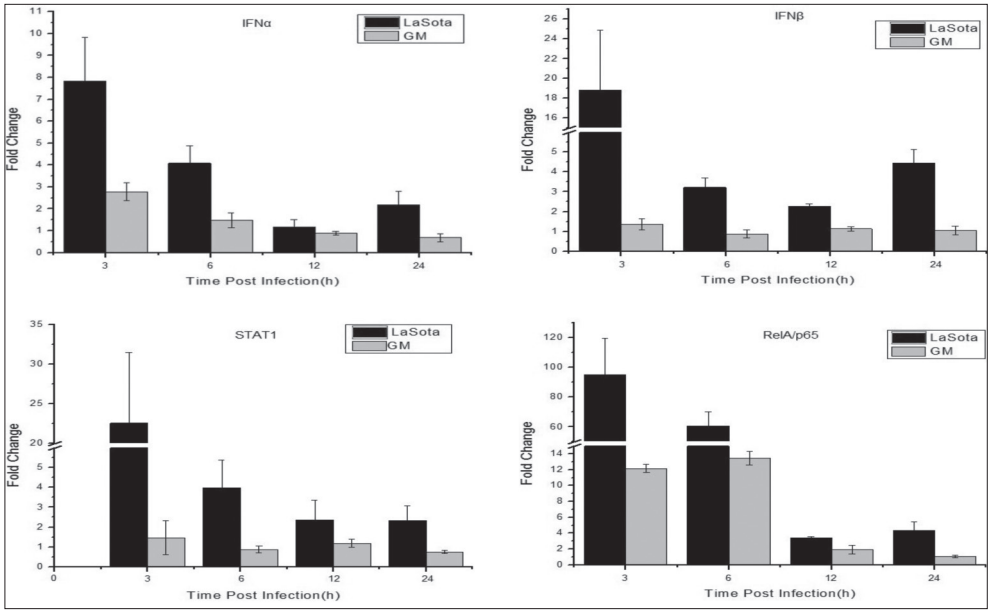


Fig 1. Two stains of NDV alter mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65 in CEF. After CEF were infected with NDV LaSota or GM at a MOI of 0.1 $TCID_{50}$/cell, the cells were harvested at 3, 6, 12 and 24 h p.i. Total RNA was extracted and analyzed by qPCR on mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65. The data were normalized by the internal control, the GAPDH gene, and the fold-change was relative to uninfected cells

Şekil 1. İki NDV suşu CEF'teki IFN-α, IFN-β, STAT1 düzeylerini ve RelA/p65'i değiştirir. CEF'nin 0.1 MOI $TCID_{50}$/hücre dozda NDV LaSota veya GM ile enfekte edilmesinin ardından, hücreler enjeksiyon sonrası (p.i) 3, 6, 12 ve 24. saatte toplandı. Total RNA mRNA üzerindeki IFN-α, IFN-β, STAT1ve RelA/p65 düzeyleri qPCR ile eksrakte edildi ve analiz edildi. Data iç kontrol ile normalize edildi, GADPH geni ve kat-değişimi non-enfekte hücrelere yakın idi

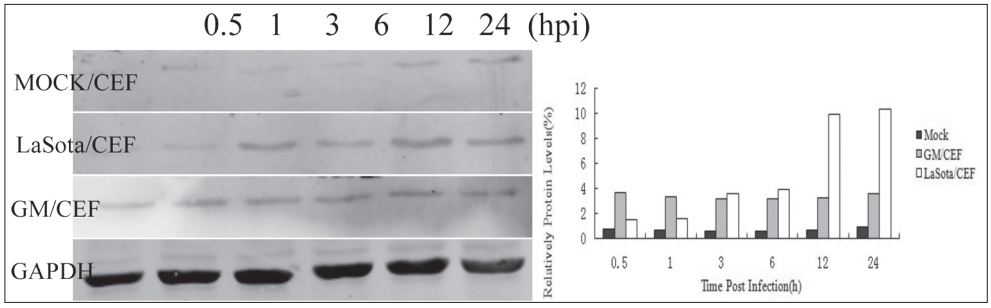


Fig 2. NDV alters the protein expression levels of RelA/p65. CEF were infected with the indicated strains of NDV LaSota or GM (MOI of 0.1 $TCID_{50}$/cell). Total cellular proteins were harvested at 0.5, 1, 3, 6, 12 and 24 h p.i and separated by SDS-PAGE. A western blotting analysis of proteins resolved by SDS-PAGE was performed with antibodies of RelA/p65 and GAPDH protein. Proteins were quantified with the Odyssey infrared imaging system. RelA/p65 protein band intensities were normalized to GAPDH

Şekil 2. NDV RelA/p65 protein ekspresyon düzeylerini değiştirir. CEF, belirtilen NDV LaSota veya GM (0,1 $TCID_{50}$/hücre MOI) suşları ile enfekte edildi. Toplam hücresel proteinler enjeksiyon sonrası 0.5, 1, 3, 6, 12 ve 24. saatte toplandı ve SDS-PAGE ile ayrıldı. SDS-PAGE ile yeniden çözülmüş proteinlerin RelA/p65 ve GAPDH protein antikorları ile Western blot analizi gerçekleştirildi. Proteinler Odyssey kızılötesi görüntüleme sistemi ile ölçüldü. RelA/p65 protein bandı yoğunlukları GAPDH için normalize edildi

results showed that RelA/p65 located predominantly in the cytoplasm (*Fig. 3*, lower two panels).

Inhibition of RelA/p65 down-regulates IFN-α, IFN-β and STAT1

To confirm the role of RelA/p65 in the NF-kB signal, we inhibited RelA/p65 in CEF by specific siRNA. The specific siRNA treatment resulted in significant decline of RelA/p65 by 78 percent at the mRNA level in CEF compared to normal cells. In order to examine whether RelA/p65 regulates the NF-kB signal, mRNA levels of IFN-α, IFN-β and STAT1 were also detected. Real time PCR showed that inhibition of RelA/p65 also led to the down-regulation of the mRNA level of IFN-α, IFN-β and STAT1 by 38, 55 and 61 percent *(Fig. 4)*. Compared to normal cells, the fold change of mRNA levels of RelA/p65, IFN-α, IFN-β and STAT1 were not apparent after treatment cells with non-targeting siRNA of RelA/p65.

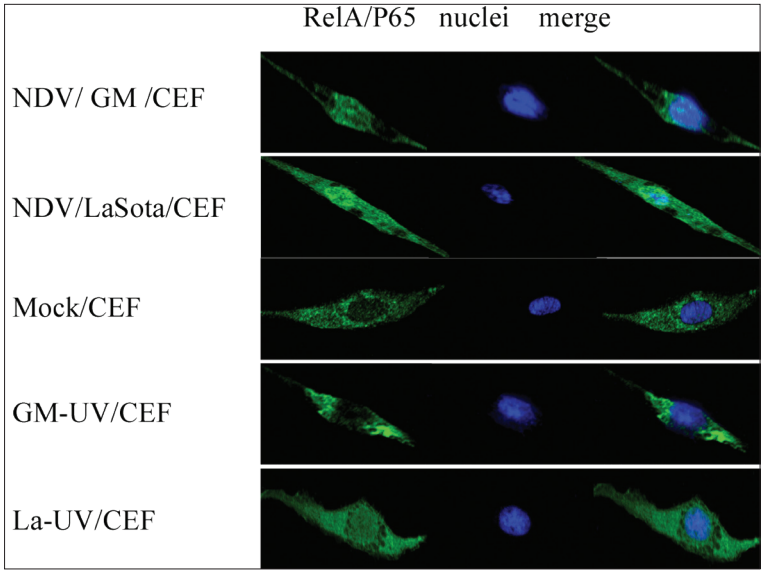
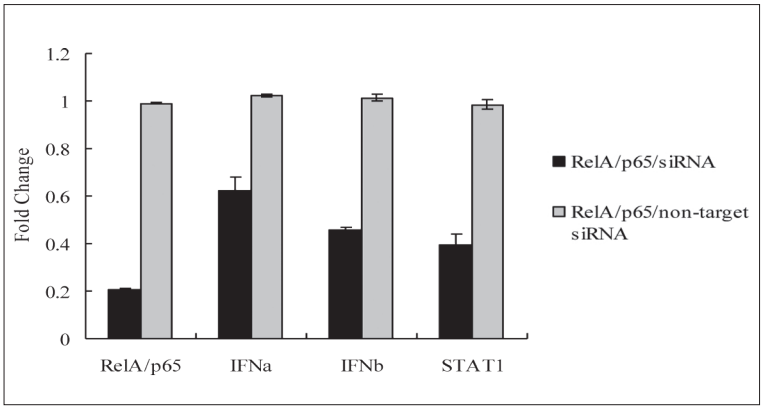


Fig 3. Subcellular localization of RelA/p65 protein in CEF. CEF were infected by NDV strain LaSota or GM with MOI of 0.1 $TCID_{50}$/cell, or were treated with UV-inactivated NDV (NDV-UV) strain LaSota or GM for 24 h. After cells were fixed with 4% paraformaldehyde for 15 min, they were incubated with rabbit RelA/p65 antibody at 4°C overnight and then stained with FITC-conjugated goat anti-rabbit antibody. To stain the nuclei, DAPI was added to CEF at room temperature for 10 min. The cells were observed under a laser copolymerization microscope

Şekil 3. CEF'de RelA/p65 proteininin hücre-içi lokalizasyonu. CEF ya 0.1 TCID50/hücre MOI doz NDV LaSota veya GM suşu ile enfekte edildi, ya da 24 saat süreyle UV ile etkisizleştirilmiş NDV (NDV-UV) LaSota veya GM suşu ile muamele edildi. Hücreler 15 dak. boyunca %4 paraformaldehit ile fikse edildikten sonra, gece boyunca tavşan RelA/p65 antikoru ile 4°C'de inkübe edildikten sonra FITC-konjügeli keçi anti-tavşan antikoru ile boyandı. Çekirdekleri boyamak amacıyla CEF'e 10 dak. için oda sıcaklığında DAPI ilave edildi. Hücreler bir lazer kopolimerizasyon mikroskobu altında gözlendi

Fig 4. Inhibition of RelA/p65 down-regulates the mRNA level of IFN-α, IFN-β, and STAT1. When CEF were grown to 70-80% confluence in 12-well plates, the cells were treated with 40 pM oligonucleotide of RelA/p65 siRNA or control siRNA for 24 h. Then the cells wereæ collected to extract total RNA, the mRNA levels of RelA/p65, IFN-α, IFN-β and STAT1 were determined by qPCR, and the fold-change was relative to normal CEF

Şekil 4. RelA/p65 inhibisyonu IFN-a, IFN-p ve STAT1 mRNA düzeyini aşağı-yöne doğru çevirir. CEF 12-yuvalı plakalar içinde %70-80 kaynaşmaya kadar büyütüldüğünde, hücreler RelA/p65 siRNA'nın 40 uM oligonükleotidi veya kontrol siRNA ile 24 saat boyunca işlemden geçirildi. Daha sonra hücreler, toplam RNA elde etmek için toplandı, RelA/p65, IFN-a, IFN-p ve STAT1 mRNA düzeyleri qPCR ile belirlendi ve kat-değişimi normal CEF'e yakındı



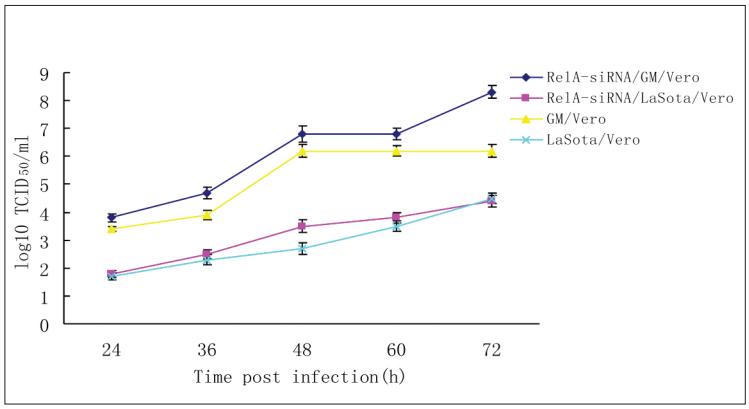


Fig 5. Inhibition of RelA/p65 enhancement of NDV production kinetics. When CEF were grown to 70-80% confluence in 6-well plates, the cells were treated with 40 pM oligonucleotide of RelA/p65 siRNA or control siRNA for 24 h and were infected by NDV strain GM with MOI of 0.1 and LaSota with MOI of 1 $TCID_{50}$/cell, respectively. The cell supernatant was collected at 24, 36, 48, 60 and 72 h p.i., and $TCID_{50}$ was determined on Vero cells

Şekil 5. NDV üretim kinetiğinin RelA/p65 artışını engellemesi. CEF 6-yuvalı plakalar içinde %70-80'lik bir kaynaşmaya kadar büyütüldüğünde, hücreler 40 uM RelA/p65 siRNA oligonükleotidi veya kontrol siRNA ile 24 saat boyunca işlemden geçirildi ve sırasıyla 0.1 MOI NDV LaSota ve 1 $TCID_{50}$/hücre MOI GM suşu ile enfekte edildi. Hücre süpernatantı enjeksiyon sonrası 24, 36, 48, 60 ve 72. saatte toplandı ve Vero hücrelerindeki $TCID_{50}$ belirlendi

Inhibition of RelA/p65 Enhance NDV Replication

To investigate the effect of RelA/p65 signals on virus production, after CEF were treated with RelA/p65 siRNA for 24 h, the cells were infected with NDV strain GM or LaSota. The cell supernatants were collected to examine the virus-production kinetics on Vero cells. In our study, whether the NDV strain velogenic GM or lentogenic LaSota, if RelA/p65 had been inhibited in advance, the virus-production kinetics were higher than that of the control group 36 h p.i *(Fig. 5)*.

DISCUSSION

The early response to a virus causes host activation of transcription factors NF-κB, interferon regulatory factors (IRFs) and activator protein-1 (AP-1) (ATF-2/c-jun) through pattern recognition receptors (PRRs), recognized pathogen-associated molecular patterns (PAMPS), and further results in secretion IFNs [23-25]. Once type I IFN are synthesized, they act in both an autocrine and paracrine manner during the innate immune response to retard virus replication by

activating the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction cascade, leading to the activation of ISGs to further limit virus replication [7,26,27].

As a subunit of the heterodimeric NF-kB protein, RelA/p65 plays an essential role in immune response. Virus entry in the host can lead to the activation of RelA/p65 and then the activation of IFN-α/β genes via the RelA/p65 signal [28-30]. However, the research of the RelA/p65 signal in animal virus infections has been limited to mammals or their cell, it is rare to study the effect of the signal on viral replication in birds, especially, the action of the RelA/p65 signal on NDV replication in CEF. Based on these considerations, we chose cell CEF as the primary cell to research the change of the RelA/p65 signal after NDV infection, and chose Vero cell which is deficient in interferon production to support efficient production of NDV for studying anti-viral responses.

In vitro or *in vivo* experiments suggest that a pronounced and rapid innate immune response may be induced by NDV. In this study, we measured mRNA levels of IFN-α, IFN-β, STAT1 and RelA/p65 in NDV-infected CEF. Our study showed that the mRNA levels of RelA/p65, IFN-α and IFN-β are increased obviously after lentogenic or velogenic NDV infection CEF, and correspondingly, the mRNA level of STAT1 was also increased. These results are in agreement with previous studies [20,21], but we also found that lentogenic and velogenic NDV have different ability to induce CEF express IFN-α, IFN-β, STAT1 and RelA/p65. An important reason perhaps interrelated to V protein of NDV which functions as an IFN-α/β antagonist by targeting STAT1 for degradation [2,5,6]. The difference of V proteins derived from the velogenic NDV-GM and the lentogenic NDV-LaSota maybe have different ability to target STAT1, and which is worthy of further study. The other reason could be interrelated to apoptosis. NDV is known to cause apoptosis in different cell types including CEF cells, Vero cells and peripheral blood mononuclear cells, NDV-induced apoptosis required virus entry and replication [31-33], different apoptosis rate induced by lentogenic and velogenic NDV maybe leading to differences of the protein production synthesized by CEF.

On the basis of the above research, Western blot analysis was carried out to demonstrate the protein level of RelA/p65 in NDV-infected CEF. The results showed that NDV could induce CEF to synthesize more RelA/p65 protein, but different virulent NDVs have different capabilities to activating RelA/p65-mediated signaling pathways. After completing above study, we performed immunofluorescence staining to confirm the subcellular localization of RelA/p65. Immunofluorescence staining demonstrated that RelA/p65 protein had a predominantly nuclear localization pattern in NDV-infected cells. While for the mock-infection CEF and NDV-UV treatment group, the RelA/p65 protein was mainly distributed to the cytoplasm. The study showed that NDV could activate the RelA/p65 signal and induce the RelA/p65 protein shift from the cytoplasm to the nucleus while inactivated NDV did not exert this function in CEF, which suggesting that the nuclear translocation of RelA/p65 is probably related to virus replication.

In order to further determine the role of the RelA/p65 signal to different NDV replications, we knocked down RelA/p65 with a specific siRNA in CEF. Real time PCR showed that the inhibition of RelA/p65 also led to down-regulating the mRNA level of IFN-α, IFN-β and STAT1. The results further indicated that RelA/p65 played an important role in the process of anti-viral actions. Subsequent studies revealed that the NDV production kinetics were enhanced after the inhibition of RelA/p65 in CEF. The results indicated that a RelA/p65-mediated signaling pathway could limit NDV replication and that RelA/p65 played an important role in the process of anti-viral actions.

This study showed that a RelA/p65-mediated signaling pathway could be activated after CEF were infected by NDV, and this change in the signal could limit the replication of NDV. Therefore, this finding will provide a theoretical foundation of the pathogenic mechanism in RelA/p65-mediated hosts' innate immune responses caused by NDV.

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Research Article

Effects of Different Levels of Essential Oil Mixed (Peppermint-Thyme-Anise Oil) Supplementation in the Drinking Water on the Growth Performance, Carcass Traits and Histologic Structure of Terminal Ileum in Quails [1]

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Abstract

This study was conducted in order to define the effects of oregofarm (peppermint, thyme and anise oil) supplementation in the drinking water on the growth performance, carcass quality and histologic structure of terminal ileum in quails. A total of 348 Japanese quail chicks (*Coturnix coturnix japonica*) of both sexes were included in this study. They were divided into one control group and two experimental groups and each of them contained 116 Japanese quail chicks. Each group was further divided into four subgroups with 29 Japanese quail chicks. This study was finalized in six weeks. All groups were fed with basal diets and received fresh water during the experiment. The control group received non-supplemented water. The group 1 and 2 received 1.0 ml/5 L and 1.5 ml/5 L oil mixture, respectively. All experimental groups were fed with water and ad-libitum. As a result of the study, there were statistically significant differences between the feed consumption and efficiency ($P<0.001$) in the end of the three weeks. Similarly, there were also statistically significant differences between same parameters ($P<0.01$; 0.05) five weeks later. The body weights of quails were not significantly different from each other ($P>0.05$). At the end of the study, there were statistically differences in the warm and cold carcass parameters ($P<0.05$). Adding essential oil mixed were not affected on histological structure of terminal ileum ($P>0.05$). Conclusively, the supplementation of oregofarm (peppermint + thyme and anise oil) has no additional effect on quail performance.

Keywords: *Quail, Essential oil mixed, Performance, Carcass, Ileum*

İçme Suyuna Farklı Düzeylerde İlave Edilen Esansiyel Yağ Karışımının (Nane-Kekik-Anason Yağı) Bıldırcınlarda Büyüme Performansı, Karkas Parametreleri ve İleumun Histolojik Yapısı Üzerine Etkileri

Özet

Bu araştırma, bıldırcın içme suyuna oregofarm (nane-kekik-anason yağı) ilavesinin büyüme ve besi performansı, karkas özellikleri ve ileumun histolojik yapısı üzerine etkisini belirlemek amacıyla yapılmıştır. Araştırmada her iki cinsiyette toplam 348 adet Japon bıldırcın (*Coturnix coturnix japonica*) civcivi kullanılmıştır. Her grupta 116 civciv bulunan 1 kontrol 2 deneme grubu oluşturulmuştur. Gruplar kendi aralarında 4'erli alt gruba ayrılmıştır ve her alt grup 29 adet civcivden oluşturulmuştur. Deneme altı hafta sürdürülmüştür. Bütün deneme grupları temel rasyonla beslenmiştir ve deneme boyunca temiz içme suyu sağlanmıştır. Kontrol grubunun içme suyuna oregofarm ilavesi yapılmamıştır. 1. deneme grubu ve 2. deneme grubu içme suyuna sırasıyla 1.0 mg/5 L ve 1.5 mg/5 L. oregofarm ilavesi yapılmıştır. Tüm deneme gruplarına yem ve su ad-libitum olarak verilmiştir. Araştırma sonunda, yem tüketimi ve yemden yararlanma oranı bakımından 3. haftada istatistiksel açıdan önemli farklılıklar ($P<0.001$) gözlenmiş olup, benzer şekilde 5. haftada da aynı değerler bakımından farklılıklar tespit edilmiştir ($P<0.01$; 0.05). Denemede canlı ağırlıklar bakımından gruplar arasında istatistiksel olarak herhangi bir farklılık gözlenmemiştir ($P>0.05$). Denemenin sonunda gruplar arasında sıcak ve soğuk karkas parametreleri bakımından istatistiksel olarak farklılıklar bulunmuştur ($P<0.05$). Esansiyel yağ ilavesinin ileumun termal yapısı üzerine etkisi görülmemiştir ($P>0.05$). Sonuç olarak, bıldırcın içme suyuna oregofarm ilavesinin performansı artırıcı ilave bir etkisinin olmadığı tespit edilmiştir.

Anahtar sözcükler: *Bıldırcın, Esansiyel yağ karışımı, Performans, Karkas, İleum*

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INTRODUCTION

For several years, the dietary use of antibiotics has been extensively used throughout the world as growth promoters in animal feeds, particularly in poultry products [1]. However, many countries have banned the use of antibiotics in animal feed as an additive since their use may lead to the antibiotic resistant bacteria which are harmful to humans [2-4]. Therefore, nutritionists and production managers have to find alternatives which have potential to alleviate the problems related to the withdrawal of antibiotics from diets and reduce enteric diseases in the poultry [5,6]. Various studies have currently focused on the research on natural feed additives in animal diets such as antioxidant [7,8], anticoccidial [9,10] or antimicrobial [11,12] plants. Recently, food additives with plant origin such as essential oils, have received considerable attention since substitutes for antibiotics are being used as a growth promoters [13]. Consequently, some plants have attracted increasing interest since they have an alternative feed additivies (antimicrobial, antiparaziter, anticancer, antioxidant, antistress and appetizing) and they can replace antibiotic growth promoters [14].

Peppermint is a member of the Labiatae family and it is possibly originated from Eastern Asia. It is used widely in herbal medicine and it is believed to be beneficial particularly for the immune system. The most abundant constituent of peppermint is menthol, which has antibacterial features [15]. Peppermint is also rich source of polyphenolic compounds and hence, it could possess strong antioxidant properties [16,17]. Anise *(Pimpinella anisum* L.) is an annual aromatic plant belonging to the Apiaceae family. It has been used over the years due to its antioxidant [18], antimicrobial [19], antibacterial [20] and antifungal [21] properties. *Thymus vulgaris* L. (thyme) is also an aromatic plant belonging to Lamiaccea family. It has been a subject of considerable interest as a medicinal and therapeutic agent worldwide [22]. The pharmacological effect of thyme is attributed to carvacrol and thymol which are main bioactive components of thyme [23]. Thymol and carvacrol have considerable antimicrobial and antifungal activity [24,25]. Furthermore, they have also been reported to have antioxidant properties as well as antibacterial activity against a wide range of bacteria [26,27].

In recent years, there have been numerous studies about essential oil supplementation of dietary on poultry [28-30]. But as we know, there have not been any study supplementation of essential oil mixed in drinking water on poultry performance. The aim of the present study is to evaluate the effects of oregofarm (peppermint, thyme and anise oils) supplementation in drinking water of quails on the growth performance and carcass quality.

MATERIAL and METHODS

This research was conducted after the approval of Kafkas University Animal Testing Local Ethics Council (Approval Number: KAÜ-HADYEK/2012-53).

Japanese quails *(Coturnix coturnix japonica)* bred in the test unit of Kafkas University Research and Application Center. A total of 348 Japanese quail chicks were used in this study. They were divided into one control group and two experimental groups each containing 116 Japanese quail chicks. Each group was divided into four subgroups and each subgroup contained 29 Japanese quail chicks. All groups were fed *ad-libitum* with a 23% crude protein and 3080 kcal/kg metabolize energy diet. The quails received fresh water during the experiment. The control group of quails received non-supplemented water. The group 1 and 2 received 1.0 and 1.5 ml/5 L oil mixture, respectively. The active components of mixture is presented in *Table 1* (Oregofarm, FARMAVET AS). It contains peppermint, thyme and anise. The composition of basal diet is presented in *Table 2*. The experimental period lasted in 42 days. The ratios were isocaloric and isonutrigenos. Diets were formulated to meet the NRC [31] nutrient requirements.

Table 1. *The active components of the essential oil mixture*
Tablo 1. *Esansiyel yağ karışımının aktif bileşenleri*

Components (mg/kg)	
Thymol (thyme oil)	2000
B-phellanderene (thymeoil)	1300
Limonene (thyme oil+pepermint oil)	3525
B-pinene (thyme oil+pepermit oil)	1977
Carvacrol (oreganum oil)	8910
Linalool (oreganum oil)	3645
Anethole (anise oil)	10712
Menthole (pepermint oil)	6375

Table 2. *Composition and calculated analysis of basal diet*
Tablo 2. *Temel rasyonun bileşimi ve hesaplanan analiz değerleri*

Ingredients (%)		Values Analysed (%)	
Corn	50.62	Dry Matter	90.90
Soybean meal	42.16	Crude Protein	23.02
Oil	4.17	Crude Extract	6.56
Limestone	1.34	Crude Ash	3.37
Dicalcium phosphate	1.09	Crude Fiber	2.37
Salt	0.18	ME, kcal/kg**	3080.88
Vitamin-mineral prem.*	0.20	Ca	0.95
Sodium Bicarbonate	0.08	P	0.66
DL-methionine	0.14		
L-Lysine	0.02		
Total	100		

** Each 1 kg of Vit-min premix includes 20.000.000 IU Vit A, 3.000.000 IU Vit D_3, 25 g Vitamin E, 4 g Vitamin B_1, 8 g Vitamin B_2,2.5 g Vitamin B_6, 20 mg Vitamin B_{12}, 20 g Nicotinamid, 12 g Calcium-D-pantothenate, 200 g Choline Chloride, 50 g Mangane, 50 g Iron, 50 g Zinc, 10 g Copper, 0.8 g Iodine, 0.15 g Cobalt, 0.15 g Selenium; ** Calculated nutritional values*

Crude nutrients in feed and experimental ratio were analyzed using AOAC [32] method. The levels of metabolic energy were calculated by using the formula developed by TSE [33]. At beginning (0 day) and on the 7th 14th 21st, 28th, 35th and 42nd day of the study, the body weight and body weight gain of the quails were recorded. Meanwhile, the whole feed residues of the subgroups were weighed weekly in order to measure feed consumptions and feed conversion ratios.

Eleven male and eleven female birds from each subgroups, a total of 132 chicks were randomly selected and slaughtered in order to determine their carcass quality. Then, animals were gained again and warm carcass weight was recorded. Cold carcass weight was determined upon keeping the carcasses at +4ºC for 18 h. Cold carcass weight divided by the slaughter weight was calculated to determine cold carcass yield.

The ileums were taken from 6 animals of each group to examine histological. The tissues were fixed in alcohol formaldehyde then were passed series of solution which are used for routine histological process, and were embedded in paraffin. Sections taken from paraffin blocks were put on the slides and were stained with Hematoxylin Eosin (HE) and Periodic Acid Schiff (PAS) then slides were examined by light microscopy (Olympus BX-51) [34]. Length and width of villusses were measured by a micrometer and for this measurements were chosen 6 adjoining villusses at random per ileum. For villus length were taken into account between top point of ileal crypts and top of villus [35]. For width of villus was taken into account in the middle of the villus.

Statistical analyses were performed and the significance of the difference between the mean scores of the groups for body weight, body weight gain, feed consumption, feed conversion ratio, carcass weight and yield were determined by using the variance analysis method. Significant differences between the mean values of different treatments were determined by using the Duncan's multiple range tests. The statistical analysis was conducted by using the SPSS 16.0 (Inc. Chicago. IL. USA) program. For ileum samples One-Way ANOVA in PASW statistic 18 programe was used for statistical calculations.

RESULTS

The effects of drinking-water supplementation with peppermint+thyme and anise oils for 42 days on growth performance are shown in *Table 3*. The body weight of quail chicks was not significantly influenced by the essential oil supplementation (P>0.05). Body weight (BW), body weight gain (BWG), feed consumption (FC) and feed conversion ratio (FCR) are shown *Table 4*. Feed consumption in control group which was not fed with essential oils was found to be higher compared to the feed consumption of experimental groups. Furthermore, the feed consumption was significantly affected by essential oils and it was found to be lower than the feed consumption of the control group (P<0.01; 0.001). In the fifth weeks, BWGs in group II were higher when compared to other experimental groups (P<0.01). When compared to other groups, feed conversion ratio was markedly low (P<0.05; 0.001) in the group II (1.5 ml/5 L oregofarm) during the 3rd and 5th weeks. The overall FCR, calculated for the 6 weeks, was significantly lower than the overall FCR of other experimental groups (P<0.05). As shown in *Table 5*, the drinking water supplementation with essential oils statistically affected the slaughter weight, warm and cold carcass (P<0.05).

It was observed that ileum tissues were histological similar in all groups *(Fig. 1, 2, 3)*. In *Table 6*, between groups, width and length of villusses were statistically determined to be similar in all groups (P>0.05).

DISCUSSION

According to our study, the oregofarm supplementation in the drinking water did not lead to statistically significant difference between experimental groups in terms of live body weights (P>0.05) *(Table 3)*. In terms of the results

Table 3. *Mean body weights of the groups (g)*
Tablo 3. *Gruplarda ortalama canlı ağırlıklar (g)*

Weeks	n	Control $X \pm S_x$	n	Group I $X \pm S_x$	n	Group II $X \pm S_x$	P
0	116	8.41±0.08	116	8.38±0.08	116	8.38±0.07	-
1	108	14.13±0.31	107	14.07±0.34	106	13.13±0.30	-
2	100	32.43±0.94	98	33.61±0.91	100	32.30±0.89	-
3	98	65.51±1.44	95	65.78±1.48	93	65.34±1.47	-
4	97	101.33±1.92	94	100.78±1.94	91	102.98±1.87	-
5	97	140.23±2.28	94	136.71±2.37	91	142.29±2.38	-
6	96	158.88±2.48	94	155.31±2.44	91	159.27±2.77	-

-: Differences among the groups were not statistically significant (P>0.05)

Table 4. *Mean feed consumption, weekly body weight gain and feed conversion rate* values of groups*
Tablo 4. *Gruplarda ortalama yem tüketimi, haftalık canlı ağırlık artışı ve yemden yararlanma oranı**

Weeks	Parameters	Control $X \pm S_x$	Group I $X \pm S_x$	Group II $X \pm S_x$	P
1	FC (g/chick) BWG (g) FCR	10.01±0.23 5.73±0.39 1.77±0.11	10.12±0.45 5.69±0.17 1.78±0.07	10.19±0.18 4.87±0.36 2.12±0.14	- - -
2	FC (g/chick) BWG (g) FCR	48.43±1.47 18.19±0.56 2.67±0.10	49.43±3.12 19.14±0.56 2.58±0.11	44.51±3.03 20.39±2.01 2.77±0.33	- - -
3	FC (g/chick) BWG (g) FCR	94.59±1.46^{a} 32.76±0.40 2.89±0.02^{a}	93.81±1.22^{a} 32.13±0.70 2.92±0.05^{a}	74.12±1.64^{b} 32.16±0.71 2.31±0.06^{b}	*** - ***
4	FC (g/chick) BWG (g) FCR	120.34±1.02 36.46±0.83 3.50±0.11	117.71±4.63 35.22±0.94 3.34±0.12	112.09±1.91 36.27±0.45 3.09±0.06	- - *
5	FC (g/chick) BWG (g) FCR	157.51±6.10^{a} 39.27±0.24^{ab} 4.01±0.14^{a}	141.99±1.50^{b} 35.98±0.80^{b} 3.95±0.08^{b}	144.17±2.32^{b} 40.46±1.89^{a} 3.58±0.12^{b}	** * *
6	FC (g/chick) BWG (g) FCR	141.16±1.01 18.72 ±1.42 7.80±0.66	139.72±4.94 18.35±1.24 7.72±0.57	137.91±1.81 20.77±4.30 7.32±1.10	- - -
1-6	FC (g/chick) BWG (g) FCR	574.04±8.15^{a} 150.44±3.02 3.82±0.09^{a}	552.77±12.12^{a} 146.94±1.67 3.76±0.05^{a}	522.97±3.25^{b} 155.34±6.89 3.38±0.13^{b}	** - *

a,b: *Mean values on the same row followed by different letters change significantly (P<0.05, 0.01, 0.001; respectively);* ***-:*** *Differences among the groups were not statistically significant (P>0.05);* ******* *(kg, feed consumption/kg, body weight gain);* ***FC:*** *Feed Consumption,* ***BWG:*** *Body Weight Gain,* ***FCR:*** *Feed Conversion Rate*

Table 5. *Mean slaughter weight , carcass weight and carcass yields of groups*
Tablo 5. *Grupların ortalama kesim ve karkas ağırlıkları ile karkas randımanları*

Parameters	Control $X \pm S_x$	Group I $X \pm S_x$	Group II $X \pm S_x$	P
Slaughter weight (g)	153.89±3.68^{a}	141.62±3.51^{b}	143.75±3.90^{ab}	*
Warm carcass (g)	114.04±2.45^{a}	105.26±2.59^{b}	106.40±2.52^{b}	*
Cold carcass (g)	111.80±2.43^{a}	102.87±2.54^{b}	103.74±2.47^{b}	*
Warm carcass percentage (%)	74.32±0.01	74.39±0.01	74.40±0.01	-
Cold carcass percentage (%)	72.84±0.01	72.67±0.01	72.53±0.01	-

a,b: *Mean values on the same row followed by different letters change significantly (P<0.05);* ***-:*** *Differences among the groups were not statistically significant (P>0.05)*

Table 6. *The effect of different levels essential oil mixed on histologic structure of terminal ileum in Japonese quails*
Tablo 6. *Japon bıldırcınlarında farklı düzeylerde essensiyel yağ karışımı ilavesinin ileumun termal yapısı üzerine etkisi*

Treatment	n	Villus Length (µm)
Control ($X \pm S_x$)	36	315.72±61.50
Group I ($X \pm S_x$)	36	298.00±43.49
Group II ($X \pm S_x$)	36	311.27±56.54
SEM		0.359
Treatment	n	Villus Width (µm)
Control ($X \pm S_x$)	36	84.47±15.40
Group I ($X \pm S_x$)	36	83.41±14.64
Group II ($X \pm S_x$)	36	79.77±13.69
SEM		0.362

Differences among the groups were not statistically significant (P>0.05)

of the live body weight gain, there was a statistically significant difference between the groups in the 5th week (P<0.05). These findings are different from the results of the study performed by Biricik et al.[13] in which they examined the effects of essential oil supplementation in the quail diets on the live body weight. However, live body weight gain results were similar to each other. In the study conducted by Parlat et al.[36] in which they searched the effects of the supplementation of virginiamycine and thyme essential oils in the diet. According to their results, the best results were obtained from quail groups which were fed with thyme essential oils. Denli et al.[37] performed a study with Japan quails and it has been shown that the thyme essential oil supplementation enhanced live body weight as well as live body weight gain according to the control group. The supplementation of the thyme + anise and rosemary extracts was shown by Tucker et al.[38]

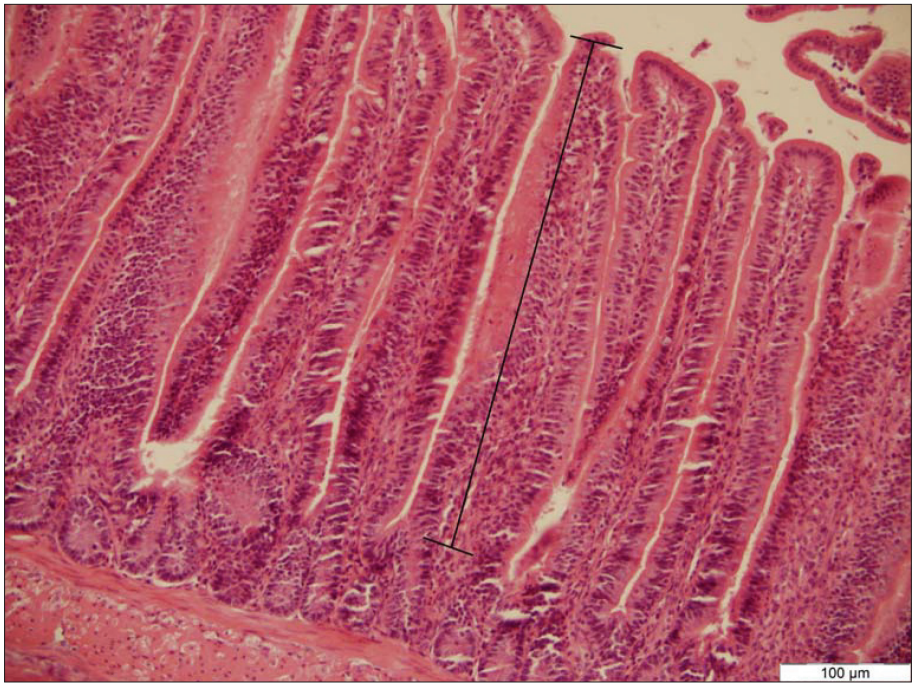
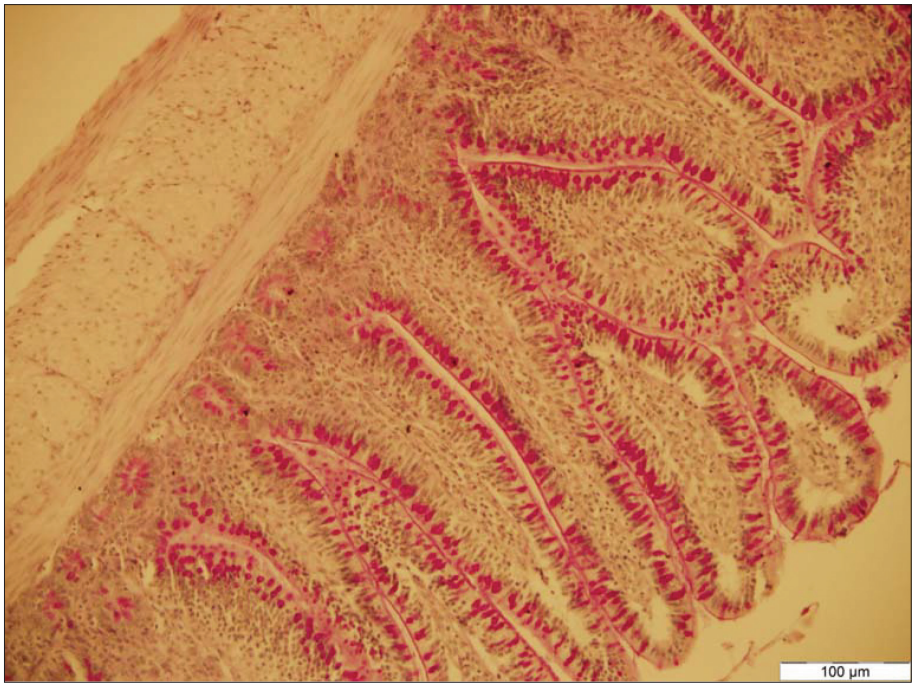


Fig 1. Villusses could be observed in group 1. HE, X20. Bar 100 µm

Şekil 1. Grup 1'de villusların görünümü. HE, X20. Bar 100 µm

Fig 2. Vilusses and goblet cells could be observed in group 2. PAS, X20. Bar 100 µm

Şekil 2. Grup 2'de villusların ve kadeh hücrelerinin görünümü. PAS, X20. Bar 100 µm



that they increased the live body weight of broilers and decrease the death rate. Celik [39] have indicated that the different amounts of essential oil mixtures (mint + juniper + rosemary + thyme) supplemented in the diet of broilers did not affect the live body weight whereas enhanced the death rate. These results are similar to the results of our study.

In a study, different doses of anise essential oil as an alternative to antibiotics were supplemented in the diet of broilers and it has been shown that the maximum live body weight was reached when the maximum dose of anise oil was supplemented. Therefore, they stated that anise oil can be used as a growth factor [40]. When the thyme oil was added to the diet as an alternative to antibiotics, the maximum live body weight was obtained in a group in which the thyme oil and antibiotics were supplemented together [41]. The differences between the studies can be due to the different species of animals, different usage of the extracts as well as the diets.

When the feed consumption and the rate of the feed of groups were assessed, it has been seen that there was a significant differences between groups starting from the 3rd

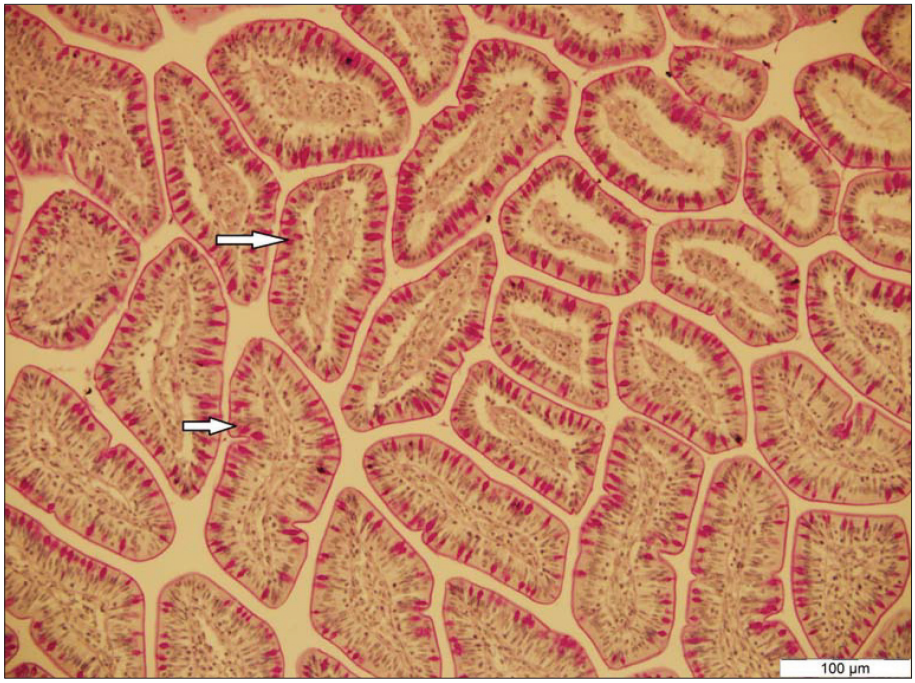


Fig 3. Cross-section of villusses, and goblet cells *(arrows)* could be observed in control group. PAS, X20. Bar 100 µm

Şekil 3. Kontrol grubu villusların enine kesiti ve kadeh hücrelerinin *(oklar)* görünümü. PAS, X20. Bar 100 µm

week (P<0.001; 0.01) *(Table 4)*. It has been detected that the feed consumption decreased with the supplementation of the mint, thyme and anise essential oil mixture in the drinking water. Harnandez et al.[42] have reported that the different two plant extract mixtures did not have a prominent effect on the feed consumption and the rate of feed. Similarly, Tucker et al.[38] have specified that the thyme and mint oil mixtures did not have an impact on the rate of feed of the broilers. Biricik et al.[13] performed a study in which they have indicated that essential oil supplementation in quail diet did not influence statistically the feed consumption. Our results are not consistent with these findings. According to the study performed by Halle et al.[43] thyme and thyme essential oil supplementation in the diets of broilers decreased the feed consumption and the essential oil prominently enhanced the rate of feed. Our study is in agreement with the study obtained by Ghazaghi et al.[44] in which they worked with quails. Similarly, Sengün et al.[45] added thyme extract in the diet of quails and they observed the improvement in the rate of feed.

When the quails were evaluated about their carcass characteristics, there was a statistically significant difference between experimental and control groups in terms of slaughter, hot and cold carcass weights (P<0.05) *(Table 5)*. According to Ghazaghi et al.[44] there was no difference between the quail groups in terms of their slaughter weights. There was no significant difference between groups related to their hot and cold carcass yields (P>0.05). Likewise, Biricik et al.[13] have reported that the essential oil supplementation in the diet of quails did not influence the hot and cold carcass yields. Because of these differences, due to adding different essential oil composition and added shape.

The length and the width of villus and villus goblet cell number are shown figures 1 to 3. It has been observed that there was no statistically significant difference between the experimental and the control groups related to the length and the width of the villus when the essential oil mixed are added to the drinking water of quails (P>0.05) *(Table 6)*. Our results are similar to the results of Cabuk et al.[46]. In contrast to the present result, Denli et al.[37] reported that including thyme and black seed essential oil in the diet increases intestinal weight and intestinal length in quails. These differences, due to adding different essential oil composition and added shape.

Conclusively, essential oils or their mixtures have been supplemented in the diet in studies have been performed till now. In these studies, it has been aimed to examine their effects by adding them in the drinking water of animals. It has been shown that the addition of the essential oil mixtures in the drinking water of the quails decreases the feed consumption, enhances the rate of feed, and decreases the death rate. The supplementation do not have so much effect on live body weight and carcass yield whereas it has positive and prominent effects on the digestive system under the poor environmental conditions and malnutrition. Thus, further studies should be performed with different doses and under different animal husbandry systems.

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Research Article

Determination of Iron Deficiency Anemia in Helicobacter Infected Dogs [1]

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Abstract

Many studies demonstrated that Helicobacter infections in humans affects iron metabolism and decreases ferritin level resulting with iron deficiency anemia. The aim of this study is the investigation of a correlation between Helicobacter infection and iron deficiency anemia in dogs. The material of the study consisted of 42 Helicobacter (+) (determined with polymerase chain reaction-PCR- following upper gastrointestinal system endoscopy) dogs (Group I) (n=42) referred with vomiting after feeding, anorexia and epigastric pain in the abdominal palpation, and 21 (Group II) (n=21) dogs negative for Helicobacter with PCR and in the routine clinical examinations. Grup I were given therapy against Helicobacter gastritis for 21 days. Blood specimen were obtained from Group I at 0. and 21. days and Group II at 0. day for blood count and ferritin levels. Iron deficiency anemia was observed in 28 dogs of 42 (66.6%) infected with Helicobacter. In conclusion, iron deficiency anemia can be a probable evidence in frequently diagnosed Helicobacter infections in dogs and must be considered in the diagnostic and therapeutic plan. This is also the first study investigating iron deficiency anemia in Helicobacter infections of dogs as far as our knowledge.

Keywords: *Helicobacter, Dog, Iron deficiency, Anemia, Serum ferritin*

Helikobakter Enfeksiyonlu Köpeklerde Demir Yetmezliği Anemisinin Belirlenmesi

Özet

Helikobakter enfeksiyonunun insanlarda demir metabolizmasını etkileyerek ferritin seviyesinin düşüşüne neden olduğu ve demir eksikliği anemisi oluşturduğu çeşitli çalışmalarla ortaya konulmuştur. Bu çalışmanın amacı, köpeklerde helikobakter enfeksiyonu ile demir eksikliği anemisi arasında bir ilişki olup olmadığının araştırılmasıdır. Çalışma materyalini, yemeyi takiben kusma, iştahsızlık ve abdominal palpasyonda epigastrik ağrı şikayeti ile getirilen ve üst gastro intestinal sistem endoskopisi sonucu polimeraz zincir reaksiyonu (PZR) ile helikobakter (+) olduğu saptanan 42 adet (Grup I) (n=42) köpek ve rutin klinik muayeneleri ve PZR'yle pozitiflik saptanmayan 21 adet (Grup II) (n=21) köpek oluşturmuştur. Grup I, Helikobakter gastritisi yönünden 21 gün süreyle tedavi edildi. Grup I'den 0. ve 21. günler, Grup II'den 0.gün kan sayımı ve serum ferritin bakıldı. Helikobakter ile enfekte 42 köpeğin 28'inde (%66.6) demir eksikliği anemisi saptandı. Sonuç olarak, köpeklerde sıklıkla gözlenen helikobakter enfeksiyonlarında demir eksikliği anemisinin kuvvetle muhtemel bir bulgu olabileceği ve hastanın teşhis ve tedavi şeması dahilinde göz önüne alınması gerekliliği ortaya konmuştur. Bu aynı zamanda, araştırmalarımız çerçevesinde köpeklerde Helikobakter enfeksiyonlarında demir eksikliği anemisini inceleyen ilk araştırmadır.

Anahtar sözcükler: *Helikobakter, Köpek, Demir yetmezliği, Anemi, Serum ferritin*

INTRODUCTION

The existence of spiral bacterium in the stomach of humans and animals has been recognized since the beginning of 1800s [1]. In addition to this, a relationship between *H. pylori* and gastric diseases in humans have been discovered [2,3].

At least 4 types of spiral organisms colonized in the stomach of cats and dogs are known. These are *H. felis* [3], *H. salomonis* [4], *H. bizzozeronii* [5] and *H. heilmannii* [2] also

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named *Gastropirillium hominis*. Many studies has been attempted for the detection of *H. pylori* in dogs, but only few reported positive results [6,7], so it is very rare.

Recently, O' Rourke et al.[8] have managed to distinguish *H. felis, H. salomonis, H. bizzozeronii* and *H. heilmannii* by conducting sequence analysis of a part of urease gene complex. In subsequent studies, it has been found out that *H. pylori* experimentally causes a similar disease in dogs [7], but Helicobacter is a frequent infection an dogs [9,10].

Although there are many studies [11-16] on helicobacter infections and iron deficiency in human medicine, it has not been completely revealed with what mechanisms this condition occurs in humans with helicobacter infections. The explanation most commonly offered for this relationship is based upon the development of H. pylori-associated chronic pangastritis with resultant achlorhydria and reduced ascorbic acid secretion leading to reduced intestinal iron absorption. Other potential explanations for an association between iron deficiency and *H. pylori* include occult blood loss from erosive gastritis and sequestration and utilization of iron by the organism [12]. In a retrospective study on 1294 patients diagnosed with iron deficiency, Ünal et al.[15], performed upper gastrointestinal endoscopy in 205 patients and found out that 84 of these patients (41%) were helicobacter (+). In their study, Serin and Serin [14], reported that Helicobacter infection should be definitely considered in patients with treatment-resistance iron deficiency anemia. Similarly, in the human literature, it is often stated that helicobacter infections may cause growth failure in children; however, this situation has not been clearly put forward. As ferritin is also an acute phase protein, the chances of inflammatory diseases, such as liver diseases and hemolytic diseases increase. In iron deficiency anemia, serum ferritin level decreases, and it increases in chronic disease anemia. Although there are many studies [11,13,14] and discussions on it in human medicine, there are no authentic studies about it in veterinary medicine as far as our knowledge. Our study aims to reveal the relationship between helicobacter infections in dogs and iron deficiency anemia.

MATERIAL and METHODS

Animal material consisted of 42 dogs (Group I) which were referred to Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Internal Diseases between 2013-2014, due to vomiting after being fed, loss of appetite and epigastric pain in the abdominal palpation.

For the diagnosis of helicobacteriosis, gastro intestinal system endoscopy materials were analysed with PCR. DNA extraction of contents were conducted with commercially available DNA extraction kits (PureLink Genomic DNA Kits, Invitrogen, Canada) according to the manufacturers' instructions. The 100 µl of extracted DNA was kept frozen at -20°C until molecular tests were carried out. Genus-spesific PCR analysis was conducted as reported by Riley et al.[17], and the observation of a 375 bpm band was considered as positive *(Fig. 1)*. The DNA of *Helicobacter pylori*, found in culture collection of our faculties' microbiology department, was used as positive control in all PCR analyses.

The dogs, diagnosed *Helicobacter* spp. positive with PCR were treated for 21 days as stated by Aytuğ [18]. On day 0 and 21, whole blood count and serum biochemistry were conducted. The group in which positivity was not detected with PCR (Group II) (n=21) was accepted healthy after routine clinical examinations and whole blood count and ferritin determinations performed on day 0.

While dogs in Group I (n=42) consisted of 22 crossbreeds, 2 labradors, 5 golden retrievers, 12 terriers, and 1 Turkish shepherd dog, Group II (n=21) consisted of 18 crossbreeds, 2 golden retrievers, and 1 German shepherd. There were 28 male and 14 female dogs in Group I (17-32 kg) and 17 female and 4 male dogs in Group II (15-25 kg). Average age of the animals were 3-7 years (5.4±1.5).

Via jugularis, 2 ml of blood was obtained from dogs into tubes with heparin and anticoagulant-free tubes. Blood samples with heparin were analysed in Abacus Vet Junior device and the results of the blood count (RBC, HGB, HCT, MCV, MCH, MCHC, RDWc, PLT) were saved separately for each animal. After anticoagulant-free blood samples were centrifuged 5.000 rpm/5 min, they were taken into eppendorf tubes with the help of serum micropipette extricated from blood. Serum was stored at -18°C for analysis.

In the serums obtained, ferritin was analysed with ELISA [19,20]. Ferritin (FE) ELISA Kit (ABIN991700) was used and evaluated according the manufacturers instructions.

For the gastroscopic examination, dogs were not given any food for a day. H_2 receptor blockers, antiacidic treatment and antibiotic were not used for a week and in the last three hours intake of water was prevented. Dogs were anaesthesized before the procedure. For gastroscopic examination, preparations were made in line with the gastroscopic examination procedure for clinically sick and healthy dogs. For gastric endoscopy, a flexible endoscopy device with Olympos® XQ20 model working channel and cold light source was used. Following the examination of front gastrointestinal channel, PCR liquid was taken from the stomach where was stored at -18°C for analysis. PCR analysis was conducted according to the method reported by Riley et al.[17] as described above. The assessment of the results have been done using one-way ANOVA and Duncan's multiple range tests in statistical package program (SPSS, 12.0). The findings have been presented as average values and standard error.

RESULTS

Iron deficiency anemia were diagnosed in 28 of 42 (66.6%) dogs in Group 1. Blood count and ferritin levels of the Group 1 dogs with iron deficiency are demonstrated in *Table 1*. As seen, results demonstrate microcytic-hypochromic anemia, characteristic of iron deficieny and low ferritin levels in dogs with Helicobacter gastritis. Species-specific PCR results are presented in *Fig. 1*.

Table 1. *Blood count and serum ferritin parameters of Helicobacter infected dogs with iron deficiency anemia*
Tablo 1. *Demir eksikliği anemisi gözlenen Helicobacter ile enfekte köpeklerin kan sayımı ve serum ferritin parametreleri*

Parameter	Helicobacter Infected Dogs with Iron Deficiency, Group I (n=28)		Group II (n=21) (Healthy Group)	Range
	Day 0	Day 21		
RBC (10^6µl)	5.08±1.70[a]	5.26±0.60[a]	6.11±1.20[b]	5.50-8.50
HGB (g/dl)	11.11±0.90[a]	12.07±1.40[b]	13.04±2.10[b]	12.00-18.00
HCT (%)	30.66±3.56[a]	31.12±2.70[a]	38.21±4.32[b]	37.00-55.00
MCV (fl)	58.27±3.30[a]	62.32±2.50[b]	62.41±5.20[b]	60-77
MCH (pg)	21±6.40[a]	23.82±40[b]	22.02±4.80[a]	22-24.50
MCHC (g/dl)	35.15±5.17[a]	37.39±4.20[b]	36.82±8.21[b]	36.00-38.00
PLT (10^3µl)	596±116[a]	289±141[b]	421±132.40[c]	200-500
Ferritin ng ml^{-1}	27±61[a]	158±36[b]	171±44[b]	36-220 [6,16]

Groups that are assigned a different letter have been found to be statistically significant at the level of P≤0.05

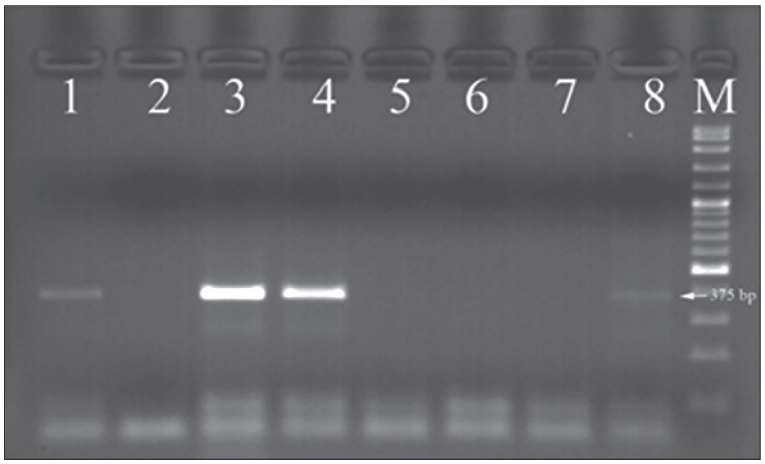

Fig 1. Species-specific PCR results . M: marker ; 1, 3, 4 and 8'th the examples are positive, the others negative

Şekil 1. Tür spesifik PZR sonuçları. M: Markır; 1, 3, 4 ve 8. örnekler pozitif, diğerleri negatif

DISCUSSION

Although helicobacter has been known for about 95 years, it was defined in real terms in the 20th century. Scientists have focused on the essential information that belongs to the bacterium for the last 20 years [8,14,17]. There are evidences on the fact that helicobacter infections cause iron deficiency [11-16]. The explanation most commonly offered for this relationship is based upon the development of H. pylori-associated chronic pangastritis with resultant achlorhydria and reduced ascorbic acid secretion leading to reduced intestinal iron absorption. Other potential explanations for an association between iron deficiency and *H. pylori* include occult blood loss from erosive gastritis and sequestration and utilization of iron by the organism. In their study, Serin and Serin [14], reported that helicobacter infection should definitely be considered in patients with treatment-resistant iron deficiency. While Bakır [21], explain helicobacter infections causing iron deficiency anemia in people with autoimmune gastritis, Michael [22], reports that hepcidin hormone, which is an inflammation mediator, prevents iron from being taken into the cell by getting on the protein that allows iron go in through the cell surface; that the hunger for iron inside the cell causes iron stores to be emptied; that iron- which is abundant in circulation- is caught by macrophages and therefore iron deficiency anemia develops. In his study, on the other hand, Arrigo [9], reports that helicobacter gastritis reduces hydrochloric acid and ascorbic acid secretion and therefore iron malabsorption develops.

In veterinary medicine, a study regarding the relationship between Helicobacter infections and iron deficiency anemia has not been reported yet as far as our literature search. Only Gazyagci and Macun [23] reported microcytic anemia due to gastric ulcer in a dog in which ferritin level was at the bottom of the reference spectrum and elevated

after the therapy, and this can be due to blood loss from the gastric ulcer.

Due to the positive correlation between serum ferritin levels in animals and their body iron stores, ferritin gives the most accurate information about the condition of iron stores in the body [24]. In iron deficiency anemia, serum ferritin level decreases, and it increases in chronic disease anemia [19,20,24], because as ferritin is also an acute phase protein, an increase is expected in inflammatory diseases such as liver diseases and haemolytic an increase. As seen in *Table 1,* serum ferritin level decreased in dogs with helicobacter gastritis (27±61), similar to the reports of human the literature [12,14,16]. Although dogs were not provided with any iron deficiency treatment, and only received helicobacter gastritis treatment, in the measurement of serum ferritin levels done on day 21, a statistical rise in ferritin levels were observed (158±36) *(Table. 1)*. In other erythrocyte index parameters in *Table 1*, on the other hand, iron deficiency anemia symptoms (microcytic-hypochromic type anemia) were detected as defined [24,25]. It has been reported that normocytic-normochromic-nonregenerative anemia appears in chronic disease anemia [24,25].

In conclusion, we had been determined a rate of 66.6% iron deficiency anemia in helicobacter infected dogs, so persistent iron deficiency anemia should definitely be considered in the diagnostic and therapeutic plan. Also, this is the first study demonstrating the relationship between iron deficiency anemia and Helicobacter infection in dogs.

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Research Article

Dimetilsülfoksit İlavesi ile Farklı Şekillerde Dondurulmuş Rumen Sıvısının *in Vitro* Sindirim Denemelerinde Kullanım Olanaklarının Araştırılması [1]

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[1] *Bu çalışma TÜBİTAK tarafından desteklenmiştir (Proje No:TOVAG-109O445)*

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Özet

Bu çalışmada, rumen sıvısı kaynağı olarak; taze rumen sıvısı, sıvı azot içerisinde dimetilsülfoksit (DMSO) katkısız ve katkılı (%5), derin dondurucuda DMSO katkısız ve katkılı (%5) dondurulmuş rumen sıvısı kullanılmıştır. Çalışmada 4 farklı kaba yem (mısır silajı, yonca kuru otu, mercimek samanı, buğday samanı), 4 farklı karma yem (sığır besi yemi, sığır süt yemi, toklu besi yemi ve buzağı büyütme yemi) ve 4 farklı yem hammaddesi (arpa, mısır, pamuk tohumu küspesi, soya küspesi) yem materyali olarak kullanılmıştır. İki aşamalı sindirim yönteminde deneme yemleri genel olarak değerlendirildiğinde gerek sıvı azot içerisinde ve gerekse derin dondurucuda dondurulan rumen sıvılarından elde edilen in vitro organik madde sindirim (İVOMS) değerleri taze rumen sıvısından elde edilmiş değerlerden düşük ($P<0.05$) bulunmuştur. Gaz üretim yönteminde ise yemlerin tamamı için %5 DMSO katkısı ile sıvı azot içerisinde dondurulmuş rumen sıvısından elde edilen İVOMS değerleri taze rumen sıvısından elde edilen değerler ile benzer ($P>0.05$) bulunmuştur. Araştırmada değerlendirilen tüm dondurulmuş rumen sıvılarındaki toplam anaerob bakteri ve toplam protozoa sayılarının, taze rumen sıvısındaki toplam anaerob bakteri ve protozoa sayılarına kıyasla azaldığı ($P<0.05$) görülmüştür. Protozoa türleri incelendiğinde %5 DMSO ilave edilerek sıvı azot içerisinde dondurulmuş rumen sıvısındaki canlı protozoa sayılarının kontrol grubu ile genel olarak benzer ($P>0.05$) olduğu belirlenmiştir. Bu araştırmadan elde edilen sonuçlara göre, %5 DMSO katkısı ile sıvı azot tankı içerisinde dondurulmuş rumen sıvılarının in vitro gaz üretim tekniğinde kullanılabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Rumen sıvısı, in vitro sindirim, Dondurma, Sıvı azot, Derin dondurucu, Dimetilsülfoksit, DMSO*

An Investigation on Usage of Frozen Rumen Fluid with Adding Dimethylsulfoxide and Different Freezing Methods for Determination of *in Vitro* Digestibility

Abstract

In this study, fresh rumen fluid, liquid nitrogen frozen rumen fluids with addition of 5% dimethylsulfoxide (DMSO) or without DMSO, deep freeze frozen rumen fluid addition of 5% DMSO or without DMSO were investigated as inoculum sources. Four roughages (corn silage, alfalfa hay, lentil straw, wheat straw), four commercial feeds (beef finishing, dairy cattle, ram lamb finishing, calf growing feeds) and four feed ingredients (barley, corn, cotton seed meal, soybean meal) were chosen as feed materials. Values of in vitro organic matter digestibility (IVOMD) of feeds using fresh rumen fluid were found generally higher than the values obtained from nitrogen or deep freeze frozen inoculum sources ($P<0.05$) in two stage in vitro procedure. Values of IVOMD via gas production technique were similar ($P>0.05$) with using liquid nitrogen frozen rumen fluids with addition of 5% DMSO. Number of the anaerobic bacteria and total protozoa were diminished with both freezing methods comparison with fresh rumen fluid ($P<0.05$). Species of protozoa count of fresh rumen fluid and nitrogen frozen with 5% DMSO were found generally similar ($P>0.05$). According to result of this study liquid nitrogen frozen rumen fluid with addition of 5% DMSO can be suggest as an alternative inoculum source for the in vitro gas production technique.

Keywords: *Rumen fluid, in vitro digestion, Freezing, Liquid nitrogen, Deep Freeze, dimethylsulfoxide, DMSO*

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GİRİŞ

Ruminant beslemede kullanılan yem maddelerinin enerji ve sindirim değerlerinin belirlenmesi genellikle *in vivo* yöntemlerle yapılmaktadır. Ancak *in vivo* yöntemlerin uygulanması zaman alıcı ve pahalı olması, araştırıcıları *in vitro* yöntemlere yöneltmiştir [1]. Yem maddelerinin *in vitro* sindirim değerlerinin belirlenmesinde mikrobiyal fermentasyon için taze rumen sıvısına ihtiyaç duyulmaktadır [2,3]. Yem analizleri yapan araştırma ve kurum laboratuarlarında rumen kanülü takılmış canlı hayvan barındırılma güçlüğü bu laboratuarlarda *in vitro* denemelerin yapılmasını engellemektedir. *İn vitro* sindirim yöntemlerinin uygulanmasında, taze rumen sıvısı temini için cerrahi operasyonla hayvanlara rumen kanülü takılması, olası sağlık problemlerinin ortaya çıkması, uzun süreli bakım masrafları ve bu hayvanların kullanımı ile ilgili etik ve hayvan refahı gibi faktörlerin bulunması bu konuda çalışanlar için dezavantaj olarak kabul edilmektedir [4]. Bu faktörleri göz önünde bulunduran araştırmacılar taze rumen sıvısına alternatif inokulant kaynakları (dışkı ve enzim) üzerine araştırmalar yapmışlar ancak elde edilen sonuçların güvenli olmadığı sonucuna varmışlardır [5,6]. Rumen mikroorganizmalarının canlılıklarını sürdürebilmeleri için özel şartlara (oksijensiz ortam, 39°C ısı, nem vb.) ihtiyaç duyarlar. Rumen mikroorganizmaları için zorunlu olan bu ortam şartları rumen sıvısının kriyoprotektan ilave edilmeksizin dondurularak saklanmasında bazı olumsuzluklar oluşturmaktadır. Rumen sıvısının dondurulmasında çeşitli kriyoprotektan maddeler (gliserol, dimetil sülfoksit, propilen glikol, etilen glikol, sukroz ve trehalose) kullanılmaktadır. Bunlardan gliserol ve dimetil sülfoksit (DMSO) en yaygın kullanım alanı bulmuştur. Kriyoprotektanlar genel olarak donma işlemi sırasında ya hücre içindeki sıvıyı hücre dışına almakta veya hücre içindeki sıvıyı çok küçük partiküller şeklinde dondurarak donma esnasında intrasellular sıvının buz kristali oluşturmasını engelleyerek hücre harabiyetini en aza indirgemektedirler [7,8]. Kriyoprotektanların bu özellikleri dondurulma ve çözme işlemi sonrasında rumen sıvısında bulunan çeşitli mikroorganizmaların aktivitelerinin devam etme olasılığını arttırmaktadır.

Gerek yemlerin iki aşamalı *in vitro* kuru madde sindirilebilirliklerinin belirlenmesinde ve gerekse gaz üretim tekniğinin uygulanmasında dondurulmuş rumen sıvısı kullanılarak yapılmış sınırlı sayıda çalışma bulunmaktadır. Zeigler ve ark.[9] iki aşamalı *in vitro* kuru madde sindirim denemesinde inokulant kaynağı olarak taze rumen sıvısı ve derin dondurucuda %5 gliserol ilave edilerek dondurulmuş rumen sıvısı kullanmışlardır. Araştırıcıların elde ettikleri sonuçlara göre derin dondurucuda katkısız olarak dondurulan rumen sıvısı ile taze rumen sıvısından elde edilen İVKMS değeri benzer bulunmuş, %5 gliserol katkısı dondurulan rumen sıvısından elde edilen İVKMS değeri ise taze rumen sıvısından elde edilen değerden düşük bulunmuştur. Cone ve ark.[10] tarafından *in vitro* gaz üretim denemesinde taze rumen sıvısını -24°C'de 1, 3, 10, 40 ve 76 gün boyunca dondurarak yaptıkları bir çalışmada, 10 günden daha uzun süre dondurulma işleminde gaz üretim miktarının olumsuz yönde etkilendiğini bildirmişlerdir. Yapılan diğer bir çalışmada [11] gaz üretim tekniğinde 0°C' de 3, 6 ve 24 saat bekletilen ile -18°C'de 24 saat süre ile dondurulmuş rumen sıvıları kullanılmıştır. Kontrol grubu ile 0°C'de 3 ve 6 saat bekletilen rumen sıvılarından elde edilen sonuçlar benzer bulunurken, -18°C'de 24 saat süre ile dondurulmuş rumen sıvısından elde edilen değerler düşük bulunmuştur. Bu farklılık donma aşamasında intrasellüler buz kristallerinin oluşumuna ve buna bağlı olarak oluşan hücre harabiyeti ile açıklanmıştır. Prates ve ark.[12] farklı şekillerde dondurulan rumen sıvısının gaz üretim tekniğinde kullanılabilirliğini araştırmak amacıyla yaptıkları bir çalışmada, gliserol katılarak sıvı azot içerisinde dondurulan rumen sıvısından en yüksek gaz üretimi sağlanmıştır. Bunun aksine -20°C'de derin dondurucuda ve gliserol katılmaksızın sıvı azot içerisinde dondurulmuş rumen sıvılarından ise en düşük gaz üretim değerleri elde edilmiştir. Abdel-Aziz ve ark.[13] rumen protozolarının sıvı azot içerisinde dondurulmalarına ilişkin yaptıkları bir çalışmada kriyoprotektan olarak %4, 5 ve 6 düzeyinde gliserol, DMSO ve etilen glikol kullanmışlardır. Dondurulma süresi sonunda çözdürülen protozoaların canlılıkları incelendiğinde, %5 DMSO ilavesinin en iyi sonuç verdiği bildirilmiştir. Benzer şekilde Nsabimana ve ark.[14] rumen protozoalarının dondurulmasında kriyoprotektan olarak %4, 5 ve 6 düzeyinde DMSO kullanmışlar, %5 DMSO katkısının protozoaların canlılıklarının korunmasında en etkili seviyenin olduğunu bildirmişlerdir. Bu çalışma, sıvı azot ve derin dondurucuda dimetil sülfoksit (DMSO) ilave edilerek dondurulmuş rumen sıvılarının, taze rumen sıvısına alternatif olarak iki aşamalı *in vitro* sindirim ve gaz üretim tekniğinde kullanılabilirliğini araştırmak amacıyla yapılmıştır.

MATERYAL ve METOT

Araştırmada rumen sıvısı alınacak 3 baş ivesi toklusuna, cerrahi operasyonla rumen kanülü takılması, bakım ve beslenmeleri ve hayvanlardan deneme süresince rumen sıvısı alınması Harran Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu (HRÜ-HADYEK)'nun 2009/6 sayılı kurul kararı doğrultusunda gerçekleştirilmiştir. Araştırmada yem materyali olarak ruminant beslenmesinde yaygın olarak kullanılan yem hammaddeleri kullanılmıştır. Bu amaçla 4 farklı kaba yem (mısır silajı, yonca kuru otu, mercimek samanı, buğday samanı), 4 farklı karma yem (sığır besi yemi, sığır süt yemi, toklu besi yemi ve buzağı büyütme yemi) ve 4 farklı yem hammaddesi (arpa, mısır, pamuk tohumu küspesi, soya küspesi) kullanılmıştır. Yem maddeleri 1 mm elekten geçecek şekilde değirmende öğütüldükten sonra kuru madde (KM), ham kül (HK), ham yağ (HY), ham protein (HP) ve ham selüloz (HS) analizleri Weende analiz sistemine göre [15], asit deterjan fiber (ADF) ve nötral deterjan fiber (NDF) analizleri Van Soest ve Robertson'a [16] göre yapılmıştır.

Araştırmada *in vitro* sindirim denemeleri iki aşamalı sindirim denemesi ve gaz üretim tekniği olarak iki deneme

halinde yürütülmüştür. İki aşamalı *in vitro* organik madde sindirim değerlerinin belirlenmesinde Tilley ve Terry'nin [17] bildirdiği, *in vitro* gaz üretim tekniği Menke ve Steingass'ın [18] bildirdiği yönteme göre uygulanmıştır. *İn vitro* sindirim denemeleri her bir yem maddesi için 5'er tekerrür olacak şekilde uygulanmıştır. Araştırmada kullanılan rumen sıvısı, günde iki öğün halinde sabah 09:00 ve akşam 17:00 saatlerinde yaşama payının %25 fazlası düzeyinde %40 kesif (toklu besi yemi) ve %60 kaba yem (yonca kuru otu) ile beslenen rumen kanülü takılmış 60 kg (±3 kg) canlı ağırlığında, bir yaşlı 3 baş İvesi erkek tokludan temin edilmiştir. Deneme hayvanlarının yeme alıştırma dönemi (20 gün) sonrasında sabah yemlemesinden 1 saat önce CO_2 gazı altında alınan rumen sıvısı içerisinde CO_2 gazı ve sıcak su ile ısıtılmış cam şişede toplanarak, termos içerisinde vakit kaybedilmeden laboratuara getirilmiştir. Laboratuara getirilen rumen sıvısı CO_2 gazı altında 4 kat tülbent bezinden süzülerek kontrol ve araştırma grupları için hazırlanan falkon tüplerine konulmuştur. Kontrol (taze rumen sıvısı) grubunda iki aşamalı *in vitro* sindirim yöntemi Tilley ve Terry'nin [17], gaz üretim tekniği ise Menke ve Steingass'ın [18] bildirdiği yönteme göre hemen başlatılmıştır. Deneme grupları sıvı azot içerisinde dimetil sülfoksit (DMSO) katkısız ve katkılı (%5), derin dondurucuda DMSO katkısız ve katkılı (%5) dondurulmuş rumen sıvısından oluşturulmuştur. Bu amaçla; araştırma gruplarındaki tüplere (%5 DMSO katılan ve katılmayan) rumen sıvısı konarak tüplerin üzerinden CO_2 gazı geçirilmiş ve bu tüpler ağızları kapalı olarak içerisinde 39°C ısıda su ve buz bulunan küvetler içerisinde buzdolabında +4°C'de 30 dak. bekletilmiştir (equilibration süresi). Buzdolabında bekletme süresi içerisinde DMSO katılan tüplere %5 oranında DMSO 10'ar dakikalık ara ile üç aşamada otomatik pipet yardımı ile ilave edilmiş ve sonrasında içerisinde rumen sıvısı bulunan tüpler CO_2 gazı ile doyurulmuştur. DMSO katılmayan tüpler ise sadece CO_2 gazı ile doyurulma işlemi uygulanmıştır. Equilibration süresi sonunda DMSO katılan ve katılmayan tüpler derin dondurucuda yatay bir şekilde –21°C'de 1 ay süre ile dondurularak saklanmışlardır. Eş zamanlı olarak sıvı azot içerisinde dondurulacak tüpler ise özel olarak hazırlanmış bir düzenek üzerinde yatay bir şekilde hareket ettirilerek önce sıvı azot buharı ile ön donmaya maruz bırakıldıktan sonra tüpler sıvı azot stok tankı içerisine atılarak donma işlemi -196°C'de sabitlenmiştir. Sıvı azot içerisinde dondurulan tüpler *in vitro* sindirim denemelerinin uygulanacağı zamana kadar (1 ay) sıvı azot tankı içerisinde saklanmıştır. DMSO katkılı ve katkısız olarak sıvı azot tankı ve derin dondurucuda bir ay süre ile dondurulan tüpler *in vitro* sindirim denemelerinin yapılacağı zamanda 39°C'deki su banyosu içerisinde 30-45 saniye içerisinde çözdürülerek kullanılmışlardır. Taze, DMSO katkılı ve katkısız sıvı azot içerisinde ve derin dondurucuda dondurulmuş rumen sıvılarındaki aktif rumen sıvısı bakterilerinin sayımı Dehority'nin [19] belirttiği metoda göre yapılmıştır. Taze, sıvı azot içerisinde ve derin dondurucuda DMSO ilave edilen ve edilmeyen dondurulmuş rumen sıvılarındaki toplam protozoaların sayımı Boyne ve ark.'nın [20] bildirdikleri yönteme göre, protozoa tiplerinin identifikasyonu ise Boyne ve ark.[20] ile Ogimoto ve Imai [21] tarafından bildirilen yöntemlere göre yapılmıştır.

Elde edilen veriler tesadüf parselleri deneme desenine göre analiz edilmiştir. Deneme sonunda elde edilen veriler deneme modeline uygun olarak varyans analizine tabii tutulmuş, ortalamaların karşılaştırılmasında Duncan çoklu karşılaştırma testi kullanılmıştır [22]. İstatistiksel analizler SAS [23] paket programı kullanılarak yapılmıştır.

BULGULAR

Araştırmada kullanılan yem maddelerinin ham besin madde içerikleri *Tablo 1*'de sunulmuştur. Çalışmada değerlendirilen yemlerin iki aşamalı *in vitro* sindirim yöntemi ile İVOMS değerlerinin belirlenmesinde dondurulma

Tablo 1. *Araştırmada kullanılan yem maddelerinin ham besin madde içerikleri (g/kg, KM)*
Table 1. *The chemical composition of the experimental feeds (g/kg, DM)*

Yemler	DM	HK	HY	HP	ADF	NDF	HS
Mısır silajı	932	63	36	71	288	532	240
Yonca kuru otu	914	87	21	139	390	529	263
Mercimek samanı	899	111	27	95	316	476	215
Buğday samanı	930	111	25	43	459	753	353
Sığır besi yemi	882	85	28	141	81	239	49
Sığır süt yemi	884	59	28	170	88	212	48
Toklu besi yemi	879	69	32	139	101	310	62
Buzağı büyütme yemi	892	94	37	175	111	299	72
Arpa	900	26	23	104	72	381	52
Mısır	873	13	30	81	45	163	21
Pamuk tohumu küspesi	898	60	10	283	291	447	164
Soya küspesi	871	65	10	476	76	130	36

KM: *Kuru madde;* ***HK:*** *Ham kül;* ***HY:*** *Ham yağ;* ***HP:*** *Ham protein;* ***ADF:*** *Asit deterjan fibre;* ***NDF:*** *Nötr deterjan fibre;* ***HS:*** *Ham seluloz*

yöntemi ile dimetil sülfoksit (DMSO) seviyesinin etkisi *Tablo 2*'de sunulmuştur. Deneme yemleri genel olarak ele alındığında gerek sıvı azot içersinde ve gerekse derin dondurucuda dondurulan rumen sıvılarından elde edilen iki aşamalı İVOMS değerleri kontrol grubundan düşük (P<0.05) bulunmuştur. Sıvı azot içerisinde DMSO katkısız olarak dondurulmuş rumen sıvısı kullanılarak elde edilen iki aşamalı İVOMS değerleri incelendiğinde bazı yemlerin (mısır silajı, buzağı büyütme yemi, arpa ve soya küspesi) haricinde elde edilen tüm yemler için bulunan İVOMS değerleri kontrol değerlerinden düşük (P<0.05) bulunmuştur. Araştırmada değerlendirilen yemlerin *in vitro* gaz üretim tekniği ile İVOMS değerlerinin belirlenmesinde dondurulma yöntemi ile dimetil sülfoksit (DMSO) seviyesinin etkisi *Tablo 3*'te sunulmuştur. Deneme yemleri genel olarak değerlendirildiğinde %5 DMSO ilavesi ile sıvı azot içerisinde dondurulmuş rumen sıvısının inokulant olarak kullanıldığı *in vitro* gaz üretim tekniğinde elde edilen İVOMS değerleri tüm yemler için kontrol değerleri ile benzer (P>0.05) bulunmuştur. Araştırmada kullanılan kontrol ve dondurulmuş rumen sıvılarına ait toplam anaerob bakteri ve protozoa sayılarına ilişkin elde edilen veriler *Tablo 4*'te

Tablo 2. *Yemlerin iki aşamalı in vitro metot ile organik madde sindirilebilirliklerinin belirlenmesinde dondurulma yöntemi ile dimetil sülfoksit (DMSO) seviyesinin etkisi*

Table 2. *The effects of freezing method and dimethyl sulfoxide (DMSO) level on in vitro organic matter digestibility (IVOMD, %) of feedstuffs via two stage method*

Yemler	Kontrol	Sıvı Azot		Derin Dondurucu		SEM
		0% DMSO	5% DMSO	0% DMSO	5% DMSO	
Mısır silajı	72.58^{a}	69.6^{ab}	68.59^{b}	59.36^{c}	54.66^{d}	1.44
Yonca kuru otu	69.04^{a}	66.03^{b}	67.00^{b}	60.03^{c}	60.66^{c}	0.75
Mercimek samanı	75.90^{a}	71.16^{b}	71.41^{b}	66.12^{c}	66.10^{c}	0.78
Buğday samanı	58.73^{a}	55.19^{b}	51.26^{c}	36.71^{e}	39.32^{d}	1.79
Sığır besi yemi	91.96^{a}	89.78^{b}	90.21^{b}	85.75^{c}	84.92^{c}	0.58
Sığır süt yemi	91.74^{a}	88.17^{b}	89.00^{b}	84.64^{c}	83.85^{c}	0.64
Toklu besi yemi	90.32^{a}	88.0^{b}	87.07^{bc}	83.35^{d}	85.58^{c}	0.50
Buzağı büyütme yemi	88.59^{a}	86.90^{ab}	85.69^{b}	82.00^{c}	81.57^{c}	0.61
Arpa	90.84^{a}	88.23^{a}	87.41^{a}	81.47^{b}	79.96^{b}	0.94
Mısır	95.33^{a}	91.72^{b}	90.30^{b}	84.40^{c}	86.41^{c}	0.83
Pamuk tohumu küspesi	70.17^{a}	65.12^{b}	63.72^{b}	61.93^{bc}	59.03^{c}	0.82
Soya küspesi	95.51^{a}	94.74^{ab}	94.04^{b}	94.65^{ab}	94.69^{ab}	0.16

DMSO: *Dimetilsülfoksit; SEM: Standart hata ortalaması,* $^{a-d}$ *Aynı satırda farklı harf taşıyan ortalama değerler arasındaki farklılık önemlidir (P<0.05)*

Tablo 3. *Yemlerin in vitro gaz üretim tekniği ile organik madde sindirilebilirliklerinin belirlenmesinde dondurulma yöntemi ile dimetil sülfoksit (DMSO) seviyesinin etkisi*

Table 3. *The effects of freezing method and dimethyl sulfoxide (DMSO) level on in vitro organic matter digestibility (IVOMD, %) of feedstuffs via gas production technique*

Yemler	Kontrol	Sıvı Azot		Derin Dondurucu		SEM
		0% DMSO	5% DMSO	0% DMSO	5% DMSO	
Mısır silajı	61.24^{a}	58.45^{a}	59.86^{a}	40.87^{c}	48.01^{b}	1.68
Yonca kuru otu	60.06^{a}	51.76^{b}	57.95^{a}	39.67^{d}	44.26^{c}	1.63
Mercimek samanı	61.07^{a}	56.67^{a}	57.30^{a}	40.66^{b}	43.49^{b}	1.77
Buğday samanı	40.64^{a}	37.02^{b}	41.75^{a}	27.03^{c}	27.26^{c}	1.33
Sığır besi yemi	73.61^{a}	73.96^{a}	71.63^{a}	62.80^{b}	64.44^{b}	1.05
Sığır süt yemi	71.44^{a}	72.73^{a}	70.31^{ab}	63.45^{c}	66.04^{bc}	0.82
Toklu besi yemi	68.56^{ab}	68.69^{ab}	70.90^{a}	67.34^{b}	65.75^{b}	0.45
Buzağı büyütme yemi	66.62^{a}	63.89^{a}	65.30^{a}	54.37^{b}	54.54^{b}	1.13
Arpa	76.11^{a}	75.35^{a}	74.42^{a}	72.25^{b}	67.50^{c}	0.66
Mısır	77.71^{a}	74.45^{b}	78.91^{a}	68.21^{c}	65.88^{c}	1.09
Pamuk tohumu küspesi	52.82^{a}	45.82^{b}	50.71^{a}	37.65^{c}	36.28^{c}	1.38
Soya küspesi	77.11^{a}	72.57^{b}	77.12^{a}	56.92^{c}	56.47^{c}	1.93

DMSO: *Dimetilsülfoksit;* ***SEM:*** *Standart hata ortalaması;* $^{a-d}$ *Aynı satırda farklı harf taşıyan ortalama değerler arasındaki farklılık önemlidir (P<0.05)*

Table 4. *Toplam canlı anaerob bakteri ve protozoa sayıları (cfu/ml) üzerine dondurulma yöntemi ile dimetil sülfoksit (DMSO) seviyesinin etkisi*
Table 4. *The effects of freezing method and dimethyl sulfoxide (DMSO) level on the number of total bacteria and protozoa (cfu/ml)*

Bakteri ve Protozoa Sayıları	Kontrol	Sıvı Azot		Derin Dondurucu		SEM
		0% DMSO	5% DMSO	0% DMSO	5% DMSO	
TABS	$104.0x10^{8a}$	$13.2x10^{8bc}$	$21.6x10^{8b}$	$6.4x10^{8c}$	$16.8x10^{8bc}$	7.46
TPS	$380.9x10^{3a}$	$121.3x10^{3b}$	$130.7x10^{3b}$	$128.9x10^{3b}$	$136.4x10^{3b}$	20.76
Entodinium spp.	$91.6x10^{3a}$	$66.8x10^{3b}$	$91.2x10^{3a}$	$60.4x10^{3c}$	$65.4x10^{3b}$	2.77
Epidinium spp.	$31.6x10^{3a}$	$29.8x10^{3a}$	$30.4x10^{3a}$	$8.4x10^{3b}$	8.80^{b}	2.22
Polipastron	$4.0x10^{3a}$	$2.2x10^{3b}$	$2.8x10^{3b}$	0.00^{c}	0.00^{c}	0.34
İsotrichia spp.	$2.0x10^{3a}$	$1.0x10^{3ab}$	$0.8x10^{3ab}$	0.00^{b}	0.00^{b}	0.21
Dastrichia spp.	$1.2x10^{3a}$	$0.2x10^{3b}$	$0.6x10^{3ab}$	0.00^{b}	0.00^{b}	0.13

TABS: *Toplam Anaerob bakteri sayısı;* ***TPS:*** *Toplam protozoa sayısı;* ***DMSO:*** *Dimetilsülfoksit;* ***SEM:*** *Standart hata ortalaması;* $^{a-c}$ *Aynı satırda farklı harf taşıyan ortalama değerler arasındaki farklılık önemlidir (P<0.05)*

sunulmuştur. Toplam anaerob bakteri ve toplam protozoa sayıları tüm dondurulmuş rumen sıvılarında kontrol denemesinden düşük (P<0.05) bulunmuştur. Ancak gerek sıvı azot içerisinde ve gerekse derin dondurucuda uygulanan dondurma işleminde %5 DMSO ilavesinin rakamsal olarak toplam anaerob bakteri ve protozoa sayısını arttırdığı görülmüştür. Protozoa türleri incelendiğinde *Polipastron* türü haricinde, sıvı azot içerisinde %5 DMSO katkısı ile dondurulmuş rumen sıvısındaki protozoa türlerinin kontrol grubu ile genel olarak benzer (P>0.05) olduğu görülmüştür.

TARTIŞMA ve SONUÇ

Bu çalışmada iki aşamalı *in vitro* yöntem ile elde edilen İVOMS değerleri incelendiğinde gerek sıvı azot içerisinde ve gerekse derin dondurucuda dondurulan rumen sıvılarından elde edilen İVOMS değerlerinin kontrol grubundan düşük (P<0.05) olduğu gözlenmiştir. Bazı yemlerin (mısır silajı, buzağı büyütme yemi, arpa ve soya küspesi) sıvı azot içerisinde DMSO ilave edilmeden dondurulmuş rumen sıvısından elde edilen İVOMS değerlerinin kontrol değerleri ile benzer (P>0.05) olduğu, ancak bu benzerliğin yemlerin genelini kapsamadığı gözlenmiştir. Zeigler ve ark.'nın [9] yapmış oldukları çalışmada yemlerin *in vitro* kuru madde sindirim değerlerinin belirlenmesinde kriyoprotektan ilave edilmeksizin dondurulmuş rumen sıvısının kullanılabileceği, %5 gliserol ilave edilerek dondurulan rumen sıvılarından elde edilen sonuçların ise taze rumen sıvısından elde edilen değerlerden düşük sonuç verdiğini bildirmektedirler. Araştırmacılar konsantre yemler için katkısız ve %5 gliserol katkılı dondurulmuş rumen sıvısından elde ettikleri *in vitro* sindirim değerlerinin taze rumen sıvısı ile benzer olduğunu, ancak yüksek seluloz içeriğine sahip kaba yemlerde *in vitro* sindirim değerinin düşük olduğunu, bu farklılığın ise dondurulma ve çözme işlemi sürecinde rumen sıvısında bulunan selülotik bakterilerin zarar görmüş olabileceği varsayımına bağlamaktadırlar [9]. Jones ve ark.'nın [24] yaptıkları bir çalışmada taze rumen sıvısının +18°C'de 48 saat boyunca etkinliğini yitirmeden saklanabileceğini bildirmektedirler. Araştırıcılar [24] kaba yemlerin iki aşamalı İVOMS değerlerinin belirlenmesinde +18°C'de 48 saat muhafaza edilen rumen sıvısının kullanılabileceğini ancak iki aşamalı *in vitro* sindirim metodunun mikrobiyal sindirim aşamasındaki 48 saatlik sürenin yetersiz olduğunu bildirmişlerdir. Bazı çalışmalarda [24-26], araştırmacılar kaba yemler için iki aşamalı *in vitro* sindirim metodunun mikrobiyal sindirim aşamasındaki 48 saatlik sürenin 72 saate çıkarılmasının gerektiğini bildirilmektedir. Benzer şekilde Denek ve ark.[27] bazı yem maddelerinin *in vitro* kuru madde sindirim değerlerinin iki aşamalı yöntem ile belirlenmesinde derin dondurucuda %5 DMSO ilave edilerek dondurulmuş rumen sıvısı kullanmışlardır. Araştırıcılar [27] kaba yemler için 48 saatlik mikrobiyal sindirim aşamasının yeterli olmadığını, bu sürenin 72 saate çıkarıldığında iki aşamalı sindirim yönteminde elde edilen sonuçların taze rumen sıvısından elde edilen sonuçlar ile benzer bulunduğu bildirmektedirler. Bu bildirimler doğrultusunda iki aşamalı *in vitro* sindirim yöntemi ile yemlerin İVOMS değerlerinin belirlenmesinde mikrobiyal inokulant kaynağı olarak sıvı azotta dondurulmuş rumen sıvısının kullanılabileceği, ancak 48 saatlik mikrobiyal inkubasyon arttırılmasına ilişkin yeni çalışmaların yapılmasına ihtiyaç duyulmaktadır.

Bu çalışmada, gaz üretim tekniği ile elde edilen İVOMS değerleri incelendiğinde, sıvı azot içerisinde %5 DMSO ilavesi ile dondurulmuş rumen sıvısından elde edilen İVOMS değerlerinin tamamı kontrol değerleri ile benzer (P>0.05) bulunmuştur. Ancak sıvı azot içerisinde DMSO ilave edilmeden dondurulmuş rumen sıvılarında ise bazı yemlerin (yonca kuru otu, mısır, pamuk tohumu küspesi ve soya küspesi) İVOMS değerlerinin kontrolden düşük (P<0.05) olduğu görülmüştür. Derin dondurucuda gerek %5 DMSO ilave edilmiş ve gerekse DMSO ilave edilmemiş gruplardan elde edilen İVOMS değerleri kontrol değerlerinden düşük (P<0.05) bulunmuştur. Prates ve ark.[12] *in vitro* gaz üretim tekniğinin uygulanmasında sıvı azotta dondurulmuş rumen sıvısının taze rumen sıvısına alternatif olabileceğini, Rumen mikroorganizmalarının etkinliği bakımından sıvı azotta dondurmanın derin dondurucuda dondurmadan daha etkin olduğunu bildirmektedirler. Bu çalışmanın sonuçları incelendiğinde elde edilen sonuç-

ların Prates ve ark.'nın [12] sonuçları ile benzer olduğu görülmektedir. Hervas ve ark.'nın [11] yapmış oldukları bir çalışmada, kriyoprotektan ilave edilmeden 0°C'de 3, 6 ve 24 saat ile derin dondurucuda veya -18°C'de 24 saat süre ile dondurulmuş rumen sıvısını gaz üretim tekniğinde, 0°C'de 3 ve 6 saat bekletilen rumen sıvılarından elde edilen gaz üretim değerlerinin kontrol grubu ile benzer bulunurken, -18°C'de 24 saat süre ile dondurulan rumen sıvısından elde edilen gaz üretim değerleri ise düşük bulunmuştur. Araştırıcılar bu farklılığın sebebini donma aşamasında rumen mikroorganizmalarında intrasellüler buz kristallerinin oluşumuna ve buna bağlı olarak hücre harabiyetine bağlamaktadırlar. Bu çalışmada benzer olarak DMSO katkılı ve katkısız olarak derin dondurucuda bir ay süre ile dondurulmuş rumen sıvılarından elde edilen İVOMS değerleri kontrol değerlerinden düşük bulunmuştur.

Araştırmada kullanılan taze ve dondurulmuş rumen sıvılarına ait toplam anaerob bakteri sayılarına ilişkin elde edilen veriler incelendiğinde, toplam canlı anaerob bakteri sayıları gerek sıvı azot ve gerekse derin dondurucuda DMSO etkisine bakılmaksızın azaldığı ($P<0.05$) tespit edilmiştir. Ancak %5 DMSO katkısının her iki dondurma işleminde de anaerob canlı bakteri sayısında rakamsal olarak artışa yol açtığı görülmektedir. Stewart ve ark.[28] rumen ortamında gram negatif bakterilerin çoğunlukta olduğunu ve bu bakterilerin dondurma ve çözme işlemlerinden olumsuz şekilde etkilendiklerini bildirmektedirler. Rumen sıvısının 0°C ve -30°C ısılarda dondurulması ile kötü sonuçlar elde edilirken, sıvı azot içerisinde (-196°C) yapılan dondurma işleminde ise mikroorganizmaların uygulanan dondurma işleminden fazla etkilenmediği bildirilmektedir [29,30]. Protozoa değerleri incelendiğinde sıvı azot içerisinde %5 DMSO ilavesi ile dondurulmuş rumen sıvısındaki canlı protozoa sayılarının kontrol grubu ile genel olarak benzer ($P>0.05$) olduğu belirlenmiştir. Bu benzerlik *Entodinium spp., Epidinium spp., İsotrichia spp.* ve *Dastrichia spp.* Türlerinde tespit edilirken, *Polipastron* türünde tespit edilememiştir. Bazı çalışmalarda [31,32] rumen protozolarının %5 DMSO ilavesi ile dondurulması ve sonrasında çözüldüğünde canlılıklarının yüksek düzeyde olduğu belirtilmektedir. Yine Abdel-Aziz ve ark.'nın [13] rumen protozolarının sıvı azot içerisinde dondurulmalarına ilişkin yaptıkları bir çalışmada kriyoprotektan olarak %4, 5 ve 6 düzeyinde gliserol, DMSO ve etilen glikol kullanmışlardır. Çözdürülen protozoaların canlılıkları incelendiğinde %5 DMSO ilavesinin en iyi sonuç verdiği görülmüştür. Benzer şekilde Nsabimana ve ark.[14] kriyoprotektan olarak %4, 5 ve 6 düzeyinde DMSO kullanmışlar ve en etkili DMSO seviyesinin %5 olduğunu bildirmişlerdir. Yapılan bazı çalışmalarda rumen mikroorganizmaları üzerine DMSO'nun diğer kriyoprotektanlardan daha etkin koruma sağlamasını hücreye daha hızlı nüfuz etmesine [33,34] ve donma işlemi sırasında hücre içerisinde buz kristalleri oluşturmamasına bağlamaktadırlar [34].

Bu araştırmanın sonuçlarına göre, yemlerin *in vitro* gaz üretim tekniği ile İVOMS değerlerinin tespitinde mikrobiyal inokulant kaynağı olarak sıvı azot içerisinde %5 DMSO katılarak dondurulmuş rumen sıvısının kullanılabileceği sonucuna varılmıştır. Bu sonuçlara göre belirli merkezlerde rumen kanülü takılmış hayvanlardan elde edilecek rumen sıvısına %5 düzeyinde DMSO ilave edilerek sıvı azot içerisinde dondurulması ile rumen kanülü açılmış donor hayvan barındırma imkânı bulunmayan araştırma merkezlerinin bu inokulantları kullanarak *in vitro* sindirim denemelerini gerçekleştirmeleri mümkün görünmektedir.

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Research Article

Evaluation of Serum and Ascitic Fluid Proteomes in Dogs with Dilated Cardiomyopathy [1]

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Abstract

The aim of the study was to investigate serum global proteomes in dogs with overt dilated cardiomyopathy (DCM) and to evaluate protein expression in serum with that in ascitic fluid. Eight healthy dogs (control group) and 8 dogs with DCM were included in the study. DCM was diagnosed based on echocardiographic evidence including increased left ventricular dimension at diastole and systole, increased E point to septal separation, and decreased fractional shortening. Serum and ascitic fluid samples were analyzed for proteomes using a label-free LC-MS/MS method. Proteome analyses revealed significantly different expressions of eight proteins in all samples. Expressions in serum of apolipoprotein (Apo) A1, Ig heavy chain V, superoxide dismutase and plasminogen were higher (P<0.001), while expressions of clusterin, hemoglobin subunit ß, Apo-CII, and β2 glycoprotein I (ß2GPI) were lower (P<0.001) in dogs with DCM than in control dogs. In addition, Apo-A1, clusterin, hemoglobin subunit ß, Ig heavy chain V, plasminogen and ß2GPI were down-regulated whereas Apo-CII and superoxide dismutase were up-regulated in ascitic fluid compared with serum in dogs with DCM. Data obtained in the present study suggest that serum and/or ascitic fluid proteomes may explain some of the pathophysiological mechanisms involved in the progression of DCM.

Keywords: *Dilated cardiomyopathy, DCM, Congestive heart failure, Proteomics, Dog*

Dilate Kardiyomiyopatili Köpeklerde Serum ve Asites Sıvısı Proteomlarının Araştırılması

Özet

Çalışmamızın amacı ileri düzeyde dilate kardiyomiyopati (DCM) tanısı konulan köpeklerde serum global proteomların araştırılması ve aynı hastaların asites sıvısı proteomları ile ilişkilerinin değerlendirilmesidir. Sekiz sağlıklı (kontrol grubu) ve 8 DCM'li köpek çalışmaya dahil edildi. DCM tanımlaması ekokardiografik olarak sistol ve diyastolde artmış sol ventriküler çap, artmış E point to septal separasyon değeri ve azalmış fraksiyonel kasılma verileri temelinde yapıldı. Serum ve asites sıvı örnekleri label-free LC-MS/MS metoduna göre analiz edilmiştir. Proteom analizi ile tüm örneklerde toplam sekiz adet proteom ekspresyonu belirlendi. DCM'li köpeklerde kontrol grubuna göre serum apolipoprotein (Apo) A1, Ig heavy chain V, superoksit dizmutaz ve plazminojen ekspresyonlarında artış (P<0.001), klasterin, hemoglobin subunit ß, Apo-CII, ve β2 glikoprotein I (ß2GPI) ekspresyonlarında ise azalma (p<0.001) belirlendi. Buna ek olarak; asites sıvısı serum örnekleri ile karşılaştırıldığında Apo-A1, klasterin, hemoglobin subunit ß, Ig heavy chain V, plazminojen ve ß2GPI azalırken, Apo-CII ve superoksit dizmutaz artış gösterdi. Çalışmada elde edilen bu verilerin; serum ve/veya asites sıvısı proteomlarının DCM gelişiminde rolü alan bazı patofizyolojik mekanizmaların açıklanmasına katkı verebileceği kanısındayız.

Anahtar sözcükler: *Dilate kardiyomiyopati, DCM, Konjestif kalp yetmezliği, Proteomik, Köpek*

INTRODUCTION

Dilated cardiomyopathy (DCM) sis one of the most common organic heart defects in dogs [1]. Echocardiographic features include ventricular dilation, atrial dilation, normal or thin wall and septal thickness, depressed systolic thickening of the free wall and septum, poor fractional shortening (FS), large E point to septal separation (EPSS), reduced aortic wall motion and global hypokinesis [1,2]. Ventricular dilation including generally the left side of the

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heart and poor myocardial function are the main findings of DCM [2]. Such abnormalities of the heart muscle result in chemical and organic reactions cause biomarker release such as natriuretic peptides and cardiac troponins [3]. Many researchers have focused on specific biomarker indicating myocardial injury in DCM [3]; however, new trend of the science is interested in such small peptides, rather than the whole proteins, called proteomics [6].

Proteomics is the large-scale study of protein expression, protein-protein interactions, or post-translational modifications [6-8]. Use of proteomics technology in veterinary medicine is presently under development. Samples and study designs are discussed due to the limitations of mass spectrometry [9]. In veterinary medicine proteomics were performed in such conditions as canine lymphoma [10] and heartworm disease [11] while canine DCM has not yet been studied using proteomics. In addition, although proteomics were performed in several canine body fluids such as cerebrospinal fluid [12], urine [13], bronchoalveolar lavage fluid [14] and follicular fluid [15], no data have yet been presented on ascitic fluid in dogs with DCM. In the present study, we hypothesized that many of the serum proteomes with low abundance and low molecular weight may have roles in the development of DCM in dogs. Besides, proteomic analyses of ascitic fluid may provide further details to explain some patho-physiologic mechanism and/or some clinical complications such as abdominal distention and pleural effusion in dogs with DCM.

Therefore, we aimed to analyze expressional proteomics in both serum and ascitic fluid samples, using label-free LC-MS/MS method, in order to present novel data that may help improve our understanding of the pathophysiology of canine DCM.

MATERIAL and METHODS

Animals

Eight dogs with DCM (5 females, 3 males) with different breed (Mixed breed n=4, Terrier n=2, Pointer n=1, Anatolian Sheepdog n=1) and an average age of 54.8±30.8 months (range: 10-96 months), and 8 clinically healthy dogs (4 females, 4 males) with different breed (Mixed breed n=5, Terrier n=2, Anatolian sheepdog n=1) and an average age of 50.2±16.4 months (range: 2 to 8 years) were included in the study. All dogs with DCM were suffering from congestive heart failure (CHF) findings such as cough, lethargy, anorexia, dyspnea, exercise intolerance, abdominal distension and/or pleural effusion.

Electrocardiographic and Echocardiographic Evaluations

After routine clinical examination, electrocardiography (ECG, Esaote, P200®- Florence, Italy), thoracic radiography and echocardiographic examinations were performed. Two-dimensional (2D) echocardiography, M-mode, color flow imaging and spectral Doppler examinations were performed using a CarisPlus® (Esoate, Florence, Italy) with a 2.5-5 MHz phased-array transducer using standard techniques [2]. The dogs were not sedated throughout the ultrasound examination, and were gently restrained in right lateral recumbency. DCM was diagnosed based on the echocardiographic findings such as increased chamber size, increased EPSS and poor FS along with ECG and thoracic radiographic findings. Diagnosis was confirmed using a scoring system for DCM proposed by European Society for Veterinary Cardiology [2]. Patients presented with all of the criteria for DCM but did not have any other cardiac pathology including congenital malformations or tumors. Dogs with primary congenital heart disease, mitral valve disease or endocrine disorders such as hypothyroidism were excluded.

Radiologic Evaluation

Assessment of cardiomegaly on thoracic radiography was based on a combination of subjective experience and the use of the vertebral heart scale system [16].

Sample Collection and LC-MS/MS Analysis

Serum and ascitic fluid samples were obtained from patients before the treatment. Samples were kept in -80ºC freezer until being sent in cold chain to the laboratory for analyses [TUBITAK, Genetical Engineering and Bio-technology Institute (GEBI), Gebze, Kocaeli, Turkey].

Protein expression analyses in serum and ascetic fluid samples were performed using a label-free nano liquid chromatography - mass spectrometry method. Extracted proteins from the samples were reduced with dithriothreitol (DTT; 5 mM, 15 min) and alkylated with iodoacetic acid (IAA; 10 mM, 30 min at dark). Tryptic peptides were generated by incubating the protein mixture at 37ºC with sequencing grade trypsin (1:50 ratio, Pierce). The peptide mixture was loaded on a nanoACQUITY UPLC Symmetry C18 Trap column (5 µm particle size, 180 µm i.d. x 20 mm length) at 5 µl/min flow rate for 5 min. Peptides were eluted from the trap column by gradient elution onto an analytical column (nanoACQUITY UPLC BEH C18 Column, 1.7 µm particle size, 75 µm i.d. x 250 mm length, Waters), at 300 nl/min flow rate with a linear gradient from 5 to 40% acetonitrile over 90 min. Data independent acquisition mode (MS^E) was carried out by operating the instrument at positive ion V mode. Mass drift was corrected by the internal mass calibrantglu-fibrinopeptide infused every 45 sec through the nanolockspray ion source at 300 nl/min flow rate. Peptide signal data between 50-1.600 m/z values were collected. Data processing parameters were set to standard operating values [17]. The Apex3D parameters were set to 0.2 min chromatographic peak width and 10.000 TOF resolution. 150, 50 and 1.200 counts were set

for low energy, elevated energy and intensity threshold, respectively. Tandem mass spectra extraction, charge state deconvolution and deisotoping were processed with ProteinLynx Global Server v2.3 software (PLGS) (Waters Corp., Milford, MA). Protein sequence database from Uniprot was used. Databank search query was set to minimum 3 fragment ion matches per peptide, minimum 7 fragment ion matches per protein, minimum 1 peptide matches per protein and 1 missed cleavage. Carbamidomethyl-cysteine fixed modification and Acetyl N-terminal, deamidation of asparagine and glutamine, oxidation of methionine variable modifications were set. Normalization of the proteins was achieved against the digest of the internal calibrant P00330.

Statistical Analyses

Data were analyzed by Student's t test using SPSS 10.0 Statistical Software (SPSS Inc), and the results were expressed as Mean ± Standard error of means (SEM). The intensity % coefficient of variation (%CV Int) were calculated to be around 14% across the identified proteins so three times the %CV Int value is set for the cut-off for statistical significance. Only proteins expressional changes showing more than 40% up-regulation or down-regulation were reported. For all comparisons, values of $P<0.05$ were considered significant.

RESULTS

Clinical Findings

Clinical charts of dogs with DCM included dyspnea (6/8), lethargy (4/8), exercise intolerance (8/8), and anorexia (5/8). Clinical examination revealed weak femoral artery pulse, distension of the jugular vein, abdominal distension, increased cardiac auscultation area and mitral and/or tricuspidal murmurs with different severities in all patients with DCM.

Electrocardiographic (ECG) Findings

ECG analysis revealed some cardiac rhythms abnormalities in dogs with DCM in which atrial fibrillation (5/8) *(Fig. 1)*, ventricular extra systoles (2/8), atrioventricular block (1/8) and sinus tachycardia (2/8) were the most common.

Radiological Findings

Enlarged heart size, deviation of the trachea, mild to severe pulmonary edema, increased vertebral heart scale (14.2±1.3), pulmonary pattern, caudal vena cava distension, and pleural and peritoneal effusions were observed on thoracic radiography in dogs with DCM *(Fig. 2)*.

Two-dimensional Echocardiographic Findings

All dogs with DCM had geometric and functional cardiac abnormalities. LA diameter (4.62±0.4 cm), LA/Ao ratio (2.08±0.4), left ventricular diastole diameter (6.3±0.7 cm) and EPSS value (1.1±0.3 cm) were above the reference limits. Poor FS (15.8±4.8%) was observed in dogs with DCM, as well *(Table 1, Fig. 3)*. Pulmonary artery flow velocity was higher ($P<0.001$) but aortic flow velocity was lower ($P<0.001$) than those of healthy controls *(Table 1)*.

Serum and Ascitic Fluids Proteomics

Expressions of 8 proteins (Apolipoprotein[Apo]A1, hemoglobin subunit ß, clusterin, Ig heavy chain V region, Apo-CII, plasminogen, b 2 glycoprotein I [ß2GPI] and superoxide dismutase) differed significantly in blood and ascitic fluid samples *(Table 2)*. Apo-A1, Ig heavy chain V, superoxide dismutase and plasminogen expressions in serum samples were higher ($P<0.05$ - 0.01), while clusterin, hemoglobin subunit ß, Apo-CII and β2GPI expressions were lower ($P<0.05$ - 0.001) in dogs with DCM than in healthy controls. Expressions of Apo-A1, clusterin, hemoglobin subunit ß, Ig heavy chain V, plasminogen, and ß2GPI were

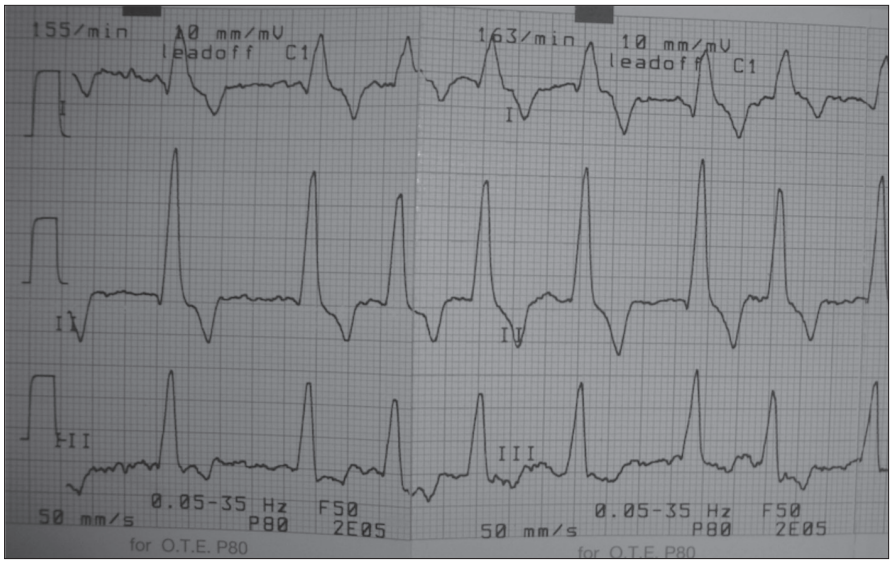

Fig 1. ECG from a dog with DCM (7 year-old, male, Turkish Shepherd Dog) revealed an atrial fibrillation due to absence of "P waves" and increased heart frequency (Calibration: 50 mm/second, 10 mm/1 mV)

Şekil 1. DCM'li bir köpek EKG'sinde (7 yaşlı, erkek, Kangal) 'P dalgalarının' olmaması ve artmış kalp frekası nedeniyle atriyal fibrilasyon belirlenmiştir (Kalibrasyon: 50 mm/saniye, 10 mm/1 mV)

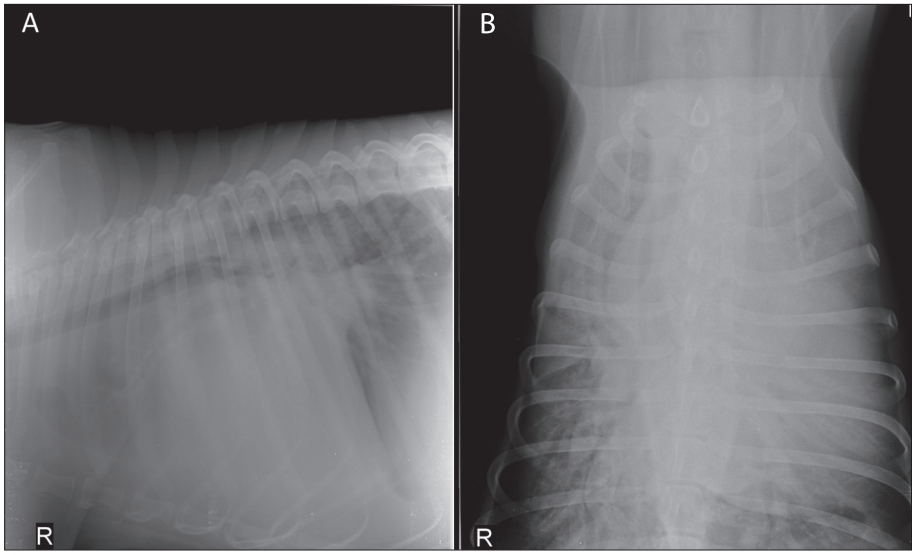

Fig 2. Radiologic evaluation of the thorax in a 8-year-old male Turkish Shepherd Dog. A: The right lateral radiograph points out the general lung tissue opacity, dorsal deviation of the trachea and the vascular hilus, bronchiectasis of the cranial lobe bronchus and enlargement of the heart borders in the thoracic cavity, B: The ventrodorsal radiograph shows general opacity, alveolar model influence of the lung lobes and unclear hearth silhouette as well as left side shift of the hearth suspected due to cardiomegaly

Şekil 2. 8 yaşlı erkek Kangal bir köpekte toraksın radyolojik olarak değerlendirilmesi. A: Sağ lateral radyografi genel akciğer dokusu opasitesindeki artışı, trakeanın dorsale deviyasyonu ve hilus vaskülarizasyonunu, kraniyal lob bronşunda bronşiyektaziyi ve torasik boşlukta kalbin sınırlarının genişlemiş olduğunu vurgulamaktadır, B: Ventrodorsal grafi genel opasite, akciğer loblarında alveoler etkilenim ve şüphelenilen kardiyomiyopati nedenli sol tarafa kaymaya ek olarak belirsiz kalp siluyeti belirtmektedir

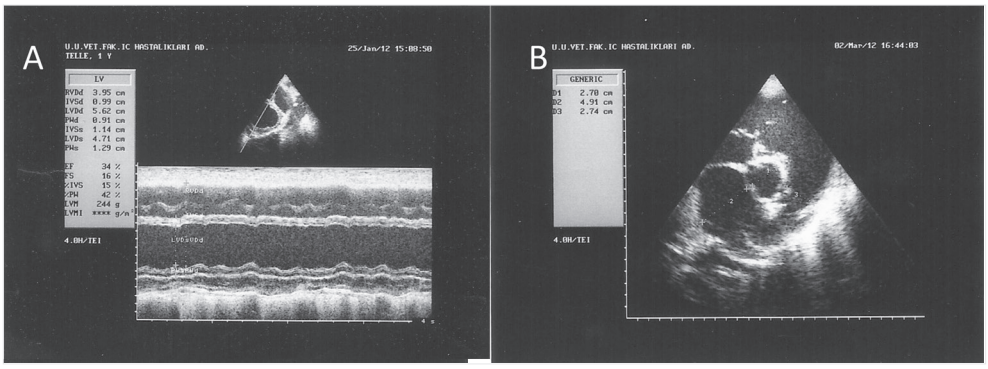

Fig 3. M-mode measurement of the left ventricle at right parasternal short axis view showed increased chamber size, poor fractional shortening and interventricular septal akinesia. Hyperechoic line in the pericardial sac indicated mild pericardial effusion (A). Left atrial dilation (left atrium/aorta ratio: 1.7) was observed on right parasternal short axis view - aortic level (B)

Şekil 3. Sol ventrikülün sağ parasternal kısa eksen M-mod görüntüsü artmış odacık büyüklüğü, zayıf fraksiyonel kısalma ve interventriküler septal akinezi olduğunu göstermiştir. Perikardiyal kesedeki hiperekoik çizgi hafif bir parikasrdiyal efüzyonu işaret etmektedir (A). Sağ parasternal kısa eksen görüntü- ortik düzeyde sol atriyal genişleme (Sol atriyum /aorta oranı: 1.7) belirlenmiştir (B)

down-regulated whereas expressions of Apo-CII and superoxide dismutase were up-regulated in ascitic fluid compared with serum in dogs with DCM.

DISCUSSION

The present study reported, for the first time, evaluation of protein expression changes in blood and ascitic fluid of dogs with DCM and made comparisons with regard to serum proteomes between dogs with DCM and healthy controls. Significant differences were detected in expressions of a total of 8 proteins (Apo-A1, hemoglobin subunit ß, clusterin, Ig heavy chain V region, Apo-CII, plasminogen, ß2GPI and superoxide dismutase) in both serum and ascitic fluid of dogs with DCM. In the study, DCM was diagnosed based on the echocardiographic

Tablo 1. *DCM'li ve sağlıklı köpeklerin ekokardiografik parametreleri (Mean ± SE)*

Table 1. *Echocardiographic parameters of the dogs with DCM and healthy dogs (Mean ± SE)*

Parameter	Dogs with DCM n=8	Healthy dogs n=8	P value
Body weight (Kg)	32.2 ±8.5	28.4±4.3	ns
IVSD (cm)	1.1±0.5	0.82±0.06	<0.01
IVSs (cm)	2.53±1.17	1.63±0.41	ns
LVDd (cm)	6.37 ±0.7	4.54±0.33	<0.001
LVDs (cm)	5.4±0.7	3.20±0.25	<0.001
PWd (cm)	0.88±0.37	0.94±0.11	<0.01
PWs (cm)	1.03±0.34	1.05±0.12	ns
Ao diameter (cm)	2.22 ±0.4	2.0±0.1	ns
LA diameter (cm)	4.62±0.4	2.3±0.0	<0.001
LA/Ao ratio	2.08±0.4	1.2±0.0	<0.001
EPSS (cm)	1.1±0.3	0.3±0.1	<0.001
FS %	15.8±4.8	30.20±2.41	<0.001
PA Vmax m/s	0.6±0.09	0.33±0.03	<0.001
AoVmax m/s	0.7±0.14	1.3±0.1	<0.001

BW: *body weight;* ***IVSD:*** *interventricular septum diastole;* ***IVSs:*** *interventricular septum systole;* ***LVDd:*** *left ventricular diameter diastole;* ***LVDs:*** *left ventricular diameter systole;* ***PWd:*** *post wall diastole;* ***PWs:*** *post wall systole;* ***Ao:*** *aorta;* ***LA:*** *left atrium;* ***LA/Ao ratio:*** *left atrium/ aorta ratio;* ***EPSS:*** *E-point to septal separation;* ***FS:*** *fractional shortening;* ***PA:*** *pulmonary artery;* **ns:** *not significant*

Table 2. *List of the protein expression results and fold changes (+ increase; - decrease) between serum from dogs with DCM (DCM) and healthy controls (H) or ascitic fluid (A)*

Tablo 2. *DCM'li (DCM) ve sağlıklı (H) köpeklerin serumları veya asites sıvıları (A) arasındaki protein salınım sonuçlarının ve son değerlerin başlangıç değerlerine oranlarının listesi (+ artış; - azalış)*

Accession #	Description	Fold Change	
		DCM *vs* H	DCM *vs* A
P02648	Apolipoprotein A 1	1.42 (+)**	1.41 (+)**
P25473	Clusterin	1.40 (-)*	1.21 (+)*
P60524	Hemoglobin subunit ß	1.40 (-)*	3.65 (+)**
P01784	Ig heavy chain V region GOM	1.41 (+)**	1.77 (+)**
P12278	Apolipiprotein C II	354.8 (-)***	299.7 (-)***
P80009	Plasminogen Fragment	1.44 (+)**	1.36 (+)**
P33703	ß 2 Glycoprotein 1	2.31 (-)**	47.6 (+)***
O54210	Superoxide dismutase Mn Fe fragment	1.65 (+)*	1.66 (-)*

vs - versus, * P<0.05, ** P<0.01, s*** P<0.001

findings along with ancillary diagnostic tests including ECG and thoracic radiography, as suggested [2].

Clusterin has a protective role on stress-mediated apoptosis, oxidative stress and protein aggregation inhibition [18]. Cardiorenal syndrome may be another reason for low serum clusterin level, since clusterin is also a renal damage biomarker in dogs [19,20]. Increased vascular permeability due to serum protein escape from the blood stream and the venous return loss in response to heart failure might be responsible of low clusterin levels in dogs with DCM studied. In the present study, the possible reason for decreased level of serum clusterin in dogs with DCM may be related with the result of excessive use of the protein in situations such as increased cell death and oxidative stress status.

Previous studies suggested that Apo-A1is responsible of cholesterol transport from tissues to the liver [11]. However *vena cava caudalis* pressure overload as a result of decreased venous return to the heart may play a role on liver-ischemia-induced loss of free cholesterol esterification. In this pathophysiology, high blood cholesterol plays a role on erythrolysis and hemoglobin release [11]. The mild increase in hemoglobin subunit ß protein detected in the present study might have resulted from increased demand of O_2 in the body in response to decreased cardiac output (lower aortic flow velocity), the myocardial contractility loss (lower FS), and decreased venous return (Jugular distention and higher pulmonary artery flow velocity) in dogs with DCM. An increased O_2 demand may be explained by superoxide anion radical formation in conjunction with increased superoxide dismutase, an anti-oxidant protein. β_2GPI molecule was classified as an Apo, and it was initially termed Apo-H [21]. Among connected results, decreased level of serum Apo-H might be a result of protection mechanism against oxidative stress induced by apoptotic ischemic cells. Consistent with this hypo-thesis, Apo-H was shown to be present in atherosclerotic plaques [22] and involved in apoptosis process at surfaces of cells undergoing apoptosis [21,23].

In our study, Apo-CII was low (354.8 fold change) in serum samples of dogs with DCM. This might be the result of increased permeability of veins and impaired circulation ended with Apo-CII escape from circulation to ascites fluid. Apo-CII was reported to be a cofactor for lipoprotein lipase and identified as a putative substrate of matrix metalloproteinases (MMP-7, MMP-14) in humans, and a deficiency in Apo-CII was associated with atherosclerosis [24]. In good accordance, Kawano *et al.*[25] showed that a mutation in the Apo-II gene caused coronary atherosclerosis. Since canine Apo-CII has not been studied yet, we can only speculate that it might be involved in pathogenesis of atherosclerosis as in humans.

Venous return loss, impaired circulation and pleural and peritoneal effusion, as observed in the present study, are the signs of congestive heart failure (CHF) in dogs with DCM [1,2]. A previous study [26] reported high plasma fibrinogen, D-dimer, thrombin-antithrombin complex and low anti-thrombin activity in dogs with chronic CHF. We showed, in the present study, changes in expressions of

proteins related with coagulation in dogs with DCM, based on serum proteomics data including increased expression of plasminogen and decreased expression of β_2GPI. It is well known that plasminogen has a fibrinolytic activity, and β_2GPI has procoagulant and anticoagulant activities in the coagulation cascade [27]. β_2GPI can also bind to plasminogen and lipoprotein A, a molecule conferring a putative risk for atherosclerotic disease in humans [21]. It may thus be suggested that plasminogen and β_2GPI act together to regulate intrinsic fibrinolytic pathways in dogs with DCM [21,27].

On the other hand, our study has several limitations. The number of the dogs is not enough to represent the whole population, because this is a pilot study in which specific breed or sex variation was not investigated. In addition, while our results seem like not related with breed type or sex, physiological states like ovulation may be involved in alterations in serum proteomics. These limitations warrant a larger-scale study.

Since hematological or endocrinological changes would change the proteomic scale, serum proteome mapping of the dogs with DCM was challenging. Despite having controlled many variables, still some other mechanisms might have affected the analysis results. Serum proteomics can easily be affected by physiological conditions therefore proteins found in this study might be altered by physiological variables [9]. In parallel with this explanation, human studies showed that patients show different proteomic patterns in pathophysiological conditions such as sepsis [28] and diabetes mellitus [29].

There are some studies on structural proteomics of the canine heart [30,31], and our study did not include brain natriuretic peptide or cardiac troponin I detection in both samples (serum and ascitic fluid). This might be associated with potential loss of high molecular bounded target proteins in protein immune-affinity depletion step of the proteomic analysis technique or binding to the depletion column [32-34]. Immune-affinity depletion is essential for eliminating most abundant proteins such as albumin, but loss of target proteins in this step narrows the data and makes more questionable [9].

In conclusion, the study presented here that DCM in dogs shows different peptide pattern in the blood stream, which are the result of increased O_2 demand, hemostatic and fibrinolytic system changes and oxygen free radicals and progressive organ damage, as well as the heart itself. In this study, observed proteomes in serum and ascitic fluid samples enhances the search for informative biomarkers in biological samples from healthy dogs or dogs with DCM, thus allowing an earlier and more precise diagnosis and a deeper comprehension of pathogenesis, development and outcome of cardiomyopathy. Proteomic analysis in dogs with DCM may be useful to evaluate dynamic pathophysiological changes of the disease process, and especially serum Apo-CII level may be accepted as a risk factor for developing DCM in dogs.

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Conflict of Interest

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Research Article

Molecular Prevalence and Haematology of Tropical Theileriosis in Cholistani Cattle from Nomadic Herds of the Cholistan Desert, Pakistan

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Abstract

This is the first report on tropical theileriosis in Cholistani cattle, with the aim of 1) assessing the reliability of PCR as a tool for diagnosis of the early/carrier state; 2) determining the prevalence of theileriosis; and 3) comparing haematological profiles of parasite-positive and parasite-negative cattle. A total of 264 cattle (142 female and 122 male; 127 adult and 137 young) were examined for tropical theileriosis through clinical examination, stained smear screening, and polymerase chain recation. No cattle showed clinical signs of the disease. Of the diagnostic tests, PCR was more sensitive for detection of the early/carrier state of theileriosis (19.3%) compared to stained thin blood smear examination (1.9%). Female (24.6%) and young animals (23.4%) showed higher prevalence than males and adults, but not significant (P>0.05). Prevalence of the disease (51.6%) was significantly higher (P<0.05) in summer. Haematological indices were not significantly different in parasite-positive compared to parasite-negative cattle, except for total protein and creatinine which were significantly higher in infected animals. The study revealed a substantial prevalence of tropical theileriosis in Cholistani cattle. Nevertheless, their adaptation to the climate and their potential for tick and disease resistance may reflect in the absence of clinical signs and in normal haematological indices.

Keywords: *Theileria annulata, Cattle, PCR, Pakistan*

Pakistan'ın Cholistan Çölünde Başıboş Dolaşan Cholistan Sığırlarında Tropikal Theileriosisin Prevalansı ve Kan Değerleri

Özet

Cholistan sığırlarında tropical theileriosis ile ilgili ilk rapor olan bu çalışma ile; 1 teşhis yöntemi olarak taşıyıcı hayvanlarda PCR metodunun güvenirliliğinin belirlenmesi; theileriosisin prevalansının tespit edilmesi; parazit pozitif ve negative bireylerde kan parametrelerinin karşılaştırılması hedeflenmiştir. Toplam 264 sığır klinik, mikroskopik ve PCR ile tropical theileriosis yönünden muayene edilmiştir. Sığırların hiç birinde klinik bulgu gözlenmemiştir. PCR (%19.3), subklinik enfeksiyonların belirlenmesinde mikroskopik bakıya (%1.9) göre oldukça duyarlı bulunmuştur. Hastalığın yaygınlığı dişi (24.6%) ve genç hayvanlarda (%23.4) erkek ve erişkinlere göre daha yüksek bulunmuş, ancak bu farklılığın istatistiksel olarak anlamlı olmadığı (P>0.05) görülmüştür. Hastalığın, yaz aylarında (%51.6) daha yüksek olduğu tespit edilmiştir. *T. annulata* yönünden pozitif ve negative hayvanların kan değerlerinde total protein ve creatinine hariç (bu değerler enfekte hanvanlarda yüksek bulunmuştur) bir farklılık gözlenmemiştir. Bu çalışma Cholistan sığırlarında hastalığın prevalansının yüksek olduğunu ortaya koymuştur. Muayene edilen hayvanların hiç birinde klinik bulguların gözlenmemesi, kan değerlerinde değişikliğin olmaması, bu sığırların kenelere ve hastalığa karşı dirençlerini yansıtmaktadır.

Anahtar sözcükler: *Theileria annulata, Sığır, PCR, Pakistan*

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INTRODUCTION

Pakistan has 15 indigenous breeds of cattle belonging to zebu (one-humped) breed *(Bos indicus)*, comprising 43% of the cattle population [1]. Cholistani cattle are considered to be ancestral to the Sahiwal and are thermotolerant, and tick-resistant [2]. Tropical theileriosis, caused by *Theileria annulata*, is an important tick-borne disease of cattle in tropical and sub-tropical regions [3-6]. The disease is transmitted by the tick *Hyalomma* [7,8]. Research has been conducted in Pakistan on various aspects of this disease in Sahiwal and crossbred cattle [6], as well as in sheep and goats [9]. It is a serious constraint to cattle production in endemic areas, causing lethal infections in exotic cattle and considerable mortality in indigenous as well as in crossbred stock [10]. Factors including Pakistan's location in a warm climate and extensive uncontrolled crossbreeding programmes have rendered it an endemic area for theileriosis [6]. Exotic cattle and their crossbreds are highly susceptible, while indigenous cattle are relatively resistant to tropical theileriosis [11].

The objectives of the study were to determine the prevalence of *T. annulata* in Cholistani cattle from Pakistan reared under desert nomadic conditions, to assess PCR as a tool for diagnosis of the carrier state of the parasite, and to compare the haematological profiles of infected and uninfected cattle.

MATERIAL and METHODS

Geo-location and Study Animals

The study was conducted from February 2013 to January 2014 in the Cholistan desert of Pakistan. Location and climatic conditions of the area have been described elsewhere [12]. This area is an extension of the Great Indian Desert, which includes the Thar Desert in Sindh Province, Pakistan and the Rajhsatan desert in India. It is located 30 km from the city of Bahawalpur, Punjab, Pakistan and covers an area of 26.000 km², from latitude 27º42´to 29º45´ North and longitude 69º52´to 75º24´ East [12]. The climate is arid subtropical continental with low/sporadic rainfall, high temperature, low relative humidity, high rate of evaporation, and strong summer winds. It is the driest and hottest area of Pakistan, with summer spanning May through October. Randomly selected artificial and natural reservoirs and ponds, called Tobas [12,13] in the desert were visited to collect the samples during the study. Two-hundred-sixty-four Cholistani cattle (142 females and 122 males; 127 adult and 137 young) were examined in a survey approved by the Ethical Review Committee for the Use of Animals, under the administrative control of the Office of Research, Innovation, and Commercialization of Bahauddin Zakariya University. Written consent was obtained from the Cholistani pastoralists involved in our study.

Blood and Tick Sampling

Animals were examined for clinical signs (fever, enlargement of superficial lymph nodes, anemia, salivation and drop in milk production) and tick infestation. Approximately 7 ml of blood was collected from the jugular vein under appropriate restraint and stored as two aliquots: clotted for harvesting serum and un-clotted (0.5 M EDTA) for DNA extraction and haematological analysis. The body of each animal was inspected by palpation for the presence of tick infestation, primarily on the ears, perineum, scrotum, udder, tail base, and along the nape of the neck. The ticks were manually removed and placed in 25 mL containers with perforated caps containing a small strip of filter or paper towel. The ticks were identified according to the standard taxonomic keys using a stereomicroscope.

Microscopic Examination

Thin blood smears were prepared, labelled, air dried, and transported to the Molecular Biology Laboratory of Bahauddin Zakariya University. The smears were fixed in absolute methanol for 5 min, stained with 10% Giemsa stain for 30 min, and examined under oil immersion (×1.000) for the presence of parasites. In each smear, 20 fields (minimum 5.000 erythrocytes) were screened for the presence of intra-erythrocytic *Theileria* piroplasms.

DNA Extraction and PCR Amplification

DNA was extracted by an inorganic method [14]. PCR amplification was carried out through an optimized method described by Shahnawaz et al.[15] in which the 30 kDa merozoite surface antigen of *T. annulata* was amplified using a set of oligonucleotide primers. The forward [N516 (5'- GTAACCTTTAAAAACGT-3')] and the reverse [N517 (5'-GTTACGAACATGGGTTT-3')] primers were as described by d'Oliveira et al.[16]. A final reaction volume of 25 µL was used for the PCR. Each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl, 0.1% Triton X-100, 200 µM (each) deoxynucleotide triphosphate, 2.5 U of Taq DNA polymerase (Merck, USA), 20 pMol of primers, and 5 µL of extracted DNA sample. Positive control DNA from *T. annulata* (previously detected by PCR from a naturally infected cow) and sterilized de-ionized water (without DNA) were run with each PCR amplification as positive and negative controls, respectively.

The sensitivity, specificity, positive predictive and negative predictive values for for blood smear examination and for PCR were determined through an online calculator available at the web link (http://www.wikihow.com/Calculate-Sensitivity,-Specificity,-Positive-Predictive-Value,-and-Negative-Predictive-Value).

Haematological Analysis

An automated Haematology Analyzer (Sysmex K21, Kobe, Japan) was used for determination of haematological

indices including haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), and total leukocyte count (TLC). Blood smears stained with Wright's stain were simultaneously prepared for differential leukocytic count (DLC). Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were calculated. The haematology analyzer was designed for human application; hence, before analysis of samples, it was validated against blood samples from 100 dogs and 100 cows as well as with manual reference methods (cynmethaemoglobin photometry, haematocrit analysis, and haemocytometry) [17]. Total protein (TP), creatinine, alanine transaminase (ALT), aspartate transaminase (AST), and triglycerides (TGs) were determined by using APEL PD-303S spectrophotometer (Japan) and diagnostic kits (Spinreact, Spain), following the manufacturer's instructions.

Statistical Analysis

The data were classified according to sex, age, and season. Animals were categorized as adolescent (≥ 24 months) or adult (<24 months). Seasons were designated as temperate spring (February through April), hot dry summer (May through July), hot humid summer (August through October), and cool dry winter (November through January). Results for sex and age are presented as odds ratio with 95% confidence intervals, and seasonal fluctuation was assessed through Mantel-Haenszel c^2 test using Minitab v. 16. Significance was considered at $P<0.05$. The difference in haematological profile of parasite-positive and parasite-negative animals was calculated through an un-paired t-test. Predictive values were determined through sensitivity and specificity of smear examination and PCR results.

RESULTS

A total of 200 ticks feding on cattle were collected. Seventy-eight of the 264 (29.5%) examined cattle carried at least one tick. The mean rate of infestation of cattle was 2.6, with the number of ticks per animal ranging from 1 to 40. Taxonomic identification revealed that all the ticks were belonged to the species *Hyalomma anatolicum*.

None of the cattle exhibited clinical signs of tropical theileriosis. Prevalence, as detected through blood smear examination and PCR, was 1.9 (5/264; CI 0.25-3.53) and 19.3% (51/264; CI 14.5-24.1) respectively *(Table 1)*. Microscopic findings were confirmed by PCR positive signals for *Theileria annulata*. All PCR positive samples produced the 721 bp fragment specific for *T. annulata*. Sensitivity, specificity, positive predictive, and negative predictive values for blood smear examination were 8.9, 45.1, 1.8, and 80.6%, respectively. Similar values for PCR were 91.0, 54.8, 19.3, and 98.1%, respectively.

Prevalence of tropical theileriosis in females was higher, but not significantly ($P>0.05$), than in males at 24.6% (35/142; CI 17.5-31.7) vs. 13.1% (16/122; CI 7.1-19.1). A similar non-significant ($P>0.05$) difference in prevalence was found between adolescent and adult cattle at 23.4% (32/137; CI 16.3-30.4) vs. 15% (19/127; CI 8.8-21.2). Significant differences in prevalence of the disease were found among seasons ($P<0.05$) *(Table 1)*. Highest prevalence was in hot dry summer at 51.6% (33/64; CI 39.2-63.8), followed by that in cold dry winter (14.5%; CI 5.7-23.3), temperate spring (7.9%; CI 1.8-13.9) and hot humid summer (4.8%; CI 0.5-10.2) *(Table 1)*.

Haematological parameters of parasite-positive and

Table 1. *Association between the presence of Theileria annulata infection in Cholistani cattle detected by PCR and the studied parameters (sex, age, season)*
Tablo 1. *Sığırlarda PCR ile tespit edilmiş Theileria annulata enfeksiyonu ile bazı parametreler (cinsiyet, yaş, mevsim) arasındaki ilişki*

Parameters	No.of Sample	Positive no. of Cattle (PCR)	Odds Ratio/*P*-value*
Gender			
Female	142	35 (24.6%; CI 17.5-31.7)**	2.17 [reciprocal =0.46]
Male	122	16 (13.1%; CI 7.1-19.1)	
Total	264	51 (19.3%; CI 14.5-24.1)	
Age			
Adult	127	19 (15%; CI 8.8-21.2)	0.58 [reciprocal =1.73]
Young	137	32 (23.4%; CI 16.3-30.4)	
Season			
Temperate spring	76	6 (7.9%; CI 1.8-13.9)	Mantel-Haenszel X^2 P=0.392
Hot Dry Summer	64	33 (51.6%; CI 39.2-63.8)	
Hot Humid Summer	62	3 (4.8%; CI 0.5-10.2)	
Cold Dry Winter	62	9 (14.5%; CI 5.7-23.3)	

** Odds Ratio/P-value analyses show the PCR results, ** 95% CI: Confidence Interval*

parasite-negative cattle showed no significant differences, except for total protein and creatinine, which were significantly higher in infected animals *(Table 2)*.

DISCUSSION

This is the first study on Cholistani cattle directed towards assessment of PCR as a reliable diagnostic tool for carrier state of theileriosis, deducing its prevalence and assessing effect of the disease on haematological indices. Absence of clinical signs of theileriosis, despite being infested with ticks, may be indicative of innate resistance of Cholistani cattle as previously reported for zebu cattle [12,13,18]. Haematological parameters showed a consistent pattern throughout the seasons, without showing variation with respect to level of weather-induced stress [2,12]. The conventional diagnostic tools for theileriosis and babesiosis are being replaced by modern, sensitive, and specific molecular diagnostic methods such as PCR and PCR-based reverse line blotting [15,19-26]. In the present study, the prevalence of *T. annulata* found was significantly higher with PCR as compared to that with blood smear examination. This is in accord with earlier reports that stained smear blood examination cannot detect all sub-clinical or chronic infections, because parasitemia is often extremely low and may be missed [4,27]. Several reports have clearly demonstrated that PCR is a more sensitive and specific test than the conventional thin blood smear [28-30] and is reliable for detecting early or carrier infections.

The prevalence of *T. annulata* is higher in exotic and cross-bred cattle than in locally-adapted zebu breeds [6,31]. The overall prevalence in Cholistani cattle in the present study as detected through PCR was lower than the 24% reported for Holstein-Friesian at Pattoki region of Pakistan [32]. A prevalence of 33% has been reported from Pakistan for cross-bred cattle through blood smear examination [6]. A prevalence of 23% has been reported in Sahiwal cattle in Pakistan using PCR [28]. An absence of clinical signs of the disease and its lower prevalence in Cholistani cattle in the present study may indicate that they are resistant to the disease.

Higher prevalence of *T. annulata* found in females is similar to previous reports [29,33]. There is only one report, from Egypt, of higher prevalence of *T. annulata* in males than in females [34]. This difference can be attributed to ecological/geographical factors and differences in housing systems. The Cholistani nomadic pastoralists are moving in search of food and water. This livestock production system could be a feature responsible for the difference between males and females, since both male and female cattle are equally exposed to tick infestation [12].

Young animals showed a higher prevalence than did adults, consistent with other reports [6,15]. Innate immunity in calves is not developed enough to combat *T. annulata* [35].

A higher prevalence of *T. annulata* in hot dry summer is in line with various reports [6,15]. High ambient temperature in this season provides an environment conducive to growth and multiplication of ticks and ultimately increases the transmission of theileriosis [6].

In this study, no haematological index showed difference in positive cattle compared to non-infected cattle. This is in contrast to most previous reports that document significantly lower TEC, Hb, and PCV values in theileriosis-affected cattle [36,37]. These alterations have been attributed to parasitaemia-induced-anaemia and immune-mediated erythrophagocytosis [37]. The lack of difference in haematological indices of *T. annulata* infection Cholistani cattle in this study may indicate an innate potential to maintain haematological parameters at consistent levels

Table 2. *Haematological profile of parasite-positive and parasite-negative blood samples from Cholistani cattle*
Tablo 2. *Cholistan sığırlarından elde edilen parazit pozitif ve negatif kan örneklerinde hematolojik profil*

Parameters	Positive (n = 51)	Negative (n = 213)	*P*-value
Total Leukocyte Count (10^{12}/L)	9.8±0.3	10.5±0.52	0.2 (NS)
Total Erythrocyte Count (10^{6}/L)	7.5±0.2	7.5±0.1	0.8 (NS)
Haemoglobin (g/dL)	10.7±0.2	11.1±0.1	0.1 (NS)
Packed Cell Volume (%)	36.0±1.7	35.8±0.6	0.9 (NS)
Mean Corpuscular Volume (fL)	48.0±1.7	48.3±0.7	0.8 (NS)
Mean Corpuscular Haemoglobin (pg)	15.2±0.7	15.3±0.2	0.8 (NS)
Mean Corpuscular Haemoglobin Concentration (g/dL)	31.1±0.7	32.1±0.3	0.1 (NS)
Total Protein (g/dL)	6.9±0.1	7.3±0.9	0.03*
Creatinine (mg/dL)	1.5±0.5	1.3±0.02	0.01*
Alanine Transaminase (U/L)	28.6±1.5	28.8±0.5	0.8 (NS)
Aspartate Transaminase (U/L)	58.8±2.7	57.1±1.2	0.5 (NS)
Triglycerides (mg/dL)	24.5±0.6	26.0±0.5	0.06 (NS)

*Values are mean ±SEM, NS: Non significant, * P<0.05*

regardless of stressors, as reported by Farooq et al.[2]. Among biochemical factors, total protein was significantly decreased in infected cattle. This is in agreement with previous reports of decreased serum proteins in affected cattle. This decrease has been attributed to effects on lymph nodes in the diseased animals, resulting in extra-vascular proteinaceous fluid in body cavities [38].

The present study revealed a substantial prevalence of theilerial infection in Cholistani cattle reared by the nomads of Cholistan. However, their adaptation to the climate and their potential for disease resistance may evident from absence of clinical signs and un-affected haematological indices as observed in present study. This could indicate the state of endemic stability, which needs further research. Uncontrolled cross-breeding and mixed farming being carried out by the nomads of Cholistan desert, Pakistan might be a source of infection-transfer from this endemically stable population to other livestock. Future research needs to be directed towards the assessment of blood biomarkers in Cholistani cattle that may be associated with *T. annulata* resistance.

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Short Communication

An Investigation on the Relationship between the Azoospermia-Like (DAZL) Gene mRNA Expression and the Infertility in Male Cattle-Yak

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Abstract

This study was conducted to study the relationship between the infertility of male cattle-yak and the expression level of *DAZL* gene mRNA. The expression profiles were obtained by RT-PCR. The *DAZL* gene was specifically expressed in cattle, yak and cattle-yak testis tissues,which confirmed its important role in the progression of cell cycle. Real-time quantitative PCR analysis indicated that the expression levels of *DAZL* gene in cattle and yak testis were higher than its in cattle-yak, also cattle-yak and yak were significantly differet than cattle, respectively (P<0.05). Therefore, the low expression level of *DAZL* gene might result in male cattle-yak infertility.

Keywords: *Male infertility, DAZL, Real-time quantitative PCR, Phylogenetic relationship*

Sığır-Yak Melezlerinde Azoospermia-Benzeri (DAZL) Gen mRNA Ekspresyon Düzeyi ile İnfertilite Arasındaki İlişki Üzerine Bir Araştırma

Özet

Bu çalışma erkek sığır-yak melezlerinde Azoospermia-Benzeri (*DAZL*) gen mRNA ekspresyon düzeyi ile infertilite arasındaki ilişkiyi araştırmak amacıyla yapılmıştır. Ekspresyon seviyeleri RT-PCR ile belirlendi. *DAZL* geninin spesifik olarak sığır, yak ve sığır-yak melezi testis dokularında eksprese edilmesi hücre siklusunun ilerlemesinde önemli rolü olduğunu teyit etmekteydi. Gerçek Zamanlı kantitatif PCR analizi sığır ve yak testis dokularında *DAZL* gen ekspresyon düzeyinin sığır-yak melezlerindekinden daha fazla olduğunu ve sığır-yak melezi ve yaklardaki seviyelerin anlamlı derecede sığırlardan farklı olduğunu göstermekteydi (P<0.05). Sonuç olarak, düşük *DAZL* gen ekspresyonu erkek sığır-yaklarda infertiliteye neden olabilir.

Anahtar sözcükler: *Erkek infertilite, DAZL, Gerçek zamanlı kantitatif PCR, Filogenetik ilişki*

INTRODUCTION

Yak is the main livestock on the Qinghai-Tibetan Plateau, which belong the unique topographic features and the original bovine. It has high coarse, cold-resistant characteristics which adapts alpine hypoxia environment, but its milk, and meat production performances are lower. In order to improve the production performance of yak, yak is crossbred with cattle. Thus the production performance of hybrid cattle-yak which were in growth, fleshy, labor force and production performance were significantly better than the those of yak, but the infertility of male cattle-yak has been greatly limited in the production and breeding [1,2].

Over the past decades, the cattle-yak males sterile were significant studied by a lot of scholars about the complex phenomenon, but the main reason about the males sterile was had not found. Genes of the *DAZ* (Deleted in Azoospermia) gene famiv, *DAZ*, *DAZL* and *BOULE*, *DAZL* was originated from *BOULE* on Chromosome 2q, *BOULE* is the ancestral gene of *DAZ* gene family on Chromosome 3q [3]. The *DAZL* gene which is being studied in animal infertility at present is the research focus [4]. A lot of studies indicates that the absence of *DAZL* gene or the base mutation of *DAZL* gene brings on meiosis arrest and spermatogenic failure, which may lead to azoospermia and male infertility [5-8].

At present, the study of the relationship between the infertility of cattle-yak and the expression level of *DAZL* gene is rare. In this study, combined with cattle and yak, the *DAZL* gene from 3 representative Qinghai-Tibetan Plateau Bovine breeds were amplified, sequenced, Real-time PCR was amplified and data was analyzed to provide theoretic

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basis which finally revealed the infertility of cattle-yak mechanism.

MATERIAL and METHODS

Specimen Collection

The tissues samples of bovine breeds were collected from the Guomaying Town in Qinghai province. Male cattle-yaks (n=10), male cattle (n=10) and male yaks (n=10) which were adult and healthy were slaughtered. Testis, epididymis, hypothalamus, pituitary, heart, liver, spleen and pectoral muscle were removed and frozen in liquid nitrogen.

Design of Primers

The β-actin gene was used as an internal control. The primers were designed according to the cattle *DAZL* gene sequence (GenBank Accession Number: EF501823.2) and *β-actin* gene sequence (GenBank Accession Number: NM_173979) using primer 3.0 software and synthesized (Sangon, Shanghai, China). The primers for DAZL gene were: 5′-TCCTCCTCCACCACAATTTC-3′and 5′-GCTCCGGTG TCAACTTCATT-3′. The primers for β-actin were: 5′-TCCCTGG AGAAGAGCTACGA-3′and 5′-TAGAGGTCCTTGCGGATGTC-3.

Total RNA Isolation and cDNA Synthesis

Total RNA from the cattle, yak and cattle-yak tissues (testis, epididymis, hypothalamus, pituitary, heart, liver, spleen and muscle) were extracted using standard methods according to the manufacturer's protocol (RNA Extraction Kit, Fastagen, Shanghai, China). The cDNA was synthesized according to the manufacturer's protocol (Reverse Transcription Kit TaKaRa, Dalian, China). Operation procedure: 10 μg of purified total RNA, Prime ScriptTM RT Enzyme Mix1 1 μL, Oligo (dT) primer 1 μL, Random primer1 μL, 5×Primer ScriptTM Buffer 4 μL and RNase Free ddH_2O in a final volume of 20 μL.The RT temperature profile was 37°C for 15min, 85°C for 15 s, and final cooling to 4°C. The cDNA was stored at -20°C until use.

PCR Amplification, Molecular Cloning and Real-time PCR Amplification

PCR was carried out in a 25 μL reaction mixture containing 5 μL RT products, 12.5 μL 2×PCR buffer (Sangon, Shanghai, China), 0.6 μL of 10 mM of each oligonucleotide primer, and ddH_2O in a final volume of 25 μL. PCR was performed on a DNA amplification machine (ABI, USA) with an initial denaturation at 94°C for 4 min, 40 cycles of 94°C for 50 s, 57°C for 30 s and 72°C for 30 s and a final extension step of 7 min at 72°C. Reaction products were run on 2% agarose gels stained with ethidium bromide, and the target bands purifed with a Gel Extraction Kit (OMEGA, Shanghai, China) according to the manufacturer's protocol. The purifed product was cloned into the pGM-T vector and then transformed into Escherichia coli DH5a (TIANGEN, Beijing, China). The positive clone plasmid which was extracted according to the manufacturer's protocol (Sangon, Shanghai, China) was identified and sequenced (Sangon, Shanghai, China).

Real-time quantitative PCR which was performed on a DNA amplification machine (ABI, USA) was used to quantitatively determine the expression level of *Dazl* gene in various bovine testical tissues. Real-time PCR was performed in a 20 μL reaction mixture containing 2 μL RT products,10 μL SYBR Premix Ex TaqTM II (2×) (TaKaRa, Dalian, China), 0.4 μL Rox Reference Dye II (50×) (TaKaRa, Dalian, China), 0.8 μL of 10 μM of each oligonucleotide primer, and 6 μL ddH_2O. Real-time PCR cycle conditions were 1cycle of 95°C for 30 s, 40 cycles of 95°C for 5 s, 57°C for 20 s and 72°C for 34 s and 1 cycle of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The plasmid of positive clone fragment was standard after gradient dilution to make the standard curve. The quantitative results were performed using SPSS17.0 software for statistical analysis.

RESULTS

The Expression Profile of DAZL Gene

According to the DNA marker and the sequencing results, the size of *DAZL* and *β-actin* expected PCR products were 270 bp and 179 bp. The expression of *DAZL* gene in cattle, yak and cattle-yak tissues (testis, epididymis, hypothalamus, pituitary, heart, liver, spleen and muscle) was detected by RT-PCR. The results showed that *DAZL* gene was expressed specifically in cattle and yak testises, but not in other tissues.

The mRNA Expression Level of DAZL Gene

The mRNA expression level of *DAZL* gene was analyzed by Real-time PCR *(Fig. 1)*. The results showed *(Table 1)* that the mRNA expression level of *DAZL* gene in cattle (8.3980±2.26146) and yak (1.2020±0.70539) testical tissues were higher than its in cattle-yak (0.9810±0.25899). Cattle-yak crossbred and yak were significantly different than cattle, respectively ($P<0.05$).

DISCUSSION

Yaks are main breeds of Qinghai-Tibet Plateau, cattle yaks, the F1 hybrid between cattle and yaks, exhibit significant hybrid vigor. However, the males are sterile, which greatly restricts the utilization of this hybrid vigor.

The *DAZL* gene, a member of the *DAZ* gene family, which shows a specific expression in germ cells, is the key regulation factors during meiosis of human and animal spermatogenesis [9,10]. The absence of *DAZL* gene brings on meiosis to arrest and failure of spermatogenesis,

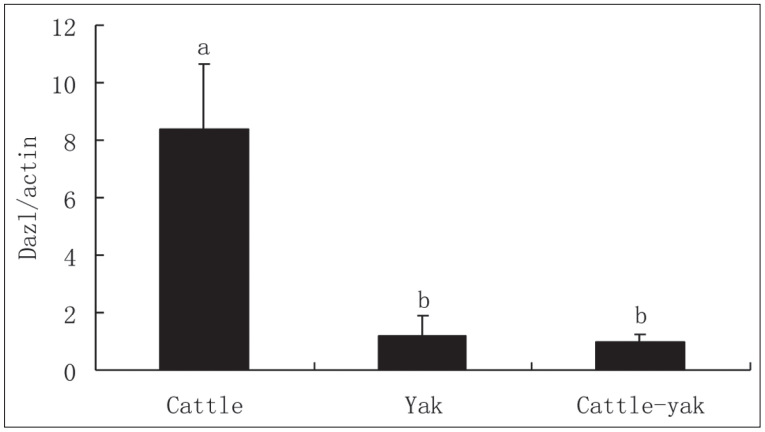

Fig 1. Developmental changes of expression level of *DAZL gene* mRNA expression in cattle, yak and cattle-yak's testises. Note: Different letters show significant differences at P<0.05

Şekil 1. Sığır, yak ve sığır-yak melezlerinin testislerindeki DAZL geninin mRNA ekspresyon seviyesindeki değişiklikler. Farklı harfler anlamlı farkın olduğunu göstermektedir (P<0.05)

Table 1. *The variance analysis of DAZL gene's mRNA expression level in cattle, yak and cattle-yak's testis tissues*
Tablo 1. *Sığır, yak ve sığır-yak melezlerinin testis dokusunda DAZL genine ait mRNA ifade seviyesinin varyans analizi*

The Source of Variation	DAZL gene			
	Sum of Squares	df	Mean Square	F
Between the species	356.144	2	178.072	94.071*
Within the category	51.110	27	1.893	
Total variation	407.253	29		

** means hows that differences significantly important*

which may lead to infertility of animal [11]. So presumably *DAZL* gene in also plays an important role in cattle-yak spermatogenesis.

In this study, to understand the function of the bovine *DAZ* gene family. The *DAZL* gene was highly expressed in bovine testis showing normal spermatogenesis but the case that mRNA level was low in testis possibly shows a defect in spermatogenesis, which suggest that *DAZL* gene might involve in spermatogenesis in the bovine testical tissue and arresting its transcription might result in infertility for male cattle-yak crossbred. Taken together with the report of *DAZL* gene [12], the result of low expression level of *DAZL* gene in cattle-yak testis suggests that *DAZL* gene might be associated with reproduction, which provide a theoretical basis of study the relationship between male sterility of cattle-yak and *DAZL* gene.

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Short Communication

Isolation and Identification of High Lactic Acid Producer Bacteria from Forage and Their Silages Grown in Different Ecologies [1]

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Abstract

In total, 695 Lactic acid bacteria isolation was made from forage crops grown in a wide part of Turkey's rangeland flora and their silages. A big majority of isolated strains (531) could regenerate. All regenerated isolates were incubated in MRS agar media containing CaCO3 in order to determine their total organic acid production. Selected 70 isolates according to their organic acid production were incubated in MRS broth media and their lactic acid productions were determined. High lactic acid producer 10 isolates were selected among treated isolates and they were identified using BIOLOG device in terms of their individual usage of different carbohydrate source during incubation period.

Keywords: *LAB, Identification, Isolation, Silage, Fermentation Products*

Farklı Ekolojilerdeki Yem Bitkilerinden ve Silajlarından Yüksek Laktik Asit Üreten Bakteri İzolasyonu ve Tanımlanması

Özet

Türkiye'nin geniş bir bölümündeki meralarda bulunan yem bitkilerinden ve bunların silajlarından 695 adet LAB izolasyonu yapılmıştır. Elde edilen izolatlardan önemli bir çoğunluğu (531 adet) rejenere olabilmiştir. Bu izolatlar CaCO3 içeren MRS agar besi yerinde inkübe edilmiş ve toplam organik asit üretimleri belirlenmiştir. Toplam asit üretimi yüksek olan 70 adet izolat MRS broth besi yerinde inkübe edilerek, laktik asit üretimleri belirlenmiştir. Bu izolatlar içerisinden laktik asit üretimi yüksek olduğu tespit edilen 10 adet izolat seçilmiştir. Seçilen 10 adet izolatın BIOLOG cihazında inkübasyon süresi boyunca farklı karbonhidrat kaynaklarını kullanma esasına göre tanımlaması yapılmıştır.

Anahtar sözcükler: *İzolasyon, LAB, Silaj, Tanımlama, Fermentasyon Ürünleri*

INTODUCTION

The objective of preserving forage resources is to ensure continuous regular feed for livestock in order to get sustainable growth, fattening or milk production when market prices of forages are highest for the dry season or for winter. One of the most important issues of quality silage making is to achieve a rapid drop of pH to a level of 4.2 in anaerobic phase of silage. Dropping in pH is closely related to lactic acid (LA) production level. The main factors affecting LA production level and speed are epiphytic lactic acid bacteria (LAB) on forage and chemical composition of crop material [1]. Silage quality is largely depends on competition between LAB and other microorganism groups [2]. Enough LA production decreases proteolysis and even can completely stop when pH level comes to below a level of 4 [3]. LAB inoculation has been using widely in recent years instead of inorganic acid or other applications in order to achieve a rapid LA fermentation in silage [4-6].

Due to microbial inoculation in silage making is not a widely used practice, some problems such as insufficient LA fermentation, bad aerobic stability and bad smell of silage are often occurred. Especially silage from legumes such as alfalfa, silage quality problems are getting bigger because of high buffering capacity, low pH level and

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relatively high proteolysis level. Isolation of LAB from rangeland and forage crops flora of some part of Turkey, selecting isolates in terms of their LA production ability and identification of selected stains were main aim of this study.

MATERIAL and METHODS

LAB Isolation Land and Plant Material

Sample collecting for LAB isolation areas were 14 points in Osmaniye, 14 points in Kahramanmaras, 12 points in Goksun, 6 points in Afsin and 2 points in Elbistan, making 48 points in total. Elevation of sample points were ranging from 39 m to 1516 m and samples were taken in different dates in order to extend isolated strain's diversity. Isolations were made not only from the fresh forage, but also from the silage made from the crops in sample points in order to increase possibility of successive strains. Approximately 500 g fresh forage was taken in all sample collecting point and immediately rinsed in ringer's solution in order to keep bacteria alive until they were incubated in MRS in laboratory. An extra 500 g of forage sample was collected and made silage with a portable vacuum machine and these silages were used for further LAB isolation from silage. Total sample replications were 347 of which 211 were from green forage while 136 from the silage made of forages in sample points. The coordinates of the sample points were recorded but not given in this paper.

LAB Isolation

Dilution series were prepared from all green forage and silage sample replications in order to count microflora (LAB, enterobacteria, yeast and moulds) and LAB isolation. LAB isolations were made from the colonies which are grown decently and well-grown ones in the petri plates. Then isolates were grown in a MRS agar media to get pure isolates. Once pure isolates were gotten, they were kept at -80°C with glycerin (15%) for the further studies. In this study, 695 isolates in total were obtained but 531 of the total isolates could be regenerated from the keeping conditions. Researches were done on these 531 regenerated isolates.

Determination of Total Organic Acid Production of Isolated LABs

Isolates that could be regenerated from the keeping conditions were firstly grown in 1 g/l $CaCO_3$ containing MRS media in order to determine total organic acid production ability of individual isolates. After the incubation period, the colonies formed two circles, one with another. Saturated colored inner circle expresses the colony size while light colored or transparent outer circle expresses the area of total organic acid produced by colony. Both circle diameters were measured by a compass and circle areas were calculated. Net acid production area was calculated by subtraction the area of inner circle from the outer one. The colonies, which have bigger acid production area, were considered as they have higher acid production ability. According to their acid production area and to rate of acid production area to colony area, 70 isolates in total were selected for the further studies and they were identified as morphologically, physiologically and biochemically. 57 of total 70 isolates which have highest acid production area (>150 mm^2) were selected directly. 13 of total isolates which have small both colony and acid production circle diameters but acid production circle is big enough compare to its colony diameter area were also selected in order to eliminate r^2 factor when calculating the area of circles. Directly the rate of two circle diameters was taken into consider when selecting 13 isolates.

Identification of Selected 70 Isolates

Selected 70 LAB isolates in total were identified morphologically (colony morphology), physiologically (catalase test) and cultural (gram reaction) then they were identified biochemically by using BIOLOG identifying system.

Determination of LA Production Ability of Selected LAB Isolates

All isolates were grown in MRS broth media for one night at 32°C then their bacterial densities were determined in spectrophotometer at 600 nm. All isolates were re-incubated for one night more in MRS broth after concentrations of all strains in per volume of MRS broth were equalized in order force all strains to produce LA under the same conditions. Bacteria were separated through filter and extract containing bacterial fermentation's end products were run at HPLC with two parallels to determine organic acids produced by LAB isolates. 10 LAB isolates were selected from 70 isolates considering amount of LA they produced. Selected high LA producer LAB strains were morphologically and physiologically examined as well as they identified by using BIOLOG (BIOLOG, Inc., Hayward, CA, USA) [7] device.

RESULTS

Isolation of LAB

Enterobacteria, yeast and mold counts were at high concentration as much as Log_{10} >7 cfu/g due to high contamination at both green and silage samples taken from the areas on which livestock was grazed in the contrast enterobacteria, yeast and mold counts were as low as Log_{10} <2 cfu/g in the samples taken from forage land and other marginal land at which animal were not grazed. LAB counts in MRS agar plate were between Log_{10} = 3 and Log_{10} = cfu/g in green forage or silage samples. LAB counts in samples taken on early spring were lower, sometimes less than Log_{10} = 2, than the samples taken in

summer time. It has been observed that epiphytic LAB concentration on green forage crops and silages increased (10^{-4}-10^{-5} for green crops and 10^{-8} for silage samples) while enterobacteria, yeast and mold counts decreased as sampling season (shown as sample number in *Table 1*) changed into summer period *(Table 1)*.

Determination of Total Acid Production Ability of LAB Isolates

Regenerated 531 LAB isolates from stock solution were point inoculated in MRS agar containing $CaCO_3$ in order to determine how much the colonies produced total organic acids during the incubation period. The best organic acid producer 70 isolates of all were selected.

Identification of Selected 70 Isolates

Selected 70 isolates according to their higher total acid production ability were identified by BILOG system. Identification probabilities of 33 isolates were higher than 70% and that of 22 isolates were between 50% and 70%. Only 15 isolates were identified with a similarity rates (probability) which are less than 50%. So, 55 of 70 isolates were assumed to be identified successfully. Genus distribution of 70 isolates according to BIOLOG system, were as follows: 43 isolates belong to *Lactobacillus*, 5 isolates belong to *Leuconostoc*, 8 isolates belong to *Enterococcus*, 3 isolates belong to *Brothotrix*, 5 isolates belong to *Corynebacterium*, 4 isolates belong to *Streptococcus*, 2 isolates belong to *Weisella* genus's.

Morphological and Biochemical Analyses of Selected 70 Isolates

All selected isolates were found as catalase (-), oksidase (-) and gram (+) concurring with LAB bacteria characters indicated by Hammes and Vogel [8]. Colony morphologies of 36, 22, 9 and 3 isolates were determined as bacillus,

Table 1. *Mean microorganism numbers. pH in silages and DM contents*
Tablo 1. *Silajda ortalama mikroorganizma sayıları. pH ve kuru madde içeriği*

Sample No	Green Forage			Silage Samples					Sample No	Green Forage			Silage Samples				
	Enterobacteria (Log_{10})	Yeast (Log_{10})	LA Bacteria (Log_{10})	Enterobacteria (Log_{10})	Yeast (Log_{10})	LA Bacteria (Log_{10})	pH	DM (%)		Enterobacteria (Log_{10})	Yeast (Log_{10})	LA Bacteria (Log_{10})	Enterobacteria (Log_{10})	Yeast (Log_{10})	LA Bacteria (Log_{10})	pH	DM (%)
1	6.34	8.94	2.00	<2	6.78	3.70	5.2	15.26	25	5.95	7.88	<2	<2	5.00	5.70	5.47	21.53
2	5.48	8.72	<2	5.72	6.73	4.11	4.86	18.36	26	<2	7.38	<2	<2	6.08	6.11	4.49	23.06
3	5.88	8.66	<2	<2	6.71	3.08	5.12	14.18	27	<2	<2	9.59	<2	6.15	5.36	4.49	19.57
4	7.00	8.79	2.00	<2	6.28	3.45	4.94	21.95	28	<2	<2	9.59	<2	4.18	3.70	4.47	15.51
5	7.20	8.68	<2	<2	6.79	3.18	5.11	16.74	29	4.95	6.88	5.08	<2	4.52	5.94	5.39	24.96
6	6.61	8.53	<2	3.70	8.04	3.41	4.89	17.74	30	<2	<2	3.72	<2	6.15	5.45	5.44	36.76
7	6.28	7.67	2.11	3.51	5.45	7.34	5.28	13.91	31	<2	<2	6.11	2.00	6.99	7.04	5.42	26.55
8	7.15	8.00	2.18	<2	5.93	6.36	3.97	22.21	32	<2	<2	5.34	<2	5.91	6.15	5.45	31.28
9	6.93	8.36	<2	5.03	6.04	8.11	5.29	18.01	33	<2	<2	5.04	2.74	6.57	6.20	5.40	26.32
10	6.79	8.40	2.67	<2	6.00	6.28	3.59	19.82	34	<2	<2	4.23	<2	5.89	4.00	4.88	32.51
11	7.30	9.08	3.41	<2	6.41	7.30	5.28	16.44	35	5.74	<2	<2	<2	4.76	<2	4.64	25.97
12	6.94	8.67	2.54	<2	3.70	4.83	4.66	23.00	36	5.41	<2	3.51	2.60	6.91	6.60	5.51	26.81
13	6.66	8.95	3.45	<2	5.74	8.74	4.26	22.65	37	5.74	<2	4.89	2.18	5.67	5.97	5.50	31.32
14	6.18	7.76	3.18	<2	4.18	6.48	3.96	24.16	38	5.67	<2	4.49	3.30	5.48	5.53	5.23	27.90
15	5.40	8.30	2.30	<2	4.00	5.71	4.04	18.73	39	<2	<2	<2	4.18	5.59	4.70	4.99	29.22
16	6.94	7.72	5.53	<2	5.43	5.54	4.81	14.25	40	6.40	7.65	<2	5.89	5.08	5.43	5.50	34.32
17	7.75	8.60	4.94	<2	5.88	5.04	5.30	13.03	41	4.30	<2	4.56	2.85	5.38	5.61	5.56	14.94
18	7.20	7.81	5.15	3.70	6.64	6.70	4.21	27.16	42	4.48	7.60	6.63	2.60	5.81	5.26	5.49	18.15
19	6.81	6.94	5.40	<2	5.00	4.78	5.50	13.39	43	<2	5.70	3.75	<2	6.00	5.83	5.51	21.08
20	7.15	8.34	<2	<2	6.38	6.71	4.49	11.56	44	5.53	6.00	5.96	<2	5.48	5.51	5.02	16.92
21	6.89	7.53	5.60	3.70	7.46	7.46	4.43	21.44	45	6.18	6.93	6.08	<2	5.26	6.74	5.42	16.35
22	7.20	8.15	3.74	<2	4.70	<2	4.06	24.65	46	5.49	5.70	4.08	<2	5.63	4.45	5.49	17.13
23	6.62	7.46	4.40	<2	6.89	6.52	5.36	15.13	47	5.54	<2	3.63	2.90	6.18	6.48	5.48	13.50
24	<2	10.80	<2	<2	5.71	5.32	5.67	15.09	48	5.67	7.27	5.69	<2	6.20	5.00	5.48	15.96

coccus, short bacillus and diplococcus, respectively.

LA and Other Organic Acid Production of Selected 70 LAB Isolates

It has been found that all isolates have produced more or less LA changing from 18.69 mmol/L to 70.02 mmol/L. The highest LA production was obtained from number 2 (LS-55-2-2) while number 61 (LS-31-1-4) produced the highest (7.78 mmol/L) acetic acid. It has been found that the most extended variation among the isolates was in terms of ethanol production ranging from 0 to 35.73 mmol/L. All isolates produced lower propionic acid (<0.2 mmol/L) except for the isolate number 7 (BOLSON-2-3) whose production was 6.81 mmol/L.

The best 10 isolates according to their LA production level among given in *Table 2* is selected *(Table 3)* and used as microbial inoculant for corn and alfalfa silages in order to determine their effects on fermentation profile in both

Table 2. *Fermentation product profile (mmol/l) and rate of LA in total fermentation product (%)*
Tablo 2. *Fermentasyon* ürün *profile (mmol/l) ve toplam fermentasyon* ürünü *içinde laktik asit oranı (%)*

No	Isolat Name	Fermentation End Products						No	Isolat Name	Fermentation End Products					
		Succinic Acid (mmol/L)	Lactic Acid (mmol/L)	Acetic Acid (mmol/L)	Propionic Acid (mmol/L)	Ethanol (mmol/L)	LA Rate in Total Products (%)			Succinic Acid (mmol/L)	Lactic Acid (mmol/L)	Acetic Acid (mmol/L)	Propionic Acid (mmol/L)	Ethanol (mmol/L)	LA Rate inTotal Products (%)
1	L-42-8	0.00	43.62	0.74	0.10	0.00	98.10	36	L-50-8	0.00	31.67	1.74	0.05	0.00	94.66
2	LS-55-2-2	0.43	70.02	6.61	0.01	8.53	81.79	37	LS-30-1	0.09	30.78	9.98	0.01	35.73	40.19
3	L-60-5	0.00	50.16	0.58	0.12	0.84	97.02	38	L-34-1-2	0.00	27.78	9.71	0.02	32.02	39.95
4	L-38-2-2	0.20	43.34	2.20	0.06	4.30	86.50	39	LS-2-2	0.30	49.49	1.85	0.19	1.33	93.10
5	L-44-4-1	0.00	32.23	0.00	0.03	0.00	99.90	40	LS-6-5-1	0.27	29.94	2.51	0.04	7.16	74.98
6	L-70-10-1	0.02	28.56	1.88	0.02	0.38	92.57	41	LS-49-2-1	0.26	51.10	1.09	0.09	3.01	92.00
7	BOLSN-2-3	0.00	48.52	0.00	6.81	0.00	87.69	42	LS-72-2	0.02	54.00	1.64	0.10	1.72	93.94
8	L-61-3-2	0.03	48.03	0.88	0.07	1.40	95.27	43	LS-6-3	0.29	35.01	3.46	0.04	0.02	90.15
9	LS-51-2-1	0.27	53.85	1.71	0.11	1.20	94.24	44	LS-39-1-3	0.00	35.30	8.58	0.05	14.36	60.56
10	LS-71-2-3	0.04	52.39	1.54	0.08	0.00	96.93	45	L-58-6-2	0.00	30.37	1.29	0.08	3.07	87.24
11	L-57-2	0.02	51.15	2.20	0.11	3.20	90.24	46	L-54-10-1	0.00	48.39	0.54	0.06	0.00	98.77
12	L-68-1	0.06	44.42	4.61	0.11	18.97	65.17	47	L-61-8-2	0.06	48.95	1.94	0.04	0.00	96.00
13	L-55-2	0.00	28.13	0.93	0.03	0.00	96.70	48	L-57-4-2	0.00	51.85	1.85	0.14	0.00	96.31
14	LS-2-4-1	0.04	52.96	2.15	0.09	2.35	91.96	49	LS-43-2-3	0.25	48.71	1.40	0.13	3.49	90.23
15	LS-3-4-2	0.35	49.64	6.00	0.00	11.17	73.91	50	LS-4-4	0.16	37.47	0.61	0.06	2.27	92.37
16	L-70-6-1	0.06	53.47	4.24	0.06	0.60	91.51	51	L-61-5-1	0.00	20.83	4.45	0.04	21.14	44.83
17	LS-8-1	0.02	52.69	2.39	0.19	1.95	92.05	52	L-61-2-1	0.02	27.40	2.87	0.02	14.67	60.92
18	LS-65-2-1	0.30	56.65	1.69	0.15	1.07	94.66	53	L-61-6-1	0.01	22.52	2.41	0.03	11.66	61.49
19	L-70-7-1	0.00	18.69	5.62	0.00	15.04	47.49	54	L-53-8-2	0.02	47.40	0.00	0.05	1.18	97.43
20	L-5-9-1	0.08	49.59	2.95	0.16	1.86	90.77	55	L-38-4	0.00	51.84	3.72	0.01	4.78	85.89
21	LS-8-5-1	0.00	22.11	2.90	0.01	25.81	43.49	56	L-51-2-1	0.03	49.22	1.83	0.09	4.04	89.15
22	L-44-2	0.00	42.05	0.00	0.12	0.14	99.37	57	L-55-8-2	0.00	49.33	6.75	0.03	7.27	77.83
23	L-50-5-1	0.00	50.01	1.56	0.11	1.43	94.17	58	L-53-1-1	0.00	24.13	0.50	0.05	0.00	97.78
24	L-57-7	0.00	43.94	0.00	0.10	4.01	91.46	59	L-51-2-2	0.05	46.49	0.50	0.05	8.44	83.72
25	LS-9-3	0.00	25.30	2.06	0.10	4.41	79.39	60	L-54-4	0.00	27.60	7.44	0.04	32.18	41.03
26	LS-8-3	0.00	41.41	4.44	0.04	2.68	85.25	61	LS-31-1-4	0.28	59.08	7.78	0.04	2.22	85.12
27	L-35-1	0.24	35.30	2.18	0.01	0.06	93.39	62	L-58-6-1	0.00	44.55	0.16	0.07	1.85	95.56
28	L-70-5	0.00	51.57	7.17	0.02	5.28	80.53	63	L-60-7	0.06	44.19	1.86	0.01	1.54	92.71
29	L-57-2-1	0.00	30.65	0.97	0.11	0.00	96.60	64	L-61-7-2	0.03	51.42	1.28	0.09	3.61	91.13
30	LS-3-3	0.22	54.59	2.57	0.10	3.01	90.26	65	LS-69-1-2	0.18	30.34	9.81	0.03	15.25	54.57
31	P710-2	0.00	35.89	4.38	0.03	28.76	51.97	66	LS-40-1-2	0.22	32.49	4.80	0.03	23.51	53.21
32	LS-8-4-1	0.00	31.87	2.45	0.01	0.56	91.34	67	L-44-11	0.00	49.80	0.34	0.15	2.35	94.62
33	L-61-9-2	0.02	50.03	0.61	0.14	1.31	96.00	68	L-61-4-1	0.06	41.75	0.22	0.12	1.03	96.68
34	L-44-8-2	0.00	30.39	5.69	0.01	30.73	45.48	69	L-57-4-1	0.00	25.47	1.44	0.11	22.29	51.65
35	LS-3-2-1	0.00	30.08	0.72	0.05	0.00	97.52	70	L-41-1-1	0.01	49.39	0.70	0.09	0.00	98.40

Table 3. *Species name. LA production (mmol/l) and LA rate in total fermentation products (%) and physiological characters of selected and transferred to further inoculation studies 10 isolates*

Tablo 3. İleriki *inokulasyon* çalışmalarında *kullanmak* üzereseçilen *10 adet izolatın tür ismi. LA* üretimi *(mmol/L). toplam fermentasyon* ürününde *LA oranı (%) ve fizyolojik karakterleri*

Isolate No	Isolate Name	Species	LA Production (mmol/L)	LA/Total Fermentation Products (%)	Physiological Character
2	LS-55-2-2	*Lactobacillus brevis*	70.02	81.79	Heterofermentative
9	LS-51-2-1	*Lactobacillus gasseri*	53.85	94.24	Homofermentative
10	LS-71-2-3	*Lactobacillus plantarum*	52.39	96.93	Homofermentative
14	LS-2-4-1	*Lactobacillus plantarum*	52.96	91.96	Homofermentative
16	L-70-6-1	*Leuconostoc citerum*	53.47	91.51	Homofermentative
17	LS-8-1	*Pediococcus citerum*	52.69	92.05	Homofermentative
18	LS-65-2-1	*Lactobacillus bifermentans*	56.65	94.66	Homofermentative
30	LS-3-3	*Lactobacillus plantarum*	54.59	90.26	Homofermentative
42	LS-72-2	*Lactobacillus plantarum*	54.00	93.94	Homofermentative
61	LS-31-1-4	*Lactobacillus buchneri*	59.08	85.12	Heterofermentative

cereal and legume silages (data not given). All selected as high LA producer isolates were belong to *Lactobacillales* family. 8, 1 and 1 of total 10 isolates scattered *Lactobacillus, Leuconostoc,* and *Pediococcus* genus', respectively. 4 isolates from *Lactobacillus* were belong to *L .plantarum*, and the others to *L. brevis*, *L. gasseri*, *L. bifermentans* and *L. buchneri* species with one each member. Among 10 selected strains, the LA production rate in total organic acid production of *L. brevis* ve *L. buchneri* species were found as 81.79% and 85.12%, respectively. LA proportions in total organic acid productions of all other isolates were higher than the critical level of 90.0% which is assumed as the lowest LA production limit of homofermentative LAB fermentation. Those two isolates which have lower LA proportion level of 90% of total fermentation products were determined as heterofermentative species while the others defined as homofermentative. Hommes and Hertel [9] stated at their Bacteriology book in which they explained widely the morphologic, physiologic and genetic characteristic of LAB, *L. brevis* and *L. buchneri* species were stated as facultative heterofermentative and they have very similar metabolic characters supporting accuracy of our identification results from the BIOLOG device.

DISCUSSIONS

It is well known that pH levels of silages made for getting LAB isolation were affected by crop factors such as DM content of raw material, growing sage of forage crops, botanical composition of forage, microbial factors such as epiphytic LAB and other microbial content of forage, LAB strains effectiveness in silo, and other environmental factors such as silo conditions [10]. pH level of all silages were lower than 5.5 in general and very low pH level values of 3.8 were also reached in some silages. DM contents of silages were varied from 11.56% to 34.32% *(Table 1)*. Incidentally LAB isolations were made in various numbers right after the plate counting then they were kept -80ºC for further use. Total 695 isolates were taken from the plates and kept in -80ºC but 531 of them can regenerate from the stock solution when the time of use. Regenerated isolates were grown at MRS media containing $CaCO_3$ in order to determine total organic acid production and 70 of them were selected.

It is found that 43 of the selected 70 isolates were isolated from green forage samples while 27 of them were from silage samples. This is because of there were much more isolates from green forage sample replications than the isolates from silage samples. Selected 70 isolates in total were transferred to further research for determining the fermentation profiles of isolates after they were identified by BIOLOG microbiological identification system. It is found that *Lactobacillus* was the most common genus (61.4% of total LAB) on epiphytic flora on plant and, as a result, on silage environment.

This is an expectable situation to consider that the most common LAB family found on crop's epiphytic flora is *Lactobacillales* family as all selected isolates were a member of a foresaid family. A great majority of selected strains (9 in 10) were isolated from silage samples while only one isolate (L-70-6-1) was from green forage. This situation indicated that exploring chance of easily grown, high competitive, high LA producer and tolerant to low pH level isolates was clearly increased by isolating directly from matured silages.

Mainly two points from this research can be deduced as follows;

1- Preselection of isolates by using $CaCO_3$ containing media in order to determine their total fermentation acid production is an appropriate method.

2- Bacteria isolation for silage inoculation from matured silage has advantages compare to that of green forage due to the bacteria already grew in silage and competed to other microorganisms. Too many isolate from green forage is needed to get successive results.

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Short Communication

Serum IL-1β, IL-6, IL-10 and TNF-α Levels in Thyroidectomized Rats [1]

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Abstract

This study was conducted to determine the serum levels of interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor alpha (TNF-α) in thyroidectomized rats. Hypothyroidism was performed by surgical thyroidectomy in rats. Four weeks after from surgical procedure, selected cytokines levels were evaluated. Hypothyroidism was confirmed by elevated thyroid stimulating hormone (TSH) and decreased free tri-iodothyronine (fT3) levels ($P<0.05$) in thyroidectomized group. Serum IL-1β, IL-6, IL-10 levels have shown slight increase, whereas only TNF-α level ($P<0.05$) were significant statistically in thyroidectomized rats compared with control group. In conclusion, the obtained data suggest that elevated levels of cytokines could be as a consequence of thyroidectomy operation.

Keywords: *Cytokine, Immune function, Rodent, Thyroid alteration, Trauma*

Tiroidektomize Ratlarda Serum IL-1β, IL-6, IL-10 ve TNF-α Seviyeleri

Özet

Bu çalışmada, tiroidektomize ratların serum interlökin (IL)-1β, IL-6, IL-10 and TNF-α düzeylerinin belirlenmesi amaçlanmıştır. Hipotiroidizm cerrahi yolla tiroidektomi yapılarak gerçekleştirildi. Cerrahi işlemden sonraki 4. haftada, seçili sitokinlerin seviyeleri ölçüldü. Hipotiroidizm, tiroidektomize grubun tiroid uyarıcı hormon (TSH) düzeyindeki artış ($P<0.05$) ve serbest tri-iyodotironin (fT3) düzeyindeki azalış ($P<0.05$) ile teyit edildi. Tiroidektomize ratların serum IL-1β, IL-6, IL-10 seviyelerinin kontrol grubuna kıyasla hafif düzeyde yükseldiği ancak, sadece TNF-α düzeyindeki artışın istatistiki olarak anlamlı ($P<0.05$) olduğu görüldü. Sonuç olarak elde edilen veriler, sitokin seviyelerindeki yükselmenin, tiroidektomi operasyonunun bir sonucu olabileceğini düşündürmektedir.

Anahtar sözcükler: *İmmun fonksiyon, Rodent, Sitokin, Tiroid bozulması, Travma*

INTRODUCTION

Cytokines are a heterogeneous group of polypeptides which have multifunctional act as modulating, triggering and regulating of inflammatory and immune responses. Most cytokines have autocrine and paracrine effects owing to multiple cellular sources. Interleukins are described as any of various compounds of low molecular weight (~17 to 60 kd) these are produced by lymphocytes, macrophages and monocytes and they act as regulate of the humoral and innate immune functions and inflammation cascades [1].

The firstly discovered interleukin was IL-1 which has extensive family consisted by 11 members. The major members of IL-1 family are IL-1α and IL-1β, these two proteins are binding to the same receptor complexes namely as IL-1 type I receptor (IL-1RI) and IL-1RII due to exert similar biological effects act as proinflommatory cytokine which has potentiating immune and inflammatory responses Multifunctional, pleiotropic cytokine IL-6 is regulated of immune and acute-phase responses, hematopoiesis and inflammation which is produced by innate immune cells (T helper 2; Th2), monocytes and macrophages, endothelial cells, fibroblasts, that promotes T-cell proliferation, B-cell differentiation and survival triggering by IL-1 and tumor necrosis factor alpha (TNF-α). When firstly described in late of the 80's an anti-inflammatory cytokine IL-10 was known as cytokine synthesis inhibiting factor. Whilst the major source of this cytokine is macrophages, Th2 cells, monocytes and keratinocytes may also produce. Another pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) is in a relationship with the physiopathologies

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of cancer, neurological, cardiovascular, autoimmune and metabolic disorders through activation of nuclear factor kappa B (*NF*-κB) pathway [2,3].

Thyroid hormones are affected nearly all metabolic processes by using different pathways. Hypothyroidism have described as absence or lacking of thyroid hormones which could cause of abnormalities on metabolic and immunological functions. Cytokines play pivotal role in autoimmune thyroid disorders namely as Graves' disease or Hashimoto's thyroiditis [4]. Various models have been widely used as congenital hypothyroid animals due to thyroid gland dysgenesis or thyroid dyshormonogenesis, thyroid hormone receptor (TR) gene mutated animals, and thyroid hormone transport or metabolism modified animals for enhancing knowledge and clarify the thyroid hormone action [5]. In order to realize adult-onset hypothyroidism in rodents, thiourea based selenium analogue antithyroid agents have been used in such as propylthiouracil (PTU) or methimazole (MMI). Nevertheless, previously studies revealed that PTU and MMI have immunomodulatory effects [6].

Therefore, hypothyroidism was induced by thyroidectomy operation due to withdrawn adverse effects of antithyroid drugs on immune system function and it was aimed to determine the serum levels of IL-1, -6, -10 and TNF-α in adult-onset hypothyroidism.

MATERIAL and METHODS

Animals

Ten male Wistar rats (12 weeks age) obtained from Selcuk University Experimental Medicine Research and Application Center. Rats were kept in a room at a constant temperature 22±2°C with 50% relative humidity, 12 h light/dark cycle period and housed in polycarbonate cages with fed by standart rat chow and tap water *ad libitum*. All experimental procedure was approved by Selcuk University Experimental Medicine Research and Application Center Local Ethics Committee (Approval number: 2014/16).

Experimental Protocol

Rats were randomly divided into two groups as control (n=5) and thyroidectomized (Tx, n=5). Hypothyroidism was generated by surgical thyroidectomy in rats with anesthetized by xylazine HCl (10 mg/kg/BW) and ketamine HCl (50 mg/kg/BW). Briefly, using a stereomicroscope (Olympus Co.,Tokyo, Japan) for better observation, the stenothyroid muscle was cut and the trachea was exposed. The parathyroid gland was found, dissected from the thyroid gland, and implanted into the surrounding neck muscle. The thyroid gland was completely excised. After surgery, carprofen (Rimadyl© Pfizer, 5 mg/kg) was injected over 3 days to alleviate pain. The control group received the pre- and post-surgery treatment. Four weeks after thyroidectomy operation blood samples were taken by cardiac puncture under deeply anesthetized with ketamine HCl and xylazine HCl collected into non-coagulant tubes and centrifuged (3.000 x g for 5 min) after that collected sera was stored -80°C until analysis.

Assessment of Thyroid Hormones and Cytokines

Serum concentrations of thyroid stimulation hormone (TSH) and free tri-iodothyronine (fT3) were determined by autoanalyser (ADVIA Centaur XP Immunoassay System, Siemens, USA) and selected cytokines IL-1β (Cat. No. BMS630), IL-6 (Cat. No. BMS625) and IL-10 (Cat. No. BMS629) and TNF-α (Cat. No. BMS622) were determined by ELISA (Bio-tek Instruments, Inc.) using sandwich enzyme-linked immunosorbent method according to manufacturer's (Ebioscience) instructions.

Statistical Analysis

Statistical analysis was performed with the SPSS 19.0 package program for Windows (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean ± standard error of the mean (SEM) Student's t-test was used for determination among the groups. P<0.05 was considered for statistically significant.

RESULTS

As shown as *Table 1*, hypothyroidism was confirmed by elevated TSH and decreased fT3 levels (P<0.05) in thyroidectomized group.

According to obtained data IL-1β, IL-6 and IL-10 levels *(Table 2)* were slightly increased but these were not statistically significance except TNF-α level in thyroidectomized animals compared with control group (P<0.05).

Tablo 1. *Tiroid hormon düzeyleri (mean±SEM, n=5)*
Table 1. *Thyroid hormone levels (mean±SEM, n=5)*

Group	TSH µIU/mL	fT3 ng/dL
Control	1.73±0.29 b	3.36±0.27 a
Tx	19.54±1.61 a	2.03±0.32 b

a,b *Different letters in the same column refers the differences between the groups (P<0.05)*

DISCUSSION

Hypothyroidism could cause of reduction in serum pro-inflammatory cytokines IL-6 and TNF-α levels in mice [7], rats [8], which were induced by PTU, MMI and thyroidectomy. Also, Kızıltunc et al.[9] reported that IL-6 and TNF-α serum levels were decreased in humans who suffered owing to hypothyroidism. Contrarily to these data, PTU did not cause any alteration in circulating levels of TNF-α in human [10] nor in rats [11].

Tablo 2. *Serum sitokin seviyeleri (mean±SEM, n=5)*
Table 2. *Serum cytokine levels (mean±SEM, n=5)*

Group	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)	TNF-α (pg/mL)
Control	28.02±2.39	30.20±2.35	36.51±1.63	39.61±2.09 b
Tx	39.05±5.49	36.04±3.73	38.93±1.85	46.23±1.01 a

a, b *Different letters in the same column refers the differences between the groups (P<0.05)*

In this regard, we have investigated the serum levels of IL-1, -6, -10 and TNF-α in thyroidectomized rats. In the present study, serum TSH levels approximately ~11 fold higher (P<0.05) and fT3 levels decreased (P<0.05) in the thyroidectomized group compared with control. The obtained data shown that hypothyroidism have been well established after thyroidectomy operation.

IL-1 inhibits TSH-induced TPO gene expression directly, thus IL-1 as a local inhibitor of THs synthesis. Additionally, IL-6 can inhibits TSH-induced TPO mRNA transcription in a dose-dependent manner [1].

Pro-inflammatory or anti-inflammatory cytokines which produced by Th1 (IL-1β, TNF-α) and Th2 (IL-6, IL-10) cells have crucial role in protecting against the autoimmune thyroid disease [4,12]. Furthermore, now it is well known that IL-1, IL-6 and TNF-α could play central role in the pathogenesis of non thyroidal illness (NTI) syndrome by acting on hypothalamus pituitary-thyroid axis [13]. IL-1, IL-6 and TNF-α are effect as suppressor mediators on thyroid hormones during NTI [14]. Also, Bartalena et al.[15] tested that hypothesis in rats with induced hypothyroidism by administration of the methimazole throughout 3 weeks and they concluded that 48 hours after a single dose recombinant human IL-6 injection could reduce serum T3, T4 and TSH concentrations due to central effects of the cytokine.

From another aspect, trauma and wound healing could lead to significant elevation in pro-and anti-inflammatory cytokines [16,17]. Especially IL-1β and TNF-α, IL-6 and C-reactive protein, which is a predictor for acute-phase response and IL-10 have been used in biomarkers for monitoring traumatic inflammation and recovery period in clinically both human and veterinary medicine [18-20]. Ozcinar et al.[21] reported the pro-inflammatory cytokines IL-1β and TNF-α levels were higher after thyroid surgery in human. However, the sample size restricted to interpret of the obtained data in the present study. Slight increased levels of pro-inflammatory cytokines IL-1, IL-6 due to their stimulator effects on TNF-α production raised the level significantly, meanwhile slight elevation on the level of anti-inflammatory cytokine IL-10 might explain as a consequence of the thyroidectomy operation in accordance with previous studies [19,21].

In conclusion, surgical thyroidectomy intervention may cause of alteration in levels of cytokines on post-operative period. Therefore, whilst used in hypothyroid animals as a model in experimental studies, these data have to take into consideration.

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Short Communication

A Survey of Crimean-Congo Hemorrhagic Fever in Livestock in Republic of Kosova

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Abstract

Crimean-Congo hemorrhagic fever (CCHF) is endemic in Kosova. The aim of our study was to detect the IgG and IgM antibodies to CCHF virus in cattle and sheep in endemic and non-endemic areas of Kosova. In total sera of 172 cattle and 95 sheep were tested by the Indirect Immunofluorescence Assay (IIA) for quantitative determination of antibodies to CCHF virus. Seven of 172 cattle sera (4.07%) and three of 95 sheep sera (3.16%) tested positive for IgG, but IgM were detected in the serum of only one sheep. The results of this study confirmed the fact that CCHF is endemic in four municipalities of Kosova and that there is a risk of the spread of viruses to other non-endemic regions through the movement of infected animals.

Keywords: *Crimean - Congo hemorrhagic fever virus (CCHFV), Cattle, Sheep, IgG, IgM*

Kosova Cumhuriyeti'nde Kırım Kongo Kanamalı Ateşi Üzerine Çiftlik Hayvanlarında Bir Araştırma

Özet

Kırım Kongo Kanamalı Ateşi (KKKA) Kosova'da endemiktir. Bu çalışmanın amacı endemik ve endemik olmayan Kosova bölgelerinde sığır ve koyunlarda KKKA virusuna karşı IgG ve IgM antikorlarını belirlemektir. Toplam 172 sığır ve 95 koyun İndirek İmmunoflorasan Tekniği ile KKKA virusuna karşı antikorların kantitatif tespiti amacıyla kullanılmıştır. 172 sığırın 70'i (%4.07) ve 95 koyunun 3'ü (%3.16) IgG pozitif olarak tespit edilirken IgM sadece 1 koyun serumunda belirlenmiştir. Bu çalışmanın sonuçları Kosova'nın 4 belediyesinde KKKA'nın endemik olduğunu doğrulamış ve enfekte hayvanların taşınması ile endemik olmayan bölgelere yayılım riskinin bulunduğunu göstermiştir.

Anahtar sözcükler: *Kırım Kongo kanamalı ateşi virusu (KKKAV), Sığır, Koyun, IgG, IgM*

INTRODUCTION

The causative agent of CCHF is a member of the Bunyaviridae family, genus Nairovirus. The disease is transmitted to humans through tick bites, by crushing infected ticks or by nosocomial contact with blood of infected animals or humans. Humans are the only known hosts of CCHF virus: the disease is manifested as an acute febrile illness followed by hemorrhagic syndrome with mortality rates of up to 50% [1]. The humans at greatest risk of infection are agricultural workers, veterinarians, abattoir workers, and other persons in close contact with animals and ticks [2]. The circulation of the virus in nature is enzootic, tick - vertebrate - tick, and *Hyalomma* ticks are considered to be the most important transmitters and source of the virus, determining distribution worldwide [2-5]. A wide variety of domestic animals and birds (cattle, sheep, goats and ostriches), as well as small wild mammals (hares and hedgehogs) can become infected with the virus, and these infections are usually asymptomatic and subclinical [6,7]. Livestock and other hosts can transmit CCHFV to humans during the viremic period [6].

CCHF is endemic in some parts of Europe, Asia and Africa. In recent years, cases of human infection have increased, and have been reported from different countries. The disease has emerged or re-emerged in Turkey, Kosova, Bulgaria, Albania and Greece. This has been attributed to mild winters and to the disruption of agricultural activities,

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both accounting for an increased tick population, as well as to the migration or transportation of tick-infested birds or animals [8,9].

Outbreaks of CCHF in Kosova usually occur in spring and summer with a reported human fatality rate up to 25.5% [10]. In 2010, 2265 patients were reported to have tick bites, with 141 suspected cases of CCHF, 29 cases were confirmed using the PCR technique, and among these, there were 8 deaths. Approximately the same number of persons with tick bites and infected patients were reported in 2013 (Report of National Institute of Public Health in Kosova, 2013). CCHF in Kosova is present in 50% of the territory with common characteristics of altitude, climate, low bush, fragmented agricultural land and the presence of the *Hyalomma* tick. Hyper-endemic zones are in the central and south west of Kosova: Malisheve, Kline, Rahovec and Suhareke. *Hyalomma marginatum* dominates in the endemic municipalities, with 90.2% versus 24.3% in the non-endemic regions. 3.6% of ticks tested positive with CCHFV by RT-PCR [11]. In 2012 a sero-prevalence of the healthy human population was 4.0% (range 0-9.3%) and in cattle 18.4%, mostly in endemic areas of Kosova [10].

The aim of this study was to detect the IgG and IgM antibodies to CCHF virus in cattle and sheep and to monitor the movement of sero-positive animals from endemic to non-endemic regions in the Republic of Kosova.

MATERIAL and METHODS

Kosova is located in the Balkans in Southeastern Europe, with continental and Mediterranean climates. It has a land area totaling 10,908 km^2 and a human population of around 2 million (Agency of Statistics of the Republic of Kosova, www.ask.rks-gov.net). Kosova has around 250.000 cattle and 120.000 sheep (Kosova Food and Veterinary Agency).

The study was conducted in CCHF-endemic regions that includes four municipalities in the central and southwest parts of Kosova, namely Malisheve, Suhareke, Prizren and Rahovec *(Fig. 1)*, and in non-endemic regions including four municipalities (Prishtina, Gjilan, Mitrovice and Peja). Sera were collected in the summer of 2008 from 267 domestic animals, 172 from cattle and 95 from sheep. Identification of the specimens included location, ownership and date of collection. Animals registered in the system of the identification unit at Kosova Food and Veterinary Agency were checked in 2014 for their movements around Kosova in the years 2010-2014. Sera were tested using the IIA for quantitative determination of IgG and IgM antibodies to CCHF virus in the Institute of Tropical Medicine "Bernhard Nocht", Hamburg, Germany. IgG and IgM antibodies to CCHF virus were detected using acetone-fixed Vero cells infected with CCHF virus (strain ArD39554, GenBank accession number DQ211641). Cultivation of the virus was carried out in a BSL 4 laboratory. Serum samples were tested in twofold steps starting at a dilution of 1:40. Cell smears were routinely counterstained with anti-CCHF nucleocapsid monoclonal antibody A4 [12] using Rhodamine-anti-mouse as a secondary antibody [2]. A chi-square test was used to determine the presence of antibodies in cattle and sheep and to establish whether there was a significant difference between endemic and non-endemic regions.

RESULTS

The study has shown that 10 from 267 tested animals (3.75%) tested positive for the presence of IgG and IgM antibodies to CCHFV. Seven of 172 (4.07%) cattle and three of 95 (3.16%) sheep sera tested positive. Nine animals were positive for IgG antibody to CCHF virus, the serum of only one sheep tested positive for IgM. The region is a factor that influences the presence of antibodies in cattle and sheep. In endemic regions, 6.67% tested positive, as opposed to 0.76% in non-endemic regions. This difference was statistically significant, $\chi 2$ (1) =6.464, P=0.011 *(Table 1)*.

Nine samples from 135 tested animals or 90% of positive cases were detected in endemic regions (Prizren, Suhareke, Rahovec and Malisheve). We estimated with 95% confidence that the proportion of ruminants in endemic regions carrying the antibodies against CCHF in sera was between 2.40% and 10.93%. One cattle serum of

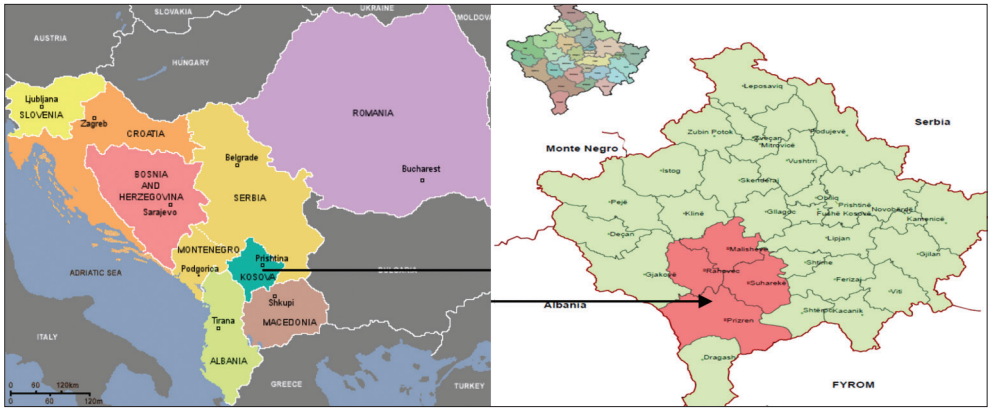

Fig 1. Map of Southeast Europe and Republic of Kosova - endemic regions of CCHF

Şekil 1. Güneydoğu Avrupa ve Kosova Cumhuriyeti Haritası - KKKA endemik bölgeler

Table 1. *Sero-prevalence of antibodies to CCHF virus in cattle and sheep in Kosova*
Tablo 1. *Kosova'da sığırlar ve koyunlarda KKKA virüsüne karşı antikor Sero-prevalansı*

Animals/Zone	Nr. of Samples	IgG Positive	IgM Positive	% Positive 95% CI	Negative Animals	Negative Animals (%)	X^2	P Value
Cattle	172	7	0	4.07 (1.09-7.05)	165	95.93	/	/
Sheep	95	2	1	3.16 (0.42-6.74)	92	96.84	/	/
Total	267	9	1	3.75 (1.45-6.04)	257	96.25	/	/
Endemic	135	8	1	6.67 (2.40-10.93)	126	93.33	6.464	0.011
Non endemic	132	1	0	0.76 (0.74-2.26)	131	99.24	/	/
Total	267	9	1	3.75 (1.45-6.04)	257	96.25	/	/

132 tested animals tested positive from the non-endemic region (Mitrovice). 29 of 267 (10.8%) tested animals were moved from endemic to non-endemic regions, where there was one positive detection of IgG to CCHFV.

DISCUSSION

In Kosova there are no data about humans infected directly by contact with animals. 54% of the humans infected and diagnosed with CCHF have evidence of being bitten by a tick, only 4% of patients were infected via contact from human to human and around 40% of infections are of unknown etiology [13]. The livestock husbandry in Kosovo is such that some households in rural areas keep livestock for family needs, where they often slaughter animals at home and may have direct contact with the blood of infected animals. Even field veterinarians during meat inspection and treatment of animals can be infected through contact with the blood of infected animals. A number of individuals became infected while removing ticks by hand from animals and crushing them. According to the Clinical Center for Infectious Diseases, the largest number of patients with CCHF is to be found in the municipalites of Malisheve with 65%, Kline with 11.5% and Rahovec, Prizren and Suhareke with a total of 15%. This study has confirmed that the vast majority of sera-positive animals are located in the endemic municipalities. This is an indication that the CCHF virus circulates in endemic municipalities, with the highest prevalence of CCHF virus found in *Hyalomma marginatum* ticks in the municipalities of Malisheva (8.6%) and Klina (4%) [11]. The fact that the domestic animal population is kept partially in pasture contributes to increase the tick population. Furthermore the fragmentation of agricultural land and the presence of wild animals are playing an influential role in increasing the tick population as they host the immature ticks (larvae and nymphs) and act as a reservoir for the viruses.

There is a need for further studies in order to identify which wild animals play an important role in hosting immature ticks *Hyalomma marginatum* in endemic regions in Kosova.

It is important to note the fact that in Kosova it is a common practice to trade domestic animals in open animal markets that are present in every municipality. In our study, 11% of animals tested from endemic regions had moved through trading in non-endemic regions and in one case we found one cow IgG seropositive for the CCHF virus. In the study conducted by Sherifi et al.[11], a tick with CCHF virus was found and removed from a cow which had been moved from an endemic municipality to a non-endemic municipality. This practice of trading and moving domestic animals with ticks infected with CCHF virus may present a great risk for the spread of the CCHF virus to other regions, where so far no reports of human infections have been received.

The results of this study are important for the Kosova national authorities that are responsible for controlling and monitoring the CCHF in Kosova. To prevent the spread of the CCHF virus during spring and summer seasons from endemic municipalities to non-endemic municipalities, it is important to treat domestic animals with Acaricides prior to taking them to the animal market for trading.

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Case Report

Treatment of Complete Urethral Obstruction by using Pneumatic Lithotripsy in a Dog: A Preliminary Report [1,2]

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Abstract

In this preliminary case presentation, use of the minimally invasive cystoscopic pneumatic lithotripsy technique in the treatment of complete urethral obstruction in a dog has been described. The animal of the case comprised a 5-year old Chihuahua presenting with difficulty during urination and inability to urinate for the previous 2 days. Post-renal azotemia and hematuria was determined. Urethral stones causing complete urethral obstruction were visualized via cystoscopy, fragmented with a pneumatic lithotripter and the stone fragments were removed using the voiding urohydropulsion method. In conclusion, the pneumatic lithotripsy method was successfully used in the treatment of complete urethral obstruction.

Keywords: *Pneumatic lithotripsy, Urethroscopy, Urethral stone, Obstruction, Dog*

Bir Köpekte Tam Üretral Obstrüksiyonun Pnömatik Litotripsi İle Tedavisi: İlk Rapor

Özet

Bu olgu sunumunda bir köpekte tam üretral obstrüksiyonun tedavisinde minimal invaziv sistoskopik pnömatik litotripsi tekniğinin kullanımı anlatıldı. Olgunun hayvan materyalini idrar yaparken zorlanma ve son iki gündür idrar yapamama şikâyetleri ile getirilen, postrenal azotemia ve hematuria tespit edilen Chihuahua ırkı, 5 yaşlı bir köpek oluşturdu. Tam üretral obstrüksiyona neden olan üretral taşlar sistoskopi ile görüntülendi, pnömatik litotriptör kullanılarak parçalandı ve taş fragmanları voiding urohydropulsiyon yöntemiyle dışarıya alındı. Sonuç olarak, pnömatik litotripsi yöntemi tam üretral obstrüksiyonun tedavisinde başarılı bir şekilde kullanıldı.

Anahtar sözcükler: *Pnömatik litotripsi, Üretroskopi, Üretral taş, Obstrüksiyon, Köpek*

INTRODUCTION

Lithotripsy derives from the Greek words “lith” stone and “tripsis” to break and is a procedure literally meaning “breaking stones”. It was first used to fragment kidney stones in humans at Munich University on 7th February 1980. This technique was named “Extracorporeal Shock Wave Lithotrpisy” (ESWL). ESWL was followed by the development of intracorporeal techniques administered directly onto the stone using endoscopic methods. Fragmenting of bladder stones became easier with the development of lithotripters applied directly onto the surface of the stone guided by a cystoscope. The electrohydrolic, ultrasonic, pneumatic and Holmium-YAG laser lithotripsy techniques are widely used in this field [1].

Pneumatic lithotripsy (PL) was developed in the 1990s. The method utilizes a rigid energy probe placed directly onto the stone and the stone is then broken into fragments by a drill-like effect due to the energy of the compressed air. The PL method provides a practical and low cost treatment approach in urethral stones due to its reusable probe [2]. Pneumatic lithotripsy is used extensively in human medicine [3-5]. In veterinary medicine, it was first demonstrated in fragmenting stones placed in the ureters of dogs and pigs in an experimental study [6].

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In this case report, the use of a pneumatic lithotripter under the guidance of a rigid cystoscope in the treatment of total urethral obstruction in a 5-year old Chihuahua dog has been described.

CASE HISTORY

The case was a 5-year old Chihuahua, brought to the Small Animal Hospital of Veterinary Faculty, Selçuk University. The case presented difficulty in urination (dysuria, stranguria), inappetence, lethargy and inability to urinate for the previous 2 days. Physical examination of the dog revealed normal body temperature, heart rate and respiratory rate. Distension and pain in the caudal abdomen along with a full urinary bladder were determined upon abdominal palpation. The dog frequently exhibited urination position but was unable to urinate. A full bladder was observed on direct radiographs with no other abnormalities detected. On ultrasound examination, structures presenting acoustic shadows were identified in the bladder and proximally to the os penis. The urethral catheterization attempt after decompressive cystocentesis was unsuccessful. Therefore, it was decided to perform cystoscopy in the dog. The patient's haematological, venous blood gases, serum biochemistry and urine analysis data are shown in *Table 1*. Haematology and venous blood gas data was within the normal range, however, post-renal azotaemia and haematuria was determined. Prior to cystoscopy, the patient was anaesthetized with xylazine (Rompun® Bayer, 2 mg/kg, IM) and ketamine (Ketasol® Interhas, 10 mg/kg, IM). On rigid cystoscopic examination, 2 yellow and rough-surfaced stones proximal to the os penis were seen to have completely obstructed the urethra *(Fig. 2)*. The stones were fragmented using a pneumatic lithotripter *(Fig. 1)* and the urethra was unblocked. The bladder was then entered and stones in the bladder were fragmented. The stone fragments were removed via urination using the voiding urohydropulsion method *(Fig. 3)* [7-9]. The voiding urohydropulsion method was repeated 3 times.

DISCUSSION

In this preliminary case report, use of minimally invasive cystoscopic pneumatic lithotripsy method in the treatment

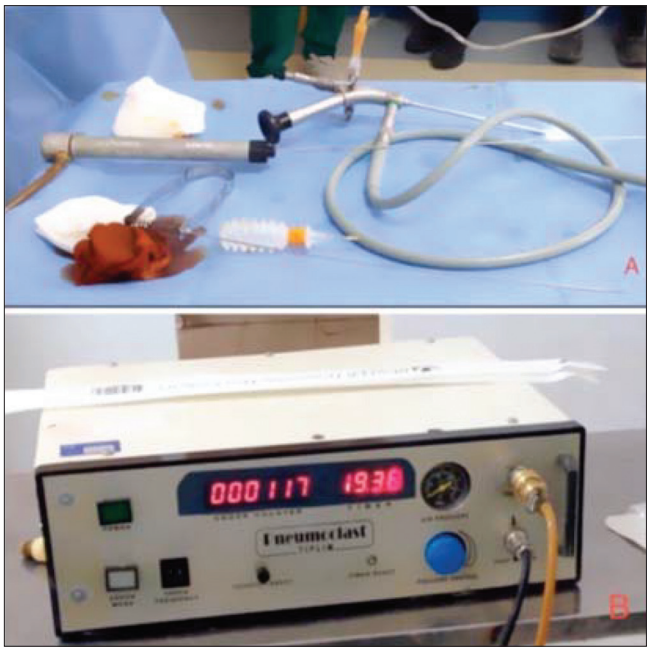

Fig 1. Pneumatic lithotripter and rigid endoscope (A), lithotripter unit (B)
Şekil 1. Pnömatik litotriptör ve rigid endoskop (A), litotriptör ünitesi (B)

Table 1. *Laboratory results*
Tablo 1. *Laboratuar sonuçlar*

Haematology		**Blood Gases (Venous)**		**Serum Biochemistry**		**Urinalysis**	
WBC (m/mm³)	12.67	pH	7.34	BUN (mg/dl)	57	Colour	Yellow
Lymphocyte (%)	52.5	pCO_2 (mmHg)	37	Creatinine (mg/dl)	2.3	Appearence	Transparancy
Monocyte (%)	21.9	pO_2 (mmHg)	37	GGT (IU/L)	11.0	SpG	1.020
Granulocyte (%)	25.6	Na^+ (mmol/L)	152	ALT (IU/L)	70	pH	6.0
Lymphocyte (m/mm³)	6.65	K^+ (mmol/L)	3.3	ALP (IU/L)	18	Protein	+1
Monocyte (m/mm³)	2.77	Ca^{++} (mmol/L)	0.61	Calcium (mg/dl)	8.8	Glucose	-
Granulocyte (m/mm³)	3.25	Glucose (mg/dl)	122	Phosphorus (mg/dl)	6.8	Keton	-
RBC (M/mm³)	8.99	Lactate (mmol/L)	3.3	Cholesterol (mg/dl)	237	Bilirubin	-
MCV (fl)	46.7	HCO_3^- (mmol/L)	20.0	Triglyceride (mg/dl)	127	Blood	-/+ intact
PCV (%)	41.9	TCO_2 (mmol/L)	21.1	Total Protein (g/dl)	6.1	Nitrite	-
MCH pg	17.2	BEecf (mmol/L)	- 5.8	Albumin (g/dl)	4.0	RBC/HPF	++ moderate
MCHC (g/dl)	36.9	BE(B) (mmol/L)	- 5.2	Glucose (mg/dl)	215	WBC/HPF	-
RDW	12.1	SO_2c (mmol/L)	66	T. Bilirubin (mg/dl)	1.8	Casts/HPF	-
Haemoglobin (g/dl)	15.5	THbc (g/dl)	14.6			Bacteria	-
Trombocyte (m/mm³)	159					Crystal	-

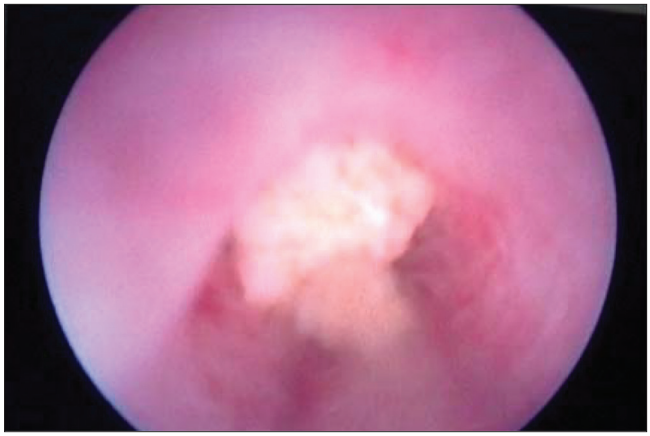

Fig 2. Endoscopic appearance of urethral stones
Şekil 2. Üretral taşların endoskopik görünümü

of complete urethral obstruction caused by stones was described in a dog. The obstruction was successfully resolved and no complications were encountered.

The movement of stones from bladder into urethra is among the most frequent causes of urinary obstruction in dogs. Small breeds are more affected [10]. The urethral obstruction is most commonly reported in the proximal part of os penis, where the urethral diameter is relatively narrow. Urethral stones can be removed using hydropulsion, lithotripsy and basket retrieval methods [9], as well as the surgical options of cystotomy and cystolithotomy [11-14]. In this case report, no stones could be visualized on radiographic examination but ultrasonographic examination revealed presence of stones in the bladder and acoustic shadows. In the light of the radioluscent stones present in the bladder and urethra, as well as the acidic nature of the urine pH, it was estimated that this could be either cysteine or urate [15]. Stone analysis could not be performed. The urethral obstruction determined to be located proximally to os penis was cleared using cystoscope-guided pneumatic lithotripsy [3-5] and voiding urohydropulsion [7-9].

Electrohydrolic (ELH) and Ho : YAG (holmium : yttrium, aluminium, garnett) laser lithotripsy methods are used to fragment stones in the urinary system [10,16-20]. ELH is recommended as a minimally invasive method in fragmenting urethral stones in males and bladder stones in females [18]. Endoscopic laser techniques remain limited in dogs weighing less than 6-8 kg and, in particular, due to the restriction of distensibility in the ventral groove of os penis [10,19]. This method has been reported to have advantageous in avoiding urethral mucosa damage [21], however, studies have also reported complications such as haematuria [19,20], superficial mucosal damage [21,22] and secondary stenosis resulting from thermal damage [6] caused by electrohydrolic/ultrasonic probes. High cost of the equipment and the need for technical experience is also among limiting factors [12].

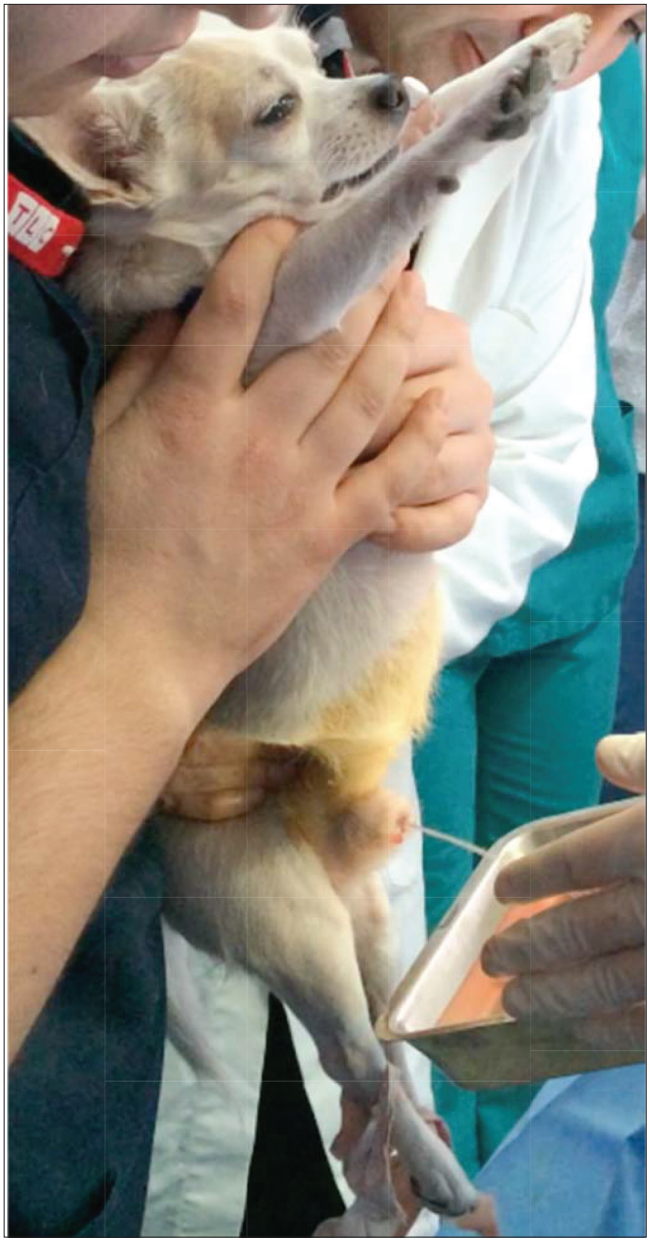

Fig 3. The application of voiding urohydropulsion
Şekil 3. Voiding urohydropulsion uygulaması

Major perioperative complications in the laser lithotripsy are death, urethra or bladder perforation and urethral obstruction; while minor perioperative complications are haematuria, leukocyturia and infection of the urinary system [20]. In studies where Ho : YAG laser lithotripsy was used, haematuria was seen in 18 of 25 dogs during the procedure and in 9 dogs after the procedure [19]. In another study, immediately after lithotripsy, focal lesions, erosions, hemorrhage and ulceration were seen in 4 of 19 dogs. These lesions were formed mostly due to the effect of the stone and tissue damage originating from laser had only developed in 1 case [21]. In another laser lithotripsy procedure performed under the guidance of a cystoscope, complication rates were found to be 17.9% and 13.3% in females and males, respectively [10].

Pneumatic lithotripsy is recommended as a useful, effective and low cost method in the removal of ureter stones in human medicine [3-5,23]. In this case report, urethral stones causing complete urethral obstruction and stones in the bladder were visualized via cystoscopy, fragmented with a pneumatic lithotripter and stone fragment were removed using the voiding urohydropulsion method *(Fig. 3)*. The pneumatic lithotripsy technique was successfully performed under the guidance of a 3-mm rigid endoscope in a relatively small dog of 3.4 kg/BW and this technique was tolerated well by the dog. Compared to other lithotripsy methods [10,17-19], no complication other than minimal mucosal bleeding observed during fragmenting the urethral stone was seen after the procedure. Protective antibiotics (Baytril® Bayer, 5 mg/kg/daily, PO) were prescribed following the procedure.

In conclusion, the pneumatic lithotripsy method was successfully used in the treatment of complete urethral obstruction. As the pneumatic lithotripsy method was a useful, low-cost, practical and minimally invasive technique in the fragmenting of urethral and bladder stones, authors suggest that based on its use in human medicine, the pneumatic lithotripsy method coupled with a flexible cystoscope may be of help in treating ureter and bladder stone. However further clinical studies are needed for this purpose.

Conflict of interest

Authors disclose no conflict of interest.

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Case Report

Morphological and Etiological Investigations in A Rotaviral Enteritis Outbreak in Calves

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Abstract

The aim of the present report was to describe the gross pathology and histopathologic findings and etiological investigations in a diarrhoeic syndrome outbreak in calves from the 24th h to 20th day of life. Clinically, affected animals exhibited profuse diarrhoea with yellow-greenish faeces mixed with mucus and blood. Rapid field tests Rainbow calf scour 5 BIO K 306 Detection of *Rotavirus, Coronavirus, E. coli* F5, *Cryptosporidium parvum* and *Clostridium perfringens* in bovine stool and ELISA was used for detection of *Rotavirus, Coronavirus, E. coli* F5, *Cryptosporidium parvum* antigens. The results of tests were positive for Group A bovine rotavirus, and negative for all other tested etiological agents. The gastrointestinal tract macroscopic lesions observed during the gross pathological examination were of inflammatory nature. Microscopic lesions confirmed the catarrhal desquamative enterocolitis through marked lymphocytic infiltration and oedema of intersitial tissues and submucosa. Non-complicated rotaviral enteritis was capable of inducing pathological alterations of the gastrointestinal tract in neonatal calves with high mortality rates.

Keywords: *Rotavirus enteritis, Diarrhoea, Group A bovine rotavirus, Calves*

Buzağılarda Gözlenen Bir Rotavirus Enteritis Salgınında Morfolojik ve Etiyolojik Araştırmalar

Özet

Bu çalışmanın amacı buzağılarda 24. saat ile 20. gün arasında şekillenen diare sendromu salgınında makroskobik ve histopatolojik bulguların tanımlanarak etiyolojinin araştırılmasıdır. Klinik olarak hasta hayvanlarda mukus ve kan ile karışık sarı yeşilimtırak renkli dışkı ile karakterize şiddetli ishal gözlemlendi. Hızlı saha testi, Rainbow calf scour 5 BIO K 306, dışkıda *Rotavirus, Coronavirus, E. coli* F5, *Cryptosporidium parvum* ve *Clostridium perfringens* antijenlerini belirlemek amacıyla kullanıldı. Testin sonuçları Grup A bovine rotavirus için pozitif ve diğer tüm etkenler için negatif olarak belirlendi. Gastrointestinal system organlarda gözlemlenen patolojik değişiklikler yangısal karakterdeydi. Mikroskobik bakıda intersitisyel dokuda ve submukozada şiddetli lenfositik infiltrasyon ve ödem ile karakterize kataral deskuamatöz enterokolitis tespit edildi. Komplike olmayan rotavirus enteritis gastrointestinal sistemde patolojik değişiklikler yapabilme ve yüksek mortalite özelliklerine sahip olabilmektedir.

Anahtar sözcükler: *Rotavirus enteritisi, Diare, Grup A bovine rotavirus, Buzağı*

INTRODUCTION

Rotaviruses provoke a number of gastrointestinal illnesses in infants, calves, pigs, foals, lamb, rabbits, antelopes, mice and exotic species (grizzly bears, red kangaroos etc.) [1,2]. Group A *(gpA)* bovine rotaviruses *(GPA BRV)* are a genus within the family of Reoviridae. On the basis of antigen specificity rotaviruses are classified into groups, subgroups and serotypes [3,4]. Group *A* viruses induce disease in men and ruminants. Group *B* affects calves, lambs and men. Group *C* affects mainly pigs and in some instances, men. Groups *D, F* and *G* provoke disease in domestic fowl [3,5]. Nevertheless, Group A rotaviruses are the main causative agents of infection in livestock [4,6,7]. Group A bovine rotaviruses are enteropathogenic and are most commonly associated with the etiology of neonatal calf diarrhoea from the birth to the 30th day of the age [8-10]. Usually, the virus affects calves around the 3rd week of age,

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with highest frequency until 6 days of age. After entering the host, the incubation period of the virus is relatively short (about 24 h) and the duration of diarrhoea - 2-5 days [5,11]. In neonatal calves, the infection is characterized with the lack of viraemic stage, short incubation period, and profuse diarrhoea combined with severe dehydration. The simultaneous infection with secondary pathogens complicates the course of the disease [12,13]. The onset of diarrhoea is due to the replication of the virus within the enterocytes and perished absorbing enterocytes with activation of enteric nervous system by the rotavirus enterotoxin [14].

The purpose of the present report was to describe the results from gross pathology and etiological investigations in a diarrhoeic syndrome outbreak in calves from the 24th h to 20th day of life.

CASE HISTORY

In the cattle farm, 13 of 40 diseased calves, 5 to 20 days of age, have died during 3-week period. The disease occurred spontaneously, and the most prominent clinical sign of disease was the diarrhoeic syndrome. Profuse diarrhoea with yellow-greenish faeces, mixed with mucus and blood in 4 of calves, was observed. The course of the disease developed within 24 to 96 h, with dehydration and fatal outcome.

A total of 32 animals were included in the study. Rectal faecal content samples were collected from 10 calves with clinical signs from 7 to 20 days of age which were analysed with rapid field test Rainbow calf scour 5 BIO K 306 Detection of Rota, Corona, *E. coli* F5, Crypto and *Clostridium perfringens* (BIOX Diagnostics, Belgium). The tests detect 5 of causative agents of neonatal calf diarrhoea *Rotavirus, Coronavirus, E. coli*-F5, *Cryptosporidium parvum* and *Clostridium perfringens* type *A.* Also, 2 g faecal samples were obtained from the rectums of 22 calves from 5 to 20 days of age for detection of antigens of Rota, Corona, *E. coli* F5, Crypto with ELISA (BIOX Diagnostics, Belgium) sandwich test for faeces.

The serological trapping on antibody-coated electron microscope grids approach of Nicolaieff et al.[15] was used for immunoelectron microscopic detection of viruses in faeces from diarrhoeic calves. Briefly, 20-40 days after the appearance of clinical signs convalescent sera were taken from 5 calves recovered from diarrhoeic syndrome. The faecal test materials for electron microscopic analyses were obtained from 5 acutely diarrhoeic calves from 6 to 12 days of age. Butvar and carbon coated 400 mesh copper grids were floated over drops of protein A solution (usually 26 µg/ml) for 20 min. Then they were washed 4 times on drops of 0.1 M sodium phosphate buffer (pH 7.0). Thereafter, the grids were floated on drops of diluted convalescent sera 1:40 in phosphate buffer for 2 h. After an intermediate rinse on drops of phosphate buffer, the SpA-convalescent sera-antibody-coated grids were left overnight on drops of supernatant of faecal probes diluted with an equal volume of 0.2 M phosphate buffer and centrifuged at 5.000× g for 30 min. Finally, the negative staining of all preparations was carried out with a 2% sodium phosphotungstate, pH 6.8. The examinations were carried out on an electron microscope JEOL 1200 EX at an accelerating voltage of 80 kV and an instrumental magnification of 6.000-70.000X.

Necropsy of 6 dead calves was done following the standard protocol. Specimens from affected gastro-intestinal compartments were collected for histopathological examination. Materials were fixed in 10% neutral buffered formalin and embedded in paraffin. The cross sections were stained with haematoxylin-eosin. From the same carcasses, samples from viscera (liver, heart, spleen, mesenteric lymph nodes) were obtained for conventional bacteriology.

The results from rapid antigenic tests proved the presence of coproantigens against *Group A bovine rotavirus* in all 10 calves. These results were also confirmed with ELISA test *(Fig. 1)* with positive results in 12 samples out of 22 examined faecal samples. The antigen tests showed that all studied 22 faecal samples were positive for *Group A bovine rotavirus (Table 1)*. There were no antigens of the other etiological agents of neonatal calf diarrhoea. The immunoelectron microscopy exhibited accumulation of rotaviral particles, distributed evenly along the grid under the form of various number of virion aggregates *(Fig. 2)*.

Gross pathology findings were similar in all necropsied animals. The external appearance of the carcass showed signs of severe dehydration and enophtalmos without other obvious changes. The perianal region was stained with yellow-greenish faeces. After dissection of the

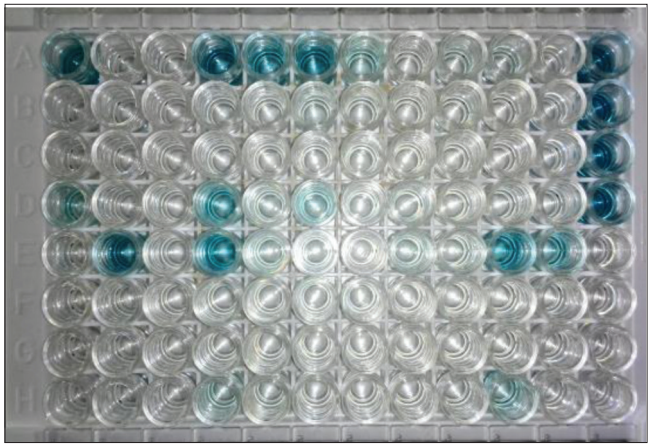

Fig 1. Positive results of sandwich ELISA test for faeces. Blue-coloured wells demonstrate the presence of antigens against rotavirus in calf faecal samples

Şekil 1. Dışkı örneklerinde sandviç ELISA pozitif sonuçları. Mavi renkli hücreler buzağı dışkı örneklerinde rotavirusa karşı antijenlerin varlığını göstermektedir

Table 1. *Summary of data from etiological antigenic tests*
Tablo 1. *Etiyolojik antijenik test sonuçları*

Test	Total Number of Faecal Samples from Calves n=32	Number of Samples Positive for BRV	Number of Samples Positive for BCoV	Number of Samples Positive for *E. coli* F5	Number of Samples Positive for a *C. parvum*
Rainbow calf scour 5 BIO K 306	10	10	0	0	0
ELISA sandwich test	22	12	0	0	0
Total number of positive samples		22	0	0	0

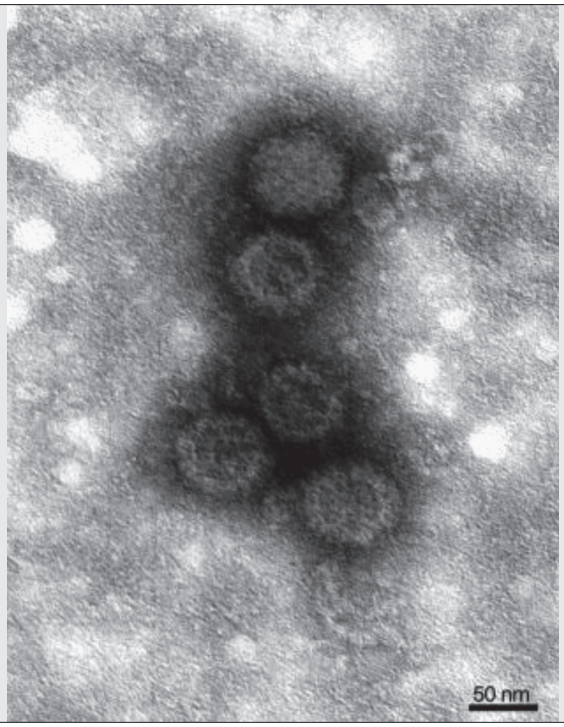

Fig 2. Immunoelectron microscopy of a faecal sample from diarrhoeic calf as per Nikolaieff et al.[15]. Rotavirus particles aggregate. Negative contrasting with 2% sodium phosphotungstate pH 6.8

Şekil 2. Nikolaieff et al.[15] tarafından tanımlanan bir ishalli buzağı dışkı örneğininde rotavirus partikülleri. 2% sodium phosphotungstate pH 6.8 ile negatif kontrast

abdomen, the abdominal cavity contained excessive amount of opaque fluid. Multiple haemorrhages on the mucosa of fore stomachs were detected. The most severe changes were observed in the small intestine, corresponding to acute catarrhal haemorrhagic enteritis. The affected compartments were dilated, with moist intestinal content and meteorism. The colour of intestinal content was yellow-greenish, mixed with numerous gas bubbles. Mesenteric lymph nodes were markedly enlarged and swollen. The abomasum was filled with milk coagula, and its mucosa - hyperaemic and spattered with multiple erosions and few ulcerations.

The liver had an enlarged gallbladder and a frail consistency. In the medullary region of both kidneys, a strong hyperaemia was seen. Petechial and ecchymotic haemorrhages of the epicardium were present. There were no relevant pathoanatomical changes on the other visceral organs.

The histological study of small and large intestine revealed strong dystrophic and necrobiotic changes in enterocytes. A large amount of desquamated cells have shed into the lumen. Some of intestinal villi were strongly atrophied, and crypts – severely dilated in some areas *(Fig. 3)*. The propria was infiltrated with numerous lymphocytes, and submucosa had a marked oedema and hyperaemia *(Fig. 4)*. The studied bacteriological samples were negative for bacterial agents.

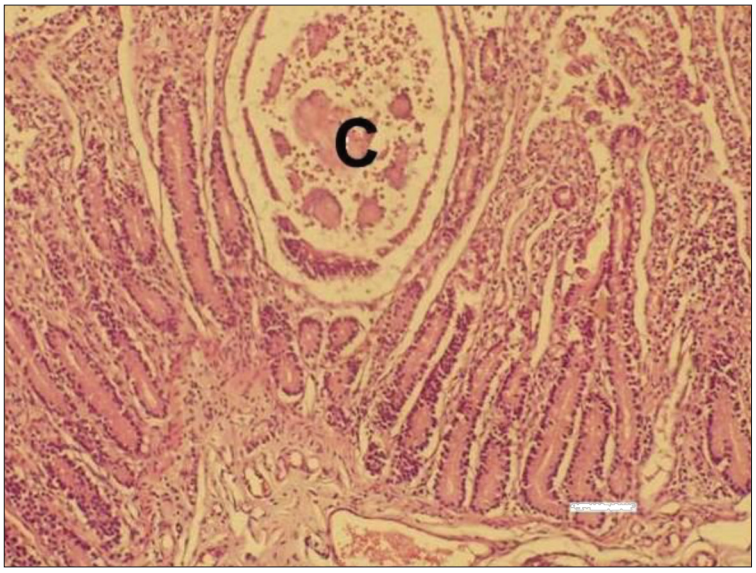

Fig 3. Transverse cross section of the jejunum. Presence of specific pseudocystic formations within the mucosa, including inflammatory cell exudate (C), H/E. Bar=50 µm

Şekil 3. Jejenumun transversal kesiti. Mukozada spesifik psödokistik oluşumlar (C) ve yangısal hücre infiltrasyonu, H/E. Bar=50 µm

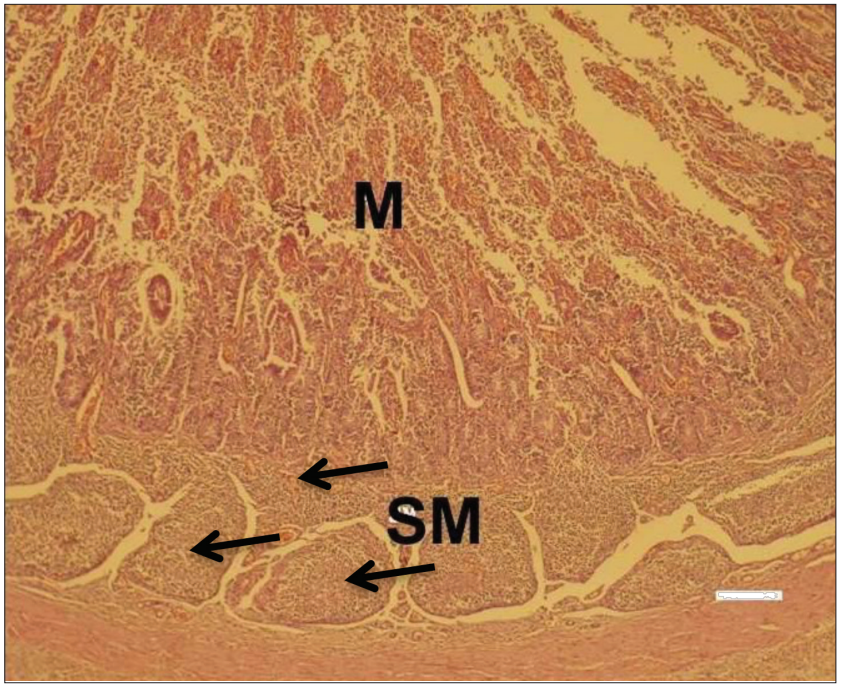

Fig 4. Transverse cross section of the ileum. Intense serous haemorrhagic and desquamative catarrh within the mucosa (M) and a diffuse inflammatory cell infiltration of the submucosa (SM), *(Black arrows),* H/E. Bar=100 µm

Şekil 4. *İleumun* transversal kesiti. Mukozada şiddetli seröz, hemorajik ve deskuamatöz kataral yangı ve submukozada diffuz yangısal hücre infiltrasyonu (SM), *(siyah oklar),* H/E. Bar=100 µm

DISCUSSION

The results from the present etiological studies proved the presence of a rotaviral monoinfection with high morbidity and mortality rate during the first 20 days of calves' life. Our results are comparable to other reports [13]. Even as a non-complicated infection, rotaviral enterites could entail high morbidity and mortality among neonatal calves.

The method of immune electron microscopy demonstrated the presence of rotavirus particles in faecal samples from calves with diarrhoea, which confirms the results of our previous studies. In 1969, the method was used to detect the viruses causing diarrhoea in calves, and named "the gold standard" in the diagnosis of viral enteritis in calves [16].

In contrast of the direct immune electron microscopy, electron microscopy has greater sensitivity, consisting in using a specific antibody for searching virus. Both methods, ELISA and immune electron microscopy have a high sensitivity ranging from 87% to 100% of the different viral agents, which gives them good diagnostic value [17].

The observed microscopic lesions in the distal compartment of small intestines characteristic for severe surface desquamation of epithelial cells and atrophy of some villi, as well as the proliferation of the propria with lymphocytes, are at the background of the pathogenesis of rotaviral enterites in calves, as also stated by other researchers [12].

Pathomorphological changes in the intestine and colon in coronavirus infection were expressed in atrophy and fusion of individual villi, with involvement of the crypts epithelium [18]. In the distal section of the small intestine (ileum) were focused histological changes in *E. coli* infection. In addition to atrophy and collection of individual villi were observed and also a large number of bacteria on their surface [19].

Compared to microscopic lesions (epithelial cell desquamation in distal small intestine and proximal colon) observed by us in an *Cryptosporidium parvum* outbreak [14], the present report from the confirmed rotaviral infection provided also evidence for damage of crypts in the distal ileum.

The performed examinations allowed concluding that the antigen diagnostic tests as Rainbow calf scour 5 BIO K 306 and ELISA (BIOX Diagnostics, Belgium) combined with gross anatomy and histopathological findings are appropriate for diagnostics and differential diagnostics of gastrointestinal diseases in neonatal and juvenile calves.

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Case Report

A Rare Complication of the Postpartum Period in a Dog: Vaginal Evisceration

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Abstract

Transvaginal intestinal evisceration was observed in a 4-year-old bitch had given birth to four puppies ten days ago, normally. For treatment, median laparotomy was performed and intestinal loops were reduced via intra-abdominal traction. Intestinal anastomosis was performed on the necrotic part of bowel and the vaginal tear was sutured. According to owner's request, the bitch underwent ovariohysterectomy. The dog was recovered without any complications. The absence of the main etiological factors in this case, suggests the presence of different factors that need to be investigated in the aetiology of the disease. To our knowledge, this case represents the first report of the successful management of transvaginal evisceration in pet practice.

Keywords: *Vaginal tear, Evisceration, Dog*

Köpekte Nadir Görülen Bir Postpartum Dönem Komplikasyonu: Vaginal Eviserasyon

Özet

On gün önce normal yolla 4 yavru doğurmuş olan 4 yaşlı bir köpekte transvaginal barsak eviserasyonu görüldü. Tedavi amacıyla median laparatomi uygulandı ve barsak segmenti abdomenden içeriye çekilerek red işlemi gerçekleştirildi. Dışarı çıkan barsak segmentinin nekrotik kısmında anostomoz uygulandı ve vaginal yırtık dikildi. Hasta sahibinin isteği üzerine ovariohisterektomi operasyonu ile hasta kısırlaştırıldı. Köpek herhangi bir komplikasyon oluşmadan iyileşti. Bu hastada vaginal rupturun bilinen nedenlerinin bulunmaması, hastalığın etiyolojisinde araştırılması gereken farklı etmenlerin varlığını düşündürmektedir. Bildiğimiz kadarıyla, bu olgu sunumu pet hekimliğinde başarıyla sağaltılmış ilk transvaginal eviserasyon raporudur.

Anahtar sözcükler: *Vaginal yırtık, Eviserasyon, Köpek*

INTRODUCTION

Obstetric injuries involving the rupture of the genital tract in animals has been seen in periparturient stage, usually during dystocia and also it need the acute surgery. Although the prevalence of obstetric injuries in dogs is not known exactly, the manual interventions [1], improper use of oxytocic drugs [2] and also oversized foetuses [3,4] are reported as high risk factors. According to previously articles, it is seen that a large portion of ruptures have been localized on the uterine wall. Meanwhile, limited reports about the complications of the vaginal tear following dystocia in dog [5-7] have been reported. Although the surgical approaches were performed immediately in both cases, postoperative prognosis was reported as the poor, with high mortality. Therefore, it can be seen that the limited knowledge about the predisposing factors and therapy of this rare emergency condition.

In this case report, the clinical and operative findings of the transvaginal small intestinal evisceration treated successfully was presented in a dog, 10 days after normal parturition.

CASE HISTORY

A 4-year-old female hound dog was referred to our clinic due to intestinal evisceration becoming in last hour.

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In patient history, it was recorded that the dog had given birth to four healthy normal-sized puppies ten days ago, normally. Parturition had taken place without any challenge and lasted about six hours. During the subsequent ten days, no complication had been observed.

On physical examination, the dog was recorded as severely underweight (15 kg), depressed with pain and dehydrated. It was clearly seen that the intestinal loops protruding from the rima vulva *(Fig. 1)*. Moreover, a part of small intestine turned to colour in dark purple and was recorded in high risk of necrosis as shown *Fig. 2*. In vaginal digital examination, the localization of the vaginal tear could not been detected. At first, the intestinal loops were washed with 0.9% saline solution and also a venous blood sample was taken for haematological analysis. Also, fluid therapy was initiated using lactated Ringers intravenously (iv). Test results were normally except the severe leucocytosis (WBC=24.10x10^9/l, RBC=5.65x10^{12}/l HGB=12.8 g/dl, HCT=37.6%). Regarding to the intestinal rupture risk at the manual reposition, median laparotomy was performed immediately. While under general anaesthesia with 7 mg/kg propofol (Propofol, Abbot®, Turkey) and 2% isoflurane (Isoflurane®, Adeka, Turkey) the intestinal loops were reduced via carefully intra-abdominal traction. The affected portion of the intestines was resected and a bowel anastomosis was performed using 2-0 Polyglactin 910 (Vicryl®, Ethicon, UK) thread with Schimiden and Lembert sutures as shown *Fig. 3*. Moreover, the vaginal tear, the cause of this evisceration was detected on the left side of vagina (9 centimetres from cervix) and it was repaired with 2-0 Polyglactin 910 (Vicryl®, Ethicon, UK) thread with simple continuous sutures *(Fig. 4)*. The abdominal cavity was flashed with warm 0.9% saline solution and intestines were repositioned in carefully. According to owner's request, the patient was spayed with ovariohysterectomy before abdominal closure. Postoperatively, cephalosporin (Sefazol®, Mustafa Nevzat, Turkey) was given for 7 days (30 mg/kg, IM) every 12 h. Also, first four days was prescribed only fluid therapy by IV. Throughout four days was used every 12 h 50 ml of 5% dextrose, lactated Ringer 75 ml, 0.9% NaCl 250 ml, and Metronidazole (Flagyl®, Eczacıbaşı, Turkey) (25 mg/kg). Next seven days was given water and diluted canine A/D

Fig 1. Macroscopic appearance of transvaginal bowel evisceration

Şekil 1. Transvaginal barsak eviserasyonunun makroskobik görünümü

Fig 2. Necrotic part of intestines after washing the loops

Şekil 2. Yıkama sonrası barsaktaki nekrotik kısım

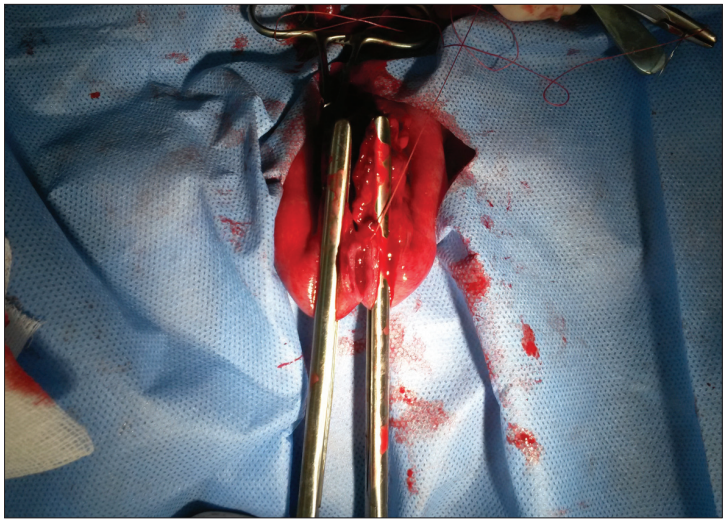
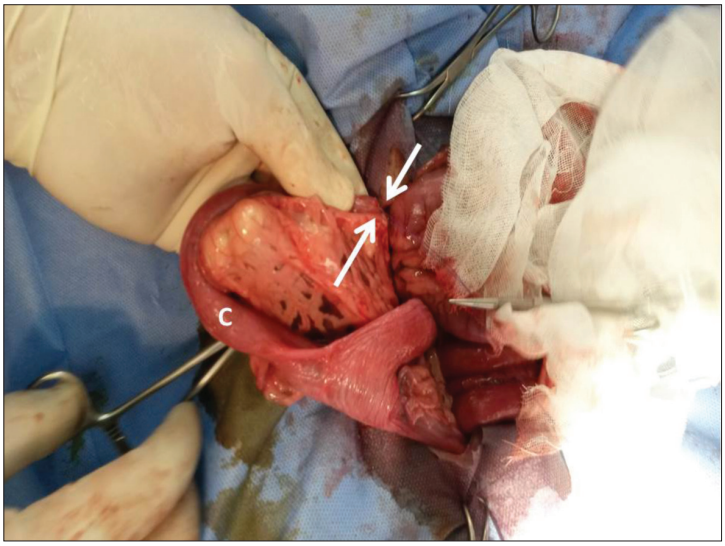


Fig 3. Termino-terminal anastomosis procedure

Şekil 3. Uçuca anostomoz uygulaması

Fig 4. Suturing of vaginal tear *(arrows),* Cervix (C)

Şekil 4. Vaginal yırtığın dikilmesi *(oklar),* Serviks (C)



prescription diet. Next seven days was given macerated canine diet was started slowly a normal feeding.

Neither general complications nor vaginal discharge occurred with surgery throughout the three days. The results of the haematological analysis were recorded normally (WBC=13.30x10^9/l, RBC=5.3x10^{12}/l, HGB=11.7 g/dl, HCT=36.4%) at postoperatively 72 h. One week later, the dog had recovered completely. At the examination after six months postoperatively, dog was normal and had no complication originated from gastrointestinal and genital organs. The dog had got weight and was in good general condition.

DISCUSSION

Vaginal evisceration characterized by internal organs prolapsing transvaginally is a rare emergency surgical condition [8-10]. Depending on women's sexual stage, this condition is associated with pelvic prolapse, previous pelvic surgery, especially vaginal hysterectomy, and also traumatic coitus [11]. To date, more than 100 vaginal evisceration cases has been previously reported in literature [10], but only few reports in veterinary medicine. Vaginal bowel evisceration cases have been recorded in several farm animals [12-14] and dogs [5,15]. But to summarize the predisposing factors is not possible due to low incidence. At breeding, the penetration of the penis to the vaginal fornix can result to this condition in two mares [13]. And also, it was reported that the similar complication in a dog following the forceful separation at mating [14]. Meanwhile, another report is associated with pregnancy and parturition. Scott [13] warned that evisceration of intestines through a tear in the dorsal vaginal wall occurs spontaneously in heavily pregnant ewes during the last month of gestation with the incidence between 2 and 5 per cent. Regarding to our presentation, two similar cases have been reported in dogs with transvaginal evisceration after parturition [5,6]. In the first case, a perineal mass containing intestinal loops, bladder and uterine horns was observed immediately after delivery of oversized foetuses resulted from tenesmus and/or due to oversized fetuses [5].

In the second case, it was observed that the vaginal rupture with bladder retroflexion and evisceration 45 days after delivery. However although the surgical treatment including ovariohysterectomy and partial vaginectomy was performed, both dogs were not survived. According to author's, deaths were attributed to the patient's poor general condition and septic shock, respectively.

In the present case, the sizes of puppies were normal and have no clinical signs related to foetal dystocia. After ten days parturition, all puppies were living without any health problem. However straining due to constipation is well-known as a risk factor for vaginal ruptures [10], there was no history about manual intervention in delivery, constipation, accidental trauma etc. according to owner's report. Therefore, this condition suggests the presence of different factors that need to be investigated in the aetiology of the disease.

The suggested surgical option is a combined abdominal-vaginal approach especially in complicated cases [11]. In this case presented, the similar surgical technique was used successfully, combined with resection the necrotic part of intestine. Regarding to the poor reducibility and high rupture risk of damaged intestinal walls, only manual reduction of loops should not be performed. Following the resection of necrotic parts ex-situ, vaginal bowel traction with abdominal laparotomy can be performed.

In human medicine, pelvic organ prolapses are described in detail under the heading "pelvic floor disorders". Normally, pelvic floor components surrounding them keep the pelvic organs in place. In reconstructive pelvic surgery, numerous surgical methods with and without graft materials have been used in pelvic support tissue according to localization of the defect [16,17]. However, the pelvic defect repair has been performed simpler in cases of pelvic organ prolapse in dogs. In our opinion, this difference can be related to the body's posture and pelvic organ localization. Pelvic organs are positioned horizontally in dogs but vertically in human body. Regarding to this different pelvic position and the need of resistance to gravity, more complex surgical approaches are inevitable in women with similar disorders. In this case, after reposition of ileum and suturing of vaginal wall, routine abdominal closure technique was performed. Likewise, no need was seen to use graft in this operation.

In obstetric injuries, many factors can be determinant for postoperative prognosis. As previously reported, poor general condition and shock have negative impact on the patient's survival [5]. Despite being severely underweight condition in dog was not a problem in this case. In beside of patient's condition, some authors pointed that the low ambient temperature could be a negatively factor on the patient's prognosis [5,18]. But the dog was referred the our hospital in a day where the temperature 23 Celsius degrees averagely in April-2015, therefore no complication related to low temperature was observed after treatment. Also it was not seen any complications throughout six months after operation.

In conclusion, it was presented that a case of transvaginal evisceration, which was seen on the postpartum tenth day, and was successfully treated surgically without any complication. Given the preoperative and postoperative findings in this case, it should be considered that the patient's survival can be increased by proper surgical treatment in this rare condition.

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Letter to the Editor

Why Systematic Examination is Important in Diagnosis of Eye Diseases? Lacrimal Punctal Atresia of a Dog Treated When He Reaches the Age of 15 Months

(Göz Hastalıklarının Tanısında Sistematik Muayene Neden Önemlidir? Bir Köpekte Ancak 15 Aylık İken Tedavi Edilebilen Atresia Punkta Lakrimalis Olgusu)

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Dear Editor,

"Punctal atresia" or "imperforate lacrimal puncta" is the most frequently diagnosed congenital anomaly of canine nasolacrimal drainage system. The most affected breeds are American Cocker Spaniels, Bedlington Terriers, Golden Retrievers, Poodles and Samoyeds. It can affect the superior, inferior, or both puncta, and it may be either unilateral or bilateral. Although superior (upper) punctal atresia is asymptomatic, a dramatic epiphora is present in inferior (lower) punctal atresia. The diagnosis is confirmed by detailed observation with a magnifying loupe or operating microscope, nasolacrimal duct cannulation and Jones test (appearance of fluorescein stain in nasal punctum after dropped into ipsilateral eye). In most cases atresia consists of a layer of conjunctiva over the punctal lumen, therefore nasolacrimal flushing through the upper punctum with a gentle pressure will cause the conjunctiva over the lower canaliculus to bulge. Surgical excision of this ballooning conjunctiva and cannulating the canaliculi to assure a patent duct during healing are main steps of treatment [1-3].

A 15 months old male Golden Retriever dog was brought to the MAKU Animal Hospital on October 2013 with complaint of chronic epiphora in right æeye. As it was learned from the patient's story, the complaint was present since he born, and treatment attempts of 3 different veterinarians (1: conjunctivitis follicularis; 2: allergic conjunctivitis and 3: bacterial conjunctivitis) give any improvement. The overall health status was good on physical examination but constant tear drainage over medial cantus and a strip of dark brown colored hair on this area *(Fig. 1A)* was obvious. Any inflammatory changes were recorded neither in cornea nor conjunctiva. The main Schirmer test value of the affected eye was 17.8 mm/min and signs such as blepharospasm, photophobia or ocular pain were absent. During the observations with a magnifying loupe, the absence of inferior lacrimal puncta was noticed *(Fig. 1B)*. With the negative result in Jones test with 2% fluorescein stain, the case was diagnosed as right inferior lacrimal punctal atresia.

The dog was anesthetized to reestablish tear inflow of the congenitally occluded puncta surgically. Nasolacrimal

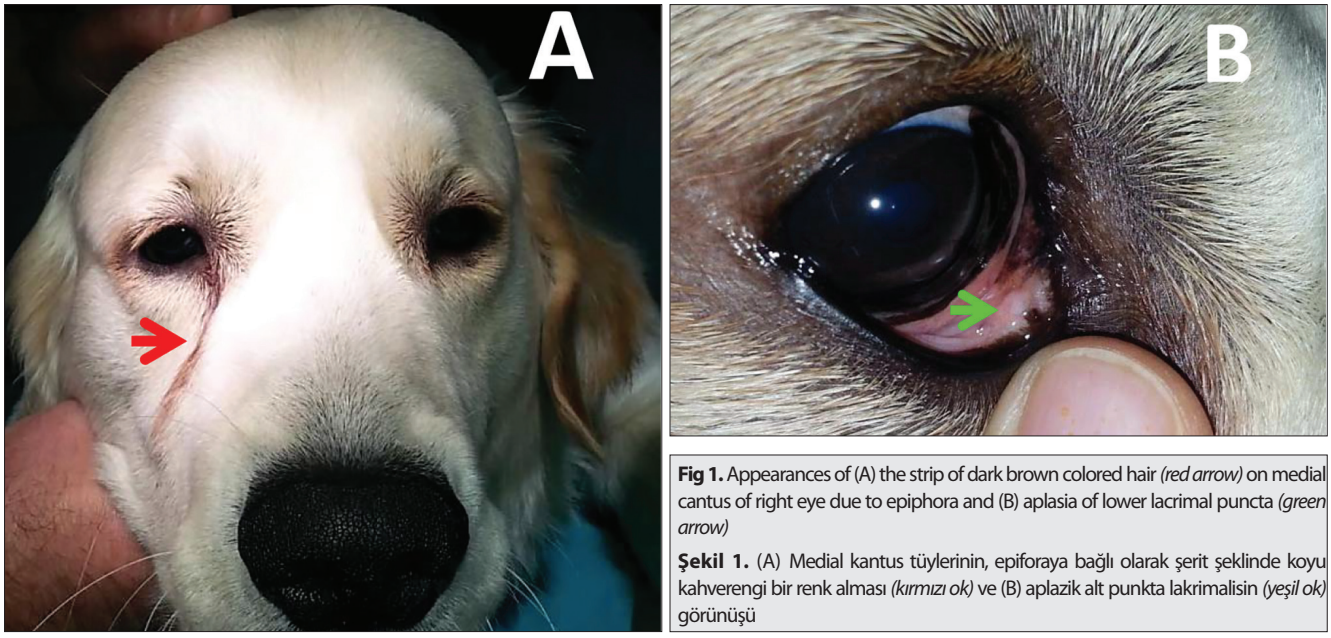

Fig 1. Appearances of (A) the strip of dark brown colored hair *(red arrow)* on medial cantus of right eye due to epiphora and (B) aplasia of lower lacrimal puncta *(green arrow)*

Şekil 1. (A) Medial kantus tüylerinin, epiforaya bağlı olarak şerit şeklinde koyu kahverengi bir renk alması *(kırmızı ok)* ve (B) aplazik alt punkta lakrimalisin *(yeşil ok)* görünüşü

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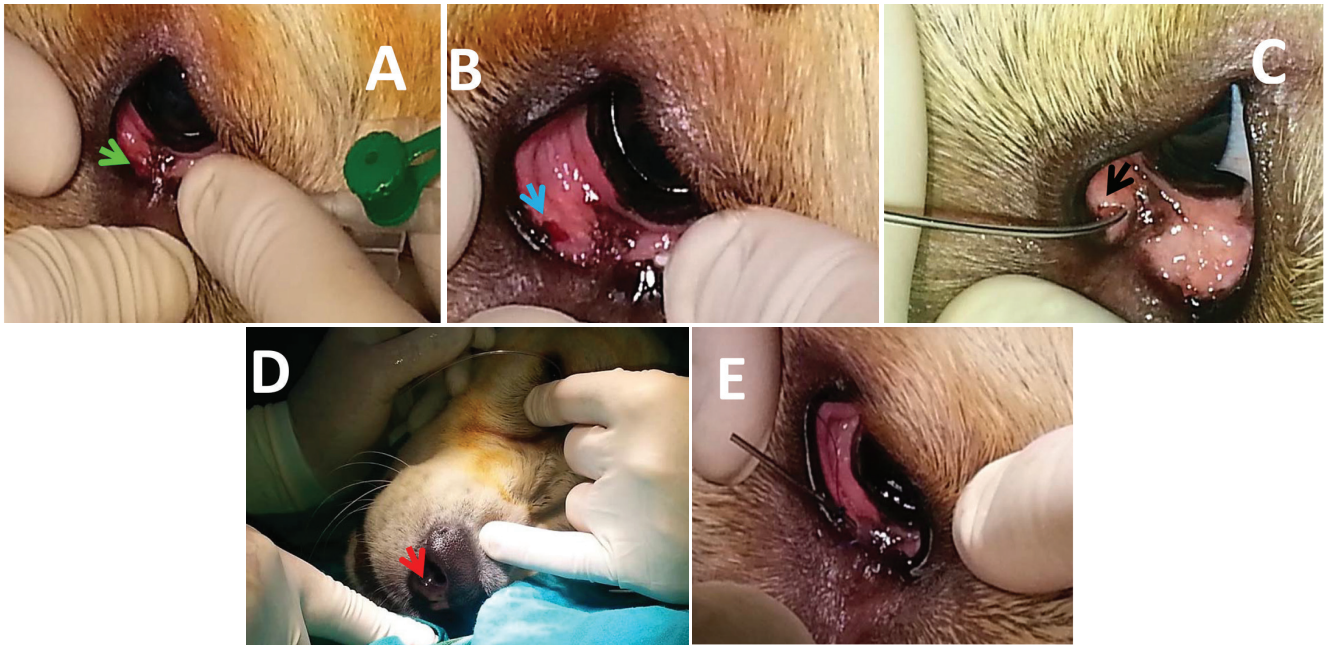

Fig 2. Appearances of (A) nasolacrimal flush performed through the superior lacrimal punctum results with ballooning of the conjunctiva over the punctal atresia *(green arrow)*; (B) circularly excised ballooning conjunctiva *(blue arrow)*; (C) catheterized excision entrance with a silicon tube *(black arrow)*; (D) tip of silicon tube passed through ductus nasolacrimalis and reached to nasal puncta *(red arrow)* and (E) upper end of silicon catheter bend and sutured over the margin of lower eyelid

Şekil 2. (A) Üst puncta lakrimalis'ten yapılan lavajın atrezik puncta üzerindeki konjunktivada yol açtığı balonlaşma *(yeşil ok)*; (B) balonlaşan konjunktivanın sirküler eksizyonu *(mavi ok)*; (C) silikon bir kateter ile yerleştirilen eksizyon girişi *(siyah ok)*; (D) ductus nazolakrimalisten ilerletilen ve puncta nazalise ulaşan *(kırmızı ok)* silikon kateterin ucu ve (E) alt göz kapağı kenarına bükülerek dikilen silikon kataterin görünüşleri

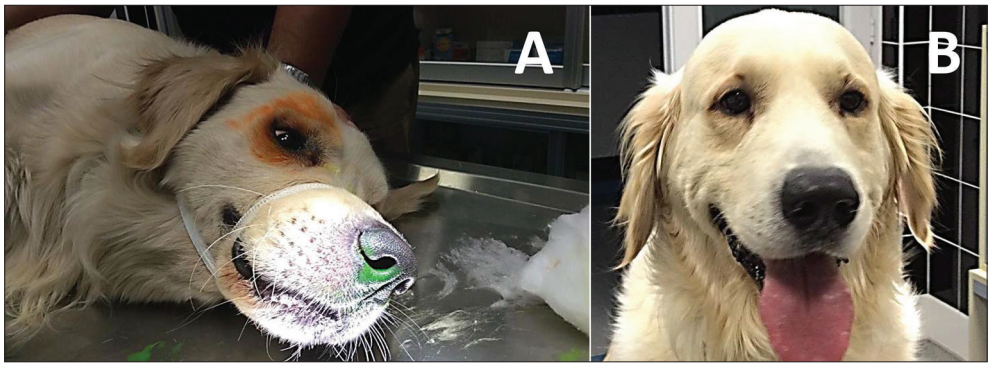

Fig 3. Appearances of (A) Jones test with 2% fluorescein resulted in staining nasal puncta and (B) disappeared discoloration of medial cantus hair

Şekil 3. (A) %2'lik fluorescein ile yapılan Jones testinde punkta nazalisin boyanması ve (B) medial kantus tüylerinde ortadan kalkan renk değişikliğinin görünüşleri

flush was performed through the upper lacrimal punctum via an 18 gauge angiocat and saline solution. Upon the positive pressure of injected saline solution, a slight ballooning of the conjunctiva over the punctal atresia was observed *(Fig. 2A)*. The ballooning conjunctiva was circularly excised with a fine scissors *(Fig. 2B)*. A silicon catheter was inserted through the excision entrance and advanced until the tip was appears in right nasal puncta *(Fig. 2C-D)*. The upper end of silicon catheter was bend over the margin of lower eyelid and sutured with 3 simple sutures parallel to eyelashes *(Fig. 2E)*. The affected eye was then treated with a topical antibiotic solution for 7 days. An Elizabeth collar was applied to ensure the inserted catheter in place for 3 weeks. After 3 weeks the catheter was removed and the patency of nasolacrimal duct was controlled periodically. In controls following 1.5 years the opening of lower canaliculi was intact, Jones test was positive *(Fig. 3A)*, and discoloration in medial cantus hair was disappeared *(Fig. 3B)*.

The adventure of this dog since birth with his right eye teaches our colleagues 3 important lessons: 1- A complete or systematical examination is important in diagnosis of eye diseases. Otherwise we may overlook a simple disorder and waste time with wrong therapies. 2- Puppies with chronic tear discharge but any ocular inflammation must be controlled for nasolacrimal drainage system anomalies. 3- Jones test seems to be an efficacious tool for nasolacrimal duct patency.

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YAZAR İNDEKSİ için tıklayınız

YAZIM KURALLARI

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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.

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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) 12 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 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.

Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.

Gözlem (Olgu Sunumu), 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 Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.

Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 2 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, Sonuç ve Kaynaklar bölümlerinden oluşmalı ve 12 sayfayı geçmemelidir.

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.

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Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.

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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.

DOI numarası bulunan kaynaklarda bu bilgi ilgili kaynak künyesinin sonuna eklenmelidir.

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