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Research Article

Effects of Organic and Inorganic Manganese Supplementation on Bone Characteristics, Immune Response to Vaccine and Oxidative Stress Status in Broiler Reared Under High Stocking Density [1] [2]

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Abstract

This study was carried out to determine the effects of organic and inorganic manganese (Mn) at different levels supplemented to diets of the broilers under stocking density stress on bone characteristics, immune response to vaccine and oxidative stress. A total of 1000, day-old broiler chicks were divided into one control group and nine treatment groups, each consisting of 100 chicks. They were randomly located at a density of 0.064 kg/m^2 until reaching seven days of age and in the compartments with 2 m^2 space at a density of 60 kg/m^2 until they are 49 days old. The control group was fed the basal diet only, whereas experimental groups were fed with basal diet supplemented with organic Mn (6.25, 12.5 and 25 mg/kg of Mn-methionine), inorganic Mn (12.5, 25 and 50 mg/kg Mn-oxide) and organic + inorganic Mn (3.125+6.25, 6.25+12.5 and 12.5+25 mg/kg Mn-methionine + Mn-oxide). There were no changes on days 25 and 49 in terms of physical properties such as humerus, femur and tibia as well as tibia fracture resistance and tibial metaphyseal chondral tissue lenght in all experimental groups (P>0.05). Tibia bone elasticity was higher in the 50 mg/kg Mn-oxide group compared to the control and other groups on day 49 (P<0.05). The serum calcium level on day 25 increased in the 6.25 and 12.5 mg/kg Mn-methionine and 6.25+12.5 mg/kg Mn-methionine + Mn-oxide groups (P<0.01). Antibody titre against Newcastle Disease Virus in Mn-methionine group on day 25 and titer against Infectious Bursal Disease in Mn-methionine and Mn-oxide groups on day 49 were higher compared to the control group (P<0.01). Serum levels of malondialdehyde and nitric oxide on day 49 decreased in all exprimental groups, whereas serum glutathione level increased in the same groups (P<0.01). As a result, dietary Mn supplementation to broilers hosted at 60 kg/m^2 of stocking density increased immune response and antioxidant activity by reducing the impact of density stress; yet, did not affect the physical properties of the bone. The effects of organic and inorganic forms of Mn on the examined properties were concluded to be similar.

Keywords: *Stocking density stress, Organic Mn, Inorganic Mn, Bone characteristic, Immune response, Oxidative stres, Broiler*

Sıklığa Maruz Bırakılmış Broylerlerde Rasyona Organik ve Inorganik Manganez İlavesinin Kemik Özellikleri, Aşılara Karşı İmmun Yanıt ve Oksidatif Stres Durumuna Etkileri

Özet

Araştırma sıklık stresi altındaki broylerlerde rasyona farklı düzeylerde organik ve inorganik manganez (Mn) ilave edilmesinin kemik özellikleri, aşılara karşı immun yanıt ve oksidatif stres üzerine etkilerinin incelenmesi amacıyla yapıldı. Araştırmada toplam 1000 adet günlük yaşta broyler civciv her biri 100 civcivden oluşan 1 kontrol ve 9 deneme grubuna ayrıldı. Civcivler 7 günlük yaşa kadar 0.064 kg/m^2'lik ve 49 günlük yaşa kadar 2 m^2'lik bölmelerde 60 kg/m^2 sıklıkta barındırıldı. Kontrol grubu temel rasyonla, deneme grupları ise temel rasyona sırasıyla 6.25, 12.5 ve 25 mg/kg düzeylerinde organik Mn (Mn-metiyonin), 12.5, 25 ve 50 mg/kg inorganik Mn (Mn-oksit), 3.125+6.25, 6.25+12.5 ve 12.5+25 mg/kg organic + inorganik Mn (Mn-metiyonin + Mn-oksit) ilave edilerek beslendi. Araştırmanın 25. ve 49. günlerinde broylerlerde humerus, femur ve tibiaya ait incelenen fiziksel özellikler ile tibia kırılma mukavemetinin ve tibial metafizyal kıkırdak doku uzunluğutüm deneme gruplarında değişmediği belirlendi (P>0.05). Tibia kemiği elastikiyeti 49. günde 50 mg/kg Mn-oksit grubunda kontrol ve diğer gruplara göre yüksek bulundu (P<0.05). Serum kalsiyum düzeyinin 25. günde 6.25 ve 12.5 mg/kg Mn-metiyonin ile 6.25+12.5 mg/kg Mn-metiyonin + Mn-oksit gruplarında arttığı belirlendi (P<0.01). Newcastle hastalığı virusuna karşı antikor titresinin 25. günde Mn-metiyonin, Enfeksiyöz Bursal hastalık virusuna karşı antikor titresinin ise 49. günde Mn-metiyonin ve Mn-oksit gruplarında kontrol grubuna göre yüksek olduğu belirlendi (P<0.01). Serumda 49. gündeki malondialdehit ve nitrik oksit düzeyleri tüm deneme gruplarında düşük iken, glutasyon düzeyinin aynı gruplarda arttığı belirlendi (P<0.01). Sonuç olarak 60 kg/m^2 yerleşim sıklığında barındırılan broylerlerde rasyona Mn ilavesinin sıklık stresinin etkisini azaltarak immun yanıtı ve antioksidan aktiviteyi artırdığı, fiziksel kemik özelliklerini etkilemediği belirlendi. Organik ve inorganik Mn formlarının incelenen özellikler üzerine etkisinin benzer olduğu kanaatine varıldı.

Anahtar sözcükler: *Sıklık stresi, Organik Mn, İnorganik Mn, Kemik özelliği, İmmun yanıt, Oksidatif stress, Broyler*

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INTRODUCTION

In the second half of the twentieth century, global poultry meat production has developed more rapidly than any farm animal meat production [1]. The poultry meat production which occurred as 103 million tons in 2010 is predicted to reach 122.5 million tons in 2020 [2]. This growth springs from modern broiler genotypes obtained from intense selections applied for juvenile growth rate, breast-meat yield and feed efficiency [3-5], and intensive production systems where high-tech is used [6].

Acoording to European Commission Directive (2007/43/EC) optimum stocking densityis 33-39 kg/m^2 when provided that the owner or keeper complies with the requirements setout this legislation. In addition, this density can be increased by a maximum of 3 kg/m^2 when further requirement are provided in the same legislation. However, when the stocking density increases the movement of broiler chickens are restricted largely in the overcrowded conditions and the opportunity to exercise required for bone development is reduced [2]. Due to the genetic rehabilitation, bones of broilers develop more slowly than the muscle tissue, and the lack of exercise resulting from high stocking density affects locomotor system reversely. Thus, major foot and leg problems and welfare loss owing to the increase in stocking density were reported [7-9]. Especially, the impacts of high stocking density in broilers on the foot health, lameness or gait disturbance, tibial dischondroplasia, gait scores, carcass bruising and scratching were reported in many researches. At finaly, the leg abnormalities result in culling, mortality and economic losses [6,10,11].

It is quite clear that, in the perspective of cost-benefit analysis, stocking density is of vital importance in producing more broiler chickens per unit area [7,11-13]. In this context, the findings that the environmental conditions are more effective on the health and welfare of the broiler chickens than the stocking density are of vital importance for the economic future of white meat industry [10,14-16]. Therefore, reducing the reverse impacts of high stocking density by the modification of environmental conditions, and increasing the upper limit set by the relevant regulations for the stocking density form a significant option [17].

In the researches conducted on the feed compositions, the importance of dietary Mn for the commercial broiler sector was especially expressed [17-21], One of the problems caused by the stocking density is the increasing lipid peroxidation in the chickens [22,23]. It specifically involves in MnSOD structure of the Manganese. In the case of oxidative stress, MnSOD steps in to protect cell mitochondrias from the degrading effects of free oxygen radicals [24]. The opportunities of benefiting from minerals taking part in enzymatic activities (Orjinase, piruvate, carboxilase and Mn superoxide dismutase etc.) are being examined in order to reduce these problems [10]. The effects of dietary nickel [25], calcium, copper, zinc and iron [26,27] in broiler diets have been examined. The importance of manganese (Mn) has been gradually increasing, since it is significant for bone formation, enzyme function and amino acid metabolism in the poultry [27,28].

In recent years, many supplementary organic Mn sources have been produced and used in feeding animals; yet, economic reasons overweights in determining dietary Mn forms being supplemented in conventional broiler diets. The notifications about the sources of Mn additives or the doses administered are conflicting. According to Berta et al.[29] and El-Husseiny et al.[30] organic forms of Mn are superior to inorganic forms of Mn in terms of utility, whereas there is no difference between the forms of Mn according to Bertechini and Hossain [30] and Conly et al.[20]. The importance of manganese (Mn) has been gradually increasing since it is significant for bone formation, enzyme function and amino acid metabolism in the poultry [28,31].

Therefore, the objective of this study was to determine the effects of organic and inorganic Mn supplementation to the diet of broilers grown under stocking density stress on bone characteristics, immune response and oxidative stress status.

MATERIAL and METHODS

Birds and Management: This study was conducted from May to July at the Afyon Kocatepe University Animal Research and Application Center with the approval of the University Ethics Committee (AKUHADYEK-37-2008). A total of 1000, day-old broiler chicks were used in this study. They were divided into one control group and nine treatment groups, each consisting of 100 chicks. Each group was subdivided into four sub-groups (replicates) with 25 chicks in each replicate; all subgroups were randomly placed in cages with 1 m^2 space. Birds were raised until 49 days of age under the same housing conditions and at a stocking density of 60 kg/m^2. Thus, a total 40 cages were used having 25 birds in each in conformity according to the management guide for Hubbard broilers [21]. Feed and water were provided *ad libitum*. A vaccination program for broilers was designed as day 0 with inactive Infectious Bursal Disease (IBD) + Newcastle Disease (ND) vaccine (Gumbopest, Merial RTA, subcutaneously), day 7 with live ND vaccine (Nobilis ND Lasota, Intervet, in drinking water) and infectious bronchitis vaccine (Nobilis, Intervet, in drinking water), day 14 with live IBD vaccine (Bursine Plus, Ford Dodge-Refarm, in drinking water), and day 21 with live ND vaccine (Nobilis ND Lasota, Intervet, in drinking water) vaccines.

Dietary Treatments: The control group was fed a basal diet including pre-dominantly of corn, soybean meal and full fat soybean *(Table 1)*. The experimental groups were fed basal diets containing three different diet treatments

(organic Mn, inorganic Mn and organic plus inorganic combination) with three different dose levels for each treatment. Each dietary treatment was comprised of 100 birds which were further sub-divided into four replicates, each replicate containing 25 birds. The organic Mn groups were fed diets supplemented with 6.25, 12.5 and 25 mg/kg Mn-methionine, inorganic Mn groups were offered diets containing 12.5, 25 and 50 mg/kg Mn-oxide and organic + inorganic Mn groups were fed diets containing 3.125 mg/kg Mn-methionine + 6.25 mg/kg Mn-oxide, 6.25 mg/kg Mn-methionine + 12.5 mg/kg Mn-oxide and 12.5 mg/kg Mn-methionine + 25 mg/kg Mn-oxide. The nutrient composition of the basal diet *(Table 1)*, including crude protein and calcium was determined according to the AOAC [32]. The metabolisable energy (ME) of the basal diet was estimated using the Carpenter and Clegg equation [33]. The available phosphorus, lysine, methionine and threonine levels of the basal diet were calculated according to the diet guide for Hubbard broilers [34].

Table 1. *Composition of the basal diets used in different periods (%)*
Tablo 1. *Farklı dönemlerde kullanılan temel rasyonun bileşimi (%)*

Ingredients	Starter (0 to12 day)	Grower (12 to 28 day)	Finisher (28 to 49 day)
Corn	47.37	50.44	51.85
Soybean meal	24.65	17.70	14.82
Full fat soybean	15.00	18.00	17.00
Poultry meal	4.00	4.00	5.00
Meat bone meal	2.50	4.00	4.00
Vegetable oil	3.50	3.52	4.91
Limestone	0.73	0.59	0.61
Dicalcium phosphate	0.86	0.46	0.35
DL-Methionine	0.36	0.33	0.34
Sodium bicarbonate	0.30	0.25	0.31
Vitamin premix*	0.20	0.20	0.20
Mineral premix**	0.10	0.10	0.15
Salt	0.14	0.12	0.07
L-Lysine	0.13	0.13	0.23
Enzyme***	0.10	0.10	0.10
Anticoccidial	0.06	0.06	0.06
Chemical composition			
ME**** (kcal/kg)	3050	3200	3360
Crude protein, %	23.20	22.70	21.80
Calcium, %	1.00	1.00	1.00
Phosphorus (available), %	0.49	0.49	0.49
Lysine, %	1.50	1.40	1.40
Methionine, %	0.75	0.70	0.70
Threonin, %	0.997	0.884	0.902

** Vitamin premix (Rovimix 124-F) provides per 2.5 kg of diets: 15.000.000 IU vitamin A, 1.500.000 IU vitamin D, 50.000 vitamin E, 5.000 mg vitamin K_3, 3.000 mg vitamin B_1, 6.000 mg vitamin B_2, 25.000 mg niacin, 12.000 mg Ca-D Pantothenate, 5.000 mg vitamin B_6, 30 mg vitamin B_{12}, 1.000 mg folic acid, 125 mg D-biotin, 300.000 mg choline chloride, 300.000 L-lysine; ** Mineral premix provides per kg of diets: 80.000 mg manganese, 30.000 mg ferric, 60.000 mg zinc, 5.000 mg cupper, 500 mg cobalt, 2.000 mg iodine, 235.680 mg calcium carbonate; *** Enzyme (Optimise M): 5.000 BU/g endo-1.3 beta-glucanai 5.000 BXU/g endo-1.4-ksilinase, 500 FYT/g phytase; ****Estimated according to the Carpenter and Clegg equation*

Bone Characteristics: At the age of 25 and 49 days, 8 birds were slaughtered (4 male and 4 female) from each group. The fresh weights of the left tibia, femur and humerus bones (after muscle, cartilage and membranes were removed) of the chicken were measured with 0.01 g precise digital scales; vertical diameters were measured using digital compass; their volumes were determined. Also, fracturing resistance of left tibia bones was determined via three-point break test performed in accordance to Park et al.[35]. Bone density was determined via the values collected with the help of a measuring cylinder and bone weight values. The Tibia weight/length index was calculated by dividing the tibia weight by its length [36].

In order to evaluate the tibial metaphyseal chondral tissue lenght, right tibial bones of 8 slaughtered birds were separated for histopathological examinations. By opening articulation genus in rear extremities, muscle and other tissues were removed. Cutting tibias from the center, extremitas proximalises were taken into buffered formalin solution. After being detected fixed for 72 h, the tissues were taken into Decastro (buffered 3% nitric acid) solution and decalcification was projected. Solutions were renewed every three days. Once the tissues softened, they were washed in running water for 48 h and embedded in paraffin after routine tissue monitoring. They were cut with microtome and stained with hematoxylin eosin, were examined in the light microscope. Slides were examined via DP20 camera set up on Olympus B51 microscope. Via Cell A program, from epiphyseal plates of the bones towards distal (metaphysis), the cartilage tissue was measured upto the area where it transforms into bone tissue *(Fig. 1)*. The length of the cartilage tissue were scored as <1000 μ=1; 1001-1500 μ=2; 1501-2000 μ=3; >2001 μ=4. The ratio of leg abnormalities in the groups were determined using percentage of severely lame birds that clinically lameness or unable to walk.

Serum Parameters: On days 25 and 49 of this study, serums obtained from the blood samples of 8 birds (4 male and 4 female) from each group were stored at -18ºC for determination of serum malondialdehyde (MDA), glutathione (GSH), nitric oxide (NO), calcium (Ca) and phosphorus (P) levels and antibody titers against Infectious Bursal Disease (IBD) and Newcastle Disease (ND). The levels of serum MDA, GSH and NO were determined by the methods described by Drappier et al.[37], Beutler et al.[38] and Miranda et al.[39], respectively. Total specific antibody production in serum against IBD and ND was performed by using commercial ELISA kits and in accordance to Puthpongsiriporn and Scheideler [40]. Serum Ca and P levels were determined with 300 Alobat Alcycon-auto analyzer.

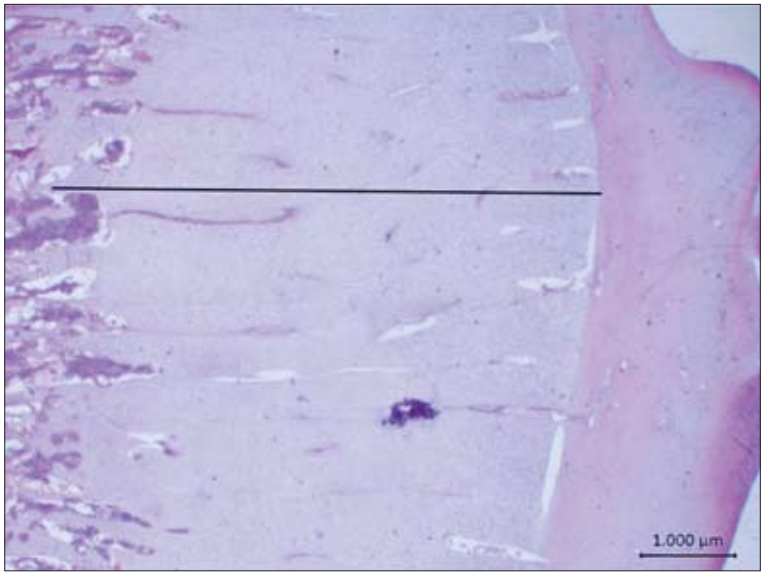

Fig 1. The line in the picture shows that the cartilage tissue was measured for metaphyseal chondral tissue in tibia, HE

Şekil 1. Resimdeki çizgi tibiadaki metafizial kıkırdak doku için ölçülen doku uzunluğunu göstermektedir, HE

Statistical Analysis: Differences among group values with regard to all parameters were examined by analysis of variance for all values and the significance of differences between groups tested by Duncan's test. Besides, Mn-methionine, Mn oxide, mixture groups and the control group each of which composing of three groups were considered as single dimensions, and the Contrast test was applied in order for each dimension to be contrasted to the block of other dimensions. A value of P<0.05 was considered as the limit for statistical significance.

RESULTS

Bone Characteristics: On days 25 and 49 of the research, physical properties such as weight and length of humerus, weight and length of femur; weight, length, thickness, density and volume of tibia and tibiotarsi index *(Table 2)* as well as tibia fracture resistance *(Table 3)* and tibial metaphyseal chondral tissue length *(Table 4)* did not change (P>0.05) between the control and experimental groups. Tibia bone elasticity was found higher in the group supplemented with 50 mg/kg Mn-oxide compared to the control and other Mn supplemented groups on day 49 (P<0.05; *Table 3*).

Serum Parameters: In this study, It was determined that antibody titre against ND in Mn-methionine group on day 25 and titer against IBD in Mn-methionine and Mn-oxide groups on day 49 were higher compared to the control group (P<0.01). At the same time, ND titer on day 25 and IBD titer on day 49 increased in the 12.5 mg/kg Mn-oxide group and in the 12.5+25 mg/kg Mn-methionine + Mn-oxide groups, respectively (P<0.01). Serum Ca level increased in the 6.25 and 12.5 mg/kg Mn-methionine and 6.25+12.5 mg/kg Mn-methionine + Mn-oxide groups compared to the control group on day 25 (P<0.01), whereas Ca level on day 49 and P level on day 25 and 49 in serum did not change in all experimental groups (P>0.05; *Table 5*).

According to the Contrast test results; In terms of ND antibody level, there were significant differences between Mn-methionine group and the other groups (P<0.01; 25 and 49 day-old chicks); Mn-methionine + Mn-oxide group and the other groups (P<0.001; 25 day-old chicks); the control group and the other groups (P<0.001; 25 and 49 day-old chicks). In terms of IBD antibody level, there were significant differences between Mn-methionine group and the other groups (P<0.05; 49 day-old chicks); the control group and the other groups (P<0.01; 49 day-old chicks). In terms of Serum Ca level, there were significant differences between Mn-methionine group and the other groups (P<0.01; 25 day-old chicks); Mn-oxide group and the other groups (p<0.05; 25day-old chicks), the control group and the other groups (P<0.01; 25 day-old chicks).

Serum GSH and NO levels on days 25 and 49 as well as serum MDA level on day 49 showed significant variations between the experimental groups. Serum levels of MDA and NO on day 49 decreased in the group supplemented with Mn, whereas serum GSH level increased in the same groups (P<0.01). Serum NO level on day 25 was lower (P<0.05) in the 25 mg/kg Mn-oxide and Mn-methionine + Mn-oxide groups compared to the control group *(Table 6)*.

According to the Contrast test results; In terms of MDA level there were significant differences between Mn-methionine + Mn-oxide group and the other groups (P<0.001; 49 day-old chickens), control group and the other groups (P<0.01; 49 day-old chickens). In terms of GSH level, the differences were significant between Mn-oxide group and the other groups (P<0.01; 25 day-old chickens), Mn-methionine + Mn-oxide group and the other groups (P<0.01; 49 day-old chickens), the control group and the other groups (P<0.001; 25 and 49 day-old chickens). In terms of NO level, the differences were significant between Mn-oxide group and the other groups (P<0.05; 25 day-old chickens), Mn-methionine + Mn-oxide

Table 2. *Effects of organic and inorganic Mn supplementation to diets of the broilers under stocking density stress on some bone characteristics*
Tablo 2. *Sıklık stresine maruz bırakılmış broyler rasyonlarına organik ve inorganik Mn ilavesinin bazı kemik özelliklerine etkisi*

Parameters	Days	Control	Mn-methionine (mg/kg)			Mn-oxide (mg/kg)			Mn-methionine + Mn-oxide (mg/kg)			SE	P
			6.25	12.5	25.0	12.5	25	50	3.125+6.25	6.25+12.5	12.5+25.0		
Humerus weight (g)	25	3.41	3.30	2.73	3.05	3.04	3.27	3.40	3.11	4.05	3.24	0.09	0.24
	49	6.55	7.84	7.93	8.58	7.42	7.89	7.77	7.60	7.42	6.36	0.21	0.36
Humerus length (mm)	25	50.35	52.37	48.84	49.19	49.97	51.11	51.68	49.01	51.91	49.26	0.36	0.23
	49	67.97	69.55	70.62	71.21	69.83	69.33	70.85	68.97	68.75	67.58	0.51	0.82
Femur weight (g)	25	5.66	5.31	5.26	5.98	5.64	5.15	5.73	6.13	5.58	5.31	0.09	0.38
	49	10.06	10.57	12.42	11.28	11.59	10.20	11.56	9.98	12.13	9.97	0.32	0.57
Femur length (mm)	25	47.77	54.71	54.48	55.75	55.07	54.51	55.87	55.54	54.70	54.51	0.67	0.26
	49	71.33	73.26	75.09	73.38	72.72	72.29	73.90	71.82	74.03	73.46	0.47	0.85
Tibia weight (g)	25	8.03	7.72	7.28	8.57	7.71	7.79	8.76	8.72	8.25	7.98	0.13	0.18
	49	14.20	14.26	14.95	16.50	16.35	14.38	17.19	12.57	15.65	13.33	0.48	0.56
Tibia length (mm)	25	75.83	75.67	74.99	77.99	76.53	75.44	77.78	77.81	76.49	76.01	0.31	0.34
	49	101.35	101.60	103.36	102.64	104.28	117.04	103.81	97.23	102.97	111.46	1.87	0.62
Tibia thickness (mm)	25	6.01	6.50	6.56	6.12	5.92	6.11	5.74	5.97	5.82	5.92	0.06	0.08
	49	7.88	8.24	8.25	8.36	8.27	7.90	8.33	8.70	8.35	8.02	0.09	0.72
Tibia density	49	1.58	1.99	1.67	2.08	2.21	1.73	1.80	1.92	2.09	1.75	0.08	0.80
Tibia volume (cm^3)	49	7.61	7.42	8.60	7.79	6.92	7.08	8.48	6.81	7.03	7.76	0.30	0.94
TibioWt/lenght index, mg/mm	25	105.92	101.82	97.05	109,50	100.75	102.98	112.48	111.79	107,83	104,56	1.41	0.26
	49	137.79	138.81	143.11	158.39	155.92	127.00	164.50	128.78	150.71	124.34	3.94	0.30

Table 3. *Effects of organic and inorganic Mn supplementation to diets of the broilers under stocking density stress on fracture characteristics of tibia bone*
Tablo 3. *Sıklık stresine maruz bırakılmış broyler rasyonlarına organik ve inorganik Mn ilavesinin tibia kemiği kırılma özellikleri üzerine etkisi*

Parameters	Days	Control	Mn-methionine (mg/kg)			Mn-oxide (mg/kg)			Mn-methionine + Mn-oxide (mg/kg)			SE	P
			6.25	12.5	25.0	12.5	25	50	3.125+6.25	6.25+12.5	12.5+25.0		
Maximum fracture height (N)	25	208.98	218.46	265.64	259.70	190.80	223.95	215.20	226.05	208.51	222.41	5.68	0.13
	49	231.79	288.00	283.96	332.44	283.69	269.00	285.82	356.66	270.85	250.76	11.18	0.39
Tibia bone elasticity (mm/s)	25	1.69	1.72	1.67	1.68	1.57	1.70	1.73	1.51	1.76	1.96	0.03	0.23
	49	1.97^{bc}	2.06^{bc}	2.07^{bc}	2.40^{ab}	1.89^{c}	1.98^{bc}	2.64^{a}	2.01^{bc}	2.14^{c}	1.88^{c}	0.05	0.04
Tibia fracture resistance (N/mm) MPa	25	73.62	67.35	72.91	87.00	71.37	76.61	82.96	79.88	80.74	83.91	1.72	0.15
	49	60.36	65.81	64.39	67.50	63.93	68.75	61.07	67.85	58.55	63.05	1.64	0.96
Tibia fracture resistance Kgf (N/9.81)	25	21.30	22.27	27.08	26.47	19.44	22.83	21.94	23.04	21.26	22.67	0.58	0.13
	49	23.62	29.36	28.95	33.89	28.92	27.42	29.13	36.36	27.61	25.57	1.14	0.40

Letters (a, b, c) in the same line indicate significant differences between different letters; Tibia bone flexibility (mm/second): The collapse amount of the bone during the fracturing process; millimeter/second; Tibia bone resistance (kgf (N/9.81): Maximum force that the bone could endure during the fracturing; kgf, N/mm

group and the other groups (P<0.05; 49 day-old chickens), the control group and the other groups (P<0.05; 25 and 49 day-old chickens).

DISCUSSION

In this study, physical properties of the bones such as humerus, femur and tibia and Ca levels in serum (other than Ca level on day 25), as well as tibia fracturing properties (other than tibia elasticity on day 49) were not affected from Mn supplementation. It was observed that the values of tibia fracturing resistance were found to have numerically increased in Mn-methionine groups. Also, tibia fracturing resistance was detected to be the lowest in the control group which Mn supplementation was not applied. The fact that a statistically significant difference was not observed in the present study may be due to the high standard error resulting from small sampling size (n). As a matter of fact our finding corresponds with the research results asserting that Mn contributes to bone properties. Ruff and Hughes [41] and El Husseiny et al.[30] also reported similar results and the researchers indicated that dietary

Table 4. *Effects of organic and inorganic Mn supplementation to diets of the broilers under stocking density stress on tibial metaphyseal chondral tissue lenght, distribution of tibial metaphyseal chondral tissue length score and ratio of leg abnormalities*

Tablo 4. *Sıklık stresine maruz bırakılmış broyler rasyonlarına organik ve inorganik Mn ilavesinin tibialarda metefizial kıkırdak doku uzunlugu, metafizial kıkırdak doku skorlarının dağılımı ve bacak problemleri oranı üzerine etkisi*

Groups		25th day					49th day					
		Cartilage Tissue Length	Cartilage Tissue Length Score				Cartilage Tissue Length	Cartilage Tissue Length Severity Score				Leg Abnormalities
		Mean (µm)	1	2	3	4	Mean (µm)	1	2	3	4	%
Control		2154.22		1	1	2	3020.85		3	2	3	5.60
Mn- meth	6.25	2382.90		1		6	1676.16	1	2	3	2	2.59
	12.5	2109.89		2		4	1934.09	1	6		1	2.73
	25	3204.90			1	6	1261.89	1	4	3		1.26
Mn-oxide	12.5	2032.29	2			5	1536.79		2	5		9.70
	25	2020.12			2	2	1613.41	1	4	1	2	4.17
	50	2499.51		1		5	1567.28		5	2	2	2.63
Mn-meth +Mn-oxide	3.125+6.25	2320.49		1	2	4	1687.73		4	2	2	2.50
	6.25+12.5	2422.62			2	4	1646.31	1	1	4	2	0
	12.5+25.0	2541.83		1		4	1411.41	1	4	3		2.70
SEM		140.73		0.11			143.94			0.01		
P		0.767		0.919			0.328			0.538		

Severity score of tibial metaphyseal chondral tissue: <1000µ=1; 1001-1500µ=2; 1501-2000µ=3; >2001µ=4

Table 5. *Effects of organic and inorganic Mn supplementation to diets of the broilers under stocking density stress on ND and IBD antibody titers and Ca (mg/dl) ve P (mg/dl) levels in serum*

Tablo 5. *Sıklık stresine maruz bırakılmış broyler rasyonlarına organik ve inorganik Mn ilavesinin serum ND ve IBD antikor titresi ile Ca (mg/dl) ve P (mg/dl) düzeyi üzerine etkisi*

Parameters	Days	Control	Mn-methionine (mg/kg)			Mn-oxide (mg/kg)			Mn-methionine+Mn-oxide (mg/kg)			SE	P
			6.25	12.5	25.0	12.5	25	50	3.125+6.25	6.25+12.5	12.5+25.0		
ND	25	2308^{c}	7287^{b}	11352^{a}	7390^{b}	7829^{b}	5259^{bc}	5313^{bc}	2320^{c}	2164^{c}	2638^{c}	457.2	0.00
	49	9266^{b}	11177^{ab}	15591^{a}	14873^{a}	11221^{ab}	14095^{ab}	9080^{b}	10391^{ab}	12260^{ab}	11672^{ab}	540.5	0.06
IBD	25	15956	16675	16207	13973	13868	14168	17738	13737	12304	11646	668.4	0.55
	49	10492^{c}	15195^{a}	17417^{a}	15903^{a}	14758^{a}	14663^{ab}	11175^{ab}	11782^{c}	16041^{cb}	15511^{a}	397.9	0.00
Ca	25	4.89^{d}	6.11^{b}	7.19^{a}	5.22^{cd}	4.90^{d}	5.07^{cd}	5.37^{bcd}	4.76^{d}	5.91^{bc}	5.16^{cd}	0.11	0.00
	49	5.91	7.73	5.96	7.54	7.50	7.76	6.65	5.98	7.00	6.16	0.22	0.28
P	25	4.24	4.07	4.19	4.18	3.80	3.92	3.72	3.57	3.70	4.22	0.07	0.33
	49	5.98	7.85	5.99	6.84	6.29	7.05	5.82	5.51	6.16	5.89	0.21	0.34

Letters (a, b, c, d) in the same line indicate significant differences between different letters; Data are means of 8 replicate cages consisting of 12 birds per replicate cage

Table 6. *Effects of organic and inorganic Mn supplementation to diets of the broilers under stocking density stress on oxidative stress status*

Tablo 6. *Sıklık stresine maruz bırakılmış broyler rasyonlarına organik ve inorganik Mn ilavesinin oksidatif stres durumu üzerine etkisi*

Parameters	Days	Control	Mn-methionine (mg/kg)			Mn-oxide (mg/kg)			Mn-methionine+Mn-oxide (mg/kg)			SE	P
			6.25	12.5	25.0	12.5	25	50	3.125+6.25	6.25+12.5	12.5+25.0		
MDA	25	280.62	252.25	258.50	250.00	247.25	260.37	240.25	212.62	227.75	225.25	6.09	0.40
	49	3.71^{a}	2.89^{bcd}	3.06^{bcd}	2.62^{d}	3.28^{abc}	2.91^{bcd}	2.77^{cd}	3.03^{bcd}	2.88^{bcd}	3.35^{a}	0.05	0.01
GSH	25	22.47^{c}	24.76^{b}	25.87^{ab}	24.87^{b}	25.75^{ab}	26.77^{a}	25.06^{ab}	25.14^{ab}	26.01^{ab}	26.04^{ab}	0.20	0.00
	49	17.43^{d}	20.32^{c}	23.24^{ab}	25.22^{a}	22.33^{c}	25.09^{ab}	23.67^{ab}	23.74^{ab}	24.93^{ab}	26.07^{a}	0.38	0.00
NO	25	13.62^{a}	13.06^{ab}	11.88^{abc}	11.73^{abc}	12.91^{ab}	11.03^{bc}	11.56^{abc}	11.35^{bc}	11.44^{bc}	10.63^{c}	0.22	0.04
	49	12.80^{a}	11.28^{b}	11.39^{b}	10.06^{bc}	9.85^{c}	10.10^{bc}	10.40^{bc}	11.20^{b}	10.22^{bc}	9.39^{c}	0.18	0.00

Letters (a, b, c, d) in the same line indicate significant differences between different letters

manganese improved bone features. Also, we found the largest tibial metaphyseal chondral tissue in control group at 49 days of age *(Table 4)*. In general, it has been known that increase in the lenght of tibial metaphyseal chondral tissue is related to a predisposing factor for tibial dyschondroplasia [42,43]. It can be argued that regarding to those results, Manganese suplementation into diet decreased the incidence of tibial dyschondroplasia in broilers reared under high stockind density.

The positive effect of manganese on tibia in this study has suggested that it enhanced Ca accumulation in tibia. In this study, Ca levels in the groups Mn was supplemented were lower than the control group without Mn, and that difference reflected on the Contrast test. Sunder et al.[44] also reported that 100 ppm of dietary Mn supplementation contributed to foot-leg health. However, these differences in calcium did not reflect upon P level. Therefore, it must be considered that dietary Mn supplementation especially during the growth period might distrupt the ratio of Ca and P. Furthermore, while the number of the chickens with clinical lameness or foot discomfort was at acceptable levels (2-3%) in the groups other than the group with 12.5 mg/kg of inorganic Mn and the control group despite the bone parameters and increasing serum Ca level, this rate was found to be numerically higher (5.6%) in the control group.

The present study revealed that broilers supplemented with dietary Mn responded to ND and IBD vaccines by producing antibodies at higher levels than chickens fed on the control feed with no Mn supplementation. Both ND and IBD antibody levels elevated more particularly in Mn methionine groups when compared to other groups *(Table 5)*. These findings suggest that Mn enhances the immune system in broilers. Especially organic manganese has a more powerful impact on that. Similarly, Sunder et al.[44] reported that dietary Mn supplementation at the level of 100 mg/kg enhanced immunity. Gajula et al.[45] also asserted that immune response became swifter as dietary Mn level increased. Moreover, as the stress caused by the exposure to stocking density was reduced to some extent by the feed with Mn supplementation, the immunity of the chickens in the control group with no Mn supplementation may have been affected from this stress more. Similarly, Bozkurt et al.[46] observed that the *bursa of fabricius* weight did not change in the groups supplemented with organic and inorganic Mn.

In this study, serum MDA and NO levels of the broilers fed with dietary Mn supplementation were found to be lower compared to the control group *(Table 6)*. Similarly, Bulbul et al.[47] reported that organic or inorganic dietary Mn supplementation reduced serum MDA. However, Lu et al.[48] reported that dietary Mn supplementation did not affect serum MDA level in broilers. In this study, high MDA level in the control group may be related to abdominal fat amount. The deterioration in oxidant-antioxidant balance in favor of antioxidants in the control group may have led to high MDA and NO levels causing high feed consumption and fat accumulation, consequently. In fact, it was observed that less abdominal fat was numerically or statistically detected in broilers fed with Mn supplemented feed than in the control group except for the group feeding with 25 mg/kg Mn-oxide [46]. It has also been reported that redundant free fatty acid intake excessing antioxidant capacity may increase oxidative stress by leading to lipid peroxidation and also bring moleculer features of fat tissue into the forefront as a major cause for oxidative stress [49]. Lu et al.[48] also reported that 100 mg/kg of dietary Mn supplementation lowered abdominal fat amount. Furthermore, Mn supplementation to the diet above the rate determined for broilers by NRC may suggest that Mn has inhibited the activities of enzymes involving in lipid peroxidation.

As a result, it was observed that dietary Mn supplementation enhanced immune response and antioxidant activity by reducing the effect of density stress for broilers having been hosted at 60 kg/m^2 stocking density; slightly increased ossification in tibia while it did not affect the physical properties of the bone or bone resistance. Considering the levels used at the study, organic and inorganic forms of Mn have similar impacts on examined properties; yet, further research on the impacts of higher doses of Mn on ossification is required.

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Research Article

An Application of Bootstrap Technique in Animal Science: Egg Yolk Color Sample

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Abstract

In this study, it was aimed to introduce the Bootstrap technique and to reveal the relationship between measurements of yolk color fan grades and digital colorimeter that is used for determining the yellow color of egg by utilizing this technique. For this purpose, a total of 1350 samples of 15 color grades of Roche yolk color fan and L* (lightness), a* (redness), b* (yellowness) values in the same samples were compared. The means, standard errors and confidence intervals for each color parameters of fan grades have been demonstrated by the Bootstrap technique. The grades of Roche yolk color fan in terms of L* values have been divided into 10 groups (P<0.01), while only divided into 9 groups in terms of b* values (P<0.01). According to the means of Redness (a*), all of the Roche yolk color fan grades (15 grades) have been determined as independent from each other (P<0.01). With the Bootstrap method, the standard error values of means were decreased by 42.03%, 35.38% and 30.24%, respectively, and the confidence intervals were narrowed by the ratio of 42.03%, 35.38% and 30.24%, respectively. The results of the study were compared with the results of the study that was conducted by using Roche yolk color fan which is cheaper but less reliable and by using digital colorimeter method which is expensive but reliable.

Keywords: *Bootstrapping, Resampling, Egg yolk color fan, Digital colorimeter*

Hayvancılık Alanında Bootstrap Tekniğinin Bir Uygulaması: Yumurta Sarı Rengi Örneği

Özet

Bu çalışmada Bootstrap tekniğinin tanıtılması ve bu metottan faydalanarak yumurta sarı renginin belirlenmesinde kullanılan yumurta sarı renk yelpazesi ve dijital renk ölçüm cihazı ölçümleri arasındaki ilişkinin ortaya konulması amaçlanmıştır. Bu amaçla Roche sarı renk yelpazesinin 15 renk sınıfına ait toplam 1350 örnek ile aynı örneklerde ölçülen L* (parlaklık), a* (kırmızılık), b* (sarılık) değerleri karşılaştırılmıştır. Yelpaze sınıflarının her renk parametresine ait ortalamaları, standart hataları ve güven aralıkları Bootstrap tekniğiyle ortaya konulmuştur. L* değerleri bakımından Roche sarı renk yelpazesi 10 gruba ayrılırken (P<0.01), b* değerleri bakımından sınıflar sadece 9 gruba ayrılmıştır (P<0.01). Kırmızılık (a*) ortalamalarına göre ise Roche sarı renk yelpaze sınıflarının tümü (15 sınıf) birbirinden bağımsız olarak tanımlanabilmiştir (P<0.01). Bootstrap yöntemi ile L*, a* ve b* ortalamalarının standart hata değerleri sırasıyla 42.03%, 35.38% ve 30.24% azalmış, güven aralıkları 48.10%, 55.43%, 34.47% oranlarında daralmıştır. Araştırmadan elde edilen sonuçlar kullanılarak mevcut literatürde daha ucuz ama güvenilirliği düşük olan Roche sarı renk yelpazesi ile yapılan çalışmalar ve pahalı ama güvenilir yöntem olan dijital renk ölçüm cihazı kullanılarak gerçekleştirilen çalışma sonuçları karşılaştırılmıştır.

Anahtar sözcükler: *Bootstrapping, Yeniden örnekleme, Yumurta sarı renk yelpazesi, Dijital renk ölçer*

INTRODUCTION

In order to estimate the parameters of any population, a set of observations (sample) which is considered to represent the mass in question has been used. Since obtaining all of the observations of the population is not possible most of the time, the sample set to make parameter estimations should be sufficient in quantity and quality [1]. There can be some problems related to the sample size in statistical analysis especially in animal

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science. Due to the failure to meet the assumptions in such cases, it is not possible to be able to obtain appropriate statistics or possible to obtain biased or erroneous results. By using resampling or Monte Carlo simulation methods, some solutions can be generated in the face of such problems. The data set is generated according to the assumptions and theoretical basis in Monte Carlo method, whereas the data set is created using real raw material in the resampling approaches [2]. Many resampling methods have been reported in the literature, but the most used ones are Randomization Exact Test, Bootstrap, Jackknife and Cross-Validation methods. By using Bootstrapping developed by Efron [3], the Bootstrap samples are being created in varying amounts and sizes by randomly changing the places of the observations in a data set of any size. Each sample in this method is treated as the actual population and an experimental distribution for the estimator is created by repeating this obtained sample many times [4].

The Bootstrap method is also advantageous to reduce the computational burden in solving intensive mathematical formulas. The Bootstrap method is quite useful in terms of demonstrating the reliability of an estimate based on sample data sets taken from the population and determining approximate confidence intervals of the parameters [5]. In case that the assumptions made regarding the error terms and the independent variables do not satisfy, the Bootstrap method which is used for the correction process. Bootstrap method has been developed in order to obtain the smaller estimation errors, and the reduction of the standard deviations, and accordingly to obtain more reliable parameter estimators and to create the confidence intervals [6]. Today, in parallel with the modern developments in statistical calculations, some progress has been made in the Bootstrap method and its use in applied statistics has become widespread. The use of the Bootstrap method has increased highly especially in the areas of molecular genetics [7-9], population genetics [10], phylogenetics [11] and virology [12].

There are limited studies conducted with the Bootstrap approach in the area of animal science. Adams and Anthony [13] who study animal behaviors analyzed their data, which does not show a normal distribution, both with non-parametric methods and with resampling approach. The researchers have stated that the Randomization test is more powerful than the Kruskal-Wallis test. In another research on animal behaviors [14] the variability of hypothesis testing results in the original data and the data obtained by resampling approach have been demonstrated. Robison et al.[15] utilized the Bootstrap method in the genetic parameters estimated for some reproductive characteristics in sheep. In the study, it has been suggested that the standard errors of genetic parameters decrease with the Bootstrap method. Takma and Atıl [16] indicated the confidence intervals of the means of egg weight of the 40th week of laying hens with the Bootstrap method. The same researchers suggested that there is a difference in terms of body weight of the 3rd week in broilers fed with two different rations at 0.05 significance level; however, after the Bootstrap performed application they demonstrated that this difference results from the Type-I Error [16].

Yolk color is one of the interior egg quality characteristics and is the subject of scientific studies for many years. In some cultures, it is believed that dark colored eggs evoke natural egg and poultry feeding studies have been carried out in this respect. The egg yolk color preferred by consumers can be provided with natural colorants in forages (carotenoids in corn, green plants, peppers, various flowers) and artificial ingredients added to the forages [17]. In this study, it was aimed to demonstrate the relationship between the two methods of yolk color fan and digital colorimeter measurements that are used in the determination of the yellow color of eggs. For this purpose, a total of 1350 samples of 15 color grades of yolk color fan have been associated with the values of L* (lightness), a* (redness), b* (yellowness). Then, the confidence intervals of the each color parameter of the scale values have been demonstrated by both the original data and the Bootstrap method.

MATERIAL and METHODS

The study was conducted at the Poultry Research Unit of Selcuk University, Faculty of Agriculture, Department of Animal Science in Konya. All color measurements were carried out in a non-air stream environment and under constant light intensity. The methods used to measure the color of the yolk and the statistical methods have been respectively indicated below.

Measurements of Egg Yolk Color

There are two approaches for the color. These are; the classification of different colors according to systems, the former approach that includes rankings and compliances and the scientific approach in which the presence of color is believed to be depended on the human eye and on light [18]. There are many color systems developed for color measurement. These are divided into two systems including the systems that are dependent on a device (RGB, CMY, HSV, HLS etc.) and the ones that are independent from a device (CIE XYZ, CIE LUV etc.). The independent systems have been developed by CIE (Commission Internationale de L'Eclairage). The scientists have produced a variety of methods for nearly 100 years to measure the color of the yolk. Some of them are "Heimann-Carver yolk color index", "Fletcher color rings" and Roche yolk color fan (12 grades) [19]. Currently, the most widely used method is the Roche yolk color fan (15 grades). The measuring system of Roche yolk color fan has been created on the basis of XYZ color system standardized by CIE. The X, Y and Z values are the total of the stimulations sent to the brain

from the nerves providing the perception of to the three primary colors (red, green, blue). The ratio of all three stimulations to the total amount of each stimulation defines the color. Accordingly, the perception rate of red is x=X/(X+Y+Z), the perception rate of green is y=Y/(X+Y+Z), the perception rate of blue is z=Z/(X+Y+Z). Theoretically, the equation of x+y+z=1 is the basis, and the equation of x=y=z=1/3 refers to white color [18]. The Chromaticity coordinate values of Roche yolk color fan are given in *Fig. 1*.

Today, electronic devices called colorimeter are used in color measurement in foodstuffs. These devices analyze the color data of the object based on a standard color calibration. In this study, Minolta Chroma-meter (Minolta corp.) color measuring device has been used. As the CIE L* a* b*color system was often used for color measurement in recent years, the data of this system has been collected with this mentioned color measurement device. The color model of CIE L* a* b* was widely used in describing all the colors that can be detected by the human eye. It has been developed specifically for this purpose by CIE. Lab color theory; Munsell color system has been built on 1948 Hunter color space and 1976 CIE color space [18]. Three parameters in the model indicate the luminousness of the color (L* refers to L*=0 black and L*=100 white values), its position between red and green (a*, negative values indicate green while positive values indicate red) its position between yellow and blue (b*, negative values indicate blue while positive values indicate yellow).

In this study, 90 samples were taken from each 15 grades of Roche yolk color fan constructed according to the XYZ color system and the samples were measured with Minolta Chroma-meter according to the CIE L* a* b* color system.

Statistical Analyses

An analysis of variance was carried out in order to compare the L*, a*, b* means of the samples taken from a total of 15 grades. The Tukey's comparison test was applied in order to demonstrate the differences of the L*, a*, b* means of the grades. After determining the distributions of the grades, the descriptive statistics and confidence intervals were obtained. After these steps, the same applications were repeated using 1000 Bootstrap samples. Each group in Bootstrap technique (n=90, N= 1350) were applied with the probability of 1/1000 by switching observations in order to obtain the $S(x_i)$ statistics within themselves. Thus, the randomized Bootstrap sample data set $x^*_i=(x_1, x_2, x_3,...,x_{1000})$ was obtained. The above-mentioned statistics were applied in each Bootstrap sample. The statistical analysis was carried out using the SAS 9.3 program [20].

RESULT

The original L*, a*, b* means of Roche yolk color fan grades, the standard error values and confidence intervals are given in *Table 1*, *Table 2*, *Table 3*, respectively. According to the performed analysis of variance results, the differences between both L*, a* and b* means were found significant (for three parameters P<0.01). The compatibility of Roche yolk color fan grades with L*, a*, b* values measured with the help of a device were found 90.12%, 99.14%, 38.82%, respectively (P<0.01 for all three). The results for Tukey's range test for L*, a*, b* means according to Roche yolk color fan grades are given in *Table 1*, *Table 2*, *Table 3*. As seen in *Table 1*, no statistically significant difference has been found between 1st-2nd, 4th-5th, 7th-8th grades in terms of L* means (P>0.01). The Roche yolk color fan grades are divided into 10 groups in terms of L* values, as well as in terms of b* values. As for yellowness (b*) values, the Roche yolk color fan grades were only divided into 9 groups (*Table 3*, P<0.01). On the contrary, all of the Roche yolk color fan grades (15 grades) according to the redness (a*) means can be identified independently of one another (*Table 2*, P<0.01).

The bias values of the means of the L*, a*, b* parameters obtained by the Bootstrap application, the standard error values of the means and confidence intervals are given in *Table 1*, *Table 2*, *Table 3*, respectively. The bias

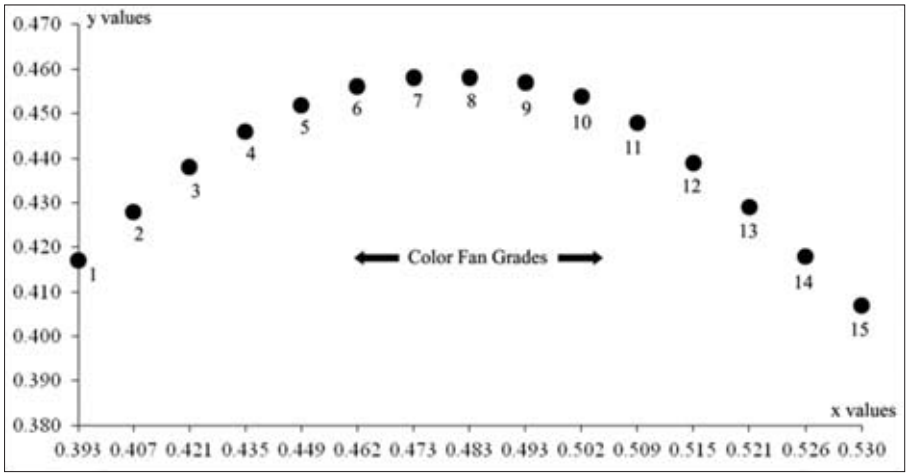

Fig 1. Chromaticity coordinates x and y for Roche yolk color fan

Şekil 1. Roche sarı renk yelpazesinin x ve y renklilik koordinatları

Table 1. *LS means and confidence intervals for original data and Bootstrap samples of lightness (L*)*
Tablo 1. *Açık renkliliğin (L*) orjinal verileri ve Bootstrap örnekleri için ortalamalar ve güven aralıkları*

Fan Grade	Original				Bootstrap			
	Mean	SE	Confidence Interval (95%)		Bias	SE	Confidence Interval (95%)	
			Lower	Upper			Lower	Upper
1	83.04^{a}	0.23	82.58	83.50	-0.0017	0.05	82.92	83.12
2	82.60^{a}	0.23	82.14	83.06	-0.0005	0.03	82.54	82.64
3	81.59^{b}	0.23	81.13	82.05	0.0005	0.08	81.41	81.73
4	79.69^{c}	0.23	79.23	80.15	-0.0037	0.81	77.79	80.52
5	80.00^{c}	0.23	79.54	80.46	-0.0005	0.22	79.49	80.25
6	78.25^{de}	0.23	77.79	78.71	0.0003	0.02	78.22	78.29
7	76.93^{ef}	0.23	76.47	77.39	-0.0014	0.05	76.81	77.02
8	77.40^{de}	0.23	76.95	77.86	0.0001	0.08	77.22	77.53
9	76.27^{fg}	0.23	75.81	76.72	0.0003	0.03	76.21	76.31
10	74.93^{h}	0.23	74.47	75.39	-0.0003	0.02	74.90	74.96
11	73.12^{i}	0.23	72.66	73.58	-0.0034	0.14	72.80	73.33
12	71.72^{j}	0.23	71.26	72.17	0.0032	0.08	71.52	71.83
13	69.76^{k}	0.23	69.31	70.22	0.0001	0.10	69.53	69.91
14	68.49^{l}	0.23	68.03	68.95	-0.0131	0.21	68.23	68.99
15	66.23^{m}	0.23	65.77	66.69	0.0011	0.08	66.04	66.35

Table 2. *LS means and confidence intervals for original data and Bootstrap samples of redness (a*)*
Tablo 2. *Kırmızılığın (a*) orjinal verileri ve Bootstrap örnekleri için ortalamalar ve güven aralıkları*

Fan Grade	Original				Bootstrap			
	Mean	SE	Confidence Interval (95%)		Bias	SE	Confidence Interval (95%)	
			Lower	Upper			Lower	Upper
1	-5.18^{o}	0.13	-5.43	-4.93	-0.0022	0.12	-5.32	-4.91
2	-3.98^{n}	0.13	-4.24	-3.73	-0.0007	0.02	-4.02	-3.95
3	-2.73^{m}	0.13	-2.98	-2.47	0.0011	0.03	-2.77	-2.67
4	-0.45^{l}	0.13	-0.70	-0.20	-0.0031	0.04	-0.52	-0.36
5	3.08^{k}	0.13	2.83	3.34	-0.0009	0.03	3.04	3.15
6	5.74^{j}	0.13	5.48	5.99	-0.0002	0.02	5.70	5.77
7	8.63^{i}	0.13	8.37	8.88	0.0019	0.03	8.56	8.67
8	12.98^{h}	0.13	12.73	13.23	-0.0004	0.04	12.89	13.04
9	15.33^{g}	0.13	15.07	15.58	-0.0015	0.03	15.27	15.37
10	19.22^{f}	0.13	18.97	19.48	-0.0016	0.22	18.98	19.75
11	20.36^{e}	0.13	20.11	20.61	0.0042	0.11	20.12	20.50
12	23.72^{d}	0.13	22.97	24.47	-0.0015	0.40	23.08	23.99
13	28.76^{c}	0.13	28.51	29.01	-0.0003	0.11	28.49	28.90
14	31.91^{b}	0.13	31.65	32.16	-0.0014	0.03	31.85	31.96
15	35.17^{a}	0.13	34.91	35.42	0.0010	0.03	35.10	35.22

means of the obtained Bootstrap samples were found quite low. This means for the L*, a*, b* parameters were found -0.0013, -0.0004, 0.0017, respectively. It has been determined that the results of the variance analyses and Tukey's range tests used original and Bootstrap data are the same.

DISCUSSION

As seen in *Table 1* and *Table 3*, the Roche yolk color fan grades cannot be completely separated from each other in terms of L* and b* values. On the contrary, all of the 15 grades were found independent of each other in

Table 3. *LS means and confidence intervals for original data and Bootstrap samples of yellowness (b*)*
Tablo 3. *Sarılığın (b*) orjinal verileri ve Bootstrap örnekleri için ortalamalar ve güven aralıkları*

Fan Grade	Original				Bootstrap			
	Mean	SE	Confidence Interval (95%)		Bias	SE	Confidence Interval (95%)	
			Lower	Upper			Lower	Upper
1	48.46^{i}	0.28	47.90	49.01	0.0048	0.12	48.17	48.64
2	55.16^{h}	0.28	54.61	55.72	-0.0001	0.01	55.14	55.19
3	59.01^{g}	0.28	58.45	59.56	-0.0031	0.12	58.74	59.17
4	62.30^{f}	0.28	61.75	62.86	0.0019	0.11	62.12	62.56
5	66.17^{e}	0.28	65.62	66.73	0.0045	0.10	65.97	66.35
6	69.61^{d}	0.28	69.05	70.16	0.0003	0.02	69.57	69.64
7	71.41^{c}	0.28	70.86	71.97	0.0022	0.55	70.16	72.07
8	74.34^{b}	0.28	73.79	74.90	-0.0003	0.21	73.88	74.65
9	77.03^{a}	0.28	76.47	77.58	0.0013	0.08	76.87	77.18
10	75.02^{b}	0.28	74.47	75.58	-0.0006	0.03	74.95	75.09
11	71.03^{c}	0.28	70.48	71.59	0.0067	0.42	70.08	71.56
12	68.85^{d}	0.28	68.29	69.40	0.0112	0.69	67.32	70.09
13	66.12^{e}	0.28	65.56	66.67	-0.0050	0.33	65.39	66.53
14	61.78^{f}	0.28	61.23	62.34	0.0011	0.12	61.50	61.96
15	59.03^{g}	0.28	58.47	59.58	0.0005	0.02	58.98	59.07

terms of a* values. Thus, it is possible to say that the Roche yolk color fan grades constructed not on luminousness or yellowness but completely on redness. In addition, because actual color occurs by a combination of all three parameters when evaluating the yolk color it is necessary to take the L* and b* parameters into consideration.

In the study, it was found consistent with statements of Simon and Bruce [21] that the results of variance analysis performed with the original data and the Bootstrap method and the results of multiple comparison tests were the same. Robison et al.[15] and Takma and Atil [16] reported that a decrease occurred in the standard error value with the Bootstrap method. In the study, the standard errors of the L*, a* and b* means with the Bootstrap method decreased by 42.03%, 35.38% and 30.24%, respectively. The results of the study have been found consistent with the findings reported by Robison et al.[15] and Takma and Atil [16]. A similar situation took place for the confidence intervals. In the confidence intervals of L*, a* and b* means obtained with Bootstrap technique narrowed by the rates of 48.10%, 55.43%, 34.47% respectively. For example, it has been seen in *Table 1* that the confidence intervals determined by the original data set for yolk color fan grade 1 and 2 are 82.58-83.50 and 82.14-83.06, respectively. The confidence intervals calculated for these grades overlap with each other, while no overlapping occurred in the Bootstrap confidence intervals (in *Table 1*, 82.92-83.12 and 82.54-82.64, respectively). This is important especially for the researchers comparing the yolk color fan and the L*, a*, b* values.

The Roche yolk color fan is used in great majority of the studies on egg yolk color. However, the L* a* b* values were obtained by measuring with the help of a device in fewer studies. Dotas et al.[22] investigated the effects of adding different proportions of dried tomato pulp and carophyll to the diets of laying hens on egg yolk color. In the study conducted in two laying periods, the Roche color fan score means of the egg yolks in the control group with no additives were found 11.32 and 10.39. The scores determined with only carophyll containing diet increased to 14.53 and 14.35. The yolk color scores of the chickens fed with diets that include dried tomato pulp and carophyll at various proportions were determined between 11.35 and 12.13. Jafari et al.[23] who conducted a similar study reported that Roche color fan score means of hen eggs fed with diets that dried tomato pulp were added at various proportions were between 4.62 and 4.79. In a study conducted by Şamlı et al.[24], the effect of the use of gluten meal and red pepper in different proportions on yolk color was investigated. It has been reported that the mean of the Roche color fan grade of the control group fed with a diet without any additives was 11.4 [24]. The reason why the egg yolk color scores of the control groups of these similar studies are quite different from each other can be the genetic difference of the flocks as well as it can be the fact that the color measurements are not objective. As is known, the Roche color fan scores are related to the perception of the human eye. It should be taken into account that the people making measurements under different light intensities can do different evaluations in such applications.

In a study carried out by Anderson et al.[25], L* a* b* values were determined by measuring the changes in egg yolk color by means of a device by adding carophyll red, carophyll yellow and the both to the laying hen diets. In the study, there was no significant differences in terms of L* values measured on 15th day, and it has been reported that the mean of the control group is 61.5, the means of the trial group are between 61.3 and 61.8. While some changes occur in the L* values on the 45th and 60th days of the experiment, significant differences in a* and b* means of the groups emerged. The a* means of the control group were found 10.1, 4.0, 2.8, respectively, on the 15th, 45th and 60th days of the experiment while the means of groups that were given carophyll red and yellow were found 9.8, 16.7, 16.2, respectively. The b* means of the control group were found 61.4, 53.7, 51.1, respectively on the 15th, 45th and 60th days of the experiment, while the means of groups that were given carophyll red and yellow were found 61.0, 59.8, 57.6 , respectively. The a* values determined by Anderson et al.[25] in the trial groups is consistent with the 5th and 8th grades of the Roche yolk color fan according to the classification given in *Table 2*. Accordingly, it can be said that the color of egg yolks of the control group reported by Anderson et al.[25] is lower than the values reported by Dotas et al.[22], but is higher than the values reported by Jafari et al.[23]. Anderson et al.[25] naturally preferred to compare the results of their study with the L*, a* and b* results of the studies conducted by Gonzales et al.[26] and Akiba et al.[27]. The similar situation is also true for the studies that used Roche yolk color fan. Generally, if the researchers used the Roche yolk color fan, they compare their results with the studies that used Roche yolk color fan. However, in this study it is possible to associate the results with the mentioned grades and the corresponding L*, a*, b* means and the confidence intervals.

In several studies, the L*, a*, b* values were used to determine the yolk color through both Roche yolk color fan and a device [28,29]. In the study conducted by Kırkpınar and Erkek [28] yolk color fan grade means were found 1.25, 4.08, 6.50, 6.08, not available, 7.00, 8.71, 8.54, respectively in 8 trial groups created by using some natural and synthetic pigment materials. In the study, the* values were reported as –4.25, –3.02, 4.08, 3.57, 31.61, 3.44, 18.15, 11.75 with the same order. It has been seen that there are quite close matches when the results reported by Kırkpınar and Erkek [28] compared with the results in *Table 2*. The negative bias is thought to be caused by the differences in L* and b* values which are the other color parameters and the error probability in the sensory analysis. Kırkpınar and Erkek [28] reported that a bad-looking pink-red color is obtained in the 5th group in which paprika meal used as a source of xanthophylls. The researchers stated that the RCF value could not be determined in this group, because there was no standard suitable for this color in the Roche color fan. When *Table 2* is analyzed, it has been seen that the Roche color fan grade that corresponds to this mean is 14. However, when the L* and b* values (59.05 and 31.64) are analyzed, it has been seen that the 14th grade does not comply with the confidence intervals, and as reported by Kırkpınar and Erkek [28] the egg yolk is far from the usual color. In another study conducted by Kırkpınar and Erkek [29] the a* values of the group that used paprika meal were found to be higher than the other groups, and reported that the Roche color fan grade had the highest mean within the groups. Kırkpınar and Erkek [29] reported that the Roche color fan and a* means of the group that used paprika meal 12.17 and 23.98, respectively. The values that reported by Kırkpınar and Erkek [29] have been found consistent with the values in *Table 2* in our study.

The egg yolk color fan is a method that easy to obtain, inexpensive method and provides simple application. Therefore, many researchers today also have been using the Roche yolk color fan grades to measure the egg yolk color. However, the reliability is low since it is evaluated by the human senses. Nevertheless, the color measuring devices obtaining the L*, a*, b* values are quite expensive and there are several difficulties in their use. However, it is a reliable method because it demonstrates numerical values. Through this study, associating both these methods was put into practice, and the L*, a*, b* values and confidence intervals corresponding to the Roche yolk color fan grades were determined. In addition to this, the Bootstrap method which has considerably less use in the scientific studies in the field of animal science has been introduced.

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Research Article

The Importance of Bioactive Feed Additives in Feeding Pigs and Their Impact on the Digestibility of Particular Nutrients [1]

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Abstract

In recent years, bioactive additives have become a subject of interest in the field of nutrition of farm animals. Therefore, the application of biosorptive properties of components is an alternative to inorganic mineral compounds and can play an important role in innovative nutritional strategies. The study presents a quantitative measurement of mineral components such as manganese, zinc, copper, and iron by using the scanning electron microscopy with an energy-dispersive X-ray spectroscopy (SEM-EDX). The research material was the feces collected from 39 growing-finishing pigs divided into 3 groups: control one, receiving inorganic mineral compounds, and experimental groups, fed organic mineral mixtures constituting 100% and 125% of the recommended daily intake. Each group of animals consisted of 13 animals (n=13). The results showed a difference in the elemental composition of the pooled samples of all three groups. The highest bioavailability of minerals had the group receiving 100% of the recommended dose of the experimental organic compounds. In the control group, the differences were statistically significant ($P<0.05$) in the case of manganese, zinc and iron. The study showed that the mineral supplements obtained in the biosorption process showed a high rate of absorption from the gastrointestinal tract and did not require the intake of doses greater than 100% of the daily requirement. The high proportion of trace elements in the second experimental group did not increase their bioavailability and contributed to the reduced digestibility of nutrients.

Keywords: *Biosorption, Trace minerals, Pig, Digestibility*

Domuz Beslenmesinde Biyoaktif Yem Katkı Maddelerinin Önemi ve Özellikle Besinlerin Sindirimi Üzerine Etkisi

Özet

Biyoaktif katkı maddeleri son yıllarda çiftlik hayvanlarının beslenme alanında önemli bir konu haline gelmiştir. Bu nedenle bioabsorptif maddeler bileşenlerinden dolayı uygulamada kullanılan inorganik mineraller için alternatif oluşturmakta ve yenilikçi besleme stratejilerinde önemli bir rol oynamaktadır. Bu çalışmada enerji dispersif X-ışın spektroskopisi (SEM-EDX) ile desteklenmiş, taramalı elektron mikroskobu kullanarak manganez, çinko, bakır ve demir gibi mineral bileşenlerin nicel bir ölçümü sunulmuştur. Araştırmada kullanılan dışkı, 39 adet ve 3 gruba ayrılmış deney hayvanlarından toplanmıştır. Kontrol 1 grubu inorganic mineral bileşenleri aldı ve 2 deney grubu, %100 ve %125 inorganik mineral içeren bileşiklerden oluşturulan yemle günlük beslendi. Her bir grup 13 hayvandan (n=13) oluşmuştur. Sonuçlar oluşturulan havuzlarda toplandı ve her üç grupta da farklılık gösterdi. Bioabsorptiflik; minerallerin önerilen dozunun %100 olduğu grupta en yüksek seviyede oldu. Kontrol grubunda, farklılıklar; manganez, çinko ve demir durumunda istatistiksel olarak anlamlı ($P<0.05$) bulundu. Çalışma, mineral takviyelerinin büyük dozlarda uygulandığında gastrointestinal sistemde bioabsorptifliğinin düşmesi nedeniyle %100 ün üzerinde alınmasının gerekli olmadığını gösterdi. İkinci deney grubunda eser elementlerin yüksek porsiyonda alımı bioabsorptifliği yükseltmemiş ve besin sindirilebilirliğini azaltmıştır.

Anahtar sözcükler: *Bioabsorpsiyon, İz mineraller, Domuz, Sindirilebilirlik*

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INTRODUCTION

Current intensive pig production is focused on maximum slaughter efficiency of these animals [1,2]. Optimal nutrition plays a decisive role in breeding, as it ensures proper growth and development of the young organism. Concentrated feeds used in the feeding of livestock, such as corn, oats or soybean meal do not fully cover the demand for essential minerals and vitamins. On the other hand, inorganic mineral supplements often contain a higher concentration of these compounds with respect to the recommended daily requirements [2-4]. Production of increased volume of manure and limited utilization area can cause environmental pollution. Nutritional strategies formulated as concepts of cost-effective diets include meeting the mineral demand, increased efficiency of feed used, and high bioavailability of minerals with their minimal excretion [5]. Nutritional deficiencies resulting from the presence of the low absorbable minerals (i.e., oxides, chlorides, sulfates, nitrates) are the cause of increased mortality of the litters, dermatological diseases, fertility disorders, increased susceptibility to infections, or diseases of the musculoskeletal system such as rickets and abnormal ossification [6-9]. In recent years, special attention is paid to the bioavailability of minerals, and thus the degree of absorbability from the gastrointestinal tract [10]. Absorption of individual micro- and macro-elements depends, among others, on their origin [6,9,11]. Since the bioavailability of inorganic salts is relatively low, there is a need to seek alternative sources of minerals, which ensure their high bioavailability [6,12]. Production of high quality chelates is difficult and expensive, which is reflected in the high price of the products based on chelate compounds. In addition, there have been reports of an irritating effect of chelates on the gastrointestinal tract of animals [12]. Utilization of the biosorprtive properties of the substrates is an alternative both for chelates and inorganic substances [13]. The technology of biosorption process is based on binding metal cations by functional groups on the surface of the biomass and the formation of a complex in the donor-acceptor system [14-16]. Nutritional analyses carried out on laying hens and growing-finishing pigs, with the use of algae biomass enriched with selected micronutrients, confirmed the beneficial health effects and increased bioavailability of trace elements applied [17]. Since the main feed in the feeding of growing-finishing pigs is soybean meal, constituting a source of valuable protein, the use of its biosorpitve properties can simultaneously contribute to an increase of the nutritional value of the mixture [7]. Increasing the degree of absorption of mineral substances from the digestive tract is reflected in digestion studies conducted to determine the amount of excreted nutrients in the feces [9]. There is also a possibility of relatively rapid implementation of a quantitative measurement of the elements in the material studied using scanning electron microscopy with an energy-dispersive X-ray spectroscopy (SEM-EDX). SEM-EDX method is now widely recognized as a tool, which is applied in many scientific disciplines [18-23]. This technique allows precise identification and imaging of the distribution of ions of chemical elements being a part of the organic material under test. Another advantage is the high sensitivity of these measurements, the possibility of repetition and data archiving. Moreover, SEM-EDX analysis, due to minimal interference with the physical and chemical properties of the test material, is referred to as a quasi-non-destructive method [18,20].

Given the current benefits of the biosorption process in obtaining bioactive additives, it was decided to enrich the post-extraction soybean meal in trace elements playing an important role in feeding the pigs. The aim of this study was to determine the influence of mineral additives on the digestibility of selected nutrients during the second period of fattening.

MATERIAL and METHODS

Biosorption Process

Soybean meal (Vetos Plant, Zębowice, Poland) was separately enriched with the following microelements: zinc (II), manganese (II), copper (II) and iron (II) via biosorption. The solutions were prepared by dissolving appropriate amounts of inorganic salts in deionized water ($ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $FeCl_2 \cdot 4H_2O$). The enrichment process was performed in a 0.1 dm^3 bed column reactor containing demineralized water, adjusted with NaOH/HCl (POCh, Gliwice, Poland) to pH 5.0, measured with pH meter equipped with an electrode (InLab413) with temperature compensation (Mettler-Toledo Seven Multi; Greifensee, Switzerland). Biosorption process was carried out at 20°C until complete saturation of the bed, controlling the concentration of the solution coming out from the column. Biomass after the process of enrichment was dried on air at 25°C for 48 h.

Feeding Experiment and Sample Collection

The feeding experiment was carried out on 39 pigs (line 990, females, 20-23 kg initial body-weight, 10-week-old) kept in individual cages in a room with controlled heating with the mean temperature 19±0.3°C and ventilation with the mean air speed 0.2±0.02 m/s. Pigs were weighed individually at the beginning and the end of the experiment. All individuals were given anthelmintic preparation and a vaccine immunizing against Porcine circovirus type 2 (PCV2). Feed and water were available semi *ad libitum* using automatic feeding systems-automatic bell drinkers and tubular feeders.

Pigs were randomly divided into three groups: two experimental and one control group. Both control and experimental groups were fed the same basic feed composition, differing only in feed premixes *(Table 1)*.

Table 1. *Composition and feeding value of mixtures*
Tablo 1. *Karışımların kompozisyonu ve besin değerleri*

Component	GROWER Content (g/kg)	FINISHER Content (g/kg)
Ground Triticale	300.0	300.0
Ground Barley	160.0	200.0
Ground Corn	200.0	200.0
Soybean Meal 46%	140.0	90.0
Rapeseed Meal	80.0	80.0
Wheat Bran	65.1	87.5
Soy Oil	20.0	9.0
Fodder Chalk	10.0	10.0
Premix	10.0	10.0
Monocalcium Phosphate	6.0	4.5
NaCl	3.5	0.3
Pell-Tech	3.0	0.3
L-Lyzine-HCl 99%	2.0	2.3
Xynalase 4000G	0.3	0.3
Phyzme XT	0.1	0.1
Chemical Composition		
Total Protein	170.0	154.0
Crude Fiber	43.0	45.0
Lysine	9.5	8.6
Methionine + Cysteine	6.0	5.6
Threonine	6.2	5.6
Tryptophan	2.1	1.9
Total Calcium	6.6	5.8
Total Phosphorus	6.2	6.0
Total Sodium	1.7	1.5
Metabolic Energy	129.0	126.0

** The composition of standard feed was established by the producer. The content of ingredients provided per kg of diet: vitamin A (retinyl acetate) 700.000 IU; vitamin D_3 (cholecalciferol) 50.000 IU; vitamin E (DL-α-tocopheryl acetate) 7.000 IU. IU- International Unit. Premix provided: Cu, 20 (as $CuSO_4 \cdot 5H_2O$); Fe, 50 (as $FeSO_4 \cdot H2O$); Mn, 20 (as MnO_2); Zn, 50 (as ZnO) mg/kg of diet*

According to different nutritional requirements for growth of the animals, two different feed compositions were used, i.e., growers during the first period of fattening (40-65 kg) were fed a standard grower feed mixture, while in the second period of fattening (65-105 kg) they were fed a standard finisher feed mixture. The composition and feeding value of mixtures is presented in *Table 1*. The source of vitamins and micro elements was a commercially available premix produced by Cargill Poland ltd. (Kiszkowo, Poland).

The control group was fed a basal diet with micro-elements in inorganic form, while experimental groups were fed a diet supplement, in which microelements in inorganic form were eliminated at the production stage and substituted for enriched soybean meal. Soybean meal was enriched with Mn, Zn, Cu and Fe microelements. The portions of microelements were established according to European Union pig nutrition standards. The demand for microelements in the control group was covered in 100%, and in experimental groups in 100% and 125%. The experiment was carried out for 13 weeks.

Feces of all animals were collected during 5 days. The samples were stored in a refrigerator at 3-4°C for 5 days from the first collecting. Next, each separate group of samples was pooled together and prepared collectively, and then chemical analyses were performed.

SEM/EDX Analysis

The feces were collected from each pig, transported to the laboratory and analyzed. All samples were kept in the freezer until analysis. The content of the microelements in each sample was analyzed by scanning electron microscopy (SEM, Zeiss Evo LS 15) combined with energy dispersive X-ray analysis (EDX). The samples were analyzed in four replicates.

Prior to the SEM/EDX analysis, samples were washed with distilled water and dehydrated in a graded series of ethanol dilutions (POCh, Gliwice, Poland), from 50% to 100%, with a 10% gradation. Dried samples were subsequently placed in the microscope chamber. The quantax detector (Brüker) with 10 kV filament tension was used for SEM/EDX analysis. The values obtained were presented as weight percentage (wt %).

Statistical Analysis

Normality of data population was determined using Shapiro-Wilk test, whereas equality of variances was assessed using Levene's. Differences between investigated groups were analyzed using one- way analysis of variance (ANOVA). The arithmetic mean values, standard deviations and statistical analysis were performed using the Statistica 7.0 software (StatSoft, Inc., Statistica for Windows, Tulsa, OK). The values of $P \leq 0.05$ were considered significant.

RESULTS

SEM-EDX analysis allowed evaluating the elemental composition and average concentrations in all samples collected. The averages of weight percentage content were presented in *Fig. 1, Table 2* and *Table 3*.

Reduced weight percentage of all microelements was observed in both experimental groups in comparison to control. Only the content of Zn (29% in comparison to control) and Cu (15% in comparison to control) were higher in samples from the group where the demand for microelements was covered in 125%. Lower concentration of microelements in experimental groups, when compared to control, was statistically significant ($P \leq 0.05$) in the case of Mn (P=0,031), Fe (P=0.0048) in Group I, and Mn (P=0.0003) in Group II.

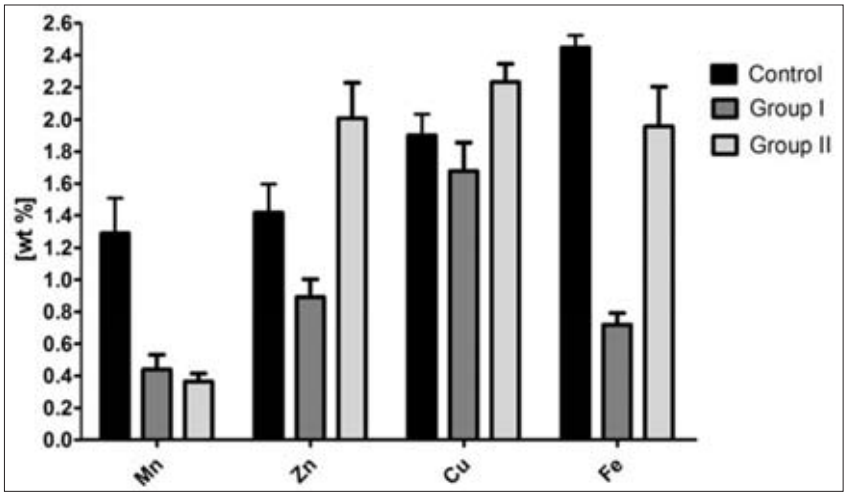

Fig 1. The averages of weight percentage content of microelements in control and experimental groups (Group I and II). The statistically significant differences were found in the experimental groups in comparison to controls

Şekil 1. Mikroelementlerin kontrol ve deneysel gruplarda (Grup I ve Grup II) ağırlıktaki oranı. Kontrol grubu deneysel grupla karşılaştırıldığında istatistiki olarak önemli farklar bulunmuştur

Table 2. *Comparison of the effects of soybean meal additive covering 100% (Group I) and 125% (Group II) requirement for the content of minerals in the sample*
Tablo 2. *Soya unu katkı maddesinin etkilerinin karşılaştırılması; % 100 (Grup I) ve % 125 (Grup II) numunelerde gereksinim için mineral içeriği*

Group	Mineral	Differences in Percentage [%]	P Value
Control and Group I	Mn	66	0.0031 significant
	Zn	37	0.10 not significant
	Cu	12	0.15 not significant
	Fe	71	0.0048 significant
Control and Group II	Mn	72	0.0003 significant
	Zn	42	0.30 not significant
	Cu	18	0.005 significant
	Fe	20	0.13 not significant
Group I and Group II	Mn	21	0.23 not significant
	Zn	56	0.007 significant
	Cu	25	0.0058 significant
	Fe	63	0.004 significant

Table 3. *The average content of microelements in swine feces in the control and experimental groups*
Tablo 3. *Deney ve kontrol gruplarında domuz dışkısı mikroelementlerinin ortalama içeriği*

Group	Mn		Zn		Cu		Fe	
	Mean wt%	SD	Mean wt%	SD	Mean wt%	SD	Mean wt%	SD
Control	1.28	0.220	1.41	0.18	1.9	0.13	2.44	0.07
Group I	0.44	0.091	0.89	0.11	1.67	0.18	0.72	0.07
Group II	0.36	0.005	2.00	0.421	2.23	0.015	1.95	0.68

The addition of microelements bound by biosorption caused a significant decrease in the content of Manganese in the pig feces; concentration of Mn was significantly higher in the control group in comparison to experimental groups. Similar results were obtained in the measurements of the content of Fe element. While the differences in the concentration of Mn between experimental groups were unnoticeable and not statistically significant, the difference in the content of Fe in group I and II was considerable. Interestingly, an increase in the content of Zn in group II was observed in comparison to control, and at the same time a decrease of wt % of zinc in group I.

The differences in the content of Zn, Cu and Fe between both experimental groups had $P \leq 0.05$ (Zn- P=0.007; Cu- P=0.0058; Fe- P=0.004) *(Table 3)*. A lower content of these microelements was clearly visible in group I. The differences between groups were readily noticeable. There

was a decreasing tendency observed in the content of Zn, Cu and Fe in the samples from group I. The concentration of the content of Zn was 56% lower in group I compared to group II. Similar situation was observed for the concentration of Cu and Fe, as their content in group I was 25% and 63% lower, respectively. Interestingly, the higher content of Mn (21% higher than in group II) was observed in the group with microelement demand covered in 100%.

DISCUSSION

Modern manufacturing technology of organic mineral compounds based on the process of biosorption has been the subject of intense research. The main advantage of the resulting biocomplexes is a significant restriction of the competition between microelements for the same site of absorption. However, interactions between trace elements, based on synergistic or antagonistic action, have not yet been fully understood and require further studies [9]. Organic forms of trace elements are readily available for the body and contribute to the growth of their average content in the material tested [24-26]. The results of the chemical analysis in relation to the control group showed an increase in the average content of copper and zinc in the experimental group receiving 125% of the recommended daily intake of minerals. This indicated a reduced absorption and assimilation of these elements from the gastrointestinal tract. Some authors noted the strong antagonism between manganese and copper [9]. The present results indicated the highest average digestibility of manganese in the groups supplemented the organic additive of this element, which can directly affect the reduced absorption of copper from the gastrointestinal tract. What is more, there is also a strong competition between zinc and copper due to similar physical and chemical properties of these elements [27]. Effect of zinc on copper metabolism is manifested by a reduced availability of copper, and thereby increased excretion of this element in feces [9,27]. Our comparative analysis of the data obtained confirmed this concept.

An important role in the control of the absorption of iron and zinc in humans is played by manganese [28]. In all growing-finishing pigs studied, the highest absorption of this element was observed in both experimental groups. The average content of zinc in the group I was approximately 56% lower compared to group II, and the differences were statistically significant. Similarly reduced values were found in the group receiving 100% of the recommended dose for copper and iron. We postulate that increasing the amount of minerals by ¼ with respect to a daily requirement, does not increase the degree of absorption and assimilation from the gastrointestinal tract. Therefore, the addition of a higher concentration of minerals in the diet may increase the competitiveness of the elements, thereby impairing their homeostasis. The results obtained confirmed better utilization of the elements in the experimental group, in which the demand was covered in 100%. Our studies also confirmed the low level of utilization of minerals in the inorganic form in the test growing-finishing pigs.

In conclusion, these data indicate a higher bioavailability of minerals obtained in the process of post-extraction biosorption of soybean meal with regard to inorganic compounds. Bioactive mineral mixtures enriched with microelement cations can complement not only the deficiencies of these elements in the diet of livestock, but also contribute to an increase in the content of trace elements in animal products. Moreover, developing pig production systems are reaching global scale and become increasingly intensified. We postulate that the diets based on bioactive minerals can increase the safety of the environment by reducing pollution and their impact on the economy of production.

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Research Article

Investigate the Effects of Non-genetic Factors on Calving Difficulty and Stillbirth Rate in Holstein Friesian Cattle Using the CHAID Analysis

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Abstract

A total number of 947 calving records from 613 Holstein Friesian cows raised at a private dairy farm in Kelkit, Turkey, from 2004 to 2006 were used to study the effect of non-genetic factors on calving difficulty and stillbirth rate using CHAID algorithm. The mean calf birth weight was 41.0±0.19 kg. The overall incidence of calving difficulty and stillbirths in the Holstein Friesian herd were 9.1% and 9.4%, respectively. Calf birth weight, birth type and calving season had the greatest impact on calving difficulty. The increase in calf birth weight was associated with a significant increase in calving difficulties (P<0.01). The calving difficulty risk in twins (18.9%) was higher than in singleton calves (5.0%). The main environmental variables affecting the stillbirth rate were parity number, calf birth weight, sex of calf, calving season and calving difficulty. Parity number was statistically the most relevant factor affecting the stillbirth rate, which was also higher in primiparous (18.7%) than in multiparous cows (5.4%). As the calf birth weight increased, a significant increase was also in the stillbirth rate (P<0.01). The stillbirth rate in winter (19.7%) was higher than in other seasons (3.6%) (P<0.01). Calving assistance was associated with an increased risk of stillbirth (P<0.05). As a result, calf birth weight, birth type and calving season had the greatest impact variables on calving difficulty, however, parity, calf birth weight, sex, calving season and calving difficulty were the most effective variables on stillbirth in Holstein Friesian Cattle.

Keywords: *Stillbirth, Calving difficulty, CHAID algorithm, Organic husbandry, Holstein Friesian*

Genetiksel Olmayan Faktörlerin Siyah Alaca Sığırlarda Güç ve Ölü Doğuma Etkilerinin CHAID Analizi İle İncelenmesi

Özet

Bu çalışmada, genetiksel olmayan bazı faktörlerin Siyah Alaca sığırlarda güç ve ölü doğuma etkilerinin CHAID algoritması ile analizi amaçlanmıştır. Bu amaçla, Gümüşhane ilinde faaliyet gösteren özel bir süt sığırı işletmesinde 2004 ile 2006 yılları arasında doğum yapan 613 Siyah Alaca ineğin 947 buzağılama kaydı kullanılmıştır. Buzağılara ait ortalama doğum ağırlığı 41.0±0.19 kg olmuştur. Sürüye ait ortalama buzağılama güçlüğü ve ölü doğum oranları sırasıyla %9.1 ve %9.4 olmuştur. Buzağılama güçlüğünü etkileyen en önemli çevresel değişkenler, buzağı doğum ağırlığı, buzağılama tipi ve buzağılama mevsimi olmuştur. Buzağılara ait doğum ağırlığı artıkça, buzağılama güçlüğü önemli oranda artmıştır (P<0.01). İkiz doğan buzağılarda güç doğum riski (%18.9), tek doğanlardan (%5.0) oldukça yüksektir (P<0.01). Ölü doğuma etkili en önemli çevresel değişkenler sırasıyla; doğum sırası, buzağının doğum ağırlığı, buzağı cinsiyeti, buzağılama mevsimi ve buzağılma güçlüğü olmuştur. Ölü doğum üzerine etkili en önemli değişken doğum sırası olup, ilkine doğum yapan ineklerde ölü doğum oranı (%18.7), çoklu doğum yapanlardan (%5.4) yaklaşık 3.5 kat daha yüksektir. Buzağıya ait doğum ağırlığı artıkça, ölü doğum oranı önemli oranda artmıştır (P<0.01). Kış mevsiminde gerçekleşen doğumlarda ölü doğum (%19.7), diğer mevsimlerden (%3.6) yüksektir (P<0.01). Güç doğan buzağılarda ölü doğum (%10.0), kolay doğanlardan (%1.9) daha yüksektir (P<0.01). Sonuç olarak, Siyah Alaca sığırlarda güç doğuma etkili değişkenler buzağı doğum ağırlığı, doğum tipi ve buzağılama mevsimi olurken ölü doğum üzerine doğum sırası, buzağının doğum ağırlığı, buzağı cinsiyeti, buzağılama mevsimi ve buzağılma güçlüğü en etkili değişkenler olmuştur.

Anahtar sözcükler: *Ölü doğum, Buzağılama güçlüğü, CHAID algoritması, Organik Hayvancılık, Siyah Alaca sığır*

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INTRODUCTION

A stillbirth is defined as the death of a calf right before, during or within 24-48 h from parturition [1-3]. Calving difficulty caused by a prolonged spontaneous calving or a prolonged or significantly assisted extraction is known as dystocia [4]. Mee et al.[5] reported that worldwide the stillbirth rate among dairy cattle varied between 2% and 10%, and dystocia ranged from 2% to 14%. However, several studies have reported an increase in stillbirth rates over the last few years in the United States and in several European countries [1,2,4,6-8].

The productivity of the beef and dairy industry is highly influenced by stillbirth and calving difficulty rates [7,9]. Meijering [10] reported that stillbirths and calving difficulties result into significant costs, which can be either direct (loss of calves, death of dams, veterinary assistance and labour) or long-term (culling rate, milk yield and fertility). It has been estimated that these losses in the dairy industry in the US and the UK amounted to US$ 125 million and £ 60 million, respectively [11]. McGuirk et al.[12]. estimated that the total cost attributable to a severe case of calving difficulty can be as high as 500 euros per case. In addition, calving difficulty is a welfare problem regarded as one of most painful conditions for calves [13].

Genetic, maternal, fetal, environmental and management factors influence stillbirths and calving difficulties [3,14], which however are generally explained by low degree of heritability. It is therefore important to determine the environmental factors causing stillbirths and calving difficulties [15,16].

The analysis of calving difficulty and stillbirth data has generally been conducted by different statistical methods, such as variance analysis [17] and logistic regression [18]. The last method is the most commonly used, because these data are binomial [19]. A potential alternative to the logistic regression is the classification tree method [20]. This technique belongs to the field of data mining, which also includes cluster analysis and artificial neural networks [21]. CHAID (Chi-Square Automatic Interaction Dedector) and CART (Classification and Regression Trees) are classified under data mining. These data mining techniques are predominantly applied in medicine, finance [22], animal farming and breeding [19,23-29]. CHAID analysis has various advantages over other statistical method. These advantages include the following [23,30]: (a) CHAID is a nonparametric method, which does not have to satisfy assumptions; (b) CHAID algorithm presents multiway splits instead of binary splits of the predictor variables; (c) CHAID can be applied for all types dependent variables (continuous, nominal and ordinal); (d) CHAID are invariant under transformations of independent variables; (e) CHAID algorithm includes the most important variables explaining the dependent variable and eliminates insignificant variables; (f) CHAID algorithm provides a graphical representation of the data and interactions within the data set can be determined and the graphical interpretation of complex results containing the interactions; (g) The model has the capability of overcoming missing values in the dependent and independent variables; (h) CHAID output is highly visual and easy to interpret.

In general, previous studies used analysis of variance and logistic regression analysis methods to determine the factors affecting calving difficulty and stillbirth rates in cattle. This study aimed to determine and classify the factors affecting calving difficulty and stillbirth rates in Holstein Friesian cattle using the CHAID algoritm.

MATERIAL and METHODS

Animals and Data Set

This study consists of a set of 947 calving records of 613 Holstein Friesian cows that calved from 2004 to 2006 at a private dairy farm located in Kelkit country in the province of Gumushane in the eastern Black Sea region of Turkey. This dairy farm was founded in the year 2003. During the time period, 5-8 month-pregnant Holstein Friesian cows were brought from farms managed under extensive conditions in the state of Wisconsin in the United States. The farm where the Holstein Friesain cattle herd under study was kept is located at an altitude of 1400 m asl. The climate in this region is relatively dry with rainfalls usually in spring and autumn. During the winter months, it snows a lot and the night temperature may drop even to -10°C. The average temperatures in this region in winter, spring, summer and fall are -1, 8.7, 18.9 and 10.9°C, respectively.

Management and Feeding

Feeding, housing and animal health were managed in compliance with the organic farming regulations issued by the Turkish Ministry of Agriculture and Rural Affairs [31,32] under the supervision of an independent control agency which ensured consistency with all legal requirements. The use of behaviour-regulating hormones and similar agents was forbidden and for mating purposes artificial insemination was mostly used. The ration of organically reared dairy cattle included 60% roughage and 40% concentrate feed. All feeds offered to the cows were grown organically at the farm. Dry meadow hay, dry alfalfa hay and corn silage were used as sources of roughage in the diets of the animals. In this farm, lactating cows were fed daily 6 kg/head of concentrate, 20 kg/head of dry meadow hay and dry alfalfa hay and 10 kg/head of corn silage. Cows were also fed a total mixed ration (TMR) throughout the year.

In this farm, all herd records were kept with great care and monitored on a daily basis. The farm staff in charge of calving management duly recorded all relevant data. The birth weights of all live-born calves were measured within 6 h from birth. Newly-born calves were allowed to suckle

from their dams untill the end of first day *post-partum*. They were housed in outdoor calf hutches for 3 months, whereas adult animals were kept in a free stall barn.

In addition to birth weights, the current study also considered a data set with data about birth date, birth season, parity number, birth type (twin, singleton), calf sex, stillbirths, calving difficulty and abortion. The data were edited a few times so as to delete missing, questionable and duplicate records. In addition, gestation periods shorter than 260 days were referred to as abortions [33] and removed from the data set.

Definitions

Deaths prior or during calving, or within 48 h from calving were classified as stillbirths [1-3]. Stillbirths were coded with a 0, whereas live births were coded with a 1. Birth weights of calves that died before or during parturition were not measured and were coded as missing values. Births occurring spontaneously and requiring no intervention were defined as normal calvings, whereas those requiring assistance from a person and/or a veterinary were classified as calving difficulties [4]. Normal births were coded with a 1, whereas calving difficulties were coded with a 0. Parity number was coded as 1., 2., *etc*. The birth season was coded as winter (December-February), spring (March-May), summer (June-August) and autumn (September-November).

Statistical Analysis

The CHAID (Chi-squared Automatic Interaction Detection) method was used for the statistical analysis. It was used to determine the relationship between a dependent and predictor variables. The CHAID is a type of decision tree technique which classifies the population into subgroups setting the variation in the dependent variable within groups with a minimum value and among the groups with a maximum value [34]. It is a multivariate analysis technique which identifies the size and rank of statistically significant differences [35]. In the CHAID analysis, if the dependent variable is nominal, ordinal and continuous, respectively Chi-square test, likelihood ratio test and F test is used to specify the best next split at each step. In this study because dependent variable is nominal, chi-squared test was used to determine each split. The significant difference is measured by the *p*-value obtained from chi-squared test. If the *p*-value for any predictor is less than or equal to $\alpha_{split\text{-}merge}$, split is performed and this process is repeated until no forward splits are found. Conversely, the *p*-value is greater than $\alpha_{split\text{-}merge}$, forwad splits is not performed and process stoped [36]. The area under the ROC curve (AUC) which was used to test the compatibility of the CHAID model shows percentage of correct classifications. The AUC vary from 0.0 to 1.0. A value of 1.0, 0.0 and 0.5 indicates an excellent positive prediction, an excellent negative prediction, and poor prediction performance, respectively. In the CHAID analysis, it was planned to find values belonging to at least 10 individuals in the parent node and 5 individuals in the child nodes in order to identify the random effects of parity, calving season, calving year, birth type, calf sex and calf birth weight, which are the dependent variables. The statistical analysis was performed using the SPSS software package [37].

RESULTS

The birth weight of calves ranged from 23 to 64.2 kg (n = 894), the average birth weight was 41.0 kg (SE=0.19 kg) and 89 calves died with the death time distribution summarized in *Table 1*. CHAID model planned that values belonging to at least 10 individuals in the parent node and 5 individuals in the child nodes be found in order to identify the random effects of parity, calving season, calf year, birth type, calf sex and calf birth weight on calving difficulty *(Fig. 1)*, and parity, calving season, birth year, birth type, calf sex, calf birth weight and calving difficulty on stilbirths *(Fig. 2)*. In the CHAID model, the significant difference is measured by the p-value obtained from a Pearson chi-square test. The α_{merge} and α_{split} values were set at 5% level.

Decision tree diagram constructed via CHAID algorithm for calving difficulty was depicted in *Fig. 1*. According to the tree diagram based on the CHAID algorithm, the number and percentage of calving difficulties were presented in the root node (Node 0) at the top of the decision tree diagram. In this node, 90.9% of the births in the herd were normal, while 9.1% were associated with calving difficulties. This node was divided into four child nodes (Node 1, Node 2, Node 3 and Node 4) according to the level of calf birth weight, which was the most important predictor variable determining calving difficulty in the CHAID model. No calving difficulty was observed when weights of the Holstein Friesian cattle at birth were below the mean calf birth weight (≤33.6 kg) (Node 1). The calving difficulty rate in Node 2, where a considerable part of parturitions within the herd can be found (68.2%), was 5.8%.

The calving difficulty rate was 14.4% when birth weights were equal to almost 5 kg or higher (>45.6 kg) than the average birth weight of the herd (41.0±0.19) (Node 3). Since Node 1 and Node 3 were terminal nodes in

Table 1. *Death time distribution*
Tablo 1. *Buzağı ölümlerinin zamana dağılımı*

Death Time	Stillbirth rate	
	n	%
Prior or during birth	53	59.6
Birth-24 h	14	15.7
24-48 h	22	24.7
Total	89	9.4

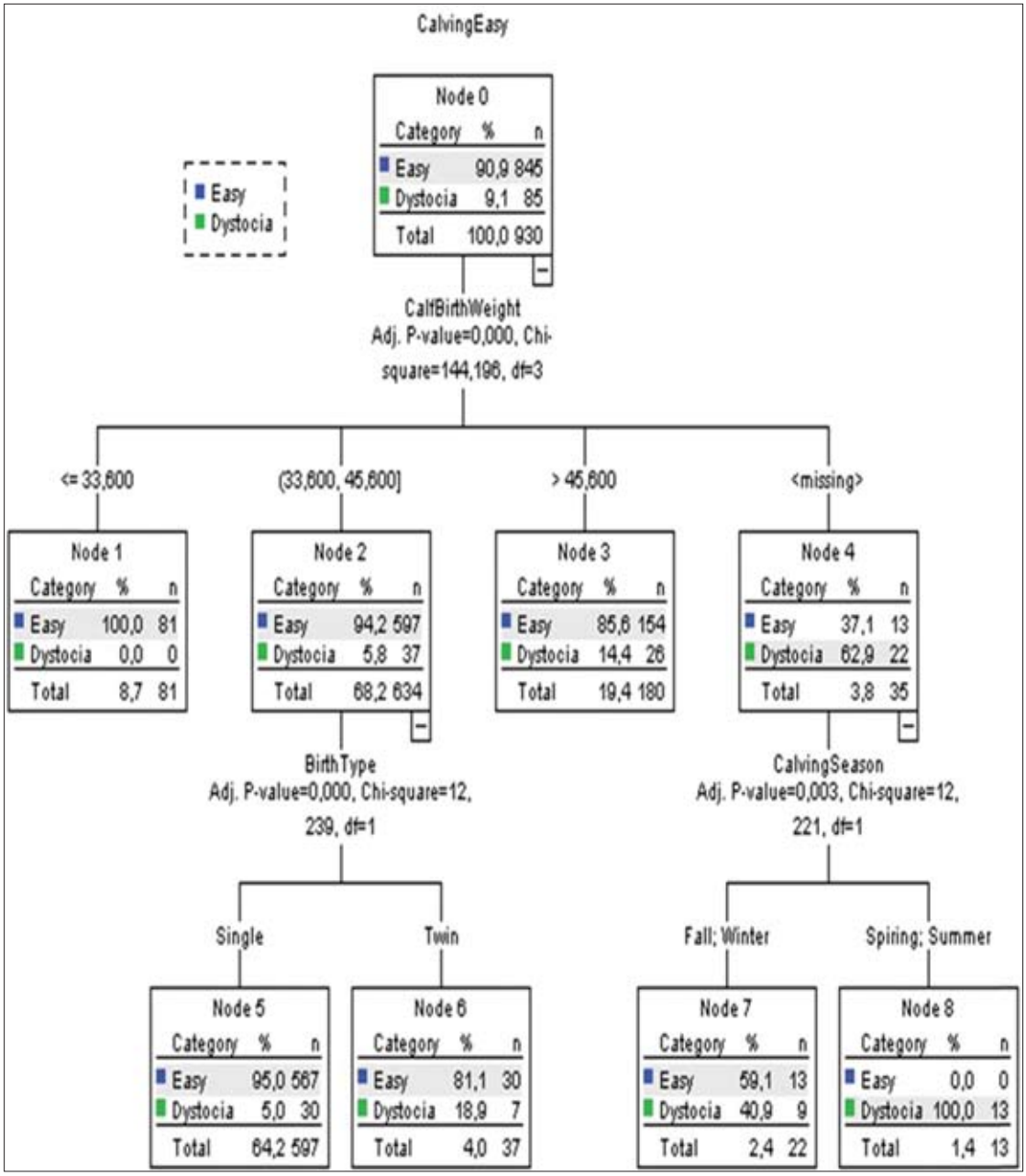

Fig 1. Diagram of the classification tree regarding the calving difficulty
Şekil 1. Buzağılama güçlüğüne ilişkin sınıflandırma ağacı diyagramı

terms of birth weight, they had a homogenous structure, as they were not separated into other nodes. Judging from CHAID diagram, a low level of calf birth weight resulted in significantly greater percentage of easy and smaller percentage of dystocia. It could be argued that when calf birth weight lower than 45.60 kg calving is easy and when it is higher than 45.60 kg the calving is dystocia.

Node 2 and Node 4, on the other hand, were not homogenous and were further divided into child nodes. Node 2, which corresponded to a calf birth weight between 33.6 and 45.6 kg, was furher broken down with respect to the birth type into two nodes, either singleton calf (Node 5) or twin calves (Node 6). The birth type was the second most important variable in causing calving difficulties *(Fig. 1)*. Node 4, which corresponded to a missing value (calves died before or during parturition), was furher split with respect to the calving season into two nodes, either fall-winter (Node 7) or spring-summer (Node 8). The calving season was the third most important variable in causing calving difficulties. While calving difficulties were observed in all stillborn calves born in the warm season, it dropped to 40.9% during the cold season *(Fig. 1)*. Since the other variables are not effective in calving difficulty determination, they are not shown in the tree diagram.

The percentage correct classification, risks, standart error of risk, AUC, standart error of AUC, and predicted

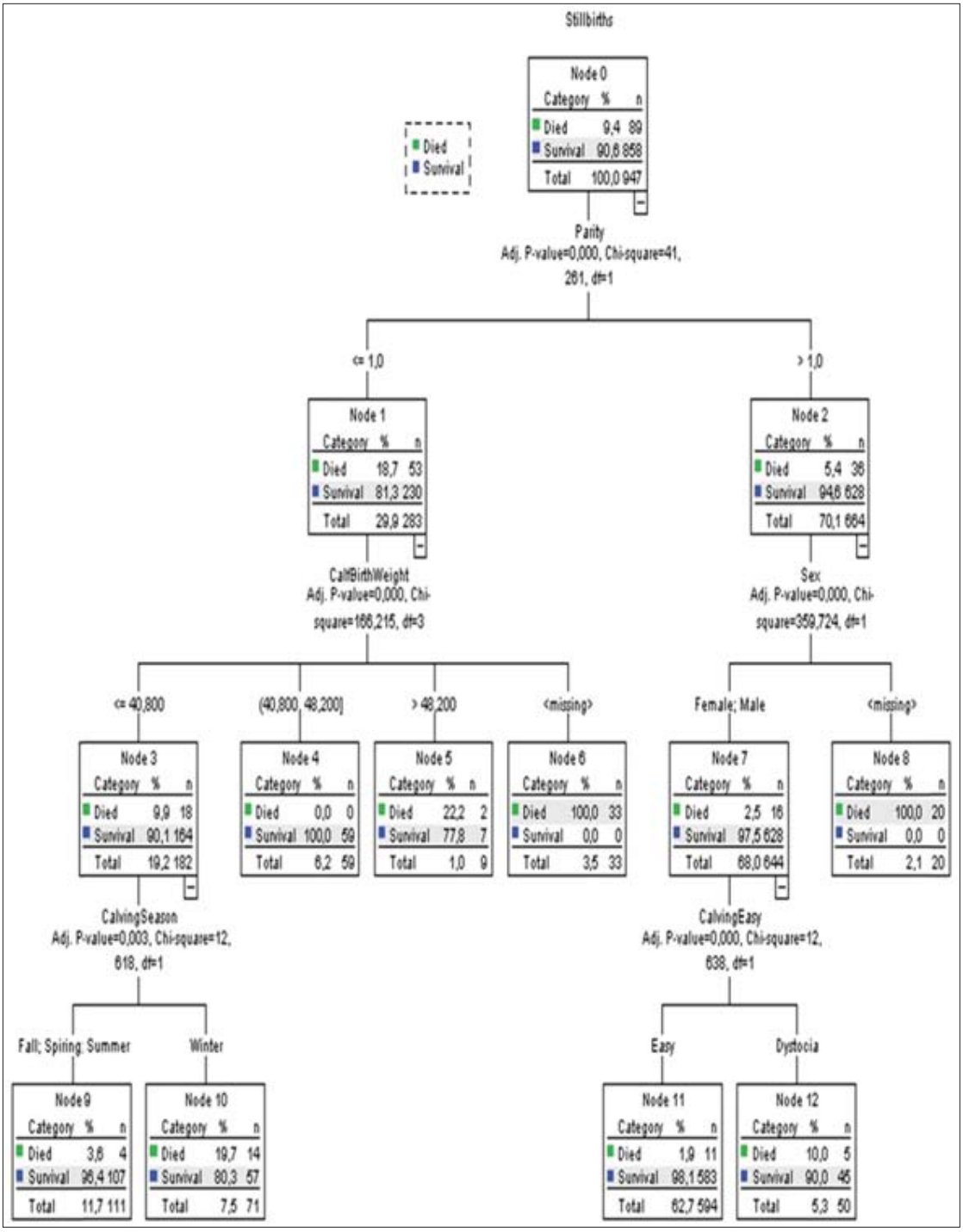

Fig 2. Diagram of the classification tree regarding stillbirth
Şekil 2. Ölü doğuma ilişkin sınıflandırma ağacı diyagramı

values obtained by the fitting CHAID model to predict the calving easy were given in *Table 2*.

According to CHAID analysis, 100% of easy calving and 15.3% of dystocia calving were correctly classified, while 0% of easy calving and 84.7% of dystocia calving were wrongly assigned by using calf birth weight, birth type and calving season variables. CHAID analysis correctly determined 92.3% of calving easy. The AUC and risk values of the model were used to test its compatibility *(Table 2)*. The compatibility of the model could be said to be favourable, because the CHAID analysis showed a fairly high efficiency (92.3%), a low risk value (7.7%), and the area under the ROC curve (AUC=0.752) significantly different from 0.5 (P<0.001) in explaining the model. These meaning that the CHAID algorihm classifies the group significantly better than by chance. AUC value close to 1.0 indicates perfect positive prediction.

Table 2. *Classification results in the CHAID analysis regarding the calving ease*
Tablo 2. *Buzağılama kolaylığı bakımından CHAID analizinde classification tree sonucu*

Calving Easy		Predicted		
		Easy	Difficult	Total
Observed	Easy	845 (100%)	0 (0%)	845
	Difficult	72 (84.7%)	13 (15.3%)	85
	Total	917 (98.6%)	13 (1.40%)	930 (100%)

Percentage Correct: *92.3%;* ***Risk:*** *0.077;* ***Standart Error of Risk:*** *0.009;* ***AUC*** *= 0.752***;* ***Standart Error of AUC*** *= 0.030; *** P<0.001*

Table 3. *Classification results of CHAID analysis regarding stillbirths*
Tablo 3. *Ölü doğum bakımından CHAID analizinde classification tree sonucu*

Stillbirths		Predicted		
		Survival	Death	Total
Observed	Survival	858 (100%)	0 (0%)	858
	Death	36 (40.4%)	53 (59.6%)	89
	Total	894 (94.4%)	53 (5.6%)	947 (100%)

Percent Correct: *96.2%;* ***Risk:*** *0.038;* ***Standart Error of Risk:*** *0.006;* ***AUC*** *= 0.740***;* ***Standart Error of AUC*** *= 0,023*

Decision tree diagram drawn for CHAID algorithm for stillbirths is shown in *Fig. 2*. In this Holstein Friesian herd, parturitions led 90.6% live births and 9.4% stillbirths. This node was divided into two child nodes (Node 1 and Node 2) according parity, which was the most important predictor variable in causin stillbirths in the CHAID model.

Node 1 was divided into four child nodes in terms of birth weight (Node 3, Node 4, Node 5, Node 6), which was the second most important predictor variable in causing stillbirths in *Fig. 2*. Node 2 was further separated with respect to sex of calf in to two child nodes, either female and male (Node 7; 628 survival and 16 died) or missing (Node 8; 0 survival and 20 died). Since the female and male calves were given in a single node (Node 7) in the CHAID diagram, no difference was found between sex of calf in terms of stillbirth rate. Node 3 was divided into two child nodes (Node 9 and Node 10) according to the calving season. The percentage of stillbirths among calves born in the winter months (19.7%) was higher than in the other seasons (3.6%).

Node 7 was divided into two child nodes: easy calving (Node 11) and difficult calving (Node 12). Among calves born normal, *i.e.* in births which required no assistance, the mortality rate was 1.9%, whereas it was 10% in births which required assistance. According to CHAID diagram, the most important variables in determination of stillbirths are parity, calf birth weight, sex, calving season and calving easy, respectively. Birth year and birth type were not effective in stillbirths determination, they are not shown in the tree diagram.

The percentage correct classification, risks, standart error of risk, AUC, standart error of AUC, and predicted values obtained by the fitting CHAID model to predict the stillbirths were given in *Table 3*.

According to CHAID analysis, 100% of survival calf and 40.4% of died calf were correctly classified, while 0% of survival calf and 59.6% of died calf were wrongly assigned by using parity, calf birth weight, sex, calving season and calving easy variables. CHAID analysis correctly determined 96.2% of stillbirths *(Table 3)*. The compatibility of the model could be said to be favourable, because the CHAID analysis had a fairly high efficiency (96.2%), a low risk value (3.8%), and the area under the ROC curve (AUC = 0.752) sifnificantly different from 0.5 (P<0.001) in explaining the model.

DISCUSSION

The average calving difficulty rate in the Holstein Friesian cows was 9.1%. This result was comparable to other study reported (5.4%-10.8%) for the same breed [1,4,5,15,16,38-40]. According to the classification tree technique, the most important variable affecting calving difficulty was the calf birth weight. This result was consistent with the outcome of earlier studies [8,14,15] that showed a increase in the calving difficulty incidence, when the birth weight increased. In dairy cattle breeds, calving difficulty caused by the disproportion between the size of calf and the pelvic area of mother was the most common. The phenotypic variations of these factors in calving difficulty were reported to be 50% and 5-10%, respectively [10].

Although the average calving difficulty rate in this study is comparable with that of Holstein Friesian cows, the calving difficulty rate with birth weights over 45.6 kg (recorded in almost 20% of the herd births) was higher (14.4%). According to the regulations for organic dairy farming in Turkey, daily rations for cattle can cointain 60% of roughage and 40% of concentrate. The use of less concentrate feed was expected to be a negative effect on the growth and development of cattle raised under organic conditions, but no difference was found in terms of age and live weight at first calving between the organic and conventional breeding systems [41-43]. However, further studies are required to reach conclusive results about this matter.

Calving difficulty for twins was almost 4 times greater (18.9%) than for singletons (5.0%). Twin births prolonged the birth process and caused pain to both the mother and the calves, therefore caused calving difficulties that required assistance. Mee et al.[5] reported that birth type was an important factor affecting calving difficulty, whereas Gundelach et al.[7] reported the contrary. The latter author reported that cows with twins had a higher risk of insufficient abdominal contractions.

In this farm, 89 calves were born within 48 h from the beginning of the parturition process. A high calving difficulty rate (62.9%) was associated with death before or during calving. In accordance with our study, Barrier et al.[11] reported that assistance was required and 57.1% of calves died before or during birth. Calving difficulties increase the likelihood of stillbirths due to trauma and anoxia [44]. The significant effect of the calving season on perinatal mortality was noted. Calving difficulties always led to stillbirths in the warm season, whereas this rate was only 40.9% in the cold season. Also earlier studies reported that the calving season is a significant factor affecting calving difficulty [3,4,9].

The average stillbirth rate in Holstein Friesian cows was 9.4%. This result was comparable to other study reported (4.06%-9.7%) for same breed [6,7,14-16,44]. In this study, the effect of parity on the stillbirth rate was significant as also mentioned by earlier authors [1,7,8,9,44]. In this respect the most important factor was the fetus-dam pelvis disproportion [1,5,8,9]. Uematsu et al.[9] reported that a high fetus weight in pregnant heifers with an immature pelvis increased the risk of calving difficulty and stillbirths. The study conducted by Gundelach et al.[7] in Holstein Friesain cows with pelvis sizes of >55 *versus* ≤55 reported stillbirth rates of 7.0% and 15.6%, respectively.

In our study, the stillbirth rate in primiparous cows (18.7%) was almost 3.5 times greater than in multiparous cows (5.4%). Although in some previous studies [1,44] differences were found in terms of parity, the differences reported for the primiparous group (7.97%-13.2%) and the multiparous group (4.51%-6.66) was lower compared those obtained in our study. These differences can be questioned in many respects. Firstly, the Holstein Friesian cattle is the dairy breed with the highest mortality rate [3], and the Northern American genotypes of this breed even have a higher mortality rate [4]. Secondly, a limited amount of concentrate feed was used in the organic dairy system. In Turkey, the daily rations for cattle can cointain 60% of roughage and 40% of concentrate, but, given the insufficient feed production and high prices, it is difficult to provide organic concentrate feed to cattle. Therefore, concentrate feed is given to cattle only in the most physiologically demanding periods. During growth and development, the diet is mainly based on roughage. This may a negative influence on the growth and development of heifers and is also thought to increase the stillbirth rate, given their narrow pelvis size at the time of calving. Further studies are required in order to reach conclusive results on this matter.

Birth weight of calf significantly affected the stillbirth rate as also reported by other authors [1,8,15]. This result was mainly attributable to a disproportion between the fetus and the dam pelvis [3]. In this study, a total 89 calves that died, more than half (n=53) died prior or during birth. This suggests that there were other problems in this farm apart from the fetus-pelvis disproportion that had a negative influence on the vitality of calves. It would be necessary to conduct an anatomical, pathological and histological examination of the calves which died before or during calving.

In accordance with previous studies [8,44,45], the stillbirth rate increased, due to the increase in dry matter intake lead to an increase in the birth weight during the winter months. Also, in cold weather increased gestation length and calf birth weight was a high risk for stillbirth, whereas in summer less intensive calving supervision and more oppurtinity for exercise at pasture was a low risk for stillbirth [5].

In accordance with the previous studies [1,3,7,11,16,44], the influence of calving difficulties on the stillbirth rate was significantly important. Some recent studies have reported that any degree of assistance (from limited to veterinary assistance) is an increased stillbirth [6] and even limited assistance (by one person) is also associated with an increased stillbirth risk [12]. It was pointed out that there is an interaction between parity and calving difficulty and that, while the stillbirth rate increased along with the prolongation of the birth process in primiparous cows, the stillbirth rate also increased in case of breech presentation and twins in multiparous cows [7].

It can be stated that in Holstein Friesian herd calving difficulty and stillbirth rates in our study is comparable to other international estimates. Calf birth weight, birth type and and calving season had the greatest impact on calving difficulty. The rates of stillbirth were significantly higher in primiparous (18.7%) than multiparous cows (5.4%). As the calf birth weight increased, a significant increase in stillbirth rate. Calving assistance was associated with an increased risk of stillbirth. Also this study demonstrates that a graphic model made with the classification tree technique makes it possible to clearly indicate factors affecting calving difficulty and stillbirth rates that the farmers and their staff may be required to manage.

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Research Article

Prevalence, Serological Typing and PCR Sensitivity Comparision of *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* spp. Isolated from Raw Chicken Carcasses [1]

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Abstract

Poultry meat is the most popular food products worldwide. *Salmonella* are important foodborne pathogens especially in poultry. Objectives in this study were to determine the presence of *Salmonella* spp. and to detect the incidence of *Salmonella* Typhimurium and *Salmonella* Enteritidis in 100 raw chicken carcasses. Carcasses which were collected from Istanbul (n=100) for the detection of the organism by conventional culture method and confirmed of strains by PCR of DNA using *inv*A and *fli*C genes. According to the results, *Salmonella* spp. was determined in 15 (15%) raw chicken carcass samples of 100 total samples analyzed due to both PCR and conventional culture method include serological tests; Four (26.6%) samples were identified as *S.* Enteritidis while 3 (20%) samples were *S.* Typhimurium of 15 total *Salmonella* spp. Sensitivity of PCR procedures for *Salmonella* spp. and S. Typhimurium were high and quite specific. However, the sensitivity of the mentioned procedure was very low for *S.* Enteritidis. It is being thought that PCR procedures can be good alternative methods to microbiological analysis procedures for *Salmonella* spp. and *S.* Typhimurium while microbiological analysis procedures have more advantages than PCR procedures for protection of the public health at the detection of S. Enteritidis.

Keywords: *Salmonella* Enteritidis, *Salmonella* Typhimurium, *PCR, Serological typing, Chicken carcass*

Çiğ Tavuk Karkaslarından İzole Edilen *Salmonella* Typhimurium, *Salmonella* Enteritidis ve *Salmonella* spp.'nin Prevalans, Serolojik Tiplendirme ve PCR Hassasiyetinin Karşılaştırılması

Özet

Kanatlı eti dünyada en popüler gıda ürünlerinden biridir. Bununla birlikte, *Salmonella* özellikle kanatlılarda önemli gıda kaynaklı patojenlerdir. Bu araştırmanın amaçları, *Salmonella* spp. varlığının belirlenmesi ve 100 adet çiğ tavuk karkasında *Salmonella* Typhimurium ve *Salmonella* Enteritidis insidensinin tespit edilmesidir. Ayrıca, izole edilen suşlarda, klasik kültürel metod ve PCR prosedürlerinin etkinliğininin karşılaştırılması da amaçlanmıştır. İstanbul'dan temin edilen karkaslarda (n=100) organizma, klasik kültürel metod ile tespit edilmiş ve tespit edilen edilen suşlar, *inv*A and *fli*C genlerinin araştırıldığı PCR analizi ile doğrulanmıştır. Elde edilen bulgulara göre *Salmonella* spp., 100 adet çiğ tavuk karkas örneğinin 15 (%15) adedinde, PCR ve serolojik testleri içeren klasik kültürel metod kullanılarak belirlenmiş ve 15 adet *Salmonella* spp.'nin 4 (%26,6) adedi *S.* Enteritidis, 3 (%20) adedi ise *S.* Typhimurium olarak serotiplendirilmiştir. *Salmonella* spp. ve *S.* Typhimurium tespit için PCR prosedürlerinin duyarlılığı yüksek ve spesifik olarak bulunmuştur. Buna karşın, söz konusu prosedürün duyarlılığı *S.* Enteritidis için oldukça düşük kalmıştır. *Salmonella* spp. ve S. Typhimurium teşhisinde PCR prosedürlerinin mikrobiyolojik analiz prosedürlerine göre göre önemli bir alternatif olabileceği düşünülmekte iken, *S.* Enteritidis tespitinde ise halk sağlığının korunması açısından mikrobiyolojik analiz prosedürlerinin PCR prosedürlerine göre daha avantajlı oldukları öngörülmektedir.

Anahtar sözcükler: *Salmonella* Enteritidis, *Salmonella* Typhimurium, *PCR, Serolojik tiplendirme, Tavuk karkası*

INTRODUCTION

Poultry meat is popular food products worldwide. Several factors such as high level protein and low fat content and favorable content of fatty acids contribute to the popularity of poultry meat and economics factors are important. Chicken meat is widely used in fast-food establishment and restaurants. Therefore, poultry meat

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comprises about two-thirds of the total production in the world [1]. Poultry meat production was 245.554.1 metric tons in 2012, Turkey. Accordingly, Turkey has been the largest 9th poultry meat producer in the world [2].

Foodborne pathogens are evaluated as serious risk factors from producing processes up to consuming for public health. Salmonellosis is one of the most common and widely distributed foodborne diseases and is caused by the bacteria *Salmonella*. It is estimated that tens of millions of human cases occur worldwide every year and the disease results in more than hundred thousand deaths [3]. Furthermore, one of the commonest causes of salmonellosis reported humans has been through the handling of raw carcasses and products, together with the consumption of undercooked poultry meat [4]. The global increase in chicken consumption stimulated by its high protein content and its accessible price has drawn the attention of producers, researchers and authorities to the necessary of controlling *Salmonella* contamination, principally during the various stages commercial production chains [5].

The prevalence of *Salmonella* spp. in chicken meat has been studied in many countries [1,5-9] including Turkey [10-13]. However, very little statistical data on *Salmonella* serotypes and infections collected from individual studies are available in Turkey [11]. *Salmonella* Typhimurium (*S.* Typhimurium) and *Salmonella* Enteritidis (*S.* Enteritidis) are the most predominant isolated organisms associated with the consumption of contaminated poultry, pork and beef meats and products [7]. Furthermore, the two most important serotypes of salmonellosis transmitted from animals to humans in most parts of the world [3]. Similarly, *S.* Typhimurium and *S.* Enteritidis are the most common serotypes isolated from humans in Turkey [14].

Many techniques, i.e. conventional culture, molecular biological and immunological, are being used for the detection of *Salmonella* spp.[10]. Culture based methods are still the most widely used detection techniques and remain the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity. Depending on the approach, standard culture methods typically require 5-7 days to obtain a result as they rely on the ability of *Salmonella* to multiply to visible colonies, which can then be characterized by performing additional biochemical and or serological tests [15]. Polymerase Chain Reaction (PCR) is a simple, rapid, very specific, and relatively inexpensive technique [5]. Currently, the use of PCR being one of the most promising approaches for the detection of *Salmonella* serotypes [5,9].

The objectives in this study were (1) to explore the prevalence of *Salmonella* spp. in the 100 raw chicken carcass samples obtained from butchers and supermarkets In Istanbul (2) to determine the incidence of *S.* Typhimurium and *S.* Enteritidis which are the two most dangerous strains for the public health among *Salmonella* spp. with PCR procedures (3) to compare the effectiveness of the PCR and conventional microbiological methods for the mentioned pathogens.

MATERIAL and METHODS

Sampling

One hundred raw chicken carcasses were collected between from different sales points (supermarkets (n=50) and butchers (n=50) in Istanbul, Turkey. Fifty samples were collected from European side (supermarkets (n=25) and butchers (n=25) and the other 50 samples were collected from Asian side of Istanbul (supermarkets (n=25) and butchers (n=25). Random sampling method was used during sampling period and middle class supermarkets and district butchers were preferred.

All the collected samples were consisted of raw chicken carcasses, the supermarket samples were packaged while the butcher samples were purchased open. Samples were transported to the laboratory after being collected in a thermobox under cold chain (+4°C) and microbiological analyses were carried out immediately.

Isolation and Identification

Samples of skin and muscle, amounting 25 g, taken from multiple parts of chicken carcasses were homogenized in a stomacher (Interscience, Saint Nom, France) with 225 ml with buffered peptone water (Oxoid CM 1049) for non–selective enrichment. After incubation at 37°C for 24 h, 0.1 ml was inoculated in 10 ml Rappaport Vassiliadis Soy (RVS) Broth (Oxoid CM 866) for selective enrichment and incubated at 42°C for 24 h. After selective enrichment procedure, a loopful of broth was streaked on Brilliant Green Phenol Red Lactose Sucrose (BPLS) Agar (Oxoid CM 263) and Xylose Lysine Desoxycholate (XLD) Agar (Oxoid CM 469) parallel and incubated at 37°C for 24 h. After the detection of presumptive colonies on agars, the colonies sub-cultured to Nutrient Agar (NA; Oxoid CM 003) were confirmed as *Salmonella* by inoculation on Triple Sugar Iron Agar (TSIA) (Oxoid CM 277), urea broth (Oxoid CM 071) and Lysine Iron Agar (LIA) (Oxoid CM 381), followed by incubation of the tubes at 35-37°C for 24-48 h. Finally, API 20E (bioMerieux® SA, Marcy l'Etoile, France) kits were used according to the manufacturer's directions for the determination of *Salmonella* spp. to the species level.

Serological Identification

Serotype identification of the 15 positive *Salmonella* strains performed according to the White-Kaufmann-Le Minor scheme with lam agglutination and serum neutralization tests [16]. According to agglutination tests, commercial phase 1 and phase 2 antisera and *Salmonella* somatic group (O) and flagella group (H) antigens provided by Difco (Becton Dickinson Co., New Jersey, USA) were used.

DNA Extraction

DNA extraction procedure was adapted from Oliveira et al.[17]. Bacteria were cultured on TSB for overnight at 37°C. 1 ml aliquot of broth was centrifuged at 2.000 x g for 4 min and the bacterial pellet resuspended in TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA) containing lysozyme (Sigma 7651) and incubated at 4°C for 30 min, after which 25 µl SDS and Proteinase K (20 mg/ml, Merck 124568) were added and incubated at 55°C for 30 min. Then, 500 µl phenol-chloroform pH 8.0 was added and DNA was precipitated with sodium acetate and cooled isopropanol and centrifuged 16.000 x g for 10 min at 4°C, following supernatant was removed and the pellet washed with 1 ml 80% cooled ethanol (Sigma 459844), the pellet being resuspended in 50 µl of TE and stored at -20°C.

PCR Analysis

PCR mix was as follows (final 25 µl); 2 µl DNA samples, 2.5 mM $MgCl_2$, 10 mM Tris–HCl pH 8.0, 5 mM KCl (0.2 mM from each nucleotide), each primer (Metabion International, Martinsried, Germany) 0.8 pmol/ml, 1 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania). The primer sequences used in PCR analysis are shown in *Table 1*. Initial denaturation heat was at 94ºC for 5 min. Then the heat treatments, 1 sec at 94ºC, 1 sec at 55ºC, and 21 sec at 72ºC for extension were applied. After 35 cycles, the procedure was completed with 7 min at 72ºC heat treatment for last elongation. Amplication products were analyzed in 1.2% (w/v) agarose gel containing 5 µl safe view (Abm, Richmond, Canada).

PCR Specification, Calculation of Specifity and Sensitivity

The relative sensitivities of PCR procedures are described as the rate of obtained PCR products to isolated cultures with reference methods [21]. Relative sensitivity (SE) and relative specific (SP) degrees of the PCR procedures applied in our study were calculated by using the formulas indicated below:

SE = PA value of PCR and reference culture methods/ N^+ X 100

PA: Positive Agreement

N^+: Number of positive samples obtained with reference isolation/identification methods

SP = NA value of PCR and reference culture methods/ N X 100

NA: Negative Agreement

N^-: Number of negative samples obtained with reference isolation/identification methods [22].

For determination of PCR sensitivity, reference *S.* Enteritidis, and *S.* Typhimurium strains were serially diluted with 0.1% peptone water (Oxoid CM 009) up to 10^{-9} concentration level (1-10 kob ml^{-1}) so that 5 replication. Grown strains were evaluated as 10^{-9} dilutions of that *Salmonella* serotypes and the strains were passage to NA, including 7 grams/liter yeast extract (Oxoid CM 019). Additionally, a non-*Salmonella* mixture consisted of 5 different non–*Salmonella* strains were treated with 0.1% peptone water up to 10^{-4} dilution concentration. For each *Salmonella* dilution, 1 ml of non-*Salmonella* mixture were added to the tubes that included *Salmonella* serotypes and the bacterial mixtures were incubated at 37ºC for 24 h. After the incubation period, each mixture was passed to RVS broth of 10 ml. Then, a last incubation at 37ºC for 24 h was applied to mixtures and 1 ml of final mixtures for each sample was stored for PCR procedures. Ten PCR replications for each dilution were applied, and the optimal dilution rate was calculated according to the procedures explained.

RESULTS

One hundred samples of retail chicken carcasses were analyzed for *Salmonella* spp. and the prevalence of *Salmonella* spp. was detected 15% in chicken carcass samples. Seven (46.6%) chicken carcasses samples obtained from Anatolian side and 8 (53.3%) chicken carcasses samples obtained from European side in Istanbul of the total 15 *Salmonella* positive carcass samples. *Table 2* shows that the *Salmonella* contamination rates were 73.3% (11/15) and 26.6% (4/15) in butcher and supermarket originated chicken carcasses, respectively.

Salmonella spp., *S.* Enteritidis and *S.* Typhimurium was determined at rate 53.3% (8/15), 26.6% (4/15), and 20% (3/15) of *Salmonella* positive samples, respectively. *S.* Enteritidis isolated only one (25%) supermarkets originated chicken carcass samples from Asian side, Istanbul. On the other hand, all the *S.* Typhimurium positive (n=3) chicken samples originated from butchers (one (33.3%) samples

Table 1. *The properties of primer sequences designed according to different Salmonella serotypes*
Tablo 1. *Farklı* Salmonella *serotiplerine göre dizayn edilen primer dizilerinin özellikleri*

Gene/bp	Virulence Factor	Primers 5′ – 3′	Target Microorganism	Reference
*inv*A/284	Invasion	F-GTGAAATTATCGCCACGTTCGGGCAA R-TCATCGCACCGTCAAAGGAACC	*Salmonella* spp.	[18]
*fli*C/620	Flagella	F-CGGTGTTGCCCAGGTTGGTAAT R-ACTGGTAAAGATGGCT	*S.* Typhimurium	[19]
*sef*A/488	Fimbria	F-GATACTGCTGAACGTAGAAGG R- GCGTAAATCAGCATCTGCAGTAGC	*S.* Enteritidis	[20]

Table 2. *Results of the Salmonella positive samples*
Tablo 2. *Salmonella pozitif örneklere ait sonuçlar*

Species	Sales Point/ *A or E	Conventional Culture Method	PCR Verification
Salmonella spp.	Supermarket (A)	+	+
Salmonella spp.	Supermarket (A)	+	+
Salmonella spp.	Supermarket (E)	+	+
Salmonella spp.	Butcher (A)	+	+
Salmonella spp.	Butcher (E)	+	+
Salmonella spp.	Butcher(A)	+	+
Salmonella spp.	Butcher (E)	+	+
Salmonella spp.	Butcher (E)	+	+
S. Typhimurium	Butcher (A)	+	+
S. Typhimurium	Butcher (E)	+	+
S. Typhimurium	Butcher (E)	+	+
S. Enteritidis	Butcher (E)	+	+
S. Enteritidis	Supermarket (A)	+	+
S. Enteritidis	Butcher (E)	+	+
S. Enteritidis	Butcher (A)	+	+

***A** or **E:** Anatolian side or European side of Istanbul*

For determining PCR sensitivity and detection limits, different concentrations of *S.* Enteritidis and *S.* Typhimurium were prepared, and the minimal detection of concentration levels were tried to determine for the mentioned strains. The minimal PCR detection limit was 8 cells for *S.* Typhimurium and $1.8x10^3$ cells for *S.* Enteritidis, respectively.

DISCUSSION

Salmonella spp. continues to be a leading cause foodborne illness. Raw poultry, meats, and meat derived products are important vehicles of human salmonellosis; however, increasingly, illnesses are associated with the consumption of fresh products and dry food products [23]. Chicken and chicken products are widely known to be an important reservoir for *Salmonella*, and they have been ascribed as vehicles of salmonellosis [6-8]. In this study the incidence of *Salmonella* in raw chicken carcasses was 15%. Similar results reported by Zahreai Salehi et al.[9] who isolated *Salmonella* spp. at rate of 15.6% (Iran), and Todd [24] reported *Salmonella* prevalence of 13.3% in retail chicken in Ethiophia. Another study conducted by Alali et al.[25] in Russia Federation showed the incidence of *Salmonella* spp. in retail chicken to be 27%. Our results of contamination rates with *Salmonella* lover than those observed by das Chagas et al.[5] 94% (Brazil), Capita et al.[6] 55% (Spain), Yang et al.[8] 52.2% (China), Abd El-Aziz [1] 44% (Egypt) and Uyttendaele et al.[7] 37% (Belgium). Not much more reference has been available on the presence of *Salmonella* in meat from Turkey, few researchers reported prevalence as low as 10% [10,11,13]. The differences in these contamination percentages are probably related to numerous factors, including the origin of the chicken lots, the sampling methods, microbiological analysis methods, the hygiene-sanitary conditions in the abattoirs, and cross-contamination that occurred during plucking, washing, cooling and wrapping [25].

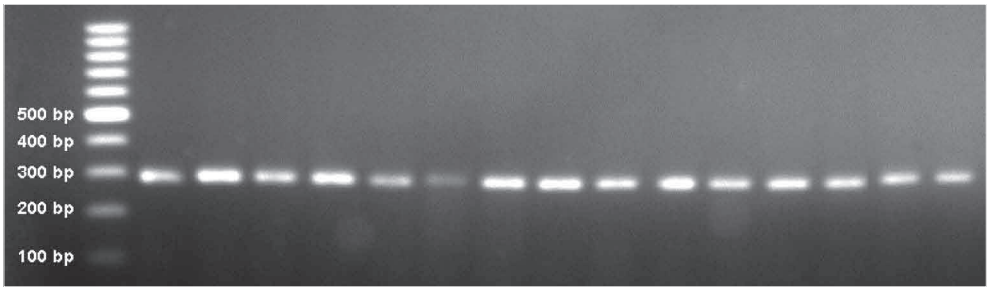

Fig 1. Agarose gel electrophoresis of the amplified products of PCR to determine strains of the *Salmonella* spp. (*inv*A gene, 284 bp). Lane 1: 100 bp marker (Thermoscientific), Lane 2-16: positive *Salmonella* strains

Şekil 1. Tespit edilen *Salmonella* spp. (*inv*A geni, 284 bp) suşlarının amplifiye PCR ürünlerinin agaroz jel elektroforezi. Sıra 1: 100 bp marker (Thermoscientific), Sıra 2-16: Pozitif *Salmonella* suşları

in Asian side and two (66.7%) samples from European side, Istanbul. Additionally, *Salmonella* spp. contamination rate were 37.5% (3/8) and 62.5% (5/8) in supermarket and butcher originated chicken carcasses, respectively. *Salmonella* spp. isolated 33.3% (1/3) supermarkets originated chicken carcass samples from European side, Istanbul. Additionally, 3 (60%) positive *Salmonella* spp. butchers originated chicken carcass samples from European side, Istanbul.

All the strains were isolated by conventional culture method and confirmed by PCR *(Table 2)*. At the PCR optimization process, the usage of magnesium chloride at a concentration of 2 mM gave the best band results for *sefA* genes. For the other two genes (*inv*A and *fli*C target genes) any significant differences was observed at the amplification period. At the end of the PCR process, 284 bp amplification products were obtained for the *invA* targets for all the *Salmonella* spp. positive samples *(Fig. 1)*.

As shown *Table 2*, the contamination rate in butcher's carcasses (73.3%) was higher than in supermarket carcasses

(26.6%). Additionally, the contamination rate of *Salmonella* was higher (53.3%) chicken carcasses originated in European side, Istanbul. Similarly, Plummer et al.[26] detected a lower number of *Salmonella* supermarkets originated carcasses (18.6%), than from shops (24.5%). Conversely, Capita et al.[6] was found the *Salmonella* prevalence 75% and 25% in supermarket carcasses and poultry shops carcasses, respectively. The differences can be related continue temperature control of refrigerators or ambient temperature in supermarkets by responsible persons.

Two different serotypes, *S.* Enteritidis and *S.* Typhimurium, were found as seen from *Table 2*. *S.* Enteritidis was the most commonly isolated serotype (26.6%) in our study. The high percentage of *S.* Enteritidis in chicken samples with the results obtained by other researchers [5,7,11,26]. Additionally, 3 (20%) samples were evaluated as positive *S.* Typhimurium. One of the most important characteristics of *S.* Typhimurium in meat and its products, is the tolerance to acidic media. The elevated presence of this serotype in chicken products agrees with *S.* Enteritidis being the predominant serotype associated with outbreaks due the consumption of eggs [6]. Accordingly, *S.* Enteritidis, *S.* Typhimurium, *S.* Paratyphi B and *S.* Typhi are the most common serotypes isolated from humans [14] and poultry [27] in Turkey.

In current study, *inv*A gene used for the detection for *Salmonella*. Similarly, Rahn et al.[18] indicated that different *Salmonella* serotype have *inv*A gene. This gene is recognized internationally as a standard for detecting the genus *Salmonella*, and its amplification has been used by many researchers to detect contamination in chicken carcasses [5,9,17,20]. For *S.* Typhimurium, DNA fragments that had, *fliC* target genes were amplified (620 bp) while, DNA fragments that had *sefA*/*inv*A target genes were amplified for *S.* Enteritidis (488 bp) *(Table 2)*. Similar to the our study, das Chagas et al.[5], Oliviera et al.[17], and Doran et al.[20], were used *inv*A gene, *fli*C and *sef*A gene for the detection of *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis, respectively. The *fli*C gene is responsible for the expression of a protein known as flagellin in *Salmonella* spp. the *sef*A gene codes for the fimbrial protein SEF14 that has unique specifity to *S.* Enteritidis and its amplification can be used identify this serotype [28].

PCR sensitivity was determined as 8 cells for *S.* Typhimurium while mentioned value was 300 cells according to Rahn et al.[18]. By using conventional microbiological methods, to isolate one or several target microorganisms, especially for *Salmonella* spp. from a food sample may be very hard. Accuracy and sensitivity of the dilution, long time of isolation procedure, a requirement of professional hand practices, and qualities of the media that is used are the main factors that complicate the isolation and identification procedures of *Salmonella* spp.[29]. Because of the reasons explained, it was thought that correct PCR procedures may be a good alternative to microbiological methods for an exact identification for *Salmonella* spp.. The PCR procedures that we used gave quite specific results for *S.* Typhimurium at a serotype level. By specification of target primers, *S.* Typhimurium positive evaluated samples by microbiological methods exactly matched with the PCR results while the other *Salmonella* strains positive results did not. Moreover, the results can be obtained average 5 days earlier by PCR procedures when compared with conventional microbiological methods.

According to the results, PCR sensitivity was about $1.8x10^3$ cells level for *S.* Enteritidis and this rate was quite low when the PCR procedure was compared with conventional microbiological methods, because, we were able to identify *S.* Enteritidis colonies from $1x10^1$ colonies concentration level by using microbiological procedures. Doran et al.[20] declared that they could identify *S.* Enteritidis from 10 cells of concentration level while Woodward and Kirwan [30] could identify the same microorganism from 4 cells. The reason explained above, may be the cause of low degree sensitivity of PCR for *S.* Enteritidis. Another possible cause may also be the hybridization of mobile DNA fragments through polymorphic proteins in spite of *sef*A primer is specific for *S.* Enteritidis. It is thought that detailed genomic DNA studies would help to clarify genomic and biochemical mechanisms of *Salmonella* strains.

The prevalence of *Salmonella* spp. was relatively high from raw chicken carcasses in Istanbul. The most predominant serotypes are *S.* Enteritidis and *S.* Typhimurium in chicken samples. On the other hand, PCR results were quite specific and sensitive for *Salmonella* spp. and *S.* Typhimurium in our study. For the identification of mentioned two strains, PCR procedures may be a good alternative to microbiological isolation and identification methods. However, results about *S.* Enteritidis were not effective as *Salmonella* spp. and *S.* Typhimurium. Because of the reason explained, using microbiological identification methods for *S.* Enteritidis, would be more effective than PCR procedures for diagnosis and public health.

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Research Article

Comparison of Flow Cytometric Analysis and Eosin-Nigrosin Staining Methods for Determining some Morphological Characteristics of Bull Epididymal Spermatozoa [1]

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Abstract

The aim of this study is to investigate necrosis and apoptosis in epididymal bull spermatozoa before freezing and after thawing using the flow cytometric method and to compare this with eosin-nigrosin dyeing, which is the conventional method used in assessing of spermatozoa. The testicles from fourteen bulls at local slaughterhouse were used for this study. The proportions of live spermatozoa, total apoptotic, necrotic and early necrotic spermatozoa levels were observed via flow cytometry. Annexin V/PI fluoruscence dyeing was used to investigate the proprotions of apoptotic, necrotic, early necrotic and live spermatozoa for flow cytometry. The proportion of dead spermatozoa and protoplasmic droplets were determined using the eosin-nigrosin conventional dyeing method in fresh and frozen-thawed spermatozoa. The average dead spermatozoa count with flow cytometry was less than with the eosin-nigrosin method ($P<0.05$). Some morphological characteristics such as protoplasmic droplets could be determined with the eosin-nigrosin method; however, sperm subpopulations entering the death process (apoptotic, necrotic and early necrotic) could be defined clearly only with the flow cytometric method. As a result, combination of eosin-nigrosin dyeing method and flow cytometric analysis of sperm morphological evaluation could give better results of bull epididymal semen in comparison to eosin-nigrosin dyeing method alone.

Keywords: *Flow cytometry, Eosin-nigrosin, Morphological characteristics, Epidydimal spermatozoa, Bull*

Epididimal boğa Spermatozoonlarının Bazı Morfolojik Özelliklerinin Belirlenmesi Amacıyla Akım Sitometri ve Eozin-Nigrozin Boyama Yöntemlerinin Karşılaştırılması

Özet

Bu çalışmanın amacı, epididimal kaynaklı boğa spermatozoonlarının taze ve dondurulup çözdürüldükten sonra akım sitometri (Flow Cytometri) yöntemiyle analiz edilmesi, nekroz/apoptoz düzeylerinin incelenmesi ve klasik boyama yöntemi olan eozin-nigrozin boyama metoduyla kıyaslanması amaçlanmıştır. Bu amaçla yerel mezbahada kesilen 14 boğanın epididimislerinden elde edilen spermatozoonlar kullanılmıştır. Canlı spermatozoa, toplam apoptotik, nekrotik ve erken nekrotik spermatozoa düzeyleri akım sitometri yöntemiyle tespit edilmiştir ve bu amaçla AnnexinV+PI (Propidium İyodür) floresan boyama yöntemi kullanılmıştır. Taze ve çözdürülmüş sperma içerisindeki, ölü spermatozoa ve protoplazmik damlacık taşıyan spermatozoonların oranları eozin-nigrozin boyama yöntemi ile belirlenmiştir. Taze spermada, akım sitometri ile ölü spermatozoon sayısı eozin-nigrozin boyamada bulunana göre daha az olduğu görülmüştür ($P<0.05$). Protoplazmik damlacık gibi bazı morfolojik parametrelerin mikroskopik muayenelerle belirlenebildiği ancak ölüm sürecine giren spermatozoonların alt kategorilerinin (apoptotik, nekrotik, erken nekrotik) akım sitometri ile net olarak tespit edilebildiği görülmüştür. Sonuç olarak, klasik yöntemlere göre spermatozoa canlılığı tespitinde akım sitometri yönteminin klasik boyama yöntemlerine destek olarak ve daha etkin bir şekilde kullanılabileceği düşünülmüştür.

Anahtar sözcükler: *Akım sitometri, Eozin-nigrozin, Morfolojik özellikler, Epididimal spermatozoa, Boğa*

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INTRODUCTION

There are many ways to investigate spermatozoa to find out their morphological intactness, and motility or diagnose whether they are dead or alive. The fertilization capability of spermatozoa largely correlates with their morphological structure and motility. Various conventional staining methods (such as eosin-nigrosin, papanicolaou, mygrunwald-giemsa, hematoxylin-eosin) are used to determine whether spermatozoa are dead or alive. There seems to be no difference among them regarding effectiveness [1].

With the eosin-nigrosin staining methods eosin stains the spermatozoa with damaged membranes while nigrosin stains the background of the spermatozoa. A darker color thus, stained spermatozoa can be examined more easily due to the contrast between these dyes [2].

Some fluorescent probes are used for flow cytometric analysis and allow many spermatologic parameters to be investigated in a short time, quantitatively. In addition, the flow cytometrical method presents data with minimal statistical errors and enables dead and live spermatozoa to be determined spermatozoa subpopulations such as early necrotic, apoptotic can be singled out with this method [3,4].

There are some contradictory views concerning the correlation between apoptosis and some spermatologic parameters. Some researchers [5-10] showed negative correlation with sperm concentration, motility and normal sperm morphology [11]. It was reported that there was no relationship between sperm morphology and apoptosis [11,12].

A fluorescent probe which dyes all of the cells and propidiumiodude (PI), which dyes only dead cells, are used together for flow cytometric analysis to determine the percentages of live and dead spermatozoa [13]. Phosphatidyl serine (PS) allocated in the plasma membrane of the cytoplasmic side normally translocate from the inner face of the plasma membrane to the cell surface. This replacing occurs early period of apoptosis for most mammalian cell types. PS translocate to the cell surface can be detected by staining with fluorescein isothiocyanate (FITC) labeled annexine V (annexine V-FITC), a protein with an affinity for PS. Apoptotic cells can be defined by Annexine-V and PI in flow cytometry and this way is also reliable, simple and fast [14,15].

The aim of this study is to investigate necrosis and apoptosis in epididymal bull spermatozoa before freezing and after thawing using the flow cytometric method and to compare this with eosin-nigrosin dyeing, which is the conventional method used in assessing of spermatozoa.

MATERIAL and METHODS

Epididymal Sperm Collection

Testicles from 14 crossbreed bulls which did not have any morphological abnormalities were provided at the local slaughterhouse. Testicles were transported to the laboratory in styrofoam boxes within two hours. Semen was collected from each epididymis separately. Epididymal semen was recovered using the modified retrograde flushing (RF) method [16]. No semen extender was used for this process except air pressure. Cauda epididymis and ductus deferens were isolated from testicle tissues and the lumen of the ductus deferens was cannulated and perfused with air. The cauda epididymis was incised to recover pure epididymal semen after being swelled by air pressure.

Sperm Evaluation

Spermatozoa concentration was determined by Neubauer haematocytometer before further processing of semen. The sperm samples from all the groups were evaluated for total motility and morphology. Total, motility was assessed subjectively (to the nearest 5%) by phase contrast microscopy (magnification 200) after dilution with the semen extender at 37°C.

Sperm morphology was evaluated with a phase contrast microscope at 1000x magnification after eosin-nigrosin staining. Two separate smears were prepared for each epididymis. A total of 200 cells were counted and the results are presented as percentages (spermatozoa viability and spermatozoa with a proximal and distal protoplasmic droplet).

Sperm Freezing

Tris-citric acid extender containing egg yolk was used as a cryodiluent (pH 7.0; osmotic pressure, -300 mOsm/kg). The spermatozoa were diluted in this extender, fructose 0.2% (wt/vol; Merck, Germany), glycerol (7%; vol/vol; Merck, Germany) and egg yolk 20% (vol/vol).

The spermatozoa collected from each pair of testicls were pooled and diluted with extender in a single step ($40x10^6$ spermatozoa/ml). Diluted semen was packed in straws (0.25 ml, IMV, France) and they cooled to 4°C for 3 h. The straws were kept above liquid nitrogen vapours for 10 min and then plunged into liquid nitrogen. Semen was used after 24 h, semen straws were thawed at 37°C for 30 sec in water bath for assessment of postthaw evaluation.

Flow Cytometric Analysis

To detect apoptosis, sperm cells were stained and analyzed by BD FACS ARIA II flow cytometry. For staining firstly epididymal semen was centrifuged at 500 g twice for 10 minutes after being diluted in PBS (FITC/Annexin V-BD Pharminge-US). The binding solution was added after removing the supernatant, and the spermatozoa concentration was adjusted to $1x10^7$ cells/ml. 100 µl of extended semen were transferred from the last mixture to 5 ml tubes and 5 µl *fluorescein* isothiocyanate (and 5 µl

propidium iodide (PI) were added to this mixture. Aliquots of semen were diluted in annexin-V-binding buffer [10 mMHepes/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$] to a concentration of $1x10^6$ cells/ml. These tubes were incubated for 15 min at room temperature in the dark after being gently mixed. An additional 400 µl of binding buffer was added and the final concentration of $1x10^5$ cells/ml was inserted into each tube prior to flow cytometric evaluation, which was conducted within 30 min.

The total spermatozoa population was detected for each sample prior to detailed evaluation, following which necrotic (Q2-1), early necrotic (Q1-1), apoptotic (Q4-1) and live spermatozoa (Q3-1) were evaluated in the charts *(Fig. 1)*.

Statistical Analysis

The data were expressed as arithmetic means and standard error (X ± SEM). Statistical analyses were performed using the SPSS 13.0 version for Windows (SPSS Inc., Chicago, IL, USA). Independent Samples *t*-test was performed to estimate the statistically significant difference between the percentages of fresh and frozen-thawed spermatozoa concentrations with total motility. Independent Samples *t*-test was performed to estimate the statistically significant difference between the percentages of dead spermatozoa with eosin-nigrosin staining and necrotic spermatozoa concentrations with flow cytometric analysis in fresh spermatozoa. The differences were considered significant when the P value was less than 0.05.

RESULTS

The average motility was 75.36% and 41.07% in fresh and frozen thawed semen samples (n=14) respectively. The difference of between fresh and frozen thawed semen was significant (P<0.05). The percentage of dead spermatozoa was 16.71% with the eosin-nigrosin staining method but 11.18% dead (necrotic), 27.36% early necrotic and 3.01% apoptotic spermatozoa were found with flow cytometric analysis in fresh semen *(Table 1)*. The percentage of dead spermatozoa was 14.00% with the eosin-nigrosin staining method after thawing. In the thawed semen, 13.62% dead (necrotic), 29.14% early necrotic and 4.89% apoptotic spermatozoa were found by flow cytometric analysis. The difference in the rates of dead sperm was statistically significant between the flow cytometric analysis and eosin-nigrozin staining methods (P<0.05) *(Table 1)*.

Dead sperm rates were found to be statistically different between fresh and frozen with eosin-nigrosin staining (P<0.05). Flow cytometric examination indicated that the number of necrotic, early necrotic and apoptotic sperm were higher in the frozen semen, but these differences were not statistically significant (P>0.05) *(Fig. 2)*.

DISCUSSION

The present study compared the findings obtained from two methods widely used for the detection of live

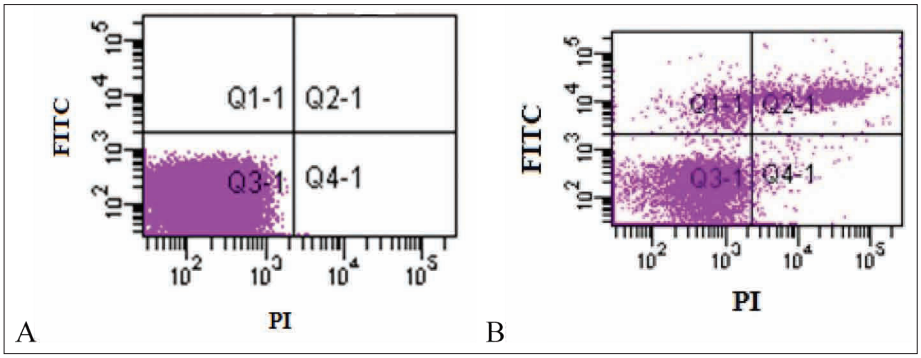
Table 1. *Comparison of eosin-nigrosin and flow cytometric analysis for fresh and after thawing epididymal spermatozoa*
Tablo 1. *Taze ve çözdürme sonrası epididimal spermatozoanın eosin-nigrozin boyama ve akım sitometrik analiz ile kıyaslanması*

Methods	Parameters (%)	Fresh (Mean±S.E.M)	After Thawing (Mean±S.E.M)
Eosin-nigrosin	Total motility	75.36±2.37	41.07±2.73
	Dead spermatozoa*	16.71[a]±1.18	14.00±0.91
	Proximal droplet	3.89±0.45	3.26±1.41
	Distal droplet	19.54±0.67	17.91±1.07
Flow cytometry	Live spermatozoa	58.24±5.18	52.37±4.42
	Necrotic sp.*	11.18[b]±1.17	13.62±1.79
	Apoptotic sp.	3.01±0.70	4.89±0.76
	Early necrotic sp.	27.36±4.31	29.14±2.72

** Means within same columns with different superscripts differ significantly (P<0.05)*

Fig 1. A- Number of total sperm cell of flow cytometric measurement, **B-** Necrotic (Q2-1), early necrotic (Q1-1), apoptotic (Q4-1) and live spermatozoa (Q3-1)

Şekil 1. A- Akım sitometrisinde toplam sperm sayıları, **B-** Nekrotik (Q2-1), erken nekrotik (Q1-1), apoptotic (Q4-1) ve canlı spermatozoa (Q3-1)



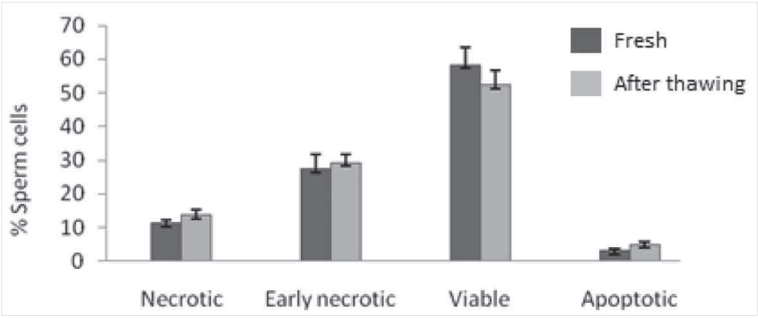


Fig 2. Different sperm subpopulations averaged fourteen bulls (mean ± SEM), fresh semen and after thawing

Şekil 2. 14 farklı boğaya ait taze ve çözdürülmüş sperm alt populasyonlarınının ortalamaları (mean ± SEM)

and dead spermatozoa (eosin-nigrosin staining and flow cytometry). The eosin-nigrosin staining method accounted for statistically higher percentage of dead sperm than the flow cytometry analysis ($P<0.05$).

Flow cytometric analysis were quantitatively clearly identified phase of cell dead processing for example early necrotic, necrotic, apoptotic sperm and live spermatozoa rates although dead spermatozoa could be determined subjectively with eosin-nigrosin staining. Some morphological characteristics, such as distal and proximal droplets on spermatozoa, can be determined with the eosin-nigrosin staining method; however, this observation was not possible with flow cytometry.

There is no significant difference among staining methods (Eosin-nigrosin, Hematoxylin-eosin, Caserette stains etc.) of spermatozoa based on the findings [17]. The eosin-nigrosin staining method is widely used for the evaluation of sperm because it is simple and rapid, but this conventional staining method for the evaluation of sperm is very subjective because only a very limited number of dead sperm can be counted [18].

Dyeing time, sperm concentration, characteristics of the semen extenders or some factors affecting the stability of the sperm membrane intactness are involved in eosin dyeing processes could alter the ratio of intact sperm [2,19].

Chalah and Brillard [20] reported that in terms of sperm membrane integrity flow cytometric analysis was a more effective method compared to eosin-nigrosin staining. Foster et al.[21] reported that number of live sperm with eosin-nigrosin was about 12.5% more compared to flow cytometric measurement. Johansson et al.[18] datas' were compatible with Foster's finding [21].

In the present study, the number of dead sperm with eosin-nigrosin staining method was higher than the data obtained by flow cytometric analysis. The reason for this was that early necrotic sperm cells were not considered to be dead sperm. When the numbers of early necrotic and necrotic sperm are accepted together as dead cell, our findings were similar to those of Foster et al.[21] and Johansson et al.[18]. The live sperm ratio can be determined inaccurately since the early necrotic sperm cell counts could vary depending on the amount of dye with eosin-nigrosin staining method.

Recent research shows apoptosis as an important mechanism that regulates spermatogenesis. Spermatozoa potential is greatly reduced during spermatogenesis and this continues throughout life. The number of germ cells can be supported by the sertoli-cells by eliminating abnormal spermatozoa via apoptosis [15,22-24].

Apoptotic cells, early necrotic and necrotic cell can be detected by flow cytometry, and but cannot be detected with conventional dyeing methods. In contrast, with the eosin-nigrosin method, morphologically abnormal spermatozoa can be distinguished. Relationships between abnormal morphological characteristics and the low fertilization ability of spermatozoa can be versatile. In this respect, the relationship between fertility capabilities and the morphological assessment of spermatozoa is difficult to define [25].

The main purpose of sperm analysis is to evaluate the sperm sample in a realistic, inexpensive, objective and quick way in terms of fertility. However, these criteria cannot be achieved in many laboratories and the findings cannot demonstrate clearly the impact on fertility [26]. Cell shape and structural features, such as morphological characteristics labeled with a fluorescent dye, can be determined by flow cytometry. Moreover, spermatozoa can be evaluated for many parameters and fertility properties simultaneously and objectively [27,28]. In addition, it is possible to evaluate a sample with tens of thousands of sperm in a few minutes by flow cytometry and it can offer a high level of reliable information compared to fluorescent microscope evaluation [29]. The subjective methods used to evaluate spermatozoa with flow cytometry may be a better estimate of fertility. In addition, conventional laboratory tests for predicting fertility in spermatozoa along with the use of flow cytometry can give better results [30].

In a study that investigated the sperm membrane using the eosin-nigrosin staining method, flow cytometric analysis, the samples with high levels sperm membrane integrity gave compatible results with these three methods. However, in cases where a high level of sperm membrane damage is observed, eosin-nigrosin staining gives more variable results compared to flow cytometric analysis [21].

In this study, the number of dead sperm obtained with eosin-nigrosin staining was higher than that found by flow cytometry (P<0.05). Our findings were similar to Foster et al.[21]. These findings may indicate that the eosin-nigrosin technique has many limitations, including a relatively low number of sperm (often only 100 or 200 cells) typically counted and subjectivity in the sperm sample evaluation as reported by Johanson et al.[18].

Klimowics-Bodys et al.[31] reported that the eosin-nigrosin staining method used in the morphological assessment of spermatozoa was cheap and practical, but this technique allows the assessment of a limited number of spermatozoa. Thousands of spermatozoa can be examined by flow cytometry in a short time; thus, it is a valuable and objective method compared to conventional methods.

In the present study, using the eosin staining method for the detection of live or dead spermatozoa was a subjective assessment that, in this respect, may result in some errors in sperm assessment depending on different levels of dye uptake. However, the apoptotic or necrotic phases of spermatozoa with flow cytometric methods can be clearly identified and the information can be obtained in more detail by this method compared to eosin-nigrosin staining method.

In this respect, determining the viability of spermatozoa with flow cytometry is more effective than conventional staining methods such as eosin-nigrosin. Flow cytometric analysis can be used to support conventional methods. As a result of this study, eosin staining method plus flow cytometry can be considered an advantage for the morphological assessment for epididymal bull spermatozoa. Further studies are necessary to determine the relationships between the morphological characteristics and motility of early necrotic and apoptotic spermatozoa.

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Research Article

Effects of Chitosan Oligosaccharides Addition to Japanese Quail's Diets on Growth, Carcass Traits, Liver and Intestinal Histology, and Intestinal Microflora

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Abstract

This research was conducted to determine effects of chitosan oligosaccharides (COS) addition to quail diets on growth, carcass traits, liver and intestinal histology, and intestinal microflora. Two hundred forty Japanese quail chicks were distributed among three treatments groups, with four replicates. A group was fed with a basal starter diet for 1-21th and a grower diet for 22-42th days (Control). The experimental groups were fed the same diets, in addition to 75 mg/kg (Trial I) or 150 mg/kg (Trial II) of COS. The final live weights of the quails in the Control and Trial I groups were higher than in the Trial II group. There were no differences among the groups in gain, feed intake, feed conversion, and carcass traits. Steatosis in the Trial II group was less than in the Control and Trial I groups. Crypt depth and villus length were higher in the Trial II group than in the other groups. The number of bacteria and yeast in the intestine were lower in the Trial I and II groups than in the Control group. In conclusion, the addition of 75 mg/kg of COS had no adverse effect on the tested parameters, and it increased the crypt depth, villus length, and beneficially on intestinal microflora.

Keywords: *Quail, Chitosan oligosaccharides, Growth performance, Villus, Steatosis, Intestinal microflora*

Bıldırcın Rasyonlarına Kitosan Oligosakkarit İlavesinin Besi Performansı, Karkas Özellikleri, Karaciğer ve Barsak Histolojisi ile Barsak Mikroflorası Üzerine Etkisi

Özet

Bu çalışma, bıldırcın rasyonlarına farklı oranlarda kitosan oligosakkarit (KOS) ilavesinin besi performansı, karkas verim özellikleri, karaciğer ve barsak histolojisi ile barsak mikroflorası üzerine etkilerini belirlemek amacıyla yapıldı. Araştırmada 240 adet Japon bıldırcını kullanıldı. Civcivler herbiri dört alt gruptan oluşan üç ana gruba ayrıldı, Kontrol grubu temel başlangıç (1-21. gün) ve büyütme (22-42. gün) yemleriyle beslendi. Deneme grupları araştırma süresince bu yemlere 75 mg/kg (Deneme I) veya 150 mg/kg (Deneme II) KOS ilave edilerek beslendi. Araştırma sonu itibariyle Kontrol ve Deneme I grubunun canlı ağırlığı Deneme II'den önemli derecede yüksek bulundu. Canlı ağırlık artışı, yem tüketimi, yemden yararlanma oranı ve karkas verim özellikleri bakımından gruplar arasında farklılık görülmedi. Deneme II grubundaki bıldırcınların karaciğerindeki yağlanmanın Kontrol ve Deneme I'den daha az olduğu gözlendi. Kript derinliği ve villus uzunluğu Deneme II grubunda diğer gruplardan önemli derecede yüksek bulundu. İnce barsaklardaki bakteri ve maya sayıları Deneme I ve II gruplarında Kontrol grubundan daha düşük bulundu. Sonuç olarak, bıldırcın rasyonlarına 75 mg/kg kitosan oligosakkarit ilavesinin incelenen parametreler üzerinde olumsuz bir etki oluşturmadığı, kript derinliğini ve villus yüksekliğini artırdığı, ince barsak mikroflorası üzerinde faydalı olduğu tespit edilmiştir.

Anahtar sözcükler: *Bıldırcın, Kitosan oligosakkarit, Besi performansı, Villus, Steatosis barsak mikroflorası*

INTRODUCTION

The use of antibiotics as feed additives, caused residues in animal tissues, and by this resulted in a decrease in the effectiveness of antibiotics in therapy. The using of antibiotics as feed additive was banned in the European Union in 2006 [1]. Since the ban, various oligosaccharides, which are natural feed additives, also called prebiotics,

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have been used in poultry feed to support growth, improve gut microbial flora, and strengthen the immune system [2-4]. Prebiotics are defined as non-digestible food ingredients that have a beneficial effect on the host animal by selectively stimulating the growth or activity, or both, one or more types of bacteria in the colon [5].

Chitosan and chitosan oligosaccharides (COS), which are recognized as prebiotics, are obtained from chitin via chemical and enzymatic hydrolysis. Chitin, a cellulose-like polymer, is found in the exoskeletons of arthropods, such as crabs, shrimps, lobsters, and insects [6]. Due to the high molecular weight, insolubility, allergic, and high viscosity of chitin, its usefulness as a natural feed additive is limited [7]. Chitosan, especially COS, has a lower molecular weight, higher solubility, and lower viscosity than chitin [8].

Studies conducted in different animals have shown that COS improves growth performance and that it has hypocholesterolemic, antidiabetic, antitumoral, antifungal, antioxidant, and immune strengthening functions [9], as well as free radical scavenging properties [10]. The addition of COS to broiler chicken diets improved their live weight, feed intake [11], and feed conversion [3]. However, Keser et al.[12] reported that the addition of COS to broiler chicken diets had no effects on their growth. Zhou et al.[11] found that the addition of this additive to broiler chicken diets had no impact on breast meat and gizzard weight but increased the liver weight.

Other studies reported that undigested oligosaccharides in the intestinal tract promoted beneficial microorganisms and increased both the villus length and crypt width in the digestive system of broiler chickens [13,14].

Chitosan and COS have antimicrobial effects on bacteria, yeast, and fungi. Chitosan and its oligomers have a stronger bactericidal effect on gram (+) bacteria. The effect of chitosan depends on its origin, molecular weight, and pH [14]. Previous studies of the effects of oligosaccharides on gut microbial flora of poultry demonstrated that they reduced colonization by *Salmonella* and *Escherichia coli* [13,15].

The present study was conducted to determine the effects of the addition of different levels of COS to quail diets on growth performance, carcass traits, liver and intestinal histology, and intestinal microflora.

MATERIAL and METHODS

Experimental Animals, Experimental Design, Diets, Management

The ethical committee approval of Kafkas University (KAÜ-HADYEK: 2014-028) was taken in order to conduct this study.

Two hundred forty 1-d-old unsexed Japanese quail chicks *(Coturnix coturnix japonica)* were used in this study. They were randomly divided into three main groups with four replicates of 20 chicks each. The study lasted for 6 weeks, with the first 3 weeks as the starter period and the last 3 weeks as the grower period. In the starter and grower periods, the birds were fed a basal diet *(Table 1)* as recommended by the NRC [16]. One of the main groups was fed these basic diets (Control), and the other groups were fed the same diets, but 75 mg/kg of COS (Trial I) or 150 mg/kg of COS (Trial II) added their diets. The COS (GlycoBio Company, Dalian, China) used in this study contained 40% COS and 60% cyclodextrine as a carrier. The birds were housed in wire cages, and feed and water were available at all times during the experimental period.

Data Collection

Live weights of quails and also feed intake of each group were recorded weekly. The feed conversion was also calculated on a group basis. At the end of the experiment, 16 quails from each group (two males and two females

Table 1. *Composition and nutrient content of the diets used in the study, %*
Tablo 1. *Araştırmada kullanılan karma yemlerin bileşimi ve besin madde içerikleri, %*

Ingredients	Starter Diet 1 to 21th days	Grower Diet 22 to 42th days
Corn	30.75	42.50
Soy bean meal	21.45	24.00
Sunflower meal	10.00	10.00
Wheat	20.00	18.25
Full fat soybean	11.00	-
Vegetable oil	3.50	2.00
DCP	1.60	1.50
Lime stone	0.66	1.00
Vit. Min. prem.*	0.35	0.35
Colin chloride	0.10	-
Salt	0.25	0.30
Methionine	0.20	0.10
Lysine	0.06	-
Threonine	0.06	-
Sodium bicarbonate	0.02	-
Nutritional content, DM basis		
Dry matter	92.20	91.08
Metabolic energy, kcal/kg**	3019	2910
Crude protein	22.25	20.22
Crude fat	7.91	4.65
Crude fibre	5.34	4.99
Crude ash	8.28	7.08

** **Per 2,5 kg containing following nutrients:** Vit A: 6.000.000 IU, Vit D_3: 32.000.000 IU, Vit E: 40.000 mg, Vit K_3: 1.600 mg, Vit B_1: 1.200 mg, Vit B_2: 3.200 mg, Niacin: 24.000 mg, Cal.D-Pantothenate: 7.200 mg, Vit B_6: 2.000 mg, Vit B_{12}: 6,4 mg, D-Biotin: 80 mg, Folic acid: 800 mg, Vit C: 40.000 mg, Manganese: 42.000 mg, Iron: 33.600 mg, Zinc: 33.600 mg, Copper: 3.600 mg, Cobalt: 80 mg, Iodine: 400 mg, Selenium: 72 mg, Molybdenum: 416 mg; ** Provided by calculation [17]*

from each subgroup) were slaughtered for determination of carcass traits. The carcasses were cut into parts using a method described previously [17].

Analyses of Feed Contents

The composition of dry matter, crude protein, crude fiber, crude fat, and crude ash of the feeds used in the experiment were determined according to the procedure in AOAC [18].

Histological Analyses

Liver and ileum tissue samples were also taken from the slaughtered quails. Cryostat (Leica CM 1510 S, USA) sections (5 µm thick) were taken to determine the amount of fat in the hepatocytes. The tissue sections were then placed onto glass slides and stained with Oil Red O. Additionally, liver and ileum samples were fixed in 10% neutral formalin solution. After routine tissue examinations, sections 5 µm thick were stained with Crossman's triple stain and hematoxylin & eosin (H & E) [19]. The stained sections were then examined and photographed under light microscopy (Olympus BX-051, Japan). The crypt depth, villus length, and villus width in the ileum were measured.

Microbiological Analysis

The contents of the small intestine of each bird were placed in a sterile flask. They were transferred to the laboratory and stored at +4°C thermal conditions. Cultures were prepared immediately. The intestinal contents were serially diluted to tenfold diagram using anaerobic dilution solution and phosphate buffer solution for enumeration of anaerobic and aerobic bacterial populations, respectively [20]. From the prepared intestinal content, 0.1 ml was inoculated in suitable medium and then incubated in aerobic and anaerobic conditions. The bacteria and fungi were identified according to the morphology of the colonies and microscopic analysis, in addition to the characteristics of the gram stain, spotting, motility, and biochemical activities.

Statistical analysis

A one-way ANOVA was used for the data analysis. Statistical significance among the groups was determined with Duncan's Multiple Range test [21]. The results are given as the average ± standard error (X ± Sx).

RESULTS

The live weight of the quails in the Trial II group was lower than in the Control and Trial I groups at the end of the study (P<0.01). Average daily gain, feed intake, and food conversion did not differ among the groups during the starter (day 1 to day 21), grower (day 22 to day 42), and overall experiment periods (day 1 to day 42) *(Table 2)*.

Table 2. *Effects of dietary chitosan oligosaccharides addition to the diet on the performance of quails*
Tablo 2. *Bıldırcın rasyonlarına kitosan oligosakkarit ilavesinin performans üzerine etkileri*

Weeks/Periods/Item	Control	Trial I	Trial II	Significance
	Live weight, g			
Hatching	8.30±0.04	8.34±0.02	8.33±0.02	NS
1	26.13±0.25a	24.85±0.23b	26.16±0.28a	**
2	54.05±0.58a	48.35±0.75b	53.06±1.08a	**
3	99.55±0.74a	94.41±0.73b	97.00±1.36ab	*
4	140.39±0.64a	133.66±0.82b	135.81±1.14b	**
5	167.80±0.81b	174.39±1.67a	171.05±0.74ab	**
6	206.23±1.21a	203.42±1.19a	195.12±1.05b	**
Starter period (from 1st to 21st day)				
Average daily gain, g	4.35±1.15	4.10±1.27	4.22±1.09	NS
Average feed intake, g	9.74±3.63	10.26±3.99	10.34±3.81	NS
Feed conversion, g feed/g gain	2.10±0.30	2.37±0.34	2.30±0.32	NS
Grower period (from 22nd to 42nd day)				
Average daily gain, g	5.08±0.59	5.19±0.53	4.67 0.63	NS
Average feed intake, g	23.11±2.93	24.28±2.01	23.75±2.87	NS
Feed conversion, g/g	4.74±0.92	4.86±0.93	5.59±1.60	NS
Overall experiment (from 1st to 42nd day)				
Average daily gain, g	4.71±0.60	4.65±0.66	4.45±0.57	NS
Average feed intake, g	16.42±3.64	17.27±3.71	17.04±3.68	NS
Feed conversion, g/g	3.42±0.73	3.62±0.71	3.94±1.03	NS

NS: *No significant;* ***a, b:*** *Values in the same row with a different letter are significantly different (* P<0.05, ** P<0.01)*

Table 3. *Effects of chitosan oligosaccharides on slaughter and carcass traits of quails*
Tablo 3. *Kitosan oligosakkarit ilavesinin bıldırcınların kesim ve karkas özelliklerine etkisi*

Item	Control	Trial I	Trial II	Significance
Slaughter weight, g	178.10±4.39	175.22±6.97	181.27±6.12	NS
Cold carcass weight, g	122.12±2.36	117.84±4.38	119.91±3.42	NS
Cold carcass percentage, %	68.73±0.65	67.44±0.85	66.42±0.91	NS
Leg percentage, %	23.69±0.32	23.61±0.33	23.99±0.35	NS
Breast percentage, %	38.28±0.76	38.58±0.68	38.80±0.43	NS
Wing percentage, %	8.91±0.18	9.34±0.20	8.69±0.20	NS
Heart percentage, %	1.43±0.04	1.36±0.05	1.39±0.04	NS
Liver percentage, %	3.68±0.28	3.70±0.32	3.67±0.27	NS
Gizzard percentage, %	3.15±0.20	3.28±0.17	3.39±0.15	NS

NS: *No significant*

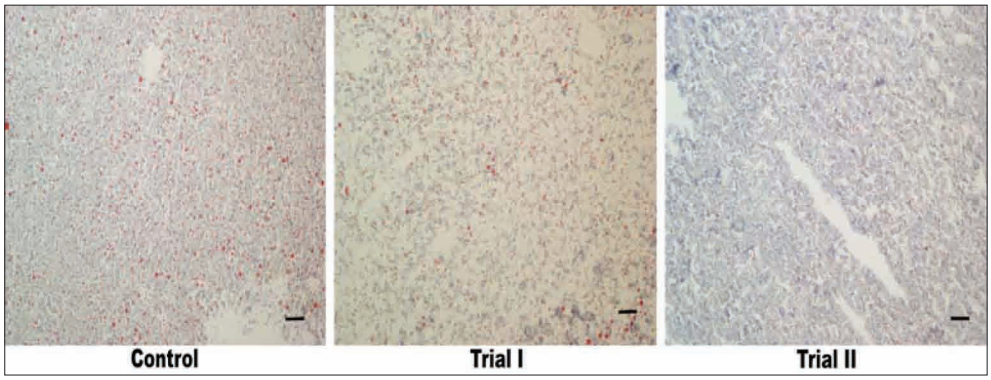
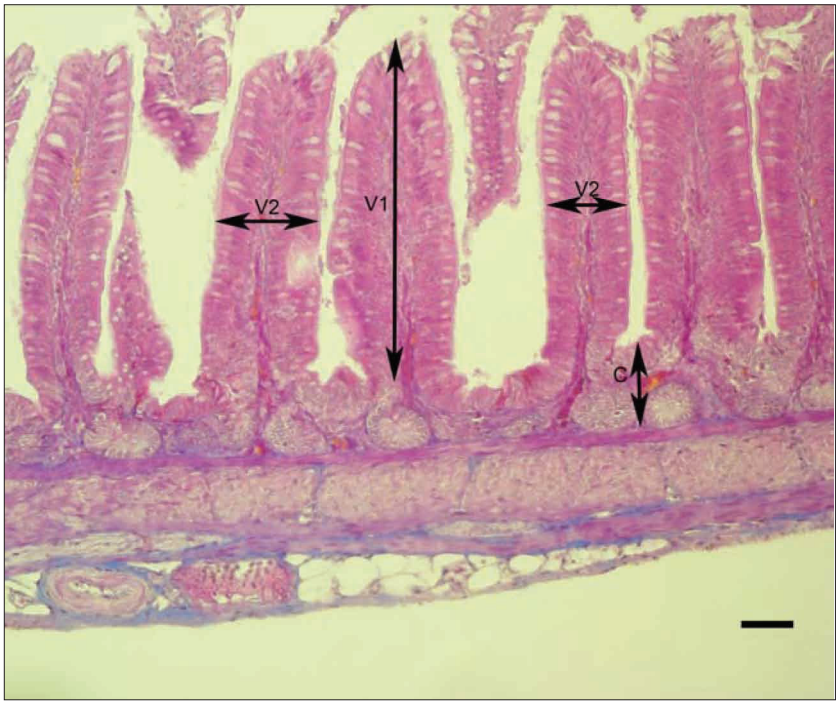


Fig 1. Microscopic aspect of liver in experimental groups. Oil Red O. Scale bar: 100 µm

Şekil 1. Araştırma gruplarındaki karaciğerin mikroskopik görünümü Oil Red O. Bar: 100 µm

Fig 2. Ileal villus length, villus width and crypt depth in the Control group. V1: Villus length, V2: Villus width, C: Crypt depth, Triple stain, Bar: 100 µm (Only measurement of the control is given, due to the other groups' measurement similar to the control)

Şekil 2. Kontrol grubunun ileum villus uzunluğu, villus genişliği ve kript derinliği. V1: Villus uzunluğu, V2: Villus genişliği, C: Kript derinliği, Üçlü boyama, Bar: 100 µm (Diğer grup ölçümleri Kontrole benzediği için sadece Kontrol grubu ölçümleri verilmiştir)



Effects of COS addition to the diets on the slaughter and carcass traits is shown in *Table 3*. As seen in *Table 3*, the slaughter and carcass traits did not differ among the groups.

In the histological examination of the liver, steatosis was observed in all the groups. Steatosis was more common in the Control and Trial I groups, and lipid deposition was higher than in the Trial II group *(Fig. 1)*.

Table 4. *Ileal crypt depth, villus length and villus width in the experimental groups, µm*
Tablo 4. *Gruplara ait ileumdaki kript derinliği, villus uzunluğu ve villus genişlikleri, µm*

Item	Control	Trial I	Trial II	Significance
Crypt depth	55.70±0.60^{b}	57.22±0.58^{b}	69.40±0.65^{a}	***
Villus length	317±3.88^{b}	312±3.67^{b}	340±5.62^{a}	***
Villus width	81.80±1.04	79.90±1.04	78.16±1.20	NS

NS: *No significant;* ***a, b:*** *Values in the same row with a different letter are significantly different (*** P<0.001)*

Table 5. *Distribution of intestinal microbial flora agents in the experimental groups, CFU/ml*
Tablo 5. *Gruplardan elde edilen ince bağırsak mikroflora etkenlerinin dağılımı*

Bacteria		Control	Trial I	Trial II	Significance
Gram (-)	*Escherichia coli*	4x10^{6}±3.28a	3.6x10^{6}±4.50a	2.3x10^{6}±2.48b	***
	Pseudomonas aeruginosa	5.4x10^{5}±1.15	5.4x10^{5}±1.09	3.6x10^{5}±0.36	NS
	Fusobacterium spp.	3.6x10^{5}±0.16a	3.6x10^{5}±0.26a	1.8x10^{5}±0.00b	***
Gram (+)	*Lactobacillus* spp.	3.6x10^{5}±0.65a	1.8x10^{5}±0.38b	0.9x10^{5}±0.00b	***
	Staphylococcus spp.	2.2x10^{6}±1.21a	2.0x10^{6}±1.55a	7.6x10^{5}±0.40b	***
	Bacillus spp.	2.0x10^{6}±1.56a	1.2x10^{6}±1.08b	8.1x10^{5}±0.85c	***
	Clostridium spp.	9.0x10^{5}±0.76a	6.3x10^{5}±0.67b	6.3x10^{5}±0.62b	**
	Streptococcus spp.	1.7x10^{6}±2.62a	8.1x10^{5}±0.83b	5.6x10^{5}±1.05b	***
	Enterococcus faecalis	1.0x10^{6}±1.41a	8.1x10^{5}±0.54a	5.4x10^{5}±0.5b	***
Yeast	*Candida* spp.	9.0x10^{5}±1.34a	2.7x10^{5}±0.33b	1.8x10^{5}±0.35b	***

NS: *Non significant;* ***a, b:*** *Values in the same row with a different letter are significantly different (** P<0.01, *** P<0.001)*

The histological structure of the ileum was similar among the groups. The crypt depth and villus length were significantly lower in the Control and Trial I groups (P<0.01) than in the Trial II group *(Table 4)*. There was no statistical difference in the villus width among the groups *(Table 4, Fig. 2)*.

The intestinal microorganisms isolated from the intestinal contents and the average amounts are given in *Table 5*. Ten different microorganisms were isolated from the intestinal contents: six gram (+) bacteria, three gram (-) bacteria, and one *Candida* yeast. The concentrations of *Enterococcus faecalis*, *Fusarium* spp., *E. coli,* and *Staphylococcus* spp. were significantly lower in the Trial II group than in the Control and Trial I groups (*P*<0.001). The concentrations of *Clostridium spp.*, *Streptococcus spp.*, *Lactobacillus* spp., *Bacillus* ssp., and *Candida* spp. were significantly lower in the Trial I and Trial II groups than in the Control group. There were no statistical differences in the concentration of *Pseudomonas aeruginosa* among the groups.

DISCUSSION

At the end of the starter period (21st day), the live weight of the quails was significantly higher in the Control group than in the Trial I group *(Table 2)*. This result is in accordance with the results of Razdan and Petterson [22]. The live weight of the quails in the Control and Trial I groups was higher than in the Trial II group at the end of the grower period (42th day). Previous studies reported that different amounts of COS added to the diet did not affect the live weight of broiler chickens [12,23]. Contrary to these results, other researchers found that the addition of COS to the diet increased the live weight of broiler chickens [4,11]. The average daily gain, feed intake, and feed conversion did not differ among the groups either in the starter and grower periods or in the entire experimental period *(Table 2)*. Similarly, other studies reported that the addition of COS to the diet did not change the daily gain of broiler chickens [2,12,23]. However, Li et al.[3] found that the addition of COS to the diet positively affected the daily gain of broiler chickens. The lack of change in the daily gain, feed intake, and feed conversion in this study might be related to the low viscosity and low molecular weight of the COS that was used. Discrepancies in the growth performance parameters between this research and other studies could be associated with the different molecular weights, deacetylation degrees, or doses of the chitosan or COS used in these experiments.

As seen in *Table 3*, slaughter and carcass traits did not differ among the groups. Our results indicated that the addition of 75 or 150 mg/kg of COS to the quail diets had neither a positive nor a negative effect on carcass traits. However, Tufan and Arslan [23] reported that 50 or 100 mg/kg of COS added to the diet of broiler chickens increased

the carcass ratio, leg and wing ratio, but not the breast, heart and gizzard ratio, and decreased the liver ratio. Zhou et al.[11] found that the addition of 14 or 28 g/kg of COS to broiler chicken diets enhanced the liver weight but did not change the breast meat ratio.

Liver steatosis was lower in the Trial II group than in the Control and Trial I groups *(Fig. 1)*. The lower fat accumulation in the Trial II group might be related to the higher level of COS added to the diet and the low fat digestibility and fat micelle binding properties of chitosan/COS. In support of this view, Razdan and Petterson [22] found that raw fat digestibility was decreased (26%) in broiler chickens fed a diet containing chitosan. Razdan et al.[24] reported that feeding chitosan reduced the concentration of bile acid in the small intestine and the total plasma cholesterol concentration. They also reported that chitosan had hypolipidemic potencies. Additionally, Genc et al.[25] established that the addition of mannan-oligosaccharide to the diets of fish reduced the accumulation of liver fat.

In this study, the crypt depth and villus length were greater in the Trial II group than in the Control and Trial I groups, which is in accordance with the results of Liu et al.[26]. The exact mode of action of COS supplementation in the diet on intestinal microflora is unknown. However, COS can selectively stimulate the growth of beneficial microorganisms, such as Lactobacillus and Bifidobacterium, thereby potentially inhibiting the growth of putrefactive and pathogenic bacteria [5]. A likely explanation for the increased villus height and crypt width could be the reduced number of pathogenic microorganisms in the small intestine following COS supplementation. In support of this idea, Mourao et al.[27] reported that COS supplementation to pigs reduced numbers of pathogen microorganisms and consequently enhanced the villus length. However, Baurhoo et al.[28] reported that the addition of mannan-oligosaccharide to broiler chicken diets had no effect on crypt depth.

In the present study, the addition of COS to the diet seemed to reduce the concentration of bacteria and *Candida* yeast *(Table 5)*. No et al.[29] and Simunek et al.[30] reported similar results. The dietary addition of 75 or 150 mg/kg of COS decreased the concentrations of intestinal pathogen microorganisms *(E. coli*, *Clostridium* spp., and *Staphylococcus* spp.), and this effect was more apparent in the group supplemented with 150 mg/kg of COS. Li et al.[3] established that the addition of 100 mg/kg of COS to broiler chicken diets reduced the cecal *E. coli* concentration. Other researchers [4,13,15] found similar results.

In conclusion, 75 mg/kg addition of COS did not change the final live weight of quails, but the addition of 150 mg/kg of COS decreased the final live weight. Overall, the results of the experiment suggest that dietary COS addition at both doses does not affect the average live weight gain, feed intake, feed conversion, or composition of the carcass traits. Given the increased ileal crypt depth and villus length and the positive effects on intestinal microflora, it conclude that 75 mg/kg of COS can be added to the diets of quails.

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Research Article

Can Melatonin Protect the Endometrium from the Adverse Effects of Caerulein?

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Abstract

We investigated the effects of a caerulein-induced acute pancreatitis (AP) on uterus and possible uterine protective effects of melatonin administration. Twenty-eight animals were divided into four groups: (1) control group (n = 7); (2) melatonin group (n = 7); (3) caerulein group (n = 7); (4) melatonin + caerulein group (n = 7). AP was induced by 4 intraperitoneal injection of caerulein given hourly (50 µg/kg) into young female animals. Melatonin (20 mg/kg) was given via intraperitoneal injection 30 min prior to the induction of AP. The rats were sacrificed by decapitation 12 h after the last injection of caerulein and their uterus were taken for histopathological evaluation. Mean body weight and uterine wet weight was recorded. The H-Score method was used to score the degree of histological changes of endo-myometrium edema, hemorrhage, necrosis, leucocyte infiltration, endometrial proliferation and endometrial thickness. There was no significant difference in the mean body weight observed after treatment in each group. The uterine wet weight differences between the control and caerulein given rats were significant ($P<0.01$). The endometrial thickness, edema, hemorrhage, necrosis and leucocyte infiltration of the caerulein group was significantly higher than the control and melatonin groups ($P<0.01$). It was observed that preteratment with melatonin normalized histological abnormalities and significantly reduced uterine wet weight as compared with the caerulein only group. Melatonin application may play an important role in the prophylaxis of uterine endometrium arising from adverse effects of caerulein.

Keywords: *Caerulein, Melatonin, Uterus, Pancreatitis, Rat*

Melatonin Serulein'in Olumsuz Etkilerine Karşı Endometriyumu Koruyabilir mi?

Özet

Serulein ile indüklenmiş akut pankreatiti (AP)'nin uterusa etkisi ve melatonin uygulamasının muhtemel uterus koruyucu etkileri araştırıldı. Yirmi sekiz rat dört gruba ayrıldı: (1) Kontrol grubu (n = 7); (2) melatonin grubu (n = 7); (3) serulein grubu (n = 7); (4) melatonin + serulein grubu (n = 7). AP, genç dişi hayvanlara saatte bir (50 µg/kg) dozunda 4 kez verilen intraperitonal serulein enjeksiyonu ile indüklenmiştir. Melatonin (20 mg/kg) AP indüksiyonundan 30 dakika önce intraperitonal enjeksiyon yolu ile verildi. Ratlar seruleinin son enjeksiyonundan 12 saat sonra sakrifiye edildi ve uterusları histopatolojik değerlendirme için alındı. Ortalama vücut ağırlığı ve uterusun ıslak ağırlıkları kaydedildi. Endo-miyometriyumdaki histolojik değişikliklerin derecesi, ödem, kanama, nekroz, lökosit infiltrasyonu, endometriyal proliferasyon ve endometrial kalınlık skorlaması için H-Puan yöntemi kullanıldı. Her grupta tedaviden sonra ortalama vücut ağırlığında anlamlı bir farkın olmadığı gözlendi. Kontrol ve serulein verilen ratlar arasındaki uterus ıslak ağırlıkları anlamlı derecede farklı ($P<0.01$) olduğu tespit edildi. Serulein grubunda endometriyal kalınlık, ödem, kanama, nekroz ve lökosit infiltrasyonu melatonin ve kontrol grubuna oranla anlamlı derecede daha fazla olduğu görüldü ($P<0.01$). Daha önceden melatonin uygulanan grupta sadece serulein uygulanan gruba oranla histolojik olarak yapısal bozuklukların normale döndüğü ve uterus ıslak ağırlığının anlamlı ölçüde azaldığı gözlendi. Melatonin uygulamasının, uterusun endometriumu üzerine seruleinden kaynaklanan olumsuz etkilerin profilaksisinde önemli bir rol oynayabileceği düşünülmektedir.

Anahtar sözcükler: *Serulein, Melatonin, Uterus, Pankreatit, Rat*

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INTRODUCTION

The nuclear factor kappa beta (NF-kB) pathway was indicated to be active in the normal endometrium of healthy women [1-3]. NF-kB has been shown to interact with the progesterone receptor and inhibit its action at endometrium [4]. Evidence supports that a physiologic amount of inflammation is necessary for endometrial receptivity and early pregnancy implantation. However, pathologic inflammation was found to interfere with the endometrial receptivity and early pregnancy implantation. AP is an inflammatory disease with wide clinical variations, which may present sepsis, multiple organ failure, and even death [5]. To date, most investigators believe that caerulein-induced rat pancreatitis is caused by the unregulated activation of NF-κB within the many organs including pancreas, gut, stomach and distand organs such as uterus and ovary leads to the local inflammation [6]. Moreover, NF-κB is activated during early stages of pancreatitis and regulates many genes that may control inflammatory activities. Most recently, a study showed that the level of NF-κB activation correlates with the severity of AP [7].

Melatonin (*N*-acetyl-5-methoxytryptamine) is the major secretion product of the pineal gland. Melatonin is a direct neutralizer of free radicals, an indirect antioxidant. It plays a role in neuroendocrine regulation, the increase of immunity, neutralization of free radicals, reduction of angiogenesis and increase of apoptosis; in studies on animals and humans, it was demonstrated that melatonin has important antineoplastic properties [8] and inversely correlated with the tumor proliferation index in patients with endometrial pathologies [9]. Previous study Turkoz et al.[10] demonstrated that melatonin protects the ovaries against oxidative damage associated with reperfusion following an ischemic insult.

This study investigates the mechanisms by which melatonin treatment in rats with caerulein-induced rat pancreatitis influences a number of factors such as: uterine weight, endometrial thickness, endometrial proliferation, endo-myometrial edema, hemorrhage, necrosis and leucocyte infiltration

MATERIAL and METHODS

Studies were performed on female Wistar rats weighing 280-350 g. Animals were housed in cages under standard conditions at room temperature on a 12 h light : 12 h dark cycle with commercial pellet chow. Rats were deprived of food 17 h prior to the start of the experiment, but drinking water was available *ad libitum*. All experiments were approved by the Ethics Committee of Inonu University Experimental Animals Production and Investigation Centre. Twenty-eight animals were divided into four groups: (1) control group (n=7); (2) melatonin group (n=7); (3) caerulein group (n=7); (4) melatonin + caerulein group (n =7). AP was induced by 4 intraperitoneal injection of caerulein given hourly (50 µg/kg/dose total of 200 µg/kg; Sigma-Aldrich Co., Taufkirchen, Germany) into animals. A total dose of caerulein (200 µg) was given at 2-h intervals, each injection was containing 50% of the doses [11]. Animal in the melatonin group was treated with 20 mg/kg body weight melatonin. Melatonin was given via intra-peritoneal (ip) injection 30 min prior to the induction of AP. Because melatonin was dissolved in absolute ethanol and further dilutions were made in saline, with a 1% final concentration of ethanol, animals in the control group received i.p. injections of 0.9% saline at 2-h intervals. Animal in the caerulein+melatonin group received same dose cerulein and melatonin. It has not been stated in any comments by the manufacturers whether caerulein has any effect on the estrous cycle. Therefore, a daily vaginal smear was monitored during the treatment period and after the permanence of the estrous cycle was confirmed. The rats were sacrificed by decapitation 12 h after the last injection of caerulein. The uterus was carefully removed by severing the attachments to the ovaries, and vagina for histo-morphological evaluation. After the uterus was rinsed and weighed (wet), pieces of it were excised from the body portion, fixed in 10% formalin and prepared for routine paraffin embedding. Paraffin-embedded specimens were cut into 5-µl sections and stained with hematoxylin eosin (H&E). Sections were examined and photographed using a Nikon Optiphot-2 light microscope and Nikon DS-L3 Image Analysis System (Nikon Corporation, Tokyo, Japan). The semi-quantitative method was used to score the degree of histological change of edema, hemorrhage, necrosis, leucocyte infiltration, endometrial proliferation and endometrial thickness for all groups.

Statistical analysis: Data distribution was tested using the Kolmogorov-Smirnov test. Comparison among the groups was performed using the Kruskal-Wallis analysis of variance and post-hoc Mann-Whitney U tests for continuous variables. Data was presented as mean and standard deviation (SD) for continuous variables. The statistical software package SPSS 21.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis.

RESULTS

There was no significant difference in the mean body weight after treatment in each group. The uterine wet weight differences between the control and caerulein given rats were significant ($P<0.01$, *Table 1*). The uterine wet weight of the caerulein group was higher than the control and melatonin group and the differences were significant ($P<0.01$). Pretreatment with melatonin demonstrated a significantly reduced uterine wet weight as compared with the caerulein only group. Evaluation of uterine histology revealed remarkable changes in endometrial thickness, edema, hemorrhage, necrosis and leucocyte infiltration in

Table 1. *The comparation of mean body weight, uterine wet weight and histological structural defects*
Tablo 1. *Islak uterus ağırlığı, ortalama vücut ağırlığı ve histolojik yapısal bozuklukların karşılaştırılması*

Parameters	Control	Melatonin	Caerulein	Melatonin+Caerulein
Mean body (g)	304.4±4.41	301.4±5.43	311.4±57.2	298.1±2.25
Uterus (g)	0.810±1.32*	0.796±2.23*	1.103±0.015	0.753±4.65*
Edema*	1.53±1.45	1.41±1.78	3.93±5.75	1.45±6.28
Hemorrhage*	1.64±4.65	1.51±0.18	3.26±3.54	1.54±8.32
Necrosis*	2.26±6.12	2.45±4.06	3.87±4.11	2.08±5.44
Leucocyte infiltration*	2.94±5.44	2.71±0.43	3.77±0.61	2.31±5.31
Endometrial proliferation	Normal	Normal	Normal	Normal
Endometrial thickness (mm)	0.124±0.11	0.128±0.45	0.144±0.31	0.126±0.01

Notes: *The data were expressed as mean ± SD; * P<0.05 compared with caerulein*

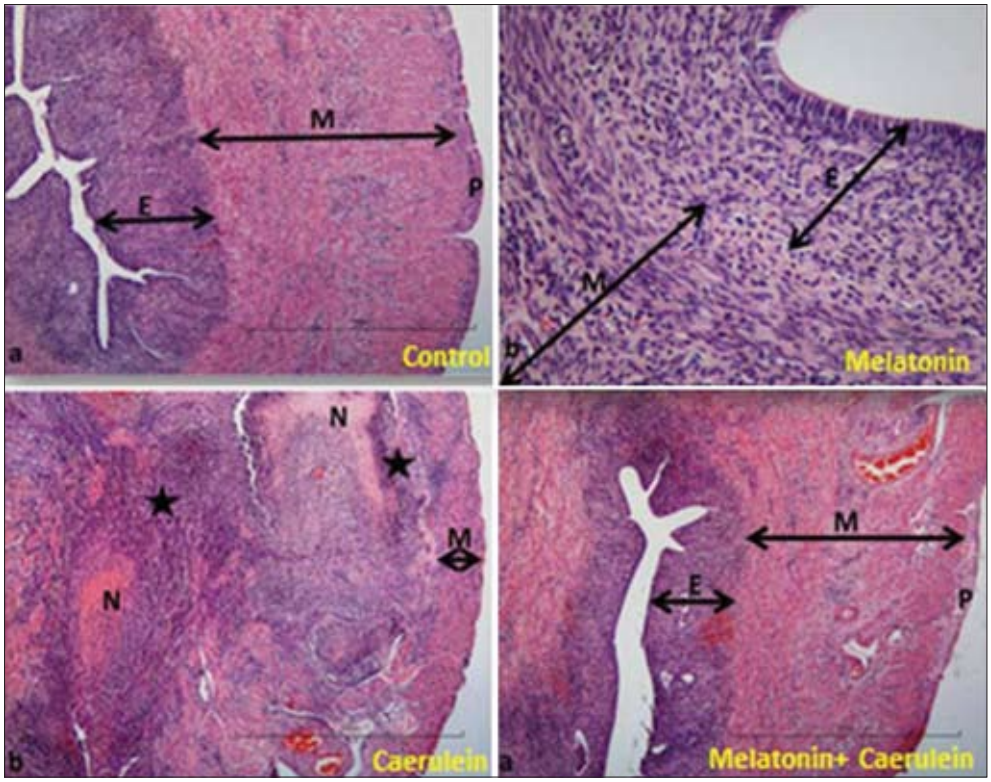

Fig 1. Histopathological appearance of uterus in all groups. Caerulein related diffuse necrosis *(black stars)* was prevented melatonin pretreatment. **N-** Necrosis and hyalinization; **M-** Muscle; **E-** Endometrium; **P-** Periton, (H&E X10)

Şekil 1. Tüm gruplarda uterusun histopatolojik görünümü. Seruleine bağlı diffüz nekroz alanları melatonin uygulaması ile önlendi. Kısaltmalar: **N-** Nekroz ve hiyalinizasyon; **M-** Kas; **E-** Endometrium; **P-** Periton (H&E X10)

the caerulein treated animals. The endometrial thickness, edema, hemorrhage, necrosis and leucocyte infiltration of the caerulein group was higher than the control and melatonin group and the differences were significant ($P<0.05$, *Fig. 1*). Morphologically, polymorphonuclear neutrophil infiltration and vascular dilatation were obvious in the caerulein given animals myometrium, and the changes also completely reversed by melatonin. Preteratment with melatonin normalized histological structural defects. Degree of endometrial proliferation was not significantly different among the groups. The addition of melatonin to caerulein treatment is associated with a decrease in endometrial thickness and prevents the edema, hemorrhage, necrosis and leucocyte infiltration in endometrium and myometrium.

DISCUSSION

Beginning with the evidence on the role of caerulein in systemic inflammation we considered that melatonin fulfills all the requirements for it to be considered as an antiinflammator drug which interact with the estrogen signaling pathways such. In the present study, morphologically, polymorphonuclear neutrophil infiltration were obvious in the caerulein given animals myometrium, and the changes also completely reversed by melatonin. Recent study demonstrated that mice developed more severe acute pancreatitis after caerulein hyperstimulation, which was explained by a decrease of apoptosis and a higher baseline proinflammatory state indicated by constitutively higher expression of inflammatory cytokines [12,13].

In our study, uterine wet weight of the caerulein group was higher than the control and melatonin group. Pretreatment with melatonin demonstrated a significantly reduced uterine wet weight. This may be results the combination of melatonin with caerulein injections increases fibrosis and loss of parenchyma. Another explanation is that melatonin decreases the endo-myometrial edema and causes reduction in uterine weight. Overuses of melatonin, which is an endogenous anti-inflammatory and antioxidative protein may attenuate uterine fibrosis. The hyperstimulation with caerulein increases edema, inflammation and necrosis ranges between 5 to 10 µg/kg/h in rats. Maximal tissue injury occurs after 12 h of continuous infusion but changes can be monitored already 15 minutes after the start of the caerulein infusion. One of the earliest consequences of hyperstimulation is the formation of edema. This edema is probably the result of several factors: increased vascular permeability, increased hydrostatic pressure from the constriction of small vessels. These events lead to a systemic inflammatory response syndrome, which includes extrapancreatic tissues including reproductive organs [12,13].

Addition of melatonin to caerulein in rats led to a decrease of endometrial thickness, necrosis and hemorrhage severe impairment of the endo-myometrial junction. Caerulein injections caused prominent histological damage with increased hemorrhage, endometrial thickness and necrosis was observed. This adverse effect of caurelein turned upside down with melatonin pretreatment. Control animals that were treated with saline or melatonin alone did not show any signs of inflammation. Melatonin had a significant anti-proliferative effect on Ishikawa cells (with estrogen receptors) at different cellular densities and different incubation times.

In this study, we used a caerulein-induced rat acute pancreatitis model to investigate the protective effects of melatonin on endometrium. Our results demonstrated that melatonin inhibited the production of proinflammatory cytokines and reversed abnormal histopathological changes of endometrium in caerulein-induced rat acute pancreatitis. In addition, rats which received melatonin treatment showed improved myometrial morphology.

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Research Article

A Morphological Study on Iridocorneal Angle and Ciliary Body of the Anatolian Shepherd Dogs *(Canis familiaris)*

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Abstract

This study was carried out to determine morphological structures of Anatolian Shepherd dog eye to compare with other animals. This important sensory organ from five adult male dogs were investigated by light microscopic and scanning electron microscopic analyses. Anatolian Shepherd dog cornea is about 435 (center) to 501 (periphery) µm thick. The dense pigmentation observed in the anterior and posterior iridal epithelium. The ciliary cleft of the iridocorneal angle was not large and well-developed. The ciliary processes, another component of the ciliary body, formed the conspicuous bundles. In conclusion, it is considered that the description of the morphological properties of both the iridocorneal region and the ciliary body would contribute to the interpretation of the functional correlation, thus to future experimental studies to be conducted in this field.

Keywords: *Dog, Eye, Iridocorneal angle, Ciliary body, Morphology*

Anadolu Çoban Köpeklerinde *(Canis familiaris)* Siliyer Cisim ve İridokorneal Açı Üzerine Morfolojik Bir Çalışma

Özet

Bu çalışma diğer hayvanlarla karşılaştırma yapmak üzere Anadolu Çoban köpeği gözündeki morfolojik yapıları belirlemek amacıyla yapılmıştır. Beş yetişkin erkek köpekten elde edilen bu önemli duyu organı ışık ve taramalı elektron mikroskobu ile incelenmiştir. Anadolu Çoban köpeğinin korneası merkezde 435, periferde ise yaklaşık 501 mikron kalınlığındaydı. İrisin anterior ve posterior epitelinde yoğun pigmentasyon gözlendi. İridokorneal açının siliyer aralığı büyük ve çok gelişmiş değildi. Siliyer cismin başka bir bileşeni olan siliyer uzantılar belirgin şekilde demetler oluşturdu. Sonuç olarak, hem iridokorneal bölgenin hem de siliyer cismin morfolojik özelliklerinin tanımlanması fonksiyonel ilişkinin yorumlanmasına katkı sağlayarak daha sonra yapılacak deneysel çalışmalara da faydalı olabileceği düşünülmektedir.

Anahtar sözcükler: *Köpek, Göz, İridokorneal açı, Siliyer cisim, Morfoloji*

INTRODUCTION

The iridocorneal angle, also referred to as the filtration or drainage angle, which is located on the anterior surface of the ciliary body, at the iridal base, in-between the borders of the cornea and sclera, and within the perimeter of the anterior chamber, is a structure responsible for the outflow of the aqueous humor from the anterior chamber of the eye [1]. In several researches, this structure has been reported to vary between animal species, depending on the adaptation of animals to terrestrial or aquatic life [2].

The aqueous humor is a nutritious fluid, generated from the ultrafiltrate of the blood plasma in the micro-circulation of the stroma of the ciliary body. This metabolically rich fluid serves as a nutritive supply for the avascular lens, cornea and trabecular meshwork. After being secreted by the non-pigmented epithelium located at the apical ends of the ciliary processes in the pars plicata, the aqueous humor enters the posterior chamber of the eye. Subsequently, it flows in-between the lens and pigmented epithelium of the iris, and passing rapidly through the pupillary opening, it enters the anterior chamber. The aqueous humor leaves the anterior chamber by two main drainage pathways, one which is conventional and referred to as the trabecular meshwork, and the other which is unconventional and referred to as the uveoscleral route. In the dog, the conventional route is mainly responsible

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for the drainage of the aqueous humor, and the uveoscleral route accounts for 10-15% of the outflow of the aqueous humor [3,4].

Several animal species have been used as a model in functional research. The anatomy of the outflow system of the aqueous humor has been studied extensively in multiple species, including horse [5], pig [6], and rabbits [7], and it has been aimed to investigate the conditions leading to the obstruction of the outflow of the aqueous fluid, which in general results in glaucoma in humans [2] .

The interpretation of eye disorders requires knowledge of the normal eye structure and physiology. To the authors' knowledge, no literature report is available on the morphology of the eye in the Anatolian Shepherd dog. Thus, this study was aimed at obtaining detailed morphological images of the normal structure of this important sensory organ by light microscopy and ultrastructural analysis. The objective of this study was to demonstrate the structures involved in the outflow of the aqueous humor in the Anatolian Shepherd dog, which is known as a guard dog and is indigenous to Turkey. It was also aimed to make a comparison of the eye morphology with that of other mammalian species on the basis of a qualitative assessment with a view to determine the potential differences, and thereby, to contribute to future clinical studies. The detailed histological investigation of the iridocorneal angle is not only a novel approach to comparative research, but is also considered to contribute to the demonstration of anatomical associations in other animal models.

MATERIAL and METHODS

Animals

Five adult male Anatolian Shepherd dogs which died because of traumas such as traffic accidents and other disorders without eye problem were used in this study and the eyes were obtained from the clinics of the Dicle University Veterinary Medicine. The properties of animals used are presented in *Table 1*.

Light Microscopy

The eyeballs were enucleated immediately before fixation. The samples were fixed in 10% neutral buffered formalin for 24 h and a small cut was made in the cornea to allow this solution to penetrate inside the eyeball to insure good fixation of the inner structures. After washed in running tap water for 24 h, the tissues were passed through a series of graded alcohols, methyl benzoate and benzole and were embedded in cross-section to long axis of body in paraffin. The 5 µm sections were cut from the paraffin blocks, mounted onto slides. The general histological structure of the eyeball was demonstrated by employing Crossman's triple staining method. The slides were examined by light microscopy and photographed using a Nikon Eclipse E400 (Nikon Instruments Inc.) microscope equipped with a digital camera (Nikon Coolpix 4500; Nikon Instruments Inc.).

Scanning Electron Microscopy (SEM)

The eyeballs were dissected into smaller pieces, after enucleated, were washed in 0.1 M chilled phosphate buffer (pH 7.4), fixed in 2.5% glutaraldehyde for 6 h and again washed twice in 0.1 M phosphate buffer (pH 7.4). Secondary fixation was carried out in 1% osmium tetroxide for 1 h, and specimens were dehydrated by acetone. Then, they were critical point dried and coated with gold palladium. The specimens were observed and photographed under a scanning electron microscope (Leica-Leo S440, Cambridge, UK). The tissues were examined under a stereomicroscope (Nikon SMZ800; Nikon Instruments Inc., Melville, NY, USA) so as to determine their general anatomical features before examination under scanning electron microscopy. Terms are used in agreement with the Nomina Anatomica Veterinaria - 2012.

RESULTS

Cornea

It was determined that the cornea was composed of an outer epithelial layer, a stromal layer in the middle, and

Table 1. *Animals used in this study*
Tablo 1. *Çalışmada kullanılan hayvanlar*

Clinical Login Information (Protocol Number)	Properties of Anatolian Shepherd Dogs			
	Age (years)	Sex	Weight (kg)	Clinical Status (Cause of Death)
09/15	1.5	Male	48	Paralysis due to traffic accident
10/24	1	Male	45	Paralysis of all foot and agony
11/181	2	Male	52	Paraplegia due to traffic accident
13/03	1.5	Male	50	L5 fracture due to traffic accident
13/14	2.5	Male	55	T12-T13 fracture due to traffic accident

an inner endothelial layer. The corneal thickness was measured as approximately 435.20±14.99 µm in the centre and as 501.43±16.81 µm in the periphery. The rather conspicuous basal membrane of the epithelium, which was approximately 31.81±5.21 µm in thickness, underlay two or three layers of epithelial cells, the outer layer of which was highly flattened, and therefore, the cell borders and nuclei of which were hardly distinguished. The inconspicuous Bowman's membrane was determined to have an approximate thickness of 6.60±1.29 µm, while the stroma, which was composed of multiple collagen fibrils organized in the form of lamellae, was determined to have an approximate thickness of 378.98±5.92 µm. The Descemet's membrane was of an approximate thickness of 5.75±0.92 µm, and the endothelium lying beneath was composed of a single layer of squamous cells *(Fig. 1 A,B)*.

Iris

The iris was composed of abundant pigment cells, blood vessels and smooth muscle cells. The surface facing the posterior chamber was lined by two-layered pigmented epithelial cells, and in the periphery, this epithelium continued with ciliary epithelial cells. The stroma of the iris was composed of pigmented, vascular and loose

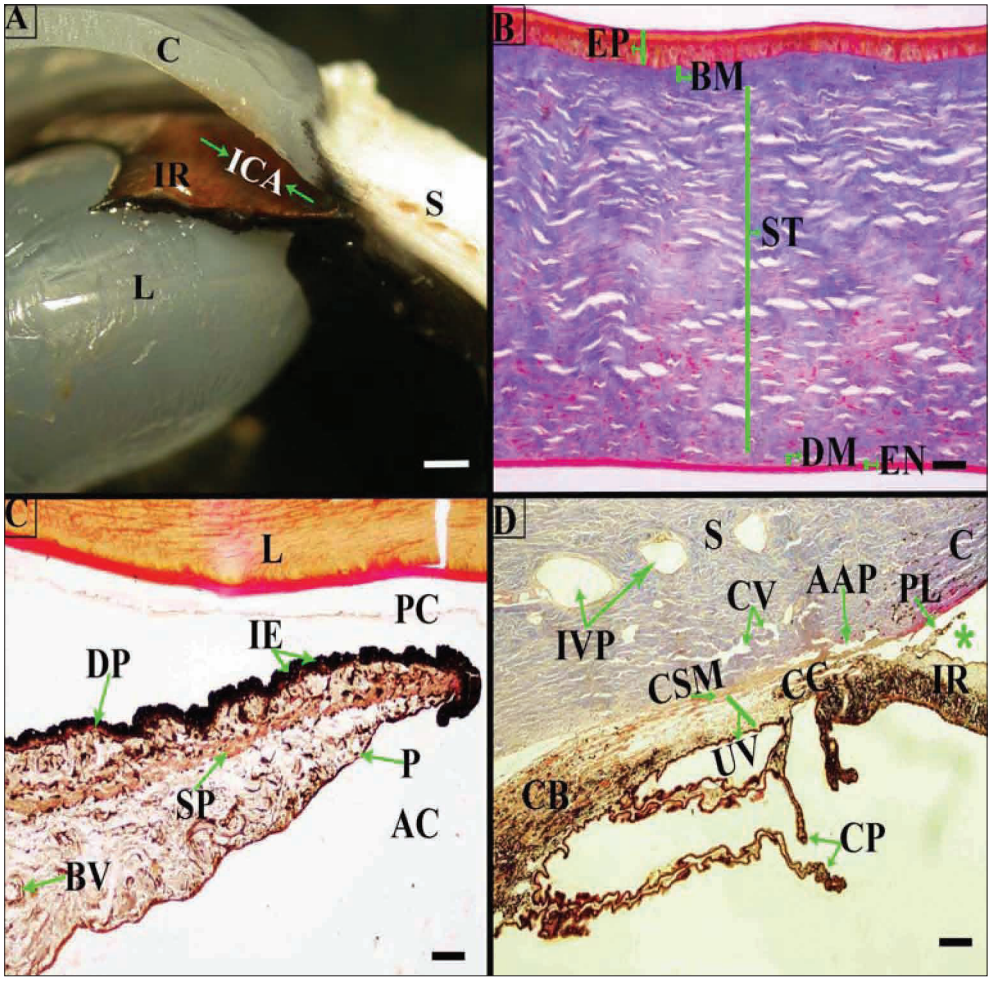

Fig 1. A- Anatomical view of the eye. C: cornea; S: sclera; L: lens; IR: iris; ICA: iridocorneal angle, **B-** Histological appearance of the cornea. EP: corneal epithelium; BM: bowman's membrane; ST: stroma; DM: descemet's membrane; EN: corneal endothelium, **C-** Histological appearance of the iris. L: lens; IE: iridal epithelium; DP: iris dilator muscle; SP: iris sphincter muscle; P: pigment cell; BV: blood vessel; PC: posterior chamber; AC: anterior chamber **D-** Histological appearance of the iridocorneal angle and ciliary body. IVP: intrascleral venous plexus; S: sclera; C: cornea; IR: iris; PL: pectinate ligament; AAP: angular aqueous plexus; CV: collecting veins; CC: ciliary cleft; CSM: corneoscleral meshwork; UV: uveal meshwork; CB: ciliary body; CP: ciliary processes; asterisk: iridocorneal angle. bar = 5 mm (A), 50 µm (B, C) and 200 µm (D)

Şekil 1. A- Gözün anatomik görünümü. C: kornea; S: sklera; L: lens; IR: iris; ICA: iridokorneal açı, **B-** Kornea'nın histolojik görünümü. EP: kornea epiteli; BM: bowman membranı; ST: stroma; DM: descemet membranı; EN: kornea endoteli, **C-** İris'in histolojik görünümü. L: lens; IE: iris epiteli; DP: irisin dilator kası; SP: irisin sphincter kası; P: pigment hücresi; BV: kan damarı; PC: arka kamara; AC: ön kamara, **D-** İridokorneal açı ve siliyer cismin histolojik görünümü. IVP: intraskleral venöz pleksus; S: sklera; C: kornea; IR: iris; PL: pektinat ligament; AAP: açısal aköz pleksus; CV: kollektör damarlar; CC: siliyer aralık; CSM: korneoskleral ağ; UV: uveal ağ; CB: siliyer cisim; CP: siliyer uzantılar; yıldız: iridokorneal açı. bar = 5 mm (A), 50 µm (B, C) ve 200 µm (D)

connective tissue. Furthermore, the stroma contained well-distributed and well-developed dilator and sphincter iridal muscles organized in bundles *(Fig. 1 A,C,D)*.

Iridocorneal Angle

The iridocorneal angle *(angulus iridocornealis)* was located in-between the iridal base, the anterior aspect of the ciliary body and the inner surfaces of the cornea and sclera. This angle appeared to be relatively small. The iridocorneal angle was observed to be associated with ciliary cleft, pectinate ligament, angular aqueous plexus, uveal and corneoscleral meshwork *(Fig. 1 D)*. The pectinate ligament, located peripheral to the anterior chamber, extended from the anterior surface of the root of the iris to the corneoscleral junction, and was observed as an isolated, fine fibre structure binding the two tissues *(Fig. 2 A)*. The pectinate ligament was 258.7±12.5 µm in length and 51.6±11.3 µm in width. The partly discontinuous epithelial cell layer lining the anterior surface of the iris also covered this ligament. The cells forming this layer presented with pigmentation, and many collagen fibrils extending in different directions were observed beneath the epithelium, which constituted the main skeleton of the ligament *(Fig. 1 D)*.

Ciliary Cleft

The ciliary cleft was bordered by the pectinate ligament at the anterior aspect, the root of the iris and the ciliary body at the posterior aspect, and the corneal limbus at the exterior aspect *(Fig. 2 B)*. In the iridocorneal angle, at which the corneal endothelium and the Descemet's membrane terminated, a dense trabecular meshwork existed, which was connected to the angular aqueous plexus. Two distinct regions were distinguished in this trabecular meshwork. The inner uveal meshwork, which originated from the anterior surface of the ciliary body and the root of the iris, was composed of loose trabecular plates. On the other hand, the corneoscleral meshwork, which originated from the sclera, was composed of leaf-like fine trabecular plates organized more tightly than that of the uveal meshwork. It was observed that, the trabecular plates forming both meshworks were made up of collagen fibrils *(Fig. 1 D)*.

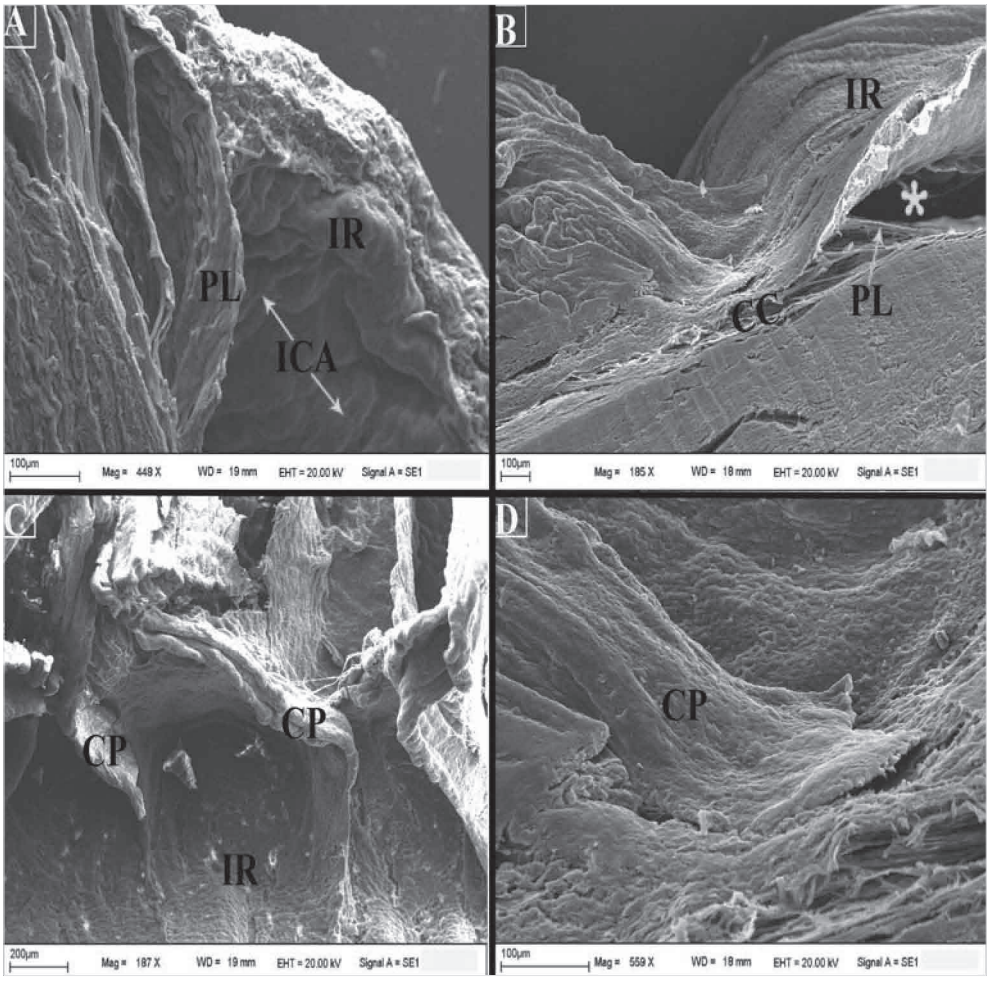

Fig 2. Scanning electron micro-graphs of the eye **(A), (B), (C), (D).** IR: iris; ICA: iridocorneal angle; PL: pectinate ligament; CP: ciliary processes; CC: ciliary cleft; asterisk: iridocorneal angle

Şekil 2. Gözün taramalı elektron fotoğrafı **(A, B, C, D)**. IR: iris; ICA: iridokorneal açı; PL: pektinat ligament; CP: siliyer uzantılar; CC: siliyer aralık; yıldız: iridokorneal açı

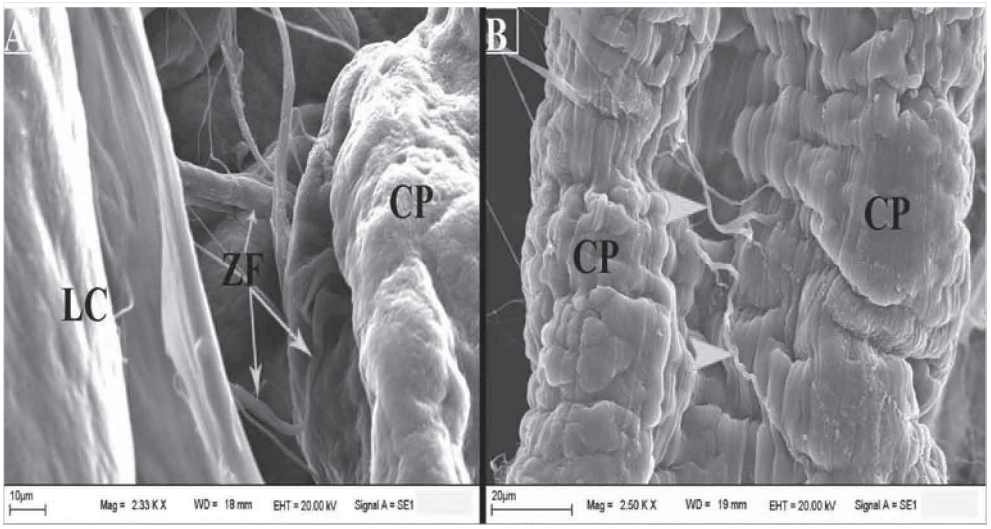

Fig 3. Scanning electron micrographs of the dog eye **(A, B)**, showing ciliary processes (CP), zonular (ZF) and collagen *(arrowheads)* fibers. The zonular fibres are connected to the lens capsule (LC)

Şekil 3. Köpek gözündeki siliyer uzantılar (CP), zonüler (ZF) ve kolajen *(okbaşları)* liflerin taramalı elektron fotoğrafı **(A, B)**. Zonüler lifler lens kapsülüne (LC) bağlanır

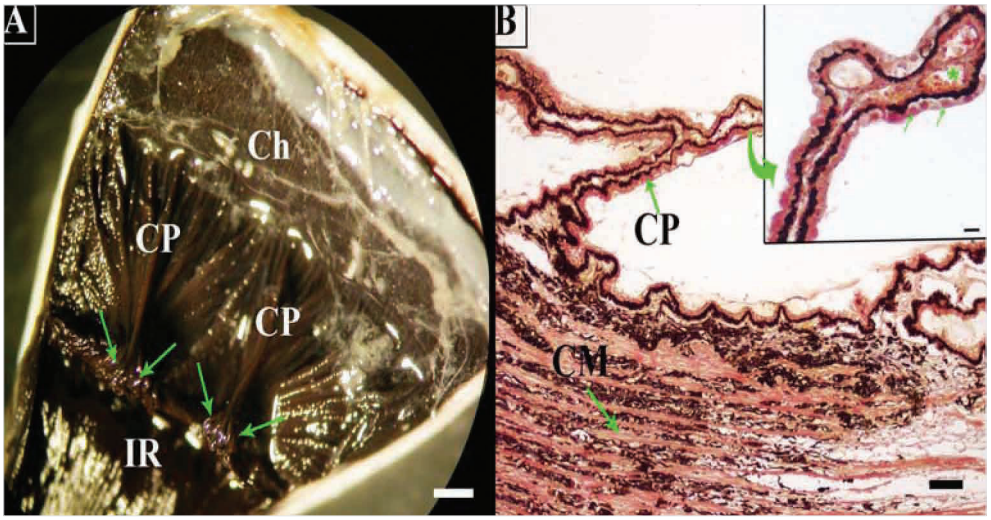

Fig 4. A- Anatomical view of the ciliary processes (CP) that form conspicuous bundles *(arrows)* at the sites of merge. Ch: choroidea; IR: iris, **B**- Histological appearance of the ciliary body. CP: ciliary processes; CM: ciliary muscle; *asterisk:* blood vessel; *arrows:* bilayered epithelium. bar=5 mm (A), 100 µm (B) and scale bar of overlapped figure is 25 µm

Şekil 4. A- Birleşme yerinde belirgin şekilde demetler *(oklar)* oluşturan siliyer uzantıların (CP) anatomik görünümü. Ch: koroid; IR: iris, **B**- Ch: koroid; IR: iris, B- Siliyer cismin histolojik görünümü. CP: siliyer uzantılar; CM: siliyer kas; *yıldız:* kan damarı; oklar: iki katmanlı epitel. bar = 5 mm (A), 100 µm (B) ve içindeki şeklin barı 25 µm

Ciliary Body

The ciliary body extended from the root of the iris to the ora serrata and was composed of two major structures, namely, the ciliary processes and ciliary muscle *(Fig. 1 D, 4 B)*. The ciliary processes originated with blunt ends at the posterior and extended anteriorly. While the ciliary processes were observed to be short and considerably close to each other at their origin, along their course to the iris their height was determined to increase progressively. Along their wavelike course, the ciliary processes were observed to merge at high points and form conspicuous bundles at the sites of merge *(Fig. 4 A)*, and the ciliary processes were reported to end separately *(Fig. 2 C)*. Furthermore, the ciliary processes, which were initially column-like at their origin, progressively increased in height and acquired a leaf-like form particularly at the level of the anterior two-thirds of the ciliary body *(Fig. 2 D)*. It was observed that while the posterior aspect of the ciliary processes lacked the zonular fibres *(lig. suspensorium lentis)*, the anterior aspect supported the lens capsule by means of the zonular fibres *(Fig. 3 A)*. The ciliary processes were composed of two-layered epithelial cells, which were non-pigmented in the inner layer and pigmented in the

outer layer *(Fig. 4 B)*. The bodies of the ciliary processes were connected to each other with collagen fibrils, and these connections were observed to be denser at the posterior aspect *(Fig. 3 B)*. Of the ciliary muscle forms, the radially organized bundles were relatively uncommon, while the well-developed meridionally arranged fibres were composed of smooth muscle cells tightly organized in bundles *(Fig. 4 B)*.

DISCUSSION

This study was aimed at the morphological investigation in the eye of the Anatolian Shepherd dogs. A moderately developed ciliary body musculature, well developed iridal muscles, small iridocorneal angle, poorly developed ciliary cleft, bundles formed by the ciliary processes, corneoscleral and uveal pathway for aqueous humor removal in Anatolian Shepherd dogs point out a marked morphological description.

The cornea, which supports the intraocular structures, refracts light owing to its convexity and allows the permeation of light owing to its transparency [8]. Similar to humans and non-human primates [9], in the Anatolian Shepherd dog the corneal layers are finer in the centre, compared to the periphery. The thickness of the corneal epithelium was determined to be less in the Anatolian Shepherd dog, compared to humans [10], and the corneal epithelium was ascertained to be composed of 2 to 3 layers, while in other domestic animals it has been reported to be composed of a greater number of layers [11]. While the Bowman's membrane, adjacent to the epithelium, is most evident in the cornea of humans and non-human primates, ruminants have been reported to possess a real-like Bowman membrane [11] and adult rabbits have been reported to lack the Bowman membrane [12]. In the present study, the Bowman membrane was inconspicuous beneath the epithelium and measured similar to that of humans [13]. The reports for the corneal stroma being composed of collagen lamellae extending parallel to the corneal surface and accounting for 90% of the total thickness of the cornea [11] have been confirmed for the dog in the present study. The thickness of the Descemet's membrane in the Anatolian Shepherd dog was found to be similar to that of humans [14]. The Descemet's membrane has been reported to serve as a semipermeable barrier for the transport of molecules between the corneal stroma and the anterior chamber of the eye, and to provide support to the posterior stroma [15]. The thickness of the corneal layers having been measured differently in various species suggests that this anatomical structure has species-specific features.

The main function of the iris is to regulate the amount of light entering into the eyeball [16]. To date, in all of the animal species investigated, the anterior surface of the iris has been determined to be lined by a monolayer of squamous epithelium, which is considered to be the continuation of the corneal endothelium [17]. Furthermore, as described by Ham [18], in the present study, at the posterior aspect, the iris was lined by two-layered pigmented epithelial cells, and the epithelium was undulated and in close contact with the dilator pupillae. While the iridal sphincter muscle has been reported to be composed of striated muscle in non-mammals and smooth muscle localized to the stroma in mammals, the localization of this muscle, which is indicated as mainly the central stroma in the horse [11], has been ascertained as the posterior stroma in the Anatolian shepherd dog. On the other hand, the iridal dilator muscle, which has been reported to be composed of smooth muscle with an exception of striated muscle in birds [11], was localized to the posterior iridal stroma in the present study. The dense pigmentation observed in the anterior and posterior iridal epithelium, apart from the iridal stroma, has been suggested to prevent the entry of light into the eyeball other than from the pupil [19], and this opinion suggests this function of the iris to be fulfilled with the contraction of both muscles.

The iridocorneal angle plays a complementary role for fluid drainage in the eye. It was observed that, in the Anatolian Shepherd dog, the ciliary cleft of the iridocorneal angle was not large and well-developed as in the pig [6]. The ciliary cleft has been reported to be deep in small diurnal herbivores, and very well-developed in large diurnal herbivores [11]. Similarly, horses have been reported to have a wide ciliary cleft, supported by both the inner part of the trabecular meshwork and the pectinate ligament, which makes the collapse of the ciliary cleft nearly impossible and results in the occurrence of glaucoma in horses only rarely [5]. Generally, in animals, this structure facilitates the drainage of the aqueous humor, and it is claimed that the iridocorneal angle of primates and carnivores being small results from the enlargement of the ciliary body musculature [20].

The pectinate ligament is composed of long strands that bind the anterior iridal base to the inner peripheral cornea. While in the present study the pectinate ligament was observed to have a fine and elongated structure, in the rabbit and pig it was observed to be shorter and thicker than in the domestic carnivores [21]. On the contrary, in the majority of ungulates, excluding the pig, the structure of the pectinate ligament ranges from moderate to thick [11]. Furthermore, reports indicate that the pectinate ligament resembles a fenestrated sheet in rabbits [21], and horses [5]. Therefore, it could be said that the structure and shape of this ligament plays an important role in both the routing of the aqueous humor and the supporting of the iridal base.

The posterior aspect of the pectinate ligament is the region of the ciliary cleft and continues with the uveal and corneoscleral meshworks. While De Geest et al.[5] reported that the corneoscleral meshwork covered only the posterior angle of the ciliary cleft in the horse, McMenamin and Steptoe [6] reported that it occupied the

entire length of the outer wall of the ciliary cleft in the pig, which was also the case in the present study. Furthermore, in the present study, the uveal meshwork was observed to be associated with the corneoscleral meshwork, and it was concluded that these structures aided in maintaining the iridocorneal angle to prevent any collapse in the drainage of the aqueous humor in the eye. Research has demonstrated that rabbits [7] lack the canal of Schlemm, but possess a sinus structure to substitute for its function. Samuelson and Gelatt [22] reported that normal drainage of the aqueous humor from the anterior chamber of the canine eye is believed to occur mainly through collecting veins in the corneoscleral meshwork, which subsequently empty into the larger intrascleral venous plexus system and to a lesser degree posteriorly through the uveoscleral route. In addition the morphology of the angular drainage channels have been demonstrated to be similar among the rat, rabbit, ox, cat, and dog with the canal of Schlemm in the primate eye. Similarly, in the present study, it was ascertained that the angular aqueous plexus existed in the eye of the Anatolian Shepherd dog, and enabled the outflow of the aqueous humor from the anterior chamber.

The caudal part of the ciliary body contains the ciliary muscle, which is a smooth muscle that alters the shape of the lens for near and far vision [23]. The rhesus monkey has an anthropoid type of eye characterized by a very well-developed ciliary body musculature and small iridocorneal angle [11]. In general, in herbivores these muscles are not well-developed and are composed of only meridionally extending muscle fibres [6]. Dellmann and Collier [24] reported that, pigs possess only circular fibres, which predominate in the nasal region of the ciliary body. It has been suggested that in some aquatic mammals, which are devoid of these muscles, an increased vascularization of the ciliary processes constitutes an alternative mechanism for the accommodation of the lens [2]. In the present study, in agreement with previous study carried out in cats [25], it was observed that these muscles were moderately developed and followed both a meridional and a radial course. The ciliary processes, another component of the ciliary body, generally do not exist in lower vertebrates [26]. Different from the results reported by Samuelson [26] suggesting the ciliary processes to be lined mostly by columnar epithelium in ungulates, the present study demonstrated that the ciliary processes were lined by a two-layered epithelium, composed of pigmented cuboidal epithelial cells in the outer layer and non-pigmented cuboidal epithelial cells in the inner layer. Thus, it can be suggested that the ciliary body plays a functional role in regulation, owing to its association with the iridocorneal angle, and that the structural differences between species are a result of adaptation to the environment.

The ciliary muscle is responsible for the optic accommodation mechanism. Its contractions move the ciliary processes and alter the tension of the zonular fibres. The two-sided convexity of the lens is modified by this mechanism, which enables focusing in the visual space [27]. In previous research on mammals, the conspicuous bundles formed by the ciliary processes were not described in detail. As can be understood from the information given above, the accommodation of the lens depends on the transfiguration capability of the lens, and in the present study it was determined that the apical ends of the ciliary processes formed bundles and adhered tightly to the lens capsule. It is considered that this anatomical feature detected in the Anatolian Shepherd dog may have a strong impact on the accommodation mechanism.

Eye is one of important organs of the body and has many roles in relationship between animal and environment [28]. In conclusion, the course followed by the ciliary processes of the ciliary body in the Anatolian Shepherd dog is considered to be interesting. Furthermore, it is considered that the description of the morphological properties of both the iridocorneal region, in which the aqueous fluid is reabsorbed, and the ciliary body, in which the aqueous humor is secreted, would contribute to the interpretation of the functional correlation, thus to future experimental studies to be conducted in this field.

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Research Article

Investigation of a Probiotic Yeast as a Cholesterol Lowering Agent on Rats Fed on a High Cholesterol Enriched Diet [1]

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Abstract

Probiotic yeast, *Cryptococcus humicola* M5-2 strain, which has high cholesterol assimilation feature and isolated from traditionally produced cheese, was investigated *in vivo*. This study was conducted to determine cholesterol assimilation ability of *C. humicola* M5-2 in vivo and specify the effect of the strain on serum total cholesterol, HDL/LDL cholesterol and triglycerides levels in rats fed on cholesterol-enriched diet. *C. humicola* had the ability to assimilate cholesterol at the rate of 73.33% in media. The strain was used with two different concentrations in animal feed (high and low doses containing 2% and 0.1% lyophilized strains, respectively). When the treatment groups were compared, low dose feeding group had the positive results in terms of testing values. According to the results of serum analysis, triglyceride and total cholesterol level were decreased by 25% and 1.34% respectively. Especially, decreasing the percentage rate of triglyceride has not obtained in other *in vivo* studies. It is thought that health promoting effect will be possible when the obtained isolate is consumed with fermented foods.

Keywords: *Cryptococcus humicola, Cholesterol, Probiotics, Animal experiment*

Probiyotik Mayanın Yüksek Kolesterol İçeren Diyetle Beslenen Sıçanlarda Kolesterolü Düşürücü Ajan Olarak Kullanılabilirliğinin Araştırılması

Özet

Yüksek kolesterol asimilasyon yeteneğine sahip probiyotik maya *Cryptococcus humicola* M5-2 suşu, geleneksel olarak üretilmiş bir peynirden izole edilmiş ve *in vivo* ortamda incelenmiştir. Çalışmada, *C. humicola* M5-2 suşunun in vivo ortamda kolesterolü asimile etme yeteneği incelenmiş ve kolesterolce zengin diyetle beslenen sıçanlarda bu suş ile beslemenin serum toplam kolesterol, HDL/LDL kolesterol ve trigliserit seviyelerine etkisi belirlenmiştir. *C. humicola*' nın besiyeri içersindeki kolesterolü asimile etme oranı %73.33'tür. Hayvanların beslenmesinde suş iki farklı dozda kullanılmıştır (yüksek ve düşük doz, sırasıyla %2 ve %0.1 liyofilize suş içermektedir). Deneme grupları karşılaştırıldığında, düşük doz besleme grubu daha olumlu sonuçlar göstermiştir. Serum analiz sonucuna göre trigliserit ve toplam kolesterol düzeyi sırasıyla %25 ve %1.34 oranında düşme göstermiştir. Özellikle trigliserit düzeyindeki düşme oranı çoğu *in vivo* çalışmada rastlanmamıştır. Elde edilen suşun fermente gıdalar ile beraber tüketiminin sağlık üzerine iyileştirici etkiler yaratacağı düşünülmektedir.

Anahtar sözcükler: *Cryptococcus humicola, Kolesterol, Probiyotikler, Hayvan denemesi*

INTRODUCTION

Today, the drugs, which can reduce the lipid ratio, are standard tools for cholesterol therapy. However, this situation disturbs patients who use cholesterol drugs along the rest of life because of the negative effects. It is estimated that some patients who take cholesterol lowering prescription drugs and follow a low-fat or low-

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cholesterol diet, do not achieve adequate reductions in their cholesterol levels [1]. Patients have sought to decrease their blood cholesterol levels with naturally.

Yeasts are widely present in dairy products and some yeast strains are used in cheese microbiology and development of new products because of its probiotic features. Yeasts, which are used technologically for producing fermented foods, have beneficial effects on human health. When the features of yeast types are investigated, it's noticed that *Cryptococcus* species are capsulated yeasts and widely found in air, soil, pigeon droppings and foods such as cheese, milk, beans and wine [2]. Topcu [3] isolated and identified yeast strains from raw milk and cheese samples and found especially *Candida*, *Geotrichum*, *Trichosporon*, *Cryptococcus*, and *Saccharomyces* genus in cheese samples. Yeast can be isolated from many types of cheese as a natural contaminate and they can be found commonly in the environment microflora of dairy plants. So, these microorganisms also found as a natural contaminates in raw milk, air, operating equipment, brine and water. Zottola et al.[4] stated that mozzarella cheese was contaminated with *Cryptococcus humicola* from hands of workers. In raw milk samples, genetically defined dairy origin yeast strains were observed and *Cryptococcus humicola* strains found [5]. It is indicated that *Cryptococcus* yeast types were found in raw milk and also in pasteurized milk as a secondary contaminate. In investigated ninety raw milk samples the ratio of *Cryptococcus* was 14% [6]. In a study *Cryptococcus humicola* was found in white cheese samples at the rate of 5.34% [7]. *Candida* spp., *Pichia* spp., *Rhodotorula* spp., *Kluyveromyces* spp. and *Cryptococcus* spp. were the most dominant types of yeasts which grow in white cheese surface and found that of strains, ratio of *Cryptococcus* was 10% [8]. There are also *Cryptococcus* strains in kefir grains which have positive effects for human health [9].

The objective of this study is to prepare lyophilized yeast culture which has probiotic properties. It is important that selected strain should have the same properties *in vivo* and *in vitro* conditions. In our previous study, yeasts were isolated and identified from several foods such as milk, cheese, yogurt, butter etc. and *in vitro* treatments about their cholesterol assimilation ratio and probiotic characteristics were researched (Research project: TUBITAK SBAG 111 S 513). The strain *Cryptococcus humicola* M5-2 was chosen because of its better probiotic characteristics and cholesterol assimilation ratio than the other examined yeast strains. In present study, we conducted to determine cholesterol assimilation ability of *Cryptococcus humicola* M5-2 *in vivo* and to specify the effect of feeding with the strain on total cholesterol, HDL/LDL cholesterol and serum triglycerides levels in rats fed cholesterol-enriched diet. In preclinical studies, experiments on animals before testing on humans are mandatory. For this reason, rats were preferred because of suitable tissue, size, anatomy, and phylogenetic properties.

MATERIAL and METHODS

Yeast Strain

Cryptococcus humicola M5-2 strain which was isolated from traditionally produced cheese was used in this study. The strain was identified with API ID 32 C test. *Cryptococcus humicola* was selected because of high cholesterol assimilation ratio (73.33%) in media. The other probiotic features of this strain were also determined. According to previous study, acid and bile tolerance of the strain was high; also the strain could survive for 90 min in artificial gastric fluid. Negative bile salt hydrolization was the other cause of this strain selection. For experimental inoculation of yeasts, the cultures of *Cryptococcus humicola* M5-2 were centrifuged at 8000 rpm for 10 min and pellets were washed with PBS-7.2 twice and lyophilized (VirTis benchtop-SLC). Finally, yeast intensities were determined by optical densitometer and $1x10^7$/mL living cells were used.

Animals

This study was approved by Local Ethics Committee of Animal Experiments of Süleyman Demirel University Hospital and performed following standard guidelines for the care and use of laboratory animals (No: B.30.2.SDÜ.0.05.06.00-65 Date: 04/10/2011). A total of 32 adult Wistar male rats (200-300 g weight) were housed under constant temperature (22±2°C) and humidity (60±5%), with 12-h dark/light cycles and allowed tap water and rat pellets *ad libitum* before and after the operation.

A pretreatment were applied to determine the highest lyophilized yeast amount before the main treatment. For this purpose, 2% lyophilized yeast were given to rats by oral gavage and effect was examined during a week. In preliminary test 3 rats were used. At the end of the week there was no negative situation in rats. Main animal treatment was carried out with 32 rats. All animals were fed on a basal diet for one week. After this adaptation period, the rats were divided randomly into 4 experimental groups of 8. The rats were housed in Euro type 4 cages. Each cage had 8 rats. Cages were cleaned 3 times a week.

Surgery

After the seven weeks feeding period, the rats were sacrificed by taking blood samples under anesthesia with 10% ketamine (Alpha, Alfas IBV) and 2% xylazine (Alfaz's, Alfas IBV). Blood samples were separated by centrifugation at 3.500 rpm for 8 min (Rotanta 460. Germany) and then serums were separated. Serums were stored at -80°C (Facis S.A. France) in medical biochemistry laboratory of Süleyman Demirel University until analysis.

Treatment

The rats were divided into four groups. One group

(negative control-Group 1) received a basal diet (cholesterol-free diet) throughout the experimental period of seven weeks and served as a negative control group. Second group (positive control-Group 2) fed on the basal diet with cholesterol and cholic acid added at a level of 1% (w/w) and 0.1% respectively (cholesterol-enriched diet). The third group (low dose lyophilized yeast supplemented-Group 3) fed on a cholesterol-enriched diet supplemented with lyophilized yeast at a level of 0.1%. The fourth group (high dose lyophilized yeast supplemented- Group 4) fed on a cholesterol-enriched diet supplemented with lyophilized yeast at a level of 2%. Cholesterol, cholic acid, and lyophilized yeast were dissolved in 2 mL water and given for all feeding period as a dose per day via oral gavage.

Determination of Cholesterol Level in Serum

Blood samples were collected from abdominal aorta, placed in sterile tubes, and vortexed (Labinco L 46, Netherlands). The obtained serum samples were analyzed for cholesterol, high density lipoprotein (HDL) cholesterol, and triglycerides. Kits were used for the analysis (Beckman Coulter AU 5800, USA). Low density lipoprotein (LDL) cholesterol levels were calculated by auto analyzer according to Fridewald formulation. Cholesterol levels were expressed in milligrams per deciliter (mg/dL).

Statistical Analyses

The generated data were analyzed by analysis of variance (ANOVA) and differences among mean values were treated with the Tukey's multiple comparison test. The statistical evaluation of the results was performed using the SPSS 17.0.0 (SPSS Inc., Chicago, IL).

RESULTS

There was no significant difference observed in point of rat's weight between the experimental groups during the seven weeks feeding period (P>0.05) *(Table 1)*. While the highest weight gain was observed in low dose yeast group, the lowest weight gain was observed in high dose yeast group.

Cholesterol supplementation to basal diet was increased the total cholesterol at a level 2.04 mg/dL. As seen in *Table 2*, there was no significant difference between the Group 2 and Group 4 in terms of the levels of total cholesterol and HDL cholesterol (P>0.05). High dose lyophilized yeast supplementation to diet had no effect on level of total cholesterol and HDL cholesterol, but it led to increase of LDL cholesterol level. In general, *in vitro* high assimilation rate was determined in the medium could not be observed *in vivo* because metabolism synthesizes cholesterol when needed. When *Table 2* was observed, feeding with low dose lyophilized yeast supplementation provide decreasing on triglyceride and total cholesterol level. Decline in the value of triglycerides was approximately 25% and reduction of total cholesterol level was 1.34%. These results could be considered as promising, because 1% reduction in cholesterol can reduce the risk of cardiovascular diseases for 2-3% [10]. In this respect, the selection of probiotic strains

Table 1. *Experimental animals weight*
Tablo 1. *Deney hayvanlarının ağırlıkları*

Days	Group 1	Group 2	Group 3	Group 4
1. day	269.75±34.59	277.13±33.15	271.88±16.74	258.63±40.32
2. week	271.88±34.93	279.13±34.10	273.63±16.85	261.00±39.80
3. week	287.13±36.38	281.63±33.73	275.88±16.87	263.75±39.73
4. week	290.25±35.76	285.63±33.76	280.38±17.37	267.38±39.26
5. week	294.13±35.87	283.43±32.66	284.13±17.50	271.38±39.24
6. week	300.38±37.14	290.29±32.89	290.00±17.49	276.75±38.76
7. week	329.38±33.32	327.71±25.74	336.88±19.36	303.75±43.65
Weight increase	22.11%	18.25%	23.91%	17.45%

Table 2. *Average level of triglyceride, total cholesterol, HDL cholesterol, LDL cholesterol*
Tablo 2. *Ortalama trigliserit, toplam kolesterol, HDL kolesterol ve LDL kolesterol değerleri*

Groups	Triglyceride (mg/dL)	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	LDL Cholesterol (mg/dL)
Group 1	44.88±7.74	38.25±5.12	23.75±5.06	4.71±2.06
Group 2	46.29±14.00	40.29±4.03	26.43±1.72	3.33±2.25
Group 3	34.75±11.63	39.75±5.52	27.50±3.89	6.00±1.29
Group 4	38.62±8.50	40.13±5.52	26.00±4.96	5.43±2.15

which have high percentage of cholesterol assimilation rates is important.

All organs of rats were investigated during the operation time. In Group 2, steatosis and caseation necrosis were observed around hearth. Rats were negative affected from high dose lyophilized yeast supplemented to diet (Group 4). In this group, the amount of blood of the rats was lower than in the other groups. Kidneys were larger than usual, color of liver was lighter. There was no observed any negative effect on Group 3 which was fed with low dose lyophilized yeast. When comparing the Group 3 and Group 4, feeding with high dose lyophilized yeast had negative effects on hematological values in parallel on organs.

DISCUSSION

Nowadays, probiotic yeasts can be delivered either in fermented foods or as lyophilized cultures administered orally. Several yeasts species, have been used in many probiotic preparations [11].

In animal studies on cholesterol assimilation, generally direct impact of lactic acid bacteria or effect of the fermented products produced therefrom have been discussed. In line with these studies different results were obtained according to used strains. Several studies have shown the ability of probiotic microorganisms to lower cholesterol levels *in vivo*. Bertazzoni Minelli et al.[12] found lower serum cholesterol (3.73%-13.49%) and triglyceride (9.30%-40.23%) ratios and stated an increase on HDL cholesterol level in fats fed on fermented milk containing *Lactobacillus casei* compared to control group after 10 days. As a result of a 3-week feeding of pigs with a mixture of probiotic, levels of total cholesterol decreased from 3.25 mmol/L to 2.74 mmol/L and triglyceride levels were not change [13]. *Lactobacillus plantarum* addition to normal diet over 14 days reduced the levels of total serum cholesterol and triglyceride of mice at a ratio 7% and 10%, respectively. Nguyen et al.[14] Beena and Prasad [15] found lower serum cholesterol in rats fed on yoghurt containing *Bifidobacterium bifidum* (1.20 g/L) compared to a positive control (1.72 g/L) after 30 days. However, the administration of fermented milk containing bifidobacteria (10^9 cfu/g) to hypercholesterolaemic human subjects resulted in a decrease in the total cholesterol level from 3 to 1.50 g/L [16]. The mechanism(s) responsible for the cholesterol-lowering effect of probiotics remains unclear, but it has been suggested that the effect could be obtained through retarded cholesterol synthesis and increased degradation of cholesterol [17].

Diets supplemented with probiotics can also significantly reduce plasma triglycerides in broilers. In a study, broilers fed with addition of 0.5% *Saccharomyces cerevisiae* triglyceride and total cholesterol levels decreased at the rate of 22.67 and 9.95% respectively after 3 weeks [18]. Cholesterol level was significantly lower in broilers supplemented with thermotolerant probiotic yeast at different levels compared to control group [17]. Oral administration of probiotics has been shown to significantly reduce cholesterol levels by as much as 22 to 33% [19]. Further, decrease in cholesterol content of eggs of laying hens and broilers diets containing yeasts was reported by Yalçın et al.[20] and Yıldız et al.[21].

Seyidoğlu and Galip [22] and Seyidoğlu et al.[23] were conducted to evaluate the effect of *Saccharomyces cerevisiae* on the serum biochemical parameters in rabbits. The diets with the yeast reduced serum HDL cholesterol and triglycerides on the 90th feeding day. Similar to our findings serum cholesterol slightly decreased by the yeast. In a study performed by Güven and Güven [24] kefir grains was caused significant suppression in serum lipids on the rabbits fed with cholesterol supplemented diet.

Numbers of studies are quite a lot which is performed with bacteria *in vivo* conditions. Some of these studies show similarities with our study results [25,26]. Jin et al.[27] were determined serum cholesterol level of broilers fed with Lactobacillus cultures (*L. acidophilus, L. fermentum, L. crispatus* and *L. brevis*). In their study, culture supplementation to diets was in levels of 0.05%, 0.1% and 0.15% in the experimental groups and the decrease in the amount of serum cholesterol was found 1.56%, 17.18% and 8.59% respectively. Similar to our findings, high dose supplementation showed lower impact. In our study, the addition of 2% lyophilized yeast (high dose) did not affect the amount of serum cholesterol in rats and has created adverse effects on tissues and organs. It may cause some tissue damage by increasing doses so these results require further studies using different types and doses of yeasts. This situation showed how important was dose adjustments in this kind of work.

In conclusion, yeast supplementation has some beneficial effect by lowering cholesterol and triglycerides. In the present study, low dose lyophilized yeast supplemented diet (Group 3) showed more positive results. It was observed that the level of triglyceride and total cholesterol were reduced at the rate of 25% and 1.34%, respectively. Especially, reducing rate of triglyceride level was higher than many *in vivo* studies. Studies in recent years have shown that yeasts also play a significant role in the spontaneous fermentation of many indigenous food products. Usage of yeasts as a co-culture is performed by researchers because of the several promoting advantages of these organisms. They have a more diverse enzymatic profile that leads to the formation of aromatic substances, also their lipolytic and proteolytic activity is high and their interactions with starter cultures is favorable. It is therefore encouraged that additional efforts are placed on exploring the health beneficial effects of yeasts. According to these informations it was thought that when obtained isolates are consumed with fermented products, positive results will be demonstrated.

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Research Article

Determination of Diagnostic Value of cELISA for the Diagnosis of Anaplasmosis in Clinically Suspected Ruminants [1]

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Abstract

The aim of this study was to determine diagnostic value of cELISA in anaplasmosis in clinically suspected animals and to compare the cELISA results with the clinical examination results. For this purpose a total of 720 ruminants (457 cattle, 146 sheep, 117 goat) were examined in terms of clinical signs. Eighty-eight ruminants consisting of 61 cattle, 11 sheep and 16 goat which had the symptoms of anemia, fever, icterus, weakness, depression and lack of appetite were selected for the study. Blood samples were collected from the jugular vein of all clinically suspected animals and serum samples were separated. A commercially available competitive enzyme-linked immunosorbent assay (C-ELISA) kit was used for determine antibodies to *Anaplasma* species. cELISA based diagnosis revealed that 47 of 88 serum samples (53.4%) were positive for anaplasmosis. In serological examination *Anaplasma* specific antibodies were determined in 45.9% of cattle, 63.6% of sheep and 56.2% of goats. Seropositivity rate was statistically differ among the age groups of cattle and the highest seropositvity rate was found in <12 month age (P<0.005). However no difference was found in the seropositivity rate of *Anaplasma* in sheep and goat in relation to age group. From the data obtained in this study it can be concluded that clinical findings are not sufficient criteria for the diagnosis of anaplasmosis and must be supported by serological examination.

Keywords: *Anaplasmosis, Ruminant, cELISA, Clinical diagnosis*

Klinik Olarak Anaplasmosis Şüphesi Olan Ruminantlarda cELISA'nın Tanısal Değerinin Belirlenmesi

Özet

Bu çalışmada cELISA'nın klinik olarak anaplasmosis şüphesi olan ruminantlardaki tanısal değerinin belirlenmesi ve klinik muayene sonuçları ile cELISA sonuçlarının karşılaştırılması amaçlanmıştır. Bunun için toplam 720 ruminant (457 sığır, 146 koyun, 117 keçi) klinik belirtiler yönünden muayene edilmiştir. Anemi, ateş, sarılık, güçsüzlük, durgunluk ve iştahsızlık belirtileri olan 61'i sığır, 11'i koyun ve 16'sı keçi olmak üzere toplam 88 ruminant çalışma için seçilmiştir. Klinik şüpheli bu hayvanların vena jugularisinden kan alınmış ve serumları çıkarılmıştır. *Anaplasma* antikorlarını belirlemek amacıyla ticari olarak temin edilen cELISA kiti kullanılmıştır. cELISA sonuçlarına göre 88 serum örneğinin 47'si (%53.4) anaplasmosis açısından pozitif bulunmuştur. Serolojik muayenede sığırların %45.9'unda, koyunların %63.6'sında ve keçilerin %56.2'sinde *Anaplasma* spesifik antikorlar saptanmıştır. İstatistiki olarak sığırlarda yaş gruplarına göre farklı seropozitivite oranları saptanmış olup, en yüksek oran 12 aydan daha küçük hayvanlarda saptanmıştır. Bununla birlikte koyun ve keçilerde yaş gruplarına göre seropozitivite oranlarında bir farklılık saptanmamıştır. Bu çalışmadan elde edilen verilere göre anaplasmosis tanısında klinik bulguların tek başına yeterli olmayacağı ve serolojik muayenelerle mutlaka desteklenmesi gerektiği sonucuna varılabilir.

Anahtar sözcükler: *Anaplasmosis, Ruminant, cELISA, Klinik tanı*

INTRODUCTION

Anaplasmosis is an important haemoricketsial disease caused by the tick-borne pathogen *Anaplasma* species [1,2]. Anaplasmosis is globaly the most prevalent tick-borne disease of ruminants and has a worldwide distribution with areas in endemicity on six continents. High prevalence rates are reported in tropical and subtropical regions of the world [2-6]. Prevalence of disease depends on distribution and density of reservoir host and tick vectors [2,5,6]. *Anaplasma* species is transmitted biologically by infected ticks, mechanically by biting flies (Tabanids) or blood contaminated fomites. Approximately 20 species of ticks have been determined as vectors worldwide

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including *Rhipicephalus spp., Ixodes spp., Hyalomma spp.,* and *Dermacentor spp.* Among these species *Rhipicephalus (Boophilus) spp.* is found to be major transmitting agent [2,7,8]. Although there are some prevalence reports of anaplasmosis in cattle with high rate (up to 80%) [1,3,9-11], information about prevalence rates in small ruminants is restricted in Turkey [12,13].

Acute phase of anaplasmosis is characterized by progressive haemolytic anemia associated with fever, jaundice, decreased milk production, weight loss, abortions, hyper excitability and in some cases sudden death [2,4,5,7]. After the first infection with *Anaplasma spp*. animals remain persistently infected carriers and serve as long term reservoirs for the maintenance of infection within herds [2,5,7]. These carrier animals are efficient sources of infection where they carry *Anaplasma* species in their bodies, but do not show any clinical signs and able to infect other animals.

Successful management of anaplasmosis in ruminants depends on accurate knowledge of prevalence and risk factors associated with transmission. For this reason diagnosis of acute infection and determination of carrier animals is vitally important. Diagnosis of disease usually based on microscopic examination of Giemsa stained blood smears in clinically suspected animals, during the acute infection [2,9,10]. However this method is not applicable for the detection of subclinical and/or latent infection in carrier animals, which serve as a reservoir for the spread of infection since the parasites are seldom detected microscopically in chronic infections [2,5]. Therefore, several serological tests have been established for the diagnosis of disease. However these tests cannot discriminate different *Anaplasma* species because of antigenic similarity. On the other hand detection of *Anaplasma* species using nucleic acid approach offers an alternative diagnostic tool [2,5,12,13].

Analyzing of diagnostic capacity of serological tests in anaplasmosis might provide further insight into the epidemiology, determination of carrier animals and may be helpful to management of disease. Although, there are few studies with respect to serologic diagnosis of this rickettsial disease in Turkey [3,9,10,12], there is no serological study conducted in clinically suspected ruminants.

Because of the scarcity of such data in the Turkish literature, the present study was undertaken to evaluate the ELISA in anaplasmosis clinically suspected ruminants from Bursa province, Turkey.

MATERIAL and METHODS

Ethical Committee Approval

The study protocols and experimental procedures were approved by the Uludag University Scientific Ethics Committee (No: 2010-05/04).

Study Area

This study was conducted at the South Marmara region of Turkey. Study area (Bursa) is located in southeast of the Marmara Sea (40°E, 28-30°N). This region is characterized by hot and dry summers with some rainfall. Winter conditions are changing mild to cool with more extended periods of light to moderate rainfall. The mean annual temperature in the area is 14-16°C with minimum and maximum averages of 5°C and 25°C. The area receives an average of 600-700 mm rain per year. There are generally in the form of chain of mountains running across the direction of east to west. Annual temperatures, rainfall distribution, are suitable for average humidity levels and forest covered areas are suitable for vector ticks [14].

Clinical Examination and Selection of Study Animals

The study was conducted during tick season between May to October, 2012 in four different districts (Alpagut, Koçuköy, Erenler, Gökçeören) of Bursa. In these districts a total of 720 ruminants (457 cattle, 146 sheep, 117 goat) were examined in terms of clinical symptoms and tick infestation. Collection of animal information such as age, breed, and origin were conducted with the help of animal owners. General physical examination was conducted on all animals in herds. Parotid, prescapular and prefemoral lymph nodes were palpated to assess whether they were enlarged. Mucous membranes of conjunctiva and mouth were examined for pallor or petechial haemorhages. All animals were examined for the presence of ocular and nasal discharges and diarrhea. The skin coat was examined any signs of roughness and ticks. According to clinic examination a total of 88 ruminants (61 cattle, 11 sheep and 16 goat) primarily having history of tick infestation, fever, jaundice and anemia were selected for the purpose of blood collection.

Blood Collection

A total of 88 ruminants were bled from the jugular vein into non-heparinised vacutainers tubes. About 5 ml of blood was taken from each animal into each tube and stored at 4°C until arrival at the laboratory. In the laboratory serum samples were separated by centrifugation at 3.000 rpm for 5 min and stored at -20°C until use.

Tick Collection and Identification

Whole bodies of animals were carefully checked for ticks and their specimens were placed into 70% ethanol in glass vials. In our laboratory ticks were identified according to the keys of Aydın [14] and recorded in data sheet.

Competitive ELISA

All sera collected from suspected animals were tested for the presence of antibodies against *Anaplasma* by competitive ELISA (cELISA). The cELISA was performed

using the *Anaplasma* antibody test kit (VMRD Inc., Pullman, WA- catalog number: 282-2) following the manufacturer's instructions. This kit can detect antibody specific to *Anaplasma marginale*, *A. ovis*, and *A. centrale* in serum samples [15]. All samples and controls were run in duplicate and the mean obligate density at 450 nm was determined.

Data Analyses

The associations of prevalence among three animal species were determined by Fischer exact test. The association of prevalence of the infection among the animals of different age groups was determined also by Fischer exact test. Results were considered to be significant at P<0.05. All results were analyzed statistically using Minitab (V-15) software package [16]. Clinical variables such as anemia, fever, weight loss, pallor of mucous membranes, diarrhea, lacrymation and lymph node enlargement were recorded as either present or absent.

RESULTS

During the study, 419 of 720 ruminants (58.16%) examined for tick infestation were carrying at least one of tick species. Tick infestation rate was found to be 62.29% in cattle, 72.27% in sheep and 68.75% in goats. As seen in the *Table 1* eight tick species belonging to five genus of Ixodidae family were identified. 26% of total ticks were *Ixodes ricinus, Rhipicephalus annulatus* 6%, *Rhipicephalus turanicus* %7.5, *Rhipicephalus bursa* 20%, *Dermacentor marginatus* 15%, *Haemaphysalis parva* 6.5%, *Hyalomma marginatum* 2%, *Hayalomma anatolicum* 3.7%, *Rhipicephalus spp nymph* 19%, *Ixodes nymph* 15.3%. *Rhipicephalus* species was the predominant tick vector followed by *Ixodes ricinus* and finally *Dermacentor marginatus*.

Clinical findings in all ruminants with anaplasmosis suspected as follows: anemia, pale mucous membrane, lack of appetite and decrease of milk production. Most animals demonstrated weakness, weight loss, depression, icterus and lethargy. Very few animals (5 cattle and 2 sheep) presented fever (>40°C) and dehydration. However there were no animal died within the study period.

cELISA based diagnosis revealed that overally 47 of 88 animals (53.4%) positive for *Anaplasma*. Serological examination of *Anaplasma* spesific antibodies proved that 45.9% of cattle, 63.6% of sheep and 56.2% of goats were positive. Detail of cELISA results according to age groups were presented in *Table 2*. According to statistical analyses results although there was a significant difference among the age groups in cattle, no difference was found in the seropositivity rate of *Anaplasma* in sheep and goat age group. In cattle the highest seropositvity rate was found in <12 age group (P<0.005). On the other hand concerning the seropositivity rate of anaplasmosis in different animal species, showed that sheep (data are not shown) had the highest infection rate. However the frequency of antibody existence were not statistically different between; cattle and sheep (P=0.279); cattle and goats (P=0.461); sheep and goats (P=1.000).

Table 1. *Tick species collected from cattle, sheep and goats in Bursa province*
Tablo 1. *Bursa yöresinde sığır, koyun ve keçilerden toplanan kene türleri*

Tick Species	Cattle	Sheep	Goat
Ixodes ricinus	+	+	+
Rhipicephalus annulatus	+	-	-
Rhipicephalus turanicus	+	+	+
Rhipicephalus bursa	+	+	-
Dermacentor marginatus	+	+	+
Haemaphysalis parva	+	+	+
Hyalomma marginatum	+	-	-
Hyalomma anatolicum	+	-	-
Ixodes spp. nymph	+	+	+
Rhipicephalus spp. nymph	+	+	+

Table 2. *Seropositivity rate obtained with cELISA related to age groups in ruminants*
Tablo 2. *Ruminantlarda yaş gruplarına göre cELISA ile elde edilen seropoziflik oranları*

Category	n	No of Positive Test Results
Cattle Age (month)		
<12	7	6[a]
13-24	18	6 [b]
25-48	21	13[ab]
>49	15	6 [ab]
P		P<0.005
Sheep Age (month)		
<12	2	1 [n.s.]
13-24	8	5 [n.s.]
>25	1	1[n.s.]
Goat Age (month)		
<12	5	3 [n.s.]
13-24	9	6 [n.s.]
>25	2	0 [n.s.]

[a, b] Values with different letters in each category are significantly different; ***n.s.*** *not significant*

DISCUSSION

Anaplasmosis is being recognized worldwide as a cause of extensive morbidity and mortality among farm animals. The disease is a major constraint to farm production in many countries and responsible for significant economic losses in endemic areas [2,4,5,9]. *Anaplasma* infections can be fatal in susceptible animals especially in cattle and partially responsible for the high rate of mortality observed in the

affected herd [17,18]. Infected animals may become carriers after recovery a long period and serving as source of infection. Naïve animals in non endemic areas may become infected with anaplasmosis following the introduction of a carrier animal from an endemic area. Therefore the reliable detection of acute infection and carriers is important issue in the epidemiology of anaplasmosis [11]. Scanning tests that are used in epidemiological studies must be reliable. In order to define whether the used test is a reliable sensitivity, specificity, false negative and false positive are calculated and it is required that these measurements have adequate level [9].

Diagnosis of anaplasmosis usually based on microscopic examination of stained blood smear. However this conventional method have some disadvantages and can only detect levels of 10^6 infected erythrosites per ml in acute infectons [19]. *A. marginale* and *A. centrale* multiplies within red blood cell of infected host, resulting in extravascular hemolysis and anemia. During this multiplying process ricketsiemia levels exceed 10^9 infected erythrocytes per ml. Recovery of acute anaplasmosis result in persistent infection characterized by $10^{2.5}$-10^7 infected erythrocytes [20]. In such cases the level of rickettsiemia is generally below the threshold level of microscopic examination. On the other hand lack of expertise among personnel performing smear examination and the occurrence of intracellular artifacts that difficult of differentiating the *Anaplasma* are other disadvantages of blood smear examination. Therefore microscopic examination of blood smears is not sufficiently sensitive and specific to detect chronic carriers. Sharma et al.[21] reported that the traditional Giemsa staining method is not applicable for determination of persistently infected cattle and buffalo. For these reasons mentioned above we have not examined blood smears of suspected animals. The present study is the first report providing serological evidence of anaplasma infections in clinical suspected animals in Turkey.

As an alternative to microscopic examination several serological tests and nucleic acid based assay can be used for detecting anaplasmosis in infected animals. Nevertheless, serological tests would be more practical for the diagnosis of large number of animals. Many authors stated that cELISA test has very high sensitivity and specificity in the diagnosis of antibodies against *Anaplasma* species [9,22]. In *Anaplasma marginale* infections cELISA can diagnose these antibodies 6 years after infection [9]. However, serodiagnostic assays did not distinguish between current infection and prior exposure. In the current study we evaluated the performance of c ELISA assay to detect infections with Anaplasma in clinically suspected animals. cELISA results indicated that overall 47 of 88 serum samples (53.4%) were positive for Anaplasma antibodies. Positivity rate was 45.9% of cattle, 63.6% of sheep and 56.2% of goats. Our finding showed that *Anaplasma* have been determined in nearly 50% of suspected animals with ELISA.

A few previous serological studies involving *A. marginale* reported that the sero-prevalence ranged from 14.86% to 59.3% in different regions of Turkey [3,9,10,12]. The differences between infection prevalence may be attributed to the changes in climatic condition, intensity of tick infestation, and also contaminated needles and instruments transmission is an efficient way of infection spreading in herds [23]. But the most important epidemiological factor for the establishment of high prevalence is the persistence of infection in the reservoirs. In this study concerning the seroprevalence of Anaplasmosis in different age groups, the results showed that adult animals (more than 12 months) of both sheep and goat had the highest seropositivity rate. This might be explained by the fact that; the age resistance which may lasts up to 12 months and as the animals get older, become more susceptible to infection. However statistical analyses revealed that seropositivity rates were not differ among age groups of sheep and goat. On the contrary; young cattle (0-12 months) showed also high infection rate, this may be due to maternal antibodies in the colostrum [24]. Our results cleared that Anaplasmosis is a disease of adults; a parallel findings were recorded by Chahan et al.[25] and Keleş et al.[26].

Meanwhile the frequency of antibody existence was not statistically different among animal species (cattle, sheep and goat). However, in our knowledge there was no any other report comparing the seroprevalence of Anaplasmosis in cattle, sheep and goat in Turkey. Therefore it was not possible for us discuss and compare our findings with others.

Farmers and veterinarians in endemic areas often suspect anaplasmosis based on a history of previous disease outbreaks and clinical signs in that locality. In this study, infected animals showed anemia, pale mucous membrane, lack of appetite, weakness, weight loss, depression, icterus, lethargy and few animals presented fever (>40°C) and dehydration. Similar findings had been reported previously by Sharma et al.[21], Birdane et al.[10] and Abao-Elnaga et al.[8]. Diagnosis of Anaplasmosis in Bursa province is based on clinical signs. However, nonspecific clinical signs (fever and anemia) could lead to misdiagnosis with other diseases such as theileriosis and babesiosis [29,30]. In addition, field veterinarians of the study region had not enough information about the symptoms of the disease. Hence, for the definitive diagnosis of the anaplasmosis the clinical findings should be supported by serological tests.

It is well known fact that ticks are biological vectors of *Anaplasma spp*. Worldwide approximately twenty species of ticks have been incriminated as biological vectors along with other mechanical means such as contaminated fomites, castration instruments and blood sucking diptera [2,5,7,10,31]. In the current study many potential tick vectors of *Anaplasma* infection were identified during the investigation in farms with seropositive animals. Overall eight tick species belonging to five genus were identified

and 58.16% of ruminants examined for tick infestation were carrying at least one of tick species. *Rhipicephalus* species was the predominant tick vector followed by *Ixodes ricinus* and *Dermacentor marginatus*. In northern part and black sea region of Turkey *I. ricinus* as the vector of Anaplasmosis was observed as the most common species [7,28]. Our findings is agreement with those of Aktas et al.[7] and Arslan et al.[27]. This results is also consistent with those of reported tick species by Aydin [28] in their comprehensive study in this region previously.

In conclusion the results obtained from the current study clearly indicated that anaplasmosis present in cattle, sheep and goat in Bursa province of Turkey. cELISA can detect 50% of anaplasma infection in clinically suspected ruminants and can serve as a valuable and practical tool under field conditions. Another important result of this study is that diagnosis of anaplasmosis only according to clinical symptoms may not be always right. Therefore clinical diagnosis of anaplasmosis must be supported by serological and molecular tests.

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Research Article

Experimental Study on Ostrich Acellular Dermal Matrix in Repair of Full-Thickness Wounds of Guinea Pig

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Abstract

ADM (Acellular dermal matrix) can be a standard treatment for full-thickness wound defects. The aim of the present study was to achieve a decellularized matrix of the ostrich as a biomedical application and assess the evaluation of wound healing, which were implanted with ADM. Eighteen (n=18) guinea pigs were conducted and they received a full- thickness 2×2 cm flank skin wounds, nine experiment were implanted with acellular ostrich dermal matrix as experimental group and nine experiment as control group, which implanted with normal ostrich skin. At days of 3, 7 and 26 were evaluated for epithelialization and the other histological characteristics (Kruskal-wallis and Mann Whitney U tests), and the wounds were assessed for wound contracture at 26 days after surgery (ANOVA and Tukey test). Each dermal substitute decreased wound contracture and show no significant different at the endpoint of study ($P>0.05$). Between groups, increasing infiltration of fibroblast and new blood vessels under collagen scaffold showed no significant different ($P=0.06$). Histological evaluation of wound epithelialization and neo-dermis which was produced by ADM, was thicker neo-dermis at the end of study in experimental group. The study revealed that ostrich acellular dermal matrix can be used as a temporary substitute with acceptable result in healing of full-thickness wounds.

Keywords: *Wound, Acellular dermal matrix, Guinea pig*

Kobaylarda Tüm-Deri Katmanlarını İçeren Yaralarda Devekuşu Asellüler Dermal Matriksin Etkisinin Araştırıldığı Deneysel Bir Çalışma

Özet

ADM (Asellüler dermal matriks) tüm katmanları kapsayan yaralarda standart bir tedavi şekli olabilir. Bu çalışmanın amacı biyomedikal bir uygulama olarak devekuşu desellüler matriksin oluşturulması ve yara iyileşmesinde ADM uygulamasının başarısının değerlendirilmesidir. On sekiz (n=18) kobay çalışmada kullanıldı ve bu hayvanların yan taraflarında 2X2 cm boyutlarında deri yaraları oluşturuldu. Deneme grubu olarak 9 hayvana asellüler devekuşu dermal matriksi uygulanırken diğer 9 hayvan kontrol grubu olarak kullanıldı ve normal devekuşu derisi uygulandı. Epitelizasyon ve diğer histolojik bulgular 3., 7. ve 26. günlerde değerlendirildi (Kruskal-wallis ve Mann Whitney U testi) ve operasyondan 26 gün sonra yara durumuna bakıldı (ANOVA ve Tukey testi). Her iki uygulama yara kontraktürünü azaltırken çalışma sonunda anlamlı bir fark tespit edilmedi ($P>0.05$). Gruplar arasında fibroblast infiltrasyonu ve kolajen çatı altında damarlaşma açısından bir fark belirlenmedi ($P=0.06$). ADM uygulanan grupta yara epitelizasyonunun ve neodermisin histolojik değerlendirmesinde çalışma süreci sonunda daha kalın neodermis oluşumu gözlemlendi. Çalışma sonunda elde edilen bulgular yara iyileşmesinde asellüler dermal matriksin geçici olarak kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Yara, Asellüler dermal matriks, Kobay*

INTRODUCTION

Skin suffers from different injuries and losses. When tissue loss is extensive, the healing process may not be enough to cure, and treatment with flaps and grafts should be considered. Skin grafts, may be classified as autologous, homologous or xenogenic, such as those of porcine or bovine origins. Different procedures for tissue replacement have been introduced with the purpose of reducing scar formation and speeding up healing time [1,2]. Different

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methods of wound closure are in use, and each has its own advantages and disadvantages. The use of xenogenic skin as a source of ADM (Acellular dermal matrix) might alleviate some problems and make ADM more readily available for use in reconstructive surgery [2]. Due to excellent success in a variety of different biomedical applications, the performance of ADM, derived from full-thickness skin treated and removing of cells and cellular components, recently has drawn the attention of researchers in many fields [3]. Acellular dermal matrices are used as a suitable tissue for covering and also treatment of extensive burns [1]. In the wound-healing process, dermal substitutes play a guiding and supporting role during the cell and blood vessel recovery process [4]. Dermal substitutes serve as a scaffold into which cells can migrate and repair the injury [5]. Acellular dermal tissues are obtained by removing cellular components that are present in the tissue, since the cells are responsible for the activation of the immunological response. The absence of cellular structures result in little inflammation, and prevents rejection of the transplanted tissue [2]. As a dermal regeneration template, they also promote the guided regeneration function of the tissue, reduce scar hyperplasia and improve the quality of wound-healing [4]. The usefulness of ADM, which is derived from full-thickness skin following of removing the cells and cellular components concentrated in skin defect and cosmetic improvements. Because of absence cell particles of skin and diminishing of its antigenicity, it has been one of the most ideal substrate for a microenvironment for the cell movement and proliferation with no rejection. This experimental study deliberate to research of the effect of ostrich ADM in healing of full-thickness wounds of guinea pig.

MATERIAL and METHODS

Eighteen (n=18) adult male guinea pigs (230±17 g) were conducted. They were housed in cages with appropriate same food, humidity, light and bedding. Animals were fasted for 6 hours before surgery. All animals were administrated subcutaneously by saline-dextrose solution (15 ml/kg) one hour before surgery for supporting energetic deficient of guinea pigs during surgery. All surgical processes were performed using aseptic technique. Anesthesia consisted of combination of acepromazine (1 mg/kg) and ketamine HCL 10% (40 mg/kg) administrated intramuscularly to maintain deep anesthesia.

Left flanks of the animals were shaved and prepared aseptically. By placing the surgical drape, surgical site was exposed. Skin excision was made as 2×2cm surgical wounds in both groups by 15 no scalpel blade. ADM and normal skin were placed on the wounds and grafts sutured by simple interrupted pattern using a 4-0 nylon suture. The wounds were dressed with cotton gauze and fine cover bandage. Enrofloxacine 0.1 mg/kg, sc. and meloxicam 0.2 mg/kg orally were administrated as antibiotic and NSAID to all animals for 3 days after surgery, respectively.

Preparation of ADM

Fresh ostrich skin was obtained from the cadaver. After complete cleaning and excision of subdermal fat tissues, the skin was used for preparation of ADM. The preparation of ADM was consisted of washing the ostrich skin with PBS (Phosphate-buffered saline) then the skin was kept in DW (Distilled Water) for 24 h and then in Triton X-100 at 4°C temperature for 24 h. After that the proceed skins were washed with DW and then skins were kept in SDS (Sodium Dodecyl Sulfate) for 15 h and then the skins were washed with DW and then were kept in SDS for 10 h. Finally the skins were washed with PBS. The cocktail of antibiotic was used after acellular protocol to preclude microbial growth.

Scanning Electron Microscopy (SEM)

Normal and ADM samples were treated with routine protocols for preparing SEM examinations and samples were loaded on to aluminum studs and coated with gold. Collagen morphologies were examined under a scanning electron microscope (Philips XL30, Netherlands). Samples were scanned, and the micrographs were recorded. Comparisons were made of morphological changes to collagen fibers before and after ADM protocols.

Biochemical Assessment of Normal and ADM Samples

Collagen and sGAG content of the tissues were quantified using the Sircol and Blyscan assay kits, respectively. Total collagen and sGAG of all tissues were quantified and expressed as µg/mg wet skin tissue.

Gross and Histologic Measurement and Assessment

Wound characteristics were measured grossly on day 26 after surgery. Direct measurements of wounds area and wound contractions were determined from photographs taken intermittently. All healed wound in each group on day 3, 7 and 26 post surgery were biopsied and then dehydrated with an increasing series of alcohol concentration and after completing the process, embedded in the paraffin finally. Paraffin-embedded samples were sectioned at a thickness of 6 µm. After H&E staining, samples were examined by light microscopy. A blinded pathologist evaluated all samples histologically for epithelization, fibroblast infiltration and degree of inflammatory cell infiltration, collagen generation and new vessels production. The DNA in samples were assessed by 4′, 6-diamidino-2-phenylindole (DAPI) staining, and then observed under light microscope.

Data Analysis and Statistics

Data were analyzed statistically by ANOVA and Tukey test, the Kruskal-wallis and Mann Whitney U tests. All

analysis was performed using SPSS software (Version 18.0). P value was considered for statistically significance.

The present study has been approved by the Animal Ethics Committee of the Iranian Laboratory animal ethic frameworks under the reference code IAEC 3-12/03.

RESULTS

There is not clinical difference in appearance of ADM and normal skin such as color, texture and size.

Histologic Observation of Normal and ADM Samples

Unprepared sample showed presence of epidermal layer in H&E and massive nucleus in ECM in DAPI staining. Decellularized sample showed no epidermal layer in H&E and few positive staining of residual nuclear materials in DAPI staining *(Fig. 1)*.

Scanning Electron Microscopy (SEM) Evaluation

The histologic evaluation using SEM examination of normal samples structure showed a dense and integral matrix. After decellularization and underwent a period of processing with Triton, PBS and SDS, the structural pattern of collagen fiber still remained without microscopic changes *(Fig. 2)*.

Biochemical Assessment of Normal and ADM Samples

The decrease of collagen content per wet weight of decellularized skin (42.11±2.5 µg/mg) was significant ($P<0.05$) in compare with native tissue (57.86±4.7 µg/mg), as well as in compare with native (1.8±0.22 µg/mg), sGAG content of decellular skin (1.03±0.05 µg/mg) was significantly decrease *(Table 1)*.

Gross Evaluation of Wound Contracture

Dimensions of the implanted graft and contracture show at day 26 after surgery for each group *(Table 2)*. Decreased wound contraction assessed by original wound area. Graft contraction in both groups manifested no statistical difference macroscopically at the end point of study ($P>0.05$).

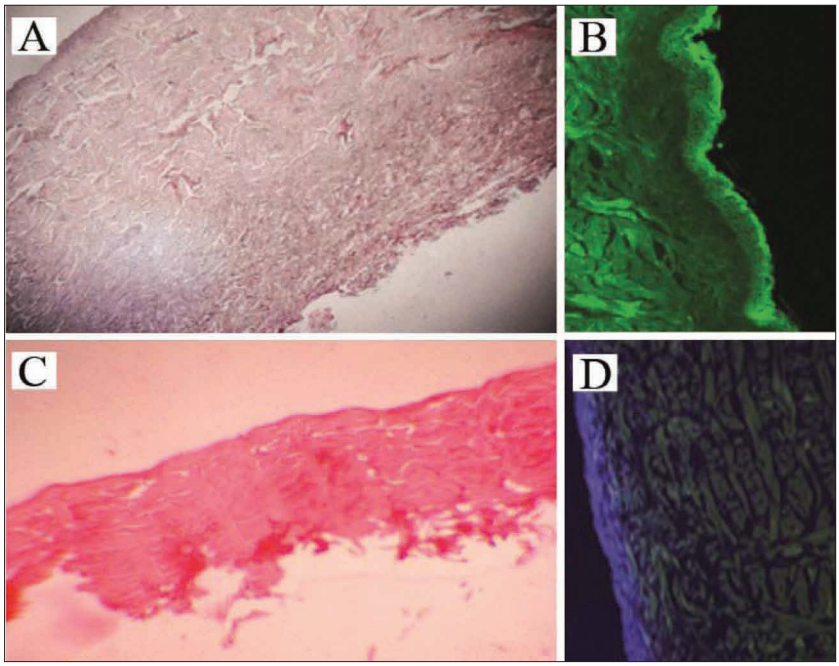

Fig 1. A- Normal skin, H&E (4×); **B-** Normal skin, DAPI (×10); **C-** Acellular skin, H&E (4×); **D-** Acellular skin, DAPI (40×)

Şekil 1. A- Normal deri, H&E (4×); **B-** Normal deri, DAPI (×10); **C-** Asellüler deri, H&E (4×); **D-** Asellüler deri, DAPI (40×)

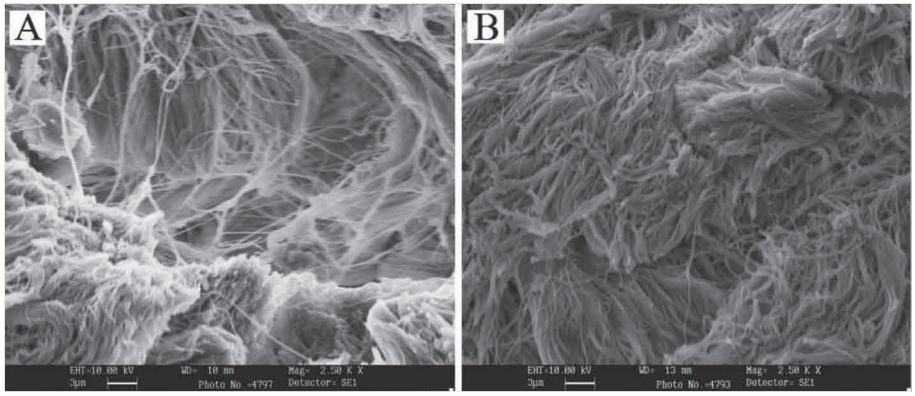

Fig 2. SEM photographs. **A-** Normal ostrich skin, **B-** Acellular ostrich skin (A, B ×2.50 kx)

Şekil 2. SEM fotoğrafları. **A-** Normal deve-kuşu derisi, **B-** Asellüler devekuşu derisi (A, B ×2.50 kx)

Table 1. *Quantification of the total collagen content and sGAG in the tissues (means ± SD)*
Tablo 1. *Dokulardaki total kolajen miktarı ve sGAG'nın kantitatif hesabı*

Grafted Tissue	Total Weight	
	Total Collagen (µg/mg wet tissue)	Total sGAG Content (µg/mg wet tissue)
Normal ostrich skin	57.86±4.7 [a]	1.8±0.22 [b]
Acelllar ostrich skin	42.11±2.5 [a]	1.03±0.05 [b]

Different signs (a,b) show signification

Table 2. *Area of grafts at day 26. Results are expressed as mean ± SD*
Tablo 2. *26. günde graftların alanı. Sonuçlar ortalama SS olarak ± verilmiştir*

Area (cm^2)	Group 1	Group 2
	1.38±0.18 [a]	3.20±0.32 [a]

(a) sign shows not significant

Histologic Evaluation of Wound in Days of Sampling

Both wounds in groups had infiltration of with fibroblast and vascularized by 3 days after surgery. Increasing in infiltration of fibroblast and neovascularization were evident in wound bed under collagen scaffold. No significant difference was shown by day 7 after surgery in infiltration of fibroblast between groups (P=0.06).

Table 3. *Evaluation of histologic factors in days 3, 7 and 26. Results are expressed as mean ± SD*
Tablo 3. *3., 7. ve 26. günde histolojik faktörlerin değerlendirilmesi. Sonuçlar ortalama SS olarak ± verilmiştir*

Factor	Day	Group 1	Group 2
Epithelization	3	0 [a]	0 [a]
	7	1.3±0.57 [a]	1.6±0.57 [a]
	26	1.6±0.57 [a]	4 [b]
Inflammation	3	1.3±0.57 [a]	3.3±1.15 [a]
	7	2.6 ±1 [a]	3.6±0.57 [a]
	26	3±0.57 [a]	3.6±1.15 [a]
Fibroblast	3	1 [a]	0.6±0.57 [a]
	7	2.3±0.57 [a]	3.6±0.57 [a]
	26	4 [a]	4 [b]
Vascular	3	1 [a]	1.6±0.57 [a]
	7	3 [a]	3.3±1.15 [a]
	26	3.3±1.15 [a]	3.6±0.57 [a]
Collagen	3	1 [a]	1 [a]
	7	1.3±0.57 [a]	2.3±0.57 [a]
	26	2.6±0.57 [a]	4 [a]

Different signs (a,b) show signification

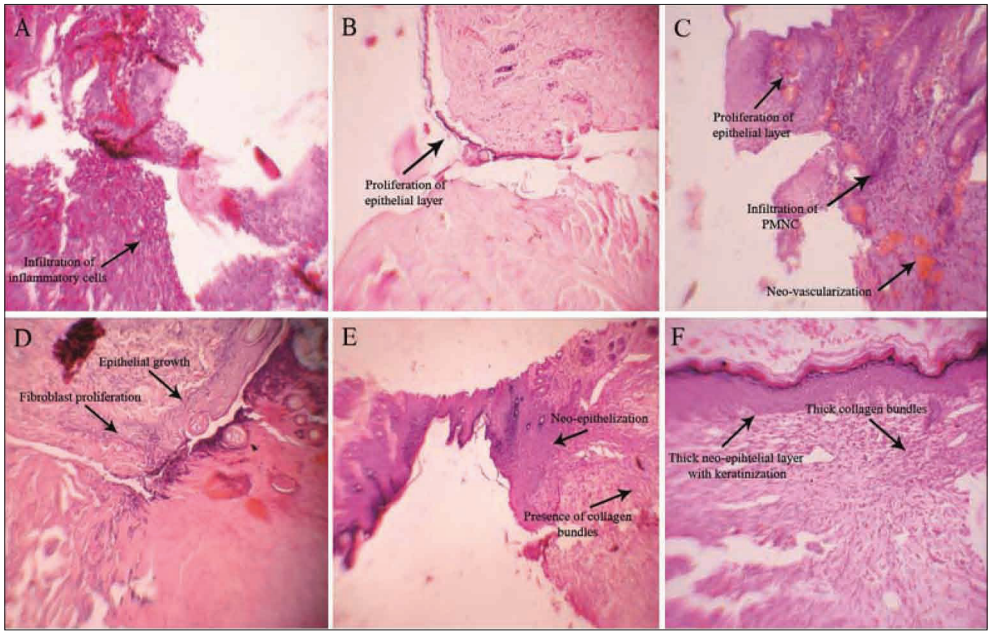

Fig 3. H&E staining of normal and ADM of ostrich skin samples. Representative histology from harvested in days 3, 7 and 26 after surgery. **A-** Day 3, control group, **B-** Day 3, experimental group, **C-** Day 7, control group, **D-** Day 7, experimental group, **E-** Day 26, control group, **F-** Day 26, experimental group (A-F ×10)

Şekil 3. Devekuşu deri örneklerinin normal ve ADM'lerinin H&E boyanmış örnekleri. Operasyondan 3, 6 ve 26 gün sonraki örnek histolojik görüntüler. **A-** 3. gün, Kontrol grubu, **B-** 3. gün, deneme grubu, **C-** 7. gün, Kontrol grubu, **D-** 7. gün, deneme grubu, **E-** 26. gün, Kontrol grubu, **F-** 26. gün, deneme grubu (A-F ×10)

Histologically, inflammation was seen in the wound samples of both groups. Comparing ADM and normal implanted skin show no significant difference between the groups (P=0.031). However extensive inflammation involving the entire biopsy specimen was observed in wound implanted with ADM and normal samples at endpoint of study. Histologic epithelialization in both groups was seen but the quantification of neo-dermis of ADM samples was thicker than normal graft *(Fig. 3)*. There is a significant difference (P<0.034) between groups in collagen bundles at endpoint of study *(Table 3)*.

DISCUSSION

In extensive deep burns and the other full-thickness skin wounds, permanent replacement of lost skin remains a major challenge. The different methods of wound closure are in use, and each has its own advantages and disadvantages. Porcine skin and preserved cadaver skin are

used for temporary wound coverage, but 1 week to 2 weeks after grafting, these tissues undergone immune-mediate rejection. Permanent wound coverage is usually accomplished using meshed split-thickness autograft harvested from undamaged regions of skin. The extensively burned patients have limited donor source, so thin split-thickness autografts are harvested repeatedly from the same sites. This results in substantial donor-site problems resulting from pain, infection, scarring and some keloid formation. Very thin meshed autografts can be used, but the lack of sufficient dermal bed often results in extensive wound contraction at the recipient site. Therefore, deepidermized xenograft porcine or human skin which cell components have been extracted and removed, as dermal substitute (Acellular Dermal Matrix) has been used with the least immuno-antigenecity and alleviate some of these problems and make ADM available for use in surgical procedure [2].

Human cadaver is expensive, limited and have economic problems, hence, available tissue are restricted as allograft. Also in islamic countries porcine products are forbidden so its skin is not used currently as xenograft. Nowadays ostrich farms spread and its products such as meat and leather are used widely in most countries. Therefore the use of xenogenic skin of ostrich as a new and wide source of ADM by alleviating of the problems make it more readily available for use in surgical procedure in skin deficit. Several previous studies have suggested using different materials for producing acellular dermal matrix of porcine or human skin. As Livesey et al.[6] extracted most or all cellular component from porcine, Srivasta et al.[2] used Dispase and Triton to produce acellular porcine skin. Mizuno et al.[7] used human skin for producing acellular matrix by using SDS (Sodium dodecyl sulfate) and hypertonic NaCl. Troung et al.[5] produced acellular human skin by using Dispase and Triton. In recent years Zuo et al.[8] in an experimental study, used EDTA-Na (Ethylenediaminetetraacetic acid), Triton and Trypsin for decellularizing rat skin. In this study, we prepared xenogenic ostrich ADM from fresh ostrich skin using SDS (Sodium dodecyl sulfate), PBS (Phosphate buffered saline) and Triton, used as xenograft in guinea pigs.

As previously mentioned, at the end point of study, wound contracture did not show significant different in two groups, that the findings agreed with Srivaste et al.[2] and Heo et al.[9] studies, which observed no significant contracture in wounds implanted with grafts. Also Walden et al.[10] study showed the derrmal grafting was prevented contracture of wound. This data was confirmed our results. Absence of prepared dermal matrix causes the fibroblast initially synthesizes an immature matrix and subsequently remodeled to form a hypertrophic scar or scar contracture [9]. Although decellularizing removes most or all of cellular components, leaves structural and functional molecules such as collagen and sulfated glycosaminoglycan (sGAG), which facilitate the communication of the adjacent cells and with external environment. GAG in ECM have special impact on delaying wound contraction and inducing regeneration [8].

Srivasta et al.[2] and Hoyama et al.[1] were observed infiltration of inflammatory cells around grafts. Srivasta et al.[2] expressed the cause of infiltration of inflammatory cells was the presence of type IV collagen, laminin, some fibronectin, and glycosaminoglycans remains in ADM, albeit in reduced amounts compared with normal skin, it seems unlikely the source of antigenicity. Histological results of presence of inflammatory cells in our study agreed with Srivasta et al.[2] and Hoyama et al.[1] studies.

Troung et al.[5] by using human acellular matrix as xenograft in rat evaluated epithelization in wound bed, also Heo et al.[9] with the aim of evaluating healing potential of different shape of ADM and comparing of the epithelialization, defined that although all full-thickness wounds implanted with/without acellular dermal matrix produced epithelialized healed wound by day 28 after surgery that the quantity of wound healing was different between the groups. Our study showed significant difference in epithelization formation between two groups. Early research with acellular dermal tissues was based on theory that if in presence of an appropriate matrix, normal cells of normal adjacent tissue would migrate towards the matrix and performing their functions as if they were in normal tissues. The other studies revealed these expectations, showing the presence of normal fibroblast, collagen and elastin with normal maturity and orientation [1]. Livessey et al. by using porcine acellular dermal matrix found that ADM attached well with the wound bed and promote the ingrowth of fibroblast on the surrounding normal tissue, also after 1 week new blood vessels were formed as a permanent dermal replacement [6]. We found that proliferation of fibroblast and formation new vessels are migrated under ostrich acellular matrix.

In conclusion, epithelization on wound bed of group implanted with acellular ostrich skin demonstrate that ostrich acellular dermal matrix as a temporary substitute may have optimal results in reconstruction of epithelial on wound bed under acellular scaffold.

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Research Article

Effects of a Short-term Supplementation with Liquid Oligofructose-enriched Inulin on Faecal Characteristics and Selected Serum Metabolites of Healthy Saanen Kids

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Abstract

The objective of this study was to evaluate the effects of a short-term supplementation with liquid oligofructose-enriched inulin on faecal characteristics and selected serum metabolites of healthy Saanen kids. Twenty-four kids (44 days of age) were allotted to a control (CG) or an experimental (EG) group. Each group consisted of 12 kids. Each kid in EG was supplemented with 0.8 and 1.6 g/d of oligofructose-enriched inulin from day 1 to 5 and from day 6 to 15, respectively. Liquid oligofructose-enriched inulin supplementation did not affect faecal score and pH (P>0.05). Faecal acetate, propionate and total SCFA concentrations did not differ (P>0.05) between CG and EG, whereas faecal butyrate concentration was higher (P<0.05) in kids supplemented with liquid oligofructose-enriched inulin. Due to trophic and antiinflammatory effects of butyrate, we hypothesize that oligofructose-enriched inulin supplementation may be useful to help tissue repair and regeneration, particularly during an intestinal infection. Faecal *Lactobacillus, Bifidobacterium* and *Clostridium perfringens* concentrations were not affected by oligofructose-enriched inulin supplementation (P>0.05). Daily dose of oligofructose-enriched inulin tended to increase serum glucose concentrations (P<0.09, P<0.08). Serum urea and albumin concentrations were similar between groups (P>0.05). Serum total protein and globulin levels were lower in EG compared with CG (P<0.05). During the experimental period lasting for 15 days, there were no differences in growth performance parameters between groups (P>0.05).

Keywords: *Liquid oligofructose-enriched inulin, Faecal characteristics, Serum metabolites, Saanen kids*

Sıvı Formdaki Oligofruktoz ile Zenginleştirilmiş İnulin Katkısının Kısa Süreli Kullanımının Sağlıklı Saanen Irkı Oğlaklarda Dışkı Özellikleri ve Bazı Serum Parametreleri Üzerine Etkisi

Özet

Bu çalışmanın amacı, sıvı formdaki oligofruktoz ile zenginleştirilmiş inulin katkısının kısa süreli kullanımının sağlıklı Saanen Irkı oğlaklarda dışkı özellikleri ve bazı serum parametreleri üzerine etkisini değerlendirmekti. Kırk dört günlük yaştaki 24 oğlak kontrol (KG) ve deneme (DG) olmak üzere 2 gruba ayrıldı. Her bir grupta 12 oğlak yer aldı. DG'deki her bir oğlağa deneme periyodunun ilk 5 günü 0.8 g/gün ve sonraki 10 gün boyunca da 1.6 g/gün oligofruktozla zenginleştirilmiş inulin verildi. Sıvı haldeki oligofruktozla zenginleştirilmiş inulin katkısının dışkı skoru ve pH'sı üzerine bir etkisi görülmedi (P>0.05). Dışkıdaki asetat, propiyonat ve toplam uçucu yağ asidi düzeyleri KG ve DG arasında farklılık göstermezken (P>0.05) dışkıdaki bütirat miktarı sıvı formdaki oligofruktozla zenginleştirilmiş inulin katkısını alan oğlaklarda daha yüksekti (P<0.05). Bütirik asidin besleyici ve antiinflamatuar etkilerinin olması sebebiyle, oligofruktozla zenginleştirilmiş inulin kullanımının özellikle intestinal enfeksiyonlar sırasında doku onarımı ve yenilenmesine yardım etmek için yararlı olabileceği ön görülmüştür. Dışkıdaki *Lactobacillus, Bifidobacterium* ve *Clostridium perfringens* konsantrasyonları oligofruktozla zenginleştirilmiş inulin uygulaması ile değişmedi (P>0.05). Oligofruktozla zenginleştirilmiş inulin katkısının günlük dozu serum glikoz konsantrasyonlarını arttırma eğilimindeydi (P<0.09, P<0.08). Serum üre ve albümin konsantrasyonları gruplar arasında benzerdi (P>0.05). DG'deki serum total protein ve globülin seviyeleri KG'dekinden daha düşüktü (P<0.05). On beş gün süren deneme periyodu sırasında gelişim performansı parametreleri bakımından gruplar arasında farklılık bulunmadı (P>0.05).

Anahtar sözcükler: *Oligofruktozla zenginleştirilmiş inulin, Dışkı özellikleri, Serum metabolitleri, Saanen Irkı oğlaklar*

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INTRODUCTION

Prebiotics are selectively fermented ingredients which can be able to modulate the composition and activity of the intestinal bacterial populations towards a healthier microflora [1,2]. Fructans are a class of the non-digestible oligosaccharides defined as a prebiotic and include short-chain fructooligosaccharides (scFOS), oligofructose and inulin [1,3].

Fructans are fermented by beneficial types of colonic bacteria *(Lactobacillus* and *Bifidobacterium)* [1,4]. *Lactobacillus* and *Bifidobacterium* are desirable colonic bacteria due to their health benefits such as inhibitory effect on the growth of pathogenic bacteria *(Escherichia coli*, *Clostridium* species and *Salmonella* species) in the colon and improving host immunity [5]. Colonic fermentation of fructans increases the production of short-chain fatty acids (SCFA), especially acetate, propionate and butyrate [6,7]. SCFA are bactericidal substances [8] and increased SCFA production reduces colonic pH, which may suppress the proliferation of potential pathogenic bacterial species [1,9]. In addition to potential effects of SCFA on the intestinal pH and pathogens, especially butyrate is associated with a trophic effect on the colonic epithelium [5,10]. Butyrate is the major energy source of colonocytes and seems to make the greatest contribution to the integrity of the colon [11,12].

Some of the previous studies in animals have shown that fructans can alter the intestinal pH [13,14], SCFA concentrations [6,7,13] and bacterial populations [15-17], as well as blood glucose [18,19] and urea [20,21] levels. In addition, it has been reported that fructans may improve the growth performance of animals [15,17,22]. But, on the other hand, a satiety effect of prebiotic supplementation can jeopardize the attempt to increase body weight gain in livestock [23].

Inulin and oligofructose are the most studied prebiotic supplements [2,24]. In addition to these fructans, a specific mixture (Synergy1, Orafti®, BENEO-Orafti S.A., Tienen, Belgium) has been developed. Synergy1 known as oligo-fructose-enriched inulin is a combination of chicory inulin molecules with selected chain lengths, enriched by a specific fraction of oligofructose. The unique chain length distribution of oligofructose-enriched inulin provides a sustained fermentation activity throughout the entire colon [24].

Although some information obtained from previous studies is available on the effects of feeding prebiotics in dogs [4], cats [16], rabbits [13], horses [7], pigs [21,25,26], calves [27,28] and lambs [29], no information has been reported, except for the results of our previous study [14], on the effects of prebiotic supplementation in kids. The objective of this study was to evaluate the effects of a short-term supplementation with liquid oligofructose-enriched inulin on the faecal responses of score, pH, SCFA concentrations and selected bacterial populations as indicators of intestinal health and serum urea and glucose levels in healthy Saanen kids.

MATERIAL and METHODS

Experimental Unit and Animals: The study was carried out at Uludag University Applied Research Center for Veterinary Faculty Unit in Bursa, Turkey. All animals were handled according to the EU directive number 86/609/EEC concerning the protection of animals used for experimental and scientific purposes. In addition, this study was conducted under an approved protocol by Animal Care and Use Committee of University of Uludag (approval number: 22.02.2012, 24/B2).

Twenty-four healthy Saanen kids with 44 days of age (12 male and 12 female) were used in the current study. Healthy kids were selected by clinical examination and monitoring in respect to general appearance and diarrhoea. The kids were sorted by parity of their dams and body weight at the beginning of the study and assigned to one of the two groups (control; CG and experimental; EG). Each group consisted of 12 kids (6 male and 6 female).

Management and Experimental Design: At 44 days of age, the kids were removed from their dams and housed in individual pens equipped with feeders and waterers throughout the experimental period lasting for 15 days. During the first 5 days of the experimental period, the kids were allowed to stay with their dams for 45 min in the morning (08.30 h to 09.15 h). The kids were weaned at 48 days of age. Water was offered *ad libitum* during the experimental period. Pelleted starter concentrate (Saf Feed Industry, Eskişehir, Turkey) was given 400 and 500 g once a day during the period from day 1 to 5 and from day 6 to 15 at 09.15 h, respectively. Nutrient analyses of pelleted starter concentrate were performed according to the AOAC [30]. Nutrient compositions of pelleted starter concentrate were presented in *Table 1*.

Each kid in EG was supplemented with 0.8 and 1.6 g/d of oligofructose-enriched inulin (Orafti®Synergy1, BENEO-Orafti S.A., Tienen, Belgium) from day 1 to 5 and from day 6 to 15 at 09.15 h, respectively, whereas the kids in CG did not receive oligofructose-enriched inulin. Synergy1 contained 93.5% of oligofructose-enriched inulin according to the certificate of analysis reported by BENEO-Orafti S.A. The daily dose of oligofructose-enriched inulin was dissolved in 10 ml of distilled water, and then administered to the kids in EG by sucking using a syringe. The kids in CG received orally only 10 ml of distilled water.

Faecal Score, the Occurrence of Diarrhoea and Faecal pH: Faecal samples were collected from each kid by retrieval from the rectum on day 1, 5 and 15 at 11.15 h. Faecal samples were scored in respect to consistency on all collection days according to the following system: 1 = watery, diarrhoea; 2 = soft, unformed; 3 = soft, formed;

Table 1. *Nutrient compositions of starter concentrate on dry matter basis*
Tablo 1. *Başlangıç yeminin kuru maddedeki besin maddesi kompozisyonu*

Item	Starter Concentrate[a, b]
Dry matter, %	92.95
CP, %	21.24
Ether extract, %	4.44
NDF, %	27.98
ADF, %	14.15
ADL, %	4.31
NFC[c], %	38.94
Ash, %	7.40

[a] Saf Feed Industry, Eskişehir, Turkey; [b] Contained the main ingredients: ground corn grain, ground barley grain, wheat bran, soybean oil, soybean meal, corn gluten meal, sunflower meal, molasses, mineral and vitamin mix, limestone, salt; [c] NFC (Nonfiber carbohydrate) = 100 - (NDF% + CP% + Ether extract% + Ash%)

4 = hard, formed; and 5 = hard, dry pellets. During the study, the kids were closely monitored daily in respect to diarrhoea. Faecal score of 1 was considered to be diarrhoea. The kids treated for diarrhoea were recorded. On day 5 and 15, each faecal sample was diluted 10-fold with distilled water as described by Verlinden et al.[31]. The mixture of faecal sample and distilled water was homogenized and faecal pH was immediately measured using an electronic pH meter (PT-10, Sartorius AG, Goettingen, Germany).

Concentrations of Faecal SCFA: After faeces collections on day 15, 2.0 g of fresh faecal samples were immediately placed in plastic tubes, and then acidified and diluted with 2 ml of 25% metaphosphoric acid and 6 ml of distilled water as described by Flickinger et al.[3]. The samples were centrifuged at 25.000 x *g* for 20 min (Eba-21, Hettich GmbH & Co.KG, Tuttlingen, Germany) within 1 h after faeces collections. The supernatants obtained were frozen at –20°C until the analysis. Before the analysis, the supernatant was thawed, centrifuged at 13.000 x *g* for 10 min and transferred into a gas chromatograph sample vial. Concentrations of acetate, propionate, butyrate and total SCFA (acetate + propionate + butyrate) were determined using a Perkin Elmer Auto System gas chromatograph (Hewlett Packard Agilent Technologies 6890N Network GC System, Serial CN10447002, China) and a glass column (30 m x 0.32 mm i.d.) packed with GP 10% SP-1200/1% H_3PO_4 on 80/100 Chromosorb (Supelco Inc., Bellefonte, PA, USA).

Selected Faecal Bacterial Populations: Sterile faecal samples could be collected from 8 of 12 and 7 of 12 kids in CG and EG, respectively, on day 15 by retrieval from rectum using sterile gloves. Faecal samples were placed in sterile sampling bags and immediately transported to the laboratory. One g of each faecal sample was homogenized with 9 ml of saline peptone water. Subsequently, serial 10-fold dilutions were made in saline peptone water and plated onto relevant selective media. *Lactobacillus* was grown on Man Rogosa Sharpe agar (MRS agar, Hypet Media, Diatek, Istanbul, Turkey) Each plate was incubated at 37°C for 48 h in anaerobic jar (Oxoid AN0035A, Basingstoke, Hampshire, UK) with gas generating sachet (Oxoid CN0020C, Basingstoke, Hampshire, UK). Non-spore-former rods, gram-positive and catalase-negative isolates were regarded as *Lactobacillus*. *Bifidobacterium* was grown on Bifidobacterium Selective Medium agar (BSM agar, Hypet Media, Diatek, Istanbul, Turkey). The plates were incubated at 37°C for 48 to 72 h in anaerobic jar with gas generating sachet. The selected bacterial colonies were investigated with regard to cell morphology by Gram staining. The colonies with gram-positive rods and characteristic bifurcated "Y" and "V" shapes were recorded as *Bifidobacterium*. *Clostridium perfringens* was grown on 4-Methylumbelliferyl phosphate-supplemented (MUP, Merck 1.00888, Darmstadt, Germany) Tryptose Sulfite Cycloserine agar (TSC agar, Hypet Media, Diatek, Istanbul, Turkey) containing egg yolk emulsion and selective supplement. Each plate was incubated at 37°C for 24 h in anaerobic jar with gas generating sachet. Each presumptive black colony were added to 10 ml of Thioglycolate Broth (Merck 1.08190, Darmstadt, Germany) and then incubated at 37°C for 16 to 18 h. Activated cultures were prepared for identifying by Gram staining and biochemical tests. The colonies with gram-positive and nonmotile rods, lactose positive, nitrate reduction positive, gelatine positive and motility negative were considered to be *Clostridium perfringens*. The bacterial counts were expressed as log 10 cfu per gram of faecal samples.

Serum Glucose, Urea, Total Protein, Albumin and Globulin Measurement: On day 15 at 2 h and 4 h after feeding, blood samples were collected from the jugular vein into serum separator tubes. Serum glucose and urea concentrations were determined using a reflotron analyzer (The Boehringer Mannheim Reflotron, Roche Diagnostics, Mannheim, Germany) with Reflotron®Glucose REF 10744948 diagnostic kit and Reflotron®Urea REF 11200666 diagnostic kit, respectively. Serum total protein, albumin and globulin levels were determined using a VetScan analyzer (Abaxis Inc., Union City, USA) with large animal profiles 500-0023 rotor.

Body Weight, Feed Intake and Feed Efficiency: The kids were weighed before feeding at day 1, 5 and 15. Daily starter concentrate feed intake was individually measured. Average daily weight gain (ADG), average daily feed intake (ADFI), and feed efficiency (ADG/ADFI) were calculated for each kid.

Statistical Analysis: Statistical analyses were conducted by using SPSS software [32]. Data for faecal score and pH were tested to determine normal distribution by Kolmogorov-Smirnov test and F-test, respectively. Faecal score and pH were analysed by independent sample T-test. Data for the amounts of individual and total SCFA in faeces and serum metabolites were tested to determine normal distribution by F-test. The analyses for faecal SCFA concentrations

and serum metabolites were performed by independent sample T-test. Faecal bacterial populations were analysed by the Mann–Whitney test. Data for growth performance parameters were tested to determine normal distribution by Kolmogorov-Smirnov test. Growth performance parameters were analysed by independent sample T-test. Differences between groups were considered significant at P≤0.05. Statistical trends were indicated as P<0.1.

RESULTS

Faecal score and faecal pH were not different (P>0.05) between groups *(Table 2)*. Diarrhoea developed in 1 of 12 and 2 of 12 kids in CG and EG, respectively, during the experimental period. The number of kids treated for diarrhoea was one in both CG and EG *(Table 2)*. The amounts of acetate, propionate and total SCFA (acetate + propionate + butyrate) in faeces did not differ (P>0.05) between groups, whereas faecal butyrate concentration was higher (P<0.05) on day 15 in EG compared with CG *(Table 2)*. No differences (P>0.05) in faecal concentrations of *Lactobacillus*, *Bifidobacterium* and *Clostridium perfringens* were found between CG and EG *(Table 3)*. Serum glucose concentrations tended to be higher (P<0.09, P<0.08) on day 15 in EG compared with CG *(Table 4)*. Serum urea concentrations were not different (P>0.05) on day 15 between groups *(Table 4)*. Serum total protein and globulin levels were lower (P<0.05) on day 15 in EG compared with CG while serum albumin levels and albumin/globulin ratios did not differ (P>0.05) between groups *(Table 4)*. During the experimental period lasting for 15 days, there were no differences (P>0.05) in body weight, ADG, ADFI and feed efficiency between groups *(Table 5)*.

DISCUSSION

***Faecal Score, the Occurrence of Diarrhoea and Faecal pH*:** Faecal scores were similar between groups in this study, which was in agreement with the results reported by Hill et al.[27], who added inulin (4 or 8 g/d) or mannanoligosaccharides (MOS; 6 g/d) to milk replacer of calves, and by Kara et al.[14], who supplemented inulin (0.6 g/d) to kids through oral gavage. In the current study, a lower faecal score would indicate formation of softer faeces. We observed that faecal score was decreased on day 5 and 15 in comparison to day 1 in EG *(Table 2)*. However, the decrease in faecal score had no clinical importance since it remained in an acceptable range and was not associated with diarrhoea. Potential adverse side effects such as loose faeces and diarrhoea may occur at high doses of fructans

Table 2. *Effect of liquid oligofructose-enriched inulin supplementation on faecal score, the occurrence of diarrhoea, faecal pH and faecal SCFA concentrations*
Tablo 2. *Sıvı haldeki oligofruktozla zenginleştirilmiş inulinin dışkı skoru, diyare oluşumu, dışkı pH'sı ve uçucu yağ asidi konsantrasyonu üzerine etkisi*

Item		Groups		SEM	P-Values
		CG[a] (n = 12)	EG[b] (n = 12)		
Faecal score[c]	day 1	2.83	3.33	0.32	0.09
	day 5	2.83	2.83	0.21	1.00
	day 15	3.00	2.83	0.24	0.50
The number of kids with diarrhoea/total kids		1/12	2/12	-	
The number of kids treated for diarrhoea/total kids		1/12	1/12	-	
Faecal pH	day 5	7.62	7.76	0.11	0.16
	day 15	7.75	7.63	0.13	0.31
Faecal SCFA concentrations (mmol/l; day 15)	Acetate	4.67	5.03	0.44	0.45
	Propionate	1.59	1.88	0.19	0.14
	Butyrate	1.42	1.71	0.14	0.05
	Total SCFA[d]	7.67	8.62	0.76	0.23

*[a] Control group; [b] Group supplemented with oligofructose-enriched inulin; [c] Faecal scoring system: **1** = watery, diarrhoea; **2** = soft, unformed; **3** = soft, formed; **4** = hard, formed; **5** = hard, dry pellets; [d] Acetate + Propionate + Butyrate*

Table 3. *Effect of liquid oligofructose-enriched inulin supplementation on faecal bacterial populations*
Tablo 3. *Sıvı haldeki oligofruktozla zenginleştirilmiş inulinin dışkıdaki bakteriyel popülasyon üzerine etkisi*

Item		Groups		SEM	P-Values
		CG[a] (n = 8)	EG[b] (n = 7)		
Faecal bacterial populations (cfu $\log_{10}$/g fresh faeces; day 15)	*Lactobacillus*	4.31	4.19	0.27	0.78
	Bifidobacterium	4.26	4.40	0.45	0.78
	Clostridium perfringens	2.24	1.74	0.35	0.64

[a] Control group; [b] Group supplemented with oligofructose-enriched inulin

Table 4. *Effect of liquid oligofructose-enriched inulin supplementation on serum glucose, urea, total protein, albumin and globulin levels and albumin/globulin ratio*

Tablo 4. *Sıvı haldeki oligofruktozla zenginleştirilmiş inulinin serum glikoz, üre, total protein, albümin ve globülin düzeyleri ve albümin/globülin oranı üzerine etkisi*

Item		Groups		SEM	P-Values
		CG[a] (n = 12)	EG[b] (n = 12)		
Glucose, mg/dl	2 h after feeding	80.87	87.15	2.53	0.09
	4 h after feeding	71.31	78.24	2.21	0.08
Urea, mg/dl	2 h after feeding	49.26	47.88	1.61	0.52
	4 h after feeding	49.73	48.39	1.70	0.61
Total protein, g/dl	2 h after feeding	6.70	6.47	0.07	0.04
	4 h after feeding	6.79	6.57	0.06	0.04
Albumin, g/dl	2 h after feeding	3.36	3.35	0.07	0.93
	4 h after feeding	3.55	3.52	0.07	0.91
Globulin, g/dl	2 h after feeding	3.34	3.12	0.06	0.02
	4 h after feeding	3.24	3.05	0.06	0.03
Albumin/globulin ratio	2 h after feeding	1.01	1.09	0.04	0.17
	4 h after feeding	1.10	1.18	0.04	0.17

[a] Control group; [b] Group supplemented with oligofructose-enriched inulin

Table 5. *Effect of liquid oligofructose-enriched inulin supplementation on growth performance*

Tablo 5. *Sıvı haldeki oligofruktozla zenginleştirilmiş inulinin büyüme performansı üzerine etkisi*

Item		Groups		SEM	P-Values
		CG[a] (n = 12)	EG[b] (n = 12)		
Body weight, kg	day 1	10.46	10.41	0.53	0.96
	day 5	10.67	11.12	0.55	0.57
	day 15	12.20	12.31	0.54	0.89
ADG[c], kg (day 1 to 15)		0.12	0.13	0.01	0.60
ADFI[c], g	day 1 to 5	218.52	228.67	18.46	0.70
	day 5 to 15	370.18	347.47	30.54	0.61
	day 1 to 15	332.03	305.26	28.46	0.52
Feed efficiency[c], g/g (day 1 to 15)		0.39	0.41	0.04	0.78

*[a] Control group; [b] Group supplemented with oligofructose-enriched inulin; [c] **ADG:** average daily weight gain; **ADFI:** average daily feed intake; Feed efficiency: ADG/ADFI*

or at moderate levels of ingestion in unadapted animals, which is due to excessive amount of fermentation of fructans by colonic bacteria [1,6]. In our study, daily dose of oligofructose-enriched inulin did not adversely affect faecal score of the kids in EG.

During the experimental period, diarrhoea occurred in 1 of 12 and 2 of 12 kids in CG and EG, respectively *(Table 2)*. The kid with diarrhoea in CG was treated with antibiotics for 5 days. While one of the kids that developed diarrhoea in EG was treated with antibiotics for 5 days, the other kid with diarrhoea in EG recovered without antibiotic treatment within 2 day. In general, the kids used in our study were healthy. Heinrichs et al.[33] reported that potential health effects of prebiotics might not be observed in healthy animals. We also observed that inulin supplementation did not affect the incidence of diarrhoea in healthy kids [14]. A previous study using piglets demonstrated that supplementation of scFOS decreased the incidence of diarrhoea during a pathogen challenge [34]. Halas et al.[26] also reported that 8% inulin added to the diet reduced the incidence of diarrhoea in pigs infected with *E. coli*. In this respect, different results of fructan supplementation on the incidence of diarrhoea may be observed in kids facing a diarrhoeal disease challenge.

Faecal pH was similar between groups in our study *(Table 2)*. The result of faecal pH was not in agreement with our previous data [14]. However, despite the fact that inulin supplementation decreased faecal pH in our previous study using kids [14], this effect of inulin was not consistent. Barry et al.[4] reported that faecal pH was not decreased in adult dogs when 0.2% or 0.4% inulin or scFOS was added to the diet. Conversely, Berg et al.[7] observed that faecal pH was lower for yearling horses supplemented with 8 or 24 g/d of scFOS in comparison to control horses.

Kanakupt et al.[16] reported that faecal pH was less for cats fed the diet supplemented with 0.5% scFOS plus 0.5% galactooligosaccharides compared with the control group. In the current study, our previous study [14] and the studies mentioned above [4,7,16], these different results for faecal pH may be caused by differences in the type of prebiotics and diets used and different dose and duration of prebiotic supplementation.

Concentrations of Faecal SCFA: Butyrate plays an essential role in maintaining colonic epithelium integrity [11,12] and exerts antiinflammatory effects [35], which may help tissue repair and regeneration [36]. Greater butyrate concentrations are thought to have an important role in intestinal health [10]. Based on the increase in faecal butyrate concentration of EG *(Table 2)*, we hypothesize that oligofructose-enriched inulin supplementation may be useful to help tissue repair and regeneration, particularly during the intestinal infections causing epithelial damage and colonic inflammation. Faecal SCFA concentrations can be used as an indicator of fermentation patterns in the colon and the amount of SCFA in faeces can provide relevant information with respect to changes in SCFA production [7], as was demonstrated in our study. Flickinger et al.[3] observed that supplementation of 1, 2 or 3 g/d scFOS did not alter concentrations of faecal acetate, propionate, butyrate and total SCFA. Xu et al.[25] reported that 0.4% fructooligosaccharides (FOS) added to the diet of piglets increased the amount of acetate in faeces while there were no significant changes in faecal propionate and butyrate concentrations of the piglets supplemented with FOS. Propst et al.[6] observed that 0.3%, 0.6% or 0.9% oligofructose and 0.3%, 0.6% or 0.9% inulin added to the diet increased the amounts of faecal acetate, propionate, butyrate and total SCFA in dogs. Berg et al.[7] observed that concentrations of faecal acetate, propionate, butyrate and total SCFA were increased in yearling horses supplemented with 8 or 24 g/d of scFOS in comparison to control horses. In the current study with oligofructose-enriched inulin and aforementioned studies [3,6,7,25], the results for faecal SCFA were different and variable, which may have been due to fermentation characteristic (the rapid or the slow fermentation) of fructans used, the amount of fructan fermentation in the colon and the type of basal diet used [1,3].

Selected Faecal Bacterial Populations: Faecal concentrations of *Lactobacillus*, *Bifidobacterium* and *Clostridium perfringens* were not affected by oligo fructose-enriched inulin supplementation *(Table 3)*. Increased SCFA concentrations and lowered pH are generally associated with a reduction in the growth of pathogenic bacteria in the intestine [1,5,12]. In this study, the result of faecal SCFA concentrations and no decrease in faecal pH supported the conclusion that oligofructose enriched inulin did not affect faecal concentration of *Clostridium perfringens* in healthy kids. In our previous study [14], it was found that supplementation of 0.6 g/d inulin did not decrease faecal concentrations of total *Clostridium* and *Escherichia coli* in healthy kids. Barry et al.[4] demonstrated that faecal concentrations of *Clostridium perfringens, Lactobacillus* and *Bifidobacterium* were not affected in healthy dogs when 0.2% or 0.4% inulin or scFOS was added to the diet. The results of prebiotic supplementation on intestinal microflora may be better during a disease challenge when the numbers of beneficial bacteria in the colon are decreased [37]. Heinrichs et al.[33] reported that the reason for the lack effect of prebiotic supplementation on faecal bacterial populations might be that calves used in their study were healthy. Since the kids used in our study were generally healthy during the experimental period, faecal bacterial populations may have not been affected by oligofructose-enriched inulin supplementation. Kanakupt et al.[16] reported that faecal concentrations of *Bifidobacterium* were increased in healthy cats supplemented with 0.5% scFOS, 0.5% galactooligosaccharides or 0.5% scFOS plus 0.5% galactooligosaccharides. Zhao et al.[17] observed that dietary supplementation with 1% and 2% fructan decreased *Escherichia coli* and increased *Lactobacillus* concentrations in faeces of healthy pigs. In contrast to ineffectiveness of fructans on colonic bacteria of healthy animals as observed in our study and some previous studies [4,14,33], it has been shown that fructans may alter faecal bacterial populations of healthy animals in the studies by Kanakupt et al.[16] and by Zhao et al.[17]. Thus, the effects of fructans on faecal microflora in healthy animals warrant further scrutiny.

Concentrations of Serum Glucose, Urea, Total Protein, Albumin and Globulin: It has been reported that fructans have the potential to decrease blood glucose level [19,20]. Conversely, in our study, serum glucose concentration tended to be higher when oligofructose-enriched inulin was supplemented to kids at the level of 0.46% (1.6 g/d) of average daily starter concentrate consumption *(Table 4)*. Similar to our result, 1% oligofructose or 1% inulin added to the diet of laying hens increased serum glucose levels [18]. In addition, Xu et al.[25] also observed that 0.4% FOS added to the diet increased serum glucose concentration in pigs. Diez et al.[38] reported that the inclusion of 7% inulin in the diet did not affect plasma glucose concentration in dogs. Diez et al.[20] observed that 2% sugar beet fiber plus 8% inulin added to the diet of dogs reduced plasma glucose level compared with the diets without additional fibre or with 1% sugar beet fiber plus 4% inulin. As observed in our study and aforementioned studies [18,20,25,38], it is likely that higher levels of dietary fibres such as fructans and beet fiber may play a role in lowering blood glucose concentration [1,20]. Nevertheless, the effect of liquid oligofructose-enriched inulin on serum glucose concentration remains unclear in kids.

The reduced pH as a result of colonic fermentation of prebiotics enhances the conversion of absorbable

ammonia into less absorbable ammonium. In addition, prebiotics serving as an energy source for intestinal bacteria may induce the trapping of nitrogen in the form of bacterial protein for extra bacterial growth, thus decreasing ammonia absorption. These effects are associated with a reduction in blood urea concentration [39,40]. Younes et al.[39] reported that FOS added to the diet decreased both pH of caecal content and plasma urea level in rats. Samal et al.[41] also demonstrated that supplementation of Jerusalem artichoke as a source of inulin reduced both pH of caecal, colonic and rectal contents and blood urea concentration in rats. In our study, serum urea concentrations were similar for both groups *(Table 4)*. The absent effect of oligofructose-enriched inulin on serum urea concentration agreed with no decrease in faecal pH and no change in faecal bacterial populations in EG.

In our study, serum total protein, albumin and globulin concentrations and albumin/globulin ratio were within reference ranges [42] in both CG and EG. These data suggest that there were no major metabolic problems at the end of the study in any group. Serum albumin concentrations and albumin/globulin ratios were similar between groups. Serum total protein and globulin levels were lower for EG in comparison to CG *(Table 4)*. Globulins are subdivided into α-, β- and γ-globulins. The γ globulins are largely composed of immunoglobulins and the α- and β-globulin fractions contain a great variety of different proteins [42]. Due to the presence of different globulin fractions identified as α, β and γ, the reason of difference in serum globulin concentration between CG and EG remained unclear.

Growth performance: A satiety effect of prebiotic supplementation can jeopardize the attempt to increase body weight gain in livestock [23]. However, in our study, liquid oligofructose-enriched inulin supplementation did not lead to a negative effect on body weight, ADG and ADFI *(Table 5)*. In contrast to a satiety effect of prebiotics, it has been shown that fructan supplementation may improve feed efficiency and growth performance of young animals [17,22,43]. Mul [44] observed an improvement in ADG and feed efficiency when 2 to 5 g/kg of oligofructose was added to milk replacer of calves. Xu et al.[15] observed that supplementation of 4.0 g/kg FOS to the diet increased ADG of broilers while 2 or 8 g/kg of FOS added to the diet had no effect on ADG. Hill et al.[27] demonstrated no improvement in ADG, ADFI and feed efficiency of calves supplemented with 4 and 8 g/d inulin. Grand et al.[28] reported that the addition of 3 or 6 g/d scFOS in the milk replacer had no effect on body weight, ADG, ADFI and feed efficiency in calves. Based on the current and previous studies, it can be seen that the effects of fructan supplementation on growth performance are inconsistent. These different results for growth performance may be related to amount of fructan compounds in the basal diet, the type of fructans used, the dose and the duration of fructan supplementation, as well as stress status of animals [17,23]. In our study, no effect of liquid oligofructose-enriched inulin on growth performance of kids may be due to the supplementation period (15 days) being short.

In conclusion, liquid oligofructose-enriched inulin supplemented to kids did not negatively affect faecal score. Supplementation of oligofructose-enriched inulin did not alter faecal pH and faecal *Lactobacillus*, *Bifidobacterium* and *Clostridium perfringens* concentrations. Higher doses than 1.6 g oligofructose-enriched inulin/d may be necessary to alter faecal pH and faecal bacterial populations of healthy kids. Faecal butyrate concentration was higher in kids supplemented with oligofructose-enriched inulin. Since butyrate exerts antiinflammatory effects and plays an essential role in maintaining colonic epithelium integrity, liquid oligofructose-enriched inulin supplementation may be useful to help tissue repair and regeneration, particularly during an intestinal infection. Daily dose of oligofructose-enriched inulin tended to increase serum glucose concentrations. Short-term supplementation with liquid oligofructose-enriched inulin did not alter growth performance parameters. The results of our study will be useful to determine the dose and duration of fructan supplementation in future studies investigating the effects of fructans on faecal characteristics, serum metabolites, health parameters and growth performance in young small ruminants.

Conflict of Interest: There are no conflicts of interest issues concerning this submission.

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Research Article

Investigation of Organizational Responsibility and Satisfaction Level of the Cattle Producers in Turkey

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Abstract

The aims of this study were to determine organizational responsibility and satisfaction levels of cattle producers according to different organizations and geographical regions of the Turkey, and to analyze the relationship between socioeconomic variables and above mentioned levels. The study was conducted with a total of 197 randomly sampled producers living in six different regions of the Turkey, between the years of 2013 and 2014. For overall Turkey, median responsibility and satisfaction values were found to be 2 (0–6) and 42% (20-100), respectively. Responsibility and satisfaction levels of the East–Southeastern region and the Milk Producers Association were significantly lower than other groups (<.01). Only eleven percent of the producers know important laws and regulations related to their own organizations. "Visiting frequency" and "meeting arrangements" components were given the lowest scores for satisfaction. Seven and four socioeconomic variables out of 11 were significantly positively correlated with the responsibility and satisfaction levels, respectively. In order to change the cattle producers' perception and attitudes farm visits and regular periodic meetings should be arranged by both livestock organizations and government. Those cattle producers, living in the East and Southeastern regions of Turkey and having low socioeconomic status, should be given priority in training programs.

Keywords: *Producer, Organization, Cattle, Responsibility, Satisfaction*

Türkiye'de Sığır Üreticilerinin Örgütsel Sorumluluk ve Memnuniyet Düzeylerinin İncelenmesi

Özet

Bu çalışmanın amaçları, farklı örgütlere ve Türkiye'nin farklı coğrafik bölgelerine göre sığır üreticilerinin memnuniyet ve sorumluluk düzeylerinin belirlenmesi ve sosyoekonomik değişkenler ile ifade edilen düzeyler arasındaki ilişkilerin analiz edilmesidir. Çalışma, Türkiye'nin 6 farklı bölgesinde tesadüfi olarak örneklenmiş toplam 197 üreticiyle 2013-2014 yılları arasında yürütülmüştür. Türkiye geneli için sorumluluk ve memnuniyet düzeylerinin medyan değerleri sırasıyla 2 (0-6) ve 42% (20-100) olarak bulunmuştur. Doğu–Güneydoğu Bölgesi ile Süt Üreticileri Birliği için sorumluluk ve memnuniyet düzeyleri diğer gruplardan anlamlı biçimde daha düşük bulunmuştur (<.01). Üreticilerin yalnızca %11'i kendi örgütleriyle ilgili yasa ve yönetmelikleri bilmektedir. En düşük memnuniyet skorlarını "ziyaret sıklığı" ve "toplantı düzenleme" bileşenleri almıştır. Toplam 11 sosyoekonomik değişkenin sırasıyla 7 ve 4 tanesi sorumluluk ve memnuniyet düzeyleriyle anlamlı pozitif bir ilişki içindedir. Sığır üreticilerinin algı ve davranışlarını değiştirmek için hayvancılık alanındaki örgütler ve kamu tarafından çiftlik ziyaretleri ve periyodik toplantılar düzenlenmelidir. Türkiye'nin Doğu ve Güneydoğu bölgelerinde yaşayan ve düşük sosyoekonomik statüdeki üreticilere eğitim programlarında öncelik verilmelidir.

Anahtar sözcükler: *Üretici, Örgüt, Sığır, Sorumluluk, Memnuniyet*

INTRODUCTION

Organizations can be defined as a group of people consciously and systematically gathered for collective goals or a particular purpose. In particular, they emerged as a result of the destruction of feudalism, the industrial revolution, and urbanization [1,2]. Many types of agricultural and livestock organizations have been established worldwide to serve the interests of members around the world. They founded by the state or by civil society for social, technical and economic reasons, and have played an important role in sustainable production. Through agricultural organizations, livestock producers increase their competitive power, and are able to provide more healthy

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and sufficient food to customers [3,4]. Also, better integration of the production and livestock-based industries can be achieved by the cooperatives [5].

In Turkey, located in Southeastern Europe and Southwestern Asia, various types of agricultural and livestock organizations have served cattle producers, such as the Cattle Breeders Associations (CBA), Beef and Lamb Producers Associations (BPA), Milk Producers Associations (MPA), Chambers of Agriculture (CA) and Agricultural Credit and Development Cooperatives (C). CBA, BPA, MPA, CA and C have served their members since 1996, 2005, 2007, 1957 and 1860, respectively. The Law of Animal Breeding (No.4631), The Law of Agricultural Producer Unions (No. 5200), The Law of Cooperatives (No. 1163), The Law of Chambers of Agriculture and Unions (No. 6964) constitute legal framework of the above-mentioned organizations [6-10]. In spite of the existence of many livestock organizations for producers in Turkey, they have not been regarded to be successful in terms of economics and policy point of view. It is pointed out that some of the important reasons for these failures are managerial weaknesses in organizations, lack of collaborations and organizational awareness amongst the members. Moreover, low levels of organizational commitment and responsibility among producers have been considered other possible reasons that may adversely affect their products quantity and quality [7,8].

Producers' organizational responsibility and their satisfaction about the services provided by the different organizations should be evaluated to better understand the current problems in a national-scale. Until now, there is no nationwide study in the literature, despite the well-known economic and political importance of the livestock organization. Previously, a similar study regarding small ruminant producers was performed by Can [8] in Hatay, Turkey. The present study is clearly different from the previous one due to following reasons: (I) it was conducted in national level, (II) it was focused on the cattle producers, and (III) its methodology was slightly changed.

The aims of the study were to determine organizational responsibility and satisfaction levels of cattle producers according to different organizations and geographical regions of the Turkey, and to analyze the relationship between socioeconomic variables with responsibility and satisfaction levels. The results of the study would be off useful for the livestock organizations and policymakers in the Ministry of Agriculture when investigating the problems and seeking the solutions about the issue.

MATERIAL and METHODS

Study Area, Sample Size and Data Collection

This study was conducted in six different geographical regions (Marmara-I, Aegean-II, Central Anatolian-III, Black Sea-IV, Mediterranean-V and East and Southeastern Anatolian-VI Regions) of the Turkey. There were a total of 12 cities (Edirne, Kırklareli, Denizli, Isparta, Hatay, Amasya, Samsun, Sinop, Çankırı, Ankara, Malatya and Gaziantep) which were represented the socioeconomic characteristics of the above mentioned regions. The minimum number of sample size [11-13] was calculated as follows;

$$n = \frac{N\ t^2\ p\ (1-p)}{d^2\ (N-1) +\ t^2\ p\ (1-p)}$$

where p = possibility of the events' occurrence of 85%, which was obtained from the from the pre-questionnaires and previous studies, N= total number of livestock enterprises in Turkey (nearly 3 million), t = 1.96 for a 95% confidence interval, d = 0.05 sampling error. Using the formula, the minimal estimated sample size was found to be 196. Then, the calculated sample size was distributed according to the number of livestock enterprises in above mentioned regions [7,8,13].

Before starting the field work, the questionnaire was pre-tested in order to remove some of the possible deficiencies and revise of the questions. Data were obtained via a questionnaire completed by 197 producers between May 2013 and November 2014.

The Items Used to Determine Producers' Satisfaction and Responsibility

The items used to determine producers' satisfaction and responsibility were modified from the study of Can [8]. Currently, Turkish livestock organizations have been working in different fields of the livestock sector and, therefore, some of the satisfaction items regarding the services were differ from each other according to the organizations. In the current study, 8 satisfaction items were expanded to 11, but nevertheless all of the six responsibility articles were same with the above mentioned study.

Data Evaluation and Statistical Analyses

In this study, commitments of the cattle producers were evaluated with the responsibility items. Each question about responsibility was answered as either "yes" or "no", and total responsibility level ranged from 0 to 6 point. Cattle producers' organizations were evaluated by their services and each question about satisfaction was ranged from 1 to 5 point. Each individual score was divided by the maximum possible level of satisfaction.

Test of normality was performed using Kolmogorov–Smirnov test. Results were analyzed using Kruskal-Wallis H test and Mann-Whitney U-test. Scatter diagrams were used to investigate the possible relationship between variables. The relationship between responsibility and satisfaction

levels with the producers' socioeconomic/demographic characteristics were analyzed using Spearman's rho [8,11,12]. All of the statistical analyses were performed with the aid of the SPSS-15.0 statistical software.

RESULTS

The distribution of the cattle producers according to different livestock organizations were presented in *Table 1*. It was found that only 15% of the producers were not member of any organization. According to the findings; majority of the producers were member of only one organization, however, very small number of producers were member of four organizations. CBA was still the most preferred occupational institution (29.95%) and has the largest share of members.

Table 1. *The distribution of the producers among different livestock organizations in Turkey*
Tablo 1. *Üreticilerin Türkiye'nin farklı hayvancılık örgütleri arasındaki dağılımı*

The Distribution of the Producers	Frequencies	
	N	%
1. Producers who are members of "one" organization	115	58.38
a. Cattle Breeders Associations	59	29.95
b. Chambers of Agriculture	27	13.71
c. Agricultural Credit and Development Cooperatives	14	7.11
d. Milk Producers Associations	11	5.58
e. Beef and Lamb Producers Associations	4	2.03
2. Producers who are members of "two" organizations	32	16.24
3. Producers who are members of "three" organizations	15	7.61
4. Producers who are members of "four" organizations	4	2.03
5. Producers who are "not members of any organization"	31	15.74
All of the producers (the sum of the above numbers)	197	100.00

In the present study, primary reason for "being a member of any organization" was asked to livestock producers. It was found that the most important factors affecting the producers' participation to any organization were "economic reasons" (54%) and "bureaucratic reasons" (23%), respectively. Other factors were as follows: "breeding and artificial insemination services" (11%), "occupational information" (7%), and veterinary services (5%). On the other hand, the reasons for "not being a member of any organization" were as follows: "to be seen as useless" (42%), "there is no sufficient information available" (37%) and "to be seen as expensive" (21%). Another finding of this study was that producers who are members of organizations had a significantly higher education level, income class, herd size and agricultural area (P<.01) compared to non-members.

A total of six responsibility components and their frequencies for five different organization are given in *Table 2*. It is understood that the majority of the producers have sufficient information about the management boards of their organizations. However, interestingly, only eleven percent of the producers know important laws and regulations related to the livestock organizations.

As it can be seen from the *Table 3*, none of the producers was "very satisfied" or "satisfied" about services provided by their organizations. The worst satisfaction scores were observed in following items: (X) "visiting frequency" and (XI) "meeting arrangements".

Producers' responsibility and satisfaction levels are summarized in *Table 4*. Considering the different organizations and regions in respect of responsibility and satisfaction levels, all of the differences are statistically significant. These levels were found to be lowest in Milk Producers Associations group and East-Southeastern region group. As a result of the statistical analysis, low level

Table 2. *Responsibility components and their frequencies for the five different organizations*
Tablo 2. *Sorumluluk bileşenleri ve bunların beş farklı örgüt için frekansları*

Responsibility Components Taken into Account	Producers who Answered "Yes" to the Questions											
	CBA (N=110)		CA (N=74)		ACDC (N=50)		MPA (N=13)		BLPA (N=14)		All Organization	
	N	%	N	%	N	%	N	%	N	%	N	%
I. No. of producers knowing important laws related to their organization	19	17	13	18	10	21	1	8	4	29	47	18
II. No. of producers reading the agreement or contract	24	22	18	25	16	33	1	8	7	50	66	26
III. No. of producers regularly vote in elections	53	48	39	54	29	60	3	23	9	64	133	52
IV. No. of producers became candidate in the elections	10	9	6	8	7	15	1	8	4	29	28	11
V. No. of producers having sufficient information about management boards	76	69	54	75	37	77	8	61	11	79	186	72
VI. No. of producers being aware of the debate topics and decisions taken	48	44	28	39	20	42	3	23	9	64	108	42

Table 3. *Satisfaction components and their median values for the five different organizations*
Tablo 3. *Memnuniyet bileşenleri ve bunların beş farklı örgüt için medyan değerleri*

Satisfaction Criterias to be Taken into Consideration	CBA (N=110)	CA (N=74)	ACDC (N=50)	MPA (N=13)	BLPA (N=14)	All Organization	
	Median	Median	Median	Median	Median	Median	Scale[1]
I. Occupational information	2	1	3	1	3	2	D
II. Required input supply	Irrelevant	Irrelevant	3	1	1	1	VD
III. Marketing of products	1	Irrelevant	2	1	3	1	VD
IV. Veterinary services	2	Irrelevant				2	D
V. Breeding services	3	Irrelevant				3	NSD
VI. Official proceedings	3	3	3	1	4	3	NSD
VII. Meeting new breeders	1	1	2	1	3	1	VD
VIII. Level of trust	3	3	3	1	3	3	NSD
IX. Speed of services	2.5	3	3	1	3	3	NSD
X. Visiting frequency	1	1	1	1	1	1	VD
XI. Meeting arrangements	1	1	1	1	1	1	VD

*[1]**1:** VD (Very Dissatisfied), **2:** D (Dissatisfied), **3:** NSD (Neither Satisfied nor Dissatisfied), **4:** S (Satisfied), **5:** VS (Very Satisfied)*

Table 4. *Responsibility and satisfaction levels acc.to different livestock organizations and geographical regions of the Turkey*
Tablo 4. *Türkiye'nin farklı hayvancılık örgütleri ve farklı coğrafik bölgelerine göre sorumluluk ve memnuniyet düzeyleri*

Different Organizations and Regions	Responsibility Level (Number)			Satisfaction Level (%)		
	Mean ± Stand. Dev.	Mean Rank	P	Mean ± Stand. Dev.	Mean Rank	P
1. Livestock Organizations						
1.a. CBA	2.09±1.65	124.18		48.57±22.38	132.55	
1.b. CA	2.19±1.63	129.01		40.78±16.24	110.12	
1.c. ACDC	2.48±1.71	140.41	< .05[1]	49.27±18.90	139.58	< .01[3]
1.d. MPA	1.31±1.44	88.04		32.38±18.18	72.04	
1.e. BLPA	3.14±1.99	165.75		51.15±25.43	138.19	
2. Geographical Regions						
2.a. Marmara	2.46±1.55	142.80		53.97±25.90	144.72	
2.b. Central Anatolian	2.71±1.43	155.03		45.90±13.98	131.72	
2.c. Black Sea	2.41±1.75	136.60	< .01[2]	48.23±19.94	134.76	< .01[2]
2.d. Aegean	2.03±2.07	114.76		45.47±24.66	117.24	
2.e. Mediterranean	1.93±1.39	120.02		43.46±15.84	120.82	
2.f. East and Southeastern	1.23±1.53	82.29		31.91±11.62	72.06	
Overall Turkey	Mean±Stand. Dev : 2.21±1.68 Median (Min-Max) : 2 (0-6)			Mean ± Stand. Dev : 46.04±20.46 Median (Min-Max) : 42 (20-100)		

[1,2,3] By Kruskal-Wallis analysis, there were significant differences among the groups; [1] Pairwise comparisons performed using the Mann-Whitney U test indicated that there were significant differences between the CBA and BLPA, between the MPA and BLPA, as well as between the MPA and ACDC, [2] Mann-Whitney U-test indicated that there were significant differences between the Central Anatolian and Mediterranean Regions, as well as between the East–Southeastern region and rest of the other regions, [3] Mann-Whitney U test indicated that there were significant differences between the ACDC and CA, between the ACDC and MPA, between the CBA and CA, as well as between the MPA and rest of the other organizations

of responsibility and medium-low level of satisfaction were found for overall Turkey.

Correlations between socioeconomic variables with the responsibility and satisfaction levels along with *p*-values were presented in *Table 5*. Seven and four socioeconomic variables out of 11 were significantly positively correlated with the responsibility and satisfaction levels, respectively. Additionally, there was a significant relationship between responsibility and satisfaction at the level of $p<.01$.

DISCUSSION

Organizational behaviors of the livestock producers are complex and multidimensional concept. In order to demonstrate organizational effectiveness of the producers

Table 5. *Correlation coefficient and P-values for responsibility and satisfaction levels*
Tablo 5. *Sorumluluk ve memnuniyet düzeyleri için ilişki katsayıları ve P-değerleri*

Socioeconomic and Demographic Variables	The unit Used	Responsibility Level		Satisfaction Level	
		Spearman Rho	P	Spearman Rho	P
1. Producers' age	(year)	-0.044	> .05	-0.040	> .05
2. Occupational experience	(year)	0.047	> .05	0.065	> .05
3. Education level	(year)	0.283	< .01	-0.006	> .05
4. Income class	(US$)	0.237	< .01	0.140	< .05
5. Herd size	(number)	0.326	< .01	0.086	> .05
6. Total number of memberships	(number)	0.193	< .05	0.195	< .01
7. The duration of membership	(year)	0.169	< .01	0.070	> .05
8. Total agricultural area	(acres)	0.374	< .01	0.291	< .01
9. Cultivated agricultural area	(acres)	0.422	< .01	0.356	< .01
Responsibility level	(number)	1		0.416	< .01
Satisfaction level	(ratio)	0.416	< .01	1	

and to understand which individual factors are correlated with the membership relations, a total of 6 responsibility and 11 satisfaction components and 9 socioeconomic variables were taken into consideration in the present study. The findings indicated that Turkish producers' responsibility and satisfaction were low and medium-low levels, respectively. It is clearly understood that responsibility of the producers is quite far from the desirable level. That is to say, they have behaved irresponsibly. Nevertheless, it should be indicated that, low level of responsibility can be also affected by the poor management and/or poor services provided by the livestock organizations.

In Turkey, majority of the producers still believe that organizational activities have not been performing effectively except providing some official documents or bureaucratic issues. In the current study, economic and bureaucratic reasons were found to be the most important factors affecting producers' decision whether membership participation in organizations or not. Indeed, the result is not surprised because producers' main purpose is already to make money and sustainable production. This result also consistent with the study of Can [8] who worked on the sheep breeders. Özüdoğru and Tatlıdil [14] determined that 93% of the farmers in member farms believe that the Cattle Breeders Association has a role in increased incomes. Another study was also reported that the most important expectations to be a member of the cooperatives were "economic reasons". Besides this, "reliable and accessible managers" was found to be important factor [15]. In the present study, nearly one-tenth of the producers were not want to be a member of an organization. The main reasons of that is the beliefs that livestock organization are not useful or required. This small proportion of producers may be underestimated or even ignored. However, it should not be forgotten that, they can be convinced with the effective training programs and/or small amount of financial support. Another finding of the study was that non-member producers had significantly lower socioeconomic status (p<.01) compared to member producers. Conversely, Can [8] did not report significant differences between members and non-members with respect to income and education levels. Alambeigi et al.[16] reported that the leading factors deterring farmer's participation were determined as lack of partnership culture and a lack of sufficient power in the cooperatives. Another noteworthy finding of the study is a lack of harmony between the objectives of villagers and the cooperatives [16]. It is indicated that without compulsory membership, organizations must appeal to members and provide valued services and opportunities [17].

The findings regarding the responsibility level clearly indicate that producers have not fulfilled their legal, democratic and/or social responsibilities. Considering the overall Turkey, forty-six percent of satisfaction may be seen as medium-low or moderate level for the organizations. Because of the fact that "visiting frequency" and "meeting arrangements" are the most negative aspects of the livestock organizations, the quantity and/or quality of these two services should be increased as much as possible. It was reported that nearly 53% of the producers were satisfied from extension services of the Livestock and Dairy Development Department, however, a majority of them indicated that extension workers never visited farms, which is the most important reason for dissatisfaction [18]. In a study, professional competency rates of Extension Agents were found to be 2.26 and 2.99 (1=very low and 4= very high) from the view of farmers and Extension Agents' perspectives, respectively [19]. Although it is indicated that agricultural organizations are not effective [6], however, according to seventy percent of the Turkish producers agricultural cooperatives are successful in their activities [15]. Another study indicate that the activities of Cattle Breeder's

Association of Manisa are generally good, but price and support policies are not sufficient [20].

It is a well-known reality that still East and Southeastern regions of Turkey has the lowest socioeconomic status and this study findings support this argument. The lowest responsibility and satisfaction levels were observed for these regions and differences were found to be significant. Although these levels are close to each other for the other five regions, it is remarkable that Marmara, Central Anatolian and Black Sea regions have the highest levels. MPA has the lowest score both for responsibility and for satisfaction. This may be due to small number of producers in this group. Another reason could be that MPA is a much younger organization than the others.

In this study, almost all of the socioeconomic variables except producers' age and occupational experience were significantly correlated with the responsibility level. Interestingly, only income class and total agricultural area were significantly correlated with satisfaction level. In general, it is normally expected that there are no close relationship between socioeconomic parameters and satisfaction because satisfaction are generally influenced by the services. Österberg and Nilsson [21] report that farmers' age has a significant effect on the organizational commitment and trust of the older producer less than younger ones. Producers who are satisfied with the profitability in their organization have a higher score than others [21]. Didier et al.[22] point out that there is a relationship between member producers' commitment and their trust which plays an important role in successful membership. According to Fulton and Giannakas [23] member commitment is linked to the cooperative's ability and there is a feedback relationship between them. Gedara et al.[24] indicated that the most influential factors of technical efficiency are membership of farmer organizations and the participatory rate in collective actions organized by farmer organizations. Ozcatalbas et al.[25] reported that there was a relationship between family sizes, experience in dairy farming, raising high yielding dairy cows with the daily milk yield, among the variables considered social factors.

Although the results of this study and official statistics clearly reveal a great number of producers are members of any professional organization in Turkey, but livestock organizations' management and financial problems could not be solved properly until now [7]. There are many different types of conflicts in economic and political areas that have been observed amongst them. In order to achieve the desirable performance, they need to be complementary to each other, rather than serving as an alternative. Can [8] indicated that high rate of member's democratic participation can help to solve current marketing problems in the sector. Idrees et al.[18] suggested that frequency of farm visits should substantially be increased and model dairy farms should be organized among the producers. Training of the organization managers are also suggested both for management skills and for pedagogical skills [21].

Basing on the findings of the present study, following recommendation can be made; (i) to change the livestock producers' perception and attitudes farm visits and regular periodic meetings should be provided by both livestock organizations and government, (ii) both member and non-member producers should be encouraged to participate in seminars and workshops, (iii) those producers living in East and Southeastern regions of Turkey and having low socioeconomic status should be given priority in training programs.

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The Melatonin Attenuates Alloxan Induced Post-Diabetic Testicular Damage and Oxidative Effects in Rats

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Abstract

The aim of this study was to examine the protective effect of melatonin (MLT) on the alloxan-induced post-diabetic testicular damage in rats. Forty-eight Sprague Dawley male rats were divided into four groups (n=12). Group C received 0.9% saline for 45 days, as control. Group DM (Diabetes Mellitus) was injected by 120 mg/kg single dose alloxan intraperitoneally (IP). Group MLT was treated by 10 mg/kg/d MLT IP for 45 d. Group DM+MLT was treated by 10 mg/kg/d MLT IP for 45 days following alloxan injection. Six animals on days 15 and the remaining 6 on days 45 were sacrificed in each group after the initiation of the study. Testis tissues and blood samples were collected to investigate blood glucose, oxidant and antioxidant statues, sperm parameters, and histopathological and 8-hydroxy-2'-deoxyguanosine (8-OHdG)-immunopositive cell examinations. MLT treatment of diabetic rats prevented the increases in malondialdehyde (MDA) levels and 8-OHdG concentrations, and the decreases in the GSH and SOD levels observed in DM groups. Alloxan administration depressed the sperm concentration, the number of progressively motile spermatozoa, premature acrosome reaction (AR) and calcium ionophore A23187-induced AR rates, without any change in dead and/or abnormal sperm rates in DM group. Histopathological and immunohistochemical observations also revealed that MLT-treated diabetic rats had an enhanced histological architecture and DNA damage in testis. These findings suggest that MLT treatment prevents the testicular damage by declining the oxidative stress, but it did not recover the depressed sperm parameters in diabetic rats.

Keywords: *Diabetes, Melatonin, Rat, Testis*

Melatonin Ratlarda Alloxan İle İndüklenen Diyabet Sonrası Oluşan Testiküler Hasarı ve Oksidatif Etkileri Hafifletir

Özet

Bu çalışmanın amacı, ratlarda alloxan ile oluşturulan diyabet sonrası gözlenen testiküler hasar üzerine melatonin'in (MLT) koruyucu etkisini incelemekti. Araştırmada kullanılan 48 adet Sprague Dawley ırkı erkek rat 4 gruba ayrıldı (n=12). Kontrol grubu olan Grup C'ye 45 gün süreyle %0.9 serum fizyolojik verildi. Grup DM'ye (Diabetes Mellitus) tek doz alloxan (120 mg/kg) intraperitoneal (IP) olarak enjekte edildi. Grup MLT'ye 45 gün süreyle 10 mg/kg/gün dozda MLT IP olarak enjekte edildi. Grup DM+MLT'ye alloxan enjeksiyonunu takiben 45 gün süreyle 10 mg/kg/gün dozda MLT IP olarak uygulandı. Her gruptan 6 hayvana araştırma başladıktan 15 gün sonra, diğer 6 hayvana ise 45 gün sonra ötenazi uygulandı. Kan glikoz seviyesi, oksidan ve antioksidan durumları, sperm parametreleri ve histopatolojik ile 8-hydroxy-2'-deoxyguanosine (8-OHdG)-immunopozitif hücre incelemeleri için testis dokusu ve kan örnekleri alındı. Diyabetik ratlara yapılan MLT uygulamasının, grup DM'de görülen malondialdehyde (MDA) seviyesi ile 8-OHdG yoğunluğundaki artışı ve glutathion (GSH) ile superoxide dismutase (SOD) seviyelerindeki azalmayı önlediği belirlendi. Alloxan uygulaması grup DM'de sperm yoğunluğu, motil sperm sayısı, prematüre akrozom reaksiyonu (AR) ve calcium ionophore A23187 ile indüklenen AR oranlarını deprese ederken, ölü ve/veya anormal sperm oranını değiştirmedi. Ayrıca, histopatolojik ve immunohistokimyasal muayeneler, diyabetik ratlara yapılan MLT uygulamasının histolojik yapı ve DNA hasarında iyileştirici bir etki gösterdiğini ortaya çıkardı. Sonuç olarak, MLT uygulamasının diyabetik ratlarda oksidatif stresi azaltarak testiküler hasar oluşumunu önlemesine rağmen, sperm parametrelerinde oluşan bozulmaları engelleyemediği gözlendi.

Anahtar sözcükler: *Diyabet, Melatonin, Rat, Testis*

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INTRODUCTION

Diabetes Mellitus (DM), with a chronic nature; i) is characterized by hyperglycaemia, ii) alters metabolic processes of carbohydrate, protein and lipid, and iii) increases the risk of complications arise from cardiovascular diseases [1]. It is associated with a high risk of atherosclerosis, thus having a destructive effect on the organs of renal, nervous, and ocular systems. Moreover, reproductive impairment is a common complication of DM in both genders [2,3]. Streptozotocin (STZ) is used in the treatment of neoplastic β-cell and in the formation of DM type-1 animal model. In virtue of the oxidative stress induced by hyperglycemia and histologic changes in testis, one feature providing the surpassing the STZ's reproductive activity has also occurred [4].

Reactive oxygen species (ROS) (e.g. hydrogen peroxide (H_2O_2), singlet oxygen (O_2^-) and superoxide anions and lipid peroxides) have been associated with the pathogenesis of many serious systemic diseases (e.g. cancer, rheumatoid arthritis, ischemic diseases, neurological disorders and diabetes). Also, the pancreatic β-cells are very sensitive to injury caused by the free radicals [5-8]. The most striking example of β-cell damage is the severe injury induced by alloxan or STZ. It has several destructive effects, including ROS generation, DNA methylation and protein modification. In all the living animal cells, there is a balance between the oxidants and antioxidant defence. When an imbalance occurs within the cells, the ROS initiate the cell death and histopathological changes. The 8-hydroxy-2′-deoxyguanosine (8-OHdG) enzyme reaction is one of the predominant forms of free radicals, and used as a biomarker of oxidative stress [9].

Dietary supplementation with antioxidants such as medicinal plants, vitamins, and flavonoids has been used to prevent the occurrence of DM [5-7]. The MLT is a free-radical scavenger and wide-spectrum antioxidant, and it stimulates the gene expression of antioxidant and pro-oxidant enzymes. MLT treatment after alloxan-induced β-cell destruction has been reported recently [10-14]. These studies suggest that MLT treatment in DM regulates the antioxidant enzymes and decreases the degenerative changes. However, no marked changes were reported in glucose level and β-cell apoptosis, while high MLT levels lead to decreased insulin release from the β-cells [15-19]. Recenly, diabetes in male rats has been induced via the STZ, but the effects of alloxan-induced diabetes on male fertility have not yet been investigated.

Herein, we aimed to determine the effect of MLT against the oxidative damage caused by alloxan injury of the testis, by measuring spermatological, testicular and biochemical parameters, and by conducting immuno-histochemical (IHC) examinations for apoptotic cells in diabetic Sprague-Dawley rats.

MATERIAL and METHODS

Animals and Groups

Forty-eight adult, 8 weeks old, male Sprague Dawley rats weighing (body weight, b.w.) about 230 g (ranging 200-250 g) were used. All procedures were carried out in accordance with the protocol approved by the Atatürk University Faculty of Veterinary Sciences, Local Board of Ethics for Animal Care and Use (Decision No: 2015/58). Pairs of rats were placed in cages under a 12 h daylight/darkness cycle with an ambient temperature of 23±2°C and humidity of 55±10% throughout the study. Standard rat chow and drinking water were provided *ad libitum*. Animals were divided into four groups (n=12, each) as follows: Control group (group C), receiving intraperitoneal (IP) saline only; MLT group, MLT injection (10 mg/kg/d, IP); DM group, diabetes induction + receiving IP saline (negative control group); DM+MLT group, diabetes induction + MLT injection (10 mg/kg/d, IP) for the 15th and 45th d. On these time points, all rats were anaesthetised with an IP injection of 60 mg/kg sodium pentobarbitone, then they were killed by taking blood samples from the intracardiac site. Thereafter, their testes and cauda epididymes were removed immediately.

Induction of Diabetes

In overnight-fasted rats, diabetes was induced by IP injection of alloxan (Sigma, St. Louis, Mo., USA) prepared freshly at a dose of 120 mg/kg b.w. in 0.9% NaCl. The inductions were verified after 24 h and non-diabetic animals (with a fasting blood glucose level lower than 220 mg/dl) were injected with the second dose of alloxan (as above). The animals were allowed 7 d for the stabilisation of blood glucose level. Blood samples were collected from the tail vein. Prior to the experiment (at zero h), the glucose levels were measured by a glucometer and test strips (On Call Pluss) on the 15th and 45th d. At the 8th d, animals having a fasting blood glucose level higher than 220 mg/dl were considered as 'diabetic'.

Drug Administration and Analyses

The MLT was supplied from Sigma (St. Louis, MO, USA). The pineal indole was dissolved freshly in ethanol and diluted with a sterile saline to give a final concentration of 0.01% ethanol and administered IP with a daily dose of 10 mg/kg. MLT treatment was commenced on the 14th d following the alloxan injection and this was considered as 1st d of treatment. This treatment is continued in the MLT and DM+MLT groups. The levels of glucose on the 15th and 45th d were measured using the commercial kits by an autoanalyser (as given above).

The right testis were removed, washed with physiological saline solution, and stored at –80°C until the analyses. The method, as described by Ohkawa et al.[20], was

used for determining the MDA levels in testis tissues. Measurements of tissue glutathione (GSH) level and superoxide dismutase (SOD) activities were performed according to the methods described previously by Tietze [21] and Sun et al.[22], respectively. The results were expressed as nmol/g protein for the MDA, nmol/g protein for the GSH and U/g protein for the SOD levels.

Sperm Collection and Evaluation

Collections of cauda epididymal sperm and the assessment of sperm parameters (progressive motility, live-dead sperm and abnormal sperm rates) were performed [under a phase contrast microscope (Carl Zeiss Axio Scope. A1) on a warm stage at 35.5±0.5°C] by modification of the method described by Akman and Aksoy [23]. The sperm concentration was determined with a Neubauer counting chamber, using modified method of Türk et al.[24], while the modified method of Larson and Miller [25] was used for acrosome reaction (AR) and staining procedures.

Histochemistry and Immunohistochemistry of Testis Tissues

The samples of left testis were fixed in Bouin's fixative fluid and processed routinely for embedding in paraffin. The series of 5-6 µm thick sections prepared from the blocks were stained by Mallor's triple staining for histopathological examination, while streptavidin-biotin-peroxidase staining method (Ventana BENCHMARK GX, automatic staining instrument) was used for immunohistochemical staining of the oxidative cells. For the former examinations, Johnsen's criteria were used to determine the spermatogenesis in testis [26]. For the latter examinations of sections, 8-hydroxy-2'-deoxyguanosine (*8-OHdG, S*anta Cruz- 66036) primary antibody was used for oxidative enzyme activity [9]. Finally, all staining sections were evaluated by high-power light microscopic examination. Image analysis system used in this study consisted of a personal computer with hardware and software camera (Kameram SLR, 1.6.1.0, Mikro Sistem Ltd. Sti., Turkey) and an optical microscope (Nikon i50).

Statistical Analysis

Results were presented as means±standard error of means (S.E.M.). One-way ANOVA and Tukey's post hoc test were used to determine differences between the groups. Paired-t test was used for the assessments between the data on the 15th and 45th d. The mean values were considered significant when P<0.05. The SPSS/PC programme (Version 10.0; SPSS, Chicago, IL) was used for the analyses.

RESULTS

Blood Glucose and Weights

On d zero, 15 and 45, the glucose levels of rats in DM group displaying hyperglycemia symptoms were significantly (P<0.05) higher (320-550 mg/dl) compared with those of the control rats (80-140 mg/dl) *(Table 1)*. Furthermore, MLT treatment significantly reduced the levels (160-300 mg/dl) in the DM+MLT group, compared to the DM group.

Table 1 shows the weights of body, testis and the cauda epididymis. The final body weights of diabetic rats were significantly lower than those of the controls on d 15 and 45. The weights were significantly improved by the MLT treatment compared with those of the DM rats.

Biochemical Results

Table 2 represents the levels of lipid peroxidation (MDA), and antioxidant enzymes (SOD and GSH) in testis of control and experimental groups. On d 15 and 45, the MDA levels were significantly (P<0.05) higher in the testis of animals that were administered alloxan alone (DM group) compared with that of the control group. This increase was attenuated by MLT treatment. Significant reductions in the GSH and SOD levels were seen in the testes of DM group

Table 1. *Blood glucose levels and the weights of body, testes and cauda epididymis (means ± S.E.M.)*
Tablo 1. *Kan glikoz seviyesi ile vücut, testis ve cauda epididimis ağırlıkları (ort. ± S.E.M.)*

Groups	Days	Glucose (mg/dl)	Total Weights		
			Body (g)	Testes (mg)	Cauda epididymis (mg)
C	15	97±10^{c}	263±25^{a}	2705.00±196.2	380.67±27.76
	45	101±10^{z}	300±28^{x}	2761.50±245.36	451.25±24.85
MLT	15	98±15^{a}	244±19^{a}	2582.25±85.07^{t}	315.75±35.35
	45	97±12^{x}	267±27^{x}	2306.80±73.40	292.80±17.33
DM	15	512±32^{a}	210±24^{b}	2044.25±378.06	318.75±90.35
	45	525±25^{x}	176±13^{z}	2176.00±520.96	319.67±87.82
DM+MLT	15	436±17^{b}	232±17^{b}	2624.00±143.42	425.50±60.71
	45	337±21^{y}	240±15^{y}	2333.40±229.13	320.00±67.02

Different superscripts a, b, c within the same column indicate significant differences for group 15 d, and x, y, z in the same column indicate significant differences for group 45 d, respectively (n=6). P<0.05; "t" on C, MLT, DM and DM+MLT groups, shows statistical change between the 15th and 45th d (n=6). P<0.05; ***C*** *= Group C;* ***MLT*** *= Group MLT;* ***DM*** *= Group DM;* ***DM+MLT*** *= Group DM+MLT*

compared with that of the controls. Treatment of diabetic rats with the MLT alleviated these DM-induced decreases. MLT treatment resulted in a significant increase in the GSH levels as accompanied with a significant decrease in the MDA and SOD activities in the MLT group as compared with that of the controls.

Spermatological Results

Table 3 shows the results of the evaluation of spermatologic parameters. On the 45th d, the sperm concentration of the DM and DM+MLT groups was found significantly (P<0.01) lower than that of group C.

The number of progressively motile sperm cells in the group DM was significantly lower than that of the group C on d 15 (P<0.05). The number of progressively motile spermatozoa in the groups DM and DM+MLT was lower than that of the group C on d 45 (P<0.01).

The premature AR rate on the 15th and 45th d significantly increased in treatment (MLT, DM and DM+MLT) groups as compared to that of the controls. Also, the rate within the MLT group significantly decreased on the 45th d as compared to that of the 15th d.

On the 15th d, the A23187-induced AR rate of C group, as similar to that of DM group, was significantly (P<0.05) higher than those in the MLT and DM+MLT groups. Further, on the 45th d, the rate in C group became significantly higher than that of all treatment groups.

On d 15 and 45, there were significant decreases in the sperm concentration, number of motile spermatozoa, and rate of A23187-induced AR in rats administered alloxan alone (DM group) as compared with that of the controls (P<0.05). This decrease was exacerbated by MLT treatment in DM+MLT rats *(Table 3)*.

Histochemical and Immunohistochemical Findings

The Johnsen's criteria of spermatogenesis and 8-OHdG immunopositivity in the testes of control and experimental groups are presented in *Table 4*.

Table 2. *The levels of MDA and activities of SOD and reduced GSH in testes tissues (means ± S.E.M.)*
Tablo 2. *Testiküler MDA, SOD ve GSH seviyeleri (ort. ± S.E.M.)*

Groups	Days	MDA (nmol/g protein)	SOD (U/g protein)	GSH (nmol/g protein)
C	15	13.13±1.50^{a}	975.69±55.75^{a}	27.30±3.50^{a}
	45	8.37±0.75^{x}	929.04±60.35^{x}	10.03±1.30^{x}
MLT	15	6.36±1.20^{b}	679.45±90.25^{b}	36.98±2.25^{b}
	45	2.94±0.70^{y}	825.60±75.45^{y}	34.51±3.25^{y}
DM	15	29.07±1.25^{c}	780.52±42.25^{b}	11.28±2.80^{c}
	45	35.10±0.50^{z}	650.20±38.75^{z}	12.50±2.25^{x}
DM + MLT	15	7.70±1.50^{b}	881.79±40.75^{a}	25.39±2.50^{a}
	45	3.92±0.50^{y}	773.36±35.50^{y}	27.62±3.20^{z}

Different superscripts a, b, c within the same column indicate significant differences for group 15 d, and x, y, z in the same column indicate significant differences for group 45 d, respectively (n=6). P<0.05; ***C*** *= Group C;* ***MLT*** *= Group MLT;* ***DM*** *= Group DM;* ***DM+MLT*** *= Group DM+MLT*

Table 3. *The cauda epididymal sperm parameters (means ± S.E.M.)*
Tablo 3. *Cauda epididimal sperm parametreleri (ort.±S.E.M.)*

Groups	Days	Cauda Epididymis		Rates (%)				
		Sperm Concentration (x10^{6})	Number of Progressively Motile Sperm (x10^{6})	Dead Sperm	Mid-piece Abnormality	Premature AR	Total AR	The A23187-Response Rate of Sperm
C	15	64.58±8.38	37.86±5.16^{a}	42.30±2.70	0.00±0.00	11.87±2.87^{a}	42.90±1.8	31.03±2.91^{a}
	45	74.41±7.21^{x}	41.49±3.25^{x}	44.77±2.53	2.05±1.20	14.10±0.83^{x}	49.35±4.50	35.25±5.07^{x}
MLT	15	41.99±10.53	22.11±5.76^{ab}	46.08±1.46	5.30±0.40	36.10±1.82^{b}	48.95±0.90	12.90±0.97^{b}
	45	39.06±4.36^{xy}	25.39±1.95^{xy}	36.26±1.71^{t}	1.64±1.00^{t}	30.76±1.01^{y}	46.36±1.76	15.62±1.24^{y}
DM	15	39.06±17.47	10.27±6.12^{b}	55.53±5.80	1.37±1.37	37.80±9.79^{b}	55.73±9.27	17.95±2.12^{ab}
	45	25.78±2.34^{y}	5.83±2.42^{y}	63.47±3.42	0.00±0.00	36.50±2.10^{y}	48.90±3.90	12.37±1.82^{y}
DM+MLT	15	29.49±8.07	14.96±3.49^{b}	47.46±3.99	1.03±1.03	31.15±3.49^{b}	47.33±4.20	16.23±3.35^{b}
	45	21.88±9.08^{y}	10.84±4.08^{y}	49.67±6.23	5.68±1.62	28.94±3.15^{y}	44.62±2.70	11.21±2.40^{y}

Different superscripts a, b, c and x, y, z within the same column indicate significant differences between groups for 15 d and 45 d, respectively (n=6). P<0.05; "t" on C, MLT, DM and DM+MLT groups, shows statistical change between the 15th and 45th d (n=6). P<0.05; ***C*** *= Group C;* ***MLT*** *= Group MLT;* ***DM*** *= Group DM;* ***DM+MLT*** *= Group DM+MLT*

Table 4. *Johnsen's criteria of spermatogenesis and 8-OHdG immunopositivity in testis (means ± S.E.M.)*
Tablo 4. *Testis dokusunda Johnsen'in spermatogenezis kriterleri ve 8-OHdG immunopozitifliği (ort. ± S.E.M.)*

Groups	Days	Johnsen's Criteria	8-OHdG Immunopositivity					
			Spermatogonia	Leptotene/ Zygotene Spermatocytes	Pachytene Spermatocytes	Spermatid	Sertoli Cell	Leydig
C	15	9.8±0.1[a]	++	+	-	-	-	-
	45	9.7±0.2[y]	++	+	-	-	-	-
MLT	15	9.7±0.1[a]	++	+	-	-	-	-
	45	9.8±0.1[x]	+	+	-	-	-	-
DM	15	7.8±0.3[c]	+++	+++	++	-	+	+
	45	7.2±0.1[z]	++++	+++	++	+	+	+
DM+MLT	15	8.4±0.2[b]	+++	++	+	-	+	+
	45	8.6±0.3[y]	++	+	-	-	+	+

Different superscripts a, b, c within the same column indicate significant differences for group 15 d, and x, y, z in the same column indicate significant differences for group 45 d, respectively (n=6). P<0.05; ***C*** *= Group C;* ***MLT*** *= Group MLT;* ***DM*** *= Group DM;* ***DM+MLT*** *= Group DM+MLT*

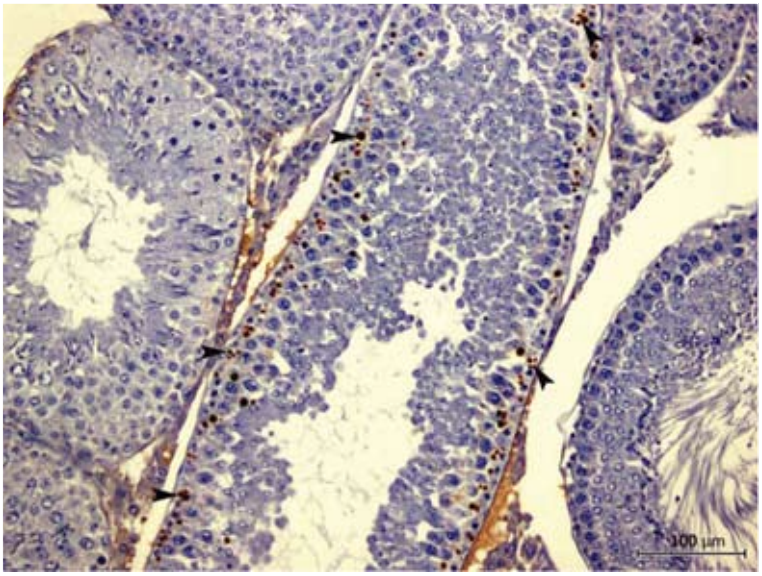


Fig 1. Oxidative stress marker 8-OHdG immunopositivity in the MLT group *(arrow heads)*. Immunopositivitiy in nuclei of leptoten or zygotene spermatocytes in the some seminiferous tubules. Streptavidin-biotin peroxidase staining

Şekil 1. MLT grubundaki oksidatif stres belirleyicisi olan 8-OHdG immunopozitiflik *(ok başları)*. Seminifer tubullerdeki leptoten veya zigoten spermatositlerin çekirdeğindeki immunopozitifliği göstermektedir. Streptavidin-biotin peroksidaz boyama

In all the groups, the seminiferous tubules (composed of germ cell layers) were ellipsoids to rounds in shape. Histochemical findings of testes showed that both the control and MLT groups had normal histological appearances that included the series of spermatogenic, Sertoli and Leydig cells *(Fig. 1)*. According to the Johnsen's criteria, the most consistent findings from the germinal cells of diabetic rats were desquamation and disorganisation, and there were considerable reductions in the spermatogenic cell series and especially in the spermatids at the metamorphose level *(Fig. 2)*. On the d 15 and 45, treatments of diabetic rats with the MLT led to a marked amelioration in the changes of seminiferous tubules as compared to those in the DM groups. The oxidative cells were demonstrated by the 8-OHdG immunohistochemical staining, located in both the nuclei and cytoplasm of germ cells. In the control and MLT groups, the 8-OHdG positivity was recorded only in the nuclei of leptotene and zygotene spermatocytes *(Fig. 1)*. However, in the DM and DM+MLT groups, the 8-OHdG concentrations were very strong in both the nuclei of leptotene and zygotene spermatocytes and the cytoplasm of pachytene spermatocytes, Sertoli and Leydig cells *(Fig. 2* and *Fig. 3)*. The 8-OHdG immunopositive cells increased in the DM groups as compared with those in the controls and MLT groups. Moreover, in the DM+MLT group, the number of 8-OHdG immunopositive cells decreased distinctly on d 45 as compared to d 15.

DISCUSSION

DM is a chronic disease characterized by hyperglycemia and markedly affects the functions of many organs and body systems. Further, it may lead to a decrease in libido and fertility, thus people's quality of life and life expectancy could become inevitablely reduced [3].

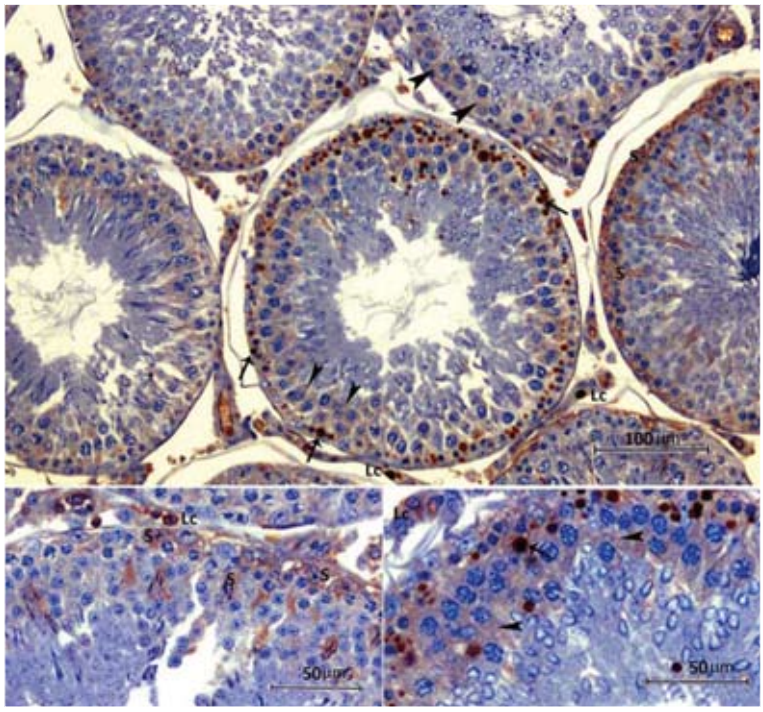
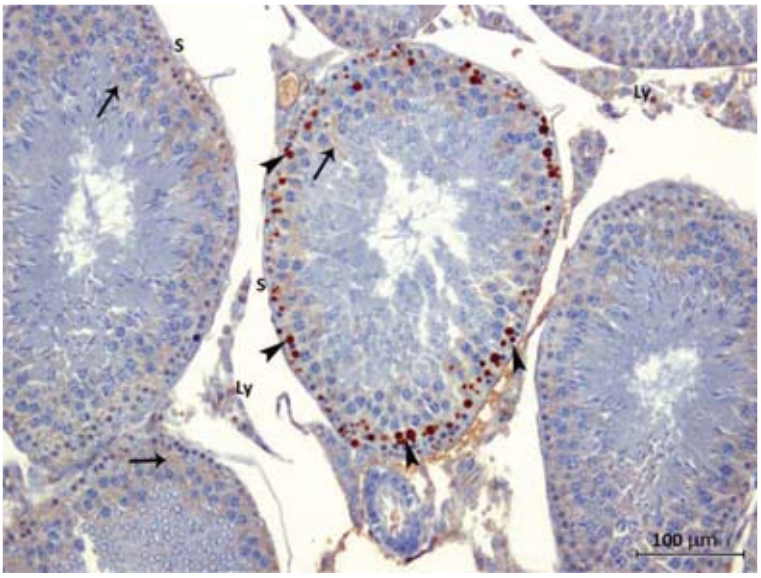


Fig 2. Oxidative stress marker 8-OHdG immunopositivity in the DM group *(arrows)*. Immunopositivity in nuclei of leptoten or zygotene spermatocytes *(arrows heads)*, immunopositivity in cytoplasm of pachytene stage spermatocytes, (S) Sertoli and (Lc) Leydig cells the some seminiferous tubules, desquamation and disorganisation in some seminiferous tubule. Streptavidin-biotin peroxidase staining

Şekil 2. DM grubundaki oksidatif stres belirleyicisi olan 8-OHdG immunopozitiflik *(oklar)*. Seminifer tubullerdeki leptoten veya zigoten spermatositlerin çekirdeğindeki immunopozitifliği *(ok başları)*, bazı seminifer tubullerdeki Leydig (Lc), Sertoli (S) hücreleri ile pakiten aşamasındaki spermatositlerin sitoplazmasındaki immunopozitifliği ve bazı seminifer tubullerdeki disorganizasyon ile deskuamasyonu göstermektedir. Streptavidin-biotin peroksidaz boyama

Fig 3. Oxidative stress marker 8-OHdG immunopositivity in the DM+MLT group *(arrows)*. Immunopositivity in nuclei of leptoten or zygotene spermatocytes *(arrows heads)*, immunopositivity in cytoplasm of pachytene stage spermatocytes, (S) sertoli and (Ly) Leydig cells the some seminiferous tubules. Streptavidin-biotin peroxidase staining

Şekil 3. DM + MLT grubundaki oksidatif stres belirleyicisi olan 8-OHdG immunopozitiflik *(oklar)*. Seminifer tubullerdeki leptoten veya zigoten spermatositlerin çekirdeğindeki immunopozitifliği *(ok başları)*, bazı seminifer tubullerdeki Leydig (Ly), Sertoli (S) hücreleri ile pachtene aşamasındaki spermatositlerin sitoplazmasındaki immunopozitifliği göstermektedir. Streptavidin-biotin peroksidaz boyama



The well-known effect of alloxan, used herein as diabetic agent, on the β-cells in pancreas is to cause the development of insufficient synthesis of insulin and, thus, increasing the blood glucose levels. Several researchers have reported that the MLT treatment in alloxan-induced diabetic rats alleviates some symptoms of diabetes, e.g. elevated blood glucose levels [27-29]. Herein, the mean levels of diabetic rats were markedly higher than those of the normal levels and this elevation in glucose was nearly constant during the course of diabetes, whereas in diabetic rats treated with the MLT, glucose level markedly reduced and become close to the normal levels ultimately.

Previous studies have shown that the ROS productions are responsible from the alloxan-induced destructive effects, particularly in the tissues of pancreas as well as kidney, retina, myocardium and testis [2,30]. These radicals could cause tissue damage as they react with the macromolecules (e.g. membrane lipids and proteins) within the testis. Herein, markedly higher MDA levels of testis were observed in the DM group than those in the controls. Treatment with the MLT markedly prevented the alloxan-induced lipid peroxidation in the testis tissue, indicating the antioxidant capacity of the MLT. This reduction in lipid peroxidation is likely to be the indication of a lesser damage of free oxygen radicals.

There are numerous natural or synthetic products that have a reasonable cytoprotective nature, presumably by influencing the antioxidant systems. The GSH is a critical

enzyme for sustainable cell viability. It modulates the cell responses to redox changes connected with the generation of reactive oxygen and nitrogen species, detoxifies the metabolites of drugs and regulates apoptosis [2]. In the presence of cytoplasmic and mitochondrial thiols such as the GSH, the alloxan produces free oxygen radicals in a redox reaction with its reduction product, dialuric acid. Oxidation of dialuric acid generates superoxide and hydrogen peroxide radicals and, in a final Fenton's reaction step, the hydroxyl radicals, as being the most reactive and toxic one. The latter radicals are responsible for final destruction of the β-cells, having a particularly low antioxidative capacity, and ensuing the state of insulin-dependent 'alloxan diabetes'. The marked decrease in the GSH levels promoted by alloxan represents a change in the cellular reduction or oxidation reactions, suggesting that the cells could have become more sensitive to the ROS. This situation would cause to a reduction in the protective effect of antioxidant defense system [31,32].

The most effective defense mechanism of the cell against various viruses, bacteria or other substances is the release of O_2^- and OH. The SOD is a key scavenging enzyme that eliminates the toxic ROS induced by the alloxan. It converts the superoxide radicals into H_2O_2 and molecular oxygen. In this respect, the decrease in SOD activity in DM group might have resulted from the inactivation either by glycolisation of the enzyme or by hydrogen peroxide [33,34]. In our study, the activities of both GSH and SOD enzymes markedly decreased in testis tissues of alloxan-treated rats. This result might be due to the increase in free radical generation or a decrease in the amounts of protecting enzymes against lipid peroxidation. However, treatment with the MLT ameliorated the alloxan-induced testis damages due to free radical production. These results suggested that the MLT might have a free radical scavenging activity and prevents pathological alteration caused by O_2^- and OH^-. Indeed, similar findings have been reported on testis tissue that alloxan injection led to lower GSH and SOD activities [35,36].

The DM is characterized by a sudden and distinct loss in body weight, a symptom as also observed herein. The decreases in body and testis weights of DM rats showed the damage or destruction of structural proteins related to diabetes [3]. When diabetic rats were treated with the MLT, a weight loss in the body was reversed. The capability of MLT to protect body weight losses seems to be a result of its ability to increase antioxidant capacity and reduce the hyperglycemia and ROS. In our study, the total testis weights (except that of the MLT group) did not show any difference between and within the groups studied, as reported by Abbasi et al.[37]. In the MLT group, total testis weight decreased markedly from 15th d to 45th d.

Alloxan-induced destructive effects lead to the productions of ROS. The 8-OHdG produced by ROS can be used as a biomarker of the DNA [38]. In normal mature rat testis, the 8-OHdG is observed in leptotene, zygotene, and pachytene spermatocytes, as immediately removed from the DNA by natural defense mechanisms within the testis [39]. In this study, histological sections from the testis of control and MLT groups were similar to histochemical structures, but the 8-OHdG level quantitatively measured in rat testis was relatively low in the MLT d 45 group as compared with those of the controls and MLT d 15 groups. However, the levels increased in the diabetic groups, and were drastically decreased by the MLT treatment in the DM+MLT group. The defense system (against the stress) by the MLT might have developed in pachytene stage spermatocytes in the DM+MLT group.

It was reported in some studies that the rats having undergone the STZ had decreased sperm motility, but without any marked change [4,37,40-44]. Similarly, herein, we did not find any difference neither between nor within the groups on the 15th and 45th d.

No marked change in total sperm number was observed by Abbasi et al.[37], while the daily sperm production in rats and the sperm concentration in mice and rats decreased in the STZ-induced diabetes [4,40-44]. Herein, the sperm concentration in the 45th d markedly decreased in the DM group as compared to those in the C group, but the MLT treatment of diabetic rats did not lead to recovering effect for a decrease in the sperm concentration.

In our study, the lower sperm concentration and the lower number of motile sperm in the DM group are consistent with the reproductive disorders [45,46]. Present findings with decreased sperm concentration in diabetic rats were similar to those of Bal et al.[4], while being slightly lower than those of Navarro-Casado et al.[43] and Scarano et al.[47]. The reduction in sperm concentration in diabetic rats might imply that the STZ had an adverse effect on the spermatogenesis [40,47]. Singh et al.[40] reported that the decrease in sperm density may be related to the decrease in the number of Leydig cells [4]. Some authors reported that the reduced epididymal and motile sperm concentrations in diabetic rats could arise from a combined effect of decreased Leydig cell function and the oxidative stress induced by diabetes [4,46,48,49]. It has been shown that the destructive effect of free oxygen radicals reduces the sperm motility and viability [49]. Shrilath and Muralidharan [44] noted that the sensitivity of sperm to the oxidative stress in the epididymis was increasingly evident around the 15th d after the STZ injection. Other researchers reported that spermatozoa are reasonably well protected by the Sertoli cells, but they are less protected against the oxidation in the epididymal milieu. Consequently, the oxidative damage to sperm may lead to DNA damage, alter the membrane functions and impair motility [50,51]. There are numerous studies that the MLT treatment increases testicular antioxidant capacity and decreases oxidative stress and thus diminishes diabetes-induced oxidative damage and leading to the restoration of sperm

parameters in the DM group rats [10-14]. The protective effects of MLT (used herein as an antioxidant) on the testis tissue are consistent with the previous results in DM models [11,12,44]. According to our results, the alloxan-induced DM in rats increases the MDA levels and leads to apoptosis in Sertoli and Leydig cells as well as spermatocytes (in leptotene, zygotene and pachytene stages) during the spermatogenesis. The sperm concentration on 45th d decreased in the DM group as compared to those in the C group, and this detrimental effect in diabetic rats was not inhibited or reversed by the MLT. Hence, we considered that the reason of low fertility in diabetic men might be due to the low sperm concentration, as observed herein with the cauda epididymal sperm in DM rats. For example, when a cow is desired to conceive by artificial insemination method, there has to be a certain number of motile sperm within the inseminate (contained within a single frozen-thawed straw). If this insemination dose within the straw is diminished per one-million sperm, the pregnancy rate in cows decreases by 2-3% from the normal values [52].

Morphological abnormalities of sperm observed herein were also reported similarly by Navarro-Casado et al.[43] unlike by Scarano et al.[47]. This discrepancy might be due to different assessment intervals used (2–3 weeks therein *vs.* 15 to 45 d herein). Clearly, their assessment intervals were too short for development of abnormalities during the spermatogenesis.

Cholesterol is a sterol playing a critical role in capacitation and contained within all mammalian sperm cells. For the capatitation and AR, a small amount of ROS is required. When cholesterol on the sperm plasma membrane is exposed to oxidation, its level diminishes, and the spermatozoon inevitably undergoes capacitation [53,54]. In a previous study, it was stated that as the temperature and time period are increased in culture medium during incubation, the rate of premature AR raises in rabbits, and that when cholesterol was added into sperm suspension, it prevented the spermatozoa from undergoing the premature AR [55]. Our study suggest that the DM might reduce the cholesterol levels of the plasma membrane because of its increasing effect on the ROS quantity within the cauda epididymis, so it might enhance the number of spermatozoa having undergone the premature AR. Lombardo et al.[56] noted that the reduction in ROS concentration by using antioxidants exerts adverse effect on male fertility. Some studies indicated that when antioxidants are used in oral pills and/or added directly into ejaculated spermatozoa, the rate of spermatozoa having undergone the AR increases [57,58]. Unlike the study of Lombardo et al.[56], herein, the increase in the premature AR rate in MLT group was found to resemble the findings of Comhaire et al.[57]. In this respect, since the MLT increased the premature AR rate as compared to those in the C group, MLT treatment in DM rats did not diminish this rate markedly as compared to those in the DM group, due likely to the outlined feature of antioxidants concerned.

Our hypothesis about the infertility of men with DM would be that the premature and A23187-induced AR rates might be critical. Indeed, a normal ejaculate must have enough spermatozoal quantity and be capable of undergoing the AR in the fertilisation area (oviduct) for the oocyte penetration in cattle [59]. In this study, the rate of spermatozoa having undergone premature AR in rats with DM was more evident than in the C groups. This case would decrease the number of sperm cells avaible for the induction (e.g. by A23187) of the AR. Since a sufficient number of sperm (with AR capability) would not reach to the oviduct, the penetration rate of fertilisable oocyte(s) might then reduce drastically, ultimately leading to male infertility.

In the experimental studies on the DM, it has been reported that the administration of some antioxidants increases the antioxidant capacity, while decreasing the oxidative stress restoring changes in the spermatological parameters [4,37]. Other antioxidants may not reverse the deterioration in these parameters, but they may restore the daily sperm production in testis, and alleviate the mitochondrial function and DNA damage [42]. Deterioration of spermatologic parameters due to the administration of various substances (with ROS generating activities within the testis), the MLT, used as an antioxidant, could ameliorate these parameters [60-63]. In our study, the MLT did not protect the sperm concentration, the number of progressively motile spermatozoa, the premature and the A23187-induced AR rates of the cauda epididymal sperm in rats with alloxan-induced DM. Possible reasons for dysfunction of MLT on the sperm parameters would be linked with; i) the adjuvant, used for solving the MLT, that might have adverse effects on spermatologic parameters [64], ii) the use of DM and MLT together that might have increased the adverse effects on spermatogenesis, and iii) the dose of MLT used that might have affected the results.

In summary, the results of this study suggest that the melatonin could reduce the blood glucose, lipid peroxidation marker (MDA levels), 8-OHdG and oxidant parameters in diabetic male rats. This may contribute to its protective effects on post-diabetic complications, such as in testicular damage. However, the melatonin did not improve the depressed sperm parameters in diabetic rats when used for the duration of 45 d. Nevertheless, it would contribute to a balanced oxidant-antioxidant status and provide a potential therapeutic choice to reduce the histologic damage of testis in diabetic patients.

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Research Article

Effect of Clinoptilolite and/or Phytase on Broiler Growth Performance, Carcass Characteristics, Intestinal Histomorphology and Tibia Calcium and Phosphorus Levels [1]

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Abstract

The effect of clinoptilolite alone or in combination with phytase on the performance, carcass characteristics, intestinal histomorphology, and tibia ash and Ca, P levels were determined in male broiler chickens. Total of 192 one-day-old male broiler chicks (Ross 308) were randomly assigned to 4 different treatments with 8 replicates containing 6 birds each. Treatment 1 (T1) was served as control fed a diet without clinoptilolite and/or phytase, Treatment 2 (T2) was supplemented with 2% clinoptilolite. Treatment 3 (T3) and Treatment 4 (T4) were (formulated Ca; 0.87, 0.80 and 0.75%, available P; 0.30, 0.28 and 0.26% for starter, grower, and finisher periods respectively) supplemented 0.01% phytase and 2% clinoptilolite + 0.01% phytase, respectively. The results showed that combined use of clinoptilolite and phytase (T4) in broiler diets decreased (P=0.044) body weight gain at 1-14 d. Dietary treatments had no effect on body weight gain, feed intake (FI) and feed conversion ratio (FCR) during the entire experimental period. Supplementation with phytase (T3) significantly (P=0.006) decreased crypt depth in duodenum on d 21. Tibia ash and phosphorus level were decreased due to combined use of clinoptilolite and phytase (T4) in comparison to those fed control (T1) and clinoptilolite (T2) diets on d 21. At 42 days of age, tibia ash, Ca, P levels and Ca/P ratio was not changed by the treatments. It is concluded that there might be a positive effect of 2% clinoptilolite on the performance of male broilers after starting period.

Keywords: *Broiler, Clinoptilolite, Carcass, Intestinal histomorphology, Performance, Tibia ash*

Klinoptilolit ve/veya Fitaz İlavesinin Broilerlerin Büyüme Performansı, Karkas Karakteristikleri, Bağırsak Histomorfolojisi ve Tibia Kalsiyum ve Fosfor Düzeylerine Olan Etkisi

Özet

Bu çalışmada erkek broiler rasyonlarında klinoptilolitin tek başına veya fitaz ile birlikte kullanılmasının performans, karkas karakteristikleri, bağırsak histomorfolojisi ve tibia külü ile Ca, P düzeyleri üzerine etkisi incelenmiştir. Toplamda 192 adet günlük yaşta erkek civciv (Ross 308) 4 deneme grubuna ve her biri 6 civcivden oluşan 8 tekerrür grubuna rasgele olacak şekilde ayrılmıştır. Birinci grup (T1) kontrol grubu olarak düzenlenerek bazal rasyon ile beslenmişlerdir. İkinci gruba (T2) %2 düzeyinde klinoptilolit ilave edilmiştir. Daha düşük düzeyde Ca ve yararlanılabilir P içeren (başlangıç, büyütme ve bitirme dönemleri için sırasıyla Ca; %0.87, 0.80 ve 0.75 yararlanılabilir P; %0.30, 0.28 ve 0.26) 3. ve 4. gruba ise sırasıyla %0.01 fitaz ve %2 klinoptilolit + %0.01 fitaz ilave edilmiştir. Araştırmanın 1-14. günlerde, klinoptilolit ve fitazın birlikte kullanıldığı grupta (T4) canlı ağırlık artışının daha düşük düşük olduğu (P=0.044) görülmüştür. Ancak tüm deneme boyunca gruplar arasında canlı ağırlık artışı, yem tüketimi ve yemden yararlanma oranı açısından farklılık tespit edilmemiştir. Araştırmanın 21. gününde yalnızca fitaz ilavesinin (T3) duodenum kript derinliğini önemli ölçüde azalttığı (P=0.006) sonucuna varılmıştır. Denemenin 21. gününde tibia külü ve fosfor düzeyinin klinoptilolit ve fitazın birlikte kullanılmasına bağlı olarak önemli ölçüde daha azaldığı görülmüştür. Ancak denemenin 42 gününde tibia parametreleri açısından gruplar arasında farklılık tespit edilememiştir. Sonuç olarak başlangıç döneminden sonra rasyonlarda kullanılacak %2 klinoptilolitin performans üzerine olumlu etkileri olabileceği düşünülmektedir.

Anahtar sözcükler: *Bağırsak Histomorfolojisi, Broiler, Karkas, Klinoptilolit, Performans, Tibia külü*

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INTRODUCTION

Zeolites are hydrated aluminosilicates of volcanic origin, which is derived from the same molecular frame containing alkali (Na, K, Li, Cs) and alkaline-earth metals (Ca, Mg, Ba, Sr), having ion exchange and adsorption characteristics and ability to lose and gain water reversibly [1]. Clinoptilolite is one of the natural zeolites used successfully as feed additive due to its specific physicochemical properties. Previous studies have revealed that the dietary clinoptilolite may increase growth performance [2], help to control ammonium in poultry houses [3], improve immune response [4], help to bind toxin [5], and improve the hygienic and sanitary conditions of litter [6]. More recently Nikolakakis et al.[7] revealed that dietary clinoptilolite supplementation improved broiler growth performance and litter quality.

Zeolites are used in animal nutrition in particular to their absorption/adsorption properties, antimicrobial effects and preventive nature against diarrheal infections [8,9]. In addition, dietary natural zeolites may improve the digestibility of the nutrients by increasing the mean retention time of the digesta in layers [10].

Zeolites can also influence Ca utilization and metabolism [11], by selectively retaining or releasing of Ca as it passes through the digestive tract [12]. Moreover zeolites may impact electrolyte balance of broilers [13]. It is well known that mineral metabolism and electrolyte balance are play a crucial role on bone formation [14,15]. Clinoptilolite has a beneficial effect on tibia bone mineralization and calcium and phosphorus utilization in broiler chicks [13]. On the other hand, Elliot and Edwards [16] and Leach et al.[17] reported that zeolites suppressed P utilization by forming an indigestible compound, and had an indirect effect on P absorption and metabolism.

Previous studies have suggested that dietary zeolite improves growth performance [17], and increase Ca utilization and bone ash deposition [13,18]. However contradictory results were reported on growth performance [16] and bone ash content [19]. Discrepancies between the studies might be partially attributable to the type of zeolite, purity and physicochemical properties of the product used in the experiments [20], supplementation level to the diets, and dietary calcium level [21].

Considerable amount of phosphorus (P) in poultry diets in the form of phyate-P, which is not utilized efficiently by monogastric animals [22,23]. In this sense inorganic phosphate sources, such as di-calcium phosphate, included to poultry diets to meet available P requirements [24]. Phytase is widely used in poultry feeds to improve P availability [25] and to reduce the cost of the ratio [24]. In addition, it may also enhance broiler performance by increasing the utilization of dietary energy and amino acids [26]. El-Sherbiny et al.[27] reported that dietary addition of 500 U/kg phytase improved body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of the birds. In contrast, Chung et al.[28] reported that both fungal and bacterial phytase supplementation had no consistent effect on broiler growth performance from d 0 to 21.

The improvement in the availability of P and other cations by dietary phytase supplementation has been generally well accepted. More recently Lalpanmawia et al.[29] concluded that laboratory phytase and commercial phytase supplementation resulted in 30% reduction in P excretion. However the effect of combined use of clinoptilolite and phytase, in diets that contain low level of P, on broiler performance and bone characteristics heretofore unreported.

The objective of the current study is to evaluate the effects of clinoptilolite addition to the broiler diets with or without phytase enzyme on the performance, carcass characteristics, intestinal histomorphology, and tibia ash, Ca and of P levels in male broiler chickens.

MATERIAL and METHODS

Experimental Design, Diets and Management

The experimental design, diet and management used in this trial were approved by the Animal Ethics Committee of the Ankara University (2012-8-60). A total of 192 one-day-old male broiler (Ross 308) chicks, with an average body weight (BW) of 42.64±0.22 g, were obtained from a commercial hatchery (Beypiliç AŞ, Turkey) and allocated to 4 treatments in a randomized complete block design with 8 replicates of 6 birds in each for a 42 day feeding trial. All diets were formulated to meet or exceed NRC [30] nutrient recommendations (except P and Ca in diets containing phytase) of broilers for starter (0-14 days), grower (14-35 days) and finisher (35-42 days) periods. Treatment 1 (T1) was served as control group fed diet without clinoptilolite and/or phytase supplementation, Treatment 2 (T2) was supplemented with 2% clinoptilolite (NAT-MIN 9000, Gördes Zeotile, Izmir, Turkey). Phytase containing diets (T3 and T4) were formulated at a low level of Ca and P (Ca; 0.87, 0.80 and 0.75%, available P; 0.30, 0.28 and 0.26% for starter, grower, and finisher periods, respectively). Treatment 3 and 4 were supplemented with 0.01% phytase (5000 FTU/g) (P500, Tempe Chemical Feed Additives, Istanbul, Turkey), and 2% clinoptilolite with 0.01% phytase, respectively. Chemical and physical characteristics of clinoptilolite (according to the manufacturer's data sheet) is shown in *Table 1*. Composition and nutrient levels of experimental diets are shown in *Table 2*.

All birds were housed in floor pens with fresh wood shavings served as litter material. Each pen (90x80x80 cm) contained one feeder and two-nipple waterer. Temperature was controlled with electrical heater, maintained at 34°C for the first three days and then gradually reduced by 2-3°C per week to final temperature of 22°C. Water and diets

Table 1. *Chemical and physical characteristics of clinoptilolite used in the experiment* [1]
Tablo 1. *Denemede kullanılan klinoptilolitin kimyasal ve fiziksel özellikleri*

Item	Composition
Chemical Formula	$[(Na_{0.5}K_{2.5})(Ca_{1.0}Mg_{0.5})(Al_6,Si_{30})O_{72}.24H_2O]$
Specific gravity	2 g/cm^3
Particle size	0-0.8 mm
Pore diameter	0.041µm
Moisture	Max 12%
Cation Exchange Capacity (NH_4^+)	1.6-2.0 meq/g

[1] *According to the manufacturer's data sheet*

(in mash form) were provided *ad libitum* throughout the experimental period.

During the experimental period, body weights were recorded for each replicate weekly to determine weight gain. Also feed intake was recorded for each pen, and feed/gain ratio (F/G) was calculated during the same periods to determine the growth performance of birds. Mortalities were recorded on as it occurred, and were used to adjust feed conversion.

Sample Collection and Procedures

At d 21 and d 42, 8 birds from each treatment (1

Table 2. *Composition of experimental diets*
Tablo 2. *Deneme yemlerinin kompozisyonu*

Item	Experimental Diets[1]											
	Starter 0 to 14 d				Grower 14 to 35 d				Finisher 35 to 42 d			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
Ingredients, %												
Corn	39.98	39.50	40.78	40.65	47.05	43.70	48.03	44.39	45.00	44.88	49.46	47.04
Soybean meal (CP, 47%)	30.00	30.00	30.00	30.00	24.00	23.36	24.00	23.37	24.65	24.55	25.00	24.00
Full fat soy (CP, 35%)	13.00	14.00	13.00	14.00	13.00	14.00	13.00	14.00	8.50	9.00	8.00	9.50
Wheat	10.00	6.50	10.00	6.54	8.00	8.00	8.00	8.00	13.30	10.00	10.00	9.00
Vegetable oil	2.50	3.50	2.50	3.10	3.90	4.90	3.60	4.80	5.00	6.00	4.60	5.50
Limestone	0.86	0.85	1.00	1.00	0.80	0.79	0.95	1.00	0.77	0.78	0.85	0.85
DCP	2.25	2.25	1.30	1.30	2.00	2.01	1.20	1.20	1.85	1.85	1.15	1.15
DL-Methionine	0.40	0.40	0.40	0.40	0.31	0.32	0.30	0.31	0.18	0.19	0.19	0.20
L-Lysine	0.26	0.25	0.26	0.25	0.19	0.17	0.16	0.17	-	-	-	-
L-Threonine	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
NaCl	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Vitamin premix[2]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mineral premix[3]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Anticoccidial	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Clinoptilolite	-	2.00	-	2.00	-	2.00	-	2.00	-	2.00	-	2.00
Phytase	-	-	0.01	0.01	-	-	0.01	0.01	-	-	0.01	0.01
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Calculated values												
Crude protein, %	23.00	22.90	23.00	23.00	20.60	20.30	20.60	20.40	19.40	19.20	19.40	19.20
ME kcal/kg	3012	3012	3037	3012	3176	3174	3177	3175	3227	3227	3226	3223
Ca, %	1.00	1.00	0.87	0.87	0.90	0.90	0.80	0.80	0.85	0.85	0.75	0.75
Available P, %	0.50	0.50	0.30	0.30	0.45	0.45	0.28	0.28	0.42	0.42	0.26	0.26
Meth+Sist, %	1.10	1.10	1.10	1.10	0.95	0.95	0.95	0.95	0.80	0.80	0.80	0.80
Lysine, %	1.44	1.44	1.44	1.44	1.24	1.22	1.22	1.22	1.01	1.01	1.01	1.01
Threonine, %	1.00	1.00	1.00	1.00	0.90	0.90	0.90	0.90	0.86	0.86	0.86	0.86
Analyzed values												
Crude protein, %	23.02	23.06	23.10	23.00	20.50	20.22	20.60	20.45	19.25	19.15	19.25	19.10
Ca, %	1.02	1.03	0.88	0.88	0.95	0.93	0.81	0.72	0.88	0.89	0.74	0.75
Total P, %	0.86	0.85	0.66	0.66	0.78	0.77	0.62	0.61	0.74	0.73	0.60	0.59

[1] ***T1:*** *Control, a standard corn-soybean meal basal diet;* ***T2:*** *2% clinoptilolite;* ***T3:*** *0.01% phytase (5000 FTU/g);* ***T4:*** *2% clinoptilolite and 0.01% phytase;* [2] *Provides per kg of diet: vitamin A, 15000 IU; vitamin D_3, 5000 IU; vitamin E, 50 mg; vitamin K_3, 10 mg; vitamin B_1, 4 mg; vitamin B_2, 8 mg; vitamin B_6, 5mg; vitamin B_{12}, 0.025mg; niacin, 50 mg; pantothenic acid, 20 mg; folic acid, 20 mg; biotin, 0.25 mg; choline,175 mg;* [3] *Provides per kg of diet: manganese, 100 mg; zinc, 150 mg; iron, 100 mg; copper, 20 mg; iodine, 1.5 mg; cobalt, 0.5 mg; selenium, 0.2 mg; molybdenum, 1mg; magnesium, 50 mg*

bird per replicate) were randomly selected for sample collection. After slaughtering, intestinal tract was removed immediately. To ensure the uniformity of samples, approximately 2 cm lengths of the mucosal segments of duodenum, jejunum and ileum were excised as follows: duodenum (from gizzard outlet to the end of the pancreatic loop), jejunum (8 cm proximal to Meckel's diverticulum), and ileum (8 cm proximal to the ileo-cecal junction). Afterwards the tissue samples were flushed with saline solution to remove adherent intestinal contents and fixed in 10% neutral buffered formaline solution for 24 h. Tissue samples were taken from formaline and dehydrated in graded ethanol solutions, cleared with xylol and embedded in paraffin, respectively. Intestinal samples were sectioned at the thickness of 6 µm with microtome. Mounted sections were stained with Mallory's modified triple staining technique [31]. Villus height was measured from the top of the villus to crypt mouth and crypt depth was defined as distance between basements of the crypt-to-crypt mouth [32]. Histological sections were examined under the light microscope (Leica DM 2500, Leica Microsystems GmbH, Wetzlar, Germany) and photographed with Leica DFC450 (Leica Microsystems, Heerbrug, Germany) digital microscope camera. The images were evaluated using ImageJ software (Image J, US National Institutes of Health, Bethesda, MD, USA).

The left tibia was removed and cleaned of adhering tissue, defatted and crushed into small particles. Ash contents were determined for each replicate (one bone from each pen) as described by the AOAC [33]. The ash percentage of each bone was determined with regard to defatted individual dry matter in tibia. Calcium was determined by gloxal-bis method according to Farese et al.[34]. Phosphorus content was measured according to the ammonium vanadate/molybdate method as described by Gericke and Kurmies [35].

Statistical Analysis

Data were analyzed as a completely randomized block design with 4 dietary treatments and 8 replicates using the ANOVA procedure [36] of the SPSS version 14.01 (SPSS Inc., Chicago, IL, USA). Significant differences among treatment groups were tested by Duncan's multiple range tests. Mortality rates were compared using a chi-squared test. Statistical differences were considered significant at $P<0.05$.

RESULTS

Growth Performance and Carcass Characteristics

The effect of dietary addition of clinoptilolite and phytase on BWG, FI, FCR and mortality rate (%) are shown in *Table 3*. Weight gain was lower in birds fed with clinoptilolite and phytase (T4) supplemented diet in comparison to control diet at 0-21 days of the study (P=0.044). Feed intake was similar among the treatment groups. The relative weight of internal organs, carcass and carcass components are shown in *Table 4*.

Table 3. *Influence of dietary treatments on performance of broiler* [1]
Tablo 3. *Deneme rasyonlarının broiler performansı üzerine etkisi*

Age (day)	Dietary Treatment[2]				Statistics	
	T1	T2	T3	T4	SEM	*P-value*
Weight gain, g						
1-14	468.62[a]	455.75[ab]	457.48[ab]	429.85[b]	5.13	0.044
1-21	977.98	974.03	997.77	941.27	8.93	0.159
1-42	3149.43	3225.14	3206.76	3147.26	21.76	0.494
Feed intake, g						
1-14	574.87	572.27	588.69	554.04	7.67	0.479
1-21	1356.74	1309.27	1346.19	1328.04	10.54	0.416
1-42	5132.50	5067.87	5105.79	5068.53	30.00	0.858
Feed intake/weight gain (FCR)						
1-14	1.23	1.26	1.29	1.29	0.01	0.335
1-21	1.39	1.34	1.35	1.41	0.01	0.161
1-42	1.63	1.57	1.59	1.61	0.01	0.071
Mortality, %						
1-42	2.08	4.16	4.16	6.25	-	0.791

[a-b] Means with different superscripts in the same row are significantly different (P<0.05); [1] Data represent mean values of 8 replicates per treatment;
*[2] **T1:** Control, a standard corn-soybean meal basal diet; **T2:** 2% clinoptilolite; **T3:** 0.01% phytase (5000 FTU/g); **T4:** 2% clinoptilolite+0.01% phytase*

Table 4. *Effect of dietary treatments on carcass components and relative organ weights on d 42* [1]
Tablo 4. *Deneme rasyonlarının karkas bileşenleri ve relatif organ ağırlıkları üzerine etkisi (42. gün)*

Item	Dietary Treatment[2]				Statistics	
	T1	T2	T3	T4	SEM	*P-value*
Dressing percentage, %	74.04	74.21	74.41	74.33	0.41	0.992
Breast weight, g	624.63	666.37	618.62	624.50	18.20	0.793
Breast % of live weight	17.28	19.44	18.46	19.00	0.34	0.876
Wings, g	210.50	226.75	222.00	228.00	3.50	0.281
Wings % of live weight	6.78	7.00	7.36	7.29	0.11	0.267
Leg quarters, g	626.25	673.00	642.50	669.71	13.51	0.579
Leg quarters % of live weight	20.20	20.78	21.00	21.41	0.22	0.296
Gizzard, g	41.37	36.62	35.37	36.12	0.95	0.098
Gizzard % of live weight	1.35	1.13	1.17	1.15	0.03	0.078
Liver, g	41.50	44.00	38.12	44.37	0.97	0.080
Liver % of live weight	1.34	1.36	1.25	1.42	0.03	0.130
Heart, g	15.00	14.00	13.62	13.75	0.42	0.675
Heart % of live weight	0.49	0.43	0.45	0.44	0.01	0.477
Abdominal fat, g	46.14	44.00	38.00	49.50	2.34	0.387
Abdominal fat % of live weight	1.06	1.14	0.98	1.13	0.07	0.307

[1] *Data represent mean values of 8 replicates per treatment;* [2] ***T1:*** *Control, a standard corn-soybean meal basal diet;* ***T2:*** *2% clinoptilolite;* ***T3:*** *0.01% phytase (5000 FTU/g);* ***T4:*** *2% clinoptilolite+0.01% phytase*

There were no significant differences among treatment groups on internal organ weights and processing characteristics.

Intestinal Histomorphology

Morphological measurements of small intestine are shown in *Table 5*. At d 21, duodenal villus height was not affected by dietary treatments, however crypt depth was significantly (P=0.006) decreased by phytase (T3) addition compared to control group (T1). On the other hand, dietary treatments have more pronounced effect on duodenal villus height at d 42. In jejunum and ileum, villus height and crypt depth were not affected by clinoptilolite and/or phytase addition either 21-d or 42-d of ages. However jejunum villus height:crypt depth ratio (VH:CD) was significantly increased (P=0.037) by phytase supplementation on d 42.

Tibia Ash, Ca and P Levels

At 21 days of age, combined use of dietary clinoptilolite and phytase reduced (P=0.002) tibia ash content of the birds compared to those fed without phytase supplemented groups *(Table 6)*.

Moreover dietary clinoptilolite and phytase (T4) reduced (P<0.001) tibia P level on d 21. However dietary treatments did not affect tibia Ca level. At 42 days of age, tibia ash, Ca, P levels and Ca/P ratio were not affected by dietary treatments.

DISCUSSION

Growth Performance

Clinoptilolite and phytase supplementation to Ca and P deficient diets decreased the growth performance of broilers at starting period. However, with the advancing age, dietary treatments did not affect broiler performance during the entire experimental period. These effects might be attributed to the level of clinoptilolite in Ca and P deficient diets. According to these results it can be assumed that dietary 2% clinoptilolite may be high at starting period. Suchy et al.[2] reported that dietary 1% clinoptilolite supplementation improved weight gain of broiler chickens than those fed with 2% clinoptilolite on d 30. However body weight was increased with both supplementation levels (1 and 2%) on d 40. In agreement with our findings, they suggested that supplementation of 2% clinoptilolite in younger chickens may have a suppressive effect on broiler performance. Moreover they concluded that the level of clinoptilolite in the diets might be increased with chickens' age. In addition, Eser et al. [37] revealed that dietary 1% sepiolite supplementation increased body weight of broiler chickens on d 42. Contrary to our results, Karamanlis et al.[38] revealed that clinoptilolite addition to the broiler diet and litter were significantly improved live weight and such differences were more pronounced after 28-d of ages. Also, there was a significant interaction between feeding and bedding treatments on

Table 5. *Effects of clinoptilolite and/or phytase addition on intestinal histomorphology of broiler chicken at d 21 and d 42* [1]
Tablo 5. *Klinoptilolite ve/veya fitaz ilavesinin 21 ve 42 günlerdeki broiler bağırsak histomorfoloji üzerine etkisi*

Item	Dietary Treatment[2]				Statistics	
	T1	T2	T3	T4	SEM	*P-value*
Duodenum						
d 21						
Villus height (µm)	1831.81	1858.69	1828.25	1785.13	35.69	0.918
Crypt depth (µm)	281.69^{a}	261.94^{ab}	233.88^{b}	260.50^{ab}	5.14	0.006
VH:CD[3] ratio	6.57	7.20	7.94	6.89	0.22	0.160
d 42						
Villus height (µm)	1760.75^{b}	2190.69^{a}	2051.13^{a}	2166.06^{a}	46.36	0.001
Crypt depth (µm)	255.50^{b}	271.19^{ab}	283.94^{ab}	305.75^{a}	6.54	0.039
VH:CD ratio	6.96	8.10	7.37	7.12	0.17	0.089
Jejunum						
d 21						
Villus height (µm)	1382.00	1399.06	1192.69	1375.00	32.53	0.104
Crypt depth (µm)	246.75	222.31	230.25	233.25	5.55	0.491
VH:CD ratio	5.65	6.38	5.31	5.79	0.18	0.213
d 42						
Villus height (µm)	1734.44	1653.06	1839.81	1740.25	43.96	0.538
Crypt depth (µm)	262.44	258.44	242.00	271.81	6.00	0.373
VH:CD ratio	6.67^{b}	6.47^{b}	7.63^{a}	6.42^{b}	0.17	0.037
Ileum						
d 21						
Villus height (µm)	788.75	907.69	834.69	949.94	24.63	0.080
Crypt depth (µm)	205.56	207.69	190.56	216.00	4.85	0.320
VH:CD ratio	3.84	4.40	4.44	4.41	0.10	0.108
d 42						
Villus height (µm)	1492.31	1347.94	1443.50	1527.31	34.54	0.443
Crypt depth (µm)	257.81	237.69	227.44	265.25	5.58	0.051
VH:CD ratio	5.80	6.08	6.35	5.79	0.12	0.326

*$^{a-b}$ Means with different superscripts in the same row are significantly different (P<0.05); [1] Data represent mean values of 8 replicates per treatment; [2] **T1:** Control, a standard corn-soybean meal basal diet; **T2:** 2% clinoptilolite; **T3:** 0.01% phytase (5000 FTU/g); **T4:** 2% clinoptilolite+0.01% phytase; [3] VH:CD; villus height:crypt depth*

days 28 (P<0.05) and 42 (P<0.001). On the other hand, dietary and bedding treatments did not affect feed conversion ratio. Similar to our results, Horniakova and Busta [39] reported that the average live weight of broiler chickens tended to increase by a feed additive containing clinoptilolite compared with the control group. In addition to the natural clinoptilolites, modified clinoptilolite [40] and zinc-bearing clinoptilolite [41] had no consistent beneficial effects on broiler overall performance from 1 to 42 d. As mentioned by the numerous reports clinoptilolite is a harmless feed additive for broilers. However discrepancies between the studies may be related to the nature, purity, concentration and composition of the clinoptilolite [40]. In this study, dietary 2% clinoptilolite and phytase supplementation decreased broiler weight gain between 1 to 14 d of the study. However, clinoptilolite and phytase combination had no pronounced effect on broiler growth performance during 42 d experimental period. It is necessary to mention that the current study was conducted under good hygienic conditions and the day old male chickens were provided from a young breeders flock. As a result, the need for dietary feed additives to increase the production performance might be decreased to minimum. In addition it can be assumed that effects of clinoptilolite on performance might be more pronounced under suboptimal environmental conditions.

Intestinal Histomorphology

The morphological changes in the small intestine, such as increasing villus height and VH:CD ratio might

Table 6. *Effect of dietary clinoptilolite and/or phytase supplementation on tibia ash, Ca and P content* [1]
Tablo 6. *Klinoptilolite ve/veya fitaz ilavesinin broiler tibia külü ile Ca ve P düzeylerine etkisi*

Item	Dietary Treatment[2]				Statistics	
	T1	T2	T3	T4	SEM	*P-value*
d 21						
Tibia ash, %	56.72[a]	57.07[a]	55.89[ab]	55.14[b]	0.21	0.002
Ca, %	20.36	20.27	19.67	19.68	0.15	0.228
P, %	10.32[a]	10.24[a]	10.07[a]	9.68[b]	0.06	<0.001
Ca/P ratio	1.97	1.98	1.95	2.03	0.02	0.326
d 42						
Tibia ash, %	59.09	60.24	58.83	58.35	0.29	0.115
Ca, %	19.98	20.47	20.29	19.80	0.18	0.538
P, %	10.15	10.30	10.56	10.17	0.07	0.099
Ca/P ratio	1.97	1.99	1.92	1.95	0.02	0.537

*[a-b] Means with different superscripts in the same row are significantly different (P<0.05); [1] Data represent mean values of 8 replicates per treatment; [2] **T1:** Control, a standard corn-soybean meal basal diet; **T2:** 2% clinoptilolite; **T3:** 0.01% phytase (5000 FTU/g); **T4:** 2% clinoptilolite+0.01% phytase*

have beneficial effects on birds performance. So that, these changes enhance the absorptive surface area that is prominent when the alternative growth stimulators are applied. Previous studies have revealed that dietary clinoptilolite can influence intestinal mucosa and protect intestinal mucosa from damage [42-44]. In the current study, duodenal villus height and crypt depth were affected by dietary treatments on d 21 and d 42 *(Table 5)*. However no significant differences were observed on jejunum and ileal histomorphology during the experimental period. Xia et al.[45] reported that dietary Cu-montmorillonite increased villus height and villus height:crypt depth ratio in small intestine. They suggested that such improvements on intestinal integrity might be related to the decreased numbers of *E. coli* and *Clostridium* spp. and increased mucus resistance. Similarly Wu et al.[40] suggested that improvement in the intestinal integrity might be related to the lower numbers of *E. coli* and Salmonella. In addition Tang et al.[41] observed an increase in villus height and villus height:crypt depth ratio of the small intestinal mucosa of the chickens fed the diet supplemented with zinc-bearing clinoptilolite. As reported by previous researchers, montmorillonite acts as a mucus stabilizer and attaches to the mucus to support the intestinal mucosal barrier and help regeneration of the epithelium [46,47]. In addition, due to the porous structure and high cation exchange capacity, clinoptilolite can bind amines, ammonia and toxins. These suggested favorable effects of clinoptilolite may help to protect gut wall to the deleterious effects of such products.

More recently, Wu et al.[4] reported that dietary addition of natural and modified clinoptilolite, at the level of 2%, may have protective effects on intestinal mucosa against LPS-mediated damage. Previous studies indicated that natural and modified clinoptilolite supplementation has beneficial effect on intestinal integrity. In addition to previous studies, our results suggested that combined use of clinoptilolite and phytase in low Ca and P diets had no detrimental effect on male broiler intestinal histomorphology.

Tibia Ash and Mineral Content

According to the previous studies [17,21] dietary zeolite supplementation improves bone ash in birds fed low Ca diets. Moreover, Leach et al.[17] reported that dietary sodium zeolite supplementation increased serum Ca level and decreased P level. They concluded that decreased serum P level might be related to the sodium zeolite addition. Contrary to that earlier study, tibia P level was decreased numerically in comparison to control on d 42. However tibia P level was significantly lower (P=0.001) for the birds fed clinoptilolite and phytase supplemented diet versus to control on d 21. It can be assumed that dietary level of 2% clinoptilolite and phytase supplementation decreased P utilization, therefore it might useful to decrease clinoptilolite level in phytase supplemented diets at starting period.

Phytase supplementation had no consistent effect on tibia parameters on d 21 and d 42. Contrary to our findings, Chung et al.[28] showed that both fungal and bacterial phytases improved bone mineral density of tibia and femur of broilers on d 21. Discrepancies between the studies might be related to the source of phytase and analytical methods.

Generally, findings from the studies were inconsistent [2,45,48] and the effect of clays is changed according to the kind of clay and obtaining area, physical or chemical properties, dose used in the experiment of the clay. Also diet type and environment (existence of pathogen or toxin etc.) may play a role on the results. It is concluded that there might be a positive effect of 2% clinoptilolite on the performance of male broilers after starting period.

Further studies are needed to determine optimum dose of clinoptilolite and/or phytase combination for starter period. Effectiveness of the clinoptilolite should be examined in suboptimal conditions such as toxin or pathogen existence. Also effect of clinoptilolite and phytase combination should be determined in diets with different Ca levels.

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Research Article

Comparison of Tuberculin Skin Test, IFN-γ Assay, Real Time PCR and Lateral Flow Rapid Test in Diagnosis of Field Outbreaks of Bovine Tuberculosis

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Abstract

Bovine tuberculosis is an important zoonotic disease transmitted by direct contact, respiratory pathway, ingestion of unpasteurised milk and milk product, raw or undercooked meat. Tuberculosis can be difficult to diagnose based only on the clinical signs. Tuberculosis is usually diagnosed in the field with the tuberculin skin test. Sputum and other body fluids may be collected for microbiological examination. Polymerase chain reaction (PCR) methods have also been described. Diagnostic blood tests include the lymphocyte proliferation assay, the interferon gamma (IFN-γ) assay, and enzyme-linked immunosorbent assays (ELISA). In this study a total of 50 animals were tested by using tuberculin skin test (TST), lateral flow rapid test, IFN-γ assay and real time PCR. The animals were selected randomly among 178 cattle in dairy farms with the aged between 3-5 years and suspected of having tuberculosis. Forty five cattle were positive out of 50 for TST while 31 for reactive by the IFN-γ assay and 28 for rapid test and 9 for real time PCR. The purpose behind such variable as age was to compare sensitivity of tuberculin skin test, the IFN-γ assay and TB lateral flow rapid test and real time PCR examination for the diagnosis of field outbreaks of bovine tuberculosis in Turkey.

Keywords: *IFN-γ assay, Real time PCR, TB lateral flow rapid test, Tuberculin skin test, Tuberculosis*

Sahada Görülen Sığır Tüberkülozunun Tanısında Kullanılan Tuberculin Skin Test, IFN-γ Assay, Gerçek Zamanlı PCR ve Lateral Flow Rapid Testlerinin Karşılaştırılması

Özet

Sığır tüberkülozu; direkt temas, solunum yolu, pastörize edilmemiş süt ve süt ürünleri, çiğ ya da az pişmiş etlerin alınmasıyla insanlara da bulaşabilen önemli bir zoonoz hastalıktır. Yalnızca klinik bulgulara bakarak tüberkülozu teşhis etmek zor olabilmektedir. Genellikle tüberkülozun tanısı sahada tüberkülin testiyle konulmaktadır. Mikrobiyolojik muayene amacıyla kraşe ve diğer vücut sıvıları temin edilmektedir. Polymerase chain reaction (PCR) yöntemleri de kullanılabilmektedir. Tanı amaçlı kan testleri arasında lenfosit proliferasyon testi, interferon gamma (IFN-γ) testi ve enzyme-linked immunosorbent assay (ELISA) bulunmaktadır. Bu çalışmada 50 hayvan tuberculin skin test (TST), lateral flow rapid test, IFN-γ assay ve gerçek zamanlı PCR kullanılarak test edilmiştir. Hayvanlar 3-5 yaş arasındaki tüberküloz şüpheli 178 sütçü sığır arasından rastgele seçilmiştir. TST sonuçlarına göre 50 sığırdan 45'i pozitifken, IFN-γ testine göre 31'i pozitif bulunmuştur. TB lateral flow rapid testte 28 pozitif, gerçek zamanlı PCR testinde ise 9 pozitif sonuç bulunmuştur. Çalışmamızda farklı testlerin uygulanmasının amacı tüberkülin skin test, IFN-γ assay, TB lateral flow rapid test ve gerçek zamanlı PCR testinin Türkiye'de karşılaşılan sığır tüberkülozundaki duyarlılıklarının karşılaştırılmasıdır.

Anahtar sözcükler: *IFN-γ assay, Real Time PCR, TB Lateral Flow Rapid Test, Tuberculin Skin Test, Tuberculosis*

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INTRODUCTION

Bovine tuberculosis is an important zoonotic disease transmitted by direct contact, respiratory pathway, ingestion of unpasteurised milk and milk product, raw or undercooked meat. In developed countries, eradication efforts have significantly reduced the prevalence of this disease, but wildlife reservoirs avoid a complete eradication [1,2]. Bovine tuberculosis is still common in less developed countries and it causes economic losses in cattle. It is also a serious threat to endangered species [3,4]. Cattle are considered to be maintenance hosts for *Mycobacterium bovis (M. bovis)*. Infections have also been described in numerous other domestic and wild animals [5].

It can be difficult to diagnose tuberculosis based only on the clinical signs [6]. In developed countries, few infections show symptoms and infections are mostly diagnosed by routine tests. Tuberculosis is usually diagnosed in the field with tuberculin skin test [7,8]. Sputum and other body fluids may be collected for microbiological examination, microscopic demonstration of acid fast bacilli, isolation of mycobacteria on selective culture media and identification by biochemical tests. Slides may be stained with Ziehl-Neelsen stain, fluorescent acid fast stain or immuno-peroxidase techniques [9,10]. PCR methods have also been described. New diagnostic blood tests include lymphocyte proliferation assay, IFN-γ assay and enzyme-linked immuno-sorbent assays (ELISA) [11,12]. IFN-γ assay is only useful in members of bovidae however lymphyocyte proliferation test may be used on other animals and wild animals. These tests are not used routinely for diagnosis [13,14].

Serological assays have shown promise as a diagnostic alternative to skin testing or culture testing for many of these species. Serological blood test based on TB assays are appealing not only for better sensitivity and specificity for captive wild animals, exotic species and other non-traditional livestock but also because they require only a single handling event, thereby minimizing capture-associated injuries [15,16]. The serological test concept is simple, rapid, easy to interpret, inexpensive and is very useful as a slaughter surveillance test or an effective and efficient trap and cull assay [17].

The purpose for conducting this study was to compare sensitivity of TST, IFN-γ assay, lateral flow rapid test and real time PCR examination for the diagnosis of field outbreaks of bovine tuberculosis in Turkey.

MATERIAL and METHODS

TST assay was applied on 178 cattle in dairy farm with the age of 3-5 years suspected from tuberculosis. Fifty samples have been selected randomly. Tuberculin skin test has been used in a herd as a first TB test and blood samples have been collected during TST. The blood samples for analysis have been collected from 50 samples selected randomly. The herd have been quarantined and cattle with positive test result have been slaughtered.

Tuberculin Skin Test

Subsequent to shaving the neck hairs with the area of 8-12 cm^2, the skin has been measured with calliper and the results have been recorded. Mammalian PPD tuberculin (0.1 ml) has been injected intradermally on shaved skin. After 72 h, injection site has been examined for pain, tenderness, warmth, swelling and skin thickness has been measured again. An increase in skin thickness was observed as follows: 0-3 mm (negative), 3-4 mm (doubtful) and 4 ≥ (positive).

IFN-γ Assay

Whole blood samples have been examined for T cell reactivity by production of IFN-γ samples stimulated with *M. bovis* and *Mycobacterium avium (M. avium)* PPDs at 20 µg/mL sample and PBS as non-stimulated controls as previously described. The blood has been incubated for 18-20 h at 37°C in a humidified atmosphere. After this period, samples have been centrifuged for 15 min at 490 g and the supernatant was collected and assayed for the presence of IFN-γ by using the ELISA (Bovigam, Product Number: 63319/63309) according to the manufacturer's instructions. Using avian and bovine PPDs for stimulation, results have been considered positive having the mean optical density at 450 nm (OD 450) stimulated with bovine PPD minus the OD 450 measured in the negative control stimulated with PBS was greater than 0.05 and greater than the OD 450 obtained in the sample stimulated with avian PPD. Additionally, a less restrictive 0.1 cut-off point was also evaluated. Each ELISA plate was validated with positive and negative controls according to the manufacturer's instructions.

Lateral Flow Rapid Test

For rapid detection of antibodies, TB lateral flow rapid test (BioNote Catalog No: RB23-02) uses selected myco-bacterial antigens immobilized on a nitrocellulose strip and a blue latex signal detection system for rapid detection of antibodies. The test was performed according to the manufacturer's instructions. A single test plate was used for each cattle. Plates were kept at room temperature. Thirty microliters of serum was distributed into the sample wells and allowed to soak into the wick. Three drops of sample buffer was added to the sample wells. The test was incubated at room temperature for 20 min and the results were recorded. A completed blue band must appear across the control line site (C-band) in order to have a valid test and a complete blue band must also appear at the test line site (T-band) in order to have a positive test.

Molecular Diagnostics

DNA extraction has been obtained from plasma samples

with commercial DNA isolation kit (Roche; Product Number: 03 038 505 001) according to the procedure [DNA extraction was performed by using MagNA Pure LC robotic isolation device according to the procedure. The isolation procedure is based on magnetic-bead technology. The samples are lysed by incubation with a special buffer containing a chaotropic salt and Proteinase K. Magnetic Glass Particles (MGPs) are added and total nucleic acids contained in the samples are bound to their surface. Unbound substances are removed by several washing steps, then the purified total nucleic acid is eluted with a low-salt buffer. The sample materials are placed into the wells of the sample cartridge. Lysis/binding buffer is added to the sample. Proteinase K is added to the samples and proteins are digested. Nucleic acids bind to the silica surface of the added MGPs due to the chaotropic salt conditions, isopropanol, and the high ionic strength of the lysis/binding buffer. MGPs with bound nucleic acids are magnetically separated from the residuallysed sample. MGPs with bound nucleic acids are washed repeatedly with wash buffer tore move unbound substances like proteins (nucleases), cell membranes, PCR inhibitors such as heparin or hemoglobin, and tore duce the chaotropic salt concentration. Again MGPs with bound total nucleic acid are magnetically separated from the wash buffer containing residual sample debris. The purified nucleic acids are eluted at +70°C from the MGPs in the wells of the elution cartridge, whereas the MGPs are retained in the reaction tip and discarded].

The DNA samples were stored at -20°C until PCR analysis. The amount of DNA determined by the measurement with spectrophotometer (ASP-3700) within 260 and 280 nm. Commercial *Mycobacterium* spp. detection kit (Way 2 Gene; Product Number: WG 40-0220- 16) was used for real-time PCR analysis. PCR was carried out according to the kit procedure. Each reaction tube contained 2 µL fast start mix 4 µL *Mycobacterium* genus primer, 1.6 µL Mg^2, 5 µL DNA template and distilled water to give a final volume of 20 µL. The thermal cycling protocol was as follows: 10 min at 95°C for denaturation, 5 s at 64°C for annealing and 40 s at 72°C for extension using a Roche Light Cycler 2.0. In all control stages, PCR as a positive standards included in the set, the distilled water was used as a negative control.

Statistical Analyses

The statistically significant difference test were associated with the chi square test. The sensitivity and specificity have been revealed as reference test IFN-γ assay. The proportions used for calculating the sensitivities and specificities of the tests were compared using SPSS 16 for Windows. The 95% confidence limits for the findings were calculated using SPSS 16.

RESULTS

In this study a total of 50 animals were tested by using tuberculin skin test, TB lateral flow rapid test, IFN-γ assay and real time PCR. Forty five were positive out of 50 for TST while 31 for reactive by the IFN-γ assay and 28 for TB lateral flow rapid test and 9 for real time PCR *(Table 1)*.

The sensitivity and specificity of TST, TB lateral flow rapid test, and real time PCR compared with IFN-γ assay were determined as 100%, 80.65% and 25.81% respectively *(Table 2)*.

DISCUSSION

The objective for conducting this analysis was to evaluate the performance on positive results of TST, IFN-γ assay, TB lateral flow rapid test and real time PCR under field conditions. Currently, definitive diagnosis of bovine tuberculosis in cattle is often made on patients' history, clinical and necropsy findings, tuberculin skin tests and abattoir meat inspection [15]. Control depends on early

Table 1. *Number of positive cattle according to TST, TB lateral flow rapid test, IFN-γ assay, real time PCR*
Tablo 1. *TST, TB lateral flow rapid test, IFN-γ assay ve gerçek zamanlı PCR testlerine göre pozitif tespit edilen sığır sayısı*

Performed Tests	Positive	Negative	Total
Tuberculin skin test	45	5	50
TB lateral flow rapid test	28	22	50
IFN-γ assay	31	19	50
Real time PCR	9	41	50

Table 2. *The sensitivity and specificity of TST, TB lateral flow rapid test and real time PCR*
Tablo 2. *TST, TB lateral flow rapid test ve gerçek zamanlı PCR testlerinin hassasiyeti ve spesifitesi*

Test	Sensitivity	Specificity	Power of the Test	P Value
TST x IFN-γ assay	100%	26.32%	72%	53.606***
TB lateral flow rapid test x IFN-γ assay	80.65%	84.21%	82%	15.489***
Real time PCR x IFN-γ assay	25.81%	94.73%	52%	

**** P>0.001*

identification and proper treatment of individuals with active disease. The tuberculin skin test is effective in the early detection of pre-clinical cases of *M. bovis* infection in cattle. This allows the rapid removal of infected animals limiting transmission of the disease and eradication of bovine tuberculosis from many countries [18]. However, a few number of ancillary tests are being used, or are currently validated. These ancillary tests are likely to provide a more accurate diagnosis following skin-testing. The blood based IFN-γ assay is a cellular immune assay which can detect early infection [19,20].

Up to date, there is no simple, rapid, sensitive and specific test that can differentiate active TB from latent infections and slowly progressive TB. A number of new antigens are being tested individually or in combinations to obtain the desired sensitivity and specificity. The search for rapid and reliable diagnostic tests for active TB based on the examination of blood and other clinical specimens has been the focus of several studies [21,22]. The tests allow early detection of latently infected individuals and are useful in contact tracing and screening of high-risk groups in a low-endemic setting. IFN-γ based tests may be important for epidemiological and surveillance studies to determine the extent of TB infection [23,24].

The initial field tirals carried out in Australia demonstrated that the IFN-γ assay had a higher sensitivity (93.6%) than the CFT (65.6%) [25]. Sensitivities ranged between 55 to 97% and the specificity was as high as 97% [7]. In this study the TST x IFN-γ ELISA sensitivity were 100% with 26.32% specificity. In the study, the agreement between TST and IFN-γ assay was determined as excellent. The sensitivities and specificities reported in the study are relatively high compared with earlier studies [7,25,26].

Furthermore, a negative result to a tuberculin test does not mean that the animal is not infected with *M. bovis* while a positive result represents and immunological response in the form of a delayed hypersensitivity reaction to mammalian tuberculin that is most commonly occur due to an infection or exposure to other bacteria that share antigens similar to those of *M. bovis*. A number of studies have shown that the tuberculin skin test and IFN-γ assay detect overlapping, but also distinct populations of *M. bovis* infected cattle [27-29]. Using these two types of cellular immune assays in quick succession can result in the removal of a greater proportion of *M. bovis* infected animals than either using this test alone [9].

Rapid test is relatively simple, inexpensive and do not require highly trained personnel or a complex technological platform. The method is suitable for use in laboratories in countries with low-income. The tests allow early detection of latently infected individuals and are useful in contact tracing and screening of high-risk groups in a low-endemic setting [17]. Results from this study so far suggest that combinations of antigens may yield the desired level of sensitivity without affecting specificity. This anti bovine TB antibody test kit has a sensitivity of 90% against bovine TB confirmed by bacterial isolation and a sensitivity of 85.1% and specificity of 98.6% against TST [17]. Also using the lateral flow rapid test, achieving sensitivity of 84% and a specificity of 84.2% for serological diagnosis of *M. bovis* infection in cattle. Similar and relatively high sensitivity (86.5% and 84.6%) and specificity (83.8% and 91.4%) have been reported with other lateral flow techniques for the diagnosis of bovine TB in farmed red deers [15]. In this study, the apparent sensitivity of both was high rapid test x IFN-γ assay sensitivity and specificity were 80.65%, 84.21% respectively and in agreement with previously authors.

PCR methods allow direct identification of *M. bovis* complex and can detect less than 10 bacteria in a clinical specimen. PCR's sensitivity ranges from 70-90% compared to the results of culture and its specificity varies between 90 and 95%. In smear of positive cases, the sensitivity of PCR is greater than 95% but in smear of negative cases, it is only 50 to 60%. Therefore, present amplification methods should not be replaced diagnostic convectional culture [4]. Molecular biological methods, such as PCR may be used to [30] diagnose TB rapidly by identifying DNA from *M. bovis* complex in clinical samples with negative microscopic results however PCR gives rapid results. Most of these report a lower sensitivity for PCR than culture [9,21], sensitivity was 25.81% while specificity 94.73% for real time PCR x IFN-γ assay. This low sensitivity occurs due to blood samples failed for PCR. These results are consistent with data obtained in other studies. This makes PCR suitable for detecting active tuberculosis.

As a result, through the development these new ancillary tests in association with skin test the means and modes proposed throughout trials will improve the detection of *M. bovis* infected cattle and reduce the unnecessary slaughter of false-positive reactors.

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Research Article

Genetic Analysis of the ORF7 Gene in Vietnamese Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) [1]

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically devastating diseases for the swine industry worldwide. The 372-bp complete nuclear capsid protein (N-protein) encoding gene (ORF7) of 36 field PRRSV isolates from Vietnam collected during 2008-2012 were sequenced and compared with certain vaccines and published strains of PRRSV. The ORF7 nucleotide sequence (nt) similarity and amino acid (aa) identity among 36 strains showed the highest and ranged from 95.1 to 100%. These isolates shared similarities with VR-2332 (nt 91.9-94.3%, aa 92.6-96.7%) and LV (nt 62.7-65.3%, aa 58.5-60.1%). There were higher level of similarity with QN07 (nt 96.2-99.1%, aa 96.7-99.1%) from the 2007 PRRS outbreak in Quang Nam province, CH-1a (nt 93.0-96.2%, aa 92.6-95.9%) isolated in China in 1995 and JXA1 (nt 96.7-99.7%, aa 97.5-100%), the highly pathogenic strain from China isolated in 2006. Six aa mutations located in both variable and conserved regions of N-protein amino acid sequence were detected in most of the 36 isolates and highly pathogenic PRRSV strains in China in comparison to the prototype strain VR-2332. Results of sequence analyses indicated that PRRSVs isolated in Vietnam during 2008-2012 were classified as North American genotype. Phylogenetic tree also clustered those 36 PRRSV isolates, other recently reported Vietnamese strains, highly pathogenic Chinese strains and JXA1-R vaccine strain in the same cluster and separated from the prototype strain (VR-2332) and Ingelvac MLV/Besta vaccine strains. The result on genetic characterization of ORF7 of circulating PRRSV strains may assist the development of effective strategies for monitoring and controlling PRRS in Vietnam.

Keywords: *Genetic variation of ORF7, North American genotype, Phylogenetic analysis, PRRSV*

Vietnam Domuz Reprodüktif ve Respiratorik Sendrom Virüsü (DRRSV) ORF7 Geni'nin Genetik Analizi

Özet

Domuz reprodüktif ve respiratorik sendromu (DRRS), dünya çapında domuz endüstrisi için ekonomik olarak en fazla kayba neden olan hastalıklardan birisidir. Vietnam'da, 2008-2012 boyunca 36 sahadaki DRRSV izolatlarının 372-bp komple nükleer kılıf proteinini (N-protein) kodlayan gen (ORF7) dizilimi yapıldı ve DSRRV'nin belli aşıları ve yayınlanmış türleri ile karşılaştırıldı. Otuzaltı 36 tür arasında, ORF7'nin nukleotid dizilim benzerliği (nt) ve aminoasit (aa) özdeşliği en yüksek olup, %95.1 ile 100 arasında değişiklik gösterdi. Bu izolatlar, VR2332 (nt %91.9-94.3, aa %92.6-96.7) ve LV (nt %62.7-65.3, aa %58.5-60.1) ile benzerlik taşıyordu. Quang Nam yöresindeki 2007 DRRS salgınında QN07 (nt %96.2-99.1, aa %96.7-99.1) ile 1995'te Çin'de izole edilen CH-1a (nt %93.0-96.2, aa %92.6-95.9) ve Çin'de 2006'da izole edilen yüksek patojenik tür JXA (nt %96.7-99.7, aa %97.5-100) ile daha yüksek düzeyde benzerlikler vardı. Prototip tür VR-2332'ye göre, her iki değişkende ve N-protein amino asit diziliminin korunaklı bölgelerinde yerleşik 6 aa mutasyonları, 36 izolatın çoğunda ve Çin'deki yüksek patojenik DRRSV türlerinde tespit edildi. Dizilim analizleri sonuçları, Vietnam'da 2008-2012 boyunca izole edilen DRRSV'lerin Kuzey Amerikan Genotipi olarak sınıflandırıldığını gösterdi. Filogenetik ağaç, ek olarak 36 DRRSV izolatlarını, diğer yakın zamanda bildirilen Vietnam türlerini, yüksek patojenik Çin türlerini ve JXA1-R aşı türlerini aynı kümede topladı ve prototip tür (VR-2332) ve Ingelvac MLV/Besta aşı türlerinden ayrıldı. Sirkülasyondaki DRRSV türlerindeki ORF7'nin genetik karakterizasyonu sonucu, Vietnam'daki DRRS'nin etkili izleme ve kontrol stratejilerinin gelişimine yardım edebilir.

Anahtar sözcükler: *ORF7'nin genetik varyasyonu, Kuzey Amerikan genotipi, Filogenetik analiz, DRRSV*

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is one of the most viral diseases caused great economic losses to pig industry. It is characterized by reproductive failure in pregnant sows, and respiratory distress in piglets and fattening pigs. PRRSV is a member of the *Arteriviridae* family, within the order *Nidovirales* [1]. PRRSV is small, an enveloped, positive, single-stranded RNA which is approximately 15 kb in size, composed of at least nine overlapping open reading frame (ORFs) [2]. ORF7 encodes for non-glycosylated nuclear capsid protein (N) with molecular size of 15 kDa, which is composed of 123 or 128 aa in the North American (NA) and European types (EU), respectively. It is the most abundant viral protein in virus-infected cell and contains important immunogenetic epitopes and immunodominant antigent in the pig immune respond to PRRS [3]. Compared to other ORFs, ORF7 is an important target for virological detection using polymerase chain reaction (PCR). Therefore, genetic analysis of ORF7 has been characterized and used for serological detection and diagnosis, genetic variation and phylogentic analysis among PRRSVs.

PRRS was first detected in the United States in 1987, and then it has spread rapidly throughout the world. In Vietnam, PRRS was first observed in outbreak of PRRS in Vietnam appeared in Hai Duong Province in March 2007 [4]. Then it spreaded to other regions of the country, affecting about 70,577 pigs with more than 20.366 pigs killed [5]. Genetic analysis of PRRS strains collected from outbreaks in China and Vietnam in 2007 indicated high nt identity (99%) among isolates [6].

Currently, both NA and EU types of PRRSV are circulating in the swine population worldwide [7], however, NA type is more common in Vietnam. In the recent years, new NA type PRRSV variants called highly pathogenic strains (HP) has emerged in Vietnam and China causing large-scale outbreaks and destructive clinical syndromes [6,8]. Those HP-PRRSV isolates share high sequence identity and have similar deletions/mutations in difference regions of viral genome such as two deletions in non-structural protein 2, one deletion in the 5'-untranslated region, and one deletion in the 3' untranslated region, and some other point mutations [9]. Recently, several studies on genetic variation and phylogenetic relationship based on major structural genes of PRRSV isolates such as ORF5, ORF7 have been done [10-14]. In this study, the 372-bp complete ORF7 of 36 PRRSV isolated from Vietnam collected during 2008-2012 were sequenced and analyzed with ORF7 sequences of PRRS vaccine viruses as well as its parental virus and other published PRRSV strains.

MATERIAL and METHODS

Total blood (n = 36) from PRRSV-infected pigs displaying clear clinical signs were sampled during 2008-2012 from swine herds in different provinces (Can Tho, Hau Giang, Dong Thap, Dong Nai, Binh Duong, Ho Chi Minh City and Dien Bien). JXA1-R vaccine from China was also sampled for analysis. All samples were stored in ice boxes and transported to the laboratory. A summary of the samples is presented in *Table 1*.

Total RNA was extracted using TRizol reagent (Invitrogen, USA) according to the manufacturer's instructions and used as template of RT-PCR to synthesize cDNA with random hexamers primers. Amplicon with molecular size of 490 bp containing complete ORF7 (372 bp) was amplified using ORF7 specific primer pairs [15]. For DNA sequencing, PCR products were purified using a QIAquick Extraction Kit (Qiagen, Germany) and directly sequenced in both directions (Macrogen, Korea).

Obtained nt sequences were identified with the Basic Local Alignment Search Tool (BLAST) [16]. Multiple nt alignments were carried out with BioEdit version 7.0.9.0 using published PRRSV sequences as references [17]. The phylogenetic tree was constructed with Mega 4.1 [18] using the neighbor-joining (NJ) method [19] and computed with the Kimura 2-parameter method [20]. Boot-strap values were calculated using 1.000 replicates of the alignment. Futhermore, the amino acid sequences deduced from ORF7 of 36 PRRSV isolates were aligned and analyzed the changes in their functional domains.

RESULTS

Sequence Analysis

The complete ORF7 sequences of 36 PRRSV strains isolated from different provinces of country during 2008-2012 have been determined. Out of 36 strains, 32 were submitted to GenBank under the following accession numbers: JQ860392-JQ860423 *(Table 1)*. All of the PRRSV sequences were the same length (372 nt), thus encoded 123 aa residues.

Nucleotide (nt) and amino acid (aa) similarities between 36 PRRSV isolates and compared to those of VR-2332, LV, 07QN, CH-1a, and vaccine viruses JXA1-R, Ingelvac MLV, BSL-PS were summarized in *Table 2*. Pairwise comparison revealed that 36 isolates shared 95.1-100% in nt identities with each other and could be separated into 13 haplotypes *(Table 1)* based on the difference in their nt sequences. There were high nt identities (91.9-94.3%) with VR-2332 but only nt 62.7-65.3% and aa 58.5-60.1% similarities with LV. The result implied that our 36 PRRSV strains were of North American genotype (NA). Compared with nt sequence of 07QN (a highly pathogenic Vietnamese strain isolated from PRRS outbreak reported in 2007), the identity was 96.2-99.1% in nt sequence. The higher nt similarity between our 36 PRRSV isolates and high pathogenic JXA1 strains from 2006 (96.7-99.7%) and lower nt identity

Table 1. *PRRSV isolates (n = 36) from Vietnam and representative PRRSV strains used for sequencing and phylogenic analyses*
Tablo 1. *Vietnam'daki DRRSV izolatları (n = 36) ve dizilim ve filojenik analizlerde kullanılan örnek DRRSV türleri*

No.	Strain*	Location-Year	Reference	Haplotype	No.	Strain	Location-Year	Reference
1	DN2008-153	VN-2008	JQ860403	1	37	JXA1-R vaccine	China-2011	-
2	DN2008-444	VN-2008	JQ860392	2	38	CH-1a	China-1996	AY032626
3	DN2008-452	VN-2008	JQ860393	2	39	NB-CH2004	China-2004	FJ536165
4	DN2008-456	VN-2008	JQ860394	3	40	GD3-CH2005	China-2005	GU269541
5	DN2008-460	VN-2008	JQ860395	1	41	JXA1-CH2006	China-2006	EF112445
6	DN2008-499	VN-2008	JQ860396	4	42	HUN4	China-2006	EF635006
7	DN2008-694	VN-2008	JQ860397	2	43	07HEN-CH2007	China-2007	FJ393457
8	DN2009-1107	VN-2009	JQ860406	2	44	EM2007	China-2007	EU262603
9	DN2009-1155	VN-2009	JQ860407	2	45	KP-CH2008	China-2008	GU232735
10	DN2009-292	VN-2009	JQ860404	2	46	HUB7-CH2009	China-2009	GU168567
11	DN2009-339	VN-2009	JQ860405	2	47	09HEN2	China-2009	JF268680
12	DN2009-42	VN-2009	JQ860398	2	48	09HUB1	China-2009	JF268682
13	DN2009-44	VN-2009	JQ860399	2	49	DC-CH2010	China-2010	JF748718
14	DN2009-59	VN-2009	JQ860400	2	50	SD16-CH2012	China-2012	JX087437
15	DN2009-73	VN-2009	JQ860401	2	51	06K805(JB)	Korea-2006	EF441853
16	DN2009-88	VN-2009	JQ860402	2	52	05K205(CN)	Korea-2006	EF441809
17	BD2010-R1	VN-2010	JQ860412	7	53	06K010(CN)	Korea-2006	EF441836
18	BD2010-X13	VN-2010	JQ860413	3	54	EDRD-1	Japan-1995	D45852
19	DN2010-1	VN-2010	JQ860408	1	55	31D.MEX4	Mexico-2003	AY209228
20	DN2010-5.2	VN-2010	JQ860409	2	56	27E.MEX3	Mexico-2003	AY209222
21	HCM2010-CC3	VN-2010	JQ860410	5	57	MN184A	USA-2005	DQ176019
22	HCM2010-D06	VN-2010	JQ860411	6	58	07QN	VN-2007	FJ394029
23	HCM2011-1P	VN-2011	-	8	59	BRVT	VN-2009	GU187019
24	CT2012-C1	VN-2012	JQ860414	9	60	T4SG	VN-2009	GU187020
25	CT2012-C2	VN-2012	JQ860415	9	61	MLV.Bestar	Singapore-2009	GU187018
26	CT2012-HS1	VN-2012	JQ860416	9	62	IngelvacPRRS	USA-1998	AF066183
27	CT2012-HS2	VN-2012	JQ860417	9	63	VR-2332	USA-1990	U87392
28	CT2012-HS3	VN-2012	JQ860418	9	64	Lelystad	Holand-1991	M96262
29	DT2012-DT7	VN-2012	JQ860419	10				
30	DT2012-DT8	VN-2012	JQ860420	10				
31	DT2012-DT9	VN-2012	JQ860421	10				
32	HG2012-RV1	VN-2012	JQ860422	11				
33	HG2012-RV2	VN-2012	JQ860423	12				
34	DB2012-1DB	VN-2012	KM659200	13				
35	DB2012-2DB	VN-2012	KM659201	13				
36	DB2012-9BD	VN-2012	KM659202	13				

**Strains in bold were isolated and used in this study; (-): sequences analysed in this study and not submit in GenBank*

(93.0-96.2%) to Chinese strains CH-1a from 1995 were observed. Among the 36 isolates, samples collected from Dien Bien exhibited unique nt changes at positions 183 (T→ C), 351 (C → T) *(data not shown)*, and both are non-synonymous substitution.

Analysis of Deduced Type II PRRSV ORF7 aa Sequences

The deduced aa sequences for ORF7 of the 36 Vietnamese isolates were aligned with representative PRRSV strains from Vietnam, China, prototype strain VR-2332 and vaccine viruses *(Fig.1)*. As shown in *Fig. 1*, all of PRRSV ORF7 sequences were the same length of 372 nt and encoded 123 aa residues. Pair-wise comparison showed that the 36 strains shared 95.1-100% aa identity with each other and high levels of aa identity with 07 QN (96.7-99.1%). The aa identities were of 92.6-96.7% in comparison

Table 2. *Nucleotide and amino acid identities (%) for ORF7 among 36 Vietnamese PRRSV isolates, and compared with LV, VR-2332, 07QN, JXA1, CH-1a strains, and MLV-Bestar/ Ingelvac/ JXA1-R vaccines*

Tablo 2. *Otuzaltı Vietnam izolatı arasında ORF7 için nukleotid ve amino asit özdeşlikleri (%), ve LV, VR-2332, 07QN, JXA1, CH-1a türleri, ve MLV-Bestar/ Ingelvac/ JXA1-R aşılarıyla karşılaştırılması*

Strains (n)		2008	2009	2010	2011	2012	07QN	VR 2332	LV	CH-1a	JXA1/ JXA1-R Vaccine	MLV-Bestar/ Ingelvac Vaccine
VN-2008 (7)	*nt*	97.8-100	97.8-100	96.7-100	98.1-99.1	96.2-99.4	97.8-99.1	93.5-94.3	63.8-64.3	94.6-96.2	98.3-99.7	93.2-94.3
	aa	99.1-100	99.1-100	96.7-100	99.1-100	96.7-100	98.3-99.1	94.3-95.1	59.3-60.1	94.3-95.1	99.1-100	94.3-95.1
VN-2009 (9)	*nt*		100	96.7-100	99.1	96.2-99.4	97.8	93.5	64.3	94.6	98.3	93.2-93.5
	aa		100	97.5-100	100	97.5-100	99.1	95.1	60.1	95.1	100	95.1
VN-2010 (6)	*nt*			96.5-99.1	96.5-99.4	95.1-99.4	97.3-98.9	92.4-94.0	63.3-65.3	94.6-96.2	97.8-98.4	92.2-94.0
	aa			95.1-100	97.5-100	95.1-100	96.7-99.1	92.6-95.9	59.3-60.1	92.6-95.9	97.5-100	92.6-95.9
VN-2011 (20)	*nt*				-	96.5-99.1	98.1	93.2	64.3	94.8	98.6	93.0-93.2
	aa				-	97.5-100	99.1	95.1	60.1	95.1	100	95.1
VN-2012 (13)	*nt*					95.1-100	96.2-97.8	91.9-94.0	62.7-64.0	93.0-95.1	96.7-98.3	91.6-94.0
	aa					97.5-100	96.7-99.1	94.3-96.7	58.5-60.1	92.6-95.1	97.5-100	94.3-96.7

(n): *number of strains;* ***nt:*** *nucleotide;* ***aa:*** *amino acid*

Fig 1. Analysis and comparison of amino acid mutations in N-protein of PRRSV. Mutations are indicated by closed boxes

Şekil 1. DRRSV'nin N-proteinindeki amino asit mutasyonlarının karşılaştırılması ve analizi. Mutasyonlar kapalı kutular tarzında gösterilmektedir

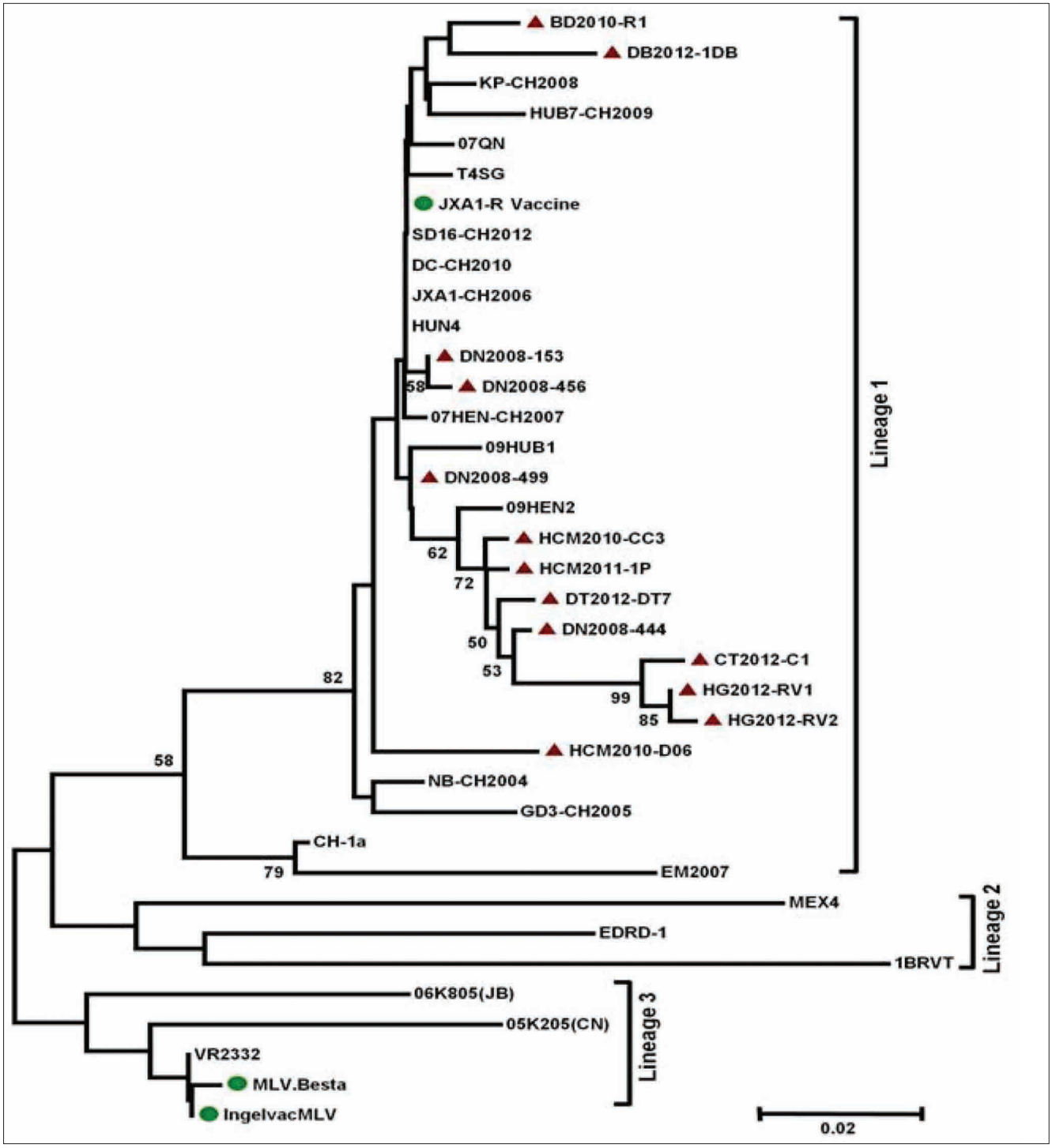

Fig 2. Phylogenetic tree containing 12 Vietnamese isolates and other representative virus strains. The comparison was based upon on the nucleotide sequence of PRRSV ORF7. The phylogenetic tree was generated by the neighbour-joining method using MEGA 4.1 with bootstrap values of 1,000 replicates. Our isolates are marked (▲). Vaccines licensed for use in Vietnam are denoted by (●)

Şekil 2. Oniki Vietnam izolatı ve diğer örnek virus türlerini kapsayan filogenetik ağaç. Karşılaştırma DRRSV ORF7'nin nükleotid dizilimi esasına göre yapıldı. Filogenetik ağaç, 1.000 kopyanın bootstrap (çekme) değerleri komşu-katılım metoduyla MEGA 4.1 kullanılarak oluşturuldu. İzolatlarımız (▲) ile işaretlendi. Vietnam'da kullanım için lisanslı aşılar (●) ile sembolize edildi

with VR-2332, 58.5-60.1% with LV, 92.6-95.9% with CH-1a, and 97.5-100% with JXA1-R. The 36 Vietnamese isolates had high aa similarity with JXA1-R (97.5-100%), the Chinese isolate used in PRRS vaccines, than that of Ingelvac MLV/BSL-PS (94.3-96.7%;) *(Table 2)*.

There were six common aa mutations located in both variable and conserved regions of N-protein amino acid sequence of the 36 PRRSV isolates compared to prototype strain LV-2332 (R11K, D15N, K46R, T91A, H109Q and V117A). In which, four mutations were observed in all strains at

position 15, 46, 109 and 117 (except isolate BD2010-R1). Out of six mutations, two aa changes at positions 11 (R→K) and 91 (T→A) were not detected in eight strains sampled from Dien Bien and Can Tho in 2012. There was a mutation at position 43 (K→R), which detected in two strains from Hau Giang only (HG2012-RV1 and HG2012-RV2). These mutations were also found in other Chinese HP-PRRSV strains [16]. Only one strain (HCM2010-D06) showed identical mutations at C-terminal (S120P and A123V).

Phylogenetic Analysis

Several genotyping studies based on ORF7 sequences have been conducted on type II PRRSVs, however, a lack of a reference sequence set, no satisfactory classification system was available. In the phylogenetic tree *(Fig. 2)*, the 36 Vietnamese PRRSV isolates were belonged to the type II (NA genotype) and were grouped closely to each others and to representative Chinese HP strains, such as HUN4, JXA1, 09HEN2, 09HUB1,... *(Lineage 1)*. They were divided into two subgroups, which is corresponding to subgroup IV and V of the previous classification [11]. The first subgroup contained 24 of our isolates belonged to eight haplotypes (2,4,5,8,9,10,11,12) and two Chinese HP reference strains (09HEN2 and 09HUB1). Other isolates belonged to haplotype 1,3,7 and 13 and representative HP strains (HUN4, HUB7 and JXA1-R) clustered in separate subgroup.

Isolates collected from Dien Bien and Binh Duong were closely grouped to HP strain 07QN, which was collected from PRRS outbreak in Quang Nam province in 2006 and Chinese vaccine JXA1-R. Seven isolates sampled from Can Tho and Hau Giang were grouped together into one branch and showed a very closely genetic relationship.

DISCUSSION

Since HP-PRRSV outbreak was first reported in 2007 in Vietnam, the virus has spread widely and is always accompanied with pig farms throughout the country. This study was to describe the genetic variation of PRRSV field isolates collected from infected pigs in different regions of Vietnam during 2008-2012 based on ORF7 sequence. Result of ORF7 sequencing confirmed that all of isolates were of NA genotype with high identity to prototype strain VR-2332 (91.4-94.3%). Within Vietnamese isolates, a highly pathogenic strain isolated from PRRS outbreak reported in 2007 (07QN) showed the the lower nt similarity with the PRRSV strains isolated in 2012 (96.2-97.8%) in compared to that of 2008 (97.8-99.1%). It implied that PRRSV isolates showed the certain genetic variation regarding the time. The nucleotide similarity of ORF7 among isolates ranged from 95.1 to 100% which was similar to these of 59 Chinese PRRSV strains (91.9-100%) [21]. Nucleotide homology analysis revealed that all isolates were closely related to the Chinese HP-PRRSV and also Chines PRRSV vaccine circulating in Vietnam (JXA1-R). High nt/aa similarities among Vietnamese and Chinese HP-PRRSV isolates *(Table 2)* correspond with findings from Feng et al.[5]. They observed 99% identity at the genomic level for Vietnamese and Chinese PRRSV isolates. With respect to ORF7 sequence, PRRSV strains circulating in Vietnam showed a greater nu/aa similarity to JXA1-R than to virus strains used in other vaccines (Ingelvac MLV and Besta-PS). It provides useful information for vaccine selection and renewal. The similar result was also obtained for ORF5 sequence of those strains [13,14].

Among viral protein, nucleocapsid protein (N-protein), encoded by ORF7, is the most abundant protein in the virion, accounting for 20-40% of the total virion protein content [22]. Because of more conserved property, N-protein has been used as target of several diagnostic tests. As shown in *Fig. 1*, analysis of the complete deduced amino acid sequence of N-protein from the 36 PRRSV isolates shared common six mutations with Chinese HP-PRRSV strains. These mutations were also detected in other HP-PRRSV strains isolated in China [11,12]. Compared to classical strain CH-1a, four mutations at position 46, 91, 109 and 117 were just observed in HP strains. Two identical mutations at position 11 and 91, which were not found in most of strains isolated in 2012. These unique variations in ORF7 gene should be considered in the development of diagnostic RT-PCR for PRRSV.

The N-terminus is presumed to play a role in the interaction with genomic viral RNA because of high composition of basic aa, such as Lysine and Arginin [23]. Therefore, the variations of R11K and D15N found in most of our strains and Chinese HP-PRRSV should be noted. N protein contains conserved nucleotide determinants such as nuclear localization signal (NLS), nuclear export signal (NES). Previous study had demonstrated that mutations at 43 and 44 within the NLS weaken viral replication [24], NLS motif such as "Pat7" located at position 41-47 (PGKKNKK) might block the recognition of the epitopes [11]. In our study, the substitution K46R observed in 36 isolates and also in Chinese HP-PRRSV strains might influence to its function. The final 11 residures at the C-terminus have been shown to be an important intermolecular reactions mediated via N-N interactions, thus is necessary for maintaining proper tertiary structure of this protein [25,26]. Two mutations at this region (postions P120S and A123V) were also deteted in isolate HCM2010-D06. Conserved mutation at position 117 (V→A) was also found in our 36 strains and other HP-PRRSV strains in compared to reference as classical strains of CH-1a and VR-2332.

Based on ORF7 sequences, Chinese HP-PRRSVs were mostly concentrated in sub-group IV and V [6]. As shown in *Fig. 2*, all of 36 isolates was also classified in those sub-groups (appeared in *Fig. 2* as Lineage 1) because they share numerous common point mutations (nu/aa). According to sequence comparison and phylogentic tree analysis, it demonstrates that a Vietnamese PRRSV strains are genetically closely related to HP-PRRSV strains that were

circulating in China at the same time. Therefore, it is very important to have effective stratergy for controlling PRRS transboundary disease and mornitoring pig movements.

Because of identical mutations at C-terminal (P120S and A123V) observed in HCM2010-D06 strain only, it was clustered separately and suggested the new genetic variants. Thus, molecular epidemiological studies of PRRSV should be carried out to provide annual genetic information for development of reliable PRRS diagnosis and disease control.

All of the 36 PRRSVs isolated during 2008-2012 in Vietnam belonged to the NA genotype. To our knowledge, this is the first report describing the genetic characterization of nucleoprotein encoding gene ORF7 of PRRSV circulating in different provinces of Vietnam collected during 2008-2012. Based on ORF7 sequence, the Vietnamese PRRSV strains were genetically closely related to each others and HP-PRRSV strains circulating in China.

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Research Article

The Protective Effects of Osajin on Ischemia/Reperfusion Injury to Rat Ovaries: Biochemical and Histopathological Evaluation

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Abstract

Reactive oxygen species (ROS) and inflammation play important roles in the pathogenesis of ischemia/reperfusion (I/R) ovarian injury. The purpose of this in-vivo study is to evaluate the effect of osajin, a prenylated flavonoid with antioxidant and anti-inflammatory properties, on oxidative balance and ovarian damage induced by unilateral I/R. The study used 48 adult, female Wistar albino rats. In the controls (CN), only laparotomy was performed. In group CN^{Osajin}, 200 mg/kg osajin was administered. In group $IR^{VEHICLE}$, an ischemic period of 3 h was followed by reperfusion for 3 h; the bilateral ovaries were then removed. In groups $IR^{Osajin100}$ and $IR^{Osajin200}$, after 3 h of ischemia, 100 and 200 mg/kg of osajin was given orally before reperfusion, respectively; after 3 h of reperfusion, the ovaries were removed. After the experiments, MPO, SOD and CAT enzyme activities and LPO levels was determined for the oxidative state and activities of PMNs. In addition, histopathological changes were examined in all rat ovarian tissues. Statistical analysis was performed using one-way ANOVA (with Duncan). According to biochemical and histopathological results, I/R increased LPO levels and MPO activities and infiltration of PMNs despite high-antioxidant SOD and CAT enzyme activity. Both dosage levels of osajin before I/R significantly decreased LPO level and MPO activity and PMN infiltration compared to those of the $IR^{VEHICLE}$ group, with the higher dosage causing greater decreases. In addition, results showed that treatment with osajin against ameliorated development of irreversible ovarian damage induced by I/R. These results suggest that osajin provides protections against ovarian I/R injury. Its mechanisms could be related to mitigation of oxidative stress and activities and to PMN infiltration.

Keywords: *Osajin, Ischemia/Reperfusion, Oxidative stress, Ovary, Rat*

Sıçan Ovaryumlarında İskemi/Reperfüzyon Hasarı Üzerine Osajin'in Koruyucu Etkileri: Biyokimyasal ve Histopatolojik Değerlendirme

Özet

İnflamasyon ve Reaktif Oksijen Sınıfları (ROS) iskemi reperfüzyon (I/R) over hasarlarında çok önemli rol oynar. Bu in vivo çalışmanın amacı tek taraflı I/R ile oluşturulan over hasarı ve antiinflamatuvar ve antioksidant özellikli bir flavonoid olan osajinin oksidatif denge üzerine etkilerini incelemektir. Çalışmada 48 yetişkin dişi Wistar albino rat kullanıldı. Kontrol (CN) grubuna yalnızca laparatomi uygulandı. CN^{Osajin} grubunda, sadece 200 mg/kg dozda osajin verildi. $IR^{VEHICLE}$ grubunda, 3 saatlik iskemik periyodu 3 saatlik reperfüzyon takip etti; daha sonra bilateral yumurtalıklar alındı. $IR^{Osajin100}$ ve $IR^{Osajin200}$ gruplarında 3 saatlik iskemiden sonra sırasıyla 100 ve 200 mg/kg osajin reperfüzyondan önce oral olarak verildi.; 3 saatlik reperfüzyondan sonra yumurtalıklar alındı. Deneylerden sonra, PMN'lerin aktiviteleri ve oksidatif durumları için MPO, SOD ve CAT enzim aktiviteleri ve LPO düzeyleri belirlendi. Ek olarak histopatolojik değişimler tüm rat ovaryum dokularında incelendi. İstatistiksel analizler one-way ANOVA kullanılarak yapıldı (Duncan ile). Biyokimyasal ve histopatolojik sonuçlara göre, yüksek antioksidan SOD ve CAT enzim aktivitesine rağmen, I/R PMN'lerin infiltrasyonunu, MPO aktivitelerini ve LPO düzeylerini artırdı. İskemik reperfüzyondan önce, osajinin her doz seviyesi büyük bir düşüşe sebep olan yüksek dozla birlikte $IR^{VEHICLE}$ grubunda olanlarla karşılaştırıldığında LPO düzeyi, MPO aktivitesi ve PMN infiltrasyonunu önemli derecede düşürdü. Ek olarak sonuçlar dönüşümsüz ovaryum hasarının iyileştirmeden gelişmesine karşın osajin ile tedavi I/R tarafından uyarılmış olduğunu gösterdi. Bu sonuçlar osajinin ovariyan iskemik reperfüzyon hasarına karşı koruma sağladığını düşündürmektedir. Bunun mekanizmaları oksidatif stresi ve aktivitelerini ve PMN infiltrasyonunu azaltması ile ilgili olabilir.

Anahtar sözcükler: *Osajin, İskemi/Reperfüzyon, Oksidatif stress, Ovaryum, Rat*

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INTRODUCTION

Ischemia/reperfusion (I/R) injury is one of the main causes of organ damage. It has been reported that certain organs, such as the kidneys, liver, ovaries, heart and brain are damaged by the I/R process [1]. I/R is a pathological situation characterized by restriction of blood flow to an organ followed by re-ensuring of perfusion and resupply of oxygen [2]. Ovarian torsion is the twisting of an ovary and/or tubular and supporting ligaments, an ischemic condition characterized by accompanying reduction or complete stoppage of blood flow [3]. Torsion of the adnexa is seen in women of all ages, however, 70-75% of patients are under the age of 30, and this condition is responsible for 2.7% of all emergency gynecological surgeries [4-6]. Signs and symptoms of ovarian torsion often resemble acute appendicitis and also are seen as abdominal and pelvic pain in young girls. Ovarian torsion often is misdiagnosed [7]. Laparoscopic ovarian-torsion surgery generally is used for ovarian detorsion and to restore blood flow to the ovary. Before surgery, painkillers usually are used for pain control [8]. In neglected or prolonged cases, necrosis develops in ovarian tissue to which blood flow has been cut for a long time. In these cases, surgical removal of the ovaries is required [9].

Ovarian detorsion may lead to increased reactive oxygen species (ROS), depending on the recovery of oxygen in the damaged ischemic tissue cells and oxidative tissue damage caused by reperfusion after ischemia [3,10]. ROS metabolics over the normal physiological levels damage cell structures, beginning with membranes (lipids) an extending to proteins and DNA [11-14]. As a result of the appearance of various stimulants via I/R, activation of macrophages in tissue increases production of pro-inflammatory cytokines, such as TNF-alpha and IL-1, and chemokines [15]. These cytokines and chemokines may induce circulating neutrophils to adhere to endothelial cells and migrate to tissues [16]. Depending on neutrophil activation, increased ROS and inflammatory products mediated by respiratory bursts may cause more oxidative damage [17,18]. Previously, it has been reported that oxidative stress and its damage mechanisms may cause infertility and early menopause [19]. In addition, some antioxidant and anti-inflammatory drugs or agents have been determined as beneficial in mitigating detorsion (reperfusion) side effects, depending on their ability to reduce oxidative stress [18].

Flavonoids are a heterogeneous group of phenolic compounds that have a variety of biological effects, such as antioxidant, anti-inflammatory, anticancer, antiviral and anti-allergic [20]. Osajin is a main flavonoid compound as is obtained from ethyl acetate extract of fruits of the *Maclura pomifera* species of the mulberry family, Moraceae [21]. Vesel´a et al.[22] showed that osajin possesses low antioxidant effects compared to peroxynitrite-scavenging activity, inhibition of lipid peroxidation and DPPH-scavenging activity. In addition, Tsao et al.[21]. observed the antioxidant activities of osajin in FRAP and β-CLAMS tests. Interestingly, Diopan et al.[23] found that, although the antioxidant capacity of osajin was lower, it strongly decreased oxidative damage of the four bases (guanine, thymine, adenine and cytosine) in DNA exposed to oxygen radicals generated by Fenton's reaction. Recently, I/R in in-vivo (animal) studies in heart and kidney tissue produced strong evidence of the antioxidant activities of osajin, in spite of its showing less activity in *in-vitro* studies [24,25]. In addition to these effects of osajin, Hošek et al. showed that inhibition of IkB-alpha degradation mediated anti-inflammatory effects of osajin in macrophage cell cultures [26]. Therefore, the present study used both biochemical and histopathological methods to investigate whether osajin could provide protective antioxidant and anti-inflammatory effects against I/R-induced ovarian damage.

MATERIAL and METHODS

Animals

The animals were housed in compliant facilities, and the experiment was conducted in accordance with international guidelines and were approved by the Institutional Animal Care and Use committee of Ataturk University. This study used 48 adult, female Wistar albino rats (210-230 g) from the Ataturk University Experimental Animal Laboratory (ATADEM-Approval No: 2013-03/96). The 48 rats were divided into eight groups of six rats each, and all rats were in the estrous phase when killed during the experiment.

Chemicals

Osajin used in this study was purified with chromatographic methods by PhD. Ahmet Çakır. Also, the chemical structure of Osajin was confirmed by spectroscopic methods in the UV-VIS, IR, 1H-NMR, 13C-NMR, 1D and 2D NMR. Thiopental sodium (Pentothal sodium) was purchased from Abbott (Istanbul, Turkey). All the other chemicals for laboratory experimentation were purchased from Sigma-Aldrich (Germany).

Surgical Technique

All surgical procedures were performed under sterile conditions using thiopental sodium as an anesthetic. During the acclimatization period, the rats were fed a diet of standard commercial rat pellets. For this procedure, the animals were anesthetized with 25 mg/kg thiopental sodium, injected intraperitoenally. A longitudinal incision of 2.5 cm was made in the midline area of the lower abdomen. A small peritoneal incision was made, and the uterine horns and adnexae were located.

A sham operation (laparotomy only) was performed

on the six rats in the control group (CN; n = 6). In the second group, a sham operation was performed after administration of 200 mg/kg osajin (CN^{Osajin}; n = 6). In the third, fourth and fifth groups, bilateral ovarian ischemia was created by applying vascular clips below the ovaries and a 3-h period of ischemia was followed by 3 h of reperfusion. Then 2 h after induction of ischemia, groups 4 and 5 were administered100 mg/kg ($IR^{Osajin100}$; n=6) and 200 mg/kg ($IR^{Osajin200}$; n = 6) doses of osajin, respectively, by oral gavage. Osajin was dissolved in a small volume of diluted, hot 0.9% NaCl to a final concentration of 1 ml. Group 3 was administered 1 ml/kg of saline solution ($IR^{VEHICLE}$; n = 6) by gavage. At the end of 3 h of reperfusion, ovaries were removed for histologic and biochemical examination.

Biochemical Analysis

Before the experiments, tissues were homogenated with liquid nitrogen in a mortar. These homogenates were stored at -80°C until the biochemical investigation, in which MPO, SOD and CAT enzyme activity and LPO levels were measured in the homogenates of rat ovary tissues, and 15 mg tissue from each group was weighed and treated with 1.5 ml of an appropriate buffer. This mixture was homogenized on ice in Eppendorf tubes with steel balls using a QIAGEN TissueLyser LT homogeniser for 15 min. Homogenates were filtered and centrifuged using a refrigerated centrifuge at 4°C. Supernatants were used to determine MPO, SOD and CAT enzyme activity and LPO amount. All assays were carried out at room temperature.

- MPO Activity: MPO activity was determined according to the modified method of Bradley et al.[27]. The homogenised tissues mixture (1.500 g) was centrifuged for 10 min at 4°C. MPO activity of ovarian tissues was determined by adding 0.1 mL supernatant to 1.9 mL of 10 mmol/L phosphate buffer (pH 6.0) and 1 mL of 1.5 mmol/L o-dianisidine hydrochloride containing 0.0005% (wt/vol) hydrogen peroxide. Absorbances of MPO activity were measured on a UV-vis spectrophotometer at 460 nm. Ovarian MPO activity was expressed as µmol/min/mg tissue.

- LPO Determination: LPO amounts were measured by estimating LPO using the thiobarbituric acid test [28]. This was done by weighing 15 mg tissue homogenized in 1.5 mL of 100 g/L KCl and adding it to a solution containing 0.2 mL of 80 g/L sodium lauryl sulfate, 1.5 mL of 200 g/L acetic acid, 1.5 mL of 8 g/L 2-thiobarbiturate and 0.3 mL of distilled water in test tubes. Homogenized tissue was incubated at 98°C water for 1 h. After cooling, 5 mL of n-Butanol: pyridine (15:l) was added to the tubes, which were then vortexed for 1 min and centrifuged for 30 min at 1875 x g. Sample absorbances were determined at 532 nm. The standard curve was achieved by using 1,1,3,3-tetramethoxypropane, and recovery was greater than 95%. LPO levels were expressed as nanomol LPO per gram of tissue (nmol/g tissue).

- SOD Activity: Measurement of SOD activity was based on generation of superoxide radicals, which react with nitro blue tetrazolium to form formazan dye, which is produced by the xanthine and xanthine-oxidase system [29]. SOD activity of ovarian tissues was determined by the degree of inhibition of this reaction at 560 nm. SOD activity was expressed as millimoles per minute per milligram (mmol/min/mg) of tissue.

- CAT Activity: The CAT catalysis decomposition of H_2O_2 was determined at 240 nm [30]. CAT activity was described as the amount of catalase required to decompose 1 nanomole of H_2O_2 per minute at 25°C and pH 7.8. CAT activity of ovarian tissue was expressed as millimoles per minute per milligram (mmol/min/mg) of tissue.

Histopathological Analyses

Ovarian tissues were fixed in 10% formaldehyde, dehydrated in a graded alcohol series, embedded in paraffin wax and sectioned using a Leica RM2125RT microtome (Leica Microsystems, Wetzlar, Germany). For histopathological examination, 5 µm thick sections of paraffin block were obtained using a systematic randomized sampling method. Sections were stained with H&E. All ovaries were examined by light microscopy for histopathological evaluation of the following parameters: H&E staining for hemorrhage, vascular destruction, inflammatory cell infiltrates, reversible cell degeneration, necrotic cell death and apoptotic cell death.

Data Analyses

Statistical analysis was performed using one-way ANOVA with Duncan post hoc test for multiple comparisons. All data are expressed as mean ± s.e, and are considered significant at a *p* level of 0.05 or less as mean. Calculations were performed using the IBM® SPSS software package, version 19.00 (SPSS, Chicago, IL, USA).

RESULTS

Biochemical Results

Fig. 1 shows the MPO enzyme activity, a significant marker of neutrophil infiltration, of the inflammatory enzyme secreted by activated neutrophils and macrophages in tissues for all treatments, I/R and control groups. Results show that administration of osajin alone did not affect ovarian MPO activity in the ovaries of sham-group rats ($P>0.05$). $IR^{Vehicle}$ ovarian tissues showed a significant increase in MPO activity compared to the control group ($P<0.05$). Osajin treatments decreased high I/R-induced MPO activity ($P<0.05$).

As *Fig. 2, 3, 4* and *5* show, for all treatments and groups, LPO levels and activity of endogenous antioxidants, including CAT and SOD, were measured to understand

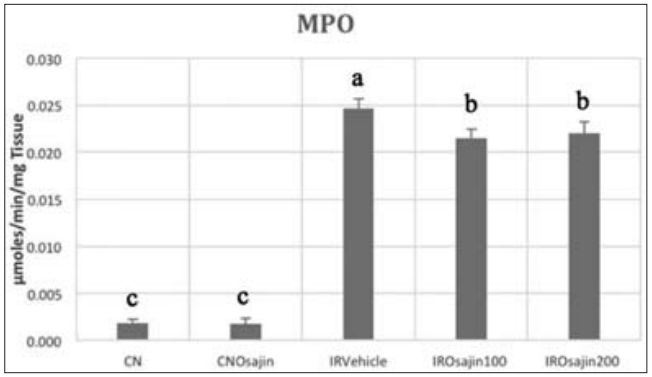


Fig 1. Effects of osajin treatments on MPO activity in I/R injury
Şekil 1. I/R hasarında MPO aktivitesi üzerine osajin tedavilerinin etkileri

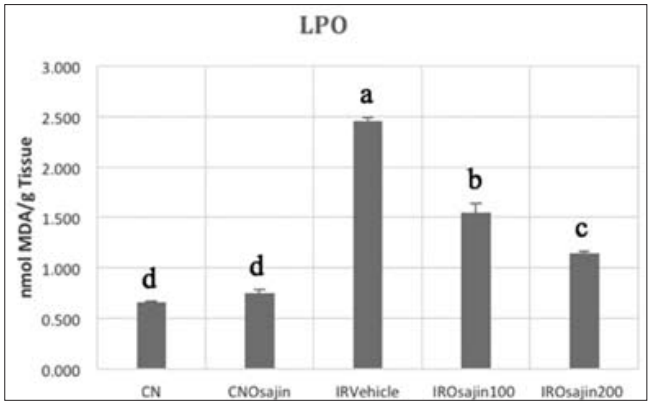


Fig 2. Effects of osajin treatments on LPO level changes in I/R injury
Şekil 2. I/R hasarında LPO seviyesindeki değişimler üzerine osajin tedavilerinin etkileri

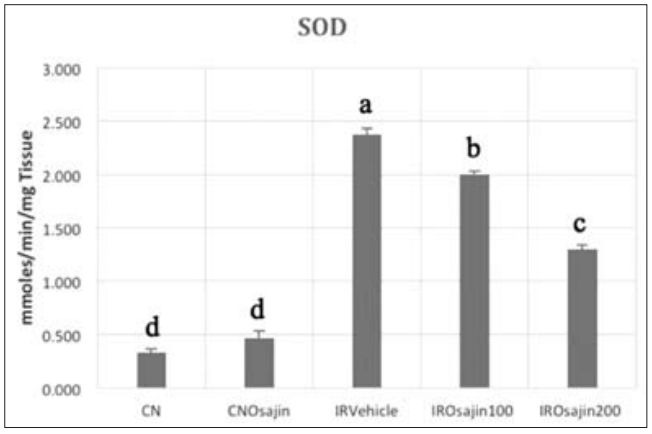


Fig 3. Effects of osajin treatments on SOD activity changes in I/R injury
Şekil 3. I/R hasarında SOD aktivitesi üzerine osajin tedavilerinin etkileri

oxidative damage and behaviours of antioxidant defense mechanisms. Ovaries in the CN and CN^{Osajin} groups had LPO values at the physiological level, and there was no statistical difference in values of LPO between them ($P>0.05$). Ovarian LPO levels in the I/R group were significantly higher than in the control group ($P<0.05$). As *Fig. 2* shows, administration of osajin significantly diminished LPO levels that had been increased in ovarian tissues by

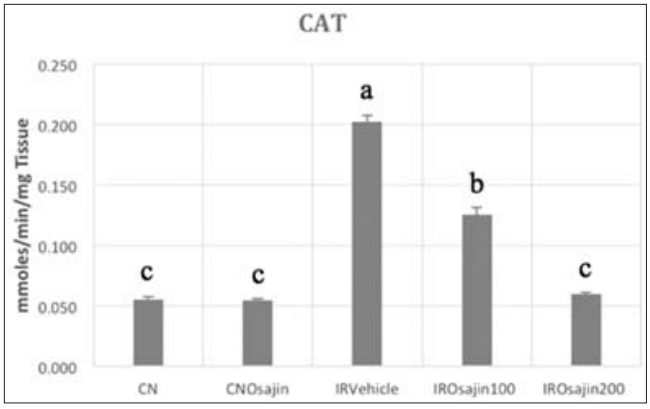


Fig 4. Effects of osajin treatments on CAT activity changes in I/R injury
Şekil 4. I/R hasarında CAT aktivitesi üzerine osajin tedavilerinin etkileri

I/R, both at the 100 mg/kg dose (36.7%) and the 200 mg/kg dose (53.4%) ($P<0.05$).

Compared to the control group and all other treatment groups, $IR^{Vehicle}$ group ovarian CAT and SOD activities were significantly higher ($P<0.05$). As *Fig. 3* and *Fig. 4* show, ovarian SOD and CAT activities were decreased in a dose-dependent manner in the $IR^{Osajin100}$ and $IR^{Osajin200}$ groups ($P<0.05$).

Histopathological Investigations

As *Fig. 5* shows, histopathological examination of ovaries in the CN and CN^{Osajin} groups revealed that ovarian follicles, the stroma in between them, the tunica albuginea containing cortex and blood vessels and the loose connective tissue, including medulla, all appeared normal. As *Fig. 6A* and *6B* show, ovarian tissues in the $IR^{Vehicle}$ group showed reversible degenerative changes, including hypertrophy, especially accompanied with prominent vacuolization in follicular cells; necrotic tissue, generally in stromal and endothelial cells; apoptotic death, especially in follicular and luteal cells; destruction of venous blood vessels and capillaries by severe hemorrhage; and dense infiltrating polymorphonuclear leukocytes in the parenchyma and stroma. As *Fig. 6C* shows, ovarian tissues in the $IR^{Osajin100}$ group demonstrated significantly preserved vascular integrity and haemorrhagic areas in the stroma and the corpus luteums, and peripheries of the ovarian follicles were reduced significantly compared to I/R groups. As *Fig. 6D* shows, in this group, apoptotic cell death accompanied by neutrophil infiltration was observed only in the luteal cells, not in stromal and follicular cells. I/R-induced necrotic cell death and reversible cell damage were reduced significantly in the stromal and parenchymal cells, respectively. As *Fig. 6E* shows, some cortex areas in the $IR^{Osajin200}$ group showed small hemorrhagic areas, but in general vascular structures close to the CN In this group, I/R-caused apoptotic cell death was not observed. In ovarian tissues in the $IR^{Osajin200}$ group, infiltration of polymorphonuclear leukocytes and hypertrophic cell degeneration were

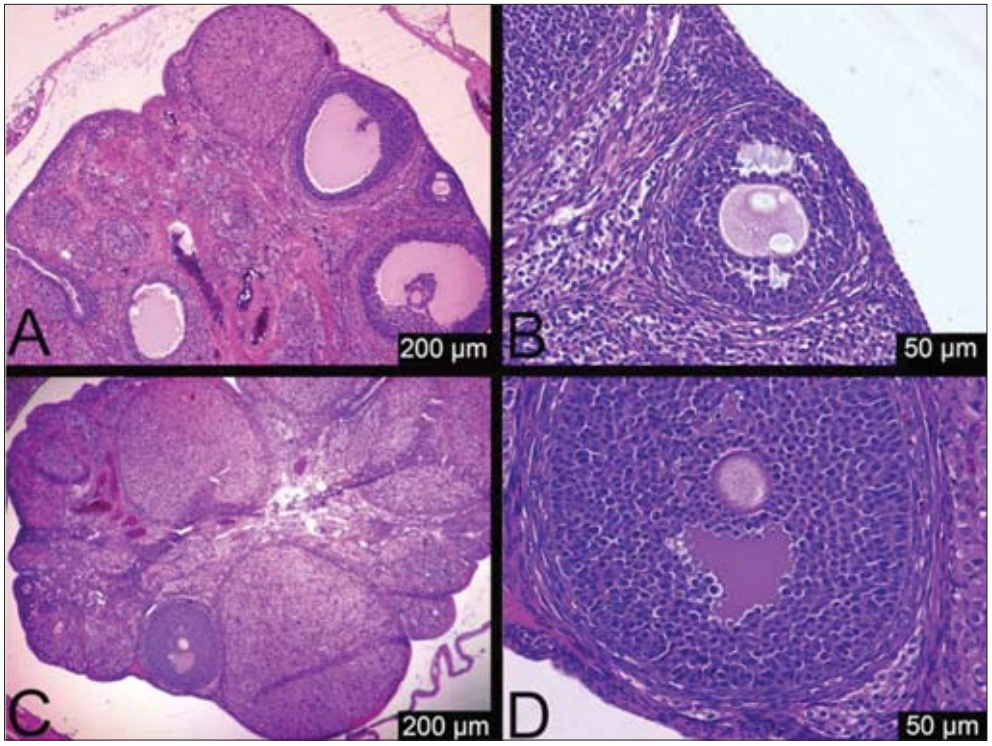
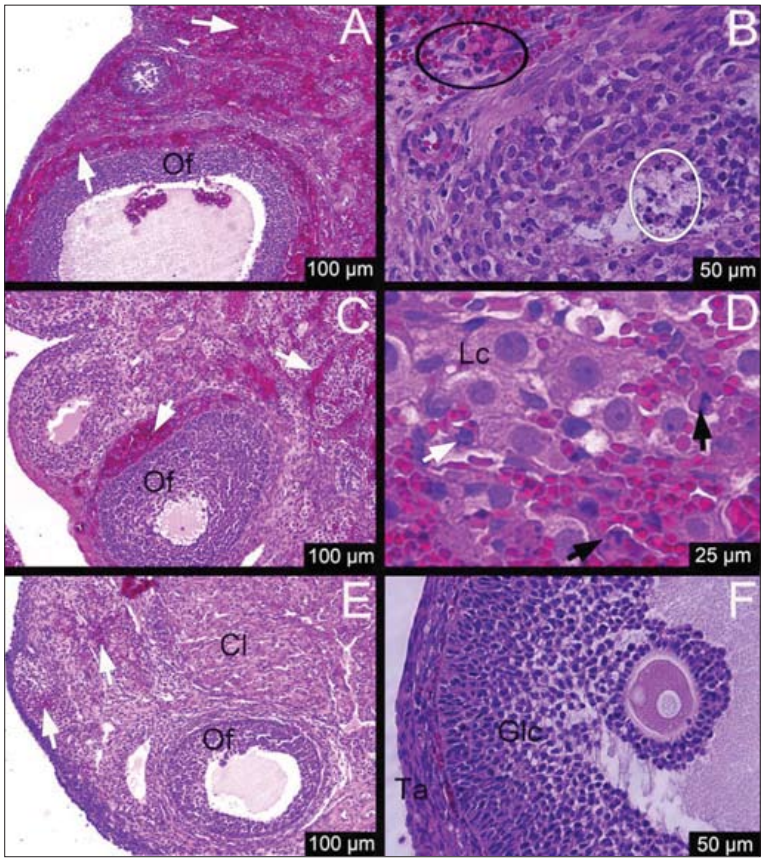


Fig 5. Histologic micrographs of ovaries of the CN (A, B) and CN^{Osajin} (C, D) groups. (A, C) Ovarian cortex and medulla, and (B, D) ovarian growing follicle and stroma on its periphery with normal appearance of ovaries in the CN and CN^{Osajin} groups

Şekil 5. CN (A, B) ve CN^{Osajin} (C, D) gruplarında overlerin histolojik mikrografları. CN ve CN^{Osajin} gruplarında ovaryum korteks ve medullası (A, C) ve büyüyen over foliküller ve onun etrafındaki stroması (B, D) ile normal görünümlü overler

Fig 6. Light micrographs of ovaries in the $IR^{Vehicle}$ (A, B), $IR^{Osajin100}$ (C, D) and $IR^{Osajin200}$ (E, F) groups. (A, C, E) Ovarian follicles (Of), Corpus luteum (Cl) and hemorrhagic cortex areas *(white arrow)* in various levels in ovaries in the $IR^{Vehicle}$, $IR^{Osajin100}$ and $IR^{Osajin200}$ groups, (D) Apoptotic cell death (white-circled area) in ovarian follicle and its periphery with PMN infiltration *(black-circled area)* in ovaries in the $IR^{Vehicle}$ group, (E) Apoptotic cell death *(black arrow)* and PMN infiltration *(white arrow)* in corpus luteum in ovaries in the $IR^{Osajin100}$ group, (F) Granulosa (Glc) or follicular cells within ovarian follicle and tunica albuginea (Ta) with normal appearance in ovaries in the $IR^{Osajin200}$ group

Şekil 6. $IR^{Vehicle}$ (A, B), $IR^{Osajin100}$ (C, D) ve $IR^{Osajin200}$ (E, F) gruplarında overlerin ışık mikrografları. $IR^{Vehicle}$, $IR^{Osajin100}$ ve $IR^{Osajin200}$ gruplarında overlerin çeşitli seviyelerdeki ovaryum folikülleri (Of), Korpus luteum (Cl) ve hemorajik korteks alanları *(beyaz ok)*, (D) $IR^{Vehicle}$ grubu ovaryum folikülündeki apoptotik hücre ölümü *(beyaz dairesel alan)* ve ovaryumda onu çevreleyen PMN infiltrasyonu *(siyah dairesel alan)*, (E) $IR^{Osajin100}$ grubu ovaryumun korpus luteumlarında apoptotik hücre ölümü *(siyah ok)* ve PMN infiltasyonu, (F) $R^{Osajin200}$ grubu ovaryumlarda granuloza (Glc) veya folikül hücreleri ile ovaryum folikülü ve tunika albugineanın (Ta) normal görünümü



reduced significantly compared to the $IR^{Vehicle}$ group. In addition, most parenchymal and stromal components were similar to those in the CN group, as *Fig. 6E* and *6F* show.

DISCUSSION

Ischemia is described as failure to achieve the oxygen and other supplements required for tissue and the lack of

removal of cellular waste by blood circulation. For tissues to use oxygen effectively in situations of hypoxia, the cells make a series of transcriptional and enzyme modifications to ensure a number of factors, such as oxygen-independent ATP production, new vessel formation and ATP catabolism [31]. Transcriptionally, inhibition of prolyl hydroxylase (PHD) uses oxygen as a cofactor in hypoxia, and this inhibition induces increased hypoxia-inducible factor 1 to play a role in synthesis of the factors responsible for glycolysis enzymes of ATP synthesis in an oxygen-independent manner and neovascularization [32-34].

In addition, continued use of ATP and reduction in its production due to the growing influence of hypoxia causes a process for meeting energy needs by converting ATP to the purine metabolites xanthine and hypoxanthine [35-37]. In normal tissues, hypoxanthine is converted into uric acid. In ischemic tissue, hypoxanthine is not further metabolized and accumulates during hypoxia [38]. At the same time, in ischemia, a large portion of xanthine dehydrogenase turns into xanthine oxidase [39]. Xanthine oxidase induces oxidation of hypoxanthine to xanthine and can further trigger conversion from xanthine to uric acid with the onset of reperfusion [40]. During this period, due to increased enzyme activity of the xanthine oxidase, there is an increase in superoxide free radicals [41]. If these increased free radicals in the tissue are not diminished by the major endogenous antioxidants, excess amounts cause oxidative degradation of macromolecules by reacting with all cellular macromolecules. This growing rate of oxidative degradation of macromolecules plays an important role in cellular degeneration and death in I/R. Lipids are important cellular components that suffer from oxidative degradation, and the main end product of this degradation, malondialdehyde, is widely used to determine lipid peroxidation and oxidative tissue damage [42-44]. That is why the present study investigated LPO levels as an indicator of oxidative tissue damage, finding that I/R significantly increased LPO levels. First, at the beginning of I/R, this increase may be associated with increased activity of xanthine oxidase-induced elevated ROS. Cellular damage and the damage-associated molecular pattern molecules (DAMPs) induced by high ROS levels can initiate an immune response in the sterile inflammatory response, and then histamine release by activated mast cells, chemokines and proinflammatory cytokines released by stimulated macrophages trigger neutrophil migration into tissue [45-48]. In later stages of I/R, both oxidative burst response and inflammatory products, along with increased neutrophil migration, play roles in oxidative tissue damage [49,50]. In the present study, biochemical and histopathological findings show increased MPO activity and neutrophil infiltrations in ovarian tissues of the I/R group, showing that neutrophils engage in the pathophysiologic process of I/R injury. Second, findings show that, in addition to xanthine oxidase-mediated ROS production, elevated LPO levels in the I/R group may be associated with ROS production induced by increased neutrophil activity.

Cells protect themselves from oxidative stress by increasing activity of endogenous antioxidants in response to increasing ROS levels [51]. The present study determined that this was responsible for the increased activity in the major endogenous antioxidant enzymes SOD and CAT. The first step of the antioxidant team, involving both SOD and CAT enzymes, eliminated damage caused by superoxide radicals and derivatives. This provides information about levels of their substrates and indicates how the defense mechanism acts [52]. In light of this information, even though there is increased antioxidant enzyme activity, high LPO levels in the tissues can be related to sufficient conversion of superoxide to H_2O_2 by SOD or to inadequate H_2O_2 degradation via CAT. Many studies have shown that oxidative stress and excessive inflammatory products, depending on their densities in I/R injuries, cause either reversible cell damage or irreversible, lethal, cell damage, such as apoptosis and necrosis [53,54].

In histopathological examination of ovarian tissue, the present study observed reversible cell damage in both stromal and parenchymal cells. In addition, apoptotic and necrotic cell death were observed mainly in the stromal and parenchymal cells. In addition, the study observed dense neutrophil migration accompanied by hemorrhage. In addition to the biochemical data, these findings suggest oxidative stress and inflammatory response at these levels can lead to irreversible cell death.

Administration of 100 and 200 mg/kg doses of osajin diminished I/R-induced increases in ovarian LPO levels by 36.7% and 53.4%, respectively. Likewise, both SOD and CAT levels decreased dose-dependently. Increased activity of any enzyme could be linked to enhanced substrate production during metabolic processes [13]. Decreased antioxidant enzyme activities in the treatment groups could be explained by reduced ROS or substrate levels. Two previous studies of renal and myocardial I/R injuries found that SOD activity in tissues increased with 60 min reperfusion [24,25]. These findings suggest that there may be a decrease in ovarian LPO levels over time, given the elevated SOD activities at the beginning of the 3-h reperfusion in the present study, which may decrease the SOD activity according to the substrate reduction level at 3 h. Interestingly, Diopan et al.[23] found that, although the antioxidant capacity of osajin was less, osajin strongly decreased oxidative damage in DNA exposed to oxygen radicals independent of antioxidant capacity. This effect shows that, in I/R injury, osajin may decrease LPO levels through pathways other than SOD and CAT activity. We have found no information in the literature about metabolites of osajin and its in-vivo metabolism. In addition, high LPO levels may be decreased by the potentially powerful antioxidant effects of osajin metabolites that may occur in organs such as the liver and kidneys. In addition to the

antioxidant effects of osajin, the present study shows anti-inflammatory effects caused by lower MPO levels and neutrophil infiltration in tissues due to osajin treatment of ovarian I/R injury. Hošek et al.[26] showed that inhibition of IkB-alpha degradation mediates anti-inflammatory effects of osajin in macrophage cell cultures. NF-κB (p65) is an essential transcription factor responsible for expression of many proinflammatory cytokines, such as TNF-a, IL-1b, IL-6 and IL-8, in macrophages [55]. IκBα inhibits NF-κB from cytoplasm to the cell nucleus via nuclear transport by binding nuclear localization signals of NF-κB. If there is any increase IkB-alpha degradation that is stimulant induced, NF-κB is released to move from the cytoplasm to the nucleus and begins expression of proinflammatory cytokines by binding to DNA as a transcription factor [56,57]. This indicates that the mitigating effects of osajin on neutrophil activities and infiltration found by the present study may be related to degradation-induced lessened release of proinflammatory cytokines from macrophages via its inhibitory effect on IkB alpha. This anti-inflammatory effect may contribute to decreased LPO levels caused by neutrophil-derived ROS. In histopathological examination of ovarian tissues in the $IR^{Osajin100}$ and $IR^{Osajin200}$ groups, density of hemorrhagic areas, infiltration of polymorphonuclear leukocytes and reversible (vacuolated hypertrophic) and irreversible (apoptotic and necrotic) cell damage all were reduced compared to the I/R group in proportion to the osajin dose. In particular, ovarian tissues in the $IR^{Osajin200}$ group generally showed parenchymal and stromal components similar to those in the CN group. Histopathological results of the present study demonstrated that osajin treatment dose-dependently promoted recovery from I/R tissue damage in torsion-detorsion models in rats.

Finally, on the basis of biochemical and histopathological findings observed for the first time in the present study, it can be said that early administration of osajin ameliorates the severity of ovarian-tissue damage induced by I/R. Therefore, osajin has been shown to be useful in preventing permanent ovarian damage caused by torsion-detorsion in women.

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Research Article

Evaluation of Acute Phase Proteins, Some Cytokines and Hemostatic Parameters in Dogs with Sepsis [1]

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Abstract

The aim of this study was to evaluate the alterations in acute phase proteins, cytokines and hemostatic parameters in dogs with sepsis and to determine the importance of these parameters in diagnosis of the sepsis. Thirty dogs with sepsis and 9 healthy dogs were used in this study. Anorexia, depression, lethargy, hyperthermia, tachycardia, tachypnea, congestion in the mucosal membranes, prolonged capillary refill time, and leukocytosis or leucopenia were identified in the dogs with sepsis. The serum interleukin-1ß (IL-1ß), tumor necrosis factor α (TNF-α), interferon γ (INF-γ), C-reactive protein (CRP), serum amyloid A (SAA), prothrombin time (PT), activated partial thromboplastin time (aPTT), antithrombin III (AT III), fibrinogen, protein C (PC), and D-dimer levels were measured in all dogs. We found that the serum IL-1ß, TNF-α, INF-γ, CRP and SAA concentrations were significantly elevated in dogs with sepsis as compared with healthy controls. In addition, the plasma PT and APTT levels were notably prolonged, the plasma fibrinogen, D-dimers and protein C concentrations were significantly increased. However, the antithrombin III activity was significantly decreased in the dogs with sepsis. In conclusion, the results of this study indicate that the SAA, IL-1ß and TNF-α parameters play important roles in the inflammatory process in dogs with sepsis. The hemostatic abnormalities observed in dogs with sepsis may be due to the development of disseminated intravascular coagulation (DIC).

Keywords: *Dogs, Sepsis, Acute phase protein, Cytokines, Coagulation profile*

Sepsisli Köpeklerde Akut Faz Proteinler, Bazı Sitokinler ve Hemostatik Parametrelerin Değerlendirilmesi

Özet

Bu çalışmanın amacı; sepsisli köpeklerde akut faz proteinler, bazı sitokinler ve hemostatik sistem parametrelerin değişimlerini değerlendirerek, hastalığın tanısında bu parametrelerin önemini ortaya koymaktır. Bu çalışmanın materyalini 30 sepsisli ve 9 sağlıklı köpek oluşturdu. Sepsisli köpeklerde iştahsızlık, durgunluk, depresyon, vücut ısısında artış, mukoz membranlarda konjesyon, kapiller dolum zamanında uzama, taşikardi, takipnea, lökositozis veya lökopeni belirlendi. Bütün köpeklerin interlökin-1ß (IL-1ß), tümör nekroz faktör α (TNF α), interferon γ (INF-γ), C-reaktif protein (CRP), serum amiloid A (SAA) ve protrombin zamanı (PT), aktive edilmiş parsiyel tromboplastin zamanı (aPTT), antitrombin III (AT III), fibrinojen, protein C ve D-dimer seviyeleri ölçüldü. Sepsisli köpeklerde serum IL-1ß, TNF-α, INF-γ, CRP ve SAA düzeylerinde önemli artış belirlendi. Sepsisli köpeklerde plazma PT ve APTT sürelerinde önemli uzama, fibrinojen, D-dimer ve protein C düzeylerinde önemli artış, AT-III düzeyinde ise önemli azalma tespit edildi. Sonuç olarak sepsisli köpeklerde SAA, IL-1ß ve TNFα paramterelerinin yangısel olaylarda önemli rol aldığı belirlendi. Sepsisli köpeklerde tespit edilen hemostatik anormallikler dissemine intravaskuler koagulasyon (DİK) gelişimi ile ilgili olabilir.

Anahtar sözcükler: *Köpek, Sepsis, Akut faz proteinleri, Sitokinler, Koagulasyon profil*

INTRODUCTION

Sepsis is defined as a systemic inflammatory response against infection and characterized by fever, tachycardia, tachypnea and leukocytosis or leukopenia [1-3]. This disease has a complex pathophsiological state and is stil associated with a high degree of mortality. Sepsis is considered to be a common cause of morbidity and mortality in both veterinary medicine and human medicine. The incidance of sepsis in dogs was increased from 1 per 1.000 hospital

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cases in 1988 to 3.5 in 1998. In addition, the mortality rates of 33-50% have been described for dogs with sepsis [4-6]. Bacterial infections are the most common cause of sepsis in dogs and cats [7]. Patient with sepsis is more likely to develop multiple organ dysfunction syndrome, which carries a high mortality rate despite recent advances in critical care [4].

Acute phase proteins (APPs), cytokines and coagulation profiles might change in dogs with sepsis. Because sepsis is the clinical menifestation of body's response to an inciting stimulus which is severe enough to cause systemic release of circulating inflammatory mediators. The acute phase reaction, which occurs in sepsis, is stimulated by the release of cytokines such as IL-1ß, interleukin-6 (IL-6) and TNF-α from macrophage and monocytes at the site of inflammatory lesions or infections [8,9]. IL-1ß, IL-6, TNF-α and IFN, produced by inflammatory cells could induce local and systemic reactions [10]. It has been reported that serum IL-1ß, IL-6 and TNF-α levels are increased in sepsis. In addition, IL-6 might serve as a valuable marker for the determination of the severity of a systemic bacterial infection [6] and the measurement of serum IL-6 and TNF-α could be useful for evaluating septic patients [7].

Using acute-phase proteins for the assessment of healthy and sick animals has greatly increased in the last decade [11]. Acute-phase proteins are synthesized in the liver in response to release of proinflammatory cytokines in diseases such as bacterial and viral infections, immuno-mediated disease, neoplasia, tissue injury (trauma), necrosis and burns [12-14]. Clinical applications for APPs have been widely demostrated for prognostication as well as for detection of clinical disease and chronic iflammation [15]. Importantly, the APP assay has repeatedly demostrated its ability to enhance the diagnostic sensitivity for inflamatory processes [15]. Furthermore, CRP and SAA have been used for diagnosing the presence of infection in dogs. It is known that CRP is a major APP in dogs. Increased CRP and SAA concentrations have been detected in dogs with systemic inflammation [10,16,17]. It was reported that CRP and SAA levels were increased in dogs with pyometra [18].

The relationship between infection and coagulation is an area of intense investigation, with studies suggesting that hypercoagulability and subsequent microvascular trombosis contribute to multiple organ dysfonction during sepsis [2]. Dissemine intravascular coagulation (DIC), an acquired syndrome representing a hypercoagulable state, haemorrhagic symptoms and multiple organ failure, might occuring sepsis [2,19]. Protrombin time, aPPT, D-dimer and fibrinogen levels, AT III activity and trombosit count should be considered regarding to DIC [20]. It was reported that hemostatic disorder occurs in dogs with sepsis [2,21].

The aim of this study was to evaluate the importance of the acute phase proteins, cytokines and haemostatic parameters for diagnosis of sepsis.

MATERIAL and METHODS

Animals and Clinical Examination

Authorization to conduct this study has been taken from S.U. Faculty of Veterinary Medicine Animal Ethics Comittee (2011/061).

The materials of this study consist of thirty dogs with sepsis (experiment group) and 9 healthy dogs (control group) aged from 1 and 4 years, were brought into the Faculty of Veterinary Medicine, Internal Medicine Department. First, routin clinical examinations were performed for all dogs. In clinical examination, body temprature, heart rate, respiratory rate, capillary refill time, mucosa, and mental and consciousness states were evaluated. Total white blood cells and thrombocyte of dogs were counted. Dogs having sepsis criteria were included in the study.

Sepsis criteria;

Hyperthermia (>39°C) or Hypothermia (<35°C)

Tachycardia (heart rate >140 per minute)

Tachypnea (respiratory rate >20 breaths per minute)

Leukocytosis (>16.000/µL, or >3% bands) or leukopenia (<6000/µL) [2]. Bacteriologic culture for confirmation of infection was not performed.

Collection of Blood Samples

Blood samples were thereafter obtained through venipuncture of the cephalic or jugular vein then placed into a citrate tube (1 part 3.8% citrate: 9 part blood) and a serum tube (without anti-coagulant). Blood (with anti-coagulant and without anti-coagulant) was centrifuged for twenty minutes collection, after seperation of blood, plasma and serum samples, which would be used in the evaluation of coagulation profiles and acute phase proteins and cytokines, were kept in -80°C deep freeze until the measurement was completed.

Measurement of Leukocyte And Trombocyte

Leukocyte and trombocyte counts were measured by hemocell counter (Haematology analyser, MS4e, CFE 279, Melet Schlosing Laboratories, France).

Measurement of Serum Acute Phase Proteins

Canine C-reactive protein (Eastbiopharm, Cat. No.CK-E90977), canine serum amyloid A (Eastbiopharm, Cat. No.CK-E90978), canine protein C (TSZ ELISA Cat. No.CA 1033) and canine fibrinogen (Eastbiopharm, Cat. No.CK-E90979 concentrations were measured by ELISA method in Synergy HT multi-mode microplate reader (BioTek Inc. USA) device. Measurable sensitivity and test interval of CRP was 0.051 mg/L, and 0.1 mg/L - 30 mg/L,respectively. In addition, measurable sensitivity of SAA was 0.047 µg/mL and test interval of SAA level was 0.1 µg/mL and 40

µg/mL. For PC, measurable sensitivity was less than 0.15 ng/mL and test interval was 0.15 ng mL and 40 ng/mL. Lastly, measurable sensitivity of fibrinogen was 0.023 mg/mL and test interval of fibrinogen level was 0.05 mg/mL and 15 mg/mL.

Measurement of Serum Cytokines

Canine interleukin 1ß (Eastbiopharm, Cat. No. CK-E90800) canine tumor necrosis factor α (Eastbiopharm, Cat. No.CK-E90806) and canine interferon γ (Eastbiopharm, Cat. No.CK-E90877) levels were measured by ELISA method in Synergy HT multi-mode microplate reader (BioTek Inc. USA) device. Measurable sensitivity of IL-1ß was 0.1 pg/mL, and the test interval of IL-1ß level was 0.2 pg/mL and 60 pg/mL, measurable sensitivity of TNF-α is 0.01 ng/L and test interval of TNF-α level was 0,03 ng/L and 9 ng/L and measurable sensitivity of INF-γ is 2.35 ng/mL and the test interval of INF-γ level was 5 ng/L and 1.000 ng/L.

Measurement of Plasma Coagulatıon Profile

Protrombin time, APPT, AT III activity and D-dimer levels were measured by coagulometric method (Coagulometric method, Sysmex CA 1500 device, Siemens, A-7799, Germany).

Statistical Analysis

Two sample student test was used to determine the differences between groups. SPSS 19.0 for Windows was used to perform the test. P values <0.05 were considered statistically significant.

RESULTS

Clinical and Hematological Findings

Anorexia, depression, lethargy, hyperthermia, tachycardia, tachpnea, congession in mucosal membrans, prolonged capillary refill time were determined in dogs with sepsis. Leucocytosis in 28 of 30 dogs with sepsis was determined, but leucopenia in 2 of them was determined.

Acute Phase Proteins and Cytokines Findings

The concentrations of serum CRP (P<0.001), SAA (P<0.01), IL-1ß (P<0.001), TNF-α (P<0.001) and INF-γ (P<0.001) were significantly increased in dogs with sepsis as compared with healty dogs *(Table 1)*.

Coagulation Profile Findings

Global coagulation times (PT and APTT, P<0.01) and plasma fibrinogen (P<0.001), D-dimer (P<0.01) and PC (P<0.001) levels were dramatically increased in dogs with sepsis as compared with healthy dogs, but AT-III (P<0.05) activity was remarkably reduced. Haemotologically, the blood leukocyte count was found to be significantly elevated in dogs with sepsis as compared with healhty dogs,

Table 1. *Serum concentrations of IL-1ß, TNF-α, INF-γ, CRP and SAA of healthy dogs and dogs with sepsis (Mean±SE)*
Tablo 1. *Sepsisli ve sağlıklı köpeklerde serum IL-1ß, TNF-α, INF-γ, CRP ve SAA düzeyleri (Mean±SE)*

Parameters	Healthy Dogs (n=9)	Dogs with Sepsis (n= 30)	P Value
IL-1ß pg/mL	9.30±0.53	22.3±3.34	P<0.001
TNF-α ng/L	0.24±0.78	2.94±0.60	P<0.001
INF-γ ng/L	104±0.86	404±68.9	P<0.001
CRP mg/L	2.27±0.19	9.89±2.10	P<0.001
SAA µg/mL	2.30±0.18	10.8±2.43	P<0.01

IL-1ß: *interleukin-1ß;* ***TNF-α:*** *tumor necrosis factor α;* ***INF-γ:*** *interferon γ;* ***CRP:*** *C-reactive protein;* ***SAA:*** *serum amyloid A*

Table 2. *Plasma coagulation parameters of of healthy dogs and dogs with sepsis (Mean±SE)*
Tablo 2. *Sepsisli ve sağlıklı köpeklerde plazma koagulasyon parametreleri (Mean±SE)*

Parameters	Healthy Dogs (n=8)	Dogs with Sepsis (n=30)	P Value
PT (Sec)	6.32±0.17	7.80±0.14	P<0.001
APTT (Sec)	39.7±3.27	68.2±5.79	P<0.01
AT III (%)	78.1±2.90	54.9±2.92	P<0.05
Fibrinogen mg/mL	3.08±0.42	9.61±1.58	P<0.001
Protein-C ng/mL	0.63±0.10	2.37±0.45	P<0.001
D-dimer mg/dL	1.27±0.87	2.64±1.59	P<0.01
PLT $x10^3$ /mm^3	235±68.1	272±27.5	P>0.05
WBC $x10^3$ /mm^3	10.2±1.16	26.53±2.04	P<0.001

PT: *prothrombin time;* ***APTT:*** *activated partial thromboplastin time;* ***AT III:*** *antithrombin III;* ***PC:*** *protein C,* ***PLT:*** *platelet;* ***WBC:*** *white blood cell*

however no significant change was seen in thrombosit count *(Table 2)*.

DISCUSSION

Sepsis has a high mortality. Therefore, a rapid diagnosis would be important to get good prognosis. This study showed significant changes of acute phase proteins, cytokines and haemostatic para-metersin dogs with sepsis. Therefore, measurement of these parameters in dogs with sepsis could be a valuable approach to evaluate septic stages.

Cytokines play an important role in the development and the regulation of immune response, thus, cytokine profiles contribute to the effect of immunity level in diseases [22]. The release of proinflammatory cytokines such as IL-1ß, IL-6 and TNF-α by monocytes/macrophages and activeted T-lymphocytes is considered to be a key event in the development of sepsis [1]. Moreover, bacterial toxins lead to release of inflammation mediators from mononuclear phagocytes in sepsis. The measurement of acute phase proteins, cytokines and coagulation profiles could be useful for determining the stage of sepsis in patients. IL-1ß and TNF-α, important inflammatory cytokines, are implicated in a variety of disease in dogs [23-26]. It has been reported that IL-6 and TNF-α levels are significantly increased in sepsis, and these parameters for evaluating of sepsis might be useful marker [6,7,27]. Serum IL-1ß and TNF-α concentrations are usually increased in inflammatory disease of dogs [28,29]. Fransson et al.[30] reported that IL-6, TNF-α and CRP levels were increased in dogs with pyometra and dogs with systemic inflammatory response syndrome. In this study, serum IL-1ß, TNF-α and INF-γ concentrations were dramatically elevated in dogs with sepsis as compared with healthy dogs *(Table 1)*. The reason of this increase in IL-1ß, TNF-α and INF-γ levels could be explained by the initiation of releasing of inflammatoric mediators against bacterial toxins that cause sepsis. Therefore, selected cytokines including IL-1ß, TNF-α and INF-γ maight be useful in assessing the clinical severity of sepsis in dogs. These results are consistent with published studies [3,6,28,29].

Acute phase proteins are synthesized in the liver in response to release of proinflammatory cytokines in diseases such as bacterial and viral infections, immuno-mediated disease, neoplasia, tissue injury (trauma), necrosis and burns [4,13]. These noninvasive markers might be useful to determine the disease severity and more likely to response the treatment or prognosis, on a disease-spesific basis [11,13,31]. CRP and SAA are useful parameters to indicate the inflammation in human and animals [32]. In addition, increased CRP and SAA concentrations have been detected in dogs with systemic inflammation [10,16,17,30]. Furthermore, CRP and SAA were shown to be significantly increased in dogs with parvovirus enteritis [33], ehrlichiosis [23] and leptospirosis [34]. C-reactive protein and SAA concentrations have been shown to be dramatically elevated in dogs with pyometra [18]. In the present study, CRP and SAA concentrations were significantly increased in dogs with suspected sepsis as compared with healthy dogs. But, CRP level was normal range. The reason for higher concentrations of SAA in septic dogs may be related to inflammatory reactions and tissue damage. Similary, several studies have reported that CRP and SAA could be useful markers for diagnosis and prognosis in various disease [10,16,17,34,35]. However, CRP and SAA do not seem to be very spesific markers for the detection of bacterial infections because it might increase in a variety of diseases, not particularly in bacterial infections [10,16,17,35]. Besides, in this study, leukocytosis was found to be a common finding in dogs with sepsis. This observation agrees with the most studies [10,16]. In our study, SAA were detected to be specific markers of systemic inflammation in dogs with sepsis.

Disseminated intravascular coagulation a hemalotical syndrome, typically defined by the activation of intra-vascular coagulation resulting in excessive fibrin formation and consumption of caogulation factors [2,18,36] is a serious problem that threats lives of both people and animals. DIC might be developed due to septic caogulation, viremia, parazitic infection, severe tissue damage, toxication, intra-vascular hemolizis, autoantibody, hepatitis, pancreatitis and neoplasma [19,21,37-40]. Prolongations in PT and APTT, increase in FDP level, and decrease in AT-III activity and thrombosit count develop in dogs with DIC [41]. Prolongation in PT and APTT, increase in D-dimer, and decrease in AT-III level are detected in dogs with sepsis [2,42], systemic inflammatory syndrome [43] or septic peritonitis [44]. In the present study, prolonged PT and APTT values as well as increased levels of fibrinogen, D-dimer and PC concentrations revealed hemostatic alteration in response to sepsis in dogs. In the present study, the indicators of the activated coagulation of DIC including prolongation of both PT and APTT as well as a decrease in AT III activity and also an increase in the level of D-dimer which shows fibrinolitic activition were defined. The occurrence of significant changes in the hemostatic system was determined. D-dimer concentration was significantly increased in dogs with sepsis, indicating the precence of fibrinolysis. Increased activity of fibrinolytic system has been associated with DIC [2,42]. Our results were in line with previous studies [2,21,42-45].

Some authors [46-48] reported that the level of fibrinogen might increase in the first period of DIC due to an inflamatory response; and then this level might decrease because of fibrinolisis. In contrast, other researchers [19,45] reported a significant decrease in fibrinogen level. In this study, a significant increase in fibrinogen level in the dogs with sepsis was observed. This dramatic increase in fibrinogen level might be related to an elevation in acute phase proteins in spite of the presence of DIC in

dogs with sepsis. Therefore, our findings were in line with findings of earlier reporters [46-48].

It was reported that PC concentration was remarkably reduced in dogs with sepsis [4,49], and dogs with systemic inflammatory syndrome [43]. Fourrier et al.[50] noticed persistent decreases in PC activity in nonsurviving patients with sepsis and septic shock. However, de Laforcade et al.[2] determined that the PC and AT activities were decreased in first 24 h and then this activities were gradually increased in dogs with sepsis. In this study, the activity of PC was dramatically increased in dogs with sepsis as compare with healthy dogs, most probably due to time dependent effects of diseases. This result was not coincidet with the results of reported by Laforcade et al.[4] and Yan and Dhainaut [49], but this result was coincidet the results of reported by de Laforcade et al.[2].

In conclusion, results of tis study indicated that SAA, IL-1ß, TNF-α , and INF- γ concentrations were significantly changed in dogs with sepsis. Particularly, SAA, IL-1ß and TNF-α parameters seem to be reliable markers for systemic inflammation in sepsis. Besides hemostatic abnormalities in dogs with sepsis may be due to developing disseminated intravascular coagulation.

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Research Article

A Comparison of Different Analysis Methods for Milk Urea Nitrogen

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Abstract

The objective of this study was to develop a reliable and cheap method to determine of milk urea nitrogen and compare some other instrumental methods. Two trials were conducted. In first trial, a milk urea nitrogen testing method was designed by modifying some methods developed for ammonia nitrogen testing method. Several studies were performed for the validation of method. By using standard solutions some applications such as determinability, recovery, repeatability, test of various analyzers and devices, limit of quantification were performed. Recovery of added urea averaged 99.38 %. In second trial, 105 individual milk samples from a dairy farm was analyzed for milk urea nitrogen by modified indophenol, infrared, and MiniFoodLab methods. The means of milk urea nitrogen obtained from three different methods were 23.87, 21.59, and 23.92 mg/dl, respectively. There was a positive correlation between modified indophenol and infrared methods.

Keywords: *Milk urea nitrogen, Enzymatic Assay*

Farklı Süt Üre Azotu Analiz Metotlarının Karşılaştırılması

Özet

Bu çalışmanın amacı süt üre azotu tayini için ekonomik ve güvenilir bir metodu geliştirmek ve bazı metotlarla karşılaştırmaktı. İki deneme halinde yürütüldü. Birinci denemede süt üre azotu analizi, amonyak ve üre analizi için geliştirilmiş bazı metotlar modifiye edilerek geliştirildi ve yöntemin doğrulanması ile ilgili çeşitli analizler yapıldı. Bu kapsamda standart eriyikler kullanılarak tespit edilebilirliği, geri alınabilirliği, süt örneklerinin tekrarlanabilirliği, değişik ölçümcülerin ve cihazların test edilmesi, tayin sınırının belirlenmesi gibi uygulamalar yapıldı. İlave edilen ürenin geri kazanımı % 99.38 idi. İkinci denemede bir çiftlikten elde edilen 105 adet bireysel süt örneği modifiye indofenol yöntemi, IR ölçüm yöntemi, MiniFoodLab ölçüm yöntemi ile üre azotu bakımından analiz edildi. Bu üç yöntemle elde edilen analiz sonuçları sırasıyla 23.87, 21.59 ve 23.92 mg/dl idi. Modifiye indofenol yöntemi ile IR yöntemi arasında orta seviyede pozitif bir korelasyon tespit edildi.

Anahtar sözcükler: *Süt üre azotu, Enzimatik ölçüm*

INTRODUCTION

While evaluating the nutritional status of a herd, the first points of consideration should be the rations and feedstuffs. The performance of an animal is the best indicator of feed quality. However, animal experiments are costly and in general, not preferred by the owners. Interpretation to a certain degree can be made through crude nutrient analysis. Another way of determining the nutritional status of cattle is to directly conduct blood tests, or in other words metabolic blood tests. However, taking blood from every animal and processing the obtained blood samples bring along some inconveniences. Monitoring herd health, milk yield and the components of milk, rather than conducting blood tests for every animal is an effective method. For this purpose, milk yield and the levels of additives, fat, protein and urea of milk are monitored [1]. In order to determine the effectiveness of the protein obtained through feed, either blood or milk urea nitrogen can be assessed. Yet, milk urea analysis (MUN) is more practical. The MUN level for Holstein dairy cattle milk is reported to be 13.7-14.0 mg/dl (10-18 mg/dl) in average. Individual MUN values, on the other hand, are considered to be normal within the range of 8-25 mg/dl [2-6].

There are several methods used for milk urea nitrogen determination. Milk urea nitrogen determination can be made from the color on the spectrophotometer by having urea directly or ammonia, as the decomposition product of urea, reaction with a reagent. In addition, milk urea level can be determined through IR spectrophotometric

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measurement, on the basis of the amount of light absorbed at a given wavelength. Furthermore, there is the standard method where measurement can be made on the basis of pH difference [7] and methods similar to the standard method [8] are used in milk urea nitrogen determination. There are also devices specially developed for milk urea nitrogen measurement. MiniFoodLab is a practical MUN measurement device that can be used on the field. However, the number of samples that can be processed with it is fairly limited, and due to the fact that its reagents and ready-to-use cuvettes are imported, it is quite costly.

With devices running with the infrared method, numerous analyses can be conducted in a short period of time. However, the initial cost of such devices is very high, they require calibration in certain time intervals and inaccurate results may be obtained in cases where the number of milk samples is low. Researchers demonstrated that the IR method may inaccurately approximate high MUN as low and low MUN as high. Wet chemical method is superior to the IR method (primarily Foss 4000) in terms of accuracy, since it completely measures urea. The main problem is that it is a rather slow analysis method and requires separate equipments.

Peterson et al.[9] analyzed the milk samples obtained from 100 Holstein dairy cattle through the use of CL-10, Skalar, Bentley, Foss 4000 and Foss 6000 equipments and reported that the respective recovery rates were 85, 95, 92, 47, and 95%, that there were differences among laboratories that used the same equipment, that the recovery decreased while the MUN level increased for Bentley and CL-10 equipments, that the recovery rate decreased with the increasing milk fat in Foss 6000, that the recovery rates obtained for the laboratories using Foss 4000 and Foss 6000 were inconsistent and therefore that with these systems the MUN results may be reported to be more or less than the actual values. Hanuš et al.[10] determined recovery to be within quite wide ranges such as 258, 80-108, and 75-140% for the analyses conducted with the IR, standard and photometric methods.

In a similar study where Kohn et al.[11] compared different equipment, it was determined that while the MUN results obtained through the Bentley, Foss 6000 and Skalar equipments were close to each other, quite different results were obtained from Foss 4000.

Luzzana and Giardino [8] developed a method for measuring MUN on the basis of pH difference, determined the repeatability and recovery of the method as 0.85 and 99.4% respectively, and reported that the developed method is simple, accurate, fast and in harmony with the standard method. Wang et al.[12] compared 3 separate methods by using Foss 4000, diacetyl monoxime and urea nitrogen kit, and reported that there were no difference among the results of MUN conducted on 50 samples of milk.

The purpose of the present project is to adapt a spectrophotometric method for analyzing urea nitrogen in milk, which is used in checking whether dairy cattle are adequately nourished in terms of protein and energy, and compare it to some existing methods.

MATERIAL and METHODS

The study was carried out in two trials.

Trial 1

The MUN in this trial was conducted in line with ammonia and urea analysis in biological fluids as implemented by Weatherburn [13] and Chaney and Marbach [14], with changes on urease enzyme [15] and the modified indophenol method. Modifications of these methods; milk was replaced with serum or plasma, incubation temperatures and times, and enzyme concentration were changed.

Reagents, solutions: Reagent 1 (A1): 50 g phenol and 0.25 g sodium nitroprusside were dissolved with deionized water in a volumetric flask and diluted to 1.000 ml. Reagent 2 (A2): 25 g sodium hydroxide and 40 ml sodium hypochlorite (5.25 %) were put into a volumetric flask and diluted to 1.000 ml with deionized water. Enzyme solution: From the lyophilized urease enzyme preserved at +4°C (5 U/mg), 0.6 g was weighted and diluted to 100 ml (30 U/ml) with deionized water in volumetric flask. Standard solution: From the dried urea in the drying chamber, 0.8576 g was taken into volumetric flask, dissolved with deionized water and diluted to 1.000 ml (40 mg/dl). By taking varying amounts from the standard solution, solutions with varying nitrogen content were prepared.

Preparation of the standard curve: 100 µl urease solutions was put into spectro cuvettes, standard solutions containing 10 µl increasing concentrations of urea were added, the mixture was shaken and kept 10 min at 40°C temperature. Later on, the cuvettes were sealed and turned upside-down following the addition of 1 ml of A1 and A2. The preparation was kept 3 min at 55°C and the absorbances were read in spectrophotometer (Schimadzu UV 1240) against the blind sample that contained deionized water on 625 nm.

Preparation and analysis of milk: 100 µl urease solution was put into spectro cuvettes, supplemented with 10 µl homogenous milk sample heated at 40°C, mixed and kept for 10 min at 40°C. 1 ml A1 and 1 ml A2 were added, then the cuvettes were sealed and turned upside-down. After keeping the preparations 3 min at 55°C, absorbance was read in the same way.

Calculation of the results: The results were calculated on the basis of the regression equation obtained through the standard solutions.

Trial 2

The results of the analyses conducted with the modified indophenol method were compared with the results obtained from IR Foss device and the MiniFoodLab device that conducts spectrophotometric measurement.

As the analysis material, milk samples of approximately 80-100 ml were taken through sampling containers put into the milking system, during the milking of 105 cattle of varying levels of milk yield in a private establishment. In order to prevent the milk from getting spoiled, 2 tablets of bronopol (2-bromo-2-nitropropane-1,3 diol) were added. After the tablets were dissolved and the milk was turned upside-down for ensuring homogeneity, the milk was portioned and half of it was analyzed with the Foss FT120. The other half of the milk was analyzed in spectrophotometer with the modified indophenol method and with the MiniFoodLab MUN measurement device.

Statistical Analyses

In the evaluation of the data obtained from the study, paired t test, correlation - regression analyses were conducted [16].

RESULTS

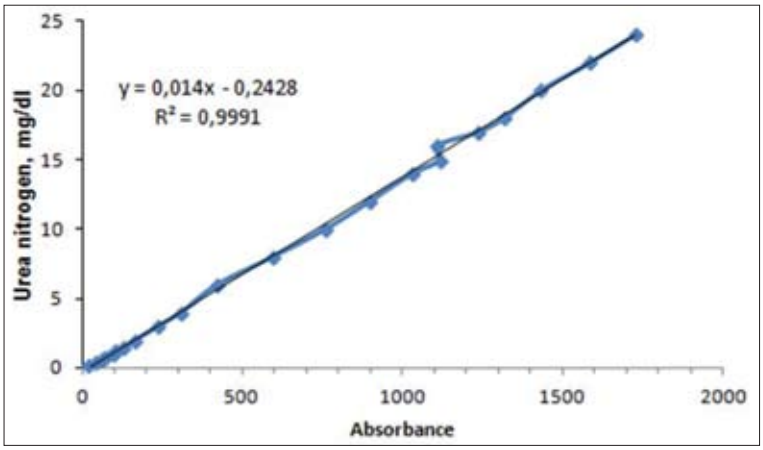
Trial 1

The standard deviation of the repeatability of the one of the standard solutions (2 mg/dl) as conducted by the same researcher, with the same equipment and with 10 iterations, was found out to be 0.083.

Fig. 1 presents the linear graphic obtained with the analysis 22 standard solutions and the regression equation. The result of readings for determinable ranges with standard solutions is shown in *Fig. 2*.

Fig 1. The linear graphic drawn with the analysis of 22 standard solutions

Şekil 1. 22 standart çözeltinin analizi ile çizilen doğrusal grafik



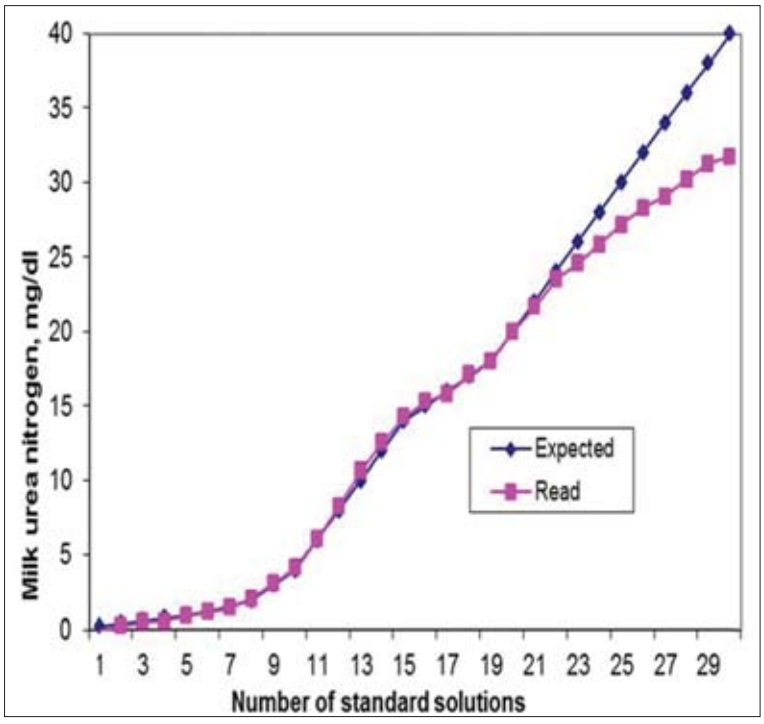


Fig 2. Expected and determined milk urea nitrogen by standard solutions

Şekil 2. Standart solusyonlara göre beklenen ve gerçekleşen süt üre nitrojeni

Table 1. *Assays related to expiration date of reagents*
Tablo 1. *Ayıraçların raf ömrü için yapılan çalışma*

Date reag.	Assay 1		Assay 2		Assay 3	
	Fresh	5 months	Fresh	7 months	Fresh	13 months
n	10		8		16	
X	6.59	6.75	4.74	4.71	9.29	9.20
Sx	1.91	1.79	1.65	1.65	2.17	2.14
P	0.332		0.688		0.330	

Table 2. *Assays related to expiration date of enzyme solution*
Tablo 2. *Enzim çözeltisinin raf ömrü için yapılan çalışma*

Date enz.	Assay 1			Assay 2	
	13 days	50 days	110 days	34 days	136 days
n	11			18	
X	4.09	3.65	3.69	6.89	6.99
Sx	1.50	1.26	1.27	1.68	1.70
P	0.968			0.418	

Table 3. *Recovery study conducted by adding urea to milk*
Tablo 3. *Süte üre ilave edilerek yapılan geri kazanım çalışması*

Sample	Read, mg/dl	Expected, mg/dl	Recovery, %
n	11	11	99.38
X	23.98	24.13	
Sx	0.94	0.93	
P	0.06		

Table 4. *Statistical values of the MUN levels obtained through three different methods*
Tablo 4. *Üç farklı yöntemle elde edilen süt üre azotu düzeylerine ait istatistik değerler*

Item	Modified Indophenol	Infrared	MiniFoodLab
Average, mg/dl	23.87 a	21.59 b	23.92 a
Standard deviation	2.68	2.17	2.05
Minimum, mg/dl	17.50	15.07	19.09
Maximum, mg/dl	30.28	26.67	29.00

a,b: *The difference between the averages that have different letters on the same line is significant (P<0.001)*

Table 5. *Linear regression equations for MUN conducted with different methods*
Tablo 5. *Farklı metotlarla yapılan süt üre azotu analizi için linear regresyon denklemleri*

Analysis Method	Regression Equation	R	Significance
MI-IR	16.0360+0.2325xIR	0.29	*
MI-MFL	25.8091-0.0791xMFL		-
IR-MI	16.2118+0.3550xMI	0.29	*
IR-MFL	20.8623+0.1416xMFL		-
MFL-MI	27.1016-0.1349xMI		-
MFL-IR	17.8030+0.1581xIR		-

MI: *Modified indophenol,* ***IR:*** *Infrared,* ***MFL:*** *MiniFoodLab, * P<0.05*

Statistical results of the expiration dates of the used reagents and enzyme solution are presented in *Table 1* and *Table 2*.

Statistical analysis results of the recovery study conducted by adding certain levels of urea to a milk sample are presented in *Table 3*.

Trial 2

Distributions of the MUN levels determined at 105 samples through the modified indophenol (MI), infrared (IR) and MiniFoodLab (MFL) methods, statistical values and linear regression equations are presented in *Table 4* and *Table 5*.

DISCUSSION

Trial 1

In this trial, standard error of repeatability is 0.08 and the variation coefficient can be calculated as 4.10%. This value is within the 95% confidence limit. With an analysis method based on pH difference and with 0.85-1.50% variation coefficient, Luzzana and Giardino [8] found the repeatability of the MUN measurement better than that found in the present study.

With the analysis of the solutions containing varying concentrations of urea, the linear regression equation was found out as Y = 0.014X-0.2428. Examining *Fig. 1* shows that the majority (0.2-24 mg/dl) of the 22 standard solutions read is above the trendline. $R^2 = 0.9991$. With the method they implemented, also Luzzana and Giardino [8] found a similar linearity and R value.

It was determined that a serial measurement consisting of the measurements of 25 different milk samples with iterations at 5 different times took about 90 min. From this, it was calculated that a single analysis took about 3 to 4 min. However, due to the fact that also standard solutions need to be analyzed every time when a milk sample is to be analyzed, it should be noted that a single analysis would take about 30 minutes with the addition of the waiting durations both with enzyme and reagents.

No significant difference could be found between the readings made on fresh and up to 13 months old reagents kept in the refrigerator, in order to determine the shelf life of the reagents used with the modified indophenol method. The important point here is that the Na hypochlorite contained in reagents should preserve its smell, or in other words its freshness. Na hypochlorite kept for long durations at room temperature loses its activity. Similarly, no difference could be found between the readings made on enzyme solutions kept in the refrigerator for 13, 34, 50, 110 and 136 days. However, Chaney and Marbach [14] reported that while reagents could stay 60 days in a cold and dark environment, enzyme solution could stay 30 days in a refrigerator. Having worked on a different enzymatic method, also Luzzana and Giardino [8] determined that reagents can be preserved in refrigerator for 6 months.

In the recovery study carried out by adding different amounts of urea to a milk sample, it was determined that the mean recovery rate was 99.38% and that there were no significant difference between the required and actually read MUN levels (P=0.06). With a method based on pH difference and similar to the standard method, also Luzzana and Giardino [8] found a recovery of 99.4%. In analyses carried out with different automatic equipments, Peterson et al.[9] found a recovery rate of 30-64% with an equipment and more that 85% with other equipments.

In consequence of the readings made on 30 standard solutions with increasing concentrations up to 40 mg/dl, in order to determine the lowest and the highest MUN level that can be accurately determined with the method of analysis in question, the lowest and highest values were determined to be 0.6 and 24 mg/dl respectively *(Fig. 2)*. These values are within the MUN values deemed normal for dairy cattle.

In other methods [13-15] which used in modification, biological fluids have been studied, therefore it has not been discussed in here.

Trial 2

According to the MUN results of 105 milk samples analyzed through the modified indophenol, infrared and MiniFoodLab methods, the values obtained from the infrared method are significantly different than the values obtained with the other two methods (P<0.001). Agreement between the modified indophenol and MiniFoodLab method was assessed statistically.

Also Arunvipas et al.[17] determined a significant difference between the results of the enzymatic method and the infrared method. Yet, on the contrary, Luzzana and Giardino [8] reported that the analysis results obtained from the two different methods were consistent with each other. On the other hand, Peterson et al.[9] reported that the MUN analysis results obtained from different models of the same IR equipment were inconsistent, and occasionally higher or lower than actual values can be obtained. Kohn et al.[11] found differences even between different laboratories using the same IR equipments.

In the present study, a significant correlation and a correlation coefficient of 0.29 (R^2=0.083) was obtained between the modified indophenol method and the infrared method. The regression equation was 16.0360+0.2325xIR for MI-IR and 16.2118+0.3550xMI for IR-MI. Hanuš et al.[10] report either high or low correlations between the IR method and varying other photometric methods.

In conclusion, the modified indophenol method that was implemented within the scope of this study is a reliable, independent on foreign and cheaper method than others for determining milk urea nitrogen level. IR apparatus is more costly and requires calibrations regularly. MiniFoodLab apparatus is also costly and its reagents from abroad. However, reader person should be carefully at sampling and titrating. Reading range was found out to be between 0.6 and 24 mg/dl, and for reading higher levels dilution is required.

A medium level and positive correlation was determined between the modified indophenol method and the infrared method. However, there is no compatibility between these two methods. Similarly, none of the methods implemented in this study was compatible with any other.

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Research Article

Comparison of Intrauterine Ozone and Rifaximine Treatment in Cows with Subclinical Endometritis

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Abstract

The aim of presented study was to compare the effect of intrauterine ozone and rifaximine treatment in cows with subclinical endometritis. The study was conducted on 53 Simmental cows with subclinical endometritis, which was diagnosed by ultrasonographic examination. According to results, interval between treatment to pregnancy (46.4±6.2 vs. 40.0±6.0), interval from calving to pregnancy (129.4±9.0 vs. 125.0±13.1), and insemination number (3.2±0.3 vs. 3.1±0.5) after treatment were similar in the groups (P>0.05). In conclusion, intrauterine ozone treatment was observed as therapeutic as rifaximine and to be an alternative treatment approach in dairy cows with subclinical endometritis.

Keywords: *Cow, Ozone, Rifaximine, Subclinical endometritis*

Subklinik Endometritisli İneklerde İntrauterin Ozon ve Rifaksimin Tedavisinin Karşılaştırılması

Özet

Sunulan çalışmanın amacı, subklinik endometritisli ineklerde uterus içi ozon ve rifaksiminin etkisini kıyaslamaktı. Çalışma, ultrasonografik muayene ile subklinik endometritis tanısı konmuş 53 adet Simental ırkı inek üzerinde yürütüldü. Sonuçlara bakıldığında; tedavi sonrası gruplar arasında tedavi gebe kalma aralığı (46.4±6.2; 40.0±6.0), buzağılama gebe kalma aralığı (129.4±9.0; 125.0±13.1) ve tohumlama sayısı (3.2±0.3; 3.1±0.5) açısından benzer sonuçlar bulundu (P>0.05). Sonuç olarak intrauterin ozon tedavisinin, rifaksimin kadar tedavi edici olduğu ve endometritisli sütçü ineklerde alternatif tedavi yöntemi olarak kullanılabileceği gözlendi.

Anahtar sözcükler: *İnek, Ozon, Rifaksimin, Subklinik endometritis*

INTRODUCTION

Reproductive efficiency is essential for sustainable dairy production and failures in reproduction cause economic losses depend on drug cost, extended interval between calving and pregnancy, decreased pregnancy per artificial insemination, reduced conception and increased culling rate [1-3]. Naturally, postpartum uterus is fully contaminated with pathogenic bacteria until 2 weeks after calving and elimination of the contamination in postpartum period has determinative effect on uterine functions and future fertility [4,5]. Most of the pathogenic bacteria (nearly 90%) that contaminate the uterus after calving are reduced until 60 d postpartum [4]. However, due to some predisposing factors such as retained placenta, subclinical hypocalcaemia, sub-optimal dry matter intake, elimination of the bacterial contamination is delayed and become a permanent infection. This permanent infection has negative and sometimes detrimental effects on ovarian cyclicity, uterine involution, endometrial regeneration and establishment of pregnancy [6,7]. Finally, the conception rate is reduced virtually 20% and interval to pregnancy is extended more 30 d in the herds with postpartum uterine infections [8].

The treatment of postpartum infections include antibacterial, antiseptic and hormone therapies [9]. Although, various systemic and intrauterine antibiotics are commonly used in postpartum infections [8,10,11], antiseptics and hormones are also suggested due to its safety for drug residue and antibacterial resistance and less inactivation in pus that can be observed in antibiotic treatment [12-15].

An alternative therapy to antibiotics, ozone is presented to use in the treatment of intrauterine infections in cows due to its safety for herd and public health [16]. Ozone is a

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gaseous and unstable molecule composed of three oxygen atoms (O_3) that shows a tendency for quickly transforming into the oxygen. In comparison with iodine and chlorine, ozone has more antibacterial activity that inactivates the bacteria, spores and viruses in a few minutes following to exposure [17,18] by its bactericidal [17], immune-stimulating [19] and anti-inflammatory [20] effects.

Although ozone has been prepared in various forms such as cream, gas, injections, paillettes, foam, pearls, boluses, the lipohydroperoxides foam and ovuli, ozone products are mostly preferred in veterinary medicine especially in the treatment of uterine infections [21].

The objective of this study was to compare the effect of intrauterine ozone and an intrauterine antibiotic, rifaximine, treatment on pregnancy rate in the cows that were not pregnant until about 90 day after calving although inseminated several times.

MATERIAL and METHODS

Animals and Experimental Groups

Fifty-three Simmental cows, which were housed in a commercial dairy farm in Erzurum, were in the 3rd lactation with average 5025±320 kg milk yield per year and were not pregnant in the 90th day after calving although artificially inseminated at least 2 times, were taken to the study from October 2013 to March 2014. The cows were fed with corn and grass silage, and concentrate from the same food producer on each farm and milked twice daily. The cows in the study had body condition score between 2.5-3.5 according the described classification by Edmonson et al.[22]. Production parameters such as fertility, milk yield, health status and vaccination in the farm, followed by a herd management software program (Nedap® Livestock Management, Netherlands)

The cows were examined by clinical examinations (vaginoscopy, rectal palpation, rectal temperature) and ultrasonography (Agroscan AL®, Noveko International Inc., Angoulême, France) to diagnose intrauterine infections. In these examinations, status of cervical orifice (erythematous and fibrotic changes), character of cervical discharge (normal, purulent, mucopurulent or pus), changes in ovarian structures (follicular development, luteal structures, or cysts) and uterine health (normal, increased heterogeneity and uterine fluid) were evaluated. Cows with subclinical endometritis was identified by detection of fluid accumulation in ultrasonographic examination as described by Kasimanickam et al.[1]. However, cytological and microbiological examinations were not performed due to inappropriate farm conditions. Clinically positive cows were not taken to the study and treated with the conventional antibiotic and hormone treatment. The remaining 53 cows, which had no clinical signs of metritis, were divided into ozone (Group 1; n=30) and rifaximine (Group 2; n=23) treatment groups.

The cows in Group 1 were treated by intrauterine ozone foam (Sanofoam Spray®, Agriprom, Netherland), whereas the cows in Group 2 were treated by intrauterine rifaximine foam spray; Fatroximin®, Vetaş, Istanbul, Turkey). All treated cows were artificially inseminated starting from the first natural oestrus following the treatment. Pregnancy was diagnosed in the 35th day after artificially insemination by ultrasonography. The cows, which were not pregnant until 200th days after calving (G1 n=4; G2 n=5), were excluded from the study and culled because of infertility.

To assess the reproductive performance for the treated cows, interval from treatment to pregnancy, interval from calving to pregnancy, and insemination number were measured. The study was performed by the permission of Animal Use and Ethics Committee of Atatürk University (Permission number: 8/151-2014).

Statistical Analysis:

The fertility parameters were analysed using Independent Samples Test t (IBM SPSS Statistics 20, 2012). Results were considered as significant when P value was <0.05.

RESULTS

At the beginning of the study, 53 cows, which were not pregnant despite consecutive inseminations until about 90 day after calving, were clinically healthy for uterine disorders. However, pregnancy was detected average 46 and 39 days after intrauterine treatment in Group 1 and Group 2, respectively. Additionally, every pregnancy could be provided by 3 consecutive inseminations, averagely *(Table 1)* in both groups. According to the result, interval between the treatment and pregnancy, interval between calving to pregnancy, and insemination number were not statistically significant between the groups *(Table 1)*. Forty-four of 53 cows were pregnant until 200th days.

Table 1. *Effects of G1 (ozone foam) and G2 (rifaximin foam) on fertility parameters in cows*
Tablo 1. *G1 (ozon köpük) ve G2' nin (rifaximin köpük) ineklerde fertilite üzerine etkisi*

Parameters	Group	n	Mean±SE
Interval from calving to treatment	G1	26	83.1±9
	G2	18	85.0±11.1
Interval between treatment to pregnancy (d)	G1	26	46.4±6.2
	G2	18	40.0±6.0
Interval from calving to pregnancy (d)	G1	26	129.4±9.0
	G2	18	125.0±13.1
Insemination number after treatment	G1	26	3.2±0.3
	G2	18	3.1±0.5

P>0.05

DISCUSSION

The presence of pathogenic bacteria in uterus causes inflammation and histological lesions in endometrium, which delays uterine involution as well as perturbs embryo survival [23]. In addition to repressing the release of pituitary LH, bacterial products or inflammations threaten postpartum follicular development and ovulation mechanism in cattle [23,24]. Thus, infections extend the period of days open and days to first service conception and decrease the conception rate [3,25,26]. According to Gautam et al.[27] metritis and endometritis are the most significant disorders for subfertility in dairy cows. As described in woman with in-vitro fertilization failure, subclinical endometrial infection has a role in implantation failure, spontaneous abortion, and preterm birth. Bacterial endotoxins and inflammatory mediators produced by the host: cytokines and chemokines [28] were accused in these failures.

The objectives of the present study were to evaluate the influence of intrauterine ozone administration on fertility in Simmental cows. The number of days open and number of artificial inseminations until pregnancy were the evaluation criteria in the study.

Intrauterine ozone administration, which was provide a potent antimicrobial activity for a wide range of micro-organisms and high oxidation potential that lead to fast transformation into free oxygen, as an antiseptic treatment approach in case of intrauterine infections was reported in previous studies [21,29-32]. Additionally, ozone also increases host immunity by activating erythrocyte metabolism and local tissue immune response. Thus, it leads to micro-environmental healing [20] and provide a cure in undiagnosed metritis cases.

Some of the disinfectant solutions, which were used in treatment of uterine infections, might cause permanent inflammations and damages and subfertility in endometrium [13,16]. On the contrary of this findings, Zobel et al.[16] suggested ozone as a non-irritant disinfectant in uterus. After contact of ozone and endometrial tissue, immunomodulative capacity and following disinfectant effect of ozone increased. Thus, days open period could be shortened in cows with endometritis [33]. Also Zobel [31] stated decreased insemination number and increased fertilization rate after ozone treatment by reducing possible spermicidal effect of endometrial inflammation.

In the current study, a difference in fertility parameter was not observed in rifaximine and ozone treatment groups. Similarly, Zobel [31] was also stated compatible cure rates between ozone and antibiotic treatment in case of intrauterine infections. Moreover, previous reports were also suggested intrauterine ozone treatment alone or combined with parenteral antibiotics was a more efficacious treatment for retained placenta in cows when compared to hormonal and parenteral antibiotic treatment modalities [15,32]. Additionally, combined treatment with prostaglandin $F_{2\alpha}$ with ozone was stated as slightly more effective than cephapirin in postpartum endometritis cases with the advantage of no drug residue in milk and meat [31]. In the presented study, interval between treatments to pregnancy were also similar in rifaximine and ozone treatment groups. Additionally, similar results in insemination number were detected in the groups.

In the current study, similar open days results with Duricic [21,34] were achieved in cows with endometritis following to ozone treatment. In these studies days open period were varied between 118 and 133. Likewise, the period was about 129 d in this study.

In recent years, the dairy herds are trying to establish new strategies such as homeotherapy, immunotherapy, and disinfectant use to minimize antibiotic consumption. These approaches become a trend especially in intra-mammary and intrauterine disease. According to results, ozone treatment was as therapeutic as rifaximine treatment during endometritis in cows. Due to ozone treatment has some advantages such as its non-irritant structure, safety for drug residue in milk, prevention against possible bacterial resistance, and inexpensiveness, the ozone treatment may be an alternative approaches to intrauterine antibiotics in dairy herds.

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Short Communication

PCR Assay for Identification of Animal Species in Different Ready to Eat Raw Meat Samples [1,2]

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Abstract

In this study, 500 ready to eat raw meat samples (minced meat, lahmacun ingredients, kebap, stew and meatball samples)analyzed for different animal originated DNA residues (pork, chicken, cattle, sheep, horse, donkey, cat, dog, mouse, cockroach and house fly) by PCR procedures. Besides, all the samples were analyzed for important foodborne pathogens (coliforms, *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes* and *Salmonella spp.*). According to the results, total of 52 samples were determined as adulterated and different originated animal DNA samples were found (chicken, horse and sheep DNA residues). Adulterated samples were also determined more risky for the consumers in microbiological aspect.

Keywords: *PCR, Species identification, Ready to eat meat products, Foodborne pathogens*

Tüketime Hazır Farklı Çiğ Et Örneklerinde PCR Prosedürleri ile Farklı Hayvan Türlerinin Araştırılması

Özet

Bu çalışmada 500 adet tüketime hazır çiğ et örneği (kıyma, lahmacun iç malzemesi, kebap) toplanılmış ve örnekler 9 adet farklı hayvana ait (domuz, tavuk, sığır, koyun, at, eşek, kedi, köpek, fare, hamamböceği ve sineği) DNA örnekleri PCR prosedürleri kullanılarak araştırılmıştır. Yanı sıra, her bir örnek halk sağlığı açısından risk teşkil edebilecek olan 5 adet gıda patojeni açısından (koliformlar, *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes* ve *Salmonella spp.*) analiz edilmiştir. 52 adet örnekte farklı hayvan türlerine ait (tavuk, at ve koyun olmak üzere) DNA kalıntıları saptanmıştır. Tüm taklit ve tağşişe maruz kalmış örnekler mikrobiyolojik olarak tüketici sağlığı açısından riskli olarak değerlendirilmiştir.

Anahtar sözcükler: *PCR, Tür tayini, Tüketime hazır çiğ et ürünleri, Gıda kaynaklı patojenler*

INTRODUCTION

The composition of food is a major concern of consumers today. In the case of adulterated meat product consumption, several factors including economic, food safety (allergy) and moral reasons (religious belief), trigger such apprehensions. Among these concerns, consumers are most sensitive because of religious factors and do not tolerate even trace amounts of adulteration of meat products with forbidden meats like pork [1]. Hygiene and right labeling notified on the label of any food stuff are very important criteria especially for public health.

This study aimed to examine various meat and meat products (kebaps, lahmacun ingredients, minced meat, stews, various meat balls etc.) which are presented in various sales points (restaurants, butcher shops, groceries etc.) in Istanbul region, to determine their ingredients

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through DNA typing method and to specify the different animal tissues/residuals in these products. Besides, all of the samples are checked for the 6 primary foodborne pathogens which can pose serious microbiological threats for consumers' health. The differences between adulterated and not adulterated products are determined by statistical methods.

MATERIALS and METHODS

Specimen Handling

Random sampling method has been used in this study. From 500 different sales points in the Istanbul region 500 meats and meat product samples have been collected.

Microbiological Analyses

The number of TAB was defined in Plate Count Agar (Oxoid, CM0325), coliforms in VRB (Oxoid, CM1082), *E. coli* in Tryptone Bile X-Glucuronide Medium Agar (Oxoid, CM0945), *S. aureus* in Baird-Parker Agar (Oxoid, CM0275) and DNASE Agar (Oxoid CM0321), *Salmonella* spp. in Xylose Lysine Desoxycholate Agar (Oxoid, CM0469) and Hectoen Enteric Agar (Oxoid, CM0419), and *L. monocytogenes* in Chromogenic Listeria Agar (ISO) Base (Oxoid, PO 5183) and Chromogenic Listeria Selcetive Supplement (ISO) (Oxoid, SR0226) and Oxford (Oxoid, CM856) and Palcam Agar (Oxoid, CM877) respectively to ISO 16649-2 2001, 4833 2003, 6888-1/A1 2004, 11290-1/A1 2005 and 6579/A1 2006 [2-7].

PCR

DNA of all isolates were extracted according to the protocol of the manufacturer (Macherey-Nagel, Nucleospin® Tissue). All the extracts were stored at -20°C until they are used as target DNA for the PCR procedure.

Statistical Analysis

In order to study the risk differences among adulterated and non–adulterated samples upon the studied microbiological parameters and to determine the statistical significance of these, Pearson correlation analysis has been used [8].

RESULTS

18 (3.6%) of the samples showed chicken DNA, 33 (6.6%) of them showed sheep DNA and 1 (0.2%) of them showed horse DNA. None of them showed pork, donkey, cat, dog, mice, cockroach and fly DNA. The detailed refraction of the results can be seen in *Table 1*. The positive results have been determined through Real-time PCR procedures.

The microbiological results are given in *Table 2*. According to coliform bacteria indications 41 (%8.2) of the samples, according to *E. coli* parameter indications 23 (4.6%) of the samples, according to *S. aureus* parameter indicators 29 (5.8%) of the samples, according to *L. monocytogenes* indications 8 (1.6%) of the samples, according to *Salmonella spp.*, 3 (0.6%) of the samples have been determined as unfit for human consumption. 70.3% of coliforms, 58.7% of *E. coli*, 72.4% of *S. aureus* and 100% of *Salmonella* spp. and *L. monocytogenes* detections are found in the adulterated samples.

DISCUSSION

In many countries, food fraud and adulteration in food products, especially in meat and meat products are done either deliberately in order to increase the profit margin or involuntarily as a result of not following the food safety standards, especially in facilities which process more than one animal species.

The main ingredient of kebap in our country is mutton and many kebap shops prepare their kebaps from a mixture of bovine meat and mutton; however, mixing meat products of different animal species either deliberately or accidentally poses a microbiological threat for the consumers, causes the consumers to consume meat products beyond their information. As a result, the consumer is deceived and retrospective follow-up, which is a very important part of food safety procedures, becomes too difficult. It is possible that especially the products containing different types of meat are deliberately adulterated or the facilities producing these in deliberately mingle different meat products.

Table 1. *Extraneous DNAs (other than cattle DNA) determined ın the samples*
Tablo 1. *Örneklerde gözlenen yabancı DNA'lar (sığır DNA'sının dışında)*

Region	Sample (raw)	Sales Point	Extraneous DNA	DNA Positive Samples
Istanbul Europe - İstanbul Asia	Lahmacun ingredients	Kebab shop	Chicken	11
İstanbul Europe - İstanbul Asia	Minced meat	Butcher shop	Chicken	5
İstanbul Europe	Kebap	Kebab shop	Chicken	2
İstanbul Europe - İstanbul Asia	Kebap	Kebab shop	Sheep	30
İstanbul Europe	Minced meat	Butcher shop	Sheep	3
Istanbul Asia	Minced meat	Butcher shop	Horse	1
TOTAL				52

Table 2. *Adulterated and unadulterated product differences according to the risks they pose for consumer health (Pearson Chi Square Method). The results show the difference between all the inadulterated samples and adulterated ones*

Tablo 2. *Tağşiş yapılan ve tağşiş yapılmayan et ürünleri arasındaki grup farklılıklarının tüketici sağlığını riske etmesi açısından analiz edilen mikrobiyolojik parametreler için sınanması (Pearson Chi Square yöntemine göre). Tablodaki sonuçlar tağşiş yapılmadığı tespit edilmiş tüm örneklerin toplamı ve tağşiş yapılmış et ürünleri arasındaki grup farklılıklarını yansıtmaktadır*

Statistical Methods	Microbiological Parameter	Relevant Variable	Value	Asymp. Sig
Pearson Chi Sq	Coliforms	Samples confirmed for adulteration/samples which don't have adulteration	11.087	.000
Pearson Chi Sq	*Escherichia coli*	Samples confirmed for adulteration/samples which don't have adulteration	1.05	.000
Pearson Chi Sq	*Listeria monocyotgenes*	Samples confirmed for adulteration/samples which don't have adulteration	12.102	.000
Pearson Chi Sq	*Staphylococcus aureus*	Samples confirmed for adulteration/samples which don't have adulteration	2.787	.000
Pearson Chi Sq	*Salmonella* spp.	Samples confirmed for adulteration/samples which don't have adulteration	3.902	.000

Values written in red are statistically relevant because they are smaller than P<0.005;Values written in red are positive for adulteration with regard to positive correlation. Adulterated meat and meat products pose a greater microbiological risk for consumer health than non-adulterated products

Medical literature states that some strains such as *S. aureus* are not very competitive and if their initial counts are lower, they cannot develop properly and their development is easily depressed in mixed cultures. Besides, lactic acid bacteria in the microflora of fermented foods and the antimicrobials they produce like the lactic acid, hydrogen peroxide and bacteriosin suppress pathogens such as *E. coli, S. aureus, L. monocytogenes and B. Cereus* [9]. It is thought that the staff hygiene practices are deficient in the facilities from which the *S. aureus* positive samples have been collected and this is the primary reason of these results.

The adulteration practices pose another risk which is often overlooked but actually important, that is food intolerance. The exogenous substances which are mixed in the adulterated products and the ingredients which might be different from the label information may cause the consumers to develop food intolerance reactions. This is considered one of the main risks of adulteration. Food intolerance may have various reasons. The prevalence of food intolerance reactions against foods and food additives is much higher than food allergies which include an immunological mechanism. Whatever the reason of the adulteration maybe, it results in deficient hygiene conditions and this is a serious threat for the facility, staff and product and consumer health. Besides, micro-organisms which reproduce in meat and meat products because of hygiene deficiency can quickly develop single or multi resistance to antibiotics through complex genetic interactions. Our study shows that adulterated products pose a statistically meaningful higher risk for consumer health than unadulterated products. Total quality management systems and food safety practices should be applied together with the official inspection of the state authorities; programs to raise consumer awareness and continuous training programs for the staff responsible for food production should also be carried into effect. All these would be beneficial to reduce the incidence of the adulteration practices.

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Case Report

Bir Kuzuda Konjenital Diyafram Fıtığı Olgusu

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Özet

Bu raporun materyalini Erciyes Üniversitesi Veteriner Fakültesi Patoloji Anabilim Dalı'na nekropsi isteği ile getirilen ve solunum güçlüğü nedeniyle öldüğü bildirilen dört günlük Akkaraman dişi bir kuzu oluşturdu. Nekropside diyaframda 13 cm uzunluğundaki yarıktan midenin bir kısmının göğüs boşluğuna geçtiği ve akciğerlerin sol lobuna baskı yaptığı görüldü. Göğüs boşluğuna geçen organ normal konumuna getirilince diyafram kasının yırtık kenarının inceldiği dikkati çekti. Akciğerlerin sol lobunun kollabe olduğu görüldü. Karaciğer üzerinde 8-12 cm büyüklüklerinde gri beyaz renkli değişikliklerin varlığı tespit edildi. Mikroskobik olarak, diyafram kasının yırtık kenarının fibröz dokudan oluştuğu gözlendi. Akciğerlerde atelektazik alanlar ile karaciğerde hepatositlerde yağ dejenerasyonu görüldü. Kuzularda ender görülen, nekropsi bulguları ve histopatolojik incelemeler sonucunda konjenital diyafram fıtığı olarak tanımlanan bu olgu Türkiye'den bildirilen ilk vakadır.

Anahtar sözcükler: *Konjenital diyafram fıtığı, Kuzu*

Congenital Diaphragmatic Hernia Case in a Lamb

Abstract

In this report, a four days old lamb was referred to Erciyes University, Pathology Department of Veterinary Medicine with a request of necropsy and reported with died from respiratory distress. At necropsy, it was seen that a portion of the stomach pass into the chest cavity from the 13 cm in length slit of diaphragm and found to compressing the left lobe of the lung. When the herniation brought to the normal position, the side in cleft part of diaphragm muscle was noticed that it was thinner. The left lobe of the lung was collapsed and also the presence of 8-12 cm sizes gray white discoloration was observed on liver. Microscopically, the rupture edges of the diaphragm muscle were composed of fibrous tissue. Atelectasis areas were seen in the lungs due from the stomach pressure and also fatty degeneration of the hepatocytes was observed in liver.This report was defined with results of necropsy and histopathological findings as a congenital diaphragmatic hernia which was the first case report in Turkey because of its rarity in lambs.

Keywords: *Congenital diaphragmatic hernia, Lamb*

GİRİŞ

Diyaframın posterolateral bir defektinden kaynaklanan ve abdominal organların toraksa geçmesine neden olan konjenital diyafram fıtığı genellikle sol tarafta şekillenmektedir [1]. Diyafram fıtığı insanlarda embriyolojik olarak gebeliğin 8-12. haftalarında şekillenmeye başlayan diyaframın geç kapanmasından kaynaklanmaktadır [2]. Diyafram fıtığı olgularında, mediastinumun karşı tarafa deplase olması ve akciğerlerin hipoplastik olmasının yanında akciğer arteriollerinin normal yapıda olmaması nedeniyle pulmoner hipertansiyon, respiratorik ve kardiyovasküler komplikasyonlar doğumda ciddi tehlikeler oluşturur ve çoğunlukla diğer malformasyonlarla birlikte seyretmektedir [1].

Hayvanlarda diyafram fıtıkları konjenital ve edinsel olarak görülmektedir. Edinsel olarak şekillenenler sık görülür ve genellikle travmatik orjinlidir [2]. Konjenital olanlar ise plöroperitoneal, peritoneoperikardial ve hiatal olarak görülebilmektedir [3].

Konjenital peritoneoperikardial diyaframatik fıtıkların gelişmesinde, özellikle kedi ve köpeklerde embriyogeneziste oluşan üç farklı teorinin etkisi düşünülmektedir. Bunlar; a) lateralplöroperitoneal kıvrımların ve ventromedial pars sternalisin, sölomun abdominal ve torasik boşluklar içinde bölünmesinin yetersizlikleri [4], b) dorsolateral septum transversumun hatalı gelişimi veya bu bölgedeki ince doku membranlarının rupturu sonucu peritoneal ve

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perikardial iletişim [5], c) doğum öncesi meydana gelen septum transversum hasarı veya septum transversum ile plöroperitoneal kıvrımlar arasında oluşan birleşme [6] olarak sıralanmaktadır.

Kedilerde, edinsel plöroperitoneal diyafram fıtıkları yaygın görülürken, konjenital diyafram fıtıkları ise çoğunlukla peritoneal perikardiyal olarak gözlenmiştir [3].

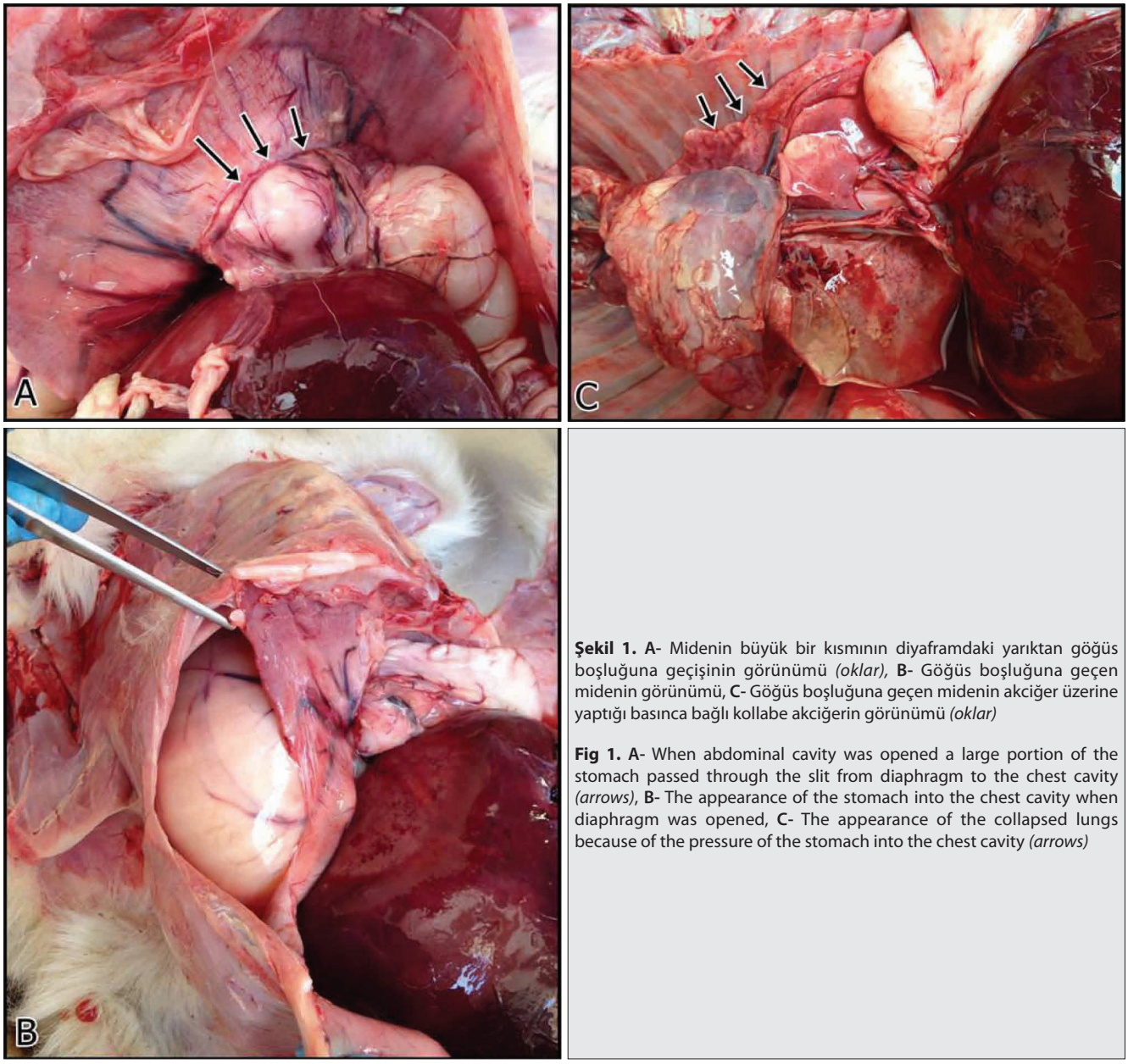
Olguya, patolojik incelemelerle konjenital diyaframa fıtığı tanısı konmuş, kuzularda ender görülmesi nedeniyle önem arz eden ve Türkiye'den bildirilen ilk rapordur.

OLGUNUN TANIMI

Bu çalışmanın materyalini Erciyes Üniversitesi Veteriner Fakültesi Patoloji Anabilim Dalı'na getirilen solunum güçlüğü nedeniyle ölen dört günlük Akkaraman dişi bir kuzu oluşturdu. Sistemik nekropsi de alınan doku örnekleri, %10'luk nötral formalin de tespit edildikten sonra rutin prosedür izlendi ve parafine gömüldü, 5-7 mikronluk kesitler alındı ve hematoksilen-eozin ile boyanarak ışık mikroskobunda değerlendirildi.

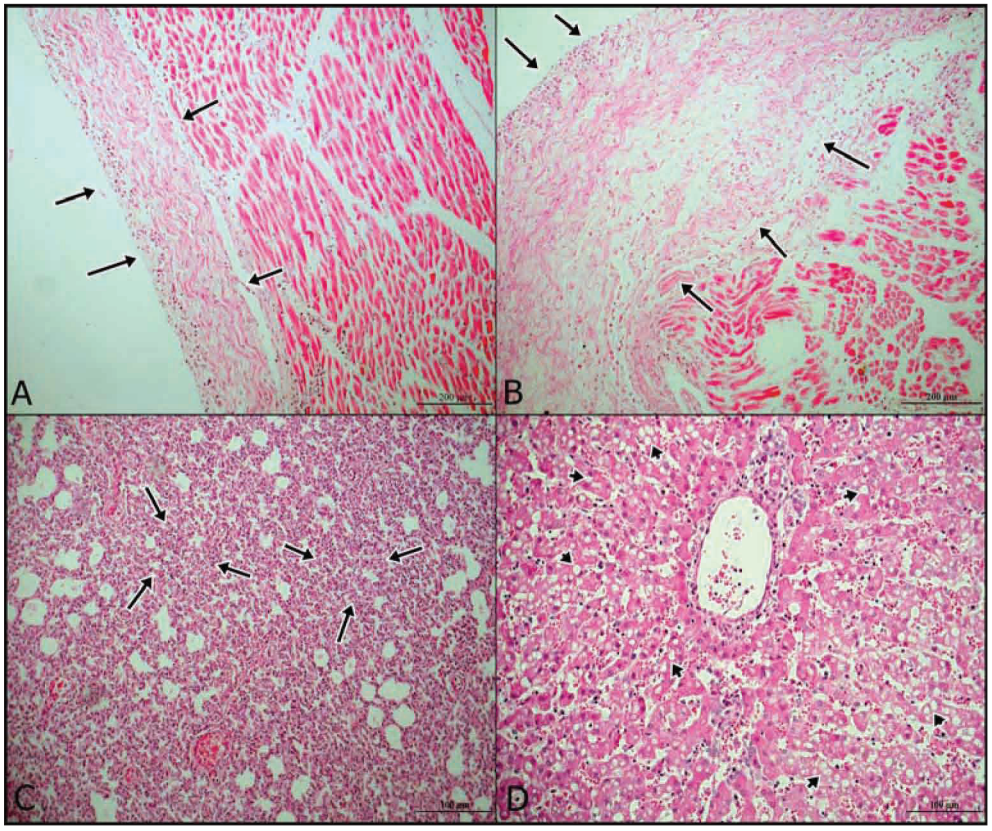
Nekropside, diyaframda 13 cm uzunluğundaki yarıktan midenin bir kısmının göğüs boşluğuna geçtiği, akciğerin sol lobu üzerine baskı yaptığı ve organın bu bölümünün kollabe olduğu görüldü *(Şekil 1)*. Göğüs boşluğuna geçen mide normal konumuna getirilince diyafram kasının yırtık kenarının inceldiği ve fibröz bir yapı haline dönüştüğü dikkati çekti. Karaciğer üzerinde 8-12 cm büyüklüklerinde gri beyaz renk değişikliklerinin varlığı tespit edildi.

Mikroskobik olarak, diyafram kasının yırtık kenarının



Şekil 1. A- Midenin büyük bir kısmının diyaframdaki yarıktan göğüs boşluğuna geçişinin görünümü *(oklar)*, **B-** Göğüs boşluğuna geçen midenin görünümü, **C-** Göğüs boşluğuna geçen midenin akciğer üzerine yaptığı basınca bağlı kollabe akciğerin görünümü *(oklar)*

Fig 1. A- When abdominal cavity was opened a large portion of the stomach passed through the slit from diaphragm to the chest cavity *(arrows)*, **B-** The appearance of the stomach into the chest cavity when diaphragm was opened, **C-** The appearance of the collapsed lungs because of the pressure of the stomach into the chest cavity *(arrows)*



Şekil 2. A,B- Diyaframdaki yırtık kenarında kas dokusu yerine oluşmuş fibröz dokunun görünümü *(oklar),* **C-** Kollabe akciğerde atelektazik alanların görünümü *(oklar),* **D-** Karaciğerde pasif hiperemi ve hepatositlerde farklı büyüklükte, keskin kenarlı vakuollerinin görünümü *(oklar)*

Fig 2. A,B- The appearance of formed fibrous tissue instead of the muscle tissue at the periphery of diaphragmatic rupture *(arrows),* **C-** The appearance of the atelectasis area of collapsed lung *(arrows),* **D-** The appearance of passive hyperemia and fat vacuoles in hepatocytes in the liver *(arrows)*

fibröz dokudan oluştuğu ve kas içermediği gözlendi *(Şekil 2A, 2B)*. Akciğerlerde özellikle midenin yaptığı basınçla ilgili alveol lümenlerinin atelektazik alanlara dönüştüğü dikkati çekti *(Şekil 2C)*. Karaciğerde hepatositlerde sitoplazmada büyüklükleri farklı, yuvarlak, keskin kenarlı vakuollerden oluşmuş yağ dejenerasyon alanları görüldü *(Şekil 2D)*.

TARTIŞMA ve SONUÇ

Konjenital diyafram fıtıkları insanlarda önemli bir problemdir. Diyaframın şekillenmemesi ve gelişiminin yetersizliği, uni veya bilateral akciğer hipoplazisi ve postnatal akciğer hipertansiyonu ile karakterizedir. Erkeklerde bayanlara oranla daha sık gözlenirken siyahi insanlarda beyazlara göre aha az şekillenmektedir [7]. Hayvanlarda konjenital diyafram fıtığı nadiren gözlenir [8], çoğunlukla kedi ve köpeklerde [3-5], yunusta [9], koyunda [10] ve sığırlarda [11] bildirilmiştir. Doğal olguların sınırlı olması ve kuzularda bildirilen vaka sayısının az olması nedeniyle olgumuzun görülme insidensi, cinsiyet ve ırk yönünden değerlendirmesi yapılamamıştır.

Diyafram fıtığı şüphelilerde göbek fıtığı, atriyal septal defekt ve damak yarığı anomalileri açısından fiziksel, radyolojik ve kardiyovasküler kontroller yapılmalıdır [8]. Olgumuz tek ve ölü olduğu için radyolojik ve kardiyovasküler muayeneler ve değerlendirme yapılamamıştır. Nekropside diyaframdaki yırtıktan başka anomali tarzında bir lezyona rastlanmamıştır. Dennis ve ark.[10] iki adet koyunda konjenital diyafram fıtığında orta derecede hidrotoraks, hidroperikardiyum ve asites bildirmişlerdir. Olgumuzda hidrotoraks, hidroperikardiyum ve asitese rastlanılmamış olup bu yönü ile araştırmacıların bulgularından farklılık göstermektedir. Ancak bu konuda literatür bilgisinin çok sınırlı olması ve olgunun tek olması bu konuda daha sağlıklı değerlendirme yapmayı engellemiştir.

Karamanoukian ve ark.[12] gebeliğin 80. günündeki koyunların kuzularında cerrahi olarak oluşturdukları diyafram fıtığında sol ve sağ ventrikül ile interventriküler septum ve atriumun ağırlıklarını ölçmüşlerdir. Sonuç olarak; total kalp ağırlığı, sol ventriküler, septal ve atrial ağırlığın kontrol kuzularıyla karşılaştırıldığında önemli ölçüde düşük olduğunu bildirmişlerdir. Sol ve sağ ventrikül duvar kalınlığı, atrioventriküler oluktan itibaren 0.5 cm ölçülmüş olup bu mesafe kontrol gruplarıyla benzer bulunmuştur. Aort (Ao) çapının kontrol gruplarıyla konjenital fıtık oluşturulan kuzularda aynı olduğu bildirilmiştir. Bununla birlikte, pulmoner arter (PA) çapının fıtık oluşturulan kuzularda artmış olduğu bildirilmiş ve bu durum Ao/PA oranının azalmasına neden olduğu belirtilmiştir. Duktus arteriosus çapının da kontrol gruplarına göre fıtık oluşturulan kuzularda arttığı kaydedilmiştir. Sunulan vakada sol ventrikül duvar kalınlığı 0.5 cm ve sağ ventrikül duvar kalınlığı 0.3 cm olarak ölçüldü. Aort çapı 0.5 cm olarak kaydedilirken deneysel çalışmada bu değer 0.37 cm olarak ölçülmüştür. Pulmoner arter çapı olguda 0.45 cm olarak belirlenirken söz konusu çalışmada bu değer 0.47 cm olarak bildirilmiştir. Sunulan olgudaki veriler sadece bir hayvandan elde edildiği için iki çalışmanın sonuçları sağlıklı olarak karşılaştırılamamıştır.

Konjenital diyafram fıtıklarında semptomların patofizyolojisi, pulmoner hipoplazi, pulmoner hipertansiyon ve kalitatif-kantitatif surfaktan yetersizliğinin kombinasyonundan oluşmaktadır [13]. Akciğer fonksiyon kaybı, gelişmesi için gerekli olan alanın kaybı ile açıklanabilmektedir. Fıtığın şekillendiği taraftaki akciğer hipoplastik olup karşı taraftaki de farklı düzeylerde etkilenebilmektedir. Akciğer ağırlığında ve yetersiz dallanma sebebiyle alveol sayısında azalma gözlenmektedir [13]. Akciğer hipoplazisi ve arteriolar kalınlaşma fıtıklaşmış iç organların akciğere prenatal dönemde yaptıkları basınç sebebiyle oluşmaktadır. Tüm bu lezyonlar cerrahi olarak tavşanlarda [14], kuzularda [15] ve primatlarda [16] oluşturulabilmektedir. Olgumuzda doğumu takip eden birkaç günlük süre içinde görülen solunum güçlüğü muhtemelen fıtığın şekillendiği sol taraftaki akciğer lobunun alveol sayısında azalmaya yol açan atelektazik alanlarında varlığıyla kendini gösteren akciğer volüm kapasitesini azaltan akciğer hipoplazisi ile açıklanabilmektedir. Ayrıca bu konu doğal olgular [3,6,10] ve muhtelif hayvanlarda yapılan deneysel çalışma [14-16] verileriyle de örtüşmektedir.

Sonuç olarak yeni doğan ve klinik olarak enfeksiyöz hastalık bulguları göstermeyen kuzularda gözlenen solunum güçlüğünün tanısında konjenital diyafram fıtığının da düşünülmesi gerektiği sonucuna varılmıştır.

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Case Report

Aortic Body Cell Tumor with Kidney Metastasis in a Dog [1]

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Abstract

In this case, a male Terrier dog, 14-year-old evaluated with a history of cardiac arrhythmias, coughing, rhinorrhagia and exercise intolerance for six mounths. The dog was died upon worsening of clinical signs. At necropsy, two masses of various sizes were observed in the heart-base region. Microscopically, cells from masses were atypic and polyhedral with eosinophilic-granular cytoplasm and basophilic nucleus with round to oval shape. The neoplastic cells were divided into lobules by connective tissue forming nests. In addition to these findings, metastasis to the left kidney was observed. Immunohistochemically, the tumor cells from both primary and metastatic tissues showed immunoreactivity to monoclonal mouse anti-neuron specific enolase antibody but were negative for cytokeratin, vimentin, chromogranin A, α smooth muscle actin and S-100. Based on the clinical, histological and immunohistochemical findings, malignant aortic body tumor with left kidney metastasis was diagnosed in the present case.

Keywords: *Aortic body tumor, Dog, Immunohistochemistry*

Bir Köpekte Böbrek Metastazlı Aortik Body Hücre Tümörü

Özet

Bu olguda 6 aydır devam eden egzersiz intoleransı, öksürük, burun akıntısı ve kardiak aritmi şikayeti olan ve klinik bulguların kötüye gitmesi sonucunda ölen 14 yaşında erkek terrier ırkı köpek değerlendirildi. Nekropside kalp bölgesinde iki kitleye rastlandı. Mikroskobik olarak bakıldığında kitlelerden alınan kesitlerdeki hücrelerin atipi gösterdiği ve ovalden yuvarlağa kadar değişen bazofilik çekirdekli, eozinofilik-granüler sitoplazmalı ve polyhedral şekilli olduğu tespit edildi. Tümoral hücrelerin etrafı sıkı bağdokuyla çevrilmişti. Bu bulgulara ilaveten sol böbrekte tümör metastazına rastlandı. İmmunohistokimyasal olarak hem primer tümör kitlesi hem de metastazlı böbrekteki neoplastik hücreler monoklonal fare anti-neuron spesifik enolaz antikoru ile pozitif reaksiyon verirken, sitokeratin, vimentin, kromagranin A, α düz kas aktin ve S-100 antikorları ile yapılan boyamalar negatifdi. Sunulan bu vaka klinik, histopatolojik ve immunohistokimyasal sonuçları ile malign aortik body tümör olarak teşhis edildi.

Anahtar sözcükler: *Aortik body tümör, Köpek, Immunohistokimya*

INTRODUCTION

Chemodectoma is a tumor arising from chemoreceptor cells which regulate level of blood pH, carbon dioxide and oxygen. Chemodectoma represents both aortic body tumors and carotid body tumors [1]. An aortic body tumor is localized in the tunica adventitia of the aortic arch whereas a carotid body tumor is arised in the carotid artery. The tumors in dogs are mostly benign but rarely malign and metastases to spleen, liver, bone, lung and myocardium [2-5]. Immunohistochemically, aortic body tumor cells usually stain for anti-neuron specific enolase (NSE), chromogranin A and S-100 [6-11] antibodies. To the best our knowledge, a case of metastatic-aortic body has not been reported in veterinary literature in Turkey. The aim of this case is to evaluate diagnostic implications for this important neoplastic condition in dogs.

CASE HISTORY

A male Terrier dog, 14-year-old, was submitted with a history cardiac arrhythmias, coughing, rhinorrhagia and

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exercise intolerance to University of Erciyes, Faculty of Veterinary Medicine. The dog died two weeks later. Systemic necropsy was performed. Macroscopically two neoplastic masses were observed on the heart-base region. The first one was found between pulmonary artery and aorta (20x38x32 cm diameter) *(Fig. 1)*. The second one was located upper the first one (40x35x34 cm diameter) *(Fig. 1)*. The walls of truncus pulmonalis, arteria subclavia sinistra and truncus brachiocephalicus which are branches of aorta were thinner by press of tumoral mass. Out of this findings, no gross lesion and metastasis was seen other organs.

The sections were fixed in neutral-buffered formalin and processed routinely. All sections were stained with Haematoxylin-Eosin and stained immunohistochemically for cytokeratin, vimentin, chromogranin A, α smooth muscle actin, S-100 and NSE antibodies. Streptavidin-biotin peroxidase (SABP) complex method with a commercial kit (Invitrogen, USA) was used for immunohistochemistry and reaction was visualized by aminoethylcarbazole chromogen (AEC, Invitrogen, USA) *(Table 1)*. Microscopically, neoplastic cells were polyhedral with eosinophilic-granular cytoplasm and basophilic nucleus with round to oval shape *(Fig. 2)* and were divided into lobules by connective tissue forming nests. The cytoplasm of some tumor cells contained vacuoles and the cytoplasmic boundaries were usually indistinct but occasionally distinct. Nuclear atypia was marked, and mitotic figures were common. By careful examination of sections of other organs, it was seen metastasis in the cortex of left kidney and cells of metastatic tumor tissue were similar to primary tumor cells *(Fig. 2)*. The tumor cells from both primary and metastatic tissues showed immunoreactivity for NSE antibody but negative for cytokeratin, vimentin, chromogranin A, α smooth muscle actin and S-100. Immunostaining for NSE antibody was diffusely cytoplasmic of the neoplastic cells within in the tumor mass.

DISCUSSION

The malignant aortic body tumor is known to prefer local invasion of the pericardium, myocardium and walls of great vessels at the base of the heart. Metastasis is infrequent and it usually spreads to lung, liver and lymph nodes [3-5]. In this case metastasis were seen in left kidney. These tumors in animals are regarded as non functional and space- occupying lesions. Therefore, tumor may lead to cardiac functional disturbance. In the present case, exercise intolerance and limping in left leg were important symptoms. This condition may occur due to the stenosis of arteria subclavia sinistra which is supplying left leg. Additionally, the construction of truncus brachiocephalicus may lead to cough. This clinical findings were not suggestive of neoplastic formation, but may relates to the neoplastic growth that leads to decreased blood flow. Aortic body tumors have been reported various brachycephalic dog breeds with sex predisposition and tumor usually seen male and 8 years-old-age or older dog [4,12]. Some brachycephalic breeds such as boxer, boston terrier and bulldog are most often affected [4,12]. Because of stenotic nares, long soft palate and distortion of pharyngeal soft tissues in

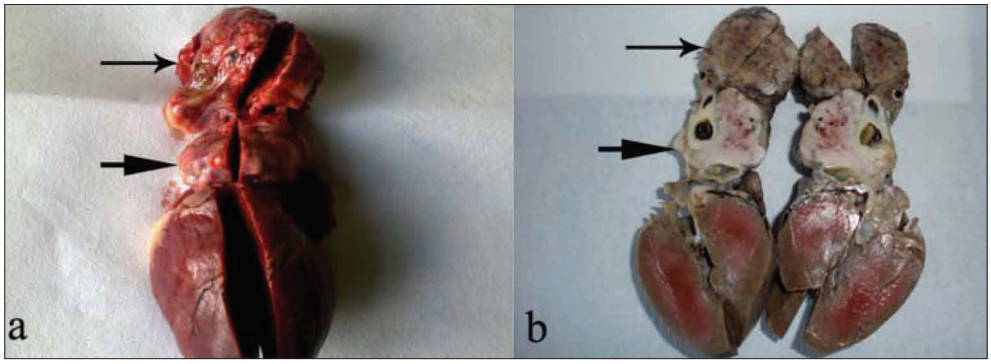

Fig 1. a- The first mass *(thick arrow)* and the second mass *(thin arrow)*, **b-** The first mass that was well-circumscribed, white-grayish in colour *(thick arrow)*, the second mass that was reddish in colour *(thin arrow)*

Şekil 1. a- Birinci kitle *(kalın ok)* ve ikinci kitle *(ince ok)*, **b-** Beyaz-gri renkli sınırlı ilk kitle *(kalın ok)*, kırmızımsı renkte ikinci kitle *(ince ok)*

Table 1. *Commercial name and dilution rate of the antibody*
Tablo 1. *Antikorların ticari ismi ve sulandırma oranları*

Specifity	Company that Antibody Purchased	Dilution and Incubation	Positivity
Vimentin (MS-129-P0)	Thermo	1/100-1 hour	_
Cytokeratin (SC81714)	Santa Cruz	1/100-1 hour	_
S-100 (PA5-16586)	Thermo	1/50-Over night	_
ChromograninA (PA1-37445)	Thermo	1/200-1 hour	_
Neuron spesific enolase (LS-C43890-1000)	Thermo	1/100-1 hour	+
α-smooth-muscle actin (MS-113-P0)	Thermo	1/200-1 hour	_

Only NSE antibody positivity was detected in primer and metastatic tumoral cells

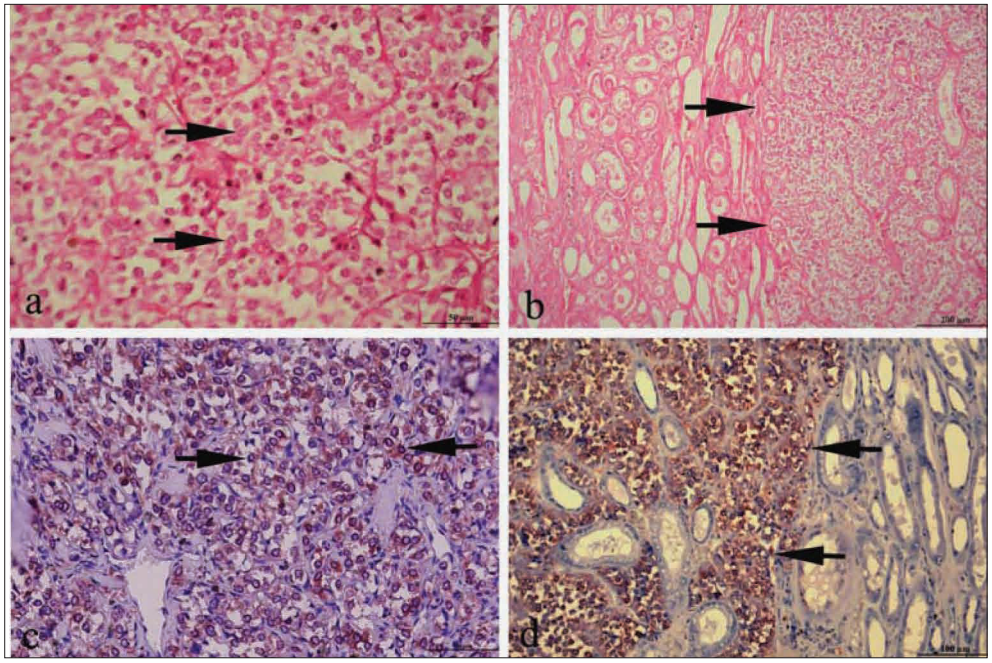

Fig 2. a- Polyhedral with eosinophilic-granular cytoplasm tumoral cells *(arrows)*, HE, x50 µ, **b-** Metastatic tumor cells in the cortex of kidney *(arrows)*, HE, x200 µ, **c-** Immunolabelling for NSE in the primer tumoral cells *(arrows)*, SABP-AEC, x100 µ, **d-** Immunolabelling for NSE in the metastatic tumoral cells *(arrows)*, SABP-AEC x100 µ

Şekil 2. a- Polihedral ve eozinofilik-granüler sitoplazmalı tümör hücreleri *(oklar)*, HE, x50 µ, **b-** Böbrek korteksinde metastatik tümöral hücreler *(oklar)*, HE, x200 µ, **c-** NSE immun-pozitif primer tümör hücreleri *(oklar)*, SABP-AEC. x100 µ, **d-** NSE immun-pozitif metastatik tümör hücreleri *(oklar)*, SABP-AEC, x100 µ

brachycephalic dog breeds may be seen chronic hipoxia and this condition possibility causes hyperplasia and afterwards, tumors of aortic body cells [13]. In the present case, 14-year-old male dog was a terrier, which was not a brachycephalic breed but morphologic, anatomic and histologic findings of this case consistent with previous descriptions of diagnosed aortic body cell tumors [8,9]. In this study, immunohistochemically, NSE, chromogranin A and S-100 antibodies were applied for aortic body tumor. On the other hand, cytokeratin, vimentin, α smooth muscle actin antibodies were used selectively to eliminate tumors which may be originated from mesenchymal, epithelial and other nervous system tumors. Both primer tumoral cells and metastastatic cells were strongly positive for NSE but negative for S 100, chromogranin A, cytokeratin, vimentin and α smooth muscle actin. But previously some studies was reported that aortic body tumoral cells were commonly positive for NSE, chromogranin A and S-100 antibodies [7-9]. The variability in negative staining for chromogranin A and S-100 may be related to grade of the neoplasia [14]. Previous studies also indicated that neuropeptides rate decreased in high grade tumors and chromogranin A and S-100 antibodies were positive staining in benign tumors while negative in high grade tumors [6,10,14]. In the present case, malignancy of tumor was high grade because of metastasis, high mitotic figure rate and pleomorphism. The findings of our study are consistent with conclusions of the previous reports. In this case, as a result of tumor localization with histopathological and immunohistochemical findings led us to diagnose aortic body tumor.

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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.
4- ***Makale Türleri***
Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır. Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 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.
Çeviri, makalenin orijinal formatı dikkate alınarak hazırlanmalıdır.
Yazarla ilgili kişisel ve kuruma ait bilgiler ana metin dosyasına değil, on-line başvuru sırasında sistemdeki ilgili yerlere unvan belirtilmeksizin eklenmelidir.
5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.
6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır. Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.
Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.
Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.
Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.
Örnek: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.
DOI numarası bulunan kaynaklarda bu bilgi ilgili kaynak künyesinin sonuna eklenmelidir.
Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi, kaynak bilgilerinin sonuna eklenmelidir.
Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.
Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.
7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.
8- Editörlük, dergiye gönderilen yazılar üzerinde gerekli görülen kısaltma ve düzeltmeleri yapabileceği gibi önerilerini yazarlara iletebilir. Yazarlar, düzeltilmek üzere yollanan yazıları online sistemde belirtilen sürede gerekli düzeltmeleri yaparak editörlüğe iade etmelidirler. Editörlükçe ön incelemesi yapılan ve değerlendirmeye alınması uygun görülen makaleler ilgili bilim dalından bir yayın danışmanı ve iki raportörün olumlu görüşü alındığı takdirde yayımlanır.
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