ISSN 1300 - 6045
e-ISSN 1309 - 2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University

(Yılda altı sayı yayımlanır)

(Published Bi-monthly)

http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Cilt / Volume : 20

Sayı / Number : 4 TEMMUZ - AĞUSTOS / JULY - AUGUST

Yıl / Year : 2014

ISSN: 1300-6045
e-ISSN: 1309-2251

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This journal is indexed and abstracted by Thomson Reuters Services beginning with Volume 13 (1) 2007 in the followings:

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Fax: +90 474 2426853
E-mail: vetdergi@kafkas.edu.tr

E-ISSN: 1309-2251

ELEKTRONİK BASKI (ELECTRONIC EDITION)
http://vetdergi.kafkas.edu.tr

ONLINE MAKALE GÖNDERME (ONLINE SUBMISSION)
http://vetdergikafkas.org

Bu dergi Kafkas Üniversitesi Veteriner Fakültesi tarafından iki ayda bir yayımlanır
This journal is published bi-monthly, by the Faculty of Veterinary Medicine, University of Kafkas

BASKI (Print)
ESER OFSET MATBAACILIK Tel: +90 442 2334667 ERZURUM

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(Contents)

Kafkas Univ Vet Fak Derg
20 (4): 481-486, 2014
DOI: 10.9775/kvfd.2013.10217

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

The Efficacy of Dietary Savory Essential Oil on Reducing the Toxicity of Aflatoxin B_1 in Broiler Chicks

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Makale Kodu (Article Code): KVFD-2013-10217

Summary

The aim of this study was to evaluate the capability of savory essential oil in counteracting the deleterious effects of aflatoxin B_1 (AFB_1) on growth performance, serum biochemistry, and humoral immune responses in broiler chickens fed 42 days of age. At a 2^2 factorial arrangement in completely randomized design, 300 day-old broiler chicks were assigned to four treatments with five replicates of 15 birds. Chickens were fed the basal diet up to day 7 of age and then fed the experimental diets. The dietary treatments involved of 0 and 0.5 mg of AFB1/kg with or without 500 mg of savory/kg dry matter. The addition of aflatoxin to diet decreased ($P<0.05$) the weight gain and feed intake and resulted in a poor feed conversion ratio. Birds in the AFB_1 group had lower level of albumin, but higher levels of creatinine and liver enzymes in the serum as compared with the control group. The addition of savory to the contaminated diet diminished ($P<0.05$) the inhibitory effects of dietary AFB_1 on the growth performance and the level of liver enzymes in serum. The addition of aflatoxin to diet caused a decrease and savory supplementation caused an increase in antibody titer against the Newcastle virus, and interaction among the factors was differ ($P<0.05$). The main effects and interaction on antibody titer against influenza virus were not differ ($P>0.05$). The addition of savory reduced the adverse effects of aflatoxin on growth performance and provided slight positive effect on serum biochemistry and humoral immune responses in broilers exposed aflatoxin.

Keywords: *Aflatoxin, Broiler, Savory, Protection*

Broyler Piliçlerde Rasyona Katılan Geyikotu Esansiyel Yağının Aflatoksin B_1 Toksisitesinin Azaltılmasındaki Etkinliği

Özet

Bu çalışma, 42 gün süreyle beslenen broyler piliçlerde yeme katılan geyioktu esansiyel yağının, aflatoksin B_1 (AFB_1)'in büyüme performansı, serum biyokimyası ve hümoral immün yanıtlar üzerine olan zararlı etkilerini yoketme kapasitesinin değerlendirilmesi amacıyla yapıldı. Randomize tasarım ile 2^2 faktöryel düzenlemede, 300 günlük broyler piliçler 15 kanatlı içeren beş tekrarlı dört tedavi grubu olacak şekilde ayarlandı. Piliçler 7. güne kadar temel rasyon ve daha sonra deneysel diyet ile beslendi. Besinsel tedaviler 500 mg kuru madde olarak geyioktu/kg içeren veya içermeyen 0 ve 0.5 mg AFB_1/kg'dan oluştu. Rasyona aflatoksin eklenmesi ağırlık kazancını ve yem tüketimini azaltırken ($P<0.05$) zayıf bir yem dönüşüm oranıyla (poor feed conversion ratio) sonuçlandı. AFB_1 grubundaki kanatlıların kontrol grubuyla kıyaslandığınıda düşük albumin düzeyine, fakat yüksek serum kreatin ve karaciğer enzimleri düzeyine sahip olduğu gözlemlendi. Kontamine rasyona geyioktu ilavesinin besinsel AFB1'in büyüme performansı ve serumdaki karaciğer enzim seviyesi üzerindeki inhibitör etkilerini azalttığı ($P<0.05$) belirlendi. Newcastle virüsüne karşı gelişen antikor titresinin rasyona aflatoksin ilavesi ile azaldığı ve geyioktu ilavesi ile arttığı ve faktörler arası etkileşimin farklı ($P<0.05$) olduğu gözlemlendi. İnfluenza virüsüne karşı gelişen antikor titresi bakımından ana etkilerin ve etkileşimin değişmediği ($P>0.05$) belirlendi. Geyioktu ilavesinin aflatoksinin büyüme performansı üzerine olumsuz etkilerini azalttığı ve aflatoksine maruz kalan broylerlerde serum biyokimyası ve hümoral immun yanıt üzerine hafif bir olumlu etki sağladığı sonucuna varıldı.

Anahtar sözcükler: *Aflatoksin, Broyler, Geyikotu, Koruma*

INTRODUCTION

Aflatoxins are mycotoxins produced by *Aspergillus* species as secondary metabolites [1]. Due to the ubiquitous nature of species *Aspergillus* in the environment, mycotoxin contamination of grains and feed is unavoidable. Toxic

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effects of aflatoxin commonly observed in animals included poor absorption of nutrients which lead to death, reduced tissue integrity, lower growth rates and poor feed conversion, reproductive problems in males and females, increasing sensitivity to extreme temperatures and reduced immune response [2]. Different approaches such as irradiation, mold inhibitors, dietary absorbents, ammoniation and ozone degradation were applied to reduce food and feed contamination by aflatoxins [3,4]. Unfortunately, most of these methods are expensive and only partially effective. Currently, the practical approach has been the addition of feed additives such as essential oils to contaminated feeds to reduce the negative effects of aflatoxin in the body.

Savory *(Satureja khuzistanica)* is an annual, herbaceous aromatic and medicinal plant belonging to the Lamiaceae family with identified essential oils named carvacrol and thymol [5].

Savory is native in western and southern part of Iran and used as an analgesic and antiseptic in folk medicine in the region. The properties of this herb were reported as antimicrobial [6], anti-inflammatory [7], antifungal [8], anti-oxidant [9,10] and anti-hyperlipemia [11]. In the literature, there was no report concerning the effect of dietary essential oil of savory on performance, serum bio-chemistry, and humeral immune responses of broilers exposed to aflatoxin. Therefore, this study was conducted to evaluate the potential of applying essential oil of savory in protecting of aflatoxicosis on performance, serum biochemistry, and humeral immune responses of broilers fed diet containing aflatoxin B_1.

MATERIAL and METHODS

The study was approved by the Ethics Committee of Islamic Azad University, Science and Research Branch of Medical and Veterinary Sciences (approval date: 12.03.2012; no: 1265, AEC 2).

Aflatoxin Production

Aflatoxin was produced from *Aspergillus parasiticus* PTTC 5286 culture through fermentation of rice using the method proposed by Shotwell et al.[12]. The sterile rice was placed in Erlenmeyer flasks and inoculated with 2 ml of the mold aqueous suspension containing 10^6 spores/ml. Cultures were allowed to grow for 7 d at 39°C in incubator. On the seventh day, Erlenmeyer flasks were autoclaved and culture materials were dried for 48 h at 40°C in a forced-air oven and ground to a fine powder. The aflatoxin B_1 levels in rice powder were measured by HPLC method [13]. Of the total aflatoxins content in the rice powder, 84.64% was aflatoxin B_1 and 15.36% was aflatoxin G_1. Aflatoxin B_2 and aflatoxin G_2 were not detected. The milled substrate was added to the basal diet to provide the level of 0.5 mg/kg of aflatoxin.

Animals and Dietary Treatments

A total of 300 day-old broiler chicks (Ross 308) were purchased from a local hatchery. On arrival, the birds were weighed and randomly allocated to one of the four treatments with five replicates of 15 birds based on a 2^2 factorial arrangement in completely randomized design, consisted of two levels of AFB_1 (0 and 0.5 mg/kg of feed) and two levels of savory (0, 500 mg/kg of feed).

Chicks up to day 7 of age are very sensitive to aflatoxins, thus they were fed diet without aflatoxin. Thereafter, until day 42 of age (8-42 days), they were fed the experimental diets. The isonitrogenous and isocaloric diets were formulated to meet the nutrients ratio and requirements of broilers as recommended by Ross [14]. Essential oils from savory were obtained by Clevenger-type water distillation. The major compounds in savory essential oils were identified using gas chromatography in another study [15] and consisted of carvacrol (80.6%), p-cymene (4.8%), myrcene (1.5%), terpinene (2.1%) and terpinene-4-ol (2.1%). The appropriate amount of savory extract for 100 kg of feed was premixed with a carrier of 1 kg in order to mix the relevant treatment diet.

Table 1 lists the basal diet formulated to meet the nutrient requirements of broilers. The chicks were raised on floor pens (150 × 150 × 80 cm) for 6 weeks and had free access to feed and water throughout the entire experimental period. The lighting program consisted of a period of 23 h light and 1 h of darkness in 1 day. The ambient temperature was gradually decreased from 33 to 25°C on day 21 and was then kept constant.

Performance and Organs Weight

The body weight was determined at days 14, 28 and 42 of age. Feed consumption and weight gain were recorded at different periods and feed conversion ratio (FCR) was calculated. At day 42 of age, two birds from each replicate were randomly selected based on the average weight of the group and sacrificed. Carcass yield was calculated by dividing eviscerated weight by live weight. Abdominal fat, liver, pancreas was separated, weighed and calculated as a percentage of live body weight.

Vaccination and Immunological Tests

The commercially available oil-adjuvant injectable emulsion against the Newcastle disease virus (NDV) and avian influenza virus (AIV) were used (H9N2 subtype) for vaccinating broiler chicks, and they were injected subcutaneously with 0.2 ml per chick at day 9 of age. Also, the chicks were orally vaccinated against the Newcastle Disease (Lasota) at 21 d of age. Antibody titers against NDV and AIV were measured as immune responses. At day 28 of age, two male broilers from each pen were randomly selected, and blood samples were taken by puncture of the brachial vein for analysis of antibody titers against

NDV and AIV. Serum antibody titers against NDV and AIV were measured by the hemagglutination inhibition test (HI), and HI antibodies were then converted to log2.

Table 1. *The ingredient and calculated composition of basal starter, grower, and finisher diets*

Tablo 1. *Temel başlangıç, büyütme ve bitiş rasyonlarının içeriği ve hesaplanmış kompozisyonu*

Item	Starter	Grower	Finisher
Ingredient, g/kg			
Corn	537.3	533	561
Soybean meal	400	395	370
Soybean oil	20	35	35
Di calcium phosphate	19.3	17.7	15.6
$CaCO_3$	10.5	8.8	8.5
NaCl	3.5	3	3
Trace mineral premix[a]	2.5	2.5	2.5
Vitamin premix[b]	2.5	2.5	2.5
DL-methionine	3.1	2	1.4
L-lysine	1.3	-	-
Savory	-	0.5	0.5
Analyzed Calculation			
Metabolizable energy (kcal/kg)	2870	2980	3000
Crude protein (g/kg)	221	220	210
Calcium (g/kg)	8.6	7.5	7
Available phosphorus (g/kg)	4.9	4.4	4.1
Methionine+cysteine (g/kg)	1.1	8.9	8
Lysine (g/kg)	13.3	11.9	11.3
Threonine (g/kg)	8.3	8.3	6.3
Tryptophan (g/kg)	3.2	3.2	3

[a] Provided the following per kg of diet: Mg, 56 mg; Fe, 20 mg; Cu, 10 mg; Zn, 50 mg; Co, 125 mg; I, 0.8 mg; [b] Provided the following per kg of diet: vit A, 10,000 IU; vit D_3, 2000 IU; vit E, 5 IU; vit K, 2 mg; riboflavin, 4.20 mg; vit B_{12}, 0.01 mg; pantothenic acid, 5 mg; nicotinic acid, 20 mg; folic acid, 0.5 mg; choline, 3 mg

Serum Biochemical Parameters

After 12 h fasting, blood samples were collected in non-heparinised tube at day 42 of age from 8 birds in each treatment by puncturing the brachial vein and the blood was centrifuged to obtain serum. Serum samples were analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), albumin, total protein, triglyceride, cholesterol and creatinine were determined using an automated analyzer (Technicon RA-1000, Tarrytown, USA) based on the commercial kit protocol (Pars Azmoon Company, Tehran, Iran).

Statistical Analysis

All data were subjected to ANOVA using the General Linear Model procedure of SAS software (SAS Inst. Inc., Cary, NC). The mean differences among different treatments were separated by Duncan's multiple range tests. A level of $P<0.05$ was used as the criterion for statistical significance.

RESULTS

Performance of Broiler Chickens

The effects of treatments on growth performance are shown in *Table 2*. During the total period, a significant interaction was observed between AFB_1 and savory for body weight and feed intake ($P<0.05$), but in terms of FCR, no significant difference was observed. The birds fed diets containing aflatoxin compared with the control group had lower body weight gain and feed intake. Chickens fed diets containing aflatoxin had lower FCR than the control group, but the difference was not significant. The inclusion of savory (500 mg/kg) to the diet of birds fed diets containing aflatoxin affected these factors and leading to a reduction in the negative effects of aflatoxin. During the total period, the feed intake, body weight gain and FCR in the birds

Table 2. *Effect of savory on growth performance for broiler chicks fed diet containing 0.5 mg aflatoxin/kg at 1 to 42 d of age*

Tablo 2. *1. - 42. günlerde 0.5 mg aflatoksin/kg içeren rasyon ile beslenen broyler piliçlerde geyikotunun büyüme performansı üzerine etkisi*

Treatments	Body Weight Gain (g)				Feed Intake (g)				Feed Conversion Ratio			
	0-14 day	14-28 day	28-42 day	0-42 day	0-14 day	14-28 day	28-42 day	0-42 day	0-14 day	14-28 day	28-42 day	0-42 day
Control	288[a]	857[a]	1030[ab]	2176[b]	436[a]	1506[a]	2237[a]	4157[a]	1.51	1.75[b]	2.19	1.91
AFB1	247[d]	652[b]	859[c]	1753[d]	379[c]	1231[c]	1869[b]	3485[c]	1.54	1.91[a]	2.19	1.98
Savory	282[b]	883[a]	1108[a]	2266[a]	414[b]	1482[ab]	2210[a]	4085[ab]	1.47	1.68[b]	1.99	1.80
AFB1+ Savory	272[c]	825[a]	980[b]	2089[c]	404[b]	1392[b]	2113[a]	3943[b]	1.49	1.69[b]	2.15	1.89
SEM	2.9	18.2	23.3	37.3	4.2	25.2	38.8	54.2	0.011	0.024	0.032	0.016
Probability												
AFB1	**	**	**	**	**	**	**	**	NS	*	NS	**
Savory	**	**	*	**	NS	NS	NS	**	NS	**	*	**
AFB1× Savory	**	**	NS	**	**	*	*	**	NS	*	NS	NS

*[a,b,c,d] Values in the same column not sharing a common superscript differ ($P<0.05$); * $P<0.05$; ** $P<0.0$*

fed savory alone were not differ (P>0.05) as compared with the control group.

Serum Biochemical Parameters

The effects of diets containing aflatoxins and savory on blood biochemical parameters of broilers are shown in *Table 3*. At day 42 of age, a significant interaction was observed between AFB_1 and savory on serum glucose, creatinine, cholesterol, albumin, ALT and ALP. However, there was no significant difference for serum true protein, triglyceride, AST and GGT among treatments (P>0.05). However, feeding the diet containing aflatoxin to broilers decreased the levels of glucose, cholesterol and albumin, and increased creatinine, ALT, and ALP as compared with the control group. The addition of savory (500 mg/kg) to the diet containing aflatoxin ameliorated the adverse effect of aflatoxin on the serum levels of glucose and ALT (P<0.05) as compared with the group fed diet containing aflatoxin without savory.

Relative Organ Weights

The relative weights of some organs are shown in *Table 4*. The main effect of aflatoxin was significantly difference, but the addition of savory to diet of broilers had no effect on the relative weight of liver and spleen. A significant interaction between savory and AFB_1 was observed in the relative weights of liver and spleen (P<0.05). The highest relative weights of liver and spleen were related to the birds fed diet containing aflatoxin. The addition of savory (500 mg/kg) to the diet containing aflatoxin decreased the relative weights of spleen, but had no significant effect on the relative weights of liver. The main effects and interaction on the relative weights of abdominal fat and pancreas were not significantly difference.

Immune Responses

The effects of experimental diets on antibody titers against NDV and AIV at day 28 of age are presented in *Table 5*. A significant interaction between savory and AFB_1 was observed for antibody titers against NDV. Antibody titers against NDV were different among treatments (P<0.05). The lowest antibody titer against NDV was found in the birds fed diet containing aflatoxin and the highest was observed in the birds fed savory alone. The addition of savory to diet containing aflatoxin significantly increased antibody titers against NDV. In the case of antibody titers against AIV, there was no difference among treatments (P>0.05).

DISCUSSION

In this study, the efficacy of savory for protection from the harmful effects of aflatoxin on the performance, health and immunity of broilers was investigated. The results indicated that ingestion of aflatoxins resulted in a significant decrease in feed intake and consequently the

Table 4. *Effect of savory on relative organ weights for broiler chicks fed diet containing 0.5 mg aflatoxin/kg at day 42 of age[1]*

Tablo 4. *42. günde 0.5 mg aflatoksin / kg içeren rasyon ile beslenen broyler piliçlerde geyikotunun göreceli organ ağırlıkları üzerine etkisi[1]*

Treatments	Abdominal Fat Weight (%BW[2])	Liver Weight (%BW)	Spleen Weight (%BW)	Pancreas Weight (%BW)
Control	1.41	2.10^{b}	0.11^{b}	0.25
AFB1	1.31	2.52^{a}	0.14^{a}	0.27
Savory	1.21	2.41^{a}	0.11^{b}	0.26
AFB1+ Savory	1.40	2.42^{a}	0.13^{ab}	0.26
SEM	0.062	0.074	0.011	0.013
Probability				
AFB1	NS	*	*	NS
Savory	NS	NS	NS	NS
AFB1× Savory	NS	*	*	NS

*^{a,b} Means within a column without a common superscript differ statistically (P<0.05); [1] Results are reported as means for 6 broilers each; [2] Body weight; * P<0.05; ** P<0.01*

Table 3. *Effect of savory on biochemical parameters for broiler chicks fed diet containing 0.5 mg aflatoxin/kg at day 42 of age[1]*

Tablo 3. *42. günde 0.5 mg aflatoksin / kg içeren rasyon ile beslenen broyler piliçlerde geyikotunun biyokimyasal parametreler üzerine etkisi[1]*

Treatments	Glucose (mg/dL)	Creatinine (µmol/L)	CHOL (mg/dL)	TRG (mg/dl)	AST (U/L)	ALT (U/L)	ALP (U/L)	TP (g/dL)	Albumin (g/dL)	GGT (U/l)
Control	243^{a}	0.32^{ab}	135^{a}	61.8	349	4.01^{b}	386^{c}	4.32	2.52^{a}	21.0
AFB1	218^{b}	0.35^{a}	126^{ab}	54.6	377	5.33^{a}	458^{a}	3.80	2.32^{b}	22.5
Savory	212^{bc}	0.29^{b}	126^{ab}	62.8	351	4.17^{b}	389^{c}	3.82	2.40^{b}	20.5
AFB1+ Savory	202^{c}	0.32^{ab}	123^{b}	61.6	356	4.33^{b}	410^{b}	4.01	2.38^{b}	21.8
SEM	2.9	0.082	2.0	2.08	7.1	0.173	16.5	0.096	0.021	0.54
Probability										
AFB1	**	**	NS	NS	NS	NS	NS	NS	**	NS
Savory	**	NS	NS	NS	NS	NS	NS	NS	NS	NS
AFB1× Savory	*	*	*	NS	NS	*	*	NS	*	NS

*^{a,b,c} Means within a column without a common superscript differ statistically (P<0.05); * P<0.05; ** P<0.01; **CHOL,** cholesterol; **TRG,** triglyceride; **AST,** aspartate aminotransferase; **ALT,** alanine aminotransferase; **ALP,** alkaline phosphatase; **TP,** total protein; **GGT,** gamma-glutamyltransferase*

Table 5. *Effect of savory on humoral immune response for broiler chicks fed diet containing 0.5 mg aflatoxin/kg at day 28 of age*

Tablo 5. *28. günde 0.5 mg aflatoksin/kg içeren rasyon ile beslenen broyler piliçlerde geyikotunun humoral immun yanıt üzerine etkisi*

Treatments	Newcastle (log2)	Influenza (log2)
Control	7.0[ab]	5.0
AFB1	5.0[b]	5.0
Savory	8.4[a]	5.6
AFB1+ Savory	6.2[b]	5.0
SEM	0.27	0.17
Probability		
AFB1	*	NS
Savory	*	NS
AFB1× Savory	*	NS

*[a,b] Values in the same row not sharing a common superscript differ significantly (P<0.05); * P<0.05; ** P<0.01*

body weight gain. In consistence with our results, Dorner et al.[16] reported that inclusion of aflatoxin in the diet of broiler chicks resulted in a decreased of daily weight gain. The reduced feed intake may be related to protein catabolism and an increase in the level of ammonia in blood. Moreover, the adverse effects of aflatoxin on growth performance have been related with a decrease in the protein and energy utilization [17], probably as a consequence of a deterioration of the digestive and metabolic efficiency of the birds.

The harmful effects of aflatoxin on the performance of birds were declined by addition of savory to aflatoxin contaminated diet. Also, in the total period birds fed savory alone had more weight gain and lower feed consumption and therefore better feed conversion ratio as compared to the control group. In consistence with our results, a study [18] showed that the addition of savory at the level of 200 mg/kg to diet of broilers could improve feed conversion ratio.

In this study the level of AST and ALP in serum, as markers of liver function, significantly increased in birds fed aflatoxin contaminated diet. Increase in the level of enzymes AST and ALP due to the addition of aflatoxin to diet has been reported by Dafalla et al.[19]. In contrast, there is a report [20] in which the addition of aflatoxin to diet has no effect on the level of these enzymes. These enzymes enter to blood from liver cells due to cell membrane damage [15]. By addition of savory to diet containing aflatoxin, the levels of AST and ALP decreased. In consistent with the results of the present study, there is a report [15], concerning the hepatoprotective effects of savory in rat that showed savory could decrease the levels of AST, ALT and ALP in serum. The major compounds in savory are the carvacrol and flavonoids [10,21]. Both of these compounds have antioxidant properties. These phenolic compounds present in the savory can prevent liver toxicity and thereby possibly reduce the release of liver enzymes into the blood. It is likely that the role of phenolic compounds present in savory is protection of the liver against toxic agents.

In the present study, the birds fed diet containing aflatoxin had lower concentrations of glucose and albumin as compared with the control group. Also, the total protein content of serum decreased as aflatoxin inclusion to the basal diet. These results agreed with the findings of previous studies [1,22], in relation to the reduction of serum total protein and albumin due to the presence of aflatoxin in diet of broilers. The reduction in total protein levels due to aflatoxin supply may result from a defection the transport of amino acids and mRNA transcription. Thus, the protein synthesis is prevented in the body of birds [23], which reflected in the serum as reduction in total protein.

In the birds fed diets containing aflatoxin the relative weights of the liver, spleen and pancreas increased as compared with the control group. The liver is a target organ for aflatoxins. It is possible that more detoxification process and more fat accumulation in the liver be main cause of increased the relative weight of liver. Significant increase in the relative weight of spleen in broilers fed diet contaminated with aflatoxin has been also reported by Bailey et al.[24]. An increase in the relative weight of the spleen can be attributed to an overactive of the spleen during feeding diets containing aflatoxins. Addition of savory to diet contaminated with aflatoxin decreased this effect on the relative weights of mentioned organs.

Serum antibody titer against NDV was the lowest in the birds fed diet containing aflatoxin. In consistent with our finding, in some of studies [25,26] chicks received aflatoxin treated ration had lower geometrical mean antibody titers against the Newcastle disease as compared to the control. Antibody titer against NDV significantly increased in group fed diet containing savory alone as compared with the control group. The savory has been reported to have antibacterial and antioxidant activities [6]. The major components savory, thyme and carvacrol have been indicated to possess potent antioxidant properties, and an increase in immune responses of chicks was anticipated. There was no report in the literature concerning the effect of savory on the immune response of animals.

The results of this study suggest that the addition of savory to diet could decrease the toxic severity of aflatoxins on broiler chicks. The protective action of this compound was particularly evident on growth performance. These findings provide a basis for further studies on the relationship between savory and protection against aflatoxins toxicity, to improve the safety and quality of poultry products.

Acknowledgments

The authors are grateful to the Islamic Azad University for research funding support. They thank Khoraman

Company for providing the savory extract and Animal Research Institute of Khoramabad for supplying the basal diet and allowing use of poultry unit. They also thank Ali Nikkhah for his assistance during laboratory works and all staffs in the poultry unit, for the assistance in the care and feeding of the chicks used in this research.

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Kafkas Univ Vet Fak Derg
20 (4): 487-492, 2014
DOI: 10.9775/kvfd.2013.10220

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effects of Various Cooking and Freezing Processes on the Residues of Sulfachlorpyridazine-Trimethoprim Combination in Broiler Tissues [1] [2]

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[1] *This research was supported by Erciyes University Scientific Research Center (Project code: VA-06-12)*
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Makale Kodu (Article Code): KVFD-2013-10220

Summary

This study was conducted to determine the effects frying, boiling and freezing processes on the levels of sulfachlorpyridazine (SCP) and trimethoprim (TMP) in broiler chest meat and liver. Male broiler chicks were assigned to two groups as control and treatment groups. Animals were fed on commercial diet not containing SCP and TMP for 35 days. At 30th day, experimental group received suspensions of SCP-TMP mixture (30 mg/kg SCP and 6 mg/kg TMP) via craw by gavage once daily for 5 days. At the end of 35th day, the chickens were sacrified and right chest tissue and the liver were taken out. A portion of the tissues were stored in -20°C freezer for 30 and 45 days. After sacrifiying, the raw tissues were exposed to frying and boiling processes. SCP and TMP analysis of tissues were performed by HPLC-DAD detector and reverse phase column. In conclusion, it has been determined that boiled and grilled processes caused a reduction of SCP and TMP residues at different rates in broiler tissues; while storing in the deep freezer did not cause a significant change on SCP and TMP residues.

Keywords: *Sulfachlorpyridazine, Trimethoprim, Broiler meat, Liver, Cooking, Freezing, HPLC*

Sülfaklorpridazin-Trimetoprim Karışımının Broyler Dokularındaki Kalıntıları Üzerine Çeşitli Pişirme ve Dondurma İşlemlerinin Etkileri

Özet

Bu araştırmayla kızartma, haşlama ve dondurma işlemlerinin piliç göğüs eti ve karaciğer dokularındaki sülfaklorpridazin (SCP) ve trimetoprim (TMP) kalıntılarına yönelik etkilerinin ortaya konulması amaçlandı. Erkek broyler civcivlerden kontrol ve deneme olmak üzere 2 çalışma grubu oluşturuldu. Hayvanlar 35 gün boyunca SCP ve TMP içermeyen yemle beslendi. Deneme grubundaki civcivlere otuzuncu günden itibaren SCP-TMP karışımı (30 mg/kg SCP, 6 mg/kg TMP) 5 gün boyunca günde 1 kez sonda ile kursağa verildi. 35. günün sonunda hayvanlar kesilerek sağ göğüs dokusu ve karaciğerleri alındı. Dokuların bir kısmı 30 ve 45 gün boyunca -20°C'lik derin dondurucuda saklandı. Kesim sonrası alınan çiğ dokulara kızartma ve haşlama işlemleri uygulandı. Dokuların SCP ve TMP analizleri DAD dedektör ve ters faz kolon ile HPLC'de gerçekleştirildi. Sonuç olarak, haşlama ve ızgara işlemlerinin broyler dokularında SCP ve TMP kalıntılarında değişik oranlarda azalmaya neden olduğu; derin dondurucuda bekletmenin ise SCP ve TMP kalıntıları üzerinde önemli bir değişime yol açmadığı belirlendi.

Anahtar sözcükler: *Sülfaklorpridazin, Trimetoprim, Etlik piliç eti, Karaciğer, Pişirme, Dondurma, HPLC*

INTRODUCTION

Sulfonamides block folic acid synthesis in bacteria and coccidia by competing with PABA. SCP is effective against many Gram-positive and Gram-negative microorganisms and coccidia *(E. necatrix, E. maxima, E. tenella, E. brunetti)*.

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SCP is used orally and well tolerated by the animals. It is used for the treatment of coli-bacteriosis, cholera and coccidiosis in drinking water for 3-6 days, at the dose of 30-50 mg/kg. If it is necessary, daily dose can be given up to 400 mg/kg [1-4]. SCP is also used in aquaculture and as a feed additive especially for pigs and cattle [5,6]. The forms of sulfonamides combined with TMP have very broad spectrum; these combinations are used diarrhea, coli-septicemia, CRD, salpingitis, coryza, cholera, staphylococcal infections and coccidiosis of poultry [1,2].

SCP are rapidly absorbed and moderate exctrected. This drug are bound to plasma proteins, especially albumin; it is distributed widely to the body and readily enter body fluids. Levels of SCP are highest in the liver, kidney and lung. Metabolism is by acetylation and glucuronidation in the liver. Like SCP, TMP are widely distributed in tissue and interstitial fluid. Concentrations of TMP are higher in tissues than serum. TMP is metabolized by the liver to oxide and hydroxylated metabolites [4]. After the sulfonamide administration of animals, sulfonamide residues can be found in meat for a long time and they migh cause allergic reactions for human and animals. Also, sulfonamide residues of meat can increase occurence of bacterial resistance [7,8]. According to international codex [7] and Turkish Food Codex [9] maximum residue limit of sulfonamides and trimethoprim are 100 µg/kg and 50 µg/kg for animal-originated food, respectively.

Today, the use of drugs for the treatment and prevention of diseases in animals has become an indispensable application. However, the medications can be harmful for human health through the residues of animal products such as allergy, carcinogenic and teratogenic effects, the development of resistancy in bacteria and decreasing the activity of antibiotics [3,10-12].

This study was conducted to determine the effects of some cooking and freezing processes on SCP and TMP residues in broiler meat and liver tissues.

MATERIAL and METHODS

In this study, 15 Ross-PM3 male chicken were used. Chicken were fed with commercial chicken diet containing all nutrients accepted by National Research Council (NRC) [13]. The diet and water were given as *ad libitum*. For this research it was taken an ethical approval by Ethical Committee of Erciyes University Veterinary Faculty (2005/056/071).

Two groups were assigned as control and SCP-TMP given group. For SCP-TMP group 10 male broiler chicks were used. Five broilers were kept as control group to study validation of SCP and TMP analysis. The chicks were fed with SCP and TMP free feed for 35 days. At the end of the 30th day, SCP-TMP combination was given by gavage to craw at the dose of 30 mg/kg SCP and 6 mg/kg TMP [3] in water, once a day for 5 days to the SCP-TMP group. 24 h after the last administration, the chicks were sacrified and right chest and livers were taken out. Broilers in control group were cut without any drug administration; right chest and liver were taken to use for validation studies. Ten grams of samples were taken from each chest tissue and livers. The remaining tissues were stored at -20°C freezer during 30 and 45 days to determine the effect of cold storage on SCP and TMP residues.

Grilling process was performed in teflon pan for 5 min, boiling process was performed on a tray with heater set to 100°C with the addition of 50 mL salin (50 mg/50 ml NaCl) on the tissues at 10 min. After boiling process, tissues and boiled water were seperated [10]. Extraction and analysis of SCP and TMP in raw, grilled, freezed tissues at 30 and 45th days, boiled tissue and meat stock were performed according to Papapanagiotou et al.[14] with several modification.

For extraction of tissue, 30 ml of dichloromethane was added to 3 g of tissue. Sulfamethazine (80 µg) was added on it as internal standard. The tube was homogenized by homogenizer for 10 min in cold water medium at 20.000 rpm. The mixture was collected by filtration through Whatman 40 filter paper. 1 mL of 3 N HCl was added on 10 ml of the filtrate and centrifuged at 3.000 rpm for 5 min. 250 µl of fluid was from the upper side and 250 µl of 3.8 M sodium acetate solution was added on it and vortexed for 15 sec. 20 µl of this solution was applied to HPLC.

Validation of SCP and TMP analysis were evaluated with the parameters of recovery, corelation coefficient, limit of detection and limit of quantitation by using chemometric techniques [15]. For preparation of calibration curve 1-80 µg/ml or g range standard solutions as different 7 concentrations were prepared. The recovery rates were calculated in drug-free tissue samples at the same range of standard solutions for each samples.

SCP and TMP analyzes were performed in HPLC equipped with DAD detector and C18 reverse phase column (ACE-121-2546, 250x4.6 mm) by using methanol:water (60:40) carrier system with pH 3 set by 10% orthophosphoric acid with 1.8 ml/min flow rate [14].

The analysis of data was performed using SPSS 15.0 software package. Data were evaluated with one-way analysis of variance (ANOVA) ($P<0.05$). Differences between groups were determined with Duncan's test.

RESULTS

Validation Results

In analyzes of samples in HPLC, retention time of

TMP, SMZ and SCP were found as 6.857 min, 8.168 min and 17.736 min, respectively (*Fig. 1* and *Fig. 2*). The standard curve showed linearity in range of 1-80 µg/ml for SCP and TMP mixtures. For SCP, recovery (%), r^2, the limit of detection (LOD; µg/g) and limit of quantitation (LOQ: µg/g) were found 93.25±4.46, 0.9992, 0.857 and 2.859 of chest; 87.95±4.5, 0.9992, 0.739, 2.464 of liver; 98.12±6.51, 0.9993, 0.512, 0.812 of boiled tissue, respectively *(Table 1)*. For TMP, recovery, r^2, the limit of detection (LOD) and limit of quantitation (LOQ) were found 97.55±3.58, 0.9923, 0.255, 0.851 of chest; 95.38±6.15, 0.9983, 0.390, 1.303 of liver; 98.87±7.49, 0.9994, 0.432, 0.502 of boiled tissue, respectively *(Table 1)*.

Experimental Results

SCP levels of chest meats in raw tissue, raw tissue frozen for 30 days, raw tissue frozen for 45 days, grilled tissue, boiled tissue were found as 49.10 µg/g, 44.93 µg/g, 48.14 µg/g, 25.33 µg/g, 21.46 µg/g, respectively; SCP levels of boiled water could not be determined *(Table 2)*.

TMP levels in chest meats in raw tissue, raw tissue frozen for 30 days, raw tissue frozen for 45 days, grilled tissue, boiled tissue and boiled water were found as 1.91 µg/g, 1.8 µg/g, 1.84 µg/g, 1.48 µg/g, 1.15 µg/g and 0.68 µg/ml, respectively *(Table 2)*.

SCP levels of liver in raw tissue, frozen tissue for 30 days, frozen tissue for 45 days, grilled tissue, boiled tissue were found as 60.31 µg/g, 61.87 µg/g, 60.91 µg/g, 20.97 µg/g and 27.33 µg/g, respectively; SCP residues could not detected in boiled water. One of the most important finding of the study is that the levels of SCP were statistically decreased in grilled and boiled tissue and boiled water as compared to raw liver tissue ($P<0.05$) *(Table 2)*.

TMP levels for liver tissue in raw tissue, frozen tissue for 30 days, frozen tissue for 45 days, grilled tissue, boiled tissue and boiled water were found as 1.56 µg/g, 1.52 µg/g, 1.59 µg/g, 1.34 µg/g, 0.97 µg/g and 0.57 µg/ml. TMP levels of liver in boiled water were reduced significantly as compared to raw tissue ($P<0.05$) *(Table 2)*.

DISCUSSION

There are several HPLC techniques for determining sulfachlorpyridazine residues in meat [7,17,18]. According to the method developed by Kowalski et al.[18] which conducted for analysis of SCP, residues in meat samples were

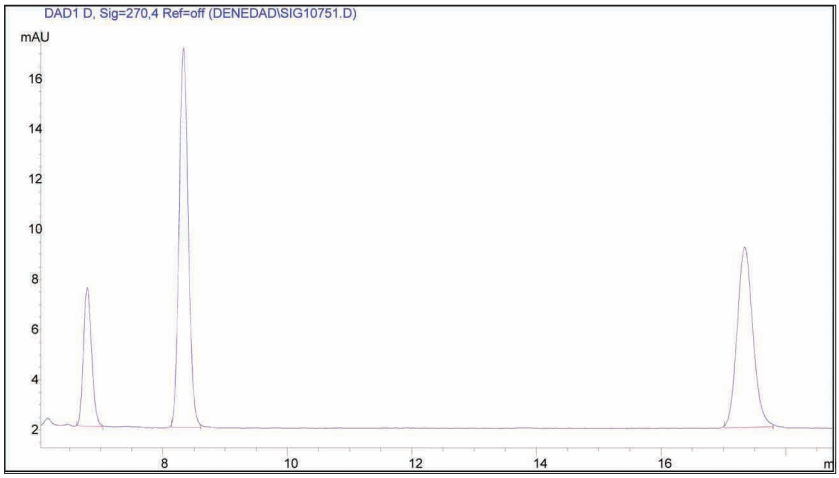

Fig 1. Chromatograms of TMP, SMZ ve SCP in chest samples at 270 nm

Şekil 1. Göğüs eti örneklerinde 270 nm'deki TMP, SMZ ve SCP'ye ait kromatogramlar

Fig 2. Chromatograms of TMP, SMZ ve SCP in liver samples at 270 nm

Şekil 2. Karaciğer örneklerinde 270 nm'deki TMP, SMZ ve SCP'ye ait kromatogramlar

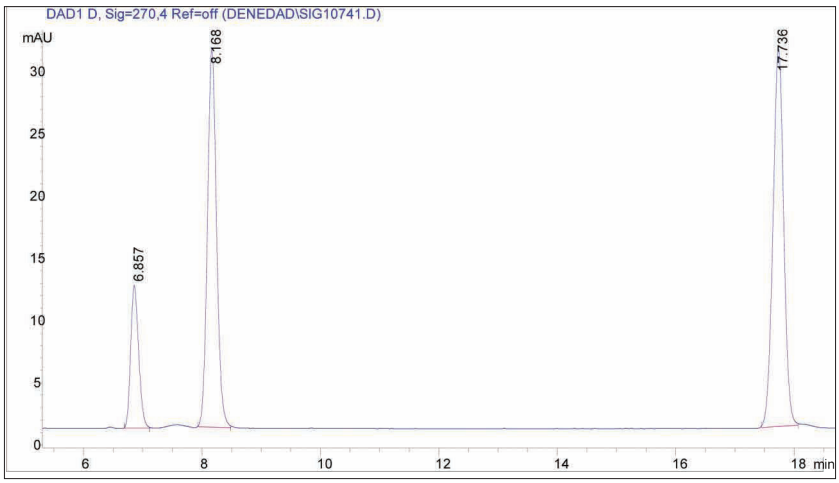

Table 1. *Validation data of SCP and TMP*
Tablo1. *SCP ve TMP analizlerine ait validasyon verileri*

Samples	Recovery (%)	r^2	LOD (µg/g)	LOQ (µg/g)
SCP (Chest)	93.25±4.46	0.9992	0.857	2.859
SCP (Liver)	87.95±4.5	0.9992	0.739	2.464
TMP (Chest)	97.55±3.58	0.9923	0.255	0.851
TMP(Liver)	95.38±6.15	0.9983	0.390	1.303
SCP (Boiled tissue)	98.12±6.51	0.9993	0.512	0.812
TMP (Boiled tissue)	98.87±7.49	0.9994	0.432	0.502

Table 2. *SCP and TMP levels of samples*
Tablo 2. *Örneklerde SCP ve TMP düzeyleri*

Samples	Chest		Liver	
	SCP	TMP	SCP	TMP
Raw tissue (µg/g)	49.10±7.26^{c}	1.91±0.19^{d}	60.31±10.47^{c}	1.56±0.67^{b}
Freezed tissue (30 day) (µg/g)	44.93±10.63^{c}	1.8±0.21^{cd}	61.87±7.24^{c}	1.52±0.33^{b}
Freezed tissue (45 day) (µg/g)	48.14±10.03^{c}	1.84±0.24^{cd}	60.91±11.62^{c}	1.59±0.63^{b}
Griled tissue (µg/g)	25.33±3.66^{b}	1.48±0.24^{bc}	20.97±8.53^{b}	1.34±0.41^{b}
Boiled tissue (µg/g)	21.46±3.68^{b}	1.15±0.57^{b}	27.33±5.84^{b}	0.97±0.07^{ab}
Boiled water (µg/ml)	00.00±00.00^{a}	0.68±0.04^{a}	00.00±00.00^{a}	0.57±0.05^{a}

deproteinized with acetonitrile, followed by treated with acetonitrile-hexane. They have obtained clean samples with dichloromethane-phosphate buffer exctraction and evaporation under nitrogen gas. In our study, tissues were extracted with dichloromethane, followed by the filtering with filter paper, treated with 3 N HCl and 3.8 M sodium acetate solution. As a result, purity of the samples is sufficient for applying to HPLC.

To previous HPLC methods conducted to determine sulfonamide residues in tissue, the wavelength was selected as 270 nm [16,19-21]. Although, the peak intensity of trimethoprim was reported to be better at 229 and 240 nm wavelengths, the wavelength of 270 nm was preferred in the analysis of TMP combined with sulfonamide [14,21,22]. In this study, the SCP, SMZ and TMP analysis were performed at the wavelength as 270 nm *(Fig. 1, Fig. 2)*. In previous studies, several solutions such as 20% acetonitrile, dichloromethane-phosphate buffer and columns such as C18, C4, C8 were used for determining sulfonamides and TMP [19-22]. In this study, the peaks for TMP, SMZ and SCP were taken as 6.857 min, 8.168 mine and 17.736 min respectively, by using methanol: water carrier system (60:40) with pH 3 (set by 10% orthophosphoric acid). Although, the retention time of SCP is longer than the other studies, obtaining the peaks of SMZ, SCP and TMP at different times and sufficient peak intensity may be considered as advantage of this method.

Kowalski et al.[18] reported that the r^2 value and recovery rate of SCP were found as 0.9997 and 72.8, respectively. Papapanagiotou et al.[14] found the recovery rate of sulphadiazine and TMP as 77.8-87.4% and 66.7-83.1%, respectively. In this study, high recovery values were obtained for both SCP and SMZ. According to the recovery and correleation coeeficient parameters of this study, Papapanagiotou's method [14] can be used for the analysis of SCP and TMP in chicken muscle and liver.

Unlike previous studies about sulfonamide analysis of tissues, it has found high recovery and, low LOD and LOQ values. This differencies can be related to modification of homogenisation procedures, high peak intensity and resolution with DAD at 270 nm, also chemometric calculation methods *(Fig. 1, Fig. 2)*.

Kostadinovic et al.[17] reported that, SCP residues in muscle tissue were eliminated faster than skin, liver, and kidney (7, 12 and 18 days respectively) in turkeys exposed to single administration of SCP; the highest tissue SCP level in liver was observed as 32.3 µg/kg. In our study, the highest SCP levels were also obtained from liver samples. The study results reveal that, the accumulation of SCP in liver was high like other studies [4].

There are several studies about the effects of cooking and storing on sulfonamides. According the studies, cooking processes have no significant effect on SMZ residues in pork meat [23,24]; ormethoprim and sulfadimethoxine residues decrease of fish meat [25]; sulfadiazine, sulfamethoxazole, sulfamonometoxin reduce of chicken meat, sulfadimethoxine, sulfaquinoxaline and sulfadoxine reduce

at different cooking and storing processes in broiler tissues [3,10,11,26]. In this study, a significant reduction was seen in the level of SCP and TMP in grilled and boiled chest tissue and boiled water as compared to raw chest tissue (P<0.05). The study results showed that, grilling and boiling processes have a reducing effect on both SCP residues in chest and TMP residues of grilling process. The SCP residues could not be identified in boiled water. This result can related to the weak passing of this drug to the water, disintegration of the drug with the effect of water and heat or transforming of the drug into different metabolites. These results compatible of several studies [10,11,26].

In the study SCP residue levels were decreased in grilled and boiled liver samples compared to raw liver samples (P<0.05), The SCP residues in boiled water were not within measurable levels. The results of the study reveal that, grilling and boiling processes have reducing effects on liver SCP residues.

In terms of TMP residue levels in liver, a significant reduction was seen only in boiled water compared to raw tissue (P<0.05), No difference was recorded between total TMP level of boiled tissue and water and raw liver TMP levels (P>0.05). These results suggested that, the TMP residue levels for liver were not affected from the storage in freezer, grilling and boiling processes.

There are many factors affecting drug residue levels in edible tissues like species, race, age and sex of animals; stability, solubility, pharmacokinetic and pharmaceutical differencies of drugs and administration route and time of drugs [10,11,23,27]. In this study, it is possible that, the reducing effects of cooking process on the residues of SCP and TMP in tissues are likely to be related to the above factors.

Although, many studies focused on the tolerance limits of drug residues in foodstuffs for raw tissues or organs, animal products such as meat, milk and eggs usually expose many process like cooking or canning before consuming. Aminoglycoside, macrolide and tetracyclines often remained in the tissues of slaughtered animals without disrupting for a long time, but some of the cooking and storing procedures could cause weak changes on drug residues [3,10,11,27]. During these processes many alterations may have seen in tissues like protein degradation, water and fat loss and change in pH. Therefore, it has been expected that, baking, roasting, frying, or cold storage processes may breakdown or convert of drugs to ineffective metabolites. So, cooking processes may be reduced drug residues taken in meat.

In conclusion, boiling and grilling processes caused a reduction in SCP and TMP residues at different proportions in broiler tissues; storing in freezer did not cause a significant change on residues of these drugs.

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Kafkas Univ Vet Fak Derg
20 (4): 493-499, 2014
DOI: 10.9775/kvfd.2013.10334

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

The Activity of IL-6 and TNF-α in Adipose Tissue and Peripheral Blood in horses Suffering from Equine Metabolic Syndrome (EMS)

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Makale Kodu (Article Code): KVFD-2013-10334

Summary

Equine metabolic syndrome (EMS) is a metabolic disorder characterized by excessive obesity and or/regional adiposity, insulin resistance (IR) and prior or current laminitis. In the course of the EMS, an important role exert systemic inflammation caused by excessive expression of proinflammatory cytokines. The aim of the current investigation was to examine relationships between expression IL-6 and TNF-α in adipose tissue and their concentration in peripheral blood in horses with EMS. On the basis of range of research procedures, horses were divided into two groups: group A (EMS horses, n=8) and group B (healthy horses with overweight, n=8). The concentration of the proinflammatory cytokines in the peripheral blood and their expression in the adipose tissue were determined. The results of the EMS group showed numerous macrophages and lymphocytes infiltration and increased diameters of adipocytes (P<0.05). The concentration of TNF-α in the serum of insulin-resistant horses was statistically higher compared to the healthy individuals. No significant differences were observed between the EMS horses and the control horses in the concentration of IL-6 in the serum. Moreover, our research revealed expression of investigated cytokines in adipose tissue. The results shows a higher expression of TNF-α in the EMS groups. What is more, macrophages infiltration were observed. In the case of IL-6, were detected the similar arrangement of this cytokines in adipocytes between both test groups. The study indicates the importance of pro-inflammatory proteins TNF- α in the equine metabolic syndrome.

Keywords: *Equine Metabolic Syndrome, TNF-α, I L-6, Insulin Resistance, Obesity*

Atlarda Metabolik Sendrom (EMS) Sancısında, Adipoz Dokuda ve Periferal Kanda IL-6 ve TNF-α nın Aktivitesi

Özet

Atların metabolik sendrom (EMS)'u aşırı obezite ve/veya bölgesel yağlanma ile karakterize, insülin direnci (IR) , ileri derecede veya aniden oluşmuş laminitis ile karakterize metabolik bir hastalıktır. EMS derslerinde, proinflamatuar sitokinlerin aşırı ekspresyonu ile oluşan sistemik inflamasyon uygulamada önemli rol oynadığı anlatılmaktadır. Bu çalışmanın amacı, adipoz dokuda sentezlenen ve periferal kanda bulunan, IL-6 ve TNF-α'nin EMS'li atlarda oluşan ilişkilerini incelemektir. Araştırmanın ana hedefine göre atlar esas olarak, iki gruba ayrıldı: Grup A (EMS atlar, n = 8) ve grup B (kilolu sağlıklı atlar, n = 8). Periferal kanda proinflamatuar sitokinlerinin konsantrasyonu ve bunların adipoz dokulardaki etkisi belirlenmiştir. EMS grubunun sonuçları, pek çok makrofaj ve lenfosit infiltrasyonu ve adipositlerin çaplarında artış (P<0.05 gösterdi. İnsüline dirençli atların serumunda TNF-α konsantrasyonu sağlıklı bireylere göre kıyaslandığında istatistiksel olarak yüksek sonuçlar görüldü. Serumdaki IL-6'nın konsantrasyonunu EMS'li atlarda ve kontrol grubu atlar arasında anlamlı farklar gözlendi. Ayrıca, araştırmada incelenen yağ dokularda sitokinlerin ekspresyonu saptandı. Sonuçlarda EMS'li grupta TNF-α nın daha yüksek anlamı olduğu görüldü. En önemlisi makrofaj infiltrasyonu gözlendi. IL-6'nın olgusunda, adipositlerde sitokinlerin benzer durumu her iki grupta tespit edildi. Çalışma atların metabolik sendromunda pro-inflamatuar proteinlerden TNF-α'nın önemini ortaya çıkardı.

Anahtar sözcükler: *Atların Metobolik Sendromu, TNF-α, I L-6, İnsülin Direnci, Obezite*

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INTRODUCTION

Recently, the obesity is becoming an increasing problem of civilization, affecting both humans and animals, and thus it becomes the subject of intense research. This issue is investigated on two main levels affecting the carbohydrate and lipid metabolism: genetic and environmental predisposition such as physical activity and nutrition/diet [1-4].

The obesity occurrence and high insulin serum level are two of the few clinical symptoms allowing to diagnose disease termed metabolic syndrome *(MetS-Metabolic Syndrome, Syndrome X)*. In the field of veterinary medicine, the concept of the equine metabolic syndrome was proposed for the first time in 2002 by Johnson et al.[5]. In 2010, the American College of Veterinary Internal Medicine, finally defined and officially recognized the Equine Metabolic Syndrome (EMS) as a disease entity [6]. The EMS is defined as a metabolic disorder that includes chronic/acute laminitis, frequent low grade laminitis, hyperinsulinemia, pathologic obesity and insulin resistance (IR). Additional features characterizing the EMS are dyslipidemia, fluctuating levels of blood adipokines and systemic inflammation [6,7]. Equine metabolic syndrome most commonly affects primitive breeds of horses, i.e., ponies and/or heavy horses, as well as those with the relatively low demand for selective nutrients (Quarter Horse, Arabian, Morgans) [8-13]. Genetic predisposition and environmental factors, such as over-feeding and lack of the exercises, contribute to initial physiological insulin resistance and subsequent chronic insulin resistance. Genetic predisposition to pathologic obesity may be caused by a specific gene mutation, i.e., melanocortin-4 receptor, which is responsible for the regulation of feed intake and insulin sensitivity [14]. Persistently high blood glucose level results in the reduction or loss of the sensitivity of cell membranes to the action of insulin. Furthermore, insulin resistance increases the risk of laminitis by stimulating the synthesis of endothelin-1 (ET-1), and activation of the sympathetic nervous system, which results in the narrowing of blood vessels. Disturbance of blood circulation within the hoof capsule is the cause of excessive accumulation of fluid in the interior, and in consequence, detachment of the hoof capsule from the coffin bone [15,16].

In horses, the symptoms of excessive obesity include regional adiposity as well as irregular fat distribution at the base of the tail area, around eyes and at the base of the neck, what is called in the literature as "cresty neck" [13,17].

Pathological adiposity in EMS individuals is strongly associated with insulin resistance, and thus adipose tissue play an important role in the course of this disease. Additionally, adipose tissue is not only an energy reservoir, but also a source of various pro-inflammatory cytokines [13,18-20]. These includes mainly tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). The TNF-α is a protein secreted by adipocytes and stromal vascular cells (primarily macrophages). The role of this protein in the pathogenesis of obesity and insulin resistance involves: (i) inhibition of the activity of genes that regulate lipid and glucose metabolism, as well as (ii) reduction of secretion of adipokines with specific anti-hyperglycemic properties. In the liver, TNF-α inhibits the expression of genes closely related to the transport of glucose into the cells [21]. Studies conducted in humans indicate a strong correlation between the TNF-α expression in adipose tissue of obese individuals and the level of hyperinsulinemia [19,22]. Another cytokine, in addition to TNF-α, playing a significant role in the EMS is IL-6, a molecule responsible for initialization and regulation of acute inflammatory response. This cytokine is also responsible for the inhibition of insulin receptor expression and the reduction of adipogenesis process. Moreover, IL-6 reduces the secretion of adiponectin, peptide hormone that has anti-inflammatory properties [22-24].

Currently, diagnosis of equine metabolic syndrome, especially at early stages of the disease development is challenging for clinicians. The diagnostic procedures are still under development and require further improvements. There is increasing interest in the towards significance of the enteroinsular axis in the pathophysiology of EMS, but still the dynamic tests, mainly the combined glucose-insulin test, are one of the main possibility of EMS diagnosis [11]. However, additional information about cytokines contribution to the physiological path of EMS is required, thus acquiring this knowledge is crucial.

The aim of this study was to determine the levels of TNF-α and IL-6 in the serum and adipose tissue derived from EMS and healthy horses. Our objective was to correlate the information about cytokine levels in the serum with their distribution in the fat tissue. We hypothesize that both information are relevant for the EMS diagnostic and can contribute to the proper assessment of animals' physiological condition.

MATERIAL and METHODS

Ethical Approval

This research was approved by the II Local Ethical Committee (No. 64/2008 and individual agreement for experiments conducted on animals No. 21/2009), localized at the Wroclaw University of Environmental and Life Sciences, Wrocław, Poland.

Experimental Horses

Sixteen heavy draft horses (Silesian breed and Haflingers), different gender, age ranging from 8-14 years were chosen for this experiment. All horses were qualified on the basis of their medical history and detail medical interview with the owners. The horses included into the

study came from the Upper Silesia region in Poland. All individuals had free access to water, sandy paddock and performed work of moderate intensity. Body weight was measured in all individuals using mobile, electronic Equine Bosh Scale (Bosh, Germany). None of the qualified mares was pregnant. Horses from the investigated group had a history of laminitis, with clearly visible deformities of the hoof. Moreover, horses qualified into the study were characterized by regional adiposity and exhibited general obesity. Body condition score (BCS) was measured in the all horses using a scoring system of 1 (emaciated) through 9 (extremely obese) to estimate fat deposition and was based on the system developed by Henneke et al.[25]. Horses, which were characterized by obesity and extreme obesity (8-9) qualified for the experimental group. The other horses with the assessment of 6-7 were assigned to the control group [25]. All investigated individuals underwent following clinical examinations: (i) measurements resting insulin level in the serum, (ii) measurements of resting glucose level (iii), the Combined Glucose-Insulin Test (CGIT test). Before performing CGIT, all horses had limited access to Timothy hay in amounts 4 kg for sixteen hours. First, blood was collected from the jugular vein for the determination of resting level glucose and insulin. Next, 50% dextrose solution (150 mg/kg bw) was injected intravenously immediately followed by application of insulin bolus (IV 0.10 U/kg bw). For the determination of glucose level by means of glucometer (Glucosens 1040), blood samples were collected at 1, 5, 15, 25, 35, 45 , 60, 75 , 90, 105, 120, 135 and 150 minutes after administration of a bolus. In total, blood samples were collected for 2.5 h [13,26,27]. Sterile technique was maintained throughout the collection process. On the basis of aforementioned procedures and the results of the study, horses were divided into two groups: group A (EMS horses, n=8) and group B (healthy horses, n=8).

Clinical Examination

All experimental animals were clinically examined by two independent veterinarians. Horses were evaluated for gait and hoof capsule using X-ray photography. Additional visual examination and palpation was performed in order to identify subclinical or clinical signs of laminitis.

Blood Samples Collection and ELISA Tests

Blood was collected from the jugular vein from all examined animals (16 individuals), into tubes with the granulate and the accelerator in an amount of 10 ml, once from each horse. Then, this material was centrifuged, preserved in liquid nitrogen and transported to the laboratory. Blood insulin level was determined using the Equine ELISA Test (BioVendor, Czech Republic), for which blood samples were centrifuged and serum was removed. Next, IL- 6 and TNF-α were detected using an equine IL6 ELISA immunoassay kit (Genorise Scientific, US) and TNF-α (Genorise Scientific, US), according to the manufactures instructions. Sterile technique was maintained throughout the collection process.

Histochemistry and Immunohistochemistry Examination of Fat Tissue

Adipose tissue sample (2 g) from the base of the mane were collected from each horse under local anesthesia (2% Lignocainum, Polfa S.A., Poland). The histological material was preserved in 10% buffered formalin for 24 h.

Next, 5 µm-thick sections were obtained on a Microm HM 340E microtome (Zeiss, Germany) and placed on histological slides. Samples were subsequently deparaffinized with xylene, ethanol (decreasing concentrations from 100 to 70 %) and washed with distilled water. Slides were stained with hematoxylin (Shandon™, Thermo Scientific US) for 8 minutes, rinsed in running tap water for 10 minutes and stained with eosin (Shandon™, Thermo Scientific US) for 5 min. Sections were dehydrated by washing with ethanol (increasing concentrations from 70 to 100%), followed by xylene and sealed with DPX mounting medium (AquaMed, Poland). Analysis was performed using light microscope (Axio Imager A1, Zeiss) [28].

The 3 µm-thick tissue sections were cut, dewaxed and rehydrated. Immunoperoxidase cell labeling was performed using polyclonal antibodies against IL-6 (Genorise Scientific, USA) and TNF-α (R&D Systems, Germany). Heat-induced antigen retrieval was performed as follows: slides were incubated in the target retrieval solution pH=9.0 (Dako, Denmark) for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and slides were washed with TBS (tris-buffered saline) for 5 min each. Tissue samples were labeled with antibody solutions. Primary antibodies were incubated for 20 min at 20°C. Detection was performed with EnVision™ Systems (Dako, Denmark). Sections were counterstained with Mayer's hematoxylin for 1 min, dehydrated and sealed. Analysis was carried out with optical microscope (Axio Imager A1, Zeiss Germany).

Morphometric Examination

Morphometric analysis of adipose tissue was performed with an Axio Imager light microscope (Zeiss, Germany) using 10 fields of view for each individual (each slide). Photographic documentation was compiled using the Axio Camera (Zeiss, Germany).

Statistical Analysis

Verification of data normality was performed using Shapiro-Wilk test, whereas the Levene's test was used to assess the equality of variances. Depending on the results of data validation, comparison analysis was performed using parametric or non-parametric statistical tests, i.e., Student's t test or Mann-Whitney U test, respectively. The P values lower than 0.05 were considered to be significant.

All statistical calculations were performed using the STATISTICA 7.0 software (StatSoft, Inc., Statistica for Windows, Tulsa, OK, USA).

RESULTS

Clinical Picture of Investigated Horses

A clinical examination of experimental animals revealed a significant degree of obesity and regional adiposity. The average body weight of horses in group A (EMS horses) was 709 kg, while in group B (control horses), the body weight was 674 kg *(Table 1)*. Horses from EMS group had unusual distribution of body fat, particularly around the base of the tail, eyes and at the base of the mane. Despite increased body weight of horses within group B, no symptoms of pathological adiposity were noticed. Horses assigned to group A were characterized by elevated levels of rest insulin (ranging from 60 to100 µU/ml). Moreover the results of CGIT test were positive. Horses assigned to group B, showed normal rest insulin level (ranging from 5 to 20 µU/ml) and the CGIT test gave negative result.

The Morphology, Morphometry, Immunohistochemistry and Immunoassays of Adipose Tissue

Adipose tissue collected from horses within group A had a dark brown color. By contrast, adipose tissue obtained from horses of group B was of pale straw color. Morphological examination of adipose tissue in group A, revealed a slight degree of mononuclear cells, abundant macrophages and lymphocytes infiltration *(Fig. 1B)*. In addition, histological examination of adipose tissue in group A exhibited a slight fibrosis *(Fig. 1B)*. In group B, no inflammatory cells infiltration was observed *(Fig. 1A)*. Adipose tissue in group B had proper histological picture *(Fig. 1A)*.

Table 1. *Body weight of individual horses qualified for the experimental and the control groups*

Tablo 1. *Deneysel ve kontrol grubundaki atların bireysel vücut ağırlıklarını kualifikasyonu*

Patient	Horses with EMS (Group A)	Healthy Horses (Group B)
1	710	625
2	684	686
3	712	680
4	680	692
5	730	720
6	710	679
7	728	645
8	721	664
X	709	674

The data of morphometric measurements of adipose tissue derived from insulin-resistant horses (group A) and the control (group B) were analyzed statistically. The results of comparative analysis showed that the average diameter of adipocytes forming the tissue obtained form group A was larger than of adipocytes in group B *(Fig. 2)*.

The immunohistochemical analysis performed to detect IL-6 distribution showed similar arrangement of this cytokine in adipocytes' membrane both in EMS and healthy horses *(Fig. 1C, D)*. The level of IL-6 in the serum was higher in group B, although statistical analysis showed no significant differences in the serum concentration of IL-6 between both test groups. The mean concentration of serum IL-6 in the group A was noted at the level of 1.893±0.0073 µg/mL, while in group B, the average level of IL-6 was 1.888±0.0079 µg/mL *(Fig. 3)*.

The analysis of TNF-α distribution showed that in adipocytes of both investigated groups this cytokine localized primarily in the nucleus *(Fig. 1E,F)*. The level of TNF-α in the serum samples was higher in group A. In this group 1.9±0.02 units of TNF-α were detected, while in group B it amounted to 1.2±0.02 units. The observed differences were statistically significant *(Fig. 4)*.

DISCUSSION

The frequency of the obesity in animals is increasing, posing a serious problem in veterinary medicine. Similarly as in the human population, the obesity in animals is considered as a medical condition of epidemic character [5,29]. The problem of obesity is especially common for small animals, i.e., dogs and cats, but it also affects other domestic animals including heavy horses (mainly draft horses). The World Horse Welfare estimates that the obesity frequency in the United Kingdom is reported in 50% of the population of pleasure riding horses [30,31]. Background for the development of horses obesity is similar to that in humans and results mainly from improper diet, physical inactivity, but also genetic predispositions. High-carbohydrate diet provides large dose of energy, which is very rarely balanced with the physical activity. The obesity affects animals by decreasing its performance, reproductive success and longevity. Moreover, in the case of horses, prolonged and untreated obesity increases the risk of development of metabolic diseases such as equine metabolic syndrome (EMS), strongly associated with insulin resistance (IR), laminitis and hyperinsulinemia. Considering these facts, the obesity in horses leads to life-threatening conditions [3,5,29,30]. The obesity in horses is often neglected by the owners, and sometimes it is difficult to determine by the veterinary clinicians. Various methods have been applied to evaluate symptoms of obesity or obesity-related diseases such as EMS. However, veterinarians are still seeking for a save diagnostic tool, helping recognize early signs of metabolic disorders,

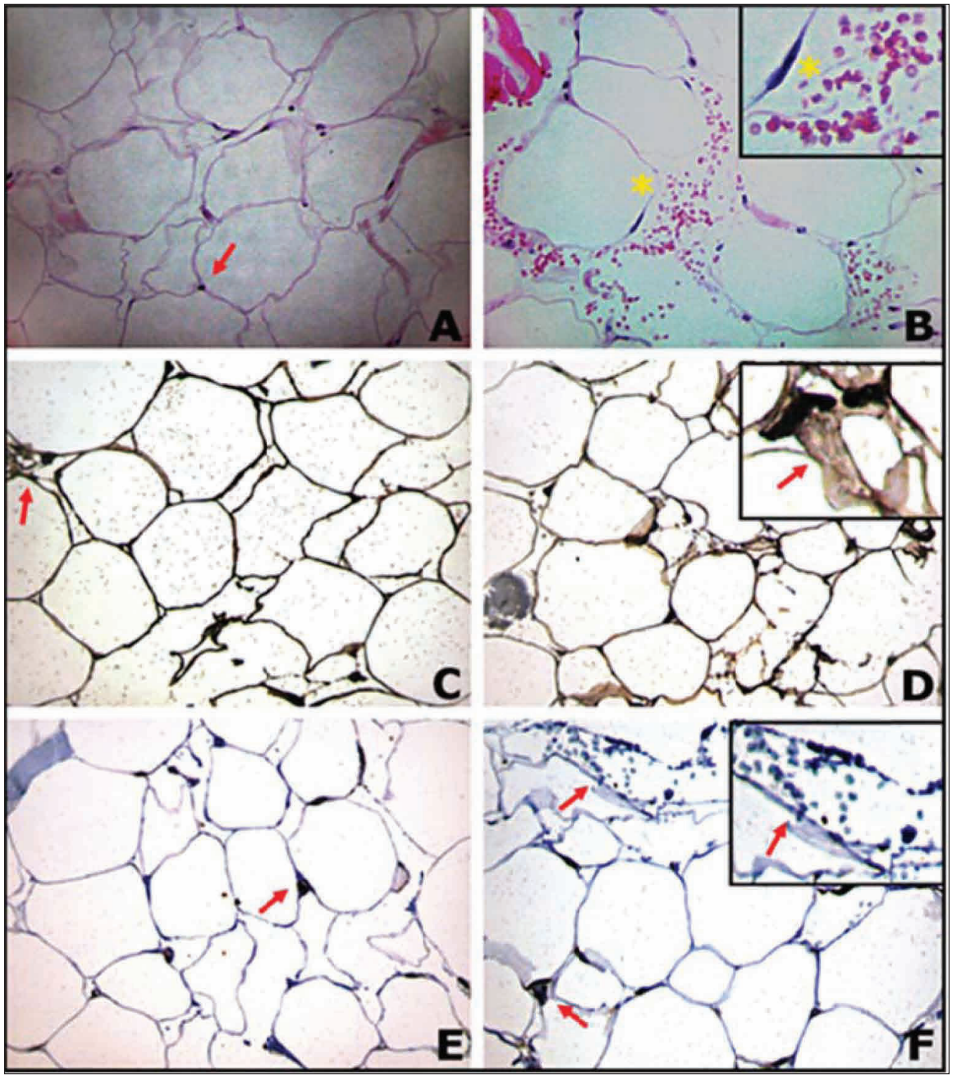

Fig 1. Histochemical and immunohistochemical analysis of adipose-tissue of EMS horses (B,D,F) and control horses (A,C,E). Inflammatory infiltration of macrophages and lymphocytes in EMS adipose tissue (E) and proper histological picture in control horses (E). High expression of TNF-α in the EMS group with additional macrophages infiltration (D) and slight degree of inflammatory cells in the control group [C]. Comparable expression of IL-6 in both study groups (A,B)

Şekil 1. EMS li atların (B,D,F) ve kontrol gruplarının (A,C,E) adipoz-dokularının histokimyasal ve immunohistokimyasal analizleri. EMS'li atların adipoz dokularındaki (E) ve uygun kontrol grubu atların (E) histolojik resimleri, makrofajların ve limfositlerin inflamatuar infiltrasyonu. EMS grupta ilave makrofaj infiltrasyonu (D) ile yüksek TNF-α oluşumu ve kontrol gruptaki (C) hafif dereceli inflamatuar hücreler. IL-6 nın her iki grupta (A,B) karşılaştırılmalı ifadesi

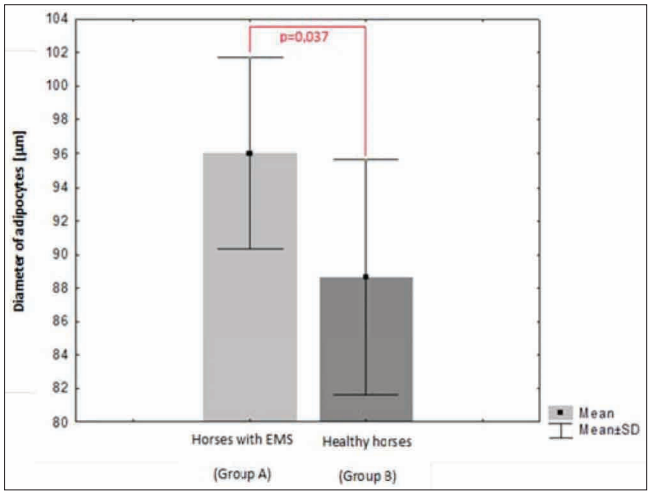

Fig 2. Statistical comparison analysis of adipocytes diameter in both groups investigated. Results were consider significant at P<0.05

Şekil 2. Her iki grupta adipozit çaplarının istatistiksel olarak karşılaştırılmalı analizi. Sonuçlar anlamlı bulundu P<0.05

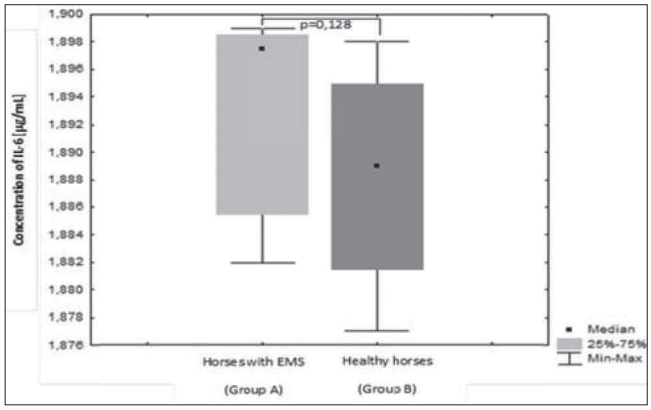

Fig 3. Comparison of IL-6 level in the serum of EMS and healthy horses

Şekil 3. EMS'li ve sağlıklı atlarda IL-6 seviyelerinin karşılaştırılması

especially when recommended methods, such as combined insulin test might be dangerous for animals' life [11,32]. Some physical characteristic of animals are crucial when diagnosing obesity. Obese horses are distinguished by the accumulation of adipose tissue especially in the tail base, area surrounding eyes and the neck region [29,32]. As it was reported previously, the obesity might correlates with the morphology of adipocytes [33]. This observation confirms

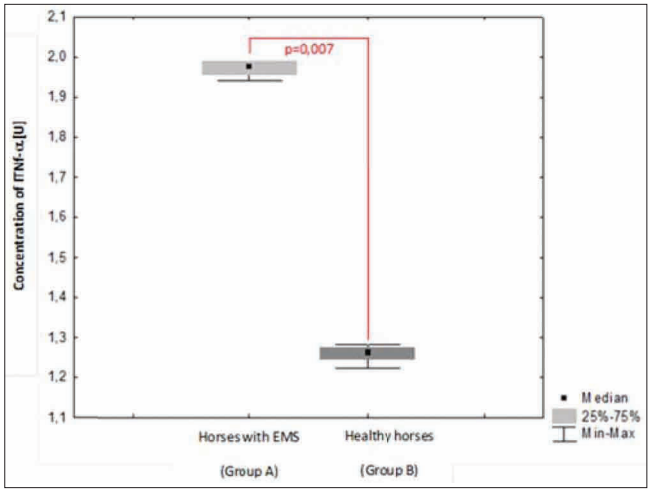

Fig 4. Comparison of TNF-α level in the serum of EMS and healthy horses

Şekil 4. EMS'li ve sağlıklı atlarda TNF-α seviyesinin karşılaştırılması

our findings where we noticed the higher adipocytes size average in EMS horses, compared to the healthy individuals with observed overweight. Due to the fact that the adipose tissue is a complex structure and may act in an endocrine manner, the disturbance of adipocytes homeostasis may have negative effect on the organism. The adipokines might act not only in an endocrine manner but also, in an autocrine and paracrine fashion, therefore, their activity might lead to adverse metabolic consequences and may give different clinical picture of disease. The body mass increasing contribute to dysfunction of secretion of adipokines, which influence on inflammation and glycemic function [34-36]. Furthermore, the hypertrophic adipocytes secrete a variety of factors that foster both peripheral and hepatic resistance to insulin. Among the adipokines, the raised activity of TNF-α and IL-6 might play a key role in the development of insulin resistance and might lead in consequence to the development of EMS [7]. Moreover the subinflammatory state, induced by obesity, can be accelerated by the macrophages infiltrating of the adipose tissue, - which additionally promotes a chronic condition [35,36]. Our research showed abundant infiltration of macrophages and lymphocytes in adipose tissue of EMS horses whereas healthy individuals did not exhibited this phenomenon. Taking under consideration that the IL- 6 and TNF-α are involved in the development of insulin resistance, we decided to investigate if there are correlation between the level of both cytokines in the serum and their distribution in in adipose-tissue. We found the elevated level of both cytokines in the serum of EMS horses (group A). However, a statistical analysis reviled only significant differences in the concentration of TNF-α between the groups. Immunohistochemical analysis showed intracellular localization of the TNF-α in adipocytes of EMS as well as in control horses. Uysal et al.[37] showed that the deletion of TNF-α 1 or TNF-α 2 receptor results in a significantly improved insulin sensitivity in the diet-induced obesity in mice but also in leptin-deficient ob/ob mice [37]. Other research reported that TNF-α may act as pivotal mediator in the insuline resistance as high TNF-α expression in human fat tissue correlated positively with BMI, percentage of body fat, and hyperinsulinemia, whereas weight loss caused a decrease of TNF-α level [38]. Based on our results, we argue that in BSC-related insulin resistance, the TNF-α may play a key role. Our results correlates with the data obtained by other research groups in animal experimental models as well as in human patients [7]. The role of IL-6 in the insulin resistance is not clear because of conflicting data. However, some research has revealed an relationship between IL-expression in the mares obesity and their age [7,39]. With the increase of BCS in mares under 20 years of age there was a decrease of IL-6. However, in human, it seems that high level of IL-6, which is typical for chronic inflammation states, is associated with the obesity and type 2 diabetes [7]. They, in turn, can result in insulin resistance and temporarily elevated level of this cytokine not affecting glucose homeostasis [40]. Our research showed similar arrangement of IL-6 distribution in adipocytes in both study groups and no significant differences in the serum concentration of IL-6 both in EMS and healthy horses. The researched horses were younger than fifteen years of age and older than seven years of age. Thus, our results might suggest the effect of age on activity IL-6 in adipose tissue and peripheral blood in obesity horses. However, the results emphasize the need for further studies to explain the role IL-6 in the course of the pathophysiology EMS. In the present research we showed correlation between the level of TNF- α in serum as well as in adipose tissue in EMS horses. We state that this information's might be used as an auxiliary tool in the process of equine metabolic syndrome diagnosis.

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Kafkas Univ Vet Fak Derg
20 (4): 501-506, 2014
DOI: 10.9775/kvfd.2013.10416

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RESEARCH ARTICLE

Histopathological Investigations on Renal Lesions in Slaughtered Camel *(Camelus dromedarius)* in North East of Iran

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Makale Kodu (Article Code): KVFD-2013-10416

Summary

Camel can tolerates water deprivation conditions more than two months. Kidneys are vital organs in this case. The objective of this study is recognition of kidney lesions. In this study 38 samples of kidney were collected from slaughter house of Tabbas and Mashhad. Specimens were fixed in 10% neutral buffered formalin for at least 24 hours and then routinely processed and then the sections were stained with Haematoxylin and Eosin (H&E), special staining and immunohistochemistry. Histopathological study revealed different lesions and the most common were glomerulonephritis. Other lesions were acute tubular necrosis (ATN), renal cyst and interstitial nephritis. Vascular calcification was significant lesions which were characterized with basophilic material in the intima of vessels. Renal adenoma was diagnosed in one camel and according to review literature it is the first report in camel.

Keywords: *Kidney, Renal Adenoma, Camelus dromedaries, Histopathology, Immunohistochemistry*

İran'ın Kuzey Doğusunda Kesimi Yapılan Develerde *(Camelus dromedarius)* Böbrek Lezyonları Üzerine Histopatolojik Araştırmalar

Özet

Deve su yoksunluğu koşullarına iki aydan fazla dayanabilmektedir. Böbrekler bu durumda hayati organlardır. Bu çalışmanın amacı, böbrek lezyonlarının belirlenmesidir. Bu çalışmada 38 böbrek numunesi Abbas ve Meşhed kesimhanelerinden toplandı. Numunelerin en az 24 saat % 10 nötral tamponlu formalinle tespit ve rutin işlemlerinin ardından kesitler Hematoksilen ve Eosin (H & E), özel boyama ve immünohistokimya ile boyandı. Histopatolojik inceleme en yaygını glomerulonefrit olmak üzere farklı lezyonları ortaya koydu. Diğer lezyonlar akut tübüler nekroz (ATN), renal kist ve interstisyel nefrit olarak belirlendi. Vasküler kalsifikasyon damarların intimasında bazofilik materyal ile karakterize önemli lezyonlardı. Renal adenom bir devede belirlendi ve bu durum literatür incelemesine göre develerde ilk kez bildirilmiştir.

Anahtar sözcükler: *Böbrek, Renal adenom, Camelus dromedaries, Histopatoloji, İmmünhistokimya*

INTRODUCTION

Camel is a comparatively hardy animal and is less susceptible to many of the diseases that affect other livestock species in the same area [1]. However, it is apparent that we know too little about the diseases from which it does suffer. In different reports, tubulonephrosis due to DNA virus [2] and Ricketsia-like organisms [3], glomerulonephritis due to aflatoxicosis [4], glomerulonephritis and interstitial nephritis [5] were described. Kidney lesions, especially nephritis, are major diseases of domestic animals. Outbreaks occur in camel, cattle, buffaloes and small ruminants in various countries of the world. This study was conducted with the objective of identifying the types of histopathological kidney lesions of slaughtered camels in north east of Iran.

Tumours have been rarely documented in captive camelids. In the Arabian dromedary *(Camelus dromedarius)* there have been reports of an ovarian teratoma [6],

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bronchioloalveolar adenocarcinoma [7], lymphocytic leukaemia [8], renal cell carcinoma [9] and osteosarcoma [10]. According to the authors knowledge no reports on renal cell adenoma have been published and this paper described the pathological and immunohistochemical findings of this tumor.

MATERIAL and METHODS

Thirty eight kidneys which exhibited grossly lesion were obtained from abattoir in Mashhad and Tabas in Iran. The tissues were fixed in 10% neutral buffered formalin for 48 h and processed according to routine procedure and sectioned at 5 µm and stained with Haematoxylin and Eosin (H&E). Selected sections were stained with von Kossa for demonstration of calcium deposition and periodic acid-schiff (PAS). Immunohistochemical staining has been carried out for cytokeratin in a kidney suspected to tumor. The sections were examined using light microscope.

RESULTS

The kidneys of slaughtered animals showed different lesions which are summarized in *Table 1*.

Glomerulonephritis was the most common lesions recorded in 29% of the camels. Glomerular tufts were enlarged and occupied the Bowman's space and also adhesions to parietal layer of Bowman's capsule were seen and the capillaries of tufts were congested. This category has been done according to histochemical staining (PAS) slides. Seven (18.4%) cases were diagnosed as membranous glomerulonephritis with increased mesangial matrix and basement membrane thickening in glomerular tufts. In most cases, the Bowman's spaces were filled with serofibrinous exudations (*Fig. 1* and *Fig. 2*) which were positive in PAS staining and were encountered as proteinuria. The amounts of mentioned materials in some cases were increased and so the cell elements of glomerules were vanished.

ATN was counted as second ranked lesions. The epithelium of proximal tubules showed pyknosis with eosinophilic cytoplasm *(Fig. 3)*.

Table 1. *Different renal lesions encountered on slaughtered camels*
Tablo 1. *Kesilen develerde karşılaşılan farklı renal lezyonlar*

Lesions Type	Number (%)
Glomerulonephritis	29 (76.3%)
Acute tubular necrosis	10 (26.3%)
Vascular calcification	5 (13.1%)
Renal cysts	2 (5.3%)
Interstitial nephritis	1 (2.6%)
Renal cell adenoma	1 (2.6%)

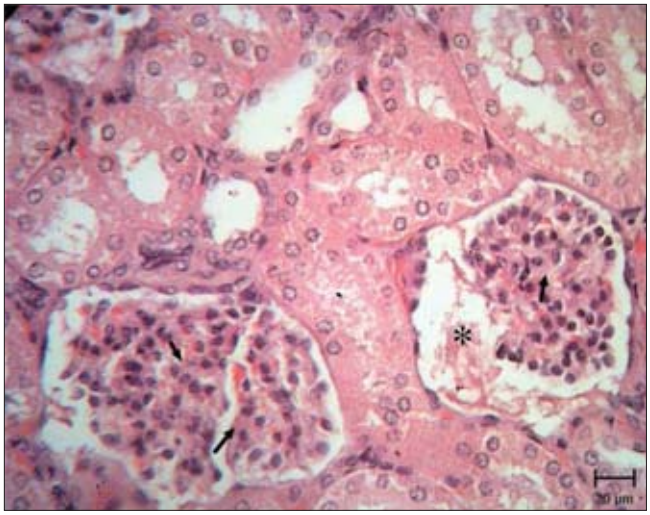

Fig 1. Camel kidney. Glomerulonephritis. Note to increasing mesangial matrix *(arrows)* and proteinuria *(star)* with amorphouse eosinophilic materials in Bowman's space (H&E)

Şekil 1. Deve böbreği. Glomerülonefrit. Bowman's alanında artan mesanjiyal matris *(oklar)* ve şekilsiz eozinofilik materyalli proteinüri *(yıldız)* görülmektedir (H&E)

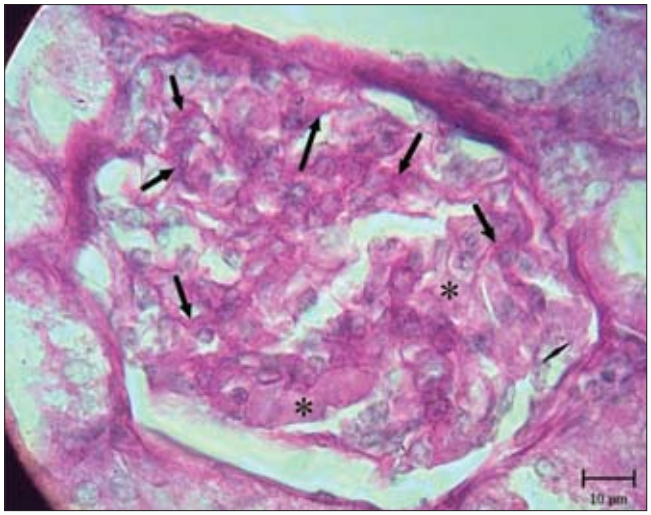

Fig 2. Camel kidney. Glomerulonephritis. Thickening of basal membrane *(arrows)* and increased mesangial matrix *(star)* is obvious (PAS staining)

Şekil 2. Deve böbreği. Glomerülonefrit. Bazal membranın kalınlaşması *(oklar)* ve artan mesanjiyal matris *(oklar)* oldukça belirgin (PAS boyama)

Mineralization of vessels walls were detected in five kidneys in various degrees. Purplish color granules with different sizes which stained black color in von Kossa staining method were deposited in vessels. The calcium salts were completely replaced the muscular fiber in affected vessels (*Fig. 4* and *Fig. 5*).

Renal cysts were seen in two cases. The cysts were single and covered by flattened epithelium. They were elliptical and minimum diameters were 600 µm. Also they located sub capsular *(Fig. 6)*.

Focal interstitial nephritis was observed in one kidney. It was characterized by accumulation of lymphocytes

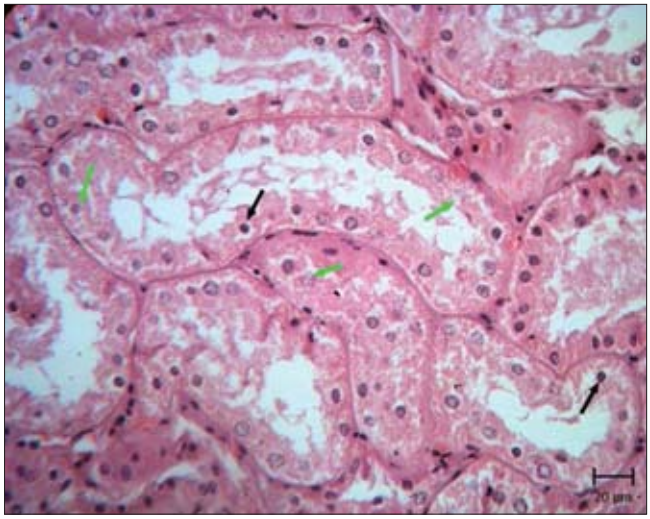

Fig 3. Camel kidney. Acute tubular necrosis. Note to pyknotic nuclei *(dark arrows)* and karyolysis *(green arrows)* which indicated coagulative necrosis of renal proximal tubules (H&E)

Şekil 3. Deve böbreği. Akut tübüler nekroz. Piknotik çekirdekler *(koyu oklar)* ve renal proksimal tübüllerin koagülatif nekrozu belirtisi olan karyoliz *(yeşil oklar)* görülmektedir (H&E)

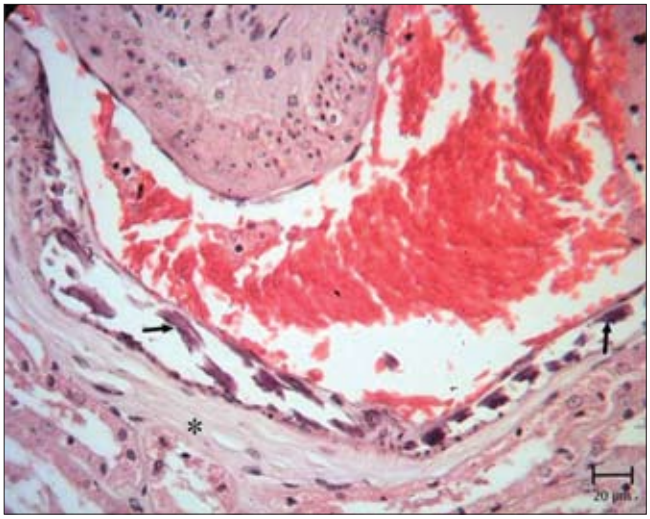

Fig 5. Part of figure 3 with high magnification. Purple precipitation *(arrows)* is obvious beneath endothelial layer and between smooth muscles of medial layer *(star)* (H&E)

Şekil 5. Yüksek büyütme ile Şekil 3'ün bir bölümü. Mor presipitasyon *(oklar)* endotel tabakasının altında ve medial katmanın düz kasları arasında belirgindir *(star)* (H&E)

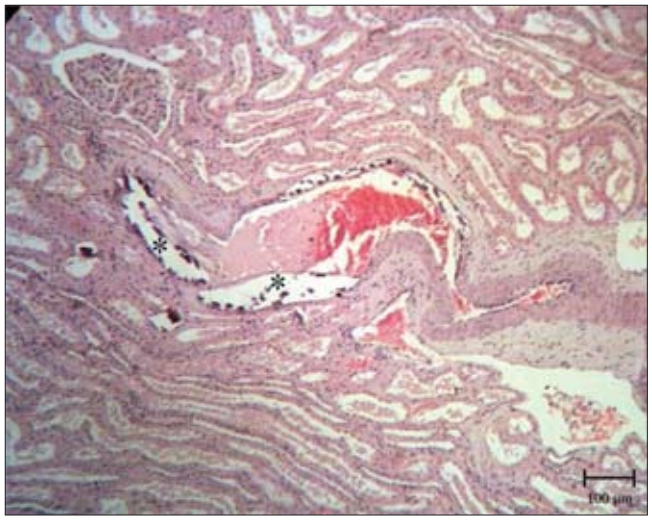

Fig 4. Camel kidney. Note to calcification of vessel wall *(star)* (H&E)

Şekil 4. Deve böbreği. Damar duvarında kalsifikasyon *(yıldız)* görülmektedir (H&E)

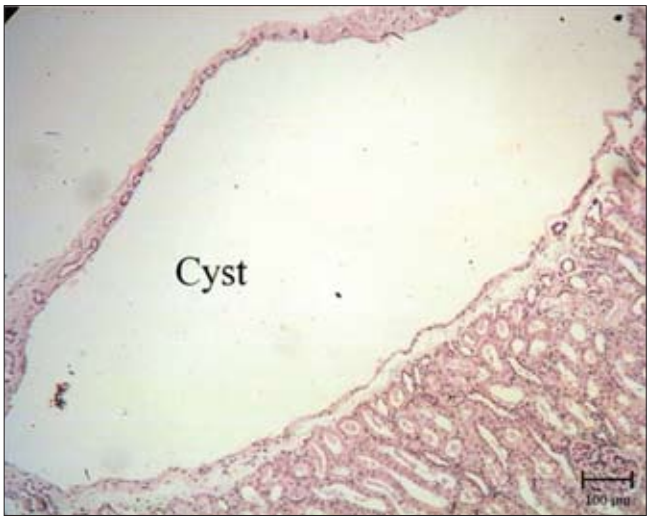

Fig 6. Camel kidney. Renal cyst is located in cortex. Note to flattened epithelium which covered the cyst wall (H&E)

Şekil 6. Deve böbreği. Böbrek kisti kortekse yerleşmiştir. Kist duvarını örten düzleşmiş epitel tabakası görülmektedir (H&E)

and plasma cells in cortico medullary area and in one case they were accumulated around glomerulous *(Fig. 7)*.

Renal adenoma with tubulopapillary pattern was diagnosed in one of the kidneys. There were multiple foci of proliferated tubules which were covered by cuboidal epithelial cells. The cells had single nuclei with single nucleolus. Also papillary projections were seen and comprised of fine, richly vascularized stroma covered with one or more layers of neoplastic cuboidal cells. In some areas the papillae were dense or finger-like projections proliferating into cystlike microcavities (*Fig. 8* and *Fig. 9*). Tubules pleomorphism, cellular atypia, mitotic figures and nuclear hyperchromatism were rare.

Immunohistochemical staining illustrated strong reaction in proliferated cells for cytokeratin. They showed dark brown staining (*Fig. 10* and *Fig. 11*).

DISCUSSION

In the present work, type and frequency of microscopic kidney lesions were encountered on camels slaughtered in north east of Iran, Mashhad and Tabas. Histopathological study of the kidneys revealed different lesions such as glomerulonephritis, acute tubular necrosis, interstitial nephritis, vascular calcification and renal adenoma. Other

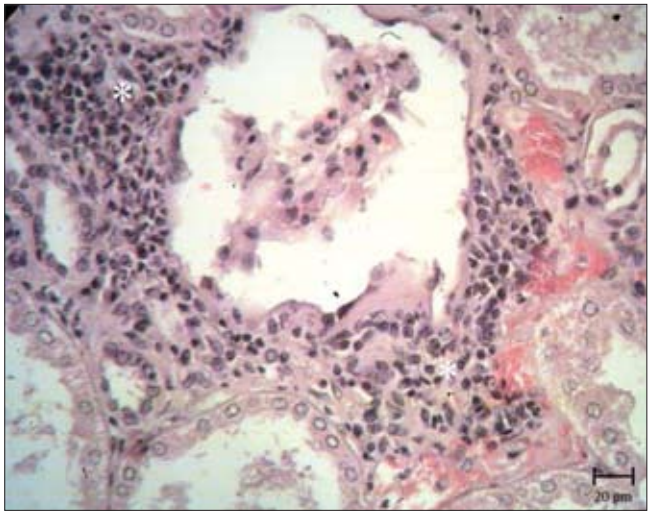

Fig 7. Camel kidney. Note to accumulation of inflammatory cells in the interstitial tissue *(star)* (H&E)

Şekil 7. Deve böbreği. İnterstisyel doku yangı hücrelerinin birikmesi *(yıldız)* görülmektedir (H&E)

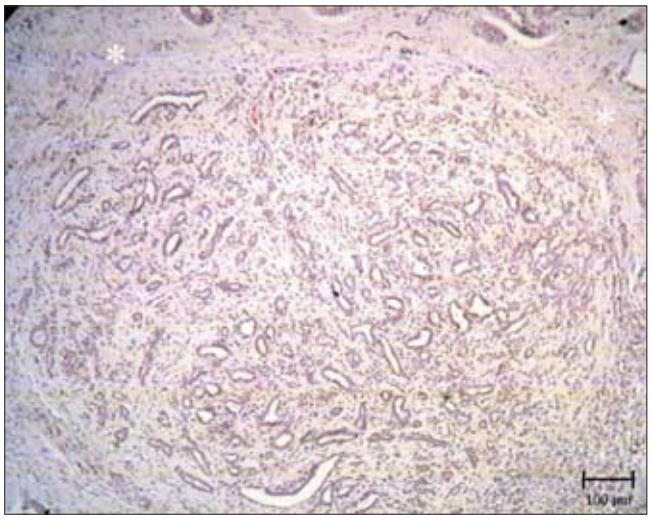

Fig 8. Camel kidney. Big foci of proliferated cells which formed tubules and surrounded by connective tissue *(star)* are seen (H&E)

Şekil 8. Deve böbreği. Tübülleri oluşturmuş ve bağ dokusu ile çevrili çoğalmış hücrelerin büyük odakları *(yıldız)* görülmektedir (H&E)

researchers also have been reported high prevalence of renal lesions in other countries [5,11]. This high prevalence of kidney lesions especially renal cell adenoma could be due to the older age of animals at slaughter with possibility of being exposed at least once to one or more agents that can induce renal disease.

The most recorded lesions of kidney were glomerulonephritis in this study. Glomerulonephritis results often from immune - mediated mechanism, which is due to involving antibodies to glomerular basement membrane or deposition of soluble immune complexes within the glomeruli. Second type of glomerulonephritis occurs in association with persistent infections or other diseases that characteristically have a prolonged antigenemia [12,13].

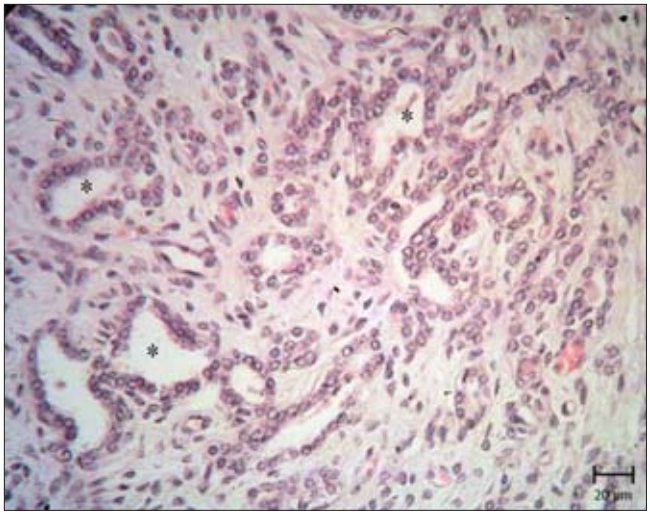

Fig 9. Part of Fig. 7 with high magnification. Note to new tubules *(star)* which covered by uniform epithelial cells and loose connective tissue around tubules (H&E)

Şekil 9. Yüksek büyütme ile şekil 7'nin bir bölümü. Aynı yapılı epitel hücreleri ile çevrili yeni tübüller *(yıldız)* ve tübüller çevresinde gevşek bağ dokusu görülmektedir (H&E)

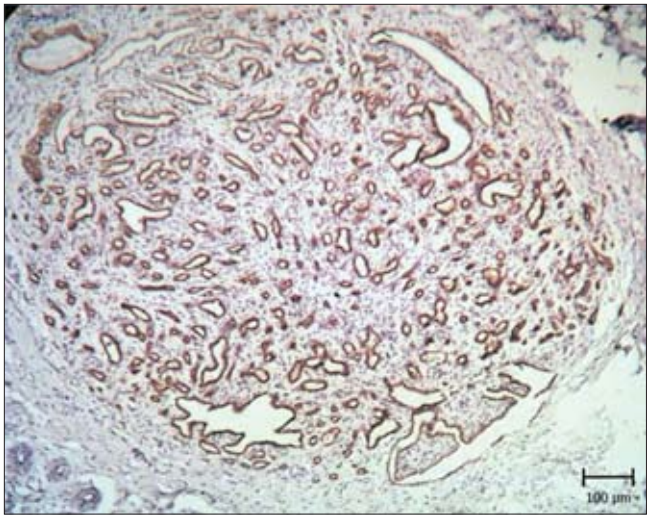

Fig 10. Renal adenoma. Note to brown tubules which are located near together. Cytokeratin immunostaining, haematoxylin counterstain

Şekil 10. Renal adenom. Yakın olarak birlikte bulunan kahverengi tübüller görülmektedir. Sitokeratin immünboyaması, hematoksilen zıt boyaması

In this study the cause of glomerulonephritis probably is prolonged infectious disease which is unclear and needs to investigate in future. Glomerulonephritis is relatively prevalent in domestic animals and represents a common form of renal disease. It also has been reported as a sequel to chronic infectious disease [14].

ATN was showed in 10 cases. It is usually due to ischemic or a toxic insult to the renal tubular epithelial cells. In first one, disruption of the tubular basement membranes occur and tubular repair in such kidneys is imperfect. The second one is due to various classes of

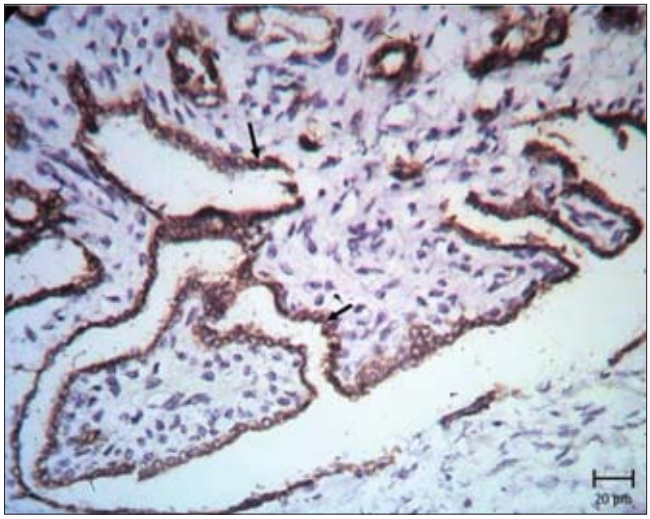

Fig 11. Part of figure 9 with high magnification. Note the brown nuclei of the epithelial cells covered the tubules *(arrows)*. Cytokeratin immunostaining, haematoxylin counterstain

Şekil 11. Yüksek büyütme ile şekil 9'un bir bölümü. Tübüller tarafından kaplanmış epitel hücrelerinin kahverengi çekirdekleri *(oklar)*. Sitokeratin immünboyaması, hematoksilen zıt boyaması

naturally occurring or synthetic compounds. Nephrotoxins usually do not damage the tubular basement membranes [12,13]. ATN which were observed in this study may be due to nephrotoxins which were naturally consumed by animals because the basement membranes of renal tubules were intact.

Renal cysts were seen in 2 cases. Renal cyst is common in pigs and calves. Taha et al.[11] reported cystic dilation of renal tubules in camels. Renal cyst may located anywhere in either cortex or medulla and they range from those barely visible to those several centimeters in diameter [12,13]. In these cases, the cysts were hardly seen in macroscopic and they were in cortex. They have thin walled and lined by flattened epithelium [12] which is in agreement with these cases characteristics. Also they are usually spherical. In this study mentioned cysts were elliptical and may be this is due to processing steps.

Vascular calcification was detected in more than 13 percent of slaughtered camels. These precipitations were seen in muscular middle layer (tunica media) of arteries. This finding is equivalent of Monckeberg's arteriosclerosis or medial calcific sclerosis of humans [15]. Minor degrees of calcification of the cardiovascular system are common in elderly people [16]. Vascular calcification results from deposition of calcium phosphate crystals (hydroxyapatite) as a consequence of disordered calcium phosphate regulation in the blood vessels. The mechanism of vascular calcification is not fully understood, but probably involves a phenotypic change in the vascular smooth muscle cells in the wall with activation of bone-forming program [17,18]. According to review literature there was no report in camelide about vascular calcification and maybe this is due to their age.

Renal adenoma is rare tumor in domestic animals and when found it usually is an incidental lesion at necropsy or slaughter because these tumors are clinically silent [19]. Also this case was related to a camel which slaughtered. They are said to occur more often in cattle and horses than in other species [12,13]. In dogs they comprise about 15% of primary renal epithelial tumors. Renal adenoma arises from epithelium of the proximal convoluted tubules. Grossly, they tend to be solitary nodules less than 2 cm across but occasionally are larger. They grow expansively. Microscopically, the tumor cells are cuboidal with moderate to abundant acidophilic cytoplasm. They form tubules and acini that may be subclassified as tubular, papillary, or solid based on the major histopathological pattern: central or elongated lumen (tubular type); papillary growth of varying sizes that project into lumens (papillary) or solid sheets. Mixtures of all three types can occur [19]. In this case mixtures of tubular and papillary form were seen and solid sheets were absent. Histologic differentiation of adenoma and renal carcinoma is sometimes impossible. A few adenomas may be small well differentiated carcinoma [13]. In this case pleomorphism, cellular atypia and mitotic figures were rare so it was detected as renal adenoma. According to histopathological and immunohistochemical testing and to the best of author's knowledge; this is the first report of renal adenoma in a *Camelus dromedarius*.

Acknowledgment

The authors wish to express their gratitude to the research council of Shahid Chamran University for their financial supports. The authors thank Miss and Mr Behdarvand for their technical assistance.

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Kafkas Univ Vet Fak Derg
20 (4): 507-511, 2014
DOI: 10.9775/kvfd.2013.10436

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RESEARCH ARTICLE

The Importance of Protein Expression SOD2 in Response to Oxidative Stress for Different Cancer Cells [1]

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[1] *This study was presented as a poster at 6th National Veterinary Biochemistry and Clinical Biochemistry Congress (25-27 June 2013, Kars - Turkey)*

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Makale Kodu (Article Code): KVFD-2013-10436

Summary

As a causal relationship between DNA damage, repair mechanism disorders and cancer demonstrated by experimental and epidemiological data. Transforming process starts from DNA damage to oxidative stress is associated with mutational inactivation of suppressor genes, activation of oncogenes. Related to this aim, it is important to indicate how cancer cells react under oxidative stress through by SOD2 expression, besides presenting correlation between NF-kB mechanism. Cell lines were maintained and cultured as recommended by ATCC (American type culture collection) resource. Cells were grouped and after detecting optimum concentration by MTT test (cell viability assay), treated with DNA damage agent (MNNG) and antioxidant (tempol) for indicated time points. Western blot analysis revealed that cell lines with comparable levels of SOD2 (Superokside dismutase 2) protein expression. However, cells were collected to measure NF-kB (Nuclear Factor kappa B) enzymatic activity using luciferase expression by transfection way. We observed high constitutive NF-kB activity by using 20 µM MNNG although decreasing NF-kB during 30 µM tempol treatment. The results showed strong correlation between SOD2 expression and NF-kB activation.

Keywords: *Antioxidant, SOD2, DNA damage*

SOD2 Salınımının Farklı Kanser Türlerinde Oksidatif Strese Karşı Değişimi ve Önemi

Özet

DNA hasarı, onarım mekanizmasındaki bozukluklar ve kanser arasındaki nedensel ilişki deneysel ve epidemiyolojik veriler ile gösterilmiştir. DNA hasarının oksidatif strese dönüşüm aşamaları baskılayıcı genlerin mutasyonal inaktivasyonu ve onkogenlerin aktivasyonu ile ilişkilendirilir. Bu amaç doğrultusunda, kanser hücrelerinin SOD2 (Superoksit dismutaz 2) değişimine bağlı olarak oksidatif stres koşullarındaki etkileri ve bu mekanizmada NF-kB (Nükleer Faktör kappa B) transkripsiyon faktörünün önemi gösterilmiştir. Hücre hatları ATCC'nin (Amerikan tipi kültür kolleksiyonu) belirttiği prosedürlere uygun olarak kültüre edilerek saklandı. Hücreler gruplara ayrıldıktan sonra, MNNG ve tempol ajanlarının optimal konsantrasyonun hücre canlılık (MTT test) yöntemiyle belirlenmesiyle belirtilen zaman aralıklarında uygulandı. Hücre hatlarındaki farklı SOD2 protein düzeyleri western blot analizleri ile gösterilerek karşılaştırıldı. Bununla birlikte transfeksiyon yoluyla yapılan luciferase yöntemi kullanılarak NF-kB enzimatik aktivitesi belirlendi. Bunun sonucunda, 20 µM MNNG uygulandığında yüksek NF-kB aktivitesi gözlenirken, 30 µM tempol tedavisi ile NF-kB seviyesinin azaldığı görüldü. Sonuçlar SOD2 ekspresyonu ve NF-kB aktivasyonu arasında güçlü korelasyon gösterdi.

Anahtar sözcükler: *Antioksidan, SOD2, DNA hasarı*

INTRODUCTION

Oxidative stress is described as imbalance between production of free radicals and reactive metabolites, is called reactive oxygen species (ROS), and their eradication by protective mechanisms, referred as antioxidants [1]. It's also important from biomedical point of view which is related to human diseases such as neurodegenerative, cardiovascular, inflammatory, allergies, immune dysfunctions, diabetes, aging and cancer [2]. Oxidative stress has been

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implicated in both apoptosis and the pathogenesis of cancer providing contrived support for two notions: free radical reactions may be increased in malignant cells and oxidant scavenging systems may be useful in cancer therapy [3,4].

ROS (Reactive oxygen species) are induced by a variety of endogenous and exogenous sources [5]. At pathologically high levels, ROS cause damage to biological molecules, including DNA. It has been estimated that around 2×10^4 DNA damaging events occur in every cell of the human body everyday. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions (8-OH-G) have been noted in various tumours, strongly implicating such damage in the etiology of cancer [6]. Importantly, ROS does not always occur products of cellular metabolism sometimes they are generated by specific plasma membrane oxidases in response to growth factors and cytokines and serve as secondary messengers in specific signaling pathways [7,8]. ROS signaling is reversible, tightly controlled through a regulatory network. This network results from a concerted assembly of protein complexes, built through protein interactions mediated by interaction modules and posttranslational modifications in the binding partners [9]. Constitutive activation of cell survival signaling is a general mechanism underlying tumor development and resistance to therapy and constitutes a major clinical problem in cancer [8,9]. Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle. If the damage is determined to be beyond repair, the cell may undergo apoptosis to prevent mutations from being propagated [10]. An optimal cellular damage response requires both repair of damage and coordination of critical cellular processes such as transcription, translation, and cell cycle progression [11,12].

Antioxidant defences are built in a complex network of nonenzymatic and enzymatic components of the cell [13-15]. Enzymatic antioxidants include SOD, catalase, peroxiredoxin, and glutathione peroxidase (GPx) [16]. Superoxide radicals formed on both sides of mitochondrial inner membranes are efficiently detoxified initially to hydrogen peroxide and then to water by Cu, Zn-SOD (SOD1, localised in the intermembrane space) and Mn-SOD (SOD2, localised in the matrix) [17-19].

Many conditions activating NF-kB are known to induce oxidative stress. In other words, these conditions increase the production of reactive oxygen species (ROS) such as superoxide (SOD), H_2O_2 and secondary reactive compounds. The reduction of SOD activity in neoplastic and transformed cells is regulated at the transcriptional level including NF-kB. Several studies have also shown different antioxidants inhibition of NF-kB in response to phosphorylation mechanism [20].

The aim of our study that cancer cells show upregulation SOD_2 under stress condition. We expect here to identify the ROS products and to gain more direct evidence for an involvement of ROS as messengers, also investigate whether changes in levels of enzymes that control intracellular ROS levels affect the activation of NF-kB.

MATERIAL and METHODS

Cell Experimental Procedures

Human cervical cancer (HeLa), human embryonic kidney cell (HEK293), human colorectal carcinoma (HCT116), human breast cancer (MDA-MB-231) cells were purchased from Dr. Brown lab (Shands cancer center FL/US) and cultured in 100 mm dish in DMEM (Invitrogen Co. USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (10.000 U/mL) at 37°C in constant atmosphere of 5% CO_2 in humidified air. All experiments were performed between passages 4 and 6.

Cell Treatments

Cells were grouped by several experimental applications. The plates were incubated for 24-48 h to allow complete 70-80% reattachment of the cells. Then detect subsequent optimum concentration on agent by MTT assay [21], cells were assigned to five groups and treated with 10 µM, 20 µM, 50 µM, 100 µM of MNNG (DNA damage agent) and control group. On the other side, cells were treated with 15 µM, 30 µM, 60 µM, 150 µM of tempol (antioxidant agent) and control group during 24 h, 48 hrs and 72 h. The absorbance of the plates was measured on ELISA microplate reader (Benchmark, BioRad) at a wavelength of 540 nm. After measurement, cell proliferation were also evaluated using inverted microscopy and then treated 20 µM MNNG for indicated time points of 48 h and 30 µM tempol for 7 days [22].

Western Blotting

Cells were washed with cold phosphate buffered saline (PBS) after treatment and lysed with RIPA buffer which contains 150 mM NaCl, 1% NP-40, 0.1% SDS, 50 mM Tris. Then the cell suspension transferred into a precooled microfuge tube, incubated on ice at 10 min and centrifuged for 10 min at 4°C. The protein concentration of each sample were determined using BCA protein assay and bovine serum albumin (BSA, Roche applied science) was used as a protein standard. Equal amounts of protein were separated by 12.5% SDS-PAGE for SOD2. Then transferred electrophoretically onto nitrocellulose membranes [23].

Clarified membranes were blocked with 5% skim milk in TBS at room temperature for 1 h, the following stage subsequently incubation with primary antibodies dilutions anti-SOD2 (1:1000, Sigma) at a predetermined optimal concentration for 2-4 h at room temperature or overnight at 4°C. Membranes washed 3 times with rinsing buffer. Afterwards they incubated in proper secondary ab (rabbit) solution during 1 h at room temperature.

After three washes with TBS, the resulting immunocomplexes were visualized by enhanced chemiluminescence (Supersignal west pico, Fisher, USA) and exposed to X-ray film (Fuji medical X-ray film, Tokyo, Japan). Differences in protein loading were monitored by reprobing stripped membranes with anti-β Tubulin antibodies [24].

Luciferase Reporter Assay

To specify NF-kB activity by Luciferase assay in addition to protein expression experiments, cells were seeded in to 12 well plates at a density of $2x10^4$ cells per well with 2 mL of FBS+DMEM and incubated for 24 h before the transfection study. NF-kB was constructed within the lentiviral plasmid vector (pLKO,1.9kb) followed by transformation into *Escherichia coli*. From consisting several colonies, picked most of them for mini-preparation of the plasmids. Then digested with restriction enzyme (EcoRI) to check for orientation of insertion. Digested plasmid was run with the uncut version and a DNA marker on an agarose gel to determine the size of plasmid. Furthermore, NF-kB concentration (266.9 ng) was measured from vector *E. coli* plasmid DNA by spectrophotometric method. Briefly, MDA-MB-231, HeLa, HEK293, HCT116 were 70% confluence transiently transfected in 12 well plates by turbofect reagent (Fisher, USA) in a 1 mL medium containing 2.0 µg NF-kB promoter with the control plasmid DNA (reference DNA, 171.29 ng). Between experimental group (NF-kB) concentration and control group (reference DNA) concentration should be 1:20 ratio in each well of total volume. Transfections were performed in triplicate. NF-kB transfected cells were either left untreated or treated with 20 µM MNNG, 30 µM tempol. Twenty four hours after treatment, the conditioned medium was removed and lysed with dual luciferase lysis buffer for 15 min.

After 48 h of transfection, cells were collected to measure NF-kB enzymatic activity using luciferase expression in the clones appeared to be constitutive. Adding luciferase detection reagent for 5 min to measure firefly activity. Additionally cells were incubated with second reagent during 5 min for renilla determination. According to luciferase assay protocol, analysis of luciferase activity driven by the synthetic along NF-kB dependent reporter using an automated bioluminescence reader [25].

Statistical Analysis

We analyzed all of the data using the graphpad prism 5 statistical programs, then evaluated the statistical significance using group comparable Tukey test [26]. We considered that our results $P<0.05$ to be statistically significant.

RESULTS

To delineate the possible mechanism by which SOD2 mediates NF-kB activation, we studied the protein change effect of SODs under stress and normal conditions. The activation of SOD2 by treatment with MNNG induced activation of NF-kB, in another experiment, we examined the effect of specific inhibitor antioxidant (tempol) on SOD2 production which in turn inhibits NF-kB transcription factor *(Fig. 1)*.

Our results shown that the most common cancer cells have increased expression of SOD2 protein level while treating with DNA damage agent correlated well with a parallel increase in the enzymatic activity. Next, we determined the influence of decreased SOD2 expression during tempol treatment *(Fig. 2, Fig. 3)*. Time course and dose response studies using cells revealed that treatment with 30 µM tempol for 10 days is optimal for suppressing stress-related protein levels. We observed high constitutive NF-kB activation by using 20 µM MNNG (DNA damage agent) while optimal period although decreasing activity of NF-kB during 30 µM tempol (antioxidant) treatment *(Fig. 4)*. The results obtain as a strong correlation between SOD2 repression and NF-kB inhibition which is given in *(Fig. 5)*.

Moreover, western blot analysis revealed for different

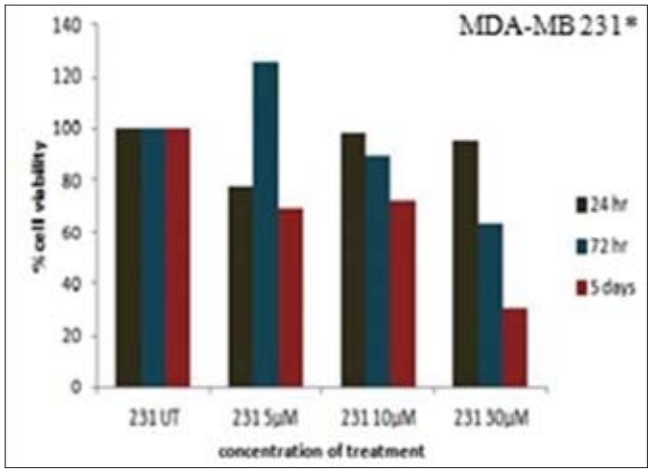

Fig 1. MTT test on MDA-MB 231 cells to detect optimal tempol concentration

Şekil 1. Optimal tempol konsantrasyonun MDA-MB 231 hücrelerinde MTT testi ile belirlenmesi

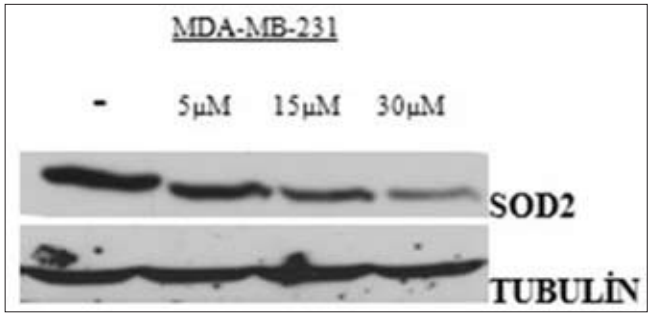

Fig 2. Different concentration of tempol effect on MDA-MB 231 cell with indicated time point

Şekil 2. Değişik zaman aralıklarında uygulanan farklı konsantrasyonlardaki tempol'ün MDA-MB 231 hücreleri üzerine etkileri

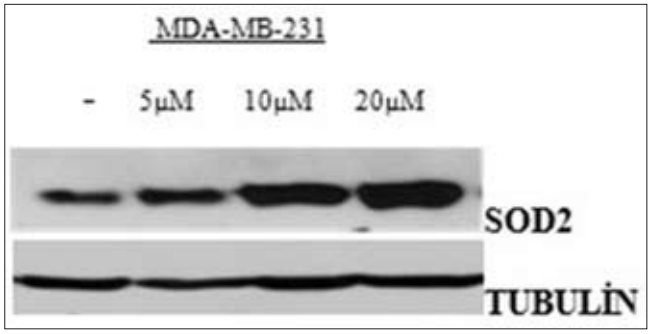

Fig 3. MNNG application on MDA-MB-231 cells to detect optimal concentration

Şekil 3. MDA-MB-231 hücrelerinde uygun MNNG konsantrasyonunun belirlenmesi

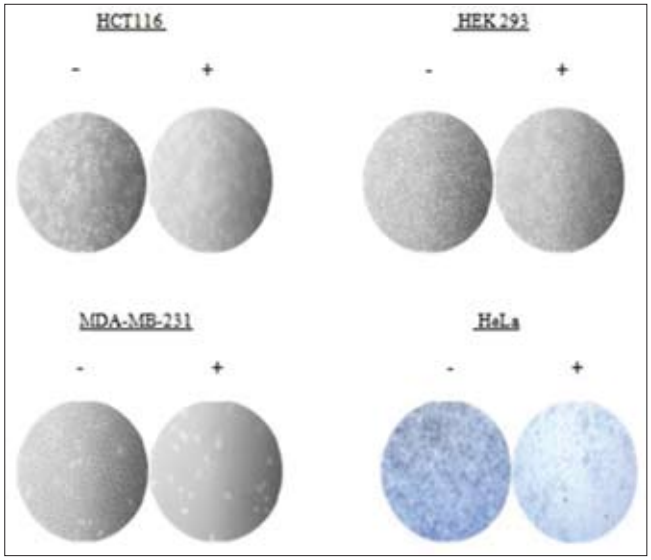

Fig 4. Cell morphology varying on antioxidant applications (30 µM tempol-48 h)

Şekil 4. Antioksidan uygulama koşullarında hücre morfolojisinin gösterilmesi (30 µM tempol- 48 saat)

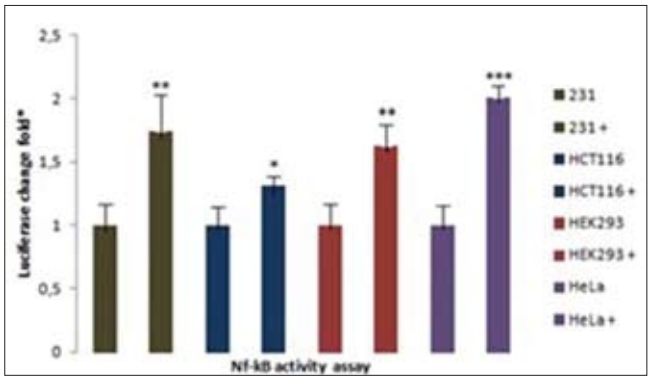

Fig 5. NF-kB transcription factor activity on different cancer cells (average fold for MDA-MB 231 cells while treat with MNNG have shown 1.7390±0.0877; for HCT116 cell 1.3111±0.0863; for HEK293 cell 1.6219±0.0845; for HeLa cell 2.0013±0.1439)

Şekil 5. Farklı kanser türlerinde NF-kB transkripsiyon faktör aktiviteleri (MNNG uygulanan MDA-MB 231 hücrelerinde ortalama değer gösterimi 1.7390±0.0877; HCT116 hücreleri için 1.3111±0.0863; HEK293 için 1.6219±0.0845; HeLa için 2.0013±0.1439)

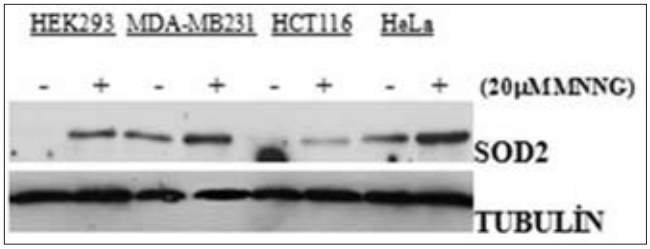

Fig 6. SOD2 expression of different cancer cell line under oxidative stress condition

Şekil 6. Oksidatif stres koşullarındaki farklı kanser türlerinde SOD2 protein ekspresyonları

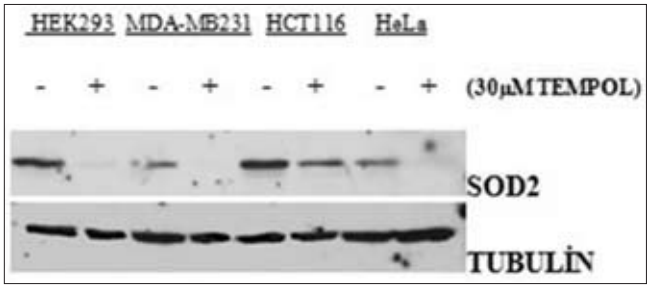

Fig 7. SOD2 expression of different cancer cell lines during antioxidant treatment

Şekil 7. Antioksidant uygulanan farklı kanser türlerinde SOD2 protein ekspresyonları

cancer cell lines have high basal levels of SOD2 protein expression. It is reasonable evidence that under oxidative stress condition, cells activate NF-kB loop and acting upregulation of SOD2 proteins *(Fig. 6, Fig. 7)*.

DISCUSSION

Our aim supports the ROS induced oxidative stress is involved in multistage process of carcinogenesis by genetic and epigenetic mechanisms [27,28]. Our study shows that when different type of cells treated by DNA damage agent, they all response to upregulation of oxidant related proteins.

The opposite affect for antioxidant treatment, we see downregulation that proteins. Evidence of our result, we detect NF-kB activity of different cancer types. The homeostasis mechanism for controlling ROS levels presented here is controlled by signalling pathways that can provide both negative and positive inputs on NF-kB [29,30]. Also have shown morphology and consistency on cells under different conditions.

Many cancer cells and cell lines have constitutive NF-kB activation, which enables malignant cells to escape apoptosis [31]. In contrast, activation of NF-kB in normal cells is transient, which prevents abnormal cell growth and survival [32].

Other studies show previously that, irrespective of their source or type, cancer cells selected for resistance to antioxidants exhibit high levels of SOD2 expression.

The data presented here suggest that SOD2 expression contributes to the development of antioxidant and metastatic phenotypes by inducing constitutive activation of NF-kB [33].

Recent research indicates that cells within a field defect characteristically have an increased frequency of epigenetic alterations and these may be fundamentally important as underlying factors in progression to cancer. Inherited germ line mutations in DNA repair genes similarly cause an increase in DNA damages due to a deficiency in repair capability, and these also cause increases in cancer risk. At least 34 inherited human DNA repair gene mutations increase cancer risk, including, for example, germ line mutations in the *BRCA1, XPC* and *MLH1* genes [34].

Controlling the cellular redox state is highly complex process governed by cellular factors and systems [35,36]. Oxidant signal, chronic inflammation, and cancer are closely linked. Antioxidants will be more useful for regulated oncogenesis and understanding pathway in future cancer research [37].

Acknowledgements

The author thanks for teaching methods, helping on experiments and suggesting about critical points to UF medical science colleagues. This research execute on Ankara University Department of Biochemistry, TR and Shands cancer center at University of Florida, US.

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Kafkas Univ Vet Fak Derg
20 (4): 513-520, 2014
DOI: 10.9775/kvfd.2013.10450

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Evaluation of Bcl-2, Bcl-X_L and Bax Expression and Apoptotic Index in Canine Mammary Tumours [1]

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Makale Kodu (Article Code): KVFD-2013-10450

Summary

Mammary tumours are the most common neoplasms in intact female dogs. Dysregulation of programmed cell death mechanisms plays an important role in the pathogenesis and progression of mammary gland tumours. The aim of this study was to investigate the relationship between some anti-apoptotic proteins (Bcl-2, Bcl-X_L and Bax), apoptotic index (AI) and histopathological diagnosis, tumour grading, tumour staging and survival time of canine mammary tumours (CMT). Twenty seven tissue samples were collected from twenty seven animals with mammary tumours. The samples were evaluated and graded histopathologically. All cases were staged according to the TNM system. The expression of Bcl-2, Bcl-X_L and Bax proteins was investigated using indirect immunoperoxidase test and apoptosis was evaluated using terminal deoxynucleotidyltransferase (TdT)-mediated nick end-labelling (TUNEL) technique. Follow-up examination and survival estimation analysis were performed. While there was a significant statistical relation between Bcl-2 expression and histopathological diagnosis ($P<0.005$), there was no considerable association between histopathological diagnosis and Bax, Bcl-X_L and AI ($P>0.05$). The differences between T1 and T5, T2 and T5 stages were statistically significant in terms of Bax expression ($P<0.05$), and Bax expressions were higher in T5 when compared with T1 or T2. No association between survival time and Bcl-2, Bax, Bcl-X_L and AI was determined ($P>0.05$). Bcl-2 was overexpressed in highly malignant tumours such as solid and tubulopapillary adenocarcinomas and Bax had high expression levels in metastatic tumours. As a result, it is concluded that Bcl-2 and Bax expression can be accessory parameters for anticipating the biologic behaviour and prognosis of CMT but these markers alone are not sufficient for the determination of survival time.

Keywords: *Canine mammary tumour, Apoptosis, Bcl-2, Bcl-X_L, Bax, Immunohistochemistry, TUNEL*

Köpek Meme Tümörlerinde Bcl-2, Bcl-X_L ve Bax Sunulumu İle Apoptotik İndeksin Değerlendirilmesi

Özet

Meme tümörleri intakt dişi köpeklerin en sık karşılaşılan tümörleridir. Programlı hücre ölüm mekanizmalarında meydana gelen düzensizlikler meme bezi tümörlerinin patogenezis ve progresyonunda önemli bir rol oynamaktadır. Bu çalışmanın amacı köpek meme tümörlerinde (KMT) bazı anti-apoptotik proteinler (Bcl-2, Bcl-X_L and Bax) ve apoptotik indeksin (AI), histopatolojik tanı, tümör derecelendirmesi, tümör evreleri ve kalan yaşam süreleri ile ilişkisini ortaya koymaktır. Meme tümörü olan yirmi yedi hayvandan yirmi yedi biyopsi örneği toplandı. Örnekler histopatolojik olarak incelendi ve derecelendirildi. Bütün olgular TNM sistemine göre evrelendi. Bcl-2, Bcl-X_L ve Bax proteinlerinin sunulumu indirect immunoperoksidaz testi ile ve apoptozis ise terminal deoksinükleotidiltransferaz (TdT)-aracılı nick end-labelling (TUNEL) tekniği ile incelendi. Hasta takibi ve hayatta kalma süresi tahmini analizleri gerçekleştirildi. Bcl-2 sunulumu ve histopatolojik tanı arasında önemli bir istatistiksel ilişki belirlenirken ($P<0.005$), histopatolojik tanı ile Bax, Bcl-X_L ve AI arasında dikkate değer bir ilişki saptanmadı ($P>0.05$). Bax sunulumu açısından T1 ve T5 ile T2 ve T5 evreleri arasında önemli istatistiksel farklılıklar belirlendi ($P<0.05$) ve T1 veya T2 ile karşılaştırıldığında Bax sunulumu T5'te daha yüksekti. Hayatta kalma süresi ile Bcl-2, Bax, Bcl-X_L sunulumları ve AI arasında hiçbir ilişki saptanmadı ($P>0.05$). Bcl-2'nin solid ve tubulopapiller adenokarsinomalar gibi malignitesi yüksek tümörlerde aşırı düzeylerde sunulduğu ve Bax'ın metastazik tümörlerde sunulumunun yüksek olduğu belirlendi. Sonuç olarak, Bcl-2 ve Bax sunulumları KMT'nin biyolojik davranış ve prognozlarını tahmin etmede yardımcı parametreler olabileceği fakat bu belirteçlerin tek başlarına hayatta kalma süresinin belirlenmesi için yeterli olmadığına karar verildi.

Anahtar sözcükler: *Köpek, Meme tümörü, Apoptozis, Bcl-2, Bcl-X_L, Bax, İmmunohistokimya, TUNEL*

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INTRODUCTION

Mammary tumours are the most common type of tumours in intact female dogs and they constitute about 50% (40-50% of which are malignant) of all neoplastic cases [1-3]. The pathogenic mechanisms behind the development and progress of canine mammary tumours (CMT) have not been completely clarified yet [4,5]. The evaluation of malignancy potential and prognosis is important for the treatment of CMT. Nevertheless the histopathological diagnosis, which is accepted as the best way for the classification of CMT, sometimes can be insufficient for the definition of prognosis because of tumours' complicated histological types and their different biological behaviours [4,6]. Thereby, in recent years the interest on novel markers such as growth and proliferation factors and apoptosis related genes and proteins is increasing in order to make more reliable diagnosis and to estimate of prognosis and biological behaviours of CMT.

Apoptosis plays a key role in the development of mammary gland. In an healthy mammary gland, cell proliferation and apoptosis are in balance. Besides, there is strong evidence that tumour growth is not only related with uncontrolled cell proliferation but also inhibition of apoptosis [7]. It has been reported that the high rate of apoptosis in human breast cancer is associated with a poor prognosis and more apoptosis is seen in tumours of high grade [8-11]. But, the significance of apoptosis in the evaluation of CMT has not been presented sufficiently yet.

The Bcl-2 family of cytoplasmic proteins plays an important role in the process of apoptosis. Bcl-2 itself is a potent cell survival agent with significant anti-apoptotic activity. Members of Bcl-2 protein family can either promote (Bax, Bid, Bcl-X_S) or supress (Bcl-2, Bcl-X_L) apoptosis in a number of cellular systems [12,13]. In CMT, Yang *et al.*[14] reported that the levels of cell proliferation and apoptosis did not appear to be correlated with the expression of Bcl-2 and Bcl-2 expression were slightly greater in benign CMTs than in their malignant counterparts. There are data stating that while expression of Bcl-2 and Bcl-X_L were increased, expression of Bax was significantly lower in both human breast cancer and CMT tissues compared to corresponding adjacent tissue [15]. Although apoptosis-associated proteins were extensively studied in human breast cancer, there are few studies on CMT. The current study is aimed to evaluate the expression of Bcl-2, Bcl-X_L, Bax, apoptotic index (AI) in different histological types of CMT and statistically investigate their association with tumour grade, tumour stage and survival time.

MATERIAL and METHODS

Twenty-seven cases of CMT routinely submitted to the Dept. of Pathology of the Faculty of Veterinary Med., Istanbul University, were included in the study. Clinical data for investigated dogs are given in *Table 1*. Tumour tissues selected for the study were all located in inguinal lobes. Along with the tumour tissues their associated superficial inguinal lymph nodes were fixed with 10% of neutral buffered formalin, processed by routine methods, and embedded in paraffin. All microscopic evaluations were recorded independently by two pathologists.

Histopathological Evaluation

From paraffin blocks, 5 µm-thick sections were cut and stained by haematoxylin and eosin (H&E) and examined under light microscope. Histologic classification and tumour grading were performed based on the protocol proposed by Goldschmidt *et al.*[16].

Tumour Staging

For staging; tumour's diameter was measured, the presence of tumoural cells in associated lymph nodes was evaluated and animals were checked for distant metastasis by thoracal and abdominal radiography. Canine mammary tumours were staged according to the TNM system, which was recommended by Owen [17]: tumour dimension (T), regional lymph node status (N), distant metastasis (M).

Follow-up

To monitor survival time, the owners of the animals were contacted 2 years later. If an animal was ex, date of its death were recorded. Because the owners of animals did not allow necropsy causes of death could not be established properly. Overall survival was defined as the time from surgery to death due to any cause.

Determination of Fragmented DNA in situ

In histopathological evaluation of tumour material Apoptotic Index (AI) is used as a measure of the extent of apoptosis. Generally it is defined as a percentage of apoptotic cells and bodies in all tumour cell population [18]. To present the apoptotic cells the fracture in DNA were labelled using Terminal deoxynucleotidyltransferase (TdT)-mediated nick end-labelling (TUNEL) technique in paraffin sections, following the procedure of applied kit (Apop Tag® Peroxidase In Situ Apoptosis Detection Kit, EMD Millipore). Sections were treated with 20 µg/ml of Proteinase K (EC 3.4.21.64, Dako Inc.) for 10 min after deparaffinization and rehydration. Later they were treated with 3% H_2O_2 in methanol for 5 min, TUNEL mixture for 1 h (in 37°C) and, subsequently, in Anti-Digoxigenin-Peroxidase for 30 min. Then 2% solution of diamino benzidin (DAB) was applied to the sections and counterstained with methyl green. By microscopic evaluation AI was detected in each section. For this purpose, in each case, TUNEL-positive cells and total cells were counted in 10 random areas, under 40x magnification objective. AI was calculated by the formula: 100x (mean number of TUNEL positive cells in 10 random fields)/(mean number of total cells in 10 random fields) [8].

Table 1. *Histopathological diagnosis and grade, clinical stage, survival (in a 2-year follow-up), Bcl-2, Bax, Bcl-X_L expressions and apoptotic index of 27 malignant canine mammary tumours*

Tablo 1. *Yirmi yedi malign köpek meme tümöründe histopatolojik tanı ve derece, klinik evre, hayatta kalma süresi (2 yıllık takip), Bcl-2, Bax, Bcl-X_L sunulumları ve apoptotik indeks*

Dog no.	Diagnosis	Histological Grade	Clinical Stage				Age	Survival (day)	Bcl-2	Bax	Bcl-X_L	Apoptotic index
			T†	N††	M†††	TNM						
1	Complex Adc	2	2	0	0	2	15	750	+	++	++	1
2	Complex Adc	2	2	0	0	2	10	750	+	+	++	0.4
3	Complex Adc	3	3	1	0	4	11	240	+	++	+	0.1
4	Complex Adc	3	3	1	0	4	13	750	+	++	++	1
5	Complex Adc	2	1	0	0	1	8	750	+	++	+++	2
6	Complex Adc	2	3	0	0	3	8	660	+	++	+	1.5
7	Complex Adc	3	2	0	0	2	13	710	+	+	+	4
8	Complex Adc	3	1	0	0	1	6	750	+	++	+++	0.5
9	Complex Adc	3	1	0	0	1	10	750	++	+	+	11
10	Tubulopapillary Adc	3	1	0	0	1	11	750	++	++	++	1
11	Tubulopapillary Adc	3	2	1	1	5	10	189	+	+++	++	0.4
12	Tubulopapillary Adc	2	1	0	0	1	13	750	+++	+	+++	0.1
13	Tubulopapillary Adc	3	2	1	1	5	12	362	+	++	++	1
14	Tubulopapillary Adc	2	1	0	0	1	10	750	+++	++	++	0.4
15	Tubulopapillary Adc	2	1	0	0	1	11	750	++	++	+++	4
16	Tubulopapillary Adc	2	2	0	0	2	13	750	++	++	++	2
17	Solid Adc	3	3	1	1	5	10	1	+++	+++	+++	1
18	Solid Adc	3	3	1	1	5	12	35	+++	++	+	0.6
19	Solid Adc	3	2	0	0	2	14	370	+++	+++	+++	2
20	Solid Adc	3	1	0	1	5	10	495	+++	+++	++	1
21	Solid Adc	3	3	1	0	4	8	750	+++	+++	+++	0.5
22	Solid Adc	3	2	0	0	2	14	480	+++	+	++	2
23	Solid Adc	3	1	1	1	5	8	170	+++	+++	+++	3
24	Spindle Cell Car	3	3	0	1	5	15	750	+	++	+	0.2
25	Spindle Cell Car	3	1	0	0	1	9	290	+++	+	+++	3
26	Spindle Cell Car	3	3	1	1	5	15	215	++	+++	++	0.5
27	Spindle Cell Car	3	3	1	1	5	12	58	++	++	++	5

† *Tumour diameter,* †† *Reginonal lymhp node metastasis,* ††† *Distant metastasis*

Immunohistochemical Staining

Sections obtained from the tumoural tissues were deparaffinized and rehydrated before treatment with 0.3% H_2O_2 solution in methanol to block the endogenous peroxidase activity at room temperature for 10 min. Then they were subjected to antigen retrieval by incubation with Citrate Buffer solution (10 mM Citric Acid, pH 6.0) in a microwave oven (750 W) for 10 min. The sections were washed three times with phosphate buffered saline (PBS; pH 7.4, 0.1M) and incubated with protein blocking agent (sc-2018, Santa Cruz Biotechnology Inc.) for 10 min to block the nonspecific immunolabelling. Subsequently in a humidity chamber, they were incubated with polyclonal anti-Bcl-2 (sc-492), polyclonal anti-Bcl-X_L (sc-634) and polyclonal anti-Bax (sc-493) antibodies (Santa Cruz Biotechnology Inc.) at a dilution of 1:300 (at room temperature for 90 min). After washing three times with PBS, the slides were treated with secondary antibody kit (sc-2018, Santa Cruz Biotechnology Inc.) containing biotinylated secondary antibody and avidin-peroxidase link (incubated at room temperature in each solution for 25 min). Finally, the sections were treated with 3,3'-diaminobenzidine (DAB) according to manufacturer's protocol (sc-2018, Santa Cruz Biotechnology Inc.), washed three times with PBS, counterstained with Mayer's haematoxylin and coverslipped. For washings steps Tween 20 (0.5 ml/l) was added to the PBS buffer. Intensity of immunolabelling was assessed by examination of 10 representative high-power fields (400x). Positive cells were indicated by brown-coloured cytoplasm. A relative staining intensity based on the proportion of immunolabelled cells was

scored as follows: 0-5%; -, 5-19%; +, 20-59%; ++, ≥60% +++. These scores were regarded as negative, mild, moderate and high respectively [14]. Both in TUNEL and IHC; for positive control, sections from a normal canine thymus were used. For negative control, the primary antibody was substituted with PBS.

Statistics

Kruskal-Wallis analysis was used to determine the significance of tumour types, grades and stages on Bcl-2, Bcl-X_L, Bax expressions and AI. In case significance was found with Kruskal-Wallis analysis, to determine the difference between tumour types, grades and stages, Mann-Whitney U analysis was used. Survival estimates for all animals according to Bcl-2, Bcl-X_L and Bax expressions, AI were analyzed with Kaplan-Meier Test. All data were analyzed with Statistical Package for Social Sciences (SPSS) 13.0 software. The level of significance was set to $P<0.05$.

RESULTS

The mean age of the dogs' at the time of the surgery was 11.15±2.44 (SE=0.47). There was no correlation between age and histopathological diagnosis, tumour grade and stage ($P>0.05$). Radiographically 9 of 27 dogs had pulmonary foci which were accepted as metastasis. From these animals 4 had solid, 3 had spindle cell and 2 had tubulopapillary adenocarcinomas. Considering histopathological diagnosis, CMT were classified into 4 groups: complex (n=9) *(Fig. 1A)*, tubulopapillary (n=7) *(Fig. 1B)*, solid (n=7) *(Fig. 1C)* and spindle cell carcinoma (n=4) *(Fig. 1D)*. Regional lymph node metastases were detected in 10 of 27 cases. All data regarding to animals, histopathological diagnosis, grading, staging, survival time, AI, and apoptotic protein expressions were given in *Table 1*.

When sections were evaluated microscopically, immunopositive labelling was observed in all tumour types with anti-Bcl-2 *(Fig. 2A)*, anti-Bcl-X_L *(Fig. 2B)* and anti-Bax *(Fig. 2C)* antibodies. Bcl-2 expression was most densely in solid carcinomas followed by tubulopapillary and spindle cell carcinomas and was weak in complex carcinomas. The Bax expression was weak in tumours which were labelled strongly with anti-Bcl-2 antibody. Additionally, it was detected that Bax expression was more intensive in tumours with lymph node metastasis. The highest AI were recorded in complex adenocarcinomas *(Fig. 2D)*.

Statistical Findings

The mean value of AI was determined to be 1.82 with ANOVA test. The tumours with AI values lower and equal to this value were accept as "low" and higher then this value were accepted as "high" (AI: low≤1.82<high).

According to Kruskal Wallis Analysis, the statistical relation between histopathological diagnosis and Bcl-2 expression was very significant ($P=0.001$). But, there was no significant association between histopathological diagnosis and Bax ($P=0.082$), Bcl-X_L ($P=0.339$) and AI ($P=0.851$).

With Mann-Whitney U test, all tumour types were compared with each other in terms of Bcl-2 expression. There were statistically significant differences between all tumour types ($P<0.05$), except tubulopapillary and spindle

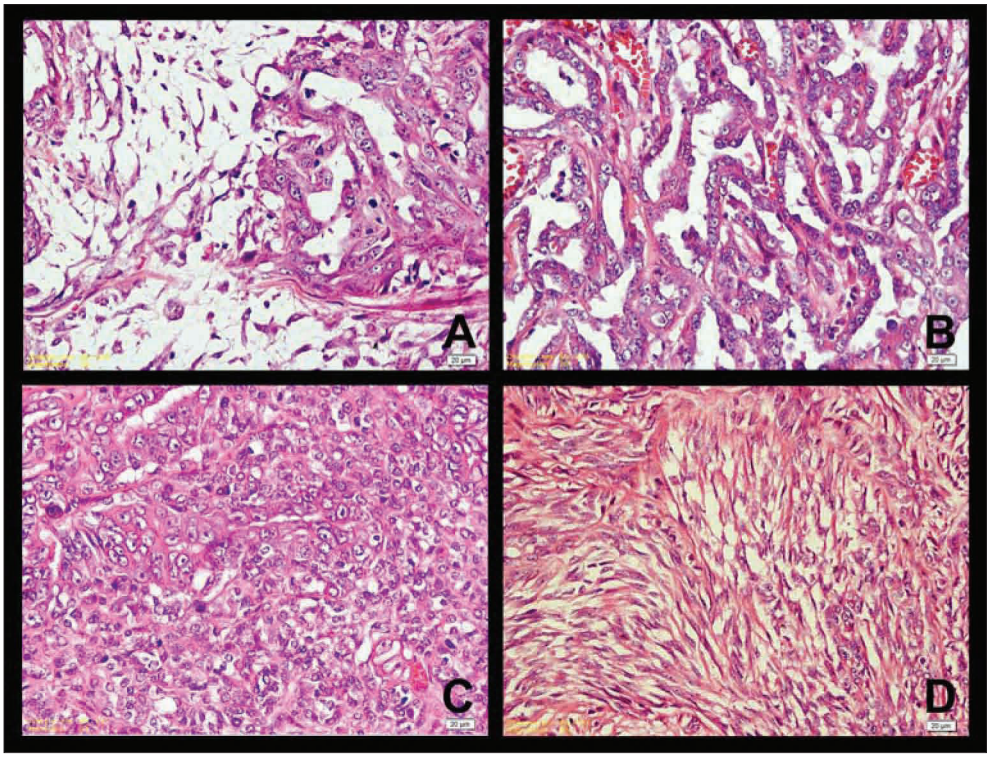


Fig 1. A- Complex adenocarcinoma, Bar=20 µm, H&E; **B**- Tubulopapillary adenocarcinoma Bar=20 µm, H&E; **C**- Solid carcinoma, Bar=20 µm, H&E; **D**-Spindle cell carcinoma, Bar=20 µm, H&E

Şekil 1. A- Kompleks adenokarsinoma, Bar=20 µm, H&E; **B**- Tubulopapiller adenokarsinoma, Bar=20 µm, H&E; **C**- Solid karsinoma, Bar =20 µm, H&E; **D**- Mekik hücreli karsinoma, Bar=20 µm, H&E

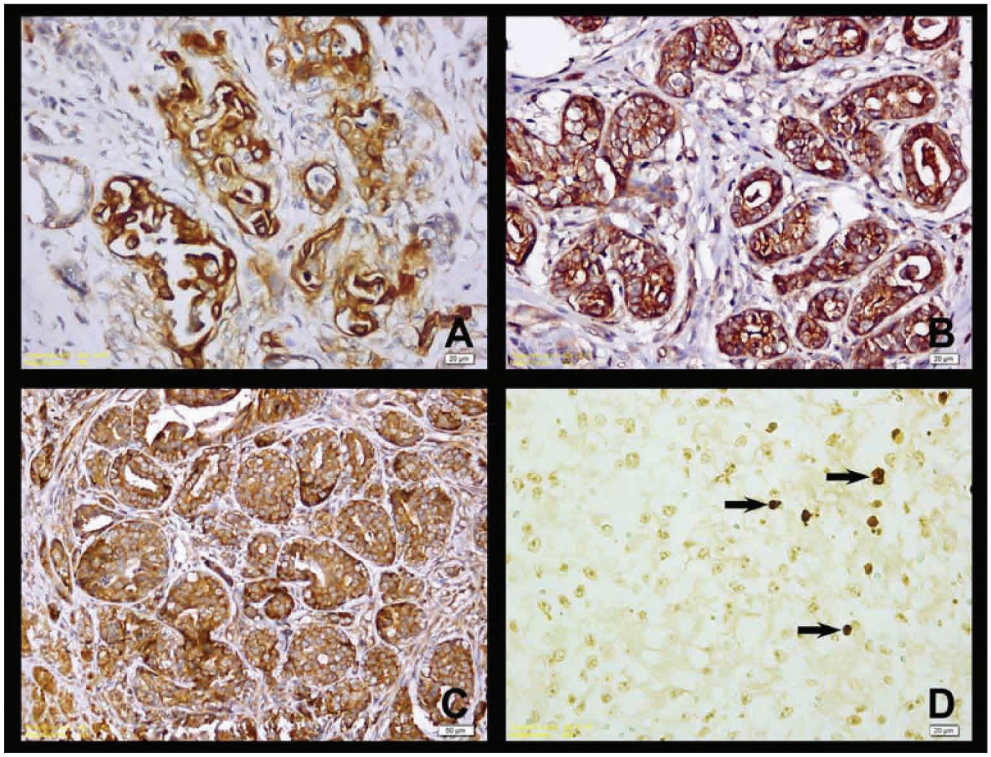
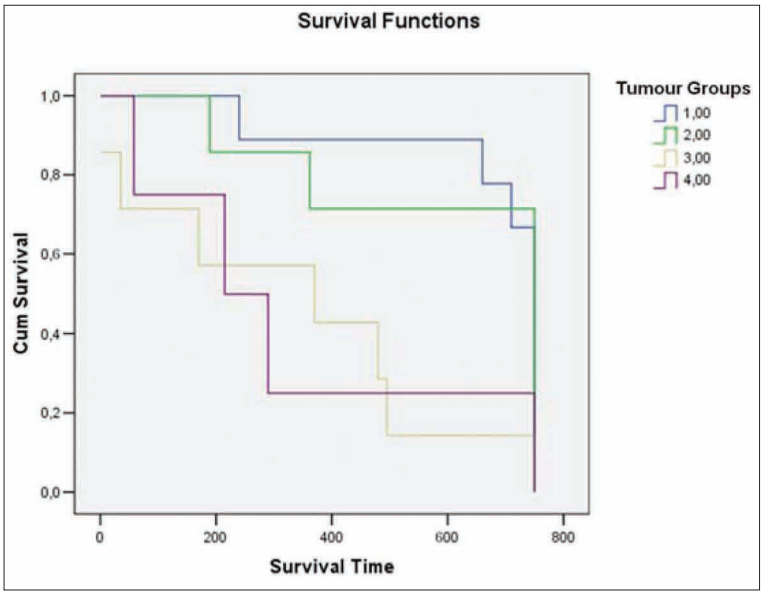


Fig 2. A- Anti-Bcl-2 antibody, high immunopositive reaction (+++), Bar=20 µm; **B-** Anti-Bcl-X_L antibody, high immunopositive reaction (+++), Bar=20 µm; **C-** Anti-Bax antibody, high immunopositive reaction (+++), Bar=50 µm; **D-** TUNEL-positive apoptotic cells (arrows), Bar=20 µm

Şekil 2. A- Anti-Bcl-2 antikoru, kuvvetli immunopozitif reaksiyon (+++), Bar=20 µm; **B-** Anti-Bcl-X_L antikoru, kuvvetli immunopozitif reaksiyon (+++), Bar=20 µm; **C-** Anti-Bax antikoru, kuvvetli immunopozitif reaksiyon (+++), Bar=50 µm; **D-** TUNEL-pozitif apoptotik hücreler (oklar), Bar=20 µm

Fig 3. Kaplan-Meier curve of survival time for dogs with mammary tumours based on histopathological diagnosis (n=27). The first (blue) line indicates dogs with complex adenocarcinomas (n=9, mean survival time 678 days; range, 240-750 days), the second (green) line indicates dogs with tubulopapillary adenocarcinomas (n=7, mean survival time 614 days; range, 189-750 days), the third (yellow) line indicates dogs with solid carcinomas (n=7, mean survival time 328 days; range, 1-750 days) and the forth (purple) line indicates dogs with spindle cell carcinomas (n=4, mean survival time 328 days; range, 58-750 days)

Şekil 3. Meme tümörlü köpeklerin histopatolojik teşhislerine göre Kaplan-Meier hayatta kalma süreleri eğrileri (n=27). Birinci çizgi (mavi) kompleks adenokarsinomalı köpekleri (n=9, ortalama hayatta kalma süresi 678 gün; aralık, 240-750 gün), ikinci çizgi (yeşil) tubulopapiller adenokarsinomalı köpekleri (n=7, ortalama hayatta kalma süresi 614 gün; aralık, 189-750 gün), üçüncü çizgi (sarı) solid karsinomalı köpekleri (n=7, ortalama hayatta kalma süresi 328 gün; aralık, 1-750 gün) ve dördüncü çizgi (mor) mekik hücreli karsinomalı köpekleri (n=4, ortalama hayatta kalma süresi 328 gün; aralık, 58-750 gün) göstermektedir



cell carcinomas (P>0.05).

In Kruskal Wallis Analysis, when the grouping variable was tumour grade, there was no important association between groups. But when the grouping variable was TNM in Kruskal Wallis Analysis, there was significance only with Bax expression (P=0.045). There was statistically significant differences between T1 and T5 (P=0.006), T2 and T5 (P=0.037) in terms of Bax expression through Mann-Whitney U test. There were no other statistically significant differences between other comparisons.

At the follow-up examination, 11 of the dogs were still alive and they were clinically assessed as described. There was no recurrence or distant metastasis in those animals. The shortest survival time periods were in spindle cell carcinoma and solid carcinoma groups *(Fig. 3)*, where 7 of 11 dogs had distant metastasis and higher percentages of Bax expression. The survival curves of histomorphologic groups which were tested daily, significant differences were found between complex and solid adenocarcinomas (P=0.01), complex and spindle cell adenocarcinomas (P=0.02), spindle cell and tubulopapillary adenocarcinomas (P=0.035) by means of Kaplan-Meier Analysis. However, when the survival curves of Bcl-2, Bcl-X_L, Bax and AI were compared, no association was determined between survival time and these parameters (P>0.05) *(Fig. 4)*.

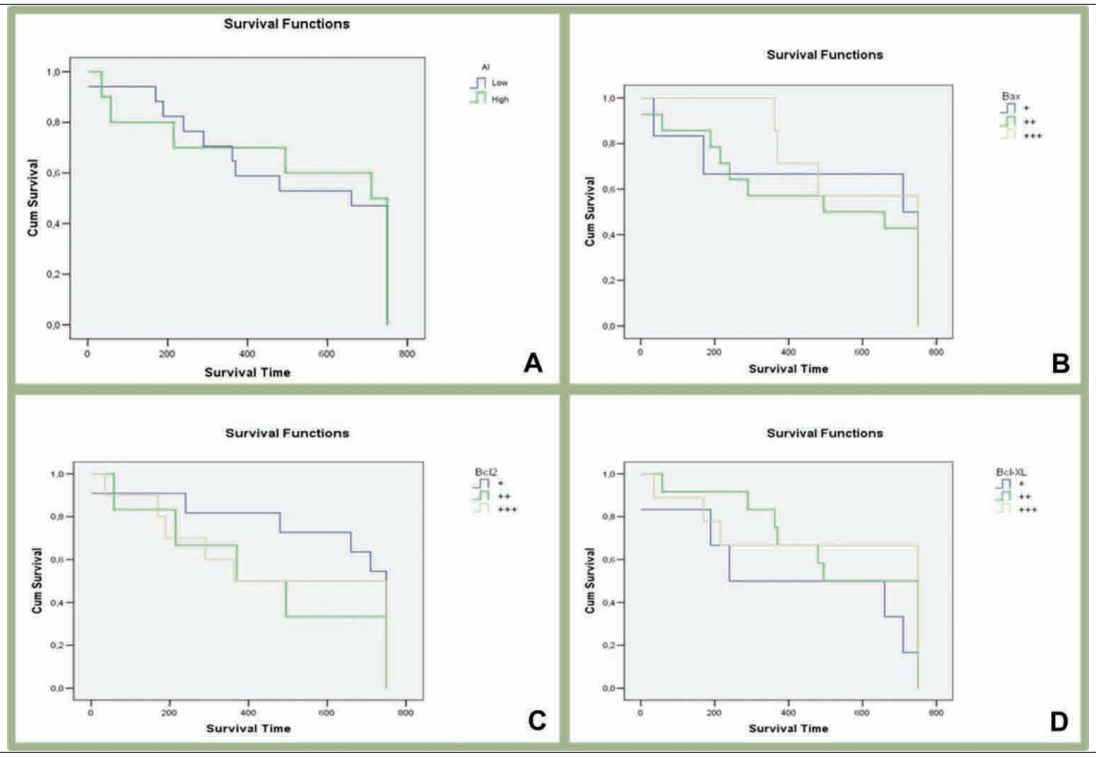


Fig 4. A- Kaplan-Meier curve of survival time for dogs with mammary tumours based on AI (n=27). The first (blue) line indicates dogs with low apoptotic index (n=17, mean survival time 515 days; range, 1-750 days), the second (green) line indicates dogs with high apoptotic index (n=10, mean survival time 526 days; range, 35-750 days); **B-** Kaplan-Meier curve of survival time for dogs with mammary tumours based on Bax expression (n=27). The first (blue) line indicates dogs with mild immunopositive reaction (+) (n=6, mean survival time 527 days; range, 35-750 days), the second (green) line indicates dogs with moderate immunopositive reaction (++) (n=14, mean survival time 474 days; range, 1-750 days), the third (yellow) line indicates dogs with high immunopositive reaction (+++) (n=7, mean survival time 601 days; range, 362-750 days); **C-** Kaplan-Meier curve of survival time for dogs with mammary tumours based on Bcl-2 expression (n=27). The first (blue) line indicates dogs with mild immunopositive reaction (+) (n=11, mean survival time 599 days; range, 1-750 days), the second (green) line indicates dogs with moderate immunopositive reaction (++) (n=6, mean survival time 439 days; range, 58-750 days), the third (yellow) line indicates dogs with high immunopositive reaction (+++) (n=10, mean survival time 479 days; range, 35-750 days); **D-** Kaplan-Meier curve of survival time for dogs with mammary tumours based on Bcl-X_L expression (n=27). The first (blue) line indicates dogs with mild immunopositive reaction (+) (n=6, mean survival time 425 days; range, 1-750 days), the second (green) line indicates dogs with moderate immunopositive reaction (++) (n=19, mean survival time 546 days; range, 58-750 days), the third (yellow) line indicates dogs with high immunopositive reaction (+++) (n=9, mean survival time 546 days; range, 35-750 days)

Şekil 4. A- Meme tümörlü köpeklerin AI'ne göre Kaplan-Meier hayatta kalma süreleri eğrileri (n=27). Birinci çizgi (mavi) düşük apoptotik indeksli köpekleri (n=17, ortalama hayatta kalma süresi 515 gün; aralık, 1-750 gün), ikinci çizgi (yeşil) yüksek apoptotik indeksli köpekleri (n=10, ortalama hayatta kalma süresi 526 gün; aralık, 35-750 gün) göstermektedir; **B-** Meme tümörlü köpeklerin Bax sunulumuna göre Kaplan-Meier hayatta kalma süreleri eğrileri (n=27). Birinci çizgi (mavi) hafif immunopozitif reaksiyonlu köpekleri (+) (n=6, ortalama hayatta kalma süresi 527 gün; aralık, 35-750 gün), ikinci çizgi (yeşil) orta immunopozitif reaksiyonlu köpekleri (++) (n=14, ortalama hayatta kalma süresi 474 gün; aralık, 1-750 gün), üçüncü çizgi (sarı) kuvvetli immunopozitif reaksiyonlu köpekleri (+++) (n=7, ortalama hayatta kalma süresi 601 gün; aralık, 362-750 gün) göstermektedir; **C-** Meme tümörlü köpeklerin Bcl-2 sunulumuna göre Kaplan-Meier hayatta kalma süreleri eğrileri (n=27). Birinci çizgi (mavi) hafif immunopozitif reaksiyonlu köpekleri (+) (n=1, ortalama hayatta kalma süresi 599 gün; aralık, 1-750 gün), ikinci çizgi (yeşil) orta immunopozitif reaksiyonlu köpekleri (++) (n=6, ortalama hayatta kalma süresi 439 gün; aralık, 58-750 gün), üçüncü çizgi (sarı) kuvvetli immunopozitif reaksiyonlu köpekleri (+++) (n=10, ortalama hayatta kalma süresi 479 gün; aralık, 35-750 gün) göstermektedir; **D-** Meme tümörlü köpeklerin Bcl-X_L sunulumuna göre Kaplan-Meier hayatta kalma süreleri eğrileri (n=27). Birinci çizgi (mavi) hafif immunopozitif reaksiyonlu köpekleri (+) (n=6, ortalama hayatta kalma süresi 425 gün; aralık, 1-750 gün), ikinci çizgi (yeşil) orta immunopozitif reaksiyonlu köpekleri (++) (n=19, ortalama hayatta kalma süresi 546 gün; aralık, 58-750 gün), üçüncü çizgi (sarı) kuvvetli immunopozitif reaksiyonlu köpekleri (+++) (n=9, ortalama hayatta kalma süresi 546 gün; aralık, 35-750 gün) göstermektedir

DISCUSSION

The imbalance between cell proliferation and apoptosis is the basis of growth, progression and regression of tumours. Disruption of apoptosis regulation can activate carcinogenesis through different pathways. Apoptosis also limits the tumour cell population at the early stages of tumour development. Decrease in apoptosis may result with cancer by uncontrolled expression of oncogenes or accumulation of malignant cells due to tumour suppressor genes [19,20]. But in some cases the accumulation of neoplastic cells is consistent with increased apoptosis ratio in certain aggressive tumours like human bladder carcinoma [18]. In literature, yet there is little published data on the value of apoptosis as a tool for diagnosis or prognosis in canine tumours. A study by Guvenc *et al.*[21] stated that AI is a useful marker for the differentiation of canine cutaneous histiocytoma and transmissible venereal tumours. Dolka *et al.*[22] reported positive correlation between AI and tumour size, histopathology, grade and proliferative activity. It has

been reported that there is no significant correlation of the survival time with the AI in malignant melanomas of dogs and cats [23]. Funakoshi *et al.*[6] reported that there are no important relationships between AI value of mammary gland tumours and clinical features like metastasis and tumour diameter. In the current study no significant association between AI and histopathological diagnosis, grading and staging of the tumours, survival time were detected. The finding of low degrees of apoptosis in highly malignant tumours or increased apoptosis in some malignant tumours diminished the value of apoptosis as a prognostic tool. So we think that this inconstancy is related to the levels of initiation, development and progression of the cancers.

As Bcl-2 protein is a prosurvival factor which blocks apoptosis one could anticipate that its overexpression would be related with aggressive tumours [24,25]. There are data reporting that overexpression of Bcl-2 is related with down-regulation of Bax and the interruption in the progression of apoptosis is dependent to the imbalance of Bcl-2/Bax, which plays a role in cancer development [15,24,26]. It was reported that in some cancer types such as myeloid leukaemia [27] and cancers of prostate [28], cervix [19] and colon [29] expression of Bcl-2 is increased. But in previous studies with human breast cancers, it was stated that Bcl-2 expression is generally observed in benign proliferative lesions and small, slowly progressing oestrogen-positive, p53-negative tumours and was found to be related with better prognosis [7,25,30]. Yang *et al.*[14] reported that, similarly human breast cancers, Bcl-2 expression is higher in benign CMT than in their malignant counterparts. Contradictory results were reported for Bcl-2 expression in CMT. Kumaraguruparan *et al.*[15,26] stated that they have found higher Bcl-2 expression in tumours than in normal mammary glands. In another study performed in CMT it was reported that no significant correspondence was found between Bcl-2 expression and histology, grade and proliferative activity of CMT [22]. In the present study it was determined that Bcl-2 expression significantly differs between histopathological types of CMT ($P<0.05$). According to the WHO classification; based on differentiation and the biologic behaviour, simple carcinomas can be graded in terms of increasing malignancy as tubulopapillary, solid and anaplastic carcinoma [1] and the simple carcinomas had worse prognosis then complex carcinoma [16]. In the current study appropriately, Bcl-2 expression was highest in solid carcinoma followed by tubulopapillary and spindle cell carcinoma which is a special type mammary gland tumour and was lowest in complex carcinoma. Some researchers reported that by expressing anti-apoptotic proteins like Bcl-2, tumour cells develop a mechanism to avoid apoptotic death signals and gain resistance against apoptosis [31,32]. We concluded that this mechanism can be considered to be the cause of overexpression of Bcl-2 in highly cellular and malignant solid CMT.

In some studies reported in human and canine cancers, the expression of Bax was found to be decreased in malignant tumours when compared to normal tissues and benign tumours [15,29]. On the contrary in the present study Bax expression was higher only in tumours in T5 stage ($P<0.05$). Although there is no sufficient data about the pathogenetic meaning of overexpression of Bax in metastatic tumours, it was reported that proapoptotic and anti-apoptotic gene transcription increases significantly in metastatic tumours [33]. This finding can be explanatory for both Bax and Bcl-2 results obtained.

There are some studies about the decreased expression of proapoptotic Bcl-X_L in canine tumours, but only one article about its relationship with CMT. Kumaraguruparan *et al.*[15] reported that Bcl-X_L expression decreases in CMT compared with normal tissues. In the current study no significant correlation between survival time, different types, grades, stages of CMT and Bcl-X_L expression were determined.

The data obtained from the comparison of survival times and histopathological diagnosis of CMT were compatible with prognostic results of previous literature [1,16]. Some studies on CMT showed that the tumour size, regional lymph node involvement, distant metastasis and histomorphologic characteristics are useful parameters for clinical prognosis [3-5,16]. In the present study, the evaluation of statistical results obtained from comparison between histopathological types, tumour grade and stage showed that the conventional diagnostic methods such as histological grading and TNM staging are reliable factors in estimating the prognosis. This finding supports the previously reported literature above mentioned.

The exact cause of death could not be determined for dogs which died during the study since the owners did not allowed for necropsy. In order to determine the most reliable result for survival time, the disease-free interval or death related to mammary cancer should be determined. Within the bounds of possibilities we could not obtain these data. Therefore we can conclude that to determine the association of survival for the parameters we evaluate, overall survival for any cause of death in dogs was not sufficient. As there was not enough published data about the relation of survival time and Bcl-2, Bcl-X_L, Bax expressions and AI, we couldn't compare with our results adequately.

Most of the studies presenting the relation between CMT, AI and apoptotic proteins are generally about the comparison of normal mammary gland tissue/benign tumours and malignant tumours. In the present study the relation between apoptotic proteins and the histologic types, grade, stage of malignant mammary tumours and survival time were evaluated. Therefore the current study is one of the first studies on this subject. Because only malignant tumours were evaluated in the current study, the diversity between previous literature about apoptotic

proteins and AI can also be explained with this difference.

In conclusion, Bcl-2 was overexpressed in highly malignant tumours such as solid and tubulopapillary adenocarcinomas and Bax had high expression levels in metastatic tumours while they have no association with tumour grade and survival time. Bcl-2 and Bax expression can be accessory parameters for anticipating the biologic behaviour and prognosis of CMT but these markers alone are not sufficient for the determination of survival time. In addition, AI and Bcl-X_L were not sufficient as dependable markers to estimate biologic behaviour and prognosis of CMT with regard to our study's result. Beside the results will contribute to the limited literature about relation between apoptotic proteins and CMT. But it is needed to further investigations with much more sample and further techniques.

Acknowledgement

We would like to express our gratitude to Prof. Bulent EKİZ from Istanbul University, Faculty of Veterinary Medicine, Zootechnics Department for the statistical analyses and to Sevgi EREN from Pathology Department for her histological technical assistance.

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Kafkas Univ Vet Fak Derg
20 (4): 521-526, 2014
DOI: 10.9775/kvfd.2013.10454

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Genetic Characterization of Turkish Cattle Breeds by Microsatellite Markers: Usefulness for Parentage Testing [1]

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[1] *This research was supported by Scientific and Technological Research Council of Turkey (TUBITAK, KAMAG - 106G114) and Selcuk University BAP (08202009)*

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Makale Kodu (Article Code): KVFD-2013-10454

Summary

Objective of this study was to evaluate microsatellite markers in paternity testing of native cattle breeds in Turkey. Blood samples were collected from Anatolian Black (n=51), Anatolian Grey (n=54), South Anatolian Red (n=51), Native Southern Anatolian Yellow (n=51), East Anatolian Red (n=45) and Zavot (n = 19) cattle. From the blood samples DNA was isolated by using a standard phenol/chloroform method. A total of 20 microsatellite loci were selected from a FAO/ISAG-suggested list. Polymerase chain reaction products were separated by capillary electrophoresis and marker genotypes were determined by fragment analysis. In statistical analyses, allel numbers, observed (Ho) and expected (He) heterozygosities, deviation from Hardy-Weinberg Equilibrium and probability of exclusion (PE) at each microsatellite locus were calculated. A total of 269 different alleles were observed and the mean allele was identified as 13.45. Mean Ho and He values were observed as 0.619-0.852 and 0.669-0.877, respectively. The results indicated that the microsatellite test panel including the most informative 7 loci had total PE value of >0.9999 in each populations and can thereby be used for parentage testing studies of native cattle breeds in Turkey.

Keywords: *Cattle, Microsatellite, Parentage testing, TURKHAYGEN-I*

Türkiye Yerli Sığır Irklarının Mikrosatellit Belirteçler ile Genetik Karakterizasyonu: Kimliklendirme Çalışmalarında Kullanılabilirliği

Özet

Bu çalışmanın amacı, mikrosatellit belirteçlerinin Türkiye yerli sığır ırklarının kimliklendirme çalışmalarında kullanılabilirliğinin araştırılmasıdır. Yerli Kara (n=51), Boz Irk (n=54), Güney Anadolu Kırmızısı (n=51), Yerli Güney Sarısı (n=51), Doğu Anadolu Kırmızısı (n=45) ve Zavot (n=19) ırkı sığırlardan alınan kan örneklerinden standart fenol/kloroform yöntemi ile DNA izolasyonu yapılmıştır. Çalışmada kullanılan 20 mikrosatellit lokusu FAO/ISAG tarafından tavsiye edilen listeden seçilmiştir. Yükseltgenen Polimeraz Zincir Reaksiyonu ürünleri kapiller elektroforez ile ayrıştırılmış ve fragman analizi ile lokus genotipleri tespit edilmiştir. İstatistiksel analizlerde, toplam allel sayısı, gözlenen (Ho) ve beklenen (He) heterezigotluk, Hardy-Weinberg Dengesine uygunluk ve dışlama gücü olasılığı parametreleri hesaplanmıştır. Toplam 269 allel gözlenmiş ve ortalama allel sayısı 13.45 olarak tespit edilmiştir. Ortalama Ho ve He değerleri sırasıyla 0.619-0.852 ve 0.669-0.877 tespit edilmiştir. Enformatif 7 lokusu içeren mikrosatellit panelinin toplam dışlama gücü olasılığının tüm ırklarda >0.9999 olacağı ve yerli sığır ırklarının kimliklendirme çalışmalarında başarıyla kullanılabileceği tespit edilmiştir.

Anahtar sözcükler: *Sığır, Mikrosatellit, Kimliklendirme, TÜRKHAYGEN-I*

INTRODUCTION

Paternity testing is widely used in criminal cases, biomedical researches, and in cases of determination of inbreeding levels in different population. While protein polymorphism, blood antigens, and tissue proteins were

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previously used for this purpose, DNA-based different test panels (RFLP, AFLP, RAPD, mtDNA etc.) were developed and found to be more efficient. Comparing with other marker systems, microsatellites [1], exist widely in the genome, have features of higher polymorphism and codominant inheritance [2]. Due to their high polymorphism and technical ease including suitability for PCR technology and capillary electrophoresis, microsatellites are widely preferred in the paternity testing efforts of various mammalian species [3]. Correct determination of genetic relationships among animal populations has critical importance for development of selection programs [3], generation of pedigree structures [4], estimation of heritability [5,6], and breeding values [7]. Significantly higher rates of paternity misidentification were reported even in the countries where herd records are performed with great care [3,5,8].

Significant levels of incorrect paternity (2.90-15%) were reported in analyses made with marker systems [5,9-11]. Moreover, misidentification rates were reported to be higher in females [9,11]. Paternity misidentification was determined to cause serious deviations (5-50%) in the estimation of genetic parameters and reduction of genetic gain in selection programs [12]. It was reported that more than 20% of paternity misidentification cases were caused by artificial insemination of more than one bull [3,11] and it can be reduced to 8% by using a quality control system which results in an increase of 1% in genetic progress [11]. It is well known that herd improvement can be performed to have extra profits by accurate estimation of parent identification [9]. In addition, even though the possibility of paternity identification for the estimation of breeding value is desired, there is still need for more cost-effective tests in commercial mean [7].

Modern dairy and beef industry focus on using a number of highly productive cattle breeds. On the other hand, local breeds are often accepted as uneconimal and certain biotechnologycal applications can not be used because of costliness. There is an increasing demand for paternity testing in breeding programs of native animal breeds. Due to population properties, special paternity testing panels are needed for some native cattle breeds in which certain loci can be uninformative. Also, an informative test panel including the lowest possible number of the most informative loci can offer economical and pratical paternity testing possibilities. Objective of this study was the evaluation of microsatellite markers in paternity testing in native cattle breeds in Turkey as part of a national project titled "In vitro Conservation and Preliminary Molecular Identification of Some Turkish Domestic Animal Genetic Resources-1 (TURKHAYGEN-I)".

MATERIAL and METHODS

A total of 271 blood samples were collected from South Anatolian Red (SAR, n = 51), Native Southern Anatolian Yellow (SAY, n = 51), Anatolian Black (AB, n = 51), Anatolian Grey (AG, n = 54), East Anatolian Red (EAR, n = 45) and Zavot (ZAV, n = 19) cattle. Genomic DNA samples were extracted by using a standard organic phenol/chloroform method [13]. A total of 20 microsatelllite loci *(Table 1)* were selected from a list [14] suggested by FAO MoDAD and International Society of Animal Genetics (ISAG).

Microsatellite genotyping procedures were described elsewhere [15]. Briefly each multiplex PCR was performed in 15 µl reaction volume including 1x Mg^{++} free PCR buffer (Fermentas), 0.125 mM dNTPs (Fermentas), 1.5 mM $MgCl^{++}$, 0.375 U of *Tag* polymerase (Fermentas), 2 - 17 pMol each primer and ~100 ng of genomic DNA.

A touchdown-PCR profile [16] was used with two steps. The first step was initial denaturation at 95°C for 4 min, followed by 16 cycles of denaturation at 94°C for 30 sec, annealing beginning at 60°C and ending at 52°C for 30 sec and extension at 72°C for 30 sec. The annealing temperature was decreased 0.5°C per cycle until it reached 52°C. At the second step, 25 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec was applied. The final extension of 72°C for 10 min was applied in all reactions. The resulting PCR products were denaturated in Hi-Di-formamide including S-400 DNA size standart and loaded onto a Beckman Coulter CEQ-8000 Genetic Analysis System for capillary electrophoresis. Genotypes were determined by fragment analysis using CEQ-8000 FragTest program. General population parameters including allele number (Na), expected (He) and observed (Ho) heterozigosities, deviation from Hardy-Weinberg Equilibrium (HWE) and probability of exclusion for each locus (PE-1=Both parents known and PE-2=Only one parent known) were calculated using GenAlEx6 [17] package program.

RESULTS

In this study, 20 microsatellite loci were separated by capillary electrophoresis and allele genotypes in each marker locus were determined by fragment analysis. Three different multiplex pool systems were formed including 7 (CSSM66, ETH03, HEL9, CSRM60, INRA023, SPS115, ILSTS006), 7 (INRA005, HAUT27, TGLA122, TGLA126, TGLA227, BM1824, HEL13) and 6 (BM2113, TGLA53, ETH225, ETH10, ETH185, BM1818) loci.

Observed allele numbers (Na), expected (He) and observed (Ho) heterozygoties deviations from Hardy-Weinberg Equilibrium (HWE) were summarized in *Table 2* and *3*. In this study, a total of 269 different alleles were detected. The mean allele number was 13.45. The maximum and minimum numbers of total alleles were observed in TGLA122 (26 alleles) and INRA005 (7 alleles), respectively. The highest average observed (Ho) and expected (He) heterozygosity values were determined as 0.619-0.852 and 0.669-0.877, respectively. HWE's were

Table 1. *Microsatellite loci used in the study*
Tablo 1. *Kullanılan mikrosatellit markör listesi*

No	Locus	Chromosome	Primer	Allele
1	BM1824	1	GAGCAAGGTGTTTTTCCAATC	170-218
			CATTCTCCAACTGCTTCCTTG	
2	BM2113	2	GCTGCCTTCTACCAAATACCC	116-146
			CTTAGACAACAGGGGTTTGG	
3	INRA023	3	GAGTAGAGCTACAAGATAAACTTC	193-235
			TAACTACAGGGTGTTAGATGAACTCA	
4	ETH10	5	GTTCAGGACTGGCCCTGCTAACA	198-234
			CCTCCAGCCCACTTTCTCTTCTC	
5	ILSTS006	7	TGTCTGTATTTCTGCTGTGG	277-309
			ACACGGAAGCGATCTAAACG	
6	HEL9	8	CCCATTCAGTCTTCAGAGGT	141-173
			CACATCCATGTTCTCACCAC	
7	ETH225	9	GATCACCTTGCCACTATTTCCT	135-165
			ACATGACAGCCAGCTGCTACT	
8	CSRM60	10	AAGATGTGATCCAAGAGAGAGGCA	79-115
			AGGACCAGATCGTGAAAGGCATAG	
9	HEL13	11	TAAGGACTTGAGATAAGGAG	178-200
			CCATCTACCTCCATCTTAAC	
10	INRA005	12	CAATCTGCATGAAGTATAAATAT	135-149
			CTTCAGGCATACCCTACACC	
11	CSSM66	14	ACACAAATCCTTTCTGCCAGCTGA	171-209
			AATTTAATGCACTGAGGAGCTTGG	
12	SPS115	15	AAAGTGACACAACAGCTTCTCCAG	235-265
			AACGAGTGTCCTAGTTTGGCTGTG	
13	TGLA53	16	GCTTTCAGAAATAGTTTGCATTCA	143-191
			ATCTTCACATGATATTACAGCAGA	
14	ETH185	17	TGCATGGACAGAGCAGCCTGGC	214-246
			GCACCCCAACGAAAGCTCCCAG	
15	TGLA227	18	CGAATTCCAAATCTGTTAATTTGCT	64-115
			ACAGACAGAAACTCAATGAAAGCA	
16	ETH03	19	GAACCTGCCTCTCCTGCATTGG	90-135
			ACTCTGCCTGTGGCCAAGTAGG	
17	TGLA126	20	CTAATTTAGAATGAGAGAGGCTTCT	104-131
			TTGGTCTCTATTCTCTGAATATTCC	
18	TGLA122	21	CCCTCCTCCAGGTAAATCAGC	134-193
			AATCACATGGCAAATAAGTACATAC	
19	BM1818	23	AGCTGGGAATATAACCAAAGG	248-278
			AGTGCTTTCAAGGTCCATGC	
20	HAUT27	26	TTTTATGTTCATTTTTTGACTGG	120-158
			AACTGCTGAAATCTCCATCTTA	

found to be insignificant mostly in ZAV (17 loci) and at least in AG (10 loci). Some loci were significantly deviated from HWE.

Power of exclusion values were calculated in the presence of one parent (PE-2) and two parents (PE-1) *(Table 4)*. PE-2 values varied between 0.328 (INRA005, TGLA126 and BM1824) and 0.806 (TGLA122). The lowest PE-1 value (0.504) was observed in SAY and ZAV populations in INRA005, TGLA126 and BM1824, the highest PE-1 value (0.893) was determined in SAR and AB populations for TGLA122 locus. The highest and the lowest average

Table 2. *The number of alleles*
Tablo 2. *Populasyonlarda gözlenen allel sayıları*

Locus	Populations						Mean	Total
	SAR	AB	AG	SAY	EAR	ZAV		
CSSM66	13	13	12	13	13	9	12.17	14
CSRM60	10	13	11	12	7	6	9.83	15
ETH03	10	11	10	11	10	11	10.50	14
INRA023	13	10	10	11	10	9	10.50	14
HEL9	11	12	12	14	11	10	11.67	16
ILSTS006	11	11	10	9	8	5	9.00	13
SPS115	9	10	8	9	8	6	8.33	10
ETH185	12	12	12	13	10	9	11.33	17
BM1818	8	10	10	11	8	7	9.00	13
ETH225	13	11	8	9	10	10	10.17	13
ETH10	8	8	8	9	7	5	7.50	9
TGLA53	18	14	18	19	11	13	15.50	23
BM2113	10	9	9	12	8	9	9.50	13
INRA005	5	6	6	4	6	4	5.17	7
HAUT27	8	9	9	8	9	7	8.33	10
TGLA122	19	19	15	17	16	12	16.33	26
TGLA126	6	8	8	9	8	4	7.17	9
TGLA227	12	12	13	13	11	11	12.00	16
BM1824	6	7	5	5	5	4	5.33	8
HEL13	8	7	7	8	6	5	6.83	9
Mean	10.50	10.60	10.05	10.80	9.10	7.80	9.81	13.45

Table 3. *Observed (Ho) and expected (He) heterozygosity and Hardy-Weinberg Equilibrium (HWE)*
Tablo 3. *Gözlenen (Ho) ve beklenen (He) heterozigotluk ile Hardy-Weinberg Dengesi (HWE)*

Locus	Mean		HWE					
	Ho	He	SAR	AB	AG	SAY	EAR	ZAV
CSSM66	0.822	0.856	ns	ns	***	ns	ns	ns
CSRM60	0.761	0.762	ns	ns	**	ns	ns	ns
ETH3	0.762	0.804	ns	ns	ns	ns	ns	ns
INRA023	0.779	0.808	ns	ns	ns	ns	***	ns
HEL9	0.793	0.834	ns	ns	ns	*	ns	ns
ILSTS006	0.673	0.755	*	ns	ns	***	ns	ns
SPS115	0.661	0.768	**	*	***	*	ns	**
ETH185	0.797	0.788	ns	ns	**	*	***	***
BM1818	0.767	0.771	ns	ns	***	ns	***	ns
ETH225	0.742	0.814	***	***	***	ns	**	ns
ETH10	0.644	0.669	***	ns	ns	*	ns	ns
TGLA53	0.801	0.877	**	**	**	**	ns	ns
BMS2113	0.806	0.840	*	ns	***	ns	ns	ns
INRA005	0.671	0.685	ns	ns	ns	ns	ns	ns
HAUT27	0.619	0.734	ns	*	***	***	***	*
TGLA122	0.794	0.842	ns	**	ns	ns	*	ns
TGLA126	0.750	0.759	ns	ns	ns	ns	ns	ns
TGLA227	0.852	0.859	ns	ns	*	**	ns	ns
BM1824	0.719	0.711	ns	ns	ns	ns	ns	ns
HEL13	0.728	0.788	ns	ns	ns	ns	ns	ns

Ho: *Observed,* ***He:*** *Expected Heterozygosity,* ***HWE:*** *Hardy-Weinberg Equilibrium,* ***ns*** *= non significant, * P<0.05, ** P<0.01, *** P<0.001*

Table 4. *Probability of Exclusion for each locus (PE-1=both parents known and PE-2= only one parent known)*
Tablo 4. *Dışlama Gücünün 1 (DG-1) ve 2 (DG-2) ebeveyn varlığında değerleri*

Locus	Populations											
	SAR		AB		AG		SAY		EAR		ZAV	
	PE-2	PE-1	PE-2	PE-1	PE-2	PE-1	PE-2	PE-1	PE-2	PE-1	PE-2	PE-1
CSSM66	0.726	0.842	0.726	0.842	0.707	0.829	0.726	0.842	0.726	0.842	0.626	0.772
CSRM60	0.657	0.795	0.726	0.842	0.684	0.813	0.707	0.829	0.542	0.707	0.486	0.660
ETH03	0.657	0.795	0.684	0.813	0.657	0.795	0.684	0.813	0.657	0.795	0.684	0.813
INRA023	0.726	0.842	0.657	0.795	0.657	0.795	0.684	0.813	0.657	0.795	0.626	0.772
HEL9	0.684	0.813	0.707	0.829	0.707	0.829	0.744	0.854	0.684	0.813	0.657	0.795
ILSTS006	0.684	0.813	0.684	0.813	0.657	0.795	0.626	0.772	0.588	0.743	0.416	0.595
SPS115	0.626	0.772	0.657	0.795	0.588	0.743	0.626	0.772	0.588	0.743	0.486	0.660
ETH185	0.707	0.829	0.707	0.829	0.707	0.829	0.726	0.842	0.657	0.795	0.626	0.772
BM1818	0.588	0.743	0.657	0.795	0.657	0.795	0.684	0.813	0.588	0.743	0.542	0.707
ETH225	0.726	0.842	0.684	0.813	0.588	0.743	0.626	0.772	0.657	0.795	0.657	0.795
ETH10	0.588	0.743	0.588	0.743	0.588	0.743	0.626	0.772	0.542	0.707	0.416	0.595
TGLA53	0.796	0.887	0.744	0.854	0.796	0.887	0.806	0.893	0.684	0.813	0.726	0.842
BM2113	0.657	0.795	0.626	0.772	0.626	0.772	0.707	0.829	0.588	0.743	0.626	0.772
INRA005	0.416	0.595	0.486	0.660	0.486	0.660	0.328	0.504	0.486	0.660	0.328	0.504
HAUT27	0.588	0.743	0.626	0.772	0.626	0.772	0.588	0.743	0.626	0.772	0.542	0.707
TGLA122	0.806	0.893	0.806	0.893	0.759	0.864	0.785	0.880	0.773	0.872	0.707	0.829
TGLA126	0.486	0.660	0.588	0.743	0.588	0.743	0.626	0.772	0.588	0.743	0.328	0.504
TGLA227	0.707	0.829	0.707	0.829	0.726	0.842	0.726	0.842	0.684	0.813	0.684	0.813
BM1824	0.486	0.660	0.542	0.707	0.416	0.595	0.416	0.595	0.416	0.595	0.328	0.504
HEL13	0.588	0.743	0.542	0.707	0.542	0.707	0.588	0.743	0.486	0.660	0.416	0.595
Mean	0.645	0.782	0.657	0.792	0.638	0.778	0.651	0.785	0.611	0.758	0.545	0.700
Total	>0.9999		>0.9999		>0.9999		>0.9999		>0.9999		>0.9999	

PE-2 and PE-1 values were determined in ZAV and AB populations. Total PE-2 and PE-1 values were calculated as >0.999 for all populations using the most polymorphic 7 (CSSM66 + CSRM60 + ETH03 + INRA023 + HEL9 + ILSTS006 + SPS115) and 5 (CSSM66 + CSRM60 + ETH03 + INRA023 + HEL9) loci, respectively.

DISCUSSION

Of 20 microsatellites used in this study, 12 loci were reported as the most commonly used for cattle parentage testing [18] and all loci were highly polymorphic. Observed high polymorphisms suggest that these loci are appropriate to be used in population genetic studies. Obtained average allele number (13.45) was found to be similar with the other studies of Turkish native cattle breeds [19-21]. The highest allele number at TGLA122 was also observed in previous studies [21-24].

Informativeness of a locus depends on the allele number. For this purpose, the parameters including heterozygosity (Ho) and probability of exclusion values were widely used and estimated by distribution of allele frequencies in populations. The Ho and He values of native cattle breeds in Turkey were determined to be higher than that reported for other breeds from different continents [19,24-26]. The reason for the higher Na, Ho and He is thought to be the number of samples used, the close localization of these populations to the domestication region and high level of genetic diversity [15,19,20,27].

Probability of exclusion (PE) is a mathematical definition of probability of excluding a random individual from the population as a potential parent. The PE is accepted as the most important criteria for genetic markers used in parentage testing studies [28]. In the present study, adequate PE values (>0.999) were observed for all cattle populations using 20 markers.

Different population genetic parameters and the misidentification rate were investigated by using 9 different microsatellite markers for Gry cows located in Brazil [29]. By using the same 7 [29] and 11 markers [3] in this study, PE values have been found to be 0.188-0.629 [29] and 0.175-0.552 [3] for Gry and Yugoslav Pied cattle, respectively. The total PE values were 0.979 [29] and 0.996 [3]. The total PE values were estimated for Holstein-Friesian, Brown Swiss

and their crosses with native cattle breeds in Turkey [30] and found to be similar (>0.9999) with this study.

The PE >0.999 was obtained with 9 [31] and 10 markers [4], however, the same PE was found in this study by only using 7 (PE-2) and 5 markers (PE-1). Recently SNPs were reported to be efficient marker system parentage testing efforts [32].

Basen on the results of this study; it was concluded that a test panel including the most informative 7 loci can provide enough power proving its usefulness for parentage testing and population genetic studies of local cattle breeds in Turkey.

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Kafkas Univ Vet Fak Derg
20 (4): 527-531, 2014
DOI: 10.9775/kvfd.2013.10521

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

An Investigation of Micronucleus Induction by Butylated Hydroxytoluene in Wistar Rat Bone Marrow Cells [1]

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Makale Kodu (Article Code): KVFD-2013-10521

Summary

This study aims to investigate whether Butylated Hydroxytoluene (BHT), the synthetic antioxidant and food additive, increases the frequency of micronucleated polychromatic erythrocytes (MNPCEs) in rat bone marrow. BHT, dissolved in corn oil, was administered intraperitoneally to 8-10 week old male and female Wistar rats (n=36) in three different doses (125, 250 and 500 mg/kg b.w.) for two different time periods. 12- and 24-h after BHT treatment, the bone marrow samples were analyzed for the frequency of MNPCEs. Additionally, by evaluating the ratio of polychromatic erythrocyte to normochromatic erythrocyte (PCE/NCE), the cytotoxic effect of BHT on bone marrow was tested. It was found that all BHT doses at two time periods significantly increased the MNPCEs frequency about 1.9-2.84 fold. On the other hand, BHT caused significant decreased in the PCE/NCE ratio, which is indicative of bone marrow cytotoxicity when compared to the control groups. This study showed that BHT increased the formation of MNPCEs in rat bone marrow and we think that this increase might be related to the applied doses and the administration way BHT.

Keywords: *Butylated hydroxytoluene, Cytotoxicity, Genotoxicity, Micronucleus*

Bütil Hidroksitoluen Tarafından Mikronükleus İndüksiyonunun Wistar Sıçan Kemik İliği Hücrelerinde Araştırılması

Özet

Bu çalışma ile sentetik antioksidan ve yiyeceklerde katkı maddesi olarak kullanılan Bütil Hidroksitoluenin (BHT) rat kemik iliğinde mikronükleuslu polikromatik eritrositlerin sayılarında herhangi bir artışa neden olup olmadığı araştırıldı. Mısır yağında çözülen BHT, iki farklı zaman periyodu ve üç farklı dozda (125, 250, 500 mg/kg v.a.), 8-10 haftalık erkek ve dişi Wistar albino sıçanlara (n=36) intraperitonal olarak verildi. BHT muamelesinden 12 ve 24 saat sonra kemik iliği örnekleri MNPCE sayısı için analiz edildi. Ayrıca, polikromatik eritrositlerin normokromatik eritrositlere oranı (PCE/NCE) değerlendirilerek BHT'nin kemik iliğindeki sitotoksik etkisine bakıldı. Bütün BHT dozları ve iki farklı zaman periyodunda MNPCE'lerin sayısında 1.9-2.84 kat önemli artışlar bulundu. Diğer taraftan BHT, kemik iliği sitotoksisite belirteci olan PCE/NCE oranını kontrol grubuna kıyasla düşürdü. Sonuç olarak bu çalışmada BHT'nin rat kemik iliğinde MNPCE oluşumunu artırdığını ve bu artışın, uygulanan dozlar ve verilme şekliyle ilişkili olabileceğini düşünmekteyiz.

Anahtar sözcükler: *Bütil Hidroksitoluen, Sitotoksisite, Genotoksisite, Mikronükleus*

INTRODUCTION

Butylated hydroxytoluene (BHT; 2,6-di-*tert*-butyl-p-cresol), a synthetic antioxidant that is widely used in the food industry around the world, is used to maintain and protect foods' color, taste, nutritive value, and freshness. It is also used to preserve drugs, fat-soluble vitamins and cosmetics for long periods of time. BHT helps to increase the life extension of rubber, elastomers and plastics [1,2].

BHT's genotoxicity was investigated in a large number of *in vitro* and *in vivo* test systems. The negative results were obtained from the mutagenicity studies performed by adding BHT to plates with different strains of *Salmonella typhimurium* [3-6]. The micronucleus test conducted by the Stanford Research Institute (S.R.I.) also presented negative results for BHT administered with doses of 30, 90, and

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1400 mg/kg (acute and subacute) in rat bone marrow cells [7]. Jung and Ryu did not find any increase in micronucleus frequency of mouse peripheral reticulocytes in BHT applications [8]. According to the World Health Organization, International Agency for Research on Cancer (WHO/IARC), BHT does not stimulate the DNA damage in *Bacillus subtilis*, mutations in *Salmonella typhimurium*, chromosomal aberrations in plants and *Drosophila melanogaster*, dominant lethal mutations in mice and micronucleus formation in bone [9]. The present study aims to investigate whether BHT is genotoxic in three different doses and two different time periods in rat bone marrow cells by applying micronucleus assay.

MATERIAL and METHODS

Animals

Healthy adult male and female *Wistar albino* rats (n=42), 8-10 weeks of age, with an average body weight of 180-200 g, were used in this study. Rats were obtained from the Experimental Medical Research Unit at Kocaeli University, Turkey. Rats were randomly selected and housed in polycarbonate cages with free access to tap water and rat chow with a 12 h dark/light cycle. The temperature value of the animal laboratory was 22±2°C and the relative humidity was 50-70%. For each dose group, six animals were used and were allowed one week to adjust to their new environment. The Ethics Committee of Kocaeli University School of Medicine gave ethical approval for this research (Ethical Approval No: 2011-24) and all procedures on animals were performed in accordance with the guidelines of this ethics unit.

Experimental Design and Doses

In this study, the food preservative BHT (Sigma - B1378) (E321) was used as the test substance. BHT was disssolved in corn oil. A total of 42 animals were randomly divided into seven groups, each including three female and three male rats (n=6 per group). Six groups (n=36) served as BHT-treated group while one group served as control group (n=6). Rats in BHT-treated groups were administered a single dose of BHT intraperitoneally at concentrations of 125, 250, and 500 mg/kg b.w. for 12 and 24 h before sacrifice. Rats in control group were given only corn oil. The control group was also referred to the solvent control. Rats were sacrificed by cervical dislocation at 12 and 24 h after BHT treatment. Femurs of each rat were bilaterally harvested and cleaned of any adhering muscle. Bone marrow cells were bilaterally collected from the rats' femurs.

Micronucleus Test

The frequency of micronucleuated erythrocytes in femoral bone marrow was evaluated according to the procedure of Schmid with slight modifications of Agarwal and Chauhan [10,11]. The bone marrow was flushed out from both femora using 1 ml of fetal calf serum and centrifuged at 336 g for 10 min and the supernatant was discarded. Evenly spread bone marrow smears were stained using the May-Grünwald and Giemsa protocol [12]. Slides were scored at a magnification of 1000x using a light microscope.

Scoring

For the analysis of MN, 2000 polychromatic erythrocytes (PCEs) per animal were scored to calculate the MN frequencies. The PCE/NCE (Normochromatic erythrocyte) ratio was also determined based on a total of 1000 erythrocytes counted. PCEs appear as blue and NCEs appear as orange/pink in bone marrow.

Statistical Analysis

The Kruskal Wallis Method was used because each group consisted of four animals and the difference between the groups was identified with the method. Then, statistical analysis was performed using the SPSS 18 package program. To determine the statistical significance between dosage and effect, depending on the time, the Mann-Whitney U Test was used. $P<0.05$ was considered as the level of significance.

RESULTS

The investigation of the genotoxic effects of BHT in rats was evaluated by the detection of micronucleus frequency in PCEs in bone marrow. No sign of sickness, decreased activity, or mortality was observed with the rats used during the study.

BHT induced a significant increase of MNPCEs for all treatment groups when compared with control groups *(Table 1, Fig. 1)* ($P<0.05$). In *Table 1*, in contrast to an increase in doses, a decrease was observed in the number of MNPCEs in 12- and 24-h experimental groups. Within the examination of the 12-h groups, it was found that the decrease in the number of MNPCEs at 500 mg/kg was statistically different from doses of 125 and 250 mg/kg. On the other hand, when 24-h experimental groups were examined, in contrast to an increase in doses, a decrease was observed in the numbers of MNPCEs. This, however, was not statistically significant. Comparing the experimental groups of the same BHT doses at 12 and 24 h, statistically no difference was found in terms of the numbers of MNPCEs.

The significant decreases in the ratio of PCE to NCE were recorded between experimental and control groups while there are significant increases in the MNPCEs frequency *(Table 1)*. In the 12-h experimental group, decreases were observed in the PCE/NCE ratio in contrast to an increase in BHT doses with 125, 250 and 500 mg/kg. These decreases were statistically significant at the 0.05 level. In the

Table 1. *The induction of micronucleus in rat bone marrow erytrocytes after butylated hydroxytoluene treatment*
Tablo 1. *Bütil hidroksitoluen muamelesinden sonra sıçan kemik iliği eritrositlerinde mikronükleus indüksiyonu*

Treatments	Dose (mg/kg)	Time (h)	Total PCE/n	MNPCE/2000 PCE	PCE/NCE
Control[a]	-	-	12000/6	6.25±1.25	1.59±0.140
BHT	125	12	12000/6	17.75±1.5*†	1.14±0.044*#
	250	12	12000/6	15.7±1.25*†	0.75±0.016*†#
	500	12	12000/6	12.25±0.96*	0.68±0.031*©
	125	24	12000/6	14.75±0.95*	0.92±0.042*
	250	24	12000/6	14.30±0.96*	0.93±0.018*
	500	24	12000/6	13.75±0.96*	0.59±0.047*†

n: *number of animals per group; MNPCE, micronucleated polychromatic erythrocyte;* ***PCE:*** *polychromatic erythrocyte;* ***NCE:*** *normochromatic erythrocyte. All data are presented as mean ± standard deviation, [a] Corn oil; * P<0.05: significantly different from control groups; †© P<0.05: significantly different from groups with BHT doses of same time periods; # P<0.05: significantly different from groups with time periods of same BHT doses*

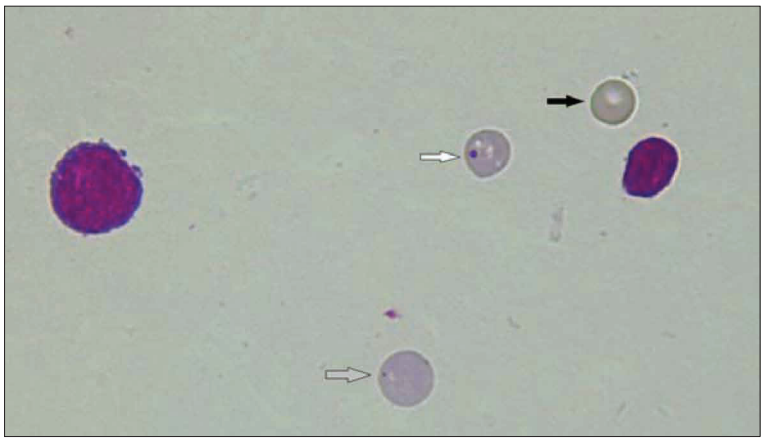

Fig 1. *White arrow* refers to MNPCE; *black arrow* refers to NCE; *gray arrow* refers to PCE

Şekil 1. *Beyaz ok* MNPCE; *siyah ok* NCE; *gri ok* PCE'yi işaret etmektedir

case of the 24-h experimental group, the statistically significant, approximately 1.5 fold decrease, was observed in the 500 mg/kg BHT dose compared with 125 and 250 mg/kg BHT doses. Comparing within the experimental groups of matching BHT doses at 12 and 24 h, the PCE/NCE ratio in animals given 125 and 250 mg/kg BHT doses was statistically significant at the 0.05 level, though no difference was found in the application of 500 mg/kg.

The PCE/NCE ratio in *Table 1* shows whether BHT had a cytotoxic effect on bone marrow. Accordingly, the PCE/NCE ratio was decreased in all applied BHT doses. This is an indication of the cytotoxicity of the bone marrow. The highest reduction was observed in rats treated with the highest dose of BHT.

DISCUSSION

A micronucleus test is a very useful biomonitoring test which is used to predict a cancer risk, screen for cancer and for researching the genotoxic and carcinogenic potentials and reabilities of all kinds of chemical substances such as physical factors, drugs, environmental pollutants and food additives that we are often exposed in our daily lives. Because of its advantages such as simplicity, reliability, validity and applicability in different cell types, the micronucleus test has been used for many years [12-17].

As reported in Bomhard et al.[6], with regard to point mutation assays, negative results were obtained in most of the genotoxicity tests with BHT. These tests include *in vitro* studies performed with various bacterial species, strains and various mammalian cell lines as well as *in vivo* studies with *Drosophila melanogaster*, silk worms and mouse specific locus tests [6,18]. According to the European Food Safety Authority Panel (EFSA Panel), effects of BHT on tumor formation reported by Olsen et al. are subject to a threshold because the genotoxicity studies generally show a lack of potential for BHT to induce point mutations, chromosomal aberrations, or to interact with or damage DNA [18,19]. The Benchmark dose *(BMD)* analysis from the Panel shows that the Benchmark dose, lower 95% confidence limit at 10% extra risk ($BMDL_{10}$) value was 247 mg/kg b.w./day for BHT derived from the data reported by Olsen et al.[18].

As reported in Bomhard et al.[6] study, there were a large number of genotoxicity studies done with BHT in *Salmonella typhimurium* in the 1980's and 1990's. The study conducted by Shelef and Chin , indicated that the addition of BHT (5-20 µg/plate) caused a 2-fold increase on mutagenic potential of aflatoxin B1 using *Salmonella typhimurium* TA98 and TA100 strains [20]. However, in a

similar study performed by Redy et al.[21] it was reported that the mutagenicity induced by 3,2'-dimethyl-4-amino-biphenyl in the presence of rat liver S-9 fraction on *Salmonella typhimurium* TA98 and TA100 strains was inhibited by BHT. Similarly, Ames test results were found negative in point mutation studies performed by adding different amounts of BHT to plates with the same bacterial species and strains [3-5].

A study performed by using Djungarian hamster fibroblast cultures transformed SV-40, it was found that a concentration as low as 10µg/ml BHT exerted a strong inhibitory effect on cell to cell dye transfer (Lucifer yellow transfer). However, it was suggested that this effect was reversible and BHT shared this effect with a series of well-known tumor promoters [22].

In the studies conducted by S.R.I. in 1972, the result of chromosomal aberration assay using 2.5-250 µg/ml BHT with human WI-38 (embryonic lung cell) was positive, and MN assay using rat bone marrow applied BHT at concentrations of 30, 90 and 1400 mg/kg (acute and sub-acute) gave negative results [7].

Jung and Ryu applied the micronucleus test in mouse peripheral reticolocytes *in vivo*. They injected single dose BHT at doses of 17.3, 34.5 and 69.0 mg/kg intraperitoneally. In the evaluation of the frequency of MNRETs at 48 h after BHT, they found that BHT administration did not cause a dose-dependent increase in the frequency of MNRETs [8].

In this study, male *Wistar albino* rats were injected intraperitoneally with BHT at doses of 125, 250 and 500 mg/kg b.w., and after 12 and 24 h. of BHT treatment the sampling of bone marrow was taken for evaluation. At the end of the study, it was found that all administered BHT doses and time periods caused an increase in the frequency of MNPCEs *(Fig. 1)*. The highest frequency of MNPCEs was in 125 mg/kg b.w dose at the 12 h time period; this increase was approximately 3 fold when compared to control groups *(Table 1)*. We might say that the dose of 500 mg/kg showed a similar effect on both MNPCEs numbers and PCE/NCE ratio at 12 h and 24 h time periods. In this study, we also determined that BHT caused a decrease in the ratio of PCE/NCE, demonstrating the cytotoxic effect of BHT in rat bone marrow cells. The ratio of PCE to NCE is an important index in showing the toxicity of chemical substances affecting bone marrow cells. The significant decrease, which was observed in the PCE/NCE ratio in the chemically treated group when compared to control group, is an indication that the administered chemical reached to bone marrow, caused a decrease in the erythrocyte formation by inhibiting the maturation and division of the nucleated erythrocyte precursor cells, and caused a toxic effect [23-26].

As presented in *Table 1*, the statistically significant increases in the number of MNPCEs were observed in all doses and two time periods when compared to control groups. However, these increases interestingly reduced with an increased dosage. The statistically significant decreases seen in PCN/NCE ratios when compare to control groups, are parallel to the decrease in the number of MNPCEs in experimental groups.

In comparison to this study in which we clearly found positive results with the studies having negative results for micronucleus frequency. Those differences depend on several factors that are range of dose, way of application, contact time, cell types, and *in vivo* or i*n vitro* study. Paschin et al. injected intraperitoneally single dose of 75 mg/kg b.w. BHT into male and female mice [27]. Jung and Ryu injected single dose BHT at doses of 17.3, 34.5 and 69. 0 mg/kg BHT into mice intraperitoneally and examined the MNRETs frequency for 48 h after BHT [8]. Contrary to all these studies, in our study, BHT was administered to *Wistar albino* rats intraperitoneally and bone marrow samples were taken 12 and 24 h after BHT treatment. The applied doses were 125, 250 and 500 mg/kg b.w. All these applications might have caused positive results identified in micronucleus test in our study.

In conclusion, this study which is performed using an *in vivo* micronucleus test demonstrated that the treated dose of BHT at experimental time periods caused an increase in the formation of MNPCEs. Given that BHT shows a promoter effect in carcinogenesis studies, solitary or combined effects of BHT should be considered as the exposure to many different chemicals in cosmetic products and food additives in our daily lives is highly probable.

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Kafkas Univ Vet Fak Derg
20 (4): 533-539, 2014
DOI: 10.9775/kvfd.2013.10550

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Investigation of the Possible Use of Black Cumin (*Nigella sativa* L.) Essential Oil on Butter Stability [1]

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Makale Kodu (Article Code): KVFD-2013-10550

Summary

The aim of this study was to investigate the effect of black cumin (*Nigella sativa* L.) essential oil on butter stability. For this aim, 0.05, 0.1 and 0.2 wt-% essential oil was added to the butter. Antioxidant activity of the essential oil was compared to that of synthetic antioxidant BHT (100 ppm). All samples were stored at 4±1°C for 90 days. Thiobarbituric acid and peroxide values of all samples containing essential oil were decreased depending on concentrations. The amount of 0.2% of essential oil had showed strong antioxidant activity, which was almost equal to that of BHT. The addition of essential oil reduced total aerobic mesophilic bacteria, lactic acid bacteria and coliform bacteria count during storage period but did not show remarkable antifungal activity. Samples containing essential oil were preferred by the panellists compared to Control sample. Present results indicate that black cumin essential oil may be considered as a new source of natural antioxidant.

Keywords: *Antioxidant, Antimicrobial effect, Butter stability, Essential oil, Nigella sativa* L.

Tereyağı Stabilitesi Üzerine Çörekotu (*Nigella sativa* L.) Uçucu Yağı Kullanılabilirliğinin Araştırılması

Özet

Bu araştırmanın amacı, çörekotu (*Nigella sativa* L.) uçucu yağının tereyağının stabilitesi üzerine etkini araştırmaktır. Bu amaç için üretimden hemen sonra tereyağına 0.05; 0.1 ve 0.2 (ağırlık/%) çörekotu uçucu yağı ilave edildi. Uçucu yağın antioksidan aktivitesi sentetik antioksidan BHT (100 ppm) ile karşılaştırıldı. Tüm örnekler 90 gün süresince 4±1°C 'de muhafaza edildi. Uçucu yağ içeren tüm örneklerin tiyobarbitürik asit ve peroksit değerleri, konsantrasyonlara bağlı olarak azaldı. %0.2 seviyesindeki uçucu yağ ilavesi, BHT ile hemen hemen eşit güçte antioksidan aktivite gösterdi. Uçucu yağ, toplam aerobik mezofilik bakteri, laktik asit bakteri ve koliform bakteri sayılarını depolama süresince azaltmış, ancak dikkate değer bir antifungal aktivite göstermemiştir. Uçucu yağ içeren örnekler kontrol örnek ile karşılaştırıldığında panelistlerce tercih edilmiştir. Sonuçlar, çörekotu uçucu yağının, yeni bir doğal antioksidan kaynağı olarak kabul edilebilir olduğunu göstermiştir.

Anahtar sözcükler: *Antioksidan, Antimikrobiyal etki, Tereyağı stabilitesi, Uçucu yağ, Nigella sativa* L.

INTRODUCTION

Butter, a food consumed all over the world, is used both directly and as an ingredient in a variety of dairy products [1]. Lipids, which are important macromolecules in foods, affect nutritional value, texture, flavour and shelf life of a product [2]. On the other hand, all fat and fat containing foods are vulnerable to oxidative deterioration, which reduce both nutritional quality and makes the food unacceptable for consumers [3]. It is reported that the oxidation of lipids is related to human diseases like cancer, heart diseases, membrane damage and ageing, so that antioxidants are used in foods to control and delay oxidation [4]. Use of natural antioxidants instead of synthetic

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ones has become popular as consumer awareness about chemical additives increased [5,6]. That is why researchers have been focusing on new natural sources that have high antioxidant activity and harmless for health to prolong shelf-life and to increase food stability [7-9].

Nigella sativa L. belongs to Ranunculaceae family, which is commonly known as "black cumin" in English and "çörekotu" in Turkish. The genus *Nigella* L. includes about 20 species [10] from the Mediterranean to Western Asia and 12 species in Turkey [11]. Black cumin (*Nigella sativa* L.) seeds have been used as a spice and the seeds or essential oils have been widely used in traditional medicinal applications [12,13]. Black cumin seed has over 100 different chemical constituents, including abundant sources of all the essential fatty acids [14]. The seeds contain essential oils, fixed oils, proteins, phospholipids, saponin and other some components [12] Essential oils, which are secondary metabolite products of aromatic plants, are strong odorous, volatile and natural complex compounds [15]. Many components are identified from the essential oil of *N. sativa* L. But the major ones are thymoquinone (27.8%-57.0%), *p*-cymene (7.1%-15.5%), carvacrol (5.8%-11.6%), *t*-anethole (0.25%-2.3%), 4-terpineol (2.0%-6.6%) and longifoline (1.0%-8.0%) [16].

Although several studies on the antimicrobial and antioxidant properties of members of *Nigella* L. genus are carried out [17-22] there are a few studies published on the use of *N. sativa* species in butter production. The aim of this study is to investigate the effects of *N. sativa* essential oils at three concentrations (0.05, 0.1 and 0.2 wt-%) on butter stability as an antioxidant according to Control and with BHT samples, and on sensorial properties as well.

MATERIAL and METHODS

Materials

Nigella sativa L. seeds were purchased from local markets in Erzurum province in the Eastern Anatolia region of Turkey. Sour raw cream used in the butter production was provided by the Leben Dairy Products Plant located in Erzurum, Turkey.

Methods

Isolation of the Essential Oils and GC-MS Analysis Conditions

The essential oils in mature seeds were subjected to steam distillation using a Clevenger-type apparatus for 4 h. The oils were extracted with $CHCl_3$ and then dried over anhydrous Na_2SO_4 and stored under N_2 atmosphere at 20°C in a sealed vial until use. The essential oil was chemically analyzed and identified by GC and GC-MS. The analysis of the essential oil was performed using a Thermofinnigan Trace GC/Trace DSQ/A1300 (EI quadrupole) equipped with an SGE-BPX5 MS capillary column (30 m x 0.25 mm i.d.. 0.25 mm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was the carrier gas, at a flow rate of 1 mL/min. Injector and MS transfer line temperatures were set at 220 and 290°C, respectively. The program used was 50 to 150°C at a rate of 3°C/min, held isothermal for 10 min, and finally raised to 250°C at 10°C/min. Diluted samples (1:100 v/v, in methylene chloride) of 1.0 mL were injected manually in splitless mode. The components were identified based on the comparison of their relative retention times and mass spectra with those of standards provided in Wiley7N library data of the GC-MS system, and data provided in the literature [23]. The results were also confirmed by comparison of the elution order of the compounds with their relative retention indices on non-polar phases reported in the literature.

Preparation of Butter Samples

The butter samples were manufactured from commercial cow milk cream under controlled condition in the Leben Dairy Plant (Erzurum, Turkey). The cream was not pasteurized to see antimicrobial effect. After butter samples were heated at 32°C, they were divided into five parts. *N. sativa* essential oil was added at 0.05, 0.1 and 0.2 wt-% levels to the first three samples, and coded NS1, NS2 and NS3, respectively. 100 ppm BHT was added to the fourth sample. The fifth sample was the Control sample which did not contain any essential oil or BHT. All samples were individually stirred carefully and homogeneously for 5 min at 40°C. Then each sample were divided into 250 g packages and stored at 4±1°C for 90 days until analysis. All experiments were performed in duplicate.

Chemical Analysis

The thiobarbituric acid (TBA) value was determined to a modified version method according to Rossell [24] of the method of Tarladgis et al.[25] and Ockerman [26]. The TBA value was calculated by using a standard curve [8]. The TBA value was the estimated as mg malonaldehyde/kg butter.

The peroxide value (PV) was determined according to the method described by Atamer [27] and Dagdemir et al.[8]. PV is expressed as milliequivalents (meq) of active oxygen per kg of butter.

Titratable acidity (lactic acid, %) was determined as suggested by Kurt et al.[28].

Microbiological Analysis

For microbiological analyses, 10 g of butter sample was weighed aseptically in 90 mL of 0.1% peptone plus 0.85% (wt/v) NaCl solution and homogenized in a sterile polyethylene bag by using a Stomacher (Mayo HG400 Stomacher, Italy) for 2 min. Serial dilutions were prepared from this solution [29]. The enumeration of total aerobic

mesophilic bacteria (TAMB) (Plate Count Agar, Merck, Darmstadt, Germany) at 30°C for 72 h [30], total coliforms (VRBA) (Violet Red Bile Agar, Merck, Darmstadt, Germany) at 35°C for 24 h [30], yeasts and moulds (Potato Dextrose Agar, Merck, Darmstadt, Germany; acidified 10% tartaric acid) at 25°C for 5 d [29], lactic acid bacteria (LAB) (de Man Rogosa Sharp Agar, Merck, Darmstadt, Germany) at 30°C for 72 h [31], were performed. All determinations were made in duplicate.

Sensory Analysis

The sensory properties of the butter samples were evaluated according to the method suggested by Bodyfelt et al.[32]. Five sensory attributes containing odour, taste, colour, texture, and general acceptability were evaluated according to a 1- (poor) to 9-point (excellent) scale by eight trained panellist. For this aim samples were removed from the refrigerator about 1 h prior to evaluation and kept at room temperature. Warm water and bread were also provided to the panellists to cleanse their palates between samples.

Statistical Analysis

The experimental design consisted of a completely randomized design in a factorial arrangement: Five treatments (butter samples: C, NS1, NS2, NS3, BHT), four storage periods (2, 30, 60, 90 days), and two replicates. Statistical analysis of the data was made using the analysis of variance (ANOVA) of the SPSS program, version 10.0.1. Means with a significant difference were compared by Duncan's multiple range tests. All analyses were made in duplicate.

RESULTS

The chemical composition of essential oil used in our study was *p*-cymene (17.40%), carvacrol (16.94%), thymoquinone (12.33%), borneol (11.55%), thymol (6.61%), linalool (5.21%), terpinen-4-ol (3.49%), β-caryophyllene (3.02%), ascoridole (2.61%), 4-terpineol (2.39%), thymol methyl ether (1.61%), α- thujene (0.56%).

TBA, peroxide and titratable acidity values of the butter samples are shown in *Table 1*. As PV and TBA values could not be determined on 2 d of storage, acidity values for the same period are not given as well. As the essential oil concentration is increased acidity values raised *(Table 1)* On the other hand, this increase was lower than the Control but higher than BHT containing sample. The primary lipid oxidation is widely measured with PV in fats, oils and fat containing foods [33]. As seen in *Table 1*, when oxidative stability of *N. sativa* L. essential oil at three concentrations were compared with the Control sample, especially, NS2 and NS3 essential oil concentrations were effective on reducing the oxidation level of butter samples. TBA values of butter samples are shown in *Table 1*. Generally, TBA values of all samples increased during the storage.

The effects of antioxidant addition and storage periods on some microbiological properties of butter samples are shown in *Table 2*. The TAMB counts decreased in all samples during storage period. TAMB counts of all samples were similar statistically on 2 and 30 days of storage. But the differences between samples were started to seen on 60 day of storage. The LAB counts of butter samples decreased during the storage period. While until 60 day of storage there were no statistical differences between samples, essential oil and BHT containing samples were lower than control sample at the end of the storage. As seen *Table 2*, while the coliform bacteria count decreased, yeast-mould count increased during the storage periods in all samples. Arici et al.[19] tested the antibacterial activity of five *N. sativa* essential oils from different regions of Turkey on some spoilage and/or pathogenic bacteria and LAB. Researchers reported that the antibacterial effects of the samples may be closely related to their high percentage

Table 1. *Effect of treatments and storage periods on acidity (lactic acid %), PV (meq O_2/kg) and TBA (mg malonaldehyde/kg) in samples of butter*
Tablo 1. *Tereyağı örneklerinde TBA (mg malonaldehyde/kg), PV (meq O_2/kg) ve asitlik (% laktik asit) üzerine depolama periyodu ve muamelelerin etkisi*

Variants	Storage Period (days)	Butters[1]				
		C	NS1	NS2	NS3	BHT
Acidity	30	0.44 ± 0.01^{aB}	0.41 ± 0.00^{abB}	0.43 ± 0.02^{aB}	0.45 ± 0.01^{aB}	0.39 ± 0.00^{bB}
	60	0.52 ± 0.03^{bB}	0.48 ± 0.01^{bAB}	0.52 ± 0.06^{bAB}	0.63 ± 0.02^{aA}	0.48 ± 0.03^{bA}
	90	0.82 ± 0.05^{aA}	0.56 ± 0.07^{bcA}	0.60 ± 0.00^{bcA}	0.67 ± 0.02^{bA}	0.55 ± 0.02^{cA}
PV	30	0.22 ± 0.03^{aC}	0.13 ± 0.02^{bC}	0.13 ± 0.01^{bC}	0.09 ± 0.01^{bC}	0.14 ± 0.04^{bC}
	60	0.72 ± 0.05^{aB}	0.57 ± 0.04^{abB}	0.35 ± 0.01^{bB}	0.33 ± 0.01^{bB}	0.65 ± 0.23^{aB}
	90	1.70 ± 0.12^{aA}	1.45 ± 0.09^{abA}	1.32 ± 0.11^{bA}	1.29 ± 0.07^{bA}	1.23 ± 0.13^{bA}
TBA	30	0.15 ± 0.01^{aC}	0.13 ± 0.01^{abB}	0.12 ± 0.01^{abB}	0.12 ± 0.00^{abB}	0.12 ± 0.01^{abB}
	60	0.25 ± 0.00^{aB}	0.22 ± 0.01^{bA}	0.20 ± 0.02^{bA}	0.22 ± 0.00^{bA}	0.18 ± 0.01^{bA}
	90	0.28 ± 0.01^{aA}	0.24 ± 0.01^{bA}	0.23 ± 0.01^{bA}	0.22 ± 0.01^{bA}	0.22 ± 0.00^{bA}

[1] ***C:*** *Control (no additives); NS1, NS2, NS3 butters with respectively 0.05, 0.1 and 0.2% of N. sativa essential oil;* ***BHT:*** *butters with 100 ppm BHT;* $^{a-c}$ *Means within a row with no common superscript differ (P<0.05);* $^{A-C}$ *Means within each column of each category followed by the different letters are significantly differ (P<0.05)*

Table 2. *Effect of treatments and storage periods on some microbiological properties of butter samples (log cfu/g)*
Tablo 2. *Tereyağı örneklerinin bazı mikrobiyolojik özellikleri üzerine depolama periyodu ve muamelelerin etkisi (log kob/g)*

Variants	Storage Period (days)	Butters[1]				
		C	NS1	NS2	NS3	BHT
TAMB	2	6.83±0.21^{aA}	6.84±0.07^{aA}	6.80±0.15^{aA}	6.84±0.14^{aA}	6.73±0.15^{aA}
	30	6.70±0.18^{aAB}	6.72±0.06^{aA}	6.65±0.08^{aA}	6.71±0.29^{aA}	6.75±0.17^{aA}
	60	6.60±0.04^{aAB}	6.36±0.09^{abcB}	6.17±0.00^{bcB}	6.13±0.01^{cB}	6.41±0.17^{abAB}
	90	6.32±0.12^{aB}	5.88±0.02^{bcC}	5.71±0.16^{cC}	5.81±0.04^{cB}	6.06±0.02^{bB}
LAB	2	6.47±0.12^{aA}	6.40±0.39^{aAB}	6.62±0.17^{aA}	6.66±0.26^{aA}	6.60±0.09^{aA}
	30	6.22±0.25^{aA}	6.62±0.09^{aA}	6.31±0.33^{aAB}	6.15±0.41^{aAB}	6.62±0.09^{aA}
	60	6.32±0.32^{aA}	6.26±0.31^{aAB}	5.97±0.01^{aBC}	5.79±0.03^{aB}	5.98±0.07^{aB}
	90	6.20±0.14^{aA}	5.75±0.02^{bB}	5.69±0.00^{bC}	5.65±0.07^{bB}	5.73±0.05^{bC}
Coliform bacteria	2	4.83±0.09^{aA}	4.54±0.10^{abA}	4.48±0.12^{bA}	4.45±0.13^{bA}	4.71±0.13^{abA}
	30	4.67±0.06^{aA}	4.42±0.03^{aA}	3.78±0.16^{bAB}	3.26±0.18^{cB}	4.43±0.36^{aAB}
	60	4.64±0.18^{aA}	4.04±0.17^{abB}	3.05±0.90^{bB}	2.95±0.41^{bB}	3.38±0.44^{abB}
	90	3.55±0.12^{aB}	2.25±0.07^{bC}	<1^{dC}	<1^{dC}	1.65±0.49^{cC}
Yeast-mould	2	4.03±0.91^{aA}	3.30±0.89^{aA}	3.57±0.27^{aB}	3.20±1.03^{aA}	3.51±0.48^{aA}
	30	4.61±0.07^{aA}	4.27±0.21^{abA}	4.18±0.14^{abAB}	3.64±0.29^{bcA}	3.49±0.38^{cA}
	60	5.37±0.33^{aA}	4.69±0.26^{abA}	4.69±0.36^{abA}	3.74±0.94^{bA}	4.18±0.09^{abA}
	90	4.69±0.34^{aA}	4.68±0.30^{aA}	4.43±0.11^{aA}	4.52±0.31^{aA}	4.16±0.19^{aA}

[1] ***C:*** *Control (no additives); NS1, NS2, NS3 butters with respectively 0.05, 0.1 and 0.2% of N. sativa essential oil;* ***BHT:*** *butters with100 ppm BHT;* $^{a-d}$ *Means within a row with no common superscript differ (P<0.05);* $^{A-C}$ *Means within each column of each category followed by the different letters are significantly differ (P<0.05)*

of thymoquinone, *p*-cymene and carvacrol which have antibacterial effect [18]. Viuda-Martos et al.[34] suggested that bacteria type is important factor on antibacterial activity because of their cell membrane differences. As seen *Table 2*, the coliform bacteria counts of the butter samples were generally lower when compared to the Control samples. The samples containing 0.1% and 0.2% essential oil had the lowest coliform bacteria count. Yeast and mould counts were increased until the 60 days of storage period but then a slight decrease was seen for the 90 days, except NS3 sample *(Table 2)*.

Sensory properties are the main measurement criteria to detect food quality and consumer enjoying. The effects of treatments and storage periods on the sensory properties of butter samples are shown *Table 3*.

DISCUSSION

Antioxidant activity of essential oils could be attributed to the high contents of some components present in the oils. One of the examples is represented by Viuda-Martus et al.[34] who found the composition as *p*-cymene (33.03%), thymoquinone (32.18%) and α-thujene (13.01%). In another study, the contents of *N. sativa* essential oil were found as thymoquionone (23.25%), *p*-cymene (32.02%), carvacrol (10.38%), α- thujene (2.40%), and thymol (2.32%) [35]. Same researchers reported that composition of black cumin could change depending on the geographic distribution, time of harvest and agronomic practices. In our study, the contents were found some differences.

In this study, at the end of the storage the acidity (%) were in this order: BHT < NS1 < NS2 < NS3 < C. *N. sativa* essential oil is mostly composed of fatty acids including linoleic (58-65%), oleic (22-24%), palmitic (13-20%) and stearic acid [36]. Our result was in agreement with Jasinska and Wasik [37], who revealed that savoury added to the butter resulted in significantly higher lipid acidity during the cold storage. Increase of acidity depending on the concentration can be due to high unsaturated fatty acid content.

Essential oil containing samples showed better anti-oxidant activity than the Control and BHT containing sample especially on 30 and 60 days of storage and there was no differences between concentrations. However, at the end of the storage, there were no statistically significant differences between essential oil containing and BHT samples but lower than Control sample. It can also be concluded that there was a negative correlation between essential oil concentrations; as the essential oil concentration increased, PV values were decreased. Among the samples, Control showed the highest PV values in all storage periods. However, PV increased statistically significantly during the storage periods in all samples. In this study 0.2% level showed the most antioxidant activity. In another study by Ozkan et al.[38] who found that 2% *Satureja clicica* essential oils showed

Table 3. *Effect of treatments and storage periods on sensory properties of butter samples*
Tablo 3. *Tereyağı örneklerinin duyusal özellikleri üzerine depolama süresi ve muamelelerin etkisi*

Variants	Storage Period (days)	Butters[1]				
		C	NS1	NS2	NS3	BHT
Colour	2	8.40±0.14^{a,A}	8.45±0.07^{aA}	8.38±0.18^{aA}	8.25±0.07^{aA}	8.40±0.14^{aA}
	30	8.15±0.21^{a,A}	8.35±0.21^{aAB}	8.35±0.21^{aA}	8.40±0.14^{aA}	8.05±0.35^{aA}
	60	8.05±0.35^{aA}	7.90±0.14^{abC}	8.00±0.28^{abA}	8.10±0.14^{aA}	7.40±0.14^{bB}
	90	7.90±0.14^{aA}	8.00±0.00^{aBC}	7.88±0.18^{aA}	7.73±0.10^{aB}	7.30±0.00^{bB}
Texture	2	8.38±0.18^{aA}	8.25±0.00^{aA}	8.13±0.18^{aA}	8.15±0.21^{aA}	8.25±0.35^{aA}
	30	8.13±0.18^{aAB}	8.25±0.35^{aA}	7.90±0.14^{aA}	7.75±0.35^{aA}	7.94±0.26^{aA}
	60	7.88±0.18^{aAB}	7.65±0.21^{aA}	7.63±0.18^{a}A	7.70±0.42^{aA}	7.75±0.35^{aA}
	90	7.63±0.18^{aB}	7.75±0.35^{aA}	7.69±0.27^{a}A	7.69±0.27^{aA}	7.60±0.57^{aA}
Odour	2	8.38±0.53^{aA}	8.50±0.00^{aA}	8.38±0.18^{aA}	8.25±0.35^{aA}	8.50±0.35^{aA}
	30	8.00±0.35^{aAB}	8.00±0.28^{aB}	8.00±0.00^{aAB}	8.00±0.28^{aA}	8.00±0.00^{aB}
	60	7.63±0.18^{aAB}	7.63±0.17^{aB}	7.69±0.27^{aB}	7.69±0.27^{aA}	7.50±0.00^{aB}
	90	7.38±0.18^{bB}	7.58±0.11^{abB}	7.63±0.18^{abB}	7.88±0.18^{aA}	7.50±0.00^{abB}
Flavour	2	8.25±0.35^{aA}	8.38±0.18^{aA}	8.38±0.18^{aA}	8.33±0.11^{aA}	8.38±0.18^{aA}
	30	7.38±0.53^{aA}	8.13±0.18^{aA}	8.15±0.21^{aA}	8.20±0.28^{aA}	8.13±0.18^{aA}
	60	8.13±0.18^{aA}	8.00±0.17^{aA}	8.00±0.28^{aA}	8.10±0.14^{aA}	8.25±0.35^{aA}
	90	7.38±0.18^{bA}	7.83±0.24^{abA}	8.00±0.00^{aA}	8.04±0.23^{aA}	7.88±0.18^{aA}
Overall Acceptability	2	8.50±0.00^{aA}	8.38±0.18^{aA}	8.50±0.00^{aA}	8.53±0.32^{aA}	8.40±0.14^{aA}
	30	7.90±0.57^{aA}	7.94±0.08^{aAB}	8.04±0.23^{aB}	7.94±0.26^{aA}	8.00±0.00^{aAB}
	60	7.75±0.35^{aA}	7.83±0.24^{aB}	7.94±0.08^{aB}	7.75±0.35^{aA}	8.05±0.35^{aAB}
	90	7.55±0.35^{aA}	7.77±0.15^{aB}	8.00±0.17^{aB}	7.94±0.08^{aA}	7.50±0.00^{aB}

[1] ***C:*** *Control (no additives); NS1, NS2, NS3 butters with respectively 0.05, 0.1 and 0.2% of N. sativa essential oil;* ***BHT:*** *butters with 100 ppm BHT;* $^{a-b}$ *Means within a row with no common superscript differ (P<0.05);* $^{A-C}$*Means within each column of each category followed by the different letters are significantly differ (P<0.05)*

more effective antioxidant activity than 0.5 and 1% level, it was reported that antiradical and antioxidant activity could vary depending on the concentration and content of essential oil. Another study revealed that both aqueous and methanolic extracts of cumin varieties containing bitter cumin, cumin and black cumin inhibited lipid peroxidation [20]. Mariod et al.[21] revealed that while black cumin seed-cake showed antioxidant activity on corn oil by decreasing PV after 72 h when compared to Control without antioxidant.

TBA values of samples containing essential oil and BHT were lower than that of Control sample. No significant differences were found between all essential oil and BHT containing samples at the end of the storage. Control sample showed the highest value *(Table 1)*. Our results were in agreement with Dagdemir et al.[8] who indicated that there were no significant differences between *Thymus haussknechtii*, *Origanum acutidens* 0.2 and 0.1%, BHT and control butter samples on the 60th day of storage. Antioxidant activity of *N. sativa* L., using two TLC screening methods showed that thymoquinone, carvacrol, *t*-anethole and 4-terpineol demonstrated important radical scavenging property [17]. It was reported that the antioxidant activity were positively correlated with phenolic content of black cumin and antioxidant properties of black cumin extracts either equal to or higher than BHA and BHT [4]. It was observed that black cumin essential oil inhibited lipid peroxidation 92.56% and DPPH radical formation at a rate of 80.25% [35].

Essential oil containing samples were statistically lower than the sample with BHT and Control on 60 and 90 days of the storage in terms of TAMB counts *(Table 2)*. A study demonstrated that TAMB counts of soft white cheeses 0.3% and 1% essential oil of *N. sativa* decreased from 7.6×10^6 to 1.34×10^6 log cfu/g and 6.2×10^6 to 9.4×10^5 log cfu/g, respectively, after 6 days of storage [40]. LAB counts were decreased depending on the concentration of essential oil. The lowest LAB count was found 5.65±0.07 in NS3 sample (0.2% essential oil) at the end of the storage period *(Table 2)*. This result was in agreement with studies by Ozkan et al.[38] and Dagdemir et al.[8] who indicated, respectively, that the LAB counts of butter samples containing *Saturaja cilicica* essential oils and *T. haussknechtii* and *O. acuditens* were decreased. It can be concluded that the concentration of essential oil was an important factor of the growth of coliform bacteria. At the end of the storage, coliform bacteria counts were in the order of NS3 = NS2 < BHT < NS1 < Control *(Table 2)*. A study conducted by Arici et al.[19]

who showed that generally, the fixed oils of the black cumin samples had higher antibacterial activity against spoilage and pathogenic bacteria than LAB. Another study showed that 0.3% and 1% essential oil of *N. sativa* decreased the *S. aureus*, *Brucella melitensis* and *Escherichia coli* in cheese samples stored for 0, 2, 4 days of storage period at refrigerator temperature [39]. On the other hand, it was reported that when essential oils were added to complex food systems, antibacterial effectiveness is diminished and sensory properties could be affected negatively [34]. Although the yeast-mould counts of essential oil and BHT containing samples were lower than the Control, there were no statistical differences in all samples at the end of the storage. A similar result was determined by Dagdemir et al.[8] who studied *T. haussknechtii* and *O. acuditens* as antioxidants. Another study has shown that while 2% black cumin added to butter, it exhibited antiyeast activity against *Candida zeylanoides* and *Candida lambica* but *Candida kefyr* [40]. Variable results from the literature on effect of essential oils on different microorganisms kinds can be attributed to kind of seed selected, essential oil composition and concentration.

The addition of the essential oils significantly affected the sensory properties of the butter samples. The colour scores of the butter samples were similar during the 90 days of storage, except for BHT which had the lowest scores. All butter samples were same statistically in terms of texture scores. There were no significant difference in the flavour scores of all butter samples during the storage period, and scores were found between 7.38 and 8.38 *(Table 3)*. The overall acceptability scores of all butter samples were same statistically ($P<0.05$). But at end of the storage, the order was BHT (7.50) < C (7.55) < NSI (7.77) < NS3 (7.94) < NS2 (8.0). Generally flavour and overall acceptability scores of samples containing essential oil were higher than those of Control. Essential oil containing samples received higher scores for each parameter. As to the general evaluation of sensory scores, all butter samples were within acceptability limits changing from 7.30 to 8.53.

N. sativa L. essential oils and its concentrations used in this study showed a higher antioxidant activity than the respective Controls. Especially the PV and TBA values of the samples containing 0.2% essential oil were similar to the BHT containing samples. It was also effective on TAMB, LAB and coliform bacteria. The yeast and mould counts of the samples containing essential oils were lower than Control sample and BHT containing sample. Besides microbiological and chemical properties, sensorial properties of foods are much more important criteria for consumer acceptance. From this point the essential oil concentration must be taken into consideration in choosing the antioxidant. That is why *N. sativa* L. and its concentration used in our study were suitable from all points. From all the evaluated studies, it is clear that natural antioxidant sources can be preferred over synthetic ones to enhance not only the shelf life of product but also for the protection of nutritional properties. Thus, the results indicate that *N. sativa* L. essential oil may be considered as new source of natural antioxidant. However, there is still need for more studies to determine the effective essential oils dose.

Acknowledgments

The authors would like to thank Dr. Ebru METE (Faculty of Science, Department of Chemistry, Atatürk University, Erzurum, Turkey) for her kind help with the GC-MS analysis. The authors also thank the Leben Dairy Factory (Erzurum, Turkey) for permission to use their facilities for the production of the butter samples.

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Kafkas Univ Vet Fak Derg
20 (4): 541-544, 2014
DOI: 10.9775/kvfd.2013.10552

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Tri-hooked Monogenean Parasite (*Heteronchocleidus buschkieli*; Ancyrocephalidae: Heteronchocleidinae) Isolated from Ornamental Fish (*Colisa lalia*) Imported into Iran

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Makale Kodu (Article Code): KVFD-2013-10552

Summary

Parasites imported with dwarf gourami fish may present risks, both to native fish populations and to the aquaculture industry. *Heteronchocleidus buschkieli* Bychowsky, 1957 is an important Anabantidae fishes parasite. A large number of imported anabantid fish originate from Southeast Asian countries. The objective of this study was to investigate the occurrence of infection with monogenean parasite in dwarf gourami fish imported from Southeast Asia into Iran. In this study, a total of 400 dwarf gouramies were randomly selected from aquaria containing this fish imported from Southeast Asia into Iran. After euthanizing, the gills were removed and arches and skin were examined microscopically for monogenean parasites. *Heteronchocleidus buschkieli* (Monogenea: Ancyrocephalidae) was the only monogenean parasite identified morphologically in 120 of 400 (30%) imported fish. The infected fish were lethargic and had hyperemic gills with small skin lesions. Prevalence, abundance and mean intensity of infection for four years were 0.3, 30 and 30±1.96, respectively. To the author's knowledge, this is the first report of dwarf gourami infection with *H. buschkieli* in Iran. In order to prevent the introduction of this parasitic pathogen from imported fishes, we recommend imported fishes should be examined and treated before domestic distribution and quarantine practices should be implemented.

Keywords: *Dwarf gourami, Ornamental fish, Heteronchocleidus buschkieli, Monogenea, Iran*

İran'a Yurt Dışından Getirilen Süs Balıklarından (*Colisa lalia*) İzole Edilen Üç-Kancalı Monogenean Parazit (*Heteronchocleidus buschkieli*; Ancyrocephalidae: Heteronchocleidinae)

Özet

Cüce gurami balık ile taşınan parazitler hem endemik balık populasyonlarına hem de akuakültür endüstrisine risk oluşturabilirler. *Heteronchocleidus buschkieli* Bychowsky, 1957 önemli bir Anabantidae balık parazitidir. Çok sayıda yurt dışından taşınan anabantid balık Güneybatı Asya ülkelerinden köken alır. Bu çalışmanın amacı İran'a Güneybatı Asya'dan ithal edilen cüce gurami balıkta monogenean parazit enfeksiyonunun mevcudiyetini araştırmaktır. Bu çalışmada, İran'a Güneybatı Asya'dan ithal edilen balıkları içeren akvaryumdan rastgele toplam 400 adet cüce gurami seçildi. Ötenaziyi takiben solungaçlar uzaklaştırıldı ve yüzgeçler ile deri monogean parazit için mikroskopta incelendi. *Heteronchocleidus buschkieli* (Monogenea: Ancyrocephalidae) ithal edilen balıklardan 400'ünden 120'sinde tespit edilen tek monogean parazitti. Enfekte balıklar halsiz olup hiperemik solungaçlara ve küçük deri lezyonlarına sahipti. Dört yıl için enfeksiyonun prevalans, çokluk ve ortalama yoğunluğu sırasıyla 0,3, 30 ve 30±1,96 idi. Yazarların bilgisi kapsamında bu çalışma İran'da *H. buschkieli* ile enfekte cüce guraminin varlığını ortaya koyan ilk rapordur. İthal edilen balıklardan bu parazitik patojenin oluşumunu engellemek amacıyla bizler ithal edilen balıkların yurt içine yayılmadan ve karantina uygulanmadan önce bu balıkların kontrol ve tedavi edilmeleri gerektiğini tavsiye ederiz.

Anahtar sözcükler: *Cüce gurami, Süs balığı, Heteronchocleidus buschkieli, Monogenea, İran*

INTRODUCTION

Until the second half of the 20th century, the Aspidogastrea, Digenea and Monogenea (Monogenoidea) were considered together as 'trematodes'. The Monogenea was converted to class level by Bychowsky [1]. Class monogenoidea is divided into 2 subclasses: 1) lower or Polyonchoinea, with 6 orders and about 30 families

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(e.g. Dactylogyridae, Gyrodactylidae, Capsalidae and Ancyrocephalidae) and 2) higher or Oligonchoinea with 5 orders and about 35 families (e.g. Discocotylidae, Mazocraeidae and Diplozoidae) [2,3].

Monogenea are microscopic to medium-sized (0.15 to 20.0 mm, rarely up to 30.0 mm) parasitic flatworm of fish generally in freshwater rivers and lakes. They have a direct life cycle and are either viviparous (e.g., Gyrodactylidae) or oviparous (e.g., Dactylogyridae) [3-6]. Monogeneans are host specific and infect only one or very few host species [2,7,8]. Oviparous species of Monogeneans cannot survive more than 2 weeks off a host (except their over-wintering eggs) [9].

Most monogeneans are ectoparasitic on specific sites of fish such as branchial arches, the fins, and the head. They attach to the host using their opisthaptor (an organ located at the posterior end of the monogen) which is usually equipped with 2 to 4 larger hooks, 12 nearly always to 16 marginal hooks and/or clamps or suckers [3-5]. The number and shape of these structures are important in monogenean classification [2,3]. Numerous species of monogeneans are known to cause morbidity and mortality in freshwater and marine fishes because of their high intensities, and significant damage at the point of attachment through their opisthaptor and by grazing on exposed organs and tissues [4]. Monogenean trematodes are serious pests of ornamental fishes particularly dactylogyrids causing damage to gill filament of fishes [5].

Heteronchocleidus Bychowsky, 1957 an Anabantidae fish parasite is a representative of infrequent tropical monogenetic trematodes related to Ancyrocephalidae (Ancyrocephalinae) [10]. The adult worms have three powerfully developed middle hooks, the fourth remains undeveloped [10,11]. The vestigial dorsal anchor (fourth hook) in all species of *Trianchoratus*, *Heteronchocleidus* and *Eutrianchoratus* those assigned to the subfamily Heteronchocleidinae Price, 1968 (previously regarding to Ancyrocephalidae Bychowsky, 1937) is comma-shaped [12]. Paperna remarked that there is an evolutionary circumstance beginning from two pairs of anchors to three anchors, by loss of one anchor [13]. Structural differences in anchors and bars are used to differentiate species, while the copulatory organ remains homologous in almost all fish species [11].

A significant portion of worldwide aquatic animals' trade consists of tropical aquarium fish such as anabantid fishes. The anabantid fishes vary in color and are found in most aquaria. They may use atmospheric air in order to breathe and an accessory breathing organ called the labyrinth organ (composed of lamella which are covered over a specific blood-soaked thin skin) is located next to the gill cavities [10,14,15]. A large number of imported anabantid fish originate from Southeast Asian countries.

Currently, there is limited information available regarding the occurrence of parasitic pathogens in imported dwarf gourami (*Colisa lalia*, Hamilton 1822) into Iran. The objective of this study was to investigate the occurrence of infection with monogenean parasite in dwarf gourami fish imported from Southeast Asia into Iran.

MATERIAL and METHODS

From July 2008 to September 2012, a total of 400 dwarf gouramies (Anabantid fish) were randomly selected from aquaria containing dwarf gouramies imported from Southeast Asia into Iran. They were held in aquaria at 27±2ºC. The fish were immediately transported to the Aquatic Laboratory, Faculty of Veterinary Medicine, Islamic Azad University. The animals were euthanized by decapitation following clove flower extract bathing. The monogenean parasites were defined and counted in fish gills and skin. The gills were removed and arches were placed one by one on separate slides. The slides were examined by light microscopy with 10 × magnification using a Laboval 4 microscope (Zeiss, Munich, Germany). The monogenean parasites were isolated and moved to another slide and were fixed and cleared by adding ammonium picrate [16]. Then, a cover slip was placed and sealed by paraffin. Finally, light microscopy at 40 × magnification was used for identification of the monogenean parasites. Using the same procedure, skin wet mount was also examined for the monogenean parasites.

Identification of monogenean species was done using morphological characteristics (shape and number of hooks and bars) according to Bychowsky [10] and Lim [17,18] identification keys. Briefly, the monogenean with three well developed anchors and one rudimentary anchor with two bars were confirmed as the genus *Heteronchocleidus*.

Key to the genera of Heteronchocleidinae Price, 1968 [18]

Monogeneans with three well developed anchors and one rudimentary anchor

With 2 bars……. *Heteronchocleidus*
With 1 bar....…….…… *Eutrianchoratus*
Without bar ……. *Trianchoratus*

All statistical analyses were performed using GraphPad software (GraphPad software, Inc., La Jolla, CA, USA) and data were expressed as mean ± standard deviation (SD).

RESULTS

Heteronchocleidus buschkieli (Monogenea: Ancyrocephalidae) *(Fig. 1)* as the only monogenean parasites was identified with its specific three hooks and two bars *(Fig. 2)* in 120 of 400 (30%) imported fish. The infected fish were lethargic and small skin lesions *(Fig. 3)*.

Overall, 7203 parasites were isolated from all 120 fish with a range of 48 to 73 parasites per each fish. In general,

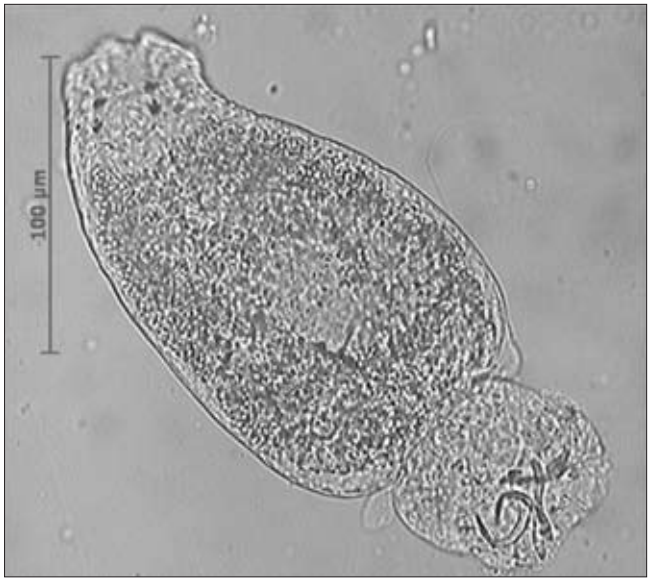

Fig 1. *Heteronchocleidus buschkieli* isolated from ornamental fish *(Colisa lalia)*

Şekil 1. Süs balığından *(Colisa lalia)* izole edilen *Heteronchocleidus buschkieli*

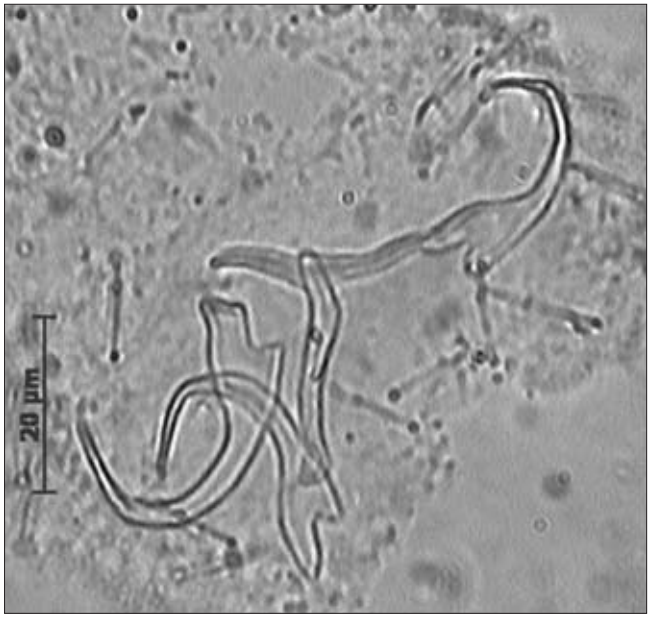

Fig 2. Three hooks and two bars of *Heteronchocleidus buschkieli* parasite isolated from ornamental fish *(Colisa lalia)*

Şekil 2. Süs balığından *(Colisa lalia)* izole edilen *Heteronchocleidus buschkieli parazitinde üç kanca ve iki bar*

5 to 9 parasites were counted in each infected gill arch and 2 to 7 parasites were counted in the skin wet mount. Mean quantity of this parasite in each fish was 60.03±7.36. With 95% Confidence Interval (CI), number of parasites in each fish was between 57.67 to 62.38. However, in the high-infected gill arches up to 25 parasites were detected per high (× 40 objective) power field.

Prevalence, abundance, mean intensity and percentage of infection for four years are shown in *Table 1*.

Fig 3. Dwarf gourami fish *(Colisa lalia)* with skin lesions

Şekil 3. Deri lezyonlu cüce gurami balığı *(Colisa lalia)*

Table 1. *Prevalence, abundance, mean intensity and percentage of infected fish*

Tablo 1. *Enfekte balıkların prevalans, sıklık, ortalama yoğunluk ve yüzdesi*

Year	Prevalence	Abundance	Mean Intensity	Percentage of Infection
1st year	0.47	47	47.04±3.45	47%
2nd year	0.25	25	24.96±1.56	25%
3rd year	0.21	21	21±1.29	21%
4th year	0.27	27	27±1.54	27%
Mean for 4 years	0.3	30	30±1.96	30%

Table 2. *Prevalence, abundance, mean intensity and percentage of parasitized fish*

Tablo 2. *Parazitli balıkların prevalans, sıklık, ortalama yoğunluk ve yüzdesi*

Year	Prevalence	Abundance	Mean Intensity	Percentage of Parasite
1st year	0.0084	61.04	61.04±6.87	0.84 %
2nd year	0.0082	59.09	59.09±7.64	0.82%
3rd year	0.0081	58.78	58.78±7.95	0.81%
4th year	0.0085	61.22	61.22±6.98	0.85%
Mean for 4 years	0.0083	60.03	60.03±7.36	0.83%

Prevalence, abundance, mean intensity and percentage of parasites for four years are shown in *Table 2*.

DISCUSSION

In the present study, the dwarf gourami imported from Southeast Asia presented a great degree of infection with *H. buschkieli*. To the author's knowledge, this is the first report of dwarf gourami infection with *H. buschkieli* in Iran. Infection with this parasite in gills of small aquarium fish *Macropodus opercularis* (paradise fish) was first reported by Bychowsky [10]. Currently, there are 29 recognized

species of heteronchocleidine monogeneans including *Trianchoratus* spp. (10 species), *Eutrianchoratus* spp. (9 species), and *Heteronchocleidus* spp. (10 species) isolated from freshwater fish species of channids (3 species) and anabantoids (13 species) [12]. But its host specificity limits to anabantid freshwater fishes.

H. buschkieli from croaking gourami *(Trichopsis vittatus)* has been found in the gills of 7 species of freshwater fishes in Malaya [17]. Chang and Ji have reported infection with *H. buschkieli* and *H. magnihamatus* in gills of *Macropodus chinensis* in China [19]. Recently, monogeneans such as *H. buschkieli*, *H. gracilis* Mizelle and Kritsky, 1969 and *H. colisai* Chandra and Yasmin, 2003 have been reported from India [11].

Heteronchocleidus sp. has been observed in imported *Trichogaster lalia* (Synonym for dwarf gourami) from Singapore [20] and in *Anabas testudineus* (climbing perch), *Channa striata* (striped snakehead) and snakehead murrel in Vietnam [21]. *Heteronchocleidus stunkardi* and *H. asymmetricus* have been identified in 85% and 4% of *Colisa lalius* in India, respectively [21,22].

It has been suggested that monogeneans have significantly high host specificity [23] which is expressed as the presence of at least one species on every species of certain host groups. Perhaps the qualitative and quantitative distinction in the patterns of host specificity of monogeneans contributes to the disparity between the sizes of the monogenean super-genera [1]. From the *Dactylogyrus* checklist, it has been calculated that each species parasitizes a mean number of about 1.45 host genera; 78% of the species are reported from a single genus [24].

Parasites imported with dwarf gourami fish may present risks, both to native fish populations and to the aquaculture industry and consequently can cause serious outbreaks of disease. In addition to the lesions in gills and skin, rapid and easy transmission can happen that indicates the necessity of quarantine, prevention, and treatment. Therefore, fish density level during export is so important to prevent such parasite infections via fish to fish transmission. It is recommended that the fishes should be examined and treated before domestic distribution and quarantine practices should be implemented. Further studies are required to determine the prevalence of parasitic pathogens in imported fishes into Iran.

Acknowledgments

The author would like to thank Dr. Mehrdad Ameri and Dr. Shahram Rabbani (Tehran Heart Center; Tehran University of Medical Sciences) for help in preparation and review of this manuscript.

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Kafkas Univ Vet Fak Derg
20 (4): 545-551, 2014
DOI: 10.9775/kvfd.2013.10555

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effects of Ankaferd Blood Stopper (ABS) and Topical Tripeptide Copper Complex (TCC) on Wound Healing in Rats: An Experimental Study

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Makale Kodu (Article Code): KVFD-2013-10555

Summary

We investigated the effects of Ankaferd Blood Stopper (ABS) and tripeptide copper complex (TCC) on wound healing in rats. A total of 24 outbred, male, Sprague-Dawley rats were randomly divided into (1) ABS, (2) TCC, and (3) control groups. Bilateral experimental wounds were created near the caudal border of the scapula. Each wound in the respective treatment group was treated daily with sponges soaked in ABS solution, topical TCC gel, or saline. On days 0, 7, 14 and 21, unhealed wound area was measured and biopsy samples were taken for histopathological analysis (except day 0). Median time for the first observable granulation tissue was not significantly different in the ABS, TCC, and control groups (5.8, 5.5, and 6.7 days, respectively) (P>0.05). Filling of the open wound with granulation tissue to skin level was significantly slower in the control group than in the ABS and TCC groups (18, 9, and 11 days, respectively). The mean unhealed wound area was significantly smaller and the mean percentage of total wound healing was significantly higher in the ABS- and TCC-treated wounds than in the control wounds on day 7, 14, and 21 (P<0.05); the average time for healing was also significantly shorter in the treatment groups than in the control group (17.4, 16.8 vs. 23.6 days, respectively) (P<0.05). Our results suggest that topical application of ABS and TCC have beneficial effects on wound healing.

Keywords: *Wound healing, Ankaferd Blood Stopper (ABS), Tripeptide copper complex (TCC), Skin, Rat*

Ankaferd Blood Stopper (ABS) ve Topikal Tripeptid Bakır Kompleksinin (TCC) Ratlarda Yara İyileşmesi Üzerine Etkisi: Deneysel Çalışma

Özet

Bu çalışmada, Ankaferd Blood Stopper (ABS) ve tripeptid bakır kompleksinin (TCC) ratlarda yara iyileşmesi üzerine etkilerinin araştırılması amaçlanmıştır. Sprague-Dawley ırkı, dişi, 24 adet rat, rastgele şekilde 1) ABS, 2) TCC, ve 3) Kontrol olmak üzere 3 gruba ayrıldı. Skapulanın kaudal sınırına yakın sahada bilateral deneysel yaralar oluşturuldu. Her bir yara günlük olarak grubuna göre ABS solüsyon emdirilmiş sponjlar, topikal TCC jel ya da serum fizyolojik ile tedavi edildi. İyileşmemiş yara alanı 0, 7, 14 ve 21. günlerde ölçüldü ve histopatolojik analiz için biyopsi örnekleri alındı (0. gün haricinde). İlk gözlenebilir granulasyon dokusu oluşumu ortalama zamanı açısından ABS, TCC ve kontrol grupları arasında anlamlı istatistiki fark yoktu (sırasıyla 5.8, 5.5 ve 6.7 gün) (P>0.05). Açık yara sahasının deri düzeyine kadar granulasyon dokusu ile dolma süresi, kontrol grubunda ABS ve TCC grubundan anlamlı şekilde daha düşüktü (sırasıyla 18, 9 ve 11 gün). ABS ve TCC ile tedavi edilen yaraların 7, 14 ve 21. günlerde yapılan ölçümlerinde ortalama iyileşmemiş yara sahasının kontrol grubuna kıyasla anlamlı şekilde daha küçük ve ortalama total yara iyileşme yüzdesinin kontrol grubuna göre anlamlı şekilde daha büyük olduğu gözlendi (P<0.05); ortalama iyileşme zamanı da uygulama yapılan gruplarda kontrol grubundan anlamlı şekilde daha kısa (sırasıyla 17.4, 16.8 ve 23.6 gün) (P<0.05) olarak bulundu. Elde edilen bulgular ışığında, ABS ve TCC topikal uygulamalarının yara iyileşmesi üzerine yararlı etkileri olduğu sonucuna ulaşılmıştır.

Anahtar sözcükler: *Yara iyileşmesi, Ankaferd Blood Stopper (ABS), Tripeptid bakır kompleksi (TCC), Deri, Rat*

INTRODUCTION

Skin wound healing is the repair process that follows injury to the skin and other soft tissues. Cutaneous wound healing is a complex process involving four major stages: hemostasis, inflammation, proliferation, and maturation/

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remodeling [1,2]. During the treatment period, the goal is to provide an optimal environment for wound contraction and epithelialization through the use of medications and bandages [3].

Ankaferd Blood Stopper® (ABS; Ankaferd Drug Cosmetic Co., Istanbul, Turkey) is a herbal medicine, ingredients of which have been used in Anatolia as a hemostatic agent for centuries for clinical hemorrhages [4,5] when the conventional control of bleeding by ligature and/or conventional hemostatic measures was ineffective [6,7]. Ankaferd is a standardized mixture of the plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*, each of which has some effects on the endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics, and/or cell mediators [4,5,8,9]. ABS is clinically effective in bleeding individuals with normal hemostatic parameters and in patients with deficient primary hemostasis and/or secondary hemostasis [10-13].

Few studies have been published evaluating the effect of ABS, which seems to be an effective hemostatic agent, on wound healing [14,15]. Isler *et al*.[14] investigated the effects of ABS on early bone healing using a rat tibia defect model. The defects treated with ABS showed more intense new bone formation and less necrosis, which may be related to increased speed of healing and decreased inflammation that is associated with anti-oxidant activity of the components of ABS. Akalin *et al*.[15] investigated the efficacy of ABS on healing of dermal wounds in a rat model. The ABS group was superior to the control group in terms of inflammatory scoring, type I/type III collagen ratio, and wound contraction rates. Except these studies, the effect of ABS on wound healing has not been investigated thus far.

Glycyl-L-histidyl-L-lysine (GHK) is a naturally occurring peptide found in several biological fluids, including plasma. A synthetic form of GHK used in the preparation of the tripeptide-copper complex (TCC) acts as a controlled delivery system for copper to the wound site. Glycyl-L-histidyl-L-lysine-Cu^{+2} (GHK-Cu) is a TCC isolated from human plasma by Pickart and Thaler in 1973 [16-18]. The efficacy of TCC in acute wound healing is well established, and it possesses many properties that promote wound healing which include increase in neo-vascularization [19], increase in epithelialization and collagen deposition [3,16,20], acceleration of wound contraction [3,21,22], and improvement of the acute wound environment by increasing proteinases [17].

The aim of this study was to evaluate the clinical and histopathological effects of ABS and TCC applied topically on open wounds created on dorsal skin of rats and to compare the results with saline treated control wounds in an experimental model.

MATERIAL and METHODS

Study Population

The study was performed at the Experimental Animal Breeding and Research Unit of the university. Upon approval of the Animal Ethics Committee (2012-01/04), the procedure was initiated.

A total of 24 outbred, male, Sprague-Dawley rats (mean weight±SD: 230±19 g; mean age: 2 months) were used. The animals were kept in individual cages, in a room with constant temperature (22±4°C) and a 12 h light/12 h dark cycle with free access to food and water. The rats were randomly divided into three groups: (1) ABS group, (2) TCC (Iamin® 5% gel; Procyte Co., Kirkland, WA, USA) group, and (3) control group (given only saline), each group with 8 rats. Prior to anaesthesia and on days 7 and 14 a complete blood cell count was performed on each rat.

Wound Creation

The rats were starved for 24 h preoperatively. On day 0, rats in all groups were anesthetized with inhalation chamber of sevoflurane (4.0-5.0%, vol). After induction, anesthesia was maintained with facial mask by inhalation of sevoflurane (2.5-3.5%, vol). Single dose of enrofloxacin (10 mg/kg, sc) (Baytril®; Bayer, Leverkusen, Germany) was administered immediately preoperatively for prophylaxis. Carprofen (4 mg/kg, sc) (Rimadyl®; Pfizer Inc., Zaventem, Belgium) was injected to all animals once just before the operation for analgesia.

The hair on the dorsum was clipped widely from the scapula to the ilium region and the clipped area was surgically prepared with polyvidone-iodine (Betadine®; Kansuk, Istanbul, Turkey). Each rat was positioned in sternal recumbency and surgically draped.

One full thickness experimental wound (1.5 cm x 1.5 cm) was created on each side, nearly 1 cm from the dorsal midline just caudal to the caudal border of the scapula on each rat. The skin, including panniculus carnosus, was excised with a no. 11 scalpel blade and scissors to create wounds perpendicular to the spine. Hemorrhage was controlled by sterile surgical sponges. After application of the topical treatments, the wound areas were left open.

Treatment Protocol

Each rat in the respective treatment groups was treated once daily, with sponges soaked in ABS solution and topical TCC gel. Enough medication to cover the wound with a thin layer was applied by delivering a segment of the medication diagonally across the square wound. In the control group, sponges soaked in saline solution was administered on wounds.

Evaluation of Wound Healing

Observations during daily wound care: Each wound was evaluated at the time of daily examination for wound healing and presence of any exudate until day 21. On days 14 and 21, any hair that had grown around the wounds was trimmed away. The day that the first granulation tissue was observed and the days that the wound was covered and then completely filled with granulation tissue and epithelialized were recorded.

Planimetry: Planimetry was performed on days 0, 7, 14, and 21 on anesthetized animals (the anesthesia protocol used to create the wounds was repeated) by tracing the perimeter of the square wounds onto a sterile piece of clear acetate film with a special marking pen. The examiner, wearing a 2.5x loupe, traced the wound margin at the border between the normal skin and the wound. The outlined area was defined as 'total wound area'. Thereafter, the examiner traced the margin at the leading edge of the advancing epithelium. The area within the margin of the advancing epithelium was defined as 'unhealed wound area'. Wound tracings were scanned and transferred to a computer, and the area (mm^2) and perimeter were calculated for each wound using the Sigma Scan® software (SPSS Inc., Chicago, IL, USA). The percentage of total wound healing was calculated for wounds on the right side by using a previously described two-step formula [3].

Step 1

Open wound day_n as percentage of original = Open wound area day_n x 100/Original wound area day_0

Step 2

% total wound healing day_n = 100 - Open wound day_n as percentage of original.

The unhealed wound area and the percentage of total wound healing were recorded at each day of measurement and used for statistical analysis.

Histopathological Examination

Four-millimeter punch biopsy instruments were used to take skin specimens from different corners of the left-side wound of each rat on days 7, 14 and 21 immediately after the planimetry was performed. Skin specimens were fixed in 10% neutral buffered formalin and processed routinely for histopathological examination. Five-micrometer sections were stained with hematoxylin and eosin (H&E). Progressive decrease in neutrophil number and progressive increase in angiogenesis were selected for monitoring the healing process histopathologically as reported in previous studies [23,24]. In every skin section, an area just beneath the epidermis or crust formation was randomly selected. Thereafter, four consecutive areas moving towards the deep dermis were selected. The five selected areas were examined under 400x magnification. The number of neutrophils was scored as 0-25=1, 26-50=2, 51-75=3, and > 75=4. The same areas were also examined for the number of vessels and the actual count was noted. All histological sections were blindly evaluated by the same investigator.

Statistical Analysis

The percentage of total wound healing, unhealed wound area, time for the first observable granulation tissue, time for the coverage of the wound with granulation tissue, and filling of the open wound to the skin level with granulation tissue for each wound at each time of measurement as compared to the original wound size were calculated and the mean values were compared among the three groups using repeated measures model for analysis of variance (ANOVA). Where differences existed, the differences were determined by Duncan's multiple-range test. Neutrophil scores and vessel counts were analysed using one-way ANOVA. When differences among the groups were significant, Mann Whitney U test was used. All analyses were performed using SPSS 13.0 (SPSS Inc.). A *P* value lower than 0.05 was considered significant.

RESULTS

Observations During Daily Wound Care

Appearances of the wounds in ABS, TCC, and control groups on day 7, 14, and 21 are shown in *Fig. 1*. The day following surgery, the wounds appeared clean; however, some of the wounds treated with TCC were covered with a bluish film reflecting the blue color of the gel. On the second and third days of treatment, TCC-treated wounds developed a tenacious, purulent appearing exudate. The tissue underlying the exudate, in most cases, was dark red or mottled on day 7 and was considered as granulation tissue. Wounds in ABS and control groups were clean and free of exudate throughout the study.

On day 7, a superficial brown colored scab was observed on the wounds in ABS group. In the control group, granulation tissue formation was less remarkable on day 7.

On day 14, the rapid growth of new tissue at the wound edges was remarkable in ABS and TCC groups. In addition, a small elevation of granulation tissue was present at the center of the wounds in TCC group *(Fig. 1E)*. The wound size in both ABS-treated and TCC-treated animals was reduced.

On day 21, all wounds were covered with granulation tissue and epithelialization in the ABS and TCC groups (mean 16.1 and 19.2 days, respectively ($P<0.05$), whereas wounds of four rats in the control group were not completely epithelialized *(Fig. 1C, 1F, 1I)*.

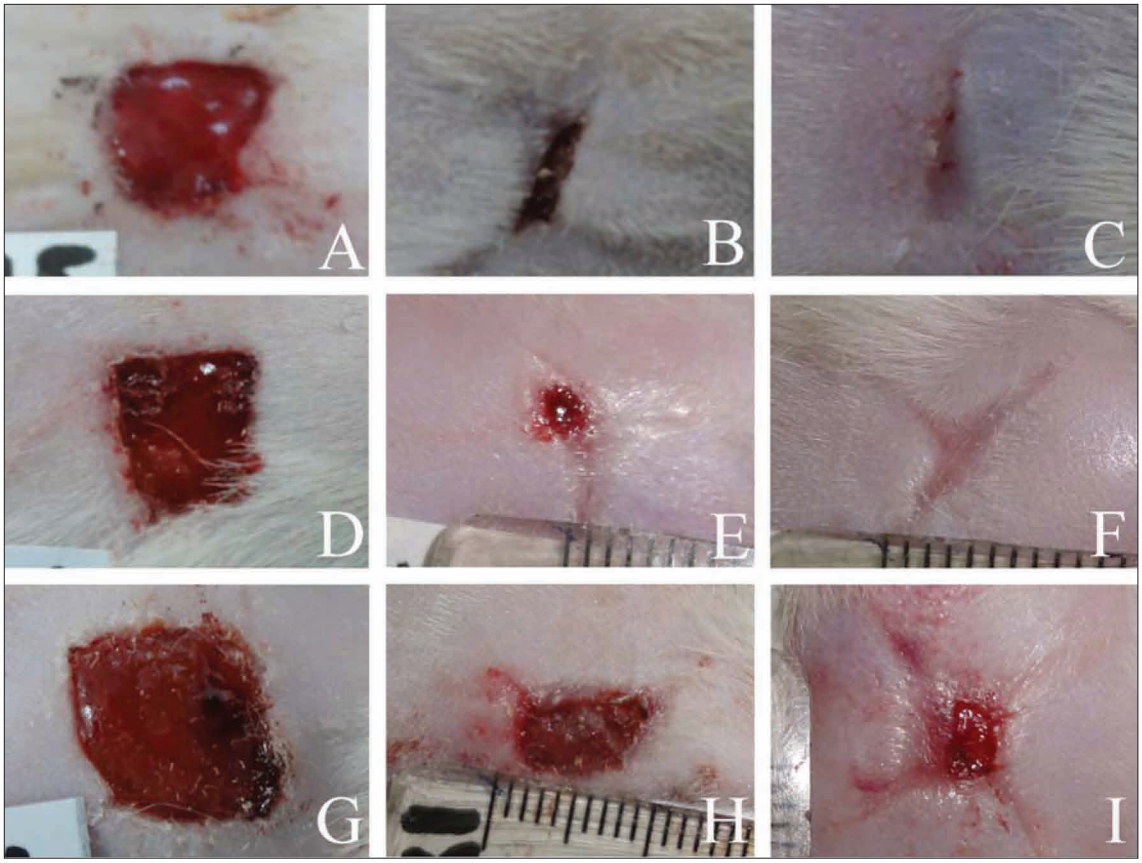

Fig 1. The figure shows the progression of wound healing in the ABS (1A to 1C), TCC (1D to 1 1F) and control (1G to 1I) groups on day 7 *(left column),* day 14 *(middle column)* and day 21 *(right column).* On day 21, all wounds in the ABS and TCC groups were covered with granulation tissue and epithelialization, whereas there were still open wounds in the control group

Şekil 1. ABS (1A-1C), TCC (1D-1F) ve kontrol (1G-1I) gruplarında 7. günde *(sol sütun),* 14. günde *(orta sütun)* ve 21. günde *(sağ sütun)* yara iyileşmesini göstermektedir. Yirmi birinci günde ABS ve TCC gruplarında tüm yaralar kapanırken, kontrol grubunda hala açık yaralar mevcuttu

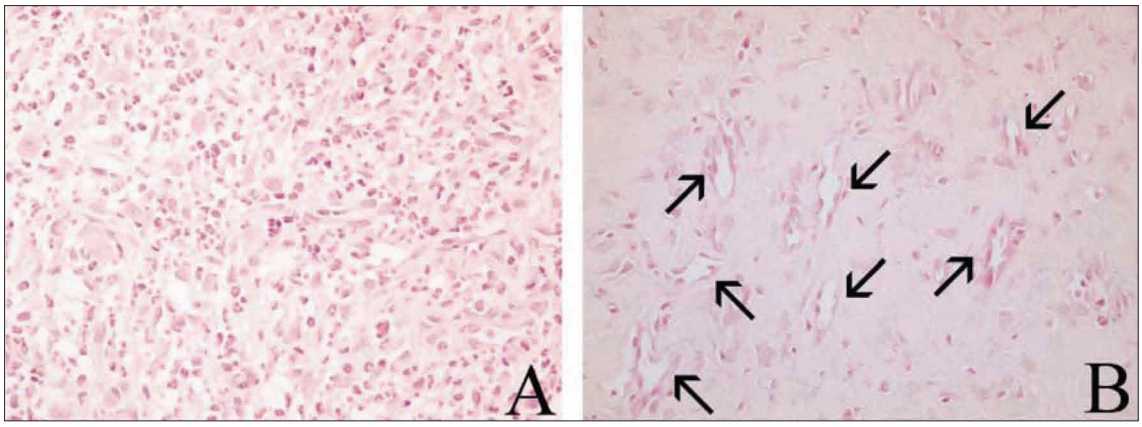

Fig 2. Histopathologically wound healing was characterized by a decrease in the neutrophil count and an increase in the number of vessels. A shows high number of neutrophils on day 7 in the ABS group, whereas B shows the increased number of new capillary vessels *(arrows)* with almost no inflammatory reaction on day 21 in the same group, H&E staining, x400 magnification

Şekil 2. Histopatolojik olarak yara iyileşmesi nötrofil sayısında azalma ve damar sayısında artış ile kendisini gösterdi. A 7. günde ABS grubunda çok sayıda nötrofili gösterirken, B aynı grupta 21. günde artan sayıdaki yeni kapillar damarları *(oklar),* ve hemen hemen hiç yangısal reaksiyonun olmadığını ortaya koymaktadır, H&E boyama, x400 büyütme

The median time for the first observable granulation tissue was not significantly different in the ABS, TCC, and control groups (5.8, 5.5, and 6.7 days, respectively) ($P>0.05$). Filling of the open wound with granulation tissue to skin level was significantly slower in the control group than in the ABS and TCC groups (18, 9, and 11 days, respectively)

Table 1. *Comparison of mean unhealed wound area and percentage of total wound healing on days 0, 7, 14, and 21 among groups of rats treated with the Ankaferd Blood Stopper (ABS), tripeptide copper complex (TCC) and saline treated controls (mean ± standard error of mean)*

Tablo 1. *Ankaferd Blood Stopper (ABS), tripeptid bakır kompleksi (TCC) ve serum fizyolojik (kontrol) uygulanan ratlarda 0, 7, 14 ve 21. günlerde ortalama iyileşmemiş yara alanı ve total yara iyileşmesi yüzdesinin karşılaştırılması (ortalama ± ortalamanın standard sapması)*

Group	Day 0	Day 7		Day 14		Day 21	
	Unhealed Wound Area (mm^2)	Unhealed Wound Area ± SE (mm^2)	Total Wound Healing (%)	Unhealed Wound Area ± SE (mm^2)	Total Wound Healing (%)	Unhealed Wound Area ± SE (mm^2)	Total Wound Healing (%)
ABS	225	102.25±34.11*	54.55±15.16*	4.50±3.12*	97.99±1.39*	0*	100*
TCC	225	124.12±47.18*	44.83±20.97*	11.12±9.08*	95.05±4.03*	0*	100*
Control	225	169.37±38.07†	24.72±16.92†	23.50±13.09†	89.55±5.82†	16.80±4.15†	95.33±4.11†

**,†Different superscripts within the same column indicate significant difference among groups (P<0.05)*

Table 2. *Mean neutrophil scores and vessel counts of open wounds in rats treated with Ankaferd Blood Stopper (ABS), tripeptide copper complex (TCC) and saline (control) for 21 days (mean ± standard error of mean)*

Tablo 2. *21 gün boyunca Ankaferd Blood Stopper (ABS), tripeptid bakır kompleksi (TCC) ve serum fizyolojik (kontrol) uygulanan ratlarda ortalama nötrofil skoru ve damar sayıları (ortalama ± ortalamanın standard sapması)*

Treatment Group¶	Neutrophil Score	Vessel Count
ABS	1.75±1.03	5.58±2.75
TCC	1.75±0.74	4.83±2.81
Control	1.91±0.83	5.00±2.32
P	0.559	0.588
Biopsy day††		
Day 7	2.38±0.92*	3.42±1.89*
Day 14	1.88±0.74*	4.25±1.67*
Day 21	1.17±0.38†	7.75±1.94†
P	0.000	0.000
Treatment group and biopsy day		
ABS- Day 7	2.50±1.20	3.75±1.28
TCC- Day 7	2.12±0.83	2.87±2.30
Control- Day 7	2.50±0.76	3.62±2.06
P	0.721	0.316
ABS- Day 14	1.75±0.89	4.63±2.39
TCC- Day 14	1.88±0.64	4.12±1.46
Control- Day 14	2.00±0.76	4.00±1.06
P	0.767	0.749
ABS- Day 21	1.0±0	8.38±1.92
TCC- Day 21	1.25±0.46	7.5±2.33
Control- Day 21	1.25±0.46	7.38±1.60
P	0.317	0.553

**, † Different superscripts within the same column indicate significant difference (P<0.05), ¶ The values are the mean ± standard error of mean (SEM) of days 7, 14, and 21 in the same group, †† The values are the mean ± standard error of mean (SEM) of the three treatment groups on the same day*

(*P*<0.05), but was similar in the ABS and TCC groups. The average time for healing was shorter in the ABS and TCC groups than in the control group (17.4, 16.8 vs. 23.6 days, respectively) (*P*<0.05), but was not different between the ABS and TCC groups. Complete blood cell count values were within normal limits for all samples taken on days 0, 7 and 14 (data not shown).

Planimetry

The mean unhealed wound area was significantly smaller and the mean percentage of total wound healing was significantly higher in the ABS- and TCC-treated wounds than in the control wounds on day 7, 14, and 21 (*P*<0.05, *Table 1*), but were similar between the ABS and TCC groups (*P*>0.05, *Table 1*).

Histopathological Examination

The mean±SEM neutrophil scores and vessel numbers are presented in *Table 2*. Neutrophil count was significantly higher and the vessel count was significantly lower on days 7 and 14 than on day 21 (*P*<0.05). In all groups, a steady increase in vessel numbers and steady decrease in neutrophil scores were observed from day 7 to day 21, but this could not be substantiated statistically (*P*>0.05) *(Fig. 2)*. The vessel count tended to be higher in the ABS group than in the other groups on all measurement days (*P*>0.05).

DISCUSSION

An understanding of the process of wound healing is essential for effective management of wounds. Topical medications should provide a specific desired effect during the appropriate stage of healing [25]. This study shows that the average time for healing was shorter and the mean total wound-healing percentage was significantly higher in the TCC- and ABS-treated wounds than in the control group.

ABS is a folkloric medicinal plant extract product, ingredients of which have been used in Turkish traditional medicine as a hemostatic agent. Its composition have been shown to have some effect on hematological and vascular parameters, and cellular proliferation [8,26-30]. Antiinfective [31,32], wound healing [14,33], antineoplastic [34], and homeostatic properties [5] have also been reported. Ozturk *et al*.[35] have shown beneficial effects of ABS in patients with osteoarthritis.

In their experimental study investigating the role of ABS on dermal wound healing, Akalin *et al*.[15] stated that all parameters that were important for effective wound healing, including polymorphonuclear leukocyte and mononuclear leukocyte infiltrations, vascularisation and fibroblast proliferation, were better in the ABS-treated group when compared with the control group [15]. Therefore, these authors concluded that the positive effects of ABS on wound healing might be attributable to these useful histopathological alterations.

Isler *et al*.[14] showed that histopathologically over sixty percent of the defects treated with ABS were free of inflammation, which is probably related to the anti-inflammatory activity of some components of this hemostatic agent. Although the occurrence of fibrosis was statistically similar in both groups, the ABS-treated group showed lower fibrosis rate than the non-treated control group, which may be attributed to the increased speed of healing in the test group.

On day 7, we observed formation of a brown colored scab over the wound area after application of ABS. This finding has been observed also by other researchers [12,36] and is believed to be due to the formation of an encapsulated protein network causing delayed degradation of erythrocytes and later hemosiderin-loaded histiocyte accumulation [12].

TCC is a naturally occurring peptide-copper complex that has been shown to possess potentially interesting properties in the wound healing process in animal [21,37] and human models [20,38]. Observation of a positive effect by TCC on wound healing in the current study is consistent with other studies [23,24] which reported that lamin, a TCC containing hydrogel, accelerates wound healing and wound contraction, and promotes epithelialization by creating a moist wound environment. The use of a hydrogel helps to produce more rapid healing by creating a moist environment which has superior features over a dry wound, such as prevention of tissue dehydration and cell death, accelerated angiogenesis, increased breakdown of dead tissue and fibrin, and potentiation of the interaction of growth factors with their target cells [39].

Swaim *et al*.[3] observed that TCC-treated wounds tended to form exuberant granulation tissue in most cases. We also observed granulation tissue formation at the center of wounds in the TCC group on day 14, but the degree was not exuberant as reported by these researchers [3]. On the other hand, we did not observe separation of the granulation tissue from the caudal edge, resulting in seroma formation and splinting of wound edges in any of the wounds as reported by Swaim *et al*.[3].

Wound healing begins as soon as the trauma has occurred. For the histopathological investigation of wound healing several parameters, such as re-epithelialization, wound cellularity [23,24,40,41], collagen deposition and blood vessel formation [23,24,42] have been used by different researchers. Decrease in neutrophil score and increase in vessel numbers were used to monitor the healing process in this study. With the progress of wound healing, a steady decrease in neutrophil scores and steady increase in vessel numbers were observed from day 7 to day 21 in all groups, although the difference was not statistically significant.

No abnormal finding was observed in the complete blood counts throughout the study, which suggests that topical applications of ABS and TCC do not result in systemic abnormalities indicated by this test. Chemistry profiles and urinalyses were not performed in this study, precluding any definitive conclusion on the safety of these topical treatments in rats. However, all rats were clinically healthy throughout the study.

In summary, our results suggest that although the median time for the first observable granulation tissue is not significantly different between the treatment groups and control, application of ABS and TCC accelerates filling of the open wound with granulation tissue to skin level and shortens the average time for healing. Histopathologically, decrease of the infiltrating neutrophil leukocytes and increase of neovascularisation are indicative of the healing process.

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Kafkas Univ Vet Fak Derg
20 (4): 553-559, 2014
DOI: 10.9775/kvfd.2013.10579

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Assessment of Prolactin, Steroid Hormone Levels in Tissue Homogenate, Serum and Receptor Distributions in Canine Mammary Tumours [1]

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Makale Kodu (Article Code): KVFD-2013-10579

Summary

The aim of the present study is to determine variations of estrone sulphate (SO4E1), progesterone (P4), 17-beta estradiol (E2), testosterone (T) and prolactin (PRL) levels in serum, mammary tissue homogenates as well as variations in the distribution of androgen (AR), estrogen alpha (ERα), progesterone (PR) and prolactin (PRL-R) receptors in mammary tumours of dogs. Thirty bitches from different breeds and ages with mammary lesions (experiment group) and 10 healthy bitches (free of any mammary lesions=control group) were used in this study. All the dogs included to the study were in anoestrus. Mammary lesions of experiment group were surgically removed and 10 normal mammary tissue samples were collected by surgical biopsy from control group dogs. Fifty-seven mammary tissue samples were obtained and were divided into two fragments for histopathology/immunohistochemistry and for hormone determinations in tissue homogenate. Besides, blood samples were collected from all dogs before the surgeries to detect hormonal variations. Student t-test was used for serum samples, one way ANOVA and tukey test were used in tissue homogenate for statistical analysis. According to current study results; PRL levels in serum were found significantly higher ($P<0.01$) in the experiment group compared to the control group. In tissue homogenate samples, T levels were found to be significantly ($P<0.01$) higher in malignant mammary tissue samples. PR was intensely expressed in 23, PRL-R was expressed in 13, ERα was expressed in 9 and AR was expressed in 6 cases of canine malignant mammary tumours (CMTs). In conclusion, PRL levels in serum, T in tissue can act on the formation of canine mammary tumours which may have different features of developments in different mammary glands of the same dog and with the predominance of PR expression in CMT.

Keywords: *Prolactin, Receptor distribution, Serum, Steroid hormone, Tissue homogenate*

Meme Tümörlü Köpeklerde Serumda, Doku Homojenatında Prolaktin ve Steroid Hormon Seviyeleri ile Reseptör Dağılımlarının Belirlenmesi

Özet

Bu çalışmanın amacı östron sülfat (SO4E1), progesteron (P4), östradiol 17-beta (E2), testosteron (T) ve prolaktin (PRL) seviyeleri değişikliklerinin serumda ve doku homojenatında, androjen (AR), östrojen alfa (ERα), progesteron (PR) ve prolaktin (PRL-R) reseptör dağılım değişikliklerinin köpeklerde meme tümörlerinde saptanmasıdır. Değişik ırk ve yaşta meme lezyonlu otuz köpek (çalışma grubu) ve 10 sağlıklı köpek (herhangi bir meme lezyonu olmayan=kontrol grubu) çalışmada kullanıldı. Çalışmaya katılan tüm köpekler anöstrustaydı. Çalışma grubu köpeklerin meme lezyonları cerrahi olarak uzaklaştırıldı ve 10 normal meme dokusu örneği kontrol grubu köpeklerden cerrahi biyopsi ile toplandı. Elli-yedi meme dokusu örneği toplandı, histopatoloji/immunohistokimya ve doku homojenatında hormon değişikliklerinin saptanması için iki parçaya ayrıldı. Bunun dışında, tüm köpeklerden ameliyat öncesi kan örnekleri toplandı. Serum örnekleri için student t-test, one way ANOVA ve tukey testi de doku homojenatında istatistiki analiz için kullanıldı. Bu çalışmanın sonuçlarına göre, serumda PRL seviyeleri çalışma grubunda önemli olarak ($P<0.01$) yüksek bulundu. Doku homojenatı örneklerinde, T seviyeleri kötü huylu meme dokusu örneklerinde önemli olarak ($P<0.01$) yüksek bulundu. Kötü huylu köpek meme tümörlerinde (KMT) PR 23, PRL-R 13, ERα 9 ve AR 6 olguda yoğun olarak ifade edildi. Sonuç olarak serumda PRL, dokuda T seviyeleri köpek meme tümörü oluşumunda rol oynayabilir bu da kötü huylu meme tümörlerinde PR baskınlığı ile aynı köpekte farklı meme bezlerinde farklı yapıda gelişimlerin oluşumunda etkili olabilir.

Anahtar sözcükler: *Prolaktin, Reseptör Dağılımı, Serum, Steroid Hormon, Doku Homojenatı*

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INTRODUCTION

Mammary tumours are the most common type of tumour in female dogs, women and cats [1-7]. Mammary tumours mostly affect middle-aged and elderly bitches [4], between the age 6 to 10 [3], median age; 10 to 11 years [6]. Approximately 53.3% of the mammary tumours in bitches are malignant [3,6,7]. Although the aetiology of canine mammary tumours is not clear, steroid hormones, some growth factors and their receptors were reported to be responsible for the occurrence of this entity [6]. High amounts of SO4E1 detected in canine inflammatory mammary carcinoma (IMC) can be due to the conversion of dehydroepiandrosterone (DHEA), androstenedione and testosterone to oestrone and oestradiol and oestradiol into SO4E1 by enzymes aromatase, steroid sulphatase and estrogen sulfotransferase respectively [8]. Estrogens produced by the tumour can act as mitogen and promote tumour growth [9]. Normal and tumoural mammary tissues contain and produce several forms of androgens [9]. Androgens may affect mammary tumour formation by binding to AR or indirectly through their transformation to estradiol [8]. The mammary tumours in dogs may be multiple and may have different histological features within or among the different tumour sites [3]. Immunohistochemistry (IHC) is an assistant diagnostic method evaluating the degree of malignancy in the tumours including canine mammary tumours [10-12]. The aim of the present study is to determine variations of estrone sulphate (SO4E1), progesterone (P4), 17-beta estradiol (E2), testosterone (T) and prolactin (PRL) levels in serum, mammary tissue homogenates as well as variations in the distribution of androgen (AR), estrogen alpha (ERα), progesterone (PR) and prolactin (PRL-R) receptors in mammary tumours of dogs.

MATERIAL and METHODS

Animals

Physical examination and vaginal cytology was performed to all dogs. Only the dogs in anoestrus were included in the study. For this study permission from "Istanbul University Animal Researches and Ethic Committee" was obtained with verdict number; 126, on date: 29.07.2010. All animals in the study (experimental group, n=30) and (the control, n=10) from different breeds were presented to our clinic. The dogs in the experimental group were 5 to 18 years of age, between 3-36.4 kg. Dogs aged 9 to 14 years and between 17-38 kg were used as controls. The age of the dogs used in the current study was decided according to the previous reports about canine mammary tumours. Mammary lesions of the experimental group were surgically removed and 10 normal mammary tissue samples were obtained by surgical biopsy from left inguinal mammary lobes of the control dogs under general anesthesia. Fifty-seven mammary tissue samples were obtained and were separated in 2 fragments for histopathology (HP)/IHC, and hormonal determinations in tissue homogenates. A sample was frozen in liquid nitrogen, stored in -86°C until homogenization. The other sample was sent to Pathology Department for HP and IHC. Fasted 10 ml blood samples were collected from 40 dogs to evaluate steroid hormones and prolactin levels before operations and serum were stored -20°C until hormone assays. Liquid nitrogen frozen tissue samples were freed from all skin and fat. For 1 mg tissue 10 ml Phosphate- buffered saline (PBS; Ph 7.4) was added and was homogenized with a homogenization device (MICCRA-D1, ART Prozess&Labortechnik GmbH&Co. KG., Germany) until there is no significant tissue mass. After 2000-3000 rpm centrifugation for 20 min supernatants were collected, hormonal analysis was performed. All of the obtained tissues were homogenized except if the tissues had the same histopathological features even collected from different mammary lobes of the same dog only one tissue was chosen and used for homogenization. In serum and tissue homogenization samples SO4E1 (Canine SO4E1 elisa kit, catalog no: CSB-E13430c), P4 (Canine P4 elisa kit, catalog no: CSB-E07285c), E2 (Canine E2 elisa kit, catalog no: CSB-E06846c), T (Canine T elisa kit, catalog no: CSB-E06893c), PRL (Canine PRL elisa kit; catalog no: CSB-E14930c) levels were detected by EIA. While P4, T, SO4E1 were determined in ng/ml, E2 was determined in pg/ml and PRL was determined in µIU/ml both in mammary tissue homogenates and serum samples.

Dogs were classified in four groups to evaluate serum hormone determinations according to their histopathological features of all mammary lesions: ***Group 1:*** Dogs with mix mammary lesions (for example; first dog had mastitis, complex adenoma, chronic active mastitis, carcinoma in situ in her different mammary lobes) (n=5); ***Group 2:*** Dogs with dysplasias; non-tumoral lesions (n=1); ***Group 3:*** Dogs with malignant mammary tumours in different mammary lobes (n= 24); ***Group 4:*** Control dogs (n=10); Total (n= 40).

Mammary tissues were classified in five groups to evaluate hormonal determinations in tissue homogenates from different mammary lobes of dogs: ***Group 1:*** Mastitis and chronic active mastitis (n= 2); ***Group 2:*** Benign mammary tumours (n=3); ***Group 3:*** Malignant mammary tumours (n=39); ***Group 4:*** Dysplasias; non-tumoral lesions (n=3); ***Group 5:*** Control dogs (n=10); Totally (n=57). Statistical measurements were not possible due to small numbers with the same characteristic of tissue samples; Group 1, 2, 4 were therefore combined and Group C (n=8) was generated. Consequently, for tissue homogenization there would be group A (malignant mammary tumours), group B (control dogs) and group C (mastitis, chronic active mastitis, benign mammary tumours, dysplasias and non-tumoural lesions).

Histopathology and Immunohistochemistry

All mammary gland samples were fixed in 10% buffered formalin and routinely processed. Histopathological diagnosis was established on H&E stained sections according to the WHO's classification for canine mammary tumours and dysplasias [13]. IHC was performed with 38 canine malignant mammary tumours using streptavidin biotin immunoperoxidase method with a commercially available HRP detection kit (ABCAM, ab94705) according to the manufacturer's instructions. Only in one case (25-6), IHC was performed on the section related to lobular hyperplasia and adenosis in order not to extract the case from the study. Antibodies against AR, PR, PRL-R and ER were used to detect the distribution and immunostaining intensity of these antigenic products *(Table 1)*. Immunoreactivity was visualised with diaminobenzidine (DAB, ABCAM, ab94665) and the sections were counterstained with Mayer's Hematoxylene. The substitution of the specific primary antibodies by PBS served as the negative control. The intensity and pattern of immunoreactivity were determined in 10 areas under 40x magnification. The intensity of staining was scored as weak (+), moderate (++) and abundant (+++).

Statistical Analysis

Student t-test was used for serum samples utilizing SPSS 13.0 programme. One-way ANOVA and tukey test were used in tissue homogenates for statistical analysis.

RESULTS

In this study, serum prolactin levels were found to be higher (P<0.01) in the experiment group when compared to the control group. Also in tissue homogenate samples T levels were found to be higher (P<0.01) in malignant mammary tumour samples when compared to the control, mastitis, dysplasias, benign mammary tumours and non-tumoural lesions. Prolactin and steriod hormone levels in serum and tissue homogenate samples are given in *Table 2, Table 3*. In current study, from 40 dogs 57

Table 1. *Details of the immunohistochemical reagents used in the study*
Tablo 1. *Çalışmada kullanılan immunohistokimyasal ayıraçların detayları*

Antigen Marker	Clone	Manufacturer	Dilution	Antigen Retrieval	Incubation Period
AR	441	Abcam	1:25	HIER	1 h at RT
ERα	33	Abcam	1:100	HIER	1 h at RT
PR	0111R	BIOS	1:200	HIER	1 h at RT
PRL-R	39279	Gene Tex	1:500	HIER	1 h at RT

AR: *Anti-androgen receptor antibody,* ***ERα:*** *Anti-Estrogen receptor alpha antibody,* ***PR:*** *Anti progesterone antibody,* ***PRL-R:*** *Prolactin antibody,* ***HIER:*** *Heat induced antigen retrieval,* ***RT:*** *Room temperature*

Table 2. *Prolactin and steriod hormone levels in serum samples*
Tablo 2. *Serum örneklerinde prolaktin ve steroid hormon seviyeleri*

Parameters	Experiment Group (n=30) (mean±SE)	Control Group (n=10) (mean±SE)	Significance
E2 (pg/ml)	70.93±12.28	51.51±14.89	NS
SO4E1 (ng/ml)	119.40±32.10	93.00±49.92	NS
P4 (ng/ml)	31.1±88.17	28.12±11.22	NS
T (ng/ml)	0.46±0.15	0.47±0.26	NS
PRL (µIU/ml)	402.63±34.69	202.79±22.92	**

NS: *P>0.05; ** P<0.01*

Table 3. *Prolactin and steroid hormone levels in tissue homogenates*
Tablo 3. *Doku homojenatında prolaktin ve steroid hormon seviyeleri*

Parameters	Group A: Malignant Mammary Tumours (n=39) (mean±SE)	Group B: Control Group (n=10) (mean±SE)	Group C: Mastitis, Chronic Active Mastitis, Benign Mammary Tumours, Dysplasias; Non-tumoral Lesio–ns (n=8) (mean±SE)	Significance
E2 (pg/ml)	99.34±3.80	85.26±3.03	110.39±13.09	NS
SO4E1(ng/ml)	7.43±1.52	1.72±0.40	8.61±1.85	NS
P4 (ng/ml)	0.65±0.10	0.30±0.45	0.97±0.22	NS
T (ng/ml)	0.14 ^{b}±0.02	0.08 ^{b}±0.02	0.36 ^{a}±0.14	**
PRL (µIU/ml)	65.41±10.23	31.77±9.10	32.02±13.37	NS

Mean values within the same row with different superscript small letters are different, ***NS:*** *P>0.05; ** P<0.01*

Table 4. *The detailed data of the mammary lesions for tissue homogenization and immunohistochemistry*
Tablo 4. *Doku homojenizasyonu ve immunohistokimya için meme lezyonlarının detaylı bilgileri*

Bitch n= 40	Lesion Localizations*, Selected Lobes for Homogenization	Lesion Type	Total Tissue Sample (n=57)	Case Number-Mammary Lobes Involved	AR 6/38	ERα 9/38	PR 23/38	PRL-R 13/38
No1	3*, 6*, 7*, 8*	3: Mastitis, 6: Complex adenoma, 7: Chronic active mastitis, 8: Carcinoma in situ	4	1-8	-	+++	+++	-
No 2	8*	8: Lobular hyperplasia and ductal hyperplasia and mastitis and ductal carcinoma (was all detected in the same lobe and evaluated as malignant mammary tumour by pathologists)	1	2-8	+	+	+++	+
No 3	4*, 5*, 8*	4: Ductal hyperplasia, 5: Dermatofibrosarcoma, 8: Carcinoma in situ	3	3-5	-	-	+++	+++
			3	3-8	-	-	+++	-
No 4	3*, 7*, 9*	3: Ductal carcinoma,7: Simple tubular carcinoma, 9: Benign mix tumour	3	4-7	-	-	+++	---
No 5	4*, 5*	4: Carcinosarcoma, 5: Tubulopapillary carcinoma	2	5-4	-	-	++	-
				5-5	-	-	+++	-
No 6	6*, 7*	6: Ductal Adenoma, 7: Chondrosarcoma and fibroadenomatosis	2	6-7	-	-	-	-
No 7	2*, 5*, 7*	2: Complex carcinoma, 5: Complex spindle carcinoma, 7: Spindle cell carcinoma	3	7-2	-	-	-	+++
				7-5	-	-	-	++
				7-7	++	-	-	+++
No 8[a]	7*	7: Complex adenocarcinoma	1	8-7	-	+	+	+++
No 9[a]	5*	5: Carcinosarcoma	1	9-5	-	-	-	+++
No 10[a]	3*	3: Complex adenocarcinoma	1	10-3	-	+++	+	+++
No 11	6*	6: Anaplastic simple carcinoma	1	11-6	-	-	-	++
No 12	8*	8: Simple tubulopapillary adenocarcinoma	1	12-8	-	-	-	+
No 13[a]	5*	5: Tubulopapillary adenocarcinoma	1	13-5	-	-	-	+
No 14[a]	5*	5: Malignant mix tumour	1	14-5	-	+	-	+++
No 15[a]	6*	6: Complex adenocarcinoma	1	15-6	-	-	-	-
No 16	5*, 7*	5: Simple adenocarcinoma 7: Adenosquamous carcinoma	2	16-5	-	-	+++	-
				16-7	-	-	+	-
No 17	5*	5: Anaplastic simple carcinoma	1	17-5	-	-	-	-
No 18[a]	5*	5: Complex carcinoma	1	18-5	+	+	++	-
No 19[a]	5*	5: Tubulopapillary carcinoma	1	19-5	-	-	+++	-
No 20	5*, 7*	5: Complex adenocarcinoma, 7: Tubulopapillary solid adenocarcinoma	2	20-5	-	-	-	-
				20-7	-	-	+++	-
No 21	4*, 8*	4: Simple Solid Carcinoma, 8: Complex carcinoma	2	21-4	-	-	-	-
				21-8	+	+	+++	-
No 22[a]	4*	4: Anaplastic simple carcinoma	1	22-4	-	-	+++	-
No 23	6*	6: Simple Tubular Carcinoma (MMT)**	1	23-6	-	-	+	-
No 24	1*, 4*	1: Complex carcinoma, 4: Solid carcinoma	2	24-1	-	+	-	+++
				24-4	+	-	+++	-
No 25	6*	6: Lobular hyperplasia and adenosis	1	25-6	-	+	++	-
No 26	2*	2: Simple tubulopapillary adenocarcinoma	1	26-2	-	-	+	-
No 27	4-5**	4-5: Solid adenocarcinoma	1	27/4-5	-	-	-	-
No 28	3*, 6*, 7*	3: Cystic tubular carcinoma, tubulopapillary type complex carcinoma, 6: Adenomatous hyperplasia, 7: Adenomatous hyperplasia and tubular adenocarcinoma	3	28-7	-	-	+++	-
No 29	4-5**	4-5: Tubulopapillary adenocarcinoma	1	29/4-5	+	-	+++	-
No 30[a]	3*	3: Complex adenocarcinoma	1	30-3	-	-	++	-
Control 1 -10	6*	6: Normal Mammary Tissue	10	C-1	-	-	-	-
				C-2	+	+	+++	+
				C-3	-	-	-	-
				C-4	-	+	-	-
				C-5	-	-	-	-
				C-6	-	-	+++	+
				C-7	-	-	-	-
				C-8	-	-	+	-
				C-9	-	+++	++	-
				C-10	-	-	-	-

**** 1:*** *right axillar,* ***2:*** *right thoracal,* ***3:*** *right cranioabdominal,* ***4:*** *right caudoabdominal,* ***5:*** *right inguinal,* ***6:*** *left inguinal,* ***7:*** *left caudoabdominal,* ***8:*** *left cranioabdominal,* ***9:*** *left thoracal,* ***10:*** *left axillar mammary glands, ** Tumoral lesions are between these two lobes (4-5),* [a]***:*** *Most of the bitches had multiple neoplastic lesions on mammary glands and their neoplasias were all in same features histopathologically, so one neoplastic gland was selected and homogenized, +: weak, ++: moderate, +++: abundant*

mammary lesions (47 had different features histopathologically and 10 normal mammary tissue) were obtained. For homogenization all of the obtained tissue samples were used. Mammary lesions, affected mammary lobes, the lobes selected for immunohistochemistry and tissue homogenization were given in *Table 4* in detail. Different than homogenization samples the sections on two of the slides for IHC were dissolved during immunohistochemistry (4-3 and 28-3) and we excluded these samples for IHC in *Table 4*. A statistically significant difference (P<0.01) was determined between the control and experimental groups for PRL levels in serum samples. With regard to T level in tissue homogenate, group C had higher mean concentration when compared to group A and B (P<0.01). In this study, PR was intensely expressed in 23, PRL-R was expressed in 13, ERα was expressed in 9 and AR was expressed in 6 cases of canine malignant mammary tumours (CMTs). Coexpression of AR/PRL-R, AR/ER, AR/PR, PR/ER, PR/PRL-R, ER/PRL-R was immunohistochemically found in 2, 3, 5, 7, 4 and 5 of the specimens evaluated within the experimental group, respectively. There was a single case which revealed (+) immunoreactivity with all receptor markers in both groups. The labeling for PR was mostly cytoplasmic *(Fig. 1)*, whereas the nuclear membrane was also stained with PRL *(Fig. 2)*, AR, and ERα antibodies.

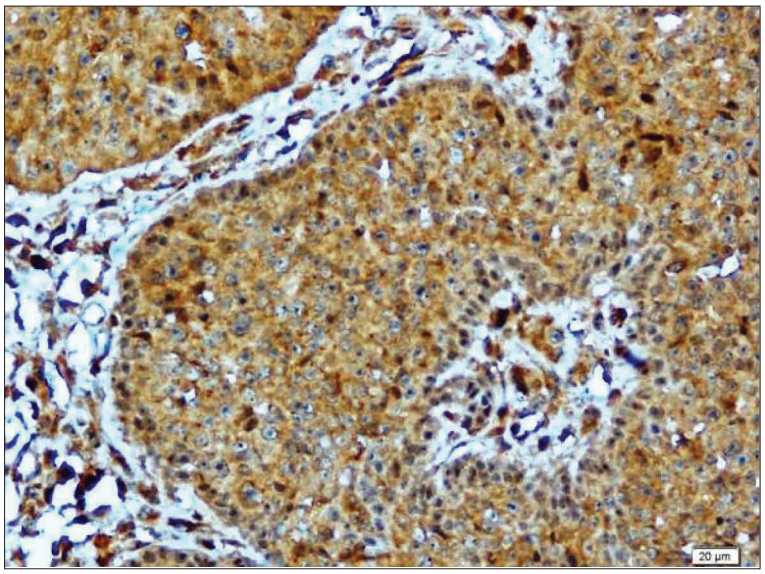
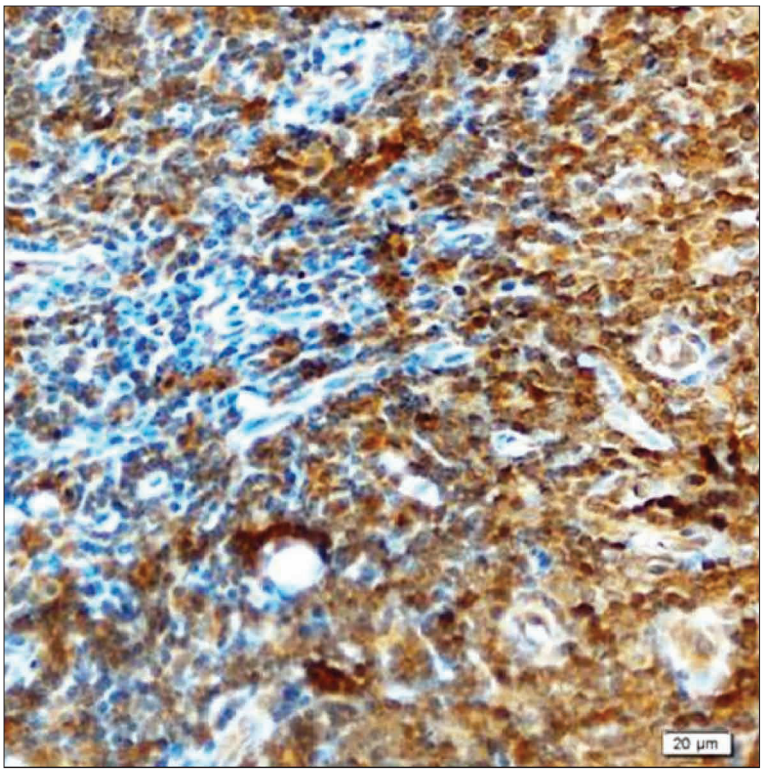


Fig 1. Complex carcinoma, Positive immunoreaction for PR cytoplasmic staining. Streptavidin-biotin immunoperoxidase. Bar: 20 µm

Şekil 1. Kompleks karsinoma, PR'nün sitoplazmik boyan-ması için pozitif immunreaksiyon, Streptavidin-biyotin immun-peroksidaz. Bar: 20 µm

Fig 2. Complex carcinoma, Positive immunoreaction for PRL-R cytoplasmic staining. Streptavidin-biotin immunoperoxidase. Bar: 20 µm

Şekil 2. Kompleks karsinoma, PRL-R'nün sitoplazmik boyan-ması için pozitif immunreaksiyon, Streptavidin-biyotin immun-peroksidaz. Bar: 20 µm



DISCUSSION

Steroid hormones are important in mammary gland development, possibly in the formation of neoplastic tissue in mammary glands [5]. In the studies conducted in dogs serum E2, SO4E1, T [5,8,14] and P4 [5,8] levels were found higher (P<0.01) in CMTs when compared to the control group. In this study E2, SO4E1 and P4 levels were not found to be different in comparison to the control group. This difference may be arisen due to the malignancy of the tumours obtained from their study of canine inflammatory mammary carcinoma [8,14]; they had compared canine inflammatory carcinoma unlike this study. Serum T level was found to be insignificant in this study in tumoural group when compared to the control group, this result contradicts with Sanchez-Archidona et al.[8] and Illera et al.[14]'s results. They found statistically significant difference between control and tumoural group and they indicated higher serum T concentrations in malignant tumours, this result may be arisen because of the histopathological features of the obtained mammary tumours in their study. Illera et al.[14] reported higher T levels (287.43±6.89 ng/ml) in tissue homogenate samples than this study's results, it might have been arisen because of the tumoural feature (inflammatory mammary carcinoma) of canine mammary tumours. According to Liao and Dickson [15] normal and cancerous mammary tissues contain and produce many kinds of androgens, and Maggiolini et al.[16] reported that androgens have effects on mammary tumour formation through binding directly to AR or indirectly by conversion of androgens to oestradiol. In this study it can be attributed to either androgens not used in the tissue or would be redirected to the blood. We found serum PRL levels of dogs with mammary tumours as 402.63±34.69 µIU/ml, which was significantly higher (P<0.01) when compared to that of the control group (202.79±22.92 µIU ml). Likewise, Queiroga et al.[5] reported lower serum PRL levels 3.086±0.8 ng/ml in control group when compared to dogs with malignant tumours (5.61±0.85 ng/ml) in which these results were statistically significant (P=0.01). Queiroga et al.[5] reported high PRL levels in malignant mammary tissue homogenates 49.61±5.21 ng/g when compared to dysplasias 16.32±3.0 ng/g, benign tumours 12.72±1.92 ng/g and control tissues 2.43±0.64 ng/g, their findings were significant (P<0.01) statistically, in this study our findings are in line with them 65.41±10.23 µIU/ml in malignant tissue homogenates; 32.02±13.37 µIU/ml in mastitis, benign tumours and dysplasias and 31.77±9.10 µIU/ml was detected in control tissues, respectively but not found statistically significant. Steroid and peptide hormones demonstrate their action by binding to their cognate receptors. In normal mammary tissues of dogs ER, PR and PRL-Rs found previously [1]. Normal and neo-plastic mammary tissues have been reported to express estrogen and progesterone receptors. The respectively low number of the cases stained positively for ER can be associated with the type of the antigen marker selected for this study. The expression of ERα was found to be lower than that of ERβ in a recent study, as well [17]. We consider that the predominance of immunoreactivity for PR confirmed the proliferative effect of progesterone hormone in the development of mammary tumours [18]. Normal canine mammary tissues and benign lesions of the mammary gland were found to express high levels of PRL-Rs. Malignancy is controversially correlated with the expression of this receptor, which was compatible with our findings [19]. The lack of immunoreactivity for AR in most of the cases was associated with the shortfall of the selected clone of the receptor marker applied in this study. Therefore we recommend a different antigenic marker for AR for further studies. Furthermore, our findings revealed a lack of correlation amongst ER, PR, PRL-R and/or AR expression, which could be expected in tumoral tissues [20]. However the relatively low number of the control samples which positively reacted with the receptor markers used in the study can be associated with the insufficient amount of mammary tissues available in the biopsy specimens. In this study different kinds of tumoural or/and non-tumoural lesions were obtained in different mammary lobes of the same dog, as explained in *Table 4*. However, the researchers did not state the exact lobular localization of the tumoral mass in previous studies [5,9,10,14,21]. Golshahi et al.[22] have used streptavidin-biotin-peroxidase technique for IHC of ERα for chondrosarcoma in mammary gland of a dog and they found this tumour was not expressed ERα like most of the cases in this study. Toniti et al.[20] used ER and PR like in this current study but they used it in different dilutions and they concluded that immunohistochemical studies did not have any correlation to estrogen and/or progesterone receptors expressions in canine mammary tumours. On the basis of our findings it can be concluded that IHC did not aid in the elucidation of the hormonal mechanism of the canine mammary tumors even to some extend although predominance of PR expression was revealed in CMT in the present study and further studies are needed. Besides there was no direct correlation between serum samples, tissue homogenates and immunohistochemical features of the evaluated samples in terms of AR, ER, PR and PRL-R expression. Therefore, it can be stated that steroid hormone levels in the sera and T levels in the tissue might have contributed to the formation of canine mammary tumours. However, only PRL and T effects were found to be significantly higher in serum and tissue homogenate samples. Further studies should be planned to detect the effects of these two hormones on the development canine mammary neoplasia. On the other hand, this study confirmed that there might be different features of developments in different mammary lobes of the same dog and IHC revealed the predominance of PR expression in CMT.

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Kafkas Univ Vet Fak Derg
20 (4): 561-564, 2014
DOI: 10.9775/kvfd.2013.10582

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Investigation of Bull Effect on *in vitro* Embryo Production

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Makale Kodu (Article Code): KVFD-2013-10582

Summary

The aim of the study was to show whether there were some differences among 9 Holstein bulls and within their replications on their ability of *in vitro* fertilization for *in vitro* embryo production as cleavage and coming into the blastocyst stage. Semen collected and frozen from nine Holstein bulls with satisfactory in vivo fertilization capabilities for artificial insemination was used for *in vitro* fertilization. Direct washing method by Brackett and Oliphant medium and 5 or 6 h incubation period were used for *in vitro* fertilization. Charles Rosenkrans medium was used for *in vitro* embryo culture. An atmosphere with a higher than 95% relative humidity, 39°C, 5% CO_2 and was used for all *in vitro* embryo production processes. Totally 2519 A and B quality oocytes were treated for *in vitro* embryo production. As a result statistically significant ($P<0.05$) variation was found for cleavage and blastocyst development among bulls. However there was no significant difference ($P>0.05$) between the replications for each bull as cleavage and coming into blastocyst stage. The results showed varied capabilities of bulls for *in vitro* fertilization and embryo production and male factor can affect success of *in vitro* embryo production.

Keywords: *Bull, In vitro, Embryo, Cleavage, Blastocyst*

In vitro Embriyo Üretimine Boğa Etkisinin Araştırılması

Özet

Dokuz farklı Holştayn boğaya ait spermanın kullanıldığı bu *in vitro* embriyo üretim çalışmasının amacı, hem boğalar arasında hem de boğaların kendi tekrarları arasında yarıklanma ve blastosiste ulaşma oranları bakımından fark olup olmadığının gösterilmesi olmuştur. Suni tohumlama boğası olarak kullanılan ve fertilite sorunu olmayan ve tatminkar düzeyde fertilite oranlarına sahip dokuz Holştayn boğadan alınan ve dondurulan spermalar *in vitro* fertilizasyon amacıyla kullanılmıştır. İn vitro fertilizasyon için Brackett ve Oliphant mediumu ile Direkt yıkama metodu ve 5-6 saat inkubasyon periyodu; *in vitro* embriyo kültürü için de Charles Rosenkrans mediumu kullanılmıştır. Kültür periyotlarında %95'in üzerinde bağıl nem, 39°C, %5 CO_2 içeren bir kültür ortamı sağlanmıştır. Toplam 2519 A ve B kalite oosit *in vitro* embriyo elde etme sürecine alınmıştır. Sonuç olarak yarıklanma ve blastosiste ulaşma oranları bakımından boğalar arasında önemli düzeyde istatistiki farklılık bulunmuştur ($P<0.05$). Fakat her bir boğanın kendi içindeki tekrarları arasında yarıklanma ve blastosiste ulaşma oranları bakımından önemli düzeyde fark görülmemiştir ($P>0.05$). Bu sonuçlar boğaların değişik düzeyde *in vitro* fertilizasyon ve embriyo üretim yeteneklerine sahip olduklarını ve *in vitro* embriyo üretim başarısı için erkek faktörünün belirleyici olduğunu göstermektedir.

Anahtar sözcükler: *Boğa, İn vitro, Embriyo, Yarıklanma, Blastosist*

INTRODUCTION

There are many factors affecting of success for *in vitro* embryo production (IVEP) such as oocyte quality, sperm preparation methods and media for *in vitro* maturation, fertilization and embryo culture, duration of fertilization. *In vitro* fertilization ability of bull semen and skills of fertility of the semen are the difference between them for bulls using for artificial insemination (AI) [1]. Semen used for IVEP, cleavage and blastocyst development rates vary depending on the bull fertilization capabilities [2-4]. However *in vitro* fertilization (IVF) capabilities of bulls can be used to predict the bulls' *in vivo* fertility capacity, in order to determine the relationship between the levels of fertilization *in vitro* and *in vivo* is unclear [5,6]. Bulls have a significant correlation in the ability of *in vivo* and *in vitro* fertilization [7]. There are wide variety of methods of IVF, sperm penetration and capacitation that are quite

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satisfactory but differences emerge between bulls in terms of IVEP success [8].

The aim of the study was to determine the variation among Holstein bulls on the individual and general yield of IVEP as cleavage and coming into the blastocyst stage rates.

MATERIAL and METHODS

Cow ovaries were obtained from the local slaughter-house and were transported to the laboratory in transport medium, consisting of physiological saline (0.9%) without antibiotics, at 25°C within 2-3 h. Cumulus oocyte complexes (COCs) (n = 2519) were provided from antral follicles (2-8 mm) by using both of aspiration and also slicing methods. *In vitro* maturation and fertilization were performed according to Kanagawa et al.[9].

A and B quality oocytes were used mixed for *in vitro* maturation (IVM) procedure. All of the A and B quality oocytes were cultured in groups of 20 oocytes per 100 µl TCM-199 (M7528/Sigma-Aldrich Co.) modified by 0.1 mg/ml L - Glutamin, 25 mM HEPES, 5% Fetal calf serum (FCS) and 2 µg/ml FSH (Folltropin-V, Bioniche, Ireland) for 22 h in petri dishes (Falcon 1008/Dickinson).

Semen collected from nine different Holstein bulls was used. One of the bull had most of capability of *in vitro* fertilization (IVF) was used as a control group. The comparisons of bull semen of *in vivo* and *in vitro* fertilization capabilities in all groups were done using this bull has high performance in *in vitro* fertilization.

Semen collected and frozen from nine Holstein bulls with satisfactory *in vivo* fertilization capabilities for artificial insemination was used for *in vitro* fertilization. All the semen was obtained from the bulls at once ejaculated in one week at autumn season and was frozen in Laciphos-477 (IVM, France) and packaged in 0.25 ml straws (IVM, France). In all cases the semen of each bull had about 50-60% motility after thawing was used.

For the purpose of fertilization BO medium modified by 5 IU/ml heparin (H3149, Sigma-Aldrich Co.) and 2 mM Caffeine (C4144, Sigma - Aldrich Co.) was prepared. Optimized amount of heparin already preferred for the laboratory routine was used. Nearly 30.000 spermatozoa were put in insemination droplets per oocyte and motile spermatozoa were at least 15.000 spermatozoa per oocyte after direct washing method of fertilization process for *in vitro* fertilization. Final concentration was adjusted as 6.25 x 10^6 spermatozoa/ml in 100 µL Brackett and Oliphant (BO) medium and covered with 400 µL of paraffin oil (Sigma - Aldrich Co.). Approximately 20 oocytes were added each one of fertilization droplets and petri dishes placed for 5 - 6 h incubation environment.

After fertilization period of oocytes, presumptive zygotes transferred droplets of Charles Rosenkrans medium (CR - 1aa) [10] and cumulus cells were removed from by pipetting with appropriate sized pipette in a few minutes. Denuded oocytes were transferred in groups of 20 in droplets of CR - 1aa. Culture medium was prepared as 100 µL and covered paraffin oil in 35 mm petri dishes (Falcon 3001/Dickinson) and was kept in same incubation environment at least 3 h earlier than culture onset. Embryos had two or more cells were transferred a new culture medium and incubated 5 days more. Culture medium was not exchanged and renewed in this culture period. Evaluation was done on hours 48 and at days 5 after cleavage (two or more cells).

Data were analyzed using general linear model (GLM) procedure of SAS (SAS Inst. Inc., Cary, NC, USA). Means were separated by Duncan test ($P<0.05$). All data were expressed as means ± standard deviation.

RESULTS

Comparison of cleavage and coming into the blastocyst stage for different bull sperm was given in *Table 1*. The highest cleavage and blastocyst development rate were 69.23±13.62% and 28.54±2.96% in control group respectively, the lowest cleavage and blastocyst development rate were 16.85±7.96% and 5.57±0.44% for "F" coded bull in experimental group *(Table 1)*. There was statistically significant difference among Bulls in terms of blastocyst rates of cleavage ($P<0.05$). Cleavage and blastocyst development rate had a wide range among bulls and 4 different groups were emerged in terms of cleavage and blastocyst development among bulls. However, among of repetitions within a bull for each bull, there was no significant difference both in terms of rates of cleavage as well as reaching the blastocyst stage ($P>0.05$).

DISCUSSION

In this study, the cleavage and blastocyst development were determined from semen collected and frozen from nine different Holstein bulls after IVF procedure. Then, they were compared for their achievement rates among bulls and their replications for each bull. A significant level of cleavage and blastocysts development rates were detected among bulls ($P<0.05$), although there was no significant difference among bulls within their own replications ($P>0.05$).

Schneider et al.[6] have found that mean cleavage rate was 57% with different levels of bull semen fertility in their IVF studies. Niwa and Ohgoda [11] identified a 68% penetration rate of spermatozoa for IVF. Brackett and Zuelke [12] were reported a range from 75% to 41% *in vitro* fertilization rates by variable methods. In this study,

Table1. *In vitro embryo production with semen of nine bulls*
Tablo1. *Dokuz boğaya ait sperma ile in vitro embriyo üretim sonuçları*

Bull	Number of Oocyte (n)	Replication of IVEP (n)	Cleavage (%)	Blastocyst (%)
Control	650	34	69.23 ± 13.62^{a}	28.54 ± 2.96^{x}
A	252	12	44.76 ± 12.97^{b}	19.36 ± 2.04^{xwy}
B	169	9	45.33 ± 10.78^{b}	16.76 ± 1.72^{wy}
C	163	9	23.44 ± 6.66^{dc}	10.65 ± 1.05^{yz}
D	232	12	46.25 ± 19.19^{b}	18.37 ± 3.32^{wy}
E	184	10	33.97 ± 14.30^{cb}	12.40 ± 1.49^{yz}
F	203	9	16.85 ± 7.96^{d}	5.57 ± 0.44^{z}
G	476	25	64.79 ± 15.91^{a}	24.97 ± 2.17^{xw}
H	190	9	34.26 ± 12.77^{cb}	11.12 ± 1.00^{yz}
SEM			±2.60	±3.39

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

cleavage rates ranged from 16% to 68% in average. Some of these rates were similar to the authors of above with some bulls but some bulls were showed cleavage rates less than those of above authors. This can be interpreted as some bulls may not be sufficient capabilities in *in vitro* fertilization.

Schneider et al.[6] and Galli and Lazzari [13] have compared cleavage rates of bulls in their IVF studies, they have not seen a statistically significant difference in the rates of cleavage. However, Lu et al.[14] reported a significant difference cleavage rates at different fertility levels of the bulls. In this study, there were significant differences (P<0.05) among some of the bulls, but not others (P>0.05) in terms of rates of cleavage. Findings of Schneider et al.[6] and Galli and Lazzari [13] supported of our results for some bulls, the results of Lu et al.[14], was compatible with our conclusions. This situation can be interpreted with the variation of *in vitro* fertilization capability among bulls. Fertilization is not the standard capabilities among any bulls. Therefore, some bulls may have shown a significant difference among *in vitro* fertilization capabilities.

Al Naib et al.[15] have compared Holstein bulls with high and low fertility capabilities and reported that the cleavage rates of high fertility Holstein bulls were significantly better than those of low fertility Holstein bulls. The varying cleavage rates obtained in this study can be due to variable fertilizing capabilities among bulls. If a bull has a high *in vivo* fertilizing capacity, he can be successful for IVF.

Otoi et al.[16] have reported that the rate of *in vitro* embryo developments can even differ among straws of semen collected and frozen at once. Contrary to statement of Otoi et al.[16], each bull did not differ among the replications in this study. This difference can be explained by the fact that due to the different source of oocytes used.

Depending on the choice of medium or protocol, up to 25-30% blastocyst development can be obtained in IVEP [17]. Brackett and Zuelke [12] reported that developing up to morulae/blastocyst stage ranged from 27% to 38% in different cultural environments. Kato and Iritani [18] and Takahashi et al.[19] reached 13.0% and 22.4% blastocyst stage respectively. In this study, blastocyst development rates were similar to the findings of Brackett and Zuelke [12] but development to the blastocyst stage rates for some of the bulls were lower than those of those authors. Blastocyst development rates of two bulls were similar to the findings of Kato and Iritani [18]. While blastocyst development rates were lower than Kato and Iritani [18] for one bull but these rates higher than those authors for other six bulls. This can be explained with the variation at *in vitro* development capacity of bull depend on their fertilization capabilities.

Leibfried-Rutledge et al.[20] reported that IVF outcomes may be directly associated with the selected bulls for IVF. Wiemer and colleagues [21] stated that a large proportion of the success of the *in vitro* culture systems, depend on oocyte selection criteria, culture conditions and additives. The differentiations in blastocyst development observed in the study may be raised from male factor and such as *in vitro* embryo production protocols, culture media involved substances, difference in sperm processing methods as reported by researcher above.

Brackett and Zuelke [12] and Lu et al.[14] reported the highly good results of some bulls and there were significant differences among bulls in terms of blastocysts development rates. Galli and Lazzari [13] reported that there were great differences between bulls for developing to the blastocyst stage. Over 90% of bulls can be used for IVF but all the sires always not give good results for *in vitro* embryo development [13]. In our study, some of the bulls in terms of achievement rates of blastocyst were statistically significant (P<0.05) differences, but others were not significant (*Table 1*; P>0.05). There was a difference in terms of reaching the blastocyst rates of some of the bulls,

which was in agreement with the findings of Barckett and Zuelke [12] and Galli and Lazzari [13]. Al Naib et al.[15] have studied with high and low fertility of the semen of Holstein bulls in IVF studies and noted that their fertility level did not affect the rates of developing to the blastocyst stage. In the present study, the great variations in terms of achievement rates of blastocysts were observed among some bulls. This could be related to the IVF capabilities of the bull semen and sources of oocytes.

Tamassia et al.[22] reported that when they reached the high percentage of cleavage, they had the high proportion of blastocyst stage after *in vitro* embryo culture period in their study. Our findings on blastocyst development are compatible with the results of Tamassia et al.[22]. Because when bulls had high cleavage rate, their coming into blastocyst stage was high in this study.

Amount of heparin and caffeine added in the sperm processing medium plays very important role for the success of IVEP [23]. In the present study, a standard IVF protocol using optimal levels of heparin and caffeine were used. Bull semen were collected and frozen in one week and straws with same lot number were used in this study and statistically significant difference was not observed between replications of the same bull *(Table 1)*.

Gordon [1] has reported that there were differences at success rate depending on the female and male own condition even using standard protocols of IVEP studies. In the present study, the source of semen was a key determining factor for the success of IVEP as reported by Gordon [1].

In conclusion, bulls ought to be known whether it is suitable to use for IVEP, because when bulls are used for the purpose of IVEP these bulls may give different ratios of cleavage and blastocyst development depending on their *in vitro* fertilization capabilities. In addition, if cleavage rate is high for a bull, developing to the blastocyst stage is usually high for that bull, in other word, if a bull has a low cleavage rate for IVEP; he has got low blastocyst development capacity.

Bull semen is one of the important factors for IVEP success. Male may not suitable for IVEP as cleavage and blastocyst development rate; this should be taken into consideration. Thus, it would be better if the bull's semen, which has high level of cleavage and blastocyst development ratio, could be put into IVEP process.

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Kafkas Univ Vet Fak Derg
20 (4): 565-570, 2014
DOI: 10.9775/kvfd.2013.10611

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effect of Different Activation Techniques on Immature and *In Vitro* Matured Cat Oocytes [1]

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[1] *The present work was supported by the Research Fund of Istanbul University (Project No: 20972 and 27728)*

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Makale Kodu (Article Code): KVFD-2013-10611

Summary

This study was conducted to determine the most successful techniques on inmature and *in vitro*-matured cat oocytes that were parhtenogenically activated using 6-dimethylaminopurine (6-DMAP) and cycloheximide (CHX), in combination with electrical stimulation and calcium ionophore. After 44 h of *in vitro* maturation, the oocytes with a polar body were separated as mature (M II) and those without a polar body were considered as immature. Four different activation treatments and two control groups were used for parthenogenetic activation with both mature and immature cat oocytes. After 48 h of activation, the oocytes were examined and the non-cleaved oocytes removed. The cleaved oocytes/embryos were cultured *in vitro* in mSOF medium for an additional four days. After six days of *in vitro* culture (IVC), embryo quality was evaluated. The results in the present study suggested that (I) both *in vitro* matured and immature cat oocytes have a potential to develop to morula and blastocyst stages after parthenogenetic activation, (II) electrical stimulation + 6-DMAP is a more useful technique for both matured and immature cat oocytes and (III) to our knowledge, this is the first report that describes morula and blastocyst formation from parthenogenetically activated immature cat oocytes.

Keywords: *Parthenogenetic, Activation, Cat, Oocyte, Immature oocyte*

Farklı Aktivasyon Tekniklerinin Olgun Olmayan ve *In Vitro* Olgunlaştırılmış Kedi Oositleri Üzerine Etkisi

Özet

Bu çalışma olgun olmayan ve *in vitro* olgunlaştırılmış kedi oositlerinin partenogenetik aktivasyonu üzerine 6-Dimetilaminopurin (6-DMAP) ve Sikloheksimidin (CHX), elektik uyarımı ve kalsiyum ionoforla birlikte farklı kullanım kombinasyonlarının denendiği bir çalışma olarak tasarlandı. *İn vitro* olgunlaştırma (IVM) aşamasının 44. saatinde polar cisimciği attığı gözlenen oositler olgun (MII), atmayanlar ise olgun olmayan (MI) olarak kabul edildi. Aktivasyon sonrası kültüre aktarılan oositler 48 saat sonra değerlendirildi ve yarıklanmayanlar kültürden çıkarıldı. Yarıklanan oositler/embriyolar Modifiye Sentetik Ovidukt Medyumu (mSOF) içerisinde dört gün daha kültüre devam ettirildi. Kültürün altıncı gününde de embriyolar kaliteleri yönünden değerlendirilerek kaydedildi. Bu çalışma sonuçları göstermiştir ki, (I) gerek olgun, gererekse de olgun olmayan kedi oositleri parthenogenetik aktivasyon sonrasında morula ve blastosist aşamasına ulaşabilmektedir, (II) elektrik ve 6-DMAP'ın birlikte kullanıldığı aktivasyon tekniği, olgun ve olgun olmayan oosit aktivasyonu gruplarının her ikisinde de en başarılı sonuçları vermiştir, (III) çalışmada olgun olmayan oositlerin aktivasyonundan elde edilen morula ve blastosist aşamasındaki embriyolar bu alanda ilktir.

Anahtar sözcükler: *Partenogenetik, Aktivasyon, Kedi, Oosit, Olgun olmayan oosit*

INTRODUCTION

Animal production by somatic cell nuclear transfer (SCNT) provides a number of opportunities for basic and applied research and genetic protection in human medicine [1]. Domestic cats are a useful research model to develop

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assisted reproductive technologies for the conservation of endangered felids and for use in biomedical research [2]. They are the preferred species for studies of normal physiology and human diseases, particularly in neuroscience and kidney diseases [3-6]. In the process of fertilization, interaction between a sperm cell and an oocyte triggers off a series of morphological and biochemical transformations, known as oocyte activation [7].

Oocyte activation is induced under natural conditions by the sperm penetrating to the egg and plays a key role in meiosis. Meiosis in the matured mammalian egg is blocked at the metaphase II (MII) stage, when the first polar body is extruded from the egg. Further progress of meiosis depends on the activating stimulus. This stimulus is brought into the egg by the sperm during fertilization and it involves mechanisms inducing the oscillation of intracellular levels of free calcium ions [8].

Artificial oocyte activation is a very important step determining the success of SCNT studies. An artificial stimulus causes pulsatile increases in intracellular calcium concentrations, induce activation and inhibit MPF activity and mitogen activation promoter factor (MAPK) of the oocytes without penetration of the sperm. These causes depolarization of the oocyte membrane, a release of cortical granule contents and a hardening of the zona pellucida [8,9]. It is suggested that activating stimulus alone is not adequate to completely activate mammalian oocytes [10,11] and it was effectively demonstrated that electroporation alone efficiently triggered the inactivation of M-phase promoting factor (MPF) but not that of MAPK. For the attainment of low MAPK activity, electrical activation should be followed by protein synthesis inhibitors [11].

Several different activating stimulants such as ionomycin, ethanol or electrical pulses, protein synthesis inhibitors such as cycloheximide (CHX) and 6-(Dimethylamino) purine (6-DMAP) are widely used to induce the artificial activation of mammalian eggs [12]. CHX and 6-DMAP are the protein synthesis inhibitors responsible for decreasing MPF and MAPK activity of the oocyte and restarting meiosis [13]. Alternatively, the effect of electrical stimulation influences movement of calcium ions in the oocyte and ionomycin establishes a complex that transports calcium ions through the membrane and into the oocytes [7,8,14].

In recent years, several different animal species have been cloned by using MII oocytes as the recipient cytoplasm in sheep [15,16], cattle [17], mouse [18], goat [19], pig [20], dog [21] and cat [22,23].

It is known that MPF activity is important for the reprogramming of the nucleus in reconstructed embryos [24,25]. It was identified that MPF activity during oocyte maturation is maximal at MII [26]. Nevertheless, it was demonstrated that MI oocytes may also reprogram somatic cell nuclei [27]. In the amphibian, although adults could not be generated from such reconstructed embryos, it was observed that the greatest output of tadpoles came from differentiated somatic cell nuclei injected into immature MI oocytes [24,28].

Although, several publications on artificial activation of cat oocytes exist [10,14,29], most of them are related to activation of MII stage oocytes during nuclear transfer studies. Only few studies had compared different methods of activation and their effectiveness, as measured by the number of activated oocytes and parthenogenetic embryos [7,13]. So far, a common activation method for cats has not been developed as the process is highly species specific. Nevertheless, there is no data in the literature about immature cat oocytes being used for parthenogenetic activation or SCNT purposes.

Because parthenogenetic activation protocols are still not well described in cats, the aim of the present study was to evaluate parthenogenetic activation in *in vitro*-matured and non-matured cat oocytes, using 6-DMAP and cycloheximide, combined with electrical stimulation and calcium ionophore.

MATERIAL and METHODS

The experiment was performed in accordance with guidelines for animal research from Istanbul University Ethics Commite on Animal Research (2011/84).

Collection of Ovaries and Recovery of Oocytes

Ovaries were collected from 26 domestic cats (crossbred of different breeds; ages 1-3 years) by routine ovariohysterectomy following anesthesia at different local veterinary clinics and maintained in Phosphate Buffered Saline (PBS) at 35°C for 3 h. The ovaries were sliced with a scalpel blade and rinsed in washing medium [30] at room temperature in order to obtain cumulus oocytes complexes (COCs). COCs were washed three times with Ham's F-10 medium [31]. The oocytes with dark homogenously pigmented ooplasm and completely surrounded by at least four layer of cumulus cells were selected for *in vitro* maturation (IVM) *(Fig. 1)*.

In vitro Maturation (IVM)

Nutrient Mixture F-10 Ham (Ham' F-10) (Sigma; N-6635) was used as a maturation medium, supplemented with 10 µg/ml FSH (Sigma; F-2293), 10 µg/ml LH (Sigma; L-5269), 20 ng/ml EFG (Invitrogen;13247-051), 0.4% BSA and antibiotics. Selected COCs were maturated at 38°C for 44 h in four-well petri dishes (NUNCR, Denmark), which included 500 µl maturation medium under mineral oil in each well [22,32]. Incubations for IVM were carried out in a humidified 5% CO_2, 5% O_2, and 90% N_2 atmosphere with 100% humidity. For each experimental group, 30-40 COCs were separately placed in each well according to the number of obtained oocytes per replication. After

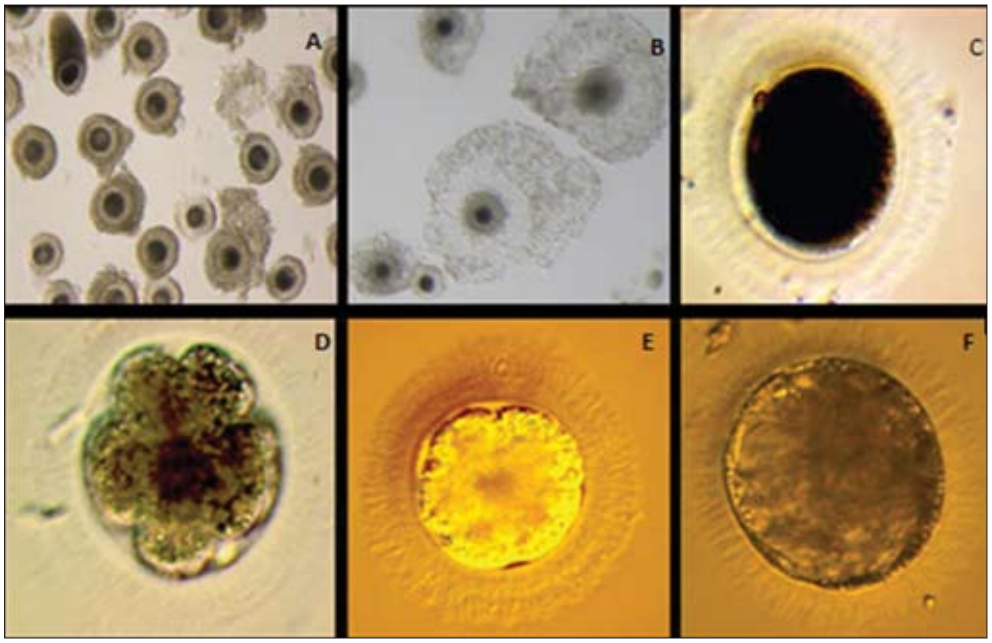

Fig 1. A- immature cat oocytes (x8), **B**- oocytes with expanded cumulus oophorous after *in vitro* maturation (x8), **C**- M II stage oocyte with extruded first polar body (x200), **D**- 8-cell stage embryo (x200), **E**- compact morula (x200), **F**- expanded blasocyst (x200)

Şekil 1. A- olgun olmayan kedi oositleri (x8), **B**- *in vitro* olgunlaşma sonrası kumulus ooforus genişlemesi gösteren oositler (x8), **C**- birinci polar cisimciği atmış M II aşamasında bulunan oosit (x200), **D**- 8-hücreli aşamada bulunan embriyo (x200), **E**- kompakt morula (x200), **F**- genişlemiş blastosist (x200)

IVM, oocytes denuded by vortexing in synthetic oviduct fluid (SOF) medium with Hepes (hSOF) plus 11.5 mg/ml hyaluronidase for 1 min allowed for the selection of intact immature and M II oocytes.

Experimental Design

After *in vitro* maturation, the oocytes were separated into two groups according to maturity. The oocytes with a first polar body were separated as mature MII and those without a polar body were considered as immatured. Five replicates were performed for each treatment.

Two control and four activation groups' experiments were performed. In the control groups, oocytes were cultured without activation treatment to confirm that parthenogenetic activation was promoted by the proposed protocols and not by the culture conditions. Both the mature and immature oocytes were subjected to the following same treatments.

Control Group 1: Oocytes were incubated in culture medium for 5 min and then transferred into the fresh culture medium (mSOF) and cultured for a further 6 days.

Control Group 2: Oocytes were incubated in fusion medium for 5 min and then transferred into the culture medium (mSOF) and cultured for a further 6 days.

In both control groups 1 and 2, oocytes were cultured in the absence of any activation agent.

The treatment groups included electrical stimulus and chemical agents as described below:

Treatment Group 1 (Electrical Stimulation + 6-DMAP): 1.0 kV/cm 20 µs DC pulses 0.1 s apart (2x) + 2 mM 6-DMAP (sigma D 2629).

Treatment Group 2 (Electrical Stimulation + CHX): 1.0 kV/cm 20 µs DC pulses 0.1 s apart (2x) + 10 µg/ml CHX (sigma C4859).

Treatment Group 3 (CaI + 6-DMAP): 5 µg/ml CaI (sigma C 7522) + 2 mM 6-DMAP.

Treatment Group 4 (CHX + 6-DMAP): 5 µg/ml CaI + 10 µg/ml CHX.

In treatment groups 1 and 2, the electricial stimulation to induce oocyte activation was performed by the cell fusion apparatus BTX 830 electro cell manipulator (BTX, San Diego, CA, USA). The chamber used contained two parallel platinum wire electrodes spaced 0.5 mm apart and overlaid with cell fusion medium including 0.3 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.05% fatty acid–free BSA at room temperature [33]. One hour after electrical stimulation, the oocytes were incubated in a synthetic oviductal fluid plus 1% essential and 1% non-essential amino acids (mSOF) supplemented with 2 mM 6-DMAP for group 1; and 10 µg/ml CHX for group 2. The incubation lasted 6 h at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. For the treatment groups 3 and 4, the oocyes were activated by using 5 µg/ml CaI for 5 min and additionally exposed to 10 µg/ml CHX and 2 mM 6-DMAP for 6 h. After 48 h of activation, the oocytes were examined and the non-cleaved oocytes accepted as non-activated and removed. The cleaved oocytes/embryos were *in vitro* cultured for an additional 4 days.

In vitro culture of activated oocytes

Activated oocytes were cultured in mSOF medium supplemented with 0.4% BSA [34]. At day 3 of culture, all cleaved embryos were transferred to a fresh SOF medium supplemented with 1.5 mM Glucose (G 6152; Sigma) and

cultured for an additional 3 days to evaluate their ability to develop to the morula and blastocyst stages.

Statistics

Each experiment was replicated 5 times. Kruskal-Wallis test was applied to compare the activation and developmental rates of oocytes *in vitro*.

RESULTS

A total of 571 immature and 422 *in vitro* matured oocytes were activated and 322 oocytes allocated to the control groups. We evaluated the cleaved embryos as the total number of parthenogenetic activated oocytes. *Table 1* and *Table 2* show the number of oocytes and the rates of cleavage of the mature and immature oocytes activated by each treatment group.

In this study, the positive effects of both activator agents (Electrical stimulus and CaI), plus two protein synthesis inhibitors (6-DMAP and Cycloheximide) on the parthenogenetic activation of *in vitro* matured (with polar body) cat oocytes was demonstrated. Although, all four treatments effectively induced parthenogenetic activation of *in vitro* matured domestic cat oocytes, the first treatment group (Electric + 6-DMAP) was the most successful group, giving higher rates for the formation of morula-blastocyst stages *(Table 1)*.

For the immature oocyte (without polar body) groups, although there were no significant differences for their activation rates among the first three groups, the 6-DMAP + Electrical stimulus and CaI treatment groups were the most successful for the formation of morula-blastocyst rates. For the Electric stimulus + CHX group no oocyte reached to morula-blastocyts stage ($P<0.05$) *(Table 2)*. Moreover, the highest degenerated oocyte rates were found in Electric stimulus + CHX groups in both mature and immature oocyte groups *(Table 1* and *Table 2)*.

DISCUSSION

In this study, no statisticall differences on parthenogenetically activated rates between all MII oocyte treatment groups were observed. Parallel to our findings, others [10,35] reported improved results when calcium ionophore was used in combination with protein synthesis inhibitors in cat and cattle.

Although there were no differences on parthenogenetic activation rate among all treatment groups in *in vitro* matured oocytes exposed to the protein synthesis inhibitor 6-DMAP after being subjected to electrical stimulus, the attainment of morula-blactocyst rate was increased (12.38%). Although researchers [13], reported satisfactory morula attainment results (22-25%) in CHX exposed cat oocytes, in our study the lowest morula-blastocyst rates were obtained in electrical stimulation and calcium ionophore

Table 1. *The results of parthenogenetic activation and in vitro embryonic development of mature (M II) stage cat oocytes*
Tablo 1. *Olgun (M II) kedi oositlerinin pathenogenetik aktivasyon ve embriyonik gelişim sonuçları*

Treatment Groups	Technique	n	Activated Oocytes (%)	Morula-Blastocyst (%)	Fragmentated Oocytes (%)	Degenerated Oocytes (%)
1	Electric + 6-DMAP	105	39 (37.14)^{a}	13 (12.38)^{a}	16 (15.24)^{bc}	6 (5.71)^{ab}
2	Electric + CHX	111	36 (32.43)^{a}	3 (2.70)^{b}	19 (17.11)^{bc}	14 (12.61)^{a}
3	Cal + 6-DMAP	95	25 (25.51)^{ab}	9 (9.47)^{ab}	16 (16.32)^{bc}	2 (2.04)^{b}
4	Cal + CHX	108	30 (27.77)^{a}	4 (3.70)^{b}	4 (3.70)^{c}	5 (4.62)^{ab}
5	Fusion Medium Control	77	8 (10.39)^{bc}	1 (1.29)^{b}	17 (22.07)^{ab}	0 (0.00)^{b}
6	Negative Control	92	6 (6.52)^{c}	1 (1.08)^{b}	34 (36.95)^{a}	2 (2.17)^{b}

Values with different superscripts in the same column are significantly different (a,b,c) (P<0.05)

Table 2. *The results of parthenogenetic activation and in vitro embryonic development of immature (MI) cat oocytes*
Tablo 2. *Olgun olmayan (M II) kedi oositlerinin pathenogenetik aktivasyon ve embriyonik gelişim sonuçları*

Groups	Technique	n	Activated Oocytes (%)	Morula - Blastosist (%)	Fragmentated Oocytes (%)	Degenerated Oocytes (%)
1	Electric + 6-DMAP	108	31 (28.70)^{a}	8 (7.40)^{a}	6 (5.55)^{bc}	6 (5.55)^{a}
2	Electric + CHX	118	23 (19.49)^{ab}	0 (0.00)^{b}	1 (0.84)^{bc}	13 (11.01)^{a}
3	Cal + 6-DMAP	220	67 (30.45)^{a}	15 (6.81)^{ab}	13 (5.90)^{bc}	13 (5.90)^{a}
4	Cal + CHX	125	16 (12.80)^{ab}	1 (0.80)^{ab}	1 (0.80)^{c}	11 (8.80)^{a}
5	Fusion Medium Control	93	11 (11.82)^{b}	0 (0.00)^{b}	18 (19.35)^{a}	6 (6.45)^{a}
6	Negative Control	70	12 (17.14)^{ab}	2 (2.85)^{ab}	7 (10.00)^{b}	5 (7.14)^{a}

Values with different superscripts in the same column are significantly different (a,b,c) (P<0.05)

groups exposed to CHX in both immature and matured oocyte groups (2.7-3.7%). These differences may depend on the use of different activator agents such as roscovitine, and strontium in the Rascadoa et al.[13] study. In contrast, others demonstrated that aging oocytes undergo artificial activation more rapidly than oocytes matured for shorter (24 h) intervals [36]. It was previously reported that cat oocytes matured *in vitro* for 24 h had concentrations of MAPK and MPF significantly higher than those matured for longer periods [37]. However, it was demonstrated that maturation of cat oocytes for longer periods (42-45 h), reduced post-fertilization embryonic development rates [22,29]. In the present study, domestic cat oocytes were matured *in vitro* for 44 h and subjected to four different activation protocols. The low morula-blastocyts rates in our study may be releated to the long maturation period.

It was previously suggested that embryo viability strongly supports the morphological observations signifying that no chromosomal damage is induced by 6-DMAP treatment after activation [38]. Our findings are supportive of these results. Although there were no statistical differences among immature oocyte treatment groups, using 6-DMAP provides higher benefit than CHX on the viability (non degeneration) of oocytes. The observed differenceses were significant among MII oocyte treatment groups.

It is reported that the process of nuclear reprogramming may be different between mature and immature oocytes, since developmental abilities of reconstructed embryos vary between the maturation stages of recipient oocytes [27]. Parallel to these statements, in the present study the morula-blastocyst rates in MII stage oocytes were higher than the same clusters of immature oocytes for all activation groups. Some researchers [12] found positive effects of the protein synthesis inhibitor CHX on the activation and subsequent parthenogenetic development of *in vitro* matured pig oocyte, activated by calcium ionophore. In our study, the protein synthesis inhibitors 6-DMAP and CHX although shown to have similar positive effects on activation rates and in terms of morula-blastocyst formation rates, 6-DMAP was more effective than CHX in both MII and imature stage oocytes.

Although the developmental abilities of reconstructed embryos vary between the maturation stages of recipient oocytes, it shown that porcine MI oocytes have a potential to develop into blastocysts after SCNT and this situation may be associated with the differencies in the process of nuclear reprogramming between MI and MII oocytes [27]. Our findings are parallel to these results where immature cat oocytes have a potential to develop to morula and blastocyst stages after parthenogenetic activation. This observation demonstrates that this was not a species-specific event. To our knowledge, this is the first report that describes morula and blastocyst formation from parhenogenetically activated immature cat oocytes.

In conclusion the current study demonstrates that (I) both matured (MII) and immature cat oocytes have a potential to develop to morula and blastocyst stages after parthenogenetic activation; (II) there was no statistical difference in the development to the morula and blastocyst stage for the CaI + 6-DMAP and Electrical stimulation + 6-DMAP group for matured (MII) and immatured cat oocytes; (III) the current study indicated that immature oocytes can develop to morula and blastocyst stage and therefore should be tested as nuclear recipients for SCNT.

Acknowledgements

We thank Veterinary Surgeons, Hakan DİNÇER, Leyla ÇAKICI KOÇOĞLU and staff from the Kadıköy/İSTANBUL Kennel for providing cat ovaries.

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Kafkas Univ Vet Fak Derg
20 (4): 571-576, 2014
DOI: 10.9775/kvfd.2013.10638

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effect of Clopidol and Amprolium/Ethopabate on Performance and Intestinal Morphology of Chickens with Experimental Coccidiosis [1]

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[1] *This work had been supported by the funds granted by Vice Chancellor for Research of Shahrekord University*

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Makale Kodu (Article Code): KVFD-2013-10638

Summary

To investigate effects of two anticoccidial drugs on growth performance and intestinal mucosa in experimental coccidiosis, broiler chicks challenged/medicated by oocytes of Eimeria/Clopidol and Amprolium + Ethopabate. There were negative effects on growth performance in chickens infected/medicated by these drugs. Most morphometric parameters of intestine were decreased in infected or medicated groups compared to negative control (P<0.05). Duodenal villus height in infected/unmedicated group and villus surface area in all infected groups were lowest between infected or medicated groups (P<0.05). Duodenal villus width was also decreased in all medicated groups compared to negative control, but this decreasing was only significant in infected / medicated groups. Jejunal villus height, width and surface area in infected/unmedicated group were lowest as compared to other uninfected / medicated groups (P<0.05). Ileal villus height in infected/unmedicated group were lowest as compared to all medicated groups. In infected/medicated groups, ileal villus surface area was significantly lower than uninfected/medicated groups. There were no significant differences between Clopidol- and Amprolium + Ethopabate-medicated groups in intestinal morphometric assessment. It is concluded that Clopidol and Amprolium + Ethopabate have adverse effects on chicken performance and intestinal morphology especially villus dimensions and absorptive surface during control or prevention of coccidiosis.

Keywords: *Intestinal morphology, Broiler chicken, Anticoccidial drug*

Deneysel Koksidiyozlu Tavuklarda Clopidol ve Amprolium/Ethopabate Uygulamasının Performans ve Barsak Morfolojisine Etkisi

Özet

İki antikoksidial ilacın deneysel koksidiyaziste büyüme performansı ve barsak mukozasına etkilerini araştırmak amacıyla broiler civcivlere Eimeria oositleri/Clopidol ve Amprolium + Ethopabate verildi. Bu uygulamaların yapıldığı tavuklarda büyüme performansının negatif olarak etkilendiği belirlendi. Barsağın çoğu morfometrik parametrelerinin uygulama gruplarında negatif kontrole oranla azaldığı tespit edildi (P<0.05). Uygulama gruplarında duodenal villus yüksekliği ve tüm enfekte hayvanlarda villus yüzey alanı uygulama grupları arasında en düşüktü (P<0.05). Duodenal villus genişliği tüm ilaç uygulama gruplarında negatif kontrole oranla azalmıştı. Ancak bu azalma sadece enfekte/ilaç uygulanan gruplarda anlamlı idi. Jejunum villus yüksekliği, genişliği ve yüzey alanı enfekte/ilaç uygulanmayan grupta diğer enfekte olmayan /ilaç uygulanan gruplara oranla en azdı (P<0.05). Enfekte/ilaç uygulanmamış grupta ileum villus yüksekliği tüm ilaç uygulanan gruplara oranla en azdı. Enfekte/ilaç uygulanan gruplarda ileum villus yüzeyi alanı infekte olmayan/ilaç uygulanan gruplara oranla anlamlı derecede daha azdı. Barsak morfometrik değerlendirmede Clopidol- ve Amprolium + Ethopabate-uygulanan gruplar arasında belirgin bir fark yoktu. Kontrol veya koksidiyozu önleme maksatlı Clopidol ve Amprolium + Ethopabate uygulamasının tavuklarda performans ve barsak morfolojisine, özellikle villus boyutları ve sindirici yüzeye, olumsuz etkileri olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Barsak morfolojisi, Broiler tavuk, Antikoksidial ilaç*

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INTRODUCTION

The coccidia as a protozoa are single celled parasites in animals in the phylum *Apicomplexa* and genus *Eimeria*. The coccidia are host-specific. The triggered place of coccidia species is the lining of the intestine or ceca in the chicken [1,2].

In the poultry industry, coccidiosis is a permanent problem; and outbreaks of coccidiosis still occur despite of the improved management conditions in broiler rearing [3,4].

High-intensity systems are leading to dependence on anticoccidial feed additives in broiler rearing to provide prophylactic control against infections due to pathogenetic species of *Eimeria*. In the modern farms, the warm humid environment due to high stocking density, provide suitable condition for *Eimeria* infection. Nine species of coccidia (from genus *Eimeria*) infect poultry [5,6]. The severity of pathogenesis caused by each species of *Eimeria* varies. The most pathogenic *Eimeria* (*E*) in chickens are *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox*, and *E. tenella* which can provide outbreaks of coccidiosis [1,4]. These parasitic agents invade the mucosa, proliferate in the intestinal epithelium and provide different pathological lesions (moderate to severe) in various segments of intestine.

Todays, in the poultry industry, effective control of coccidiosis is still important to obtain profitable production. In this disease, because of oocyst resiliency, chemoprophylaxis by the use of anticoccidial drugs would be necessary. Since the utilization of the sulphonamides as the first chemical anticoccidial drugs, the development and introduction of a continual succession of these and other drugs have been more or less successful in prophylaxis of the disease [7]. At the present study, the effects of two anticoccidial drugs (Clopidol and Amprolium/Ethopabate as chemical drug) were evaluated on the performance and intestinal morphology of healthy and chaleged broiler chicken. The used drugs in this experiment are the usual anticoccidials in the poultry industry, used as prophylactic agents or against clinical and subclinical forms of coccidiosis.

MATERIAL and METHODS

Animals, Management and Treatments

Two hundred and sixteen, one-day-old fast-growing chickens from Ross 308 breed were randomly divided into six equal groups with three replicates per group (36 birds per group). Chicks were reared in floor pens on wood shaving litter at standard condition for six weeks and provided *ad libitum* access to water and a standard basal diet. The basal diets were in mash form and formulated for starter (1-14 d), grower (15-29 d), and finisher (30-42 d) growth periods and the composition is shown in *Table 1* [8]. The drugs were fed to the birds from 21 days of age. Three groups of chickens in this experiment were infected orally at day 21 by a mix of four species of sporulated oocytes, consist of 2.5×10^4 *E. acervulina*, 2×10^4 *E. maxima and* 2.5×10^4 *E. necatrix* and 8×10^4 *E. tenella*. Experimental groups were designed as following:

Group A: As negative control, uninfected/unmedicated control.

Group B: As uninfected/clopidol, uninfected while medicated with 125 ppm clopidol.

Group C: As uninfected/amprolium + ethopabate, uninfected while medicated with 125 ppm Amprolium/8 ppm ethopabate.

Group D: As positive control, infected with 1.5×10^5 mixed oocytes while unmedicated.

Group E: As infected/amprolium + ethopabate, infected with 1.5×10^5 mixed oocytes and medicated with 125 ppm Amprolium/8 ppm ethopabate .

Group F: As infected/clopidol, infected with 1.5×10^5 mixed oocytes and medicated with 125 ppm clopidol.

Feed consumption and body weight were recorded in the each group, feed conversion were calculated at the end of experiment. Excreted oocysts were counted from 5 to 11 days after infection according to Pirali Kheirabadi et al.[9] and offered as OPG (oocytes per gram feces). The mortality rate of chickens in each group was recorded during rearing. The study was approved by the Ethics Committee of Shahrekord University.

Morphometric Analysis of the Intestine

At 42 days of age, 6 chicks from each group were killed by decapitation and their intestinal morphometric variables i.e., villus sizes (height, width, surface area and lamina propria thickness) evaluated according to Zamani Moghaddam et al.[10] in the duodenum, jejunum and ileum. Briefly, midpoint segments of the duodenum, jejunum, and ileum were dissected. The segments were fixed in Clark fixative for 45 min, and then left in ethyl alcohol for longer storage. Each segment was divided into 3 sections along its length. Sections were left in periodic acid Schiff (PAS) reagent for 2-3 min, rows of villi were cut in thickness of the sections, transferred over the glass slides and covered with a cover-slip. These samples were examined by a microscope with eyepiece graticules (10×) and magnification of ×100 [10,11]. The villus height was measured from the top of the villus to the top of the lamina propria. Surface area was calculated using the formula = $(\pi) \times (VW) \times (VL)$ in which VW = villus width and VL = villus length [12]. The lamina propria thickness was measured at the space between the base of the villus and the top of the muscularis mucosa. The muscle layer was measured from the top of the muscularis propria to the serosa [13]. In each bird, three segments of each one of the duodenum, jejunum and ileum was examined.

Statistical Analysis

All data are represented as mean ± SE. Results of OPG, intestinal and performance parameters were compared between groups using one way ANOVA (SPSS Institute Inc.). All data were checked to have a normal distribution and log transformed if necessary. Any data requiring log transformation were back-transformed for presentation of data. *P* values less than 0.05 were significant.

RESULTS

Estimation of Oocyst

The numbers of OPG of feces from the infected/medicated groups of chickens were counted from days 5 to 11 post challenge that are shown in *Fig. 1*. The shedding of oocysts was recorded as early as on the 5th day post challenge in experimentally infected groups (D, E and F) while there was not any oocyst in the feces of uninfected groups (A, B and C) (data not shown). There was significant decreasing of OPG in the groups E and F in days 5, 6, 7, 8 and 9 as compared to other infected group (D, positive control) (P<0.05). There was also significant decreasing of OPG in the group F in days 6, 7 and 8 in compared to group E (P<0.05) *(Fig. 1)*.

Growth Performance in the Experimental Groups

Effects of Clopidol and Amprolium/Ethopabate on the performance of infected and uninfected chickens are presented in *Table 2* and *Table 3*. The parameters of bird performance were presented weekly during rearing. The mean body weight of chickens in the infected or medicated groups was significantly (P<0.05) decreased in weeks 4, 5 and 6 as compared to negative control (Group A). The mean body weight of chickens in group D was lowest in the weeks 4 (P<0.05) and 5 in comparison to other groups *(Table 2)*. Conversely, The FCR of infected or medicated groups was higher than negative control (Group A) in five last weeks of rearing, which this increasing was only significant (P<0.05) in week 4. In this week, chickens in group D had highest FCR (P<0.05) *(Table 3)*.

There were no significant (P>0.05) differences between Clopidol-medicated and Amprolium + Ethopabate medicated groups in growth performance of chickens (*Table 2* & *3*).

The mortality was only observed in group D (Infected/unmedicated) that its rate was about 11.1%.

Intestinal Morphometric Assessment

Intestinal morphometric parameters were compared

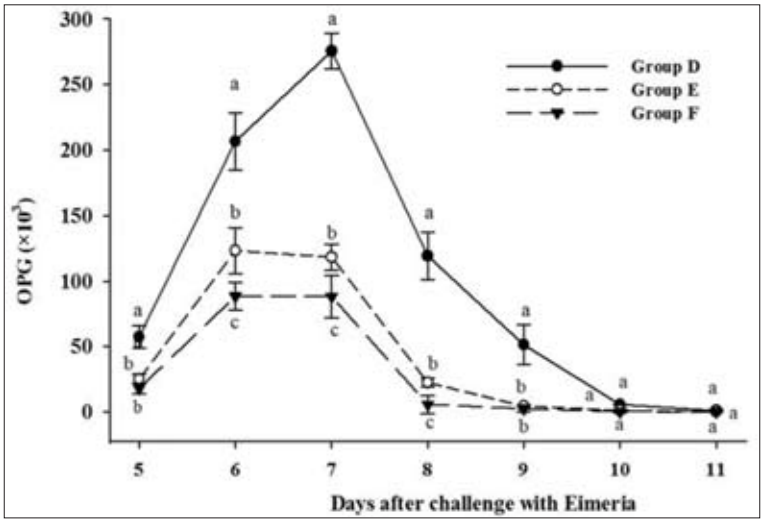

Fig 1. Comparison of number of oocytes per gram feces (OPG) between chicken groups, 5 to 11 days after experimental challenge with *Eimeria,* [a,b,c] Means with the different indices between groups at each day are significantly different for P<0.05. Group D, positive control (infected/unmedicated); Group E, infected/amprolium; Group F, infected/clopidol

Şekil 1. *Eimeria* uygulamasından 5 ile 11 gün sonrasında gruplar arasında her gram dışkıda oosit sayılarının karşılaştırılması, [a,b,c] belirteçleri gruplar arasında her bir gün için anlamlı derecede P<0.05 için farklılığı göstermektedir. Grup D, pozitif kontrol (enfekte/ilaç uygulanmamış); Grup E, enfekte/amprolium; Grup F, enfekte/clopidol

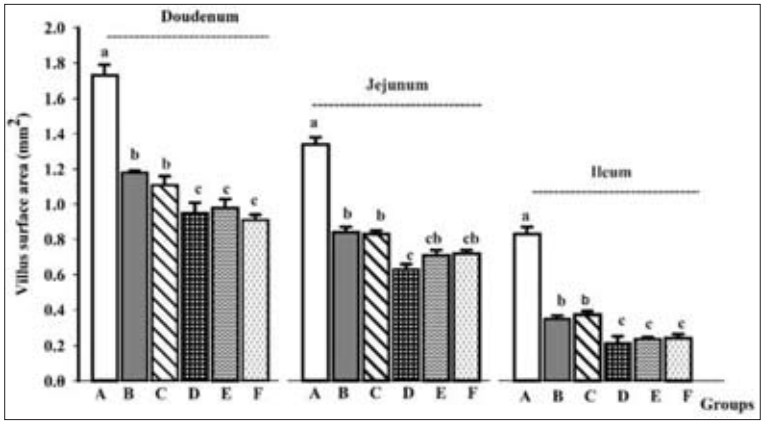

Fig 2. Villus surface area in three intestinal parts in the experimental groups of chickens at 42 days of age, [a,b,c] Means with the different indices between groups are significantly different for *P<0.05*. Group A, negative control (uninfected/unmedicated); Group B, uninfected/Clopidol; Group C, uninfected/amprolium; Group D, positive control (infected/unmedicated); Group E, infected/amprolium; Group F, infected/clopidol

Şekil 2. 42 günlük deneysel gruplardaki tavuklarda üç farklı barsak bölgesinin villus yüzey alanı, [a,b,c] belirteçleri gruplar arasında anlamlı derecede P<0.05 için farklılığı göstermektedir. Grup A, negatif kontrol (enfekte olmamış/ilaç uygulanmamış); Grup B, ilaç uygulanmamış/clopidol; Grup C, enfekte olmamış/amprolium; Group D, pozitif kontrol (enfekte/ilaç uygulanmamış); Grup E, enfekte/amprolium; Group F, enfekte/clopidol

between experimental groups of chickens at 42 days of age that are shown in *Table 4* and *Fig. 2*.

The duodenal villus height, lamina propria thickness and surface area were significantly lower in the infected or medicated groups than negative control (group A) (P<0.05; *Table 4; Fig. 2*). The duodenal villus height in group D (infected/unmedicated) and villus surface area in all infected groups (D, E and F) were lowest between infected or medicated groups (P<0.05). The duodenal width was also decreased in all treated groups compared to negative control (group A), but this decreasing was only significant in groups E and F (P<0.05).

The jejunal villus height, width, lamina propria thickness and surface area were significantly lower in the infected or medicated groups than negative control (group A) (P<0.05; *Table 4; Fig. 2*). The jejunal villus height, width and surface area in group D (infected/unmedicated) were lowest as compared to other uninfected/medicated groups (B, C) (P<0.05).

The ileal villus height, width and surface area were significantly decreased in the infected or medicated groups than negative control (group A) (P<0.05; *Table 4; Fig. 2*).

The ileal villus height in group D (infected/unmedicated) were lowest as compared to groups B, C (P<0.05), E and F. The ileal lamina propria thickness was not different among experimental groups (P>0.05; *Table 4*). In the infected/medicated groups (D, E and F), the ileal villus surface area was significantly lower than uninfected/medicated groups (B and C) (P<0.05; *Fig. 2*).

There were no significant (P>0.05) differences between Clopidol- and Amprolium + Ethopabate-medicated groups in the intestinal morphometric assessment *(Table 4; Fig. 2)*.

Table 1 .*Composition of basal diets*
Tablo 1. *Bazal diyetin kompozisyonu*

Ingredients	Starter (1-14 d)	Grower (15-29 d)	Finisher (30-42 d)
Corn	53.01	58.65	63.95
Soybean meal (44% CP)	39.01	33.51	27.85
Soybean oil	2.89	3.21	3.82
Limestone	1.44	1.35	1.33
Dicalcium phosphate	2.27	2.00	1.83
Vitamin mixture [1]	0.50	0.50	0.50
Mineral mixture [2]	0.30	0.25	0.25
Salt	0.30	0.30	0.30
DL-methionine L- lysine Vitamin E	0.23 0.03 0.02	0.20 0.01 0.02	0.14 0.01 0.02
Calculated Chemical Composition			
ME (Kcal/kg)	2900	3000	3100
Crude protein (%)	22	20	18

[1] Supplied per kg diet: vitamin A, 90.00IU; cholecalciferal, 1.500 IU; vitamin E, 10 IU; vitamin K, 0.5mg; cobalamin, 0.007 mg; thiamin 0.4 mg; riboflavin, 6 mg; folic acid, 1 mg; biotin, 0.15 mg; pantothenic acid 12 mg; niacin, 35 mg; pyridoxine, 4 mg; cholin chloride, 1.000 mg; [2] Supplied per kg diet: Mn, 60 mg; Cu, 5 mg; Zn, 50 mg; I, 0.35 mg; Se, 0.1 mg; iron 40 mg

DISCUSSION

Maintaining bird health, regarding diseases or agents acting on the gastrointestinal tract, is crucial in broiler production, since this is the entry route of nutrients for bird development. The small intestine is responsible for the digestion and absorption of nutrients from food, and the duodenal segment mainly for absorption [14]. Broilers exhibiting shortening of villi have impaired nutrient absorption [14,15]. Cell divisions in the intestine of birds, unlike mammals, are not restricted to crypts, occurring also along the villi [16]. According to Nabuurs [17], the ideal intestinal morphometry in birds are long villi and shallow crypts. That is, length of villi is related to the digestive capacity and intestinal absorptive area [16,18]. However, factors such as dietary supplements, drugs or pathogens can cause changes in the intestinal morphology [19,20].

Table 2. *Comparison of chicken body weights (g) in the experimental groups during rearing*
Tablo 2. *Yetiştirme süresince deneysel gruplarda tavuk vücut ağırlıklarının (g) karşılaştırılması*

Group	n	Weeks					
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Group A	36	107.9	323.1	659.5	1136.6^{a}	1649.2^{a}	2158.2^{a}
Group B	36	104.3	306.9	634.7	986.2^{b}	1431.6^{b}	1948.6^{b}
Group C	36	109.8	299.4	615.5	976.9^{b}	1444.3^{b}	1982.9^{b}
Group D	36	114.3	310.6	668.3	845.9^{c}	1276.4^{c}	1913.9^{b}
Group E	36	106.1	309.2	629.1	946.5^{b}	1389.0^{bc}	1934.6^{b}
Group F	36	107.3	307.6	643.5	930.0^{b}	1360.7^{bc}	1915.6^{b}
Pooled SEM	-	3.7	6.3	20.6	20.7	38.1	38.3

^{a,b,c} Means with the different indices between groups at end of each week are significantly different for P<0.05. Group A, negative control (uninfected/unmedicated); Group B, uninfected/Clopidol; Group C, uninfected/Amprolium; Group D, positive control (infected/unmedicated); Group E, infected/Amprolium; Group F, infected/Clopidol

Table 3. *Comparison of feed conversion rate (FCR) in the experimental groups of chickens during rearing*
Tablo 3. *Yetiştirme süresince deneysel gruplarda yem dönüşüm oranlarının (FCR)karşılaştırılması*

Group	n	Weeks					
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Group A	36	0.98	1.25	1.57	2.04^{a}	2.18	2.33
Group B	36	0.97	1.26	1.65	2.30^{b}	2.38	2.44
Group C	36	0.99	1.24	1.70	2.26^{ab}	2.37	2.34
Group D	36	0.99	1.23	1.59	2.69^{c}	2.49	2.52
Group E	36	1.00	1.24	1.60	2.35^{b}	2.46	2.39
Group F	36	1.01	1.28	1.66	2.40^{b}	2.43	2.50
Pooled SEM	-	0.03	0.01	0.03	0.06	0.07	0.05

^{a,b,c} Means with the different indices between groups at each week are significantly different for P<0.05. Group A, negative control (uninfected/unmedicated); Group B, uninfected/Clopidol; Group C, uninfected/Amprolium; Group D, positive control (infected/unmedicated); Group E, infected/Amprolium; Group F, infected/Clopidol

Table 4 .*Comparison of intestinal morphology between experimental groups of chickens at 42 days of age*
Tablo 4. *42 günlük tavuklarda deneysel grupların barsak morfolojilerini karşılaştırılması*

Intestine	Group	n	Villus Dimensions		
			Height (mm)	Width (mm)	Lamina Propria (mm)
Duodenum	Group A	6	0.79^{a}	0.69^{a}	0.33^{a}
	Group B	6	0.64^{b}	0.58^{ab}	0.23^{b}
	Group C	6	0.62^{b}	0.57^{ab}	0.23^{b}
	Group D	6	0.52^{c}	0.57^{ab}	0.21^{b}
	Group E	6	0.57^{bc}	0.54^{b}	0.21^{b}
	Group F	6	0.57^{bc}	0.51^{b}	0.21^{b}
	Pooled SEM	-	0.01	0.02	0.01
Jejunum	Group A	6	0.61^{a}	0.69^{a}	0.30^{a}
	Group B	6	0.49^{b}	0.54^{b}	0.25^{b}
	Group C	6	0.50^{b}	0.53^{b}	0.25^{b}
	Group D	6	0.45^{c}	0.44^{c}	0.22^{b}
	Group E	6	0.47^{bc}	0.47^{bc}	0.25^{b}
	Group F	6	0.48^{bc}	0.47^{bc}	0.24^{b}
	Pooled SEM	-	0.02	0.01	0.01
Ileum	Group A	6	0.37^{a}	0.72^{a}	0.22
	Group B	6	0.21^{bc}	0.53^{b}	0.22
	Group C	6	0.25^{b}	0.48^{b}	0.20
	Group D	6	0.14^{c}	0.49^{b}	0.22
	Group E	6	0.17^{bc}	0.44^{b}	0.21
	Group F	6	0.18^{bc}	0.43^{b}	0.21
	Pooled SEM	-	0.02	0.02	0.01

^{a,b,c} Means with the different indices between groups at each segment of intestine are significantly different for P<0.05. Group A, negative control (uninfected/unmedicated); Group B, uninfected/Clopidol; Group C, uninfected/Amprolium; Group D, positive control (infected/unmedicated); Group E, infected/Amprolium; Group F, infected/Clopidol; ***n:*** *number of chickens*

Nowadays, it is common that anicoccidials are used prophylactically throughout the entire growing period in chicken to achieve total continual prevention of occurrence or suppression of coccidiosis.

In the present study, we used two anticoccidial drugs, Clopidol and Amprolium + Ethopabate as protection in the diets of healthy chickens for 3 weeks and found their adverse effects on the bird performance i.e. body weight and feed conversion rate. It has also been reported that with no *Eimeria* present in the chicken, each intake of an anticoccidial drug lead to a negative effect on growth or feed conversion of a bird [21]. Anticoccidial drugs have a narrow margin of safety and some of them are toxic [22]. In

fact, in the most experiments, a negative influence is seen. This is different for each drug and depends on the dosage administered. However, as soon as *Eimeria* infections build up in the flocks, the possible growth depressing effect of the drug will be neutralized by effective control of the infection. The compensatory growth seen after withdrawal of the drug from the feed, could also be explained by the growth depression effect of the drug used in their study [23]. Brake *et al.*[24] also reported adverse effects of semduramicin, on broiler breeders. They found that semduramicin causes dose-related decrease in egg production, percentage shell, fertile hatchability and increase in early embryonic mortality.

Hassanpour et al.[21] reported when diclazuril, semduramicin, salinomycin and maduramycin were used as prophylactic drugs against chicken coccidiosis, intestinal morphometric parameters especially villus dimensions and absorptive surface were severely diminished.

To our knowledge, the effects of Clopidol and Amprolium + Ethopabate on intestinal mucosa have not been studied previously in the intact and infected chickens. In this experiment, we found that these drugs decreased villous length, width and surface area, in the duodenum and jejunum and ileum. However these data is probably the evidence of impaired nutrition absorption of intestine and reduced enteric function due to anticoccidial drugs.

In this study, we also reported the reduction of lamina propria thickness in the duodenum and jejunum by anti-coccidials, which apparently show a deminished Lieberkühn's glands in these segments of intestine. Thus, it could be predictable that these drugs may influence intestinal secretion in chickens. Of course, it needs further studies to clarify the effect of anticoccidials on the intestinal secretions in broiler chickens.

The functions of Clopidol and Amprolium + Ethopabate on controlling of *Eimeria* infection, growth performance and intestinal morphology were compared together in the present study. In regarding to reduction of shedding oocysts in the infected chickens, Clopidol had better effect than Amprolium + Ethopabate while adverse effects of these drugs on the body performance and intestine were similar.

It is concluded that Clopidol and Amprolium + Ethopabate have adverse effects on chicken performance and intestinal morphology especially villus dimensions and absorptive surface during control or prevention of coccidiosis. There was also evidence for involvement of these drugs in the intestinal secretion that need more studies to confirm.

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Kafkas Univ Vet Fak Derg
20 (4): 577-581, 2014
DOI: 10.9775/kvfd.2013.10661

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Doğal Olarak Enfekte Olmuş Farklı Tür Hayvanlardan, Post-Mortem Olarak Elde Edilen Beyin Numunelerinde, Klasik Kuduzun Rutin Laboratuvar Teşhisinde Real Time RT-PCR Testinin Değerlendirilmesi

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Makale Kodu (Article Code): KVFD-2013-10661

Özet

Bu çalışmada, doğal olarak enfekte olmuş farklı hayvan türlerine ait beyin numunelerinde klasik kuduzun teşhisi için real-time RT-PCR testi değerlendirildi. Çalışma materyalini Elazığ Veteriner Kontrol Enstitüsü'ne 2011 yılı boyunca Türkiye'nin Doğu ve Güneydoğu Anadolu Bölgelerinde bulunan Elazığ, Malatya, Diyarbakır, Mardin, Tunceli, Muş, Bingöl, Batman, Şırnak, Hakkâri ve Bitlis illerinden getirilen 36 köpek, 13 sığır, 6 koyun, 6 eşek, 4 at, 4 tilki, 3 kurt, 2 kedi ve 1 keçiye ait toplam 75 adet kuduz hastalığı şüpheli beyin numunesi oluşturmuştur. Farklı hayvan türlerine ait beyin numunelerinden kuduz virüsü genomunu tespit etmek amacıyla klasik kuduz virüsüne özgü primer ve TaqMan prob seti kullanıldı. Bu primer ve prob seti, beyin numunelerine real-time RT-PCR testi optimize edilerek uygulandı. Laboratuvara ulaşan beyin numunelerine floresan antikor tekniği (FAT) ve deneme hayvanı inokulasyonu (MIT) testleri ile birlikte real time RT-PCR testi de yapıldı. Real-time RT-PCR testi ile 75 beyin numunesinin 53'ünde kuduz virusuna ait spesifik viral RNA tespit edildi. Otoliz olmuş 3 adet köpek beyni numunesine FAT ve MIT uygulanamazken, real-time RT-PCR testi sonucunda bu numunelerin birinde kuduz virusuna ait spesifik viral RNA tespit edildi. Taze (n=72) beyin dokularından etçillerde *C. ammonis*, otçullarda *Cerebellum*'dan elde edilen homojenatlara direkt FAT testi uygulandı, 72 beyin numunesinin 51'inde viral antijenler tespit edildi. Aynı homojenatlardan MIT yöntemiyle 72 beyin numunesinin 52'inde virüs izolasyonu sağlandı. Taze beyin numunelerinden elde edilen MIT testi sonuçlarının, real-time RT- PCR testi sonuçları ile tam uyum gösterdiği (%100) görüldü. Sonuç olarak, klasik kuduz teşhisinde direkt beyin dokusuna uygulanan real-time RT-PCR testinin, hem FAT ve MIT uygulanamayan otoliz olmuş numunelerde güvenle kullanılabileceği ve hem de alt yapısı sağlanmış laboratuvarlarda invitro şartlarda FAT için doğrulayıcı test olarak da kullanılabileceği tespit edilmiştir.

Anahtar sözcükler: *Kuduz Teşhisi, Klasik Kuduz Virüsü, Real-Time RT-PCR*

Evaluation of Real-Time RT-PCR Assay for Routine Laboratory Diagnosis of Classical Rabies in Post-Mortem Brain Samples from Naturally Infected Different Species of Animals

Summary

In this study; the real-time RT- PCR assay was evaluated for laboratory diagnosis of classical rabies in post mortem brain samples from naturally infected different animals species. The material consisted of 75 suspected brain samples brought from 11 different provinces, belonging Eastern and Southeastern Anatolia region, including Elazig, Malatya, Diyarbakir, Mardin, Sirnak, Batman, Tunceli, Muş, Bingol, Hakkari and Bitlis in year of 2011. These samples were from 36 dogs (33 normal, 3 autolytic), 13 cattle, 6 sheep, 6 donkeys, 4 horses, foxes, 3 wolves, 2 cats , and one goat. The samples were autolytic in 3 of 75 (4%), whereas 72 (96%) of 75 samples were freshly arrived to the laboratory. Due to badly autolysis of 3 samples, they could not be examined by fluorescent antibody test (FAT) and mouse inoculation test method (MIT). Seventy two of 75 samples were tested with both fluorescent antibody test (FAT) and mouse inoculation test method (MIT), whereas all the samples (75 of 75) were examined by optimizing real-time RT- PCR assay in which the primary for classical rabies virus and TaqMan prob set were used. Fresh brain homogenates from *Cornu ammonis* in carnivores and *Cerebellum* in herbivores were used for FAT and MIT. As a result, 51 of 72 samples were positive with FAT, 52 of 72 samples with MIT, whereas 52 of 72 were positive in real-time RT-PCR. In fresh brain samples, with MIT results in mouse brain, real time RT- PCR test results showed complete relation each other. As both fluorescent antibody test (FAT) and mouse inoculation test method (MIT) could not be applied to the autolytic samples, the real time RT-PCR assay was the only choice. Consequently, real-time RT-PCR assay could be utilized in autolytic samples conveniently as well as confirmatory to FAT in in vitro conditions.

Keywords: *Rabies Diagnosis, Classical Rabies Virus, Real-Time RT-PCR*

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GİRİŞ

Kuduz, *Rhabdoviridae* familyasında farklı Lyssavirus türlerinin neden olduğu, insanlığın en eski ve en korkulan hastalığı olarak bilinen öldürücü enzootik bir hastalığıdır. Virüs mermi şeklinde, tek iplikcikli negatif polaritede RNA taşımaktadır [1]. Karnivorlar ve yarasalar virüsün temel konakçılarıdır. İnsan ve evcil hayvanlar, enfekte olmuş hayvanların ısırması veya tükürük salgısı ile yaraların bulaşması sonucu enfeksiyona yakalanmaktadır [2].

Virüs, merkezi sinir sistemi (MSS) dışında birçok organda yayılma gösterdiği, kuduz virüsü antijeninin veya RNA'sının hayvanların tükürük bezlerinde, akciğer, böbrek, kalp ve karaciğerinde saptandığı bildirilmiştir [3]. Klasik kuduz virüsü (RABV), genotip 1 olarak da ifade edilmektedir. Antartika, Japonya, Avusturalya, Hawaii, Yeni Zelanda, İngiltere gibi yerler dışında, yeryüzünün hemen her tarafında yaygın olarak bulunmaktadır [1].

İnsan ve hayvanlarda kuduzun kesin teşhisi, post-mortem laboratuar bulgularına dayanılarak yapılmaktadır. Histolojik olarak Negri cisimciklerinin tespiti yerine, floresan antikor tekniği (FAT) rutin kullanım alanı bulmuştur. Ayrıca, deneme hayvanı inokulasyonu (MIT) ve hücre kültüründe (RTCIT) virüs izolasyonu yöntemleri de doğrulayıcı test olarak kullanılmaktadır. Ancak bu yöntemlerle kuduz teşhisi uzun süre gerektirmektedir. Daha sonraları, kuduz virüsü ile ilgili epidemiyolojik çalışmalar ve teşhis çalışmalarında kullanılmak üzere hızlı, güvenilir ve spesifik Polimeraz Zincir Reaksiyonu (PZR) yöntemleri geliştirilmiştir [4-6]. Rutin kuduz teşhisinde, The World Organisation for Animal Health (OIE) ve World Health Organization (WHO), FAT testi ile Lyssavirus antijenlerinin tespit edilmesini, FAT testinde negatif veya şüpheli olan vakalarda teyit için MIT yapılmasını önermektedir [5].

Bu çalışma, viroloji laboratuarına getirilen farklı hayvan türlerine ait beyin numunelerinde, kuduz hastalığını teşhis etmek için tek tüp real-time RT-PCR testinin optimize edilmesi, kullanılması, test sonuçlarının konvansiyonel testlerle karşılaştırılması ve viroloji laboratuarlarında kuduz hastalığının teşhisinde rutin kullanımında uygulanabilirliğini belirlemek amacıyla yapılmıştır.

MATERYAL ve METOT

Materyal

Bu çalışmada materyal olarak Elazığ Veteriner Kontrol Enstitüsü'ne, 2011 yılı boyunca Doğu ve Güneydoğu Anadolu Bölgelerinde yer alan Elazığ, Malatya, Diyarbakır, Mardin, Tunceli, Muş, Bingöl, Batman, Şırnak, Hakkâri ve Bitlis illerinden gönderilen farklı hayvan türlerine *(Tablo 1)* ait kuduz şüpheli beyin numuneleri kullanıldı. Kuduz virüsünün teşhisi için farklı hayvan türlerine ait 72 adet taze ve 3 adet köpeğe ait otoliz olmuş numune olmak üzere toplam 75 adet kuduz şüpheli beyin numunesi incelendi. Hayvan türlerine göre etçillerde *Cornu ammonis*, otçullarda *Cerebellum*'un farklı bölgelerinden doku parçacıkları alındı ve testler yapılıncaya kadar -20ºC'de muhafaza edildi.

Metot

Floresan Antikor Testi (FAT)

Etçillerde *C. ammonis,* otçularda *Cerebellum* dokularının kesit yüzü lama bastırılarak tuşe preparatlar hazırlandı ve oda ısısında kurutuldu. Kurutulmuş olan lamlar daha sonra -20ºC'de aseton içinde yarım saat süreyle tespit edildi. Tespit işleminden sonra, asetondan çıkarılan preparatlar oda ısısında 4-5 dk. bekletilerek asetonu uçuruldu. Fluorescein isotiosiyanat işaretli monoklonal anti-kuduz antikor, konjugat (Fujirebio Diagnostics, Inc. Sweden) ile boyanarak floresan mikroskopta incelendi. Filizi veya parlak yeşil floresan veren, yuvarlak veya oval şekilli merkezine doğru parlaklıkları azalan boyanma alanlarının (Negri cisimciği) görülmesi, kuduz pozitif sonuç olarak kabul edildi [5-7].

Deneme Hayvanı İnokulasyonu Testi (MIT)

Üç veya 4 haftalık en az 5-10 fareye, etçillerde *C. ammonis*

Tablo 1. *Örneklenen materyallere göre tanı yöntemlerinin sonuçları*
Table 1. *Results of diagnostic methods according to the materials sampled*

Hayvan Türü		Materyal Sayısı	Tanı Yöntemi		
			FAT	MIT	Real Time RT-PCR
Etçil Hayvanlar	Köpek	33+3*	23	24	24+1*
	Kedi	2	4	4	4
	Tilki	4	2	2	2
	Kurt	3	-	-	-
Otçul Hayvanlar	Sığır	13	12	12	12
	Koyun	6	2	2	2
	Keçi	1	1	1	1
	At	4	3	3	3
	Eşek	6	4	4	4
Toplam		72+3*	51	52	52+1*

** Otolize olmuş materyal*

ve otçullarda *Cerebellum*'dan olmak üzere şüphelenilen beyin numunesinden alınan ve %20 (w/v) oranında antibiyotikli izotonik buffer solüsyonundan hazırlanan homojenat, intracerebral olarak her bir fareye 0.03 ml olacak şekilde enjekte edildi ve bu fareler 21 gün boyunca günaşırı olarak kuduz belirtileri (farelerde bacaklarda gevşek paralizi, merkezi sinir sistemi semptomları ve ölüm) gösterip göstermedikleri yönünden kontrol edildi. Semptom göstererek ölen farelerin beyin dokusundan hazırlanan preparatlarda FAT boyama ile viral antijenlerin olup olmadığı yönünden incelendi [5,7].

Real-Time RT-PCR Testi

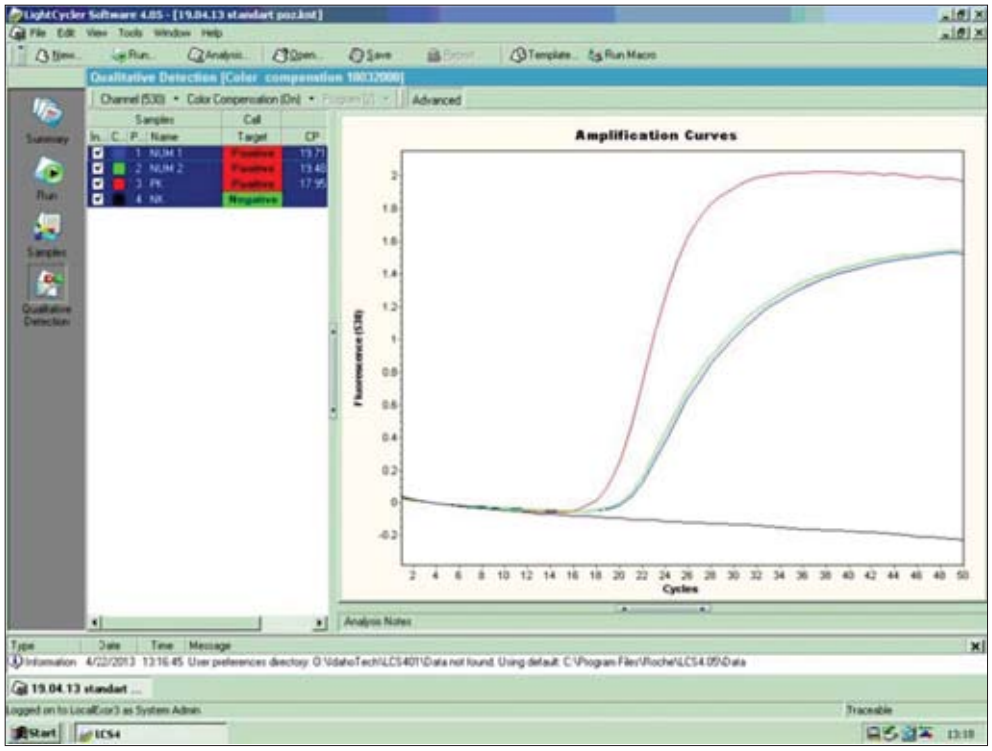
Beyin doku örnekleri steril koşullarda hassas terazide tartılıp MagNA Lyser doku homojenizatöründe parçalandı. Qiagen RNeasy Mini Kit 250 (Qiagen, Hilden, Almanya, Cat No 74106) [8] kullanılarak kit prosedürüne uygun RNA izolasyonları yapıldı. Ekstrakte edilen RNA 50 µl RNase- ve DNase-enzimleri içermeyen su ile sulandırıldı. Elde edilen RNA'lar daha sonra kullanılmak üzere -80ºC'de saklandı.

Klasik Kuduz Virusu oligonükleotid primer ve TaqMan prob dizinleri Hoffmann ve ark.'larının [9] bildirdiği şekilde hazırlandı. Real-time RT-PCR testi, One-Step RT-PCR kit (Kat No:210212, Qiagen, Hilden, Almanya) ile 20 µl reaksiyon hacminde tek tüpte gerçekleştirildi [9]. PCR reaksiyon hacimleri, SDW 7.4 µl, 5X buffer 4 µl, $MgCl_2$ 1 µl, dNTP 0.8 µl, Enzim 0.8 µl, Primer R (5pmol/ul) 1 µl, Primer F (5 pmol/ul) 1 µl, Prob (5 pmol/ul) 1 µl olmak üzere toplam 17 µl mix, 3 µl kontrol RNA veya örnek RNA konularak 20 µl LC kapillerlere konuldu. Hazırlanan kapillerler, 3.000 rpm'de 15 sn santrifüj edildi. Daha sonra Light Cycler 2.0 (Roche Diagnostics Corporation, USA) cihazına konularak 50ºC'de 30 dk. bekletildi ve cDNA sentezi gerçekleştirildi. İlk denatürasyon 95ºC'de 15 dk. bekletilerek, takip eden 45 siklus için ısıtma ve soğutma programı 95ºC'de 25 sn, 55ºC'de 25 sn ve 72ºC'de 25 sn süreyle gerçekleştirildi. Threshold cycle number (Ct) değerleri ile sonuçların değerlendirilmesi, Real-time TaqMan RT-PCR cihazının kantitatif tespit programı ile yapıldı. 18-35 değerleri arasında Ct değeri gösteren numuneler pozitif olarak değerlendirildi *(Şekil 1)*.

FAT, MIT ve real-time RT-PCR testleri için pozitif ve negatif kontrol standart numuneleri, Etlik Veteriner Kontrol Merkez Enstitüsü Ulusal Kuduz Referans Laboratuvarından elde edildi. Kontrollerde, negatif standartta FAT'da herhangi bir floresan ışıma gözlenmedi; MIT'nda 21. gün sonunda ölüm gözlenmedi; real-time RT-PCR'da amplifikasyon eğrisi görülmedi. Pozitif standartta FAT yönteminde filizi veya parlak yeşil floresan ışıldamalar gözlendi; MIT testinde 9. günden sonra farelerde klinik belirtiler ve ölüm görüldü. Ölen farelerin beyinlerinden yapılan FAT testinde, filizi veya parlak yeşil floresan ışıldamalar gözlendi. Real-time RT-PCR testinde ise amplifikasyon eğrisi görüldü.

BULGULAR

Bu çalışmada 2011 yılı içerisinde, Türkiye'nin Doğu ve Güneydoğu Anadolu Bölgelerinde bulunan değişik iklim



Şekil 1. Real-Time RT-PCR test amplifikasyon eğrileri
Fig 1. Amplification curves of Real-Time RT-PCR test

ve coğrafik şartlara sahip 11 ilden rutin kuduz teşhisi için laboratuvara gelen 75 beyin numunesinden hazırlanan homojenatlar analiz edildi.

Real-time RT-PCR test sonucunda numunelerin 53/75 kuduz virusuna ait spesifik viral RNA tespit edildi. Bu örneklerin üç tanesi otoliz olduğundan, FAT ve Fare Virüs İzolasyonu testi yapılamadı. Otoliz olmuş 3 adet köpek beyni numunesinden sadece birinde viral nükleik asit tespiti yapıldı. Taze beyin numunelerinde, direkt FAT testi ile beyin dokularının 51/72 viral antijenler tespit edilebilirken, MIT testi sonucunda dokuların 52/72 virüs izolasyonu gerçekleştirildi.

TARTIŞMA ve SONUÇ

Hayvanlarda görülen kuduz Türkiye'de toplum sağlığını tehdit etmeye devam etmektedir [10,11]. Hızlı, doğru ve güvenilir teşhis, kuduz hayvanlarla temasta bulunmuş insanlar için oldukça önemlidir. Elazığ Veteriner Kontrol Enstitüsü Kuduz Teşhis Laboratuvarına teşhis amacıyla doku numuneleri kabul edilmektedir. Bazen bu numuneler otoliz olmuş olarak laboratuarımıza gelmektedir. Klasik kuduz teşhisinde, kokuşmuş numunelere OIE ve WHO'nin önerdiği direkt FAT testi uygulanamamakta, bunun yerine moleküler tekniklere [12,13] ihtiyaç duyulmaktadır. Son yıllarda kuduzun rutin teşhisinde moleküler metotların kullanımında önemli gelişmeler olduğunu bildiren araştırmacılar [1,9], real-time RT-PCR testinin, FAT ve MIT gibi klasik metotlara iyi bir alternatif olduğunu bildirmişlerdir. Bu metotlar pahalı ekipman ve uzmanlaşmış personel gerektirmesine rağmen, 3-4 saat gibi kısa bir sürede teşhis imkanı sağlamaktadır. Bu çalışmada, klasik kuduz virüsünün teşhis edilmesi amacıyla postmortem beyin numunelerine FAT ve MIT gibi konvansiyonel testlere ilaveten, tek tüp real-time RT-PCR testi uygulandı. Kuduz hastalığının teşhisinde tek tüp real time RT-PCR testinin laboratuvarda optimize edilmesi, sonuçlarının konvansiyonel testlerle karşılaştırılması ve laboratuvarda rutin kullanımına uygunluğunun belirlenmesi amaçlandı.

Postmortem kuduz teşhisinde FAT, kuduz teşhisinde çok yaygın kullanılmakta ve OIE ile WHO tarafından da önerilmektedir [5]. Ancak FAT testi ile mikroskopta antijen varlığının belirlenmesinde, personel deneyiminin olmadığı durumlarda yanlış sonuçlar da alınabilmektedir [14]. Tespit edilmemiş doku numunelerinde Seller's metotla boyama ile kuduz teşhisi bir saatten az sürerken, tespit edilmiş dokulardan, histolojikal testlerle kuduz teşhisi, üç günde yapılabilmektedir. Seller's metodunun, çok düşük sensitivitesinden dolayı terk edilmesi gerektiği de bildirilmektedir [15]. FAT aynı zamanda direkt doku numunelerine de uygulanabilmekte ve bu metotla kuduz virüsü antijen varlığı araştırılmaktadır. FAT testi, taze beyin numunelerinin %95-99'unda 1-2 saat içerisinde güvenilir sonuç vermektedir. MIT, doku süspansiyonunda, infektiviteyi tespit etmek için yapılmaktadır. FAT testinde şüpheli ve negatif sonuç alınan durumlarda ise, hücre kültürü yapılarak veya MIT testi uygulanarak teşhisin tamamlanması gerekmektedir. Ancak MIT testi, OIE ve WHO tarafından doğrulayıcı test olarak önerilmesine rağmen oldukça pahalı ve fare beyninden virüs izolasyonu için uzun bir süreye ihtiyaç duyulmaktadır. MIT testinde kuduza bağlı ölümler en erken inokulasyondan sonraki 9. günden sonra başlamaktadır. Ayrıca, hayvan deneyleri etik kuralları gereği yaşama saygı ilkesi her tür hayvan için geçerli kabul edildiğinden, invitro teşhis metotlarının, zorunlu olmadıkça hayvan deneylerine dayalı invivo teşhis metotlarına tercih edilmesi önerilmektedir [16]. Rutin klasik kuduz teşhisinde kullanılan Hücre Kültüründe Virus İzolasyonu (RTCIT) yönteminde ise canlı hayvan kullanılmamakta olup, aynı zamanda hem MIT testi kadar duyarlı olduğu ve hem de daha ucuz ve daha hızlı sonuç verdiği bildirilmektedir [5]. Buna karşın, postmortem kuduz hastalığının rutin teşhisinde, adı geçen konvansiyonel testler karşısında çabukluğu, sensitivitesinin yüksekliği ve güvenilirliği ve kısa sürede sonuç alma gibi nedenlerden dolayı, moleküler teşhis metotlarından PCR ve real-time RT-PCR testlerinin daha çok tercih edildiği görülmektedir [15,17,18]. Bu çalışmada, rutin kuduz teşhisinde, saha şartlarında laboratuara ulaştırılan 75 numunenin tamamına tek tüp real-time RT-PCR testi uygulanmış ve 3-4 saat gibi kısa bir sürede sonuçlar alınmıştır.

Doku kültüründe virüs izolasyonu testi ve MIT test sonuçları ile real-time RT-PCR testi sonuçlarının karşılaştırıldığı bir çalışmada [17] real-time RT-PCR testinin daha duyarlı olduğu tespit edilmiş ve alt yapısı sağlanmış laboratuvarlarda real-time RT-PCR testinin kuduz teşhisinde klasik testleri tamamlayacak bir yaklaşım olacağını belirtilmiştir. Dantas ve ark.[13] tarafından yapılan bir çalışmada da, attan elde edilmiş bir beyin numunesinde FAT ile negatif sonuç alınmasına rağmen, MIT ve RT-PCR testinde pozitif sonuç alındığı bildirilmektedir. Çalışmamızda, FAT ile negatif bulunan Diyarbakır Dicle'den gelen bir köpek beyni numunesi, MIT ve tek tüp real-time RT-PCR testlerinde pozitif bulunmuştur. Çalışmamızda elde edilen bu bulgu, Dantas ve ark.'ları [13] tarafından yapılan çalışmadan elde edilen sonuçla benzerlik göstermektedir. Real-time RT-PCR ile MIT'in karşılaştırıldığı Wakeley ve ark.[1] yaptığı başka bir çalışmada da, pozitif numunenin 1/128 sulandırmasında FAT testi ile pozitiflik bulunamadığı halde, real time RT-PCR ve MIT testlerinde pozitiflik bulgusu elde edilmiştir. Düşük konsantrasyonda antijen bulunan numunelerde FAT testi ile sonuç alınamayan durumlarda, real-time RT-PCR testi ile sonuç alınmıştır. Bu nedenle araştırıcılar, kuduz hastalığının hızlı, doğru ve güvenilir teşhisi için, real-time RT-PCR testinin yararlı ve duyarlı bir metot olarak kuduz teşhis laboratuvarlarında rutinde kullanılabileceğini belirtmişlerdir [19,20].

Klasik kuduz virüsünün teşhisi için real-time RT-PCR testi optimizasyonu sağlamak için Hoffmann ve ark.[9] tarafından yapılan bir araştırmada, labratuvar standartları sağlandıktan sonra klasik kuduz virüsünü teşhis etmek

için, RT-PCR testinin ve özelliklede real-time TaqMan RT- PCR testinin otoliz olmuş numunelerde teşhis amaçlı kullanılabileceği gibi tam potansiyel doğrulayıcı teşhis metodu olarak da kuduz teşhis laboratuvarlarında kullanılabileceğini önermektedirler. Bu çalışmamızda da, otoliz olmuş şekilde laboratuarımıza ulaşmış 3 adet köpek numunesine FAT ve MIT testi uygulanamazken, yapılan tek tüp real-time RT-PCR testi sonucunda da bu numunelerin birinde pozitif sonuç elde edilmiştir.

Sonuç olarak, evcil ve yabani hayvanlarda görülen kuduz hastalığı, Türkiye'de toplum sağlığını ciddi manada tehdit etmektedir. Kuduz hastalığında hızlı ve doğru teşhisi, şüpheli hayvanlarla temas sonucu [10,11] oluşabilecek hastalık riskini azaltma açısından da önemlidir. Saha şartlarında değişik iklim ve coğrafik şartlara bağlı olarak bazan otoliz olmuş olarak laboratuvara ulaşan beyin numunelerine OIE ve WHO'nin önerdiği FAT ve MIT testleri uygulanamamaktadır. Bu çalışma ile altyapısı kurulmuş laboratuvarlarda tek tüp real-time RT-PCR testinin kuduz hastalığının hızlı ve güvenilir teşhisinde rutinde kullanılabileceği, FAT ve MIT testlerinin yapılmasına uygun olmayan kokuşmuş numunelerde uygulanabileceği, böylece oluşabilecek belirsizliğin ortadan kalkmasının sağlanacağı ve sonuçlarının MIT testi ile %100 uyum göstermesinden dolayı FAT testi sonuçlarını doğrulamak için alternatif doğrulayıcı bir test olarak rutinde kullanılabileceğinin daha güvenilir olacağı sonucuna varılmıştır.

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Kafkas Univ Vet Fak Derg
20 (4): 583-590, 2014
DOI: 10.9775/kvfd.2014.10459

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effects of Nonylphenol on Motion Kinetics, Acrosome and Mitochondrial Membrane Potential in Frozen-Thawed Bull Sperm [1]

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Makale Kodu (Article Code): KVFD-2014-10459

Summary

Recent reports have shown that the Nonylphenol (NP), a well-known toxic environmental chemical has adverse effects on sperm in different species. Therefore, the current study was aimed to determine the deleterious effect of NP on frozen-thawed bull sperm. Sperm were exposed to different concentrations of NP (1, 10, 100, 250 and 500 µg NP/mL) dissolved either in DMSO or Ethanol and incubated for 4 h at 37°C. Sperm parameters were assessed at 0, 1st, 2nd, 3rd, and 4th h of incubation. Both percent motility and mitochondrial membrane potential (MMP) of sperm have decreased significantly ($P<0.05$) at dose level of ≥ 250 µg NP/mL. Acrosome reaction was induced ($P<0.05$) even at minimal dose concentrations of 1µg NP/mL ($P<0.05$). However, the percentage of acrosome reacted sperm increased ($P<0.05$) with increase in dose level. In conclusion, NP impairs the sperm characteristics by adversely affecting motion kinetics, mitochondrial membrane potential and inducing premature acrosome reaction in bovine.

Keywords: *Acrosome, Bull sperm, DNA, Flow cytometry, Mitochondria, Nonylphenol*

Nonylphenol'ün Dondurulmuş-Çözdürülmüş Boğa Sperma Hareket Kinetiğine, Akrozom ve Mitokondrial Membran Potansiyeline Etkisi

Özet

Son çalışmalar çevre toksini olarak bilinen Nonylpfenol (NP)'in, değişik türlerin sperm parametreleri üzerine zararlı etkisinin olduğunu göstermiştir. Bu çalışma NP'in dondurulmuş-çözdürülmüş boğa spermaası üzerine etkisinin tespit edilmesi amacı ile yapılmıştır. Sperma örnekleri Ethanol veya DMSO içerinde değişik yoğunlukta (1, 10, 100, 250 ve 500 µg/ml) çözdürülen NP ile 4 saat 37°C'de inkübe edildi. Sperm parametreleri inkübasyonun 0., 1., 2., 3. ve 4. saatlerinde ölçüldü. Hem motilite ve hem de mitokondrial membran potansiyeli 250 µg NP/ml ≥ dozlara sahip gruplarda önemli derecede düşmüştür ($P<0.05$). Minimal doz olan 1µg/ml NP'de akrozom reaksiyonunu indüklenmiştir ($P<0.05$), ancak dozun artmasına bağlı olarak akrozom reaksiyonu geçirmiş sperm oranında da artışlar olduğu tespit edilmiştir ($P<0.05$). Sonuç olarak NP; spermatozoa hareket kinetiği, mitokondrial membran potansiyeli ve akrozom reaksiyonu üzerine negatif etki yaparak sperma parametrelerine zarar verdiği saptanmıştır.

Anahtar sözcükler: *Akrozom, Boğa spermaası, DNA, Flow sitometri, Mitokondri, Nonylphenol,*

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INTRODUCTION

There has been a serious concern in the industrial world that many living organisms on earth, from lower vertebrates to mammals, are likely to be directly or indirectly exposed to environmental chemicals such as pesticides and other industrial chemicals used in livestock production [1-3]. Among these industrial chemicals alkyl-phenolpolyethoxylates (APEs), also called estrogenic environmental endocrine disrupters, are widely used as non-ionic surfactants and anti-oxidants in detergents, herbicides, pesticides, paints, plastic ware, emulsifiers [4] and intra vaginal spermicides [5]. About 650.000 metric tons of APEs is produced in a year around the world and it has been shown that 60% are accumulated in the streams, rivers, lakes, and seas [6]. APEs undergo biodegradation processes to give short side chain derivatives such as nonylphenol (NP), octylphenol (OP) and butylphenol (BP) in anaerobic conditions in water [7]. NP, one of the most abundant derivatives of APEs, has been demonstrated to stay in a biologically active state in the body longer natural estrogen [8]. NP has an estrogenic activity posing a potential danger to living organisms in various ways. For example, nonoxynol-9 consisting NP is used as an intravaginal spermicide and condom lubricant [3]. When administered to lab animals it is metabolized to free NP inhibiting testicular development and decreasing sperm motility, viability and sperm count [9-16].

The estrogenic effects of chemicals might emerge by (1) mimicking the effect of endogenous hormones, (2) antagonizing the effect of endogenous hormones, (3) disrupting the synthesis and metabolism of endogenous hormones or (4) disrupt the synthesis and metabolism of hormone receptors [3]. To date, the adverse effects of NP on many different species including mice [14,17], rats [13,18], quails [19] and aquatic species including fish, and sea urchins [1,10,11,20-23] have been reported. Cattle are the most widely raised livestock and bull sperm is used for artificial insemination worldwide. Thus, determining the effect of environmental pollutants such as NP on sperm is important for bulls' reproductive health.

Concentrations of NP in air, soil and water are not lethal to living organisms. However NP have been reported to accumulate in especially fat tissue up to 750 folds more than its environmental concentrations [20,21,24-26]. Numerous studies have shown that APEs, especially NP, is found in foodstuffs including fresh fruits vegetables and rice [6,27,28,29], human milk [30-32] aquatic as well as livestock products [22,23]. Kawaguchi et al.[33] demonstrated that feedstuffs used in animal nutrition might contain about 200 µg NP/kg. Thus APEs particularly NP may pose serious threat on the health, reproduction and fertility of terrestrial organisms through the intake of NP in foodstuffs. Therefore, the current study was aimed to determine the deleterious effect of NP on motion kinetics, acrosome reaction and mitochondrial membrane potential of frozen-thawed bull sperm.

MATERIAL and METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

Sperm Samples

Frozen sperm samples from Holstein bulls were thawed and diluted in HEPES buffered Tyrode's lactate (TL-HEPES) solution (~290 mOsm and pH 7.4) containing 3 mg/mL bovine serum albumin (BSA). For each treatment eight straws of frozen sperm samples were thawed and pooled in a 10 ml tube. Sperm samples were washed twice by centrifugation (275 x g) for 15 min each time, re-suspended in culture medium to 100 x 10^6 sperm/mL, and held at 20-25°C for not more than 1 h before incubations were initiated. Initial motility was determined by a computer-assisted sperm motility analysis (CASA) system (Hamilton Thorne Biosciences, Inc., M2030, Beverly, MA). Sperm samples having 80% or higher motility were used. The experiment was replicated three times.

Sperm Incubation with NP and Motility Assessment

To determine the effects of NP on sperm parameters in treatment groups, two different solvents, namely ethanol (ETOH) and dimethyl sulfoxide (DMSO), were used to dissolve and deliver NP (4-Nonylephenol with 99% purity) to the sperm within the medium. Both solvents were also tested alone for their ability to alter sperm quality. Aliquots of 0.5 mL with concentration of 100x10^6 sperm/mL were transferred in one set of 12x55 mm polypropylene tubes containing 0 (control), 1% ETOH (solvent control), 1 µg 17β-estradiol /mL, and 1, 10, 100, 250 and 500 NP/mL. Aliquots of 0.5 mL with concentration of 100x10^6 sperm/mL were transferred to another set of 12x55 mm propylene tubes containing 0 (control), 1% DMSO (solvent control), 1 µg 17β-estradiol /mL, and 1, 10, 100, 250 and 500 µg NP/mL. NP and 17β-estradiol used in the previous set were dissolved in ethanol, while NP and 17β-estradiol used in the latter set were dissolved in DMSO. While attaining the above final concentrations of NP or 17β-estradiol, neither ETOH nor DMSO exceeded the amount of 1%. Therefore, either 1% ETOH or DMSO was also added in the previous and latter set, respectively. All samples were then incubated for 4hr at 37ºC and 5% CO_2. At the end of incubation time point 1, 2, 3 and 4 h, sperm motility characteristics (*i.e.* total motility, progressive motility and average path velocity; VAP) were assessed and recorded for each treatment using CASA. Briefly, Aliquots of 10 µl bull sperm suspension were put onto a pre-warmed 10 µm deep Makler Counting Chamber (Sefi-Medical Instruments, Haifa, Israel) and sperm motility characteristics were determined by the

CASA system at 37°C. At least 6 fields were counted and the measurements were replicated 2 times for each donor. Minimums of about 200 cells were analyzed in 6 fields. Motility estimates were validated manually by the video playback option of the instrument. The setting parameters of measured sperm motion parameters for the CASA were: frames per second; the duration of the tracking time (1 sec), average path velocity cutoff (50 µm/sec) and low VAP cutoff (20 µm/sec).

Fluorescent Microscopic Evaluation of Acrosomal Integrity

At the end of 4 h exposure of bull sperm to various concentrations (0, 1, 10, 100, 250, and 500 µg/mL dissolved NP in either DMSO or ETOH), aliquots' of 3 µl samples from each treatment were smeared onto microscope slides to determine the effects of NP on the acrosomal status of bull sperm. Epifluorescent microscopy was used to assess acrosomal integrity after staining with Alexa Fluor-488-PNA (peanut agglutinin) conjugate (catalog no: L-21409, Molecular Probes, Eugene, OR, USA). The treated and control samples were smeared onto microscope slides and air-dried. The specimens were fixed with 99% methanol and kept at room temperature until fluorescence staining. For staining, slides were incubated with 20 µg/mL Alexa Fluor-488-PNA at 37°C for 30 min, washed with PBS, and analyzed by epifluorescent microscopy (Zeiss Axiophot, Germany, using a appropriate fluorescence filter). The images of stained sperm samples were classified into 2 groups: Sperm displaying strong and moderate bright fluorescence in the acrosomal region were considered to be intact, whereas sperm displaying weak, patchy, or no fluorescence in the acrosomal region were considered to be damaged. 100 sperm on each slide were evaluated to determine the proportion of sperm with intact acrosomes [13].

Flow Cytometric Analysis of Mitochondrial Membrane Potential

The lipophilic cationic probe JC-1 (Molecular Probes, Eugene, OR, USA) was used to assess the mitochondrial status of the sperm before and after treatments following the manufacturer's guidelines. Essentially, the fluorescence of JC-1 changes reversibly from green (monomeric status to orange (multimeric status) when mitochondrial membrane potential is high [34]. The sperm samples were exposed to NP for different time points (1 and 4 h) and concentrations 0 (control), 1, 10, 100, 250, and 500 µg/mL. The samples were then diluted with TL-HEPES and 300 µl were transferred to 12x55 mm polypropylene tubes. 3 µl of JC-1 stock solution (2 µM JC-1 in DMSO) were added and the tubes were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air for 30 min. At the end of each incubation period, the stained samples were brought to the Flow Cytometry Core Facility for analysis using a FACS analyzer flow cytometry (FACSCalibur, Becton-Dickinson, and San Hose CA). The generated data were examined using Cell Quest software (version 3.3) to determine the cells with high mitochondrial membrane potential (orange stained) using the appropriate gating parameters. Ten thousand spermatozoa from each treatment were used in the evaluation of mitochondrial membrane potential.

Flow Cytometric Analysis of Chromatin Integrity

The sperm samples were exposed to NP for different time points (1 and 4 h) and concentrations 0 (control), 1, 10, 100, 250, and 500 µg/mL. We used the exact method that was previously described for mouse [35], bull, deer and dog sperm [36] to assess chromatin stability using metachromatic staining with Acridine Orange (AO) (In vitrogene, Molecular Probes, Eugene, OR, USA). Essentially, AO dye is known to fluorescence in green when incorporated into intact double stranded DNA helix, and in red when incorporated into denatured DNA. Briefly, samples were diluted with TNE buffer (pH 7.4) into polypropylene tubes at a final sperm concentration of $30x10^6$ cells/mL. For analysis, samples kept in an ice water bath at 4°C were mixed with 400 µl of an acid-detergent solution (pH 1.4). Exactly 30 sec later, 1.20 mL of acridine orange staining solution (pH 6.0, 4°C) containing 6 µg/mL purified AO was added. The stained samples were analyzed at 30 min after AO staining. Samples were then analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson Immunochemistry Systems; San Jose, CA, USA), equipped with standard optics and an argon laser tuned at 488 µm. The generated data were examined using Cell Quest software (version 3.3) to determine the cells with damaged chromatin using the appropriate gating parameters. Ten thousands spermatozoa from each treatment used in the evaluation of chromatin status.

Statistical Analysis

The data were analyzed by analysis of variance. MINITAB was used to analyze the data. The time effect, treatment effect, and their interaction were determined. Tukey's test was used to compare means using pair-wise comparison. For all statistical tests, α-value of 0.05 and 0.01 were chosen as the level of significance.

RESULTS

Bull sperm motility values (mean±SEM) after exposure to different concentrations of NP dissolved in either ethanol or in DMSO at the end of each incubation period are shown in *Table 1* and *Table 2*, respectively. The initial motility the sperm samples was 90±3%. Treatment of the sperm samples with1 µg 17β-estradiol/mL dissolved in ETOH or DMSO for 3 or 4 h did not have any effect on bull sperm motility ($P>0.05$). Sperm motility was not significantly affected ($P>0.05$) by 1 µg to 10 µg NP/mL concentrations. However, there was a significant decrease ($P<0.05$) in the sperm motility in 100, 250, and 500 µg NP/

Table 1. *Effects NP and 17β-E_2 diluted in ETOH on the motility of bull sperm*

Tablo 1. *ETOH içerisinde çözdürülen NP ve 17β-E_2'nin boğa sperma motilitesi üzerine etkisi*

Treatment	n	Percent Motility (mean±SEM)			
		1 h	2 h	3 h	4 h
Control	3	$90\pm1^{a,*}$	$85\pm2^{a,*}$	$76\pm6^{a,*}$	$79\pm8^{a,*}$
ETOH	3	$91\pm2^{a,*}$	$83\pm3^{a,*}$	$78\pm6^{a,*}$	$74\pm8^{a,*}$
17β- Estradiol	3	$91\pm1^{a,*}$	$81\pm6^{a,*}$	$74\pm9^{a,*}$	$73\pm10^{a,*}$
1 µg NP/mL	3	$93\pm2^{a,*}$	$92\pm1^{a,*}$	$86\pm3^{a,*}$	$83\pm5^{a,*}$
10 µg NP/mL	3	$92\pm1^{a,*}$	$86\pm2^{a,*}$	$78\pm5^{a,*}$	$81\pm4^{a,*}$
100 µg NP/mL	3	$80\pm5^{a,*}$	$69\pm8^{a,*}$	$65\pm9^{a,*}$	$51\pm8^{a,**}$
250 µg NP/mL	3	$49\pm3^{a,**}$	$50\pm5^{a,**}$	$33\pm7^{a,**}$	$12\pm4^{a,***}$
500 µg NP/mL	3	$9\pm4^{a,***}$	$4\pm2^{a,***}$	$3\pm1^{a,***}$	$3\pm1^{a,****}$

*^{a,b} Different letters indicate significance in rows at (P<0.05); * Numbers indicate significance in columns at (P<0.05)*

Table 3. *Progressive motility and velocity values of bull sperm that were subjected to different NP concentrations dissolved in ETOH*

Tablo 3. *ETOH içerisinde çözdürülen değişik konsantrasyonlardaki NP'nin, boğa sperması prograsiv motilite ve hızına etkisi*

Treatment	n	Progressive Motility (%) (means ± SEM)		Velocity (VAP) mic/sec (means ± SEM)	
		1 h	4 h	1 h	4 h
Control	3	70 ± 7^{a}	54 ± 1^{ab}	105 ± 6^{a}	105 ± 5^{a}
ETOH	3	59 ± 3^{a}	62 ± 10^{a}	98 ± 5^{ab}	103 ± 13^{a}
17β- Estradiol	3	61 ± 6^{a}	51 ± 4^{ab}	97 ± 2^{ab}	79 ± 7^{b}
1 µg NP/mL	3	66 ± 7^{a}	58 ± 2^{a}	97 ± 3^{ab}	92 ± 6^{ab}
10 µg NP/mL	3	63 ± 9^{a}	55 ± 2^{ab}	98 ± 3^{ab}	86 ± 5^{ab}
100 µg NP/mL	3	53 ± 1^{ab}	38 ± 6^{b}	90 ± 1^{ab}	87 ± 4^{ab}
250 µg NP/mL	3	4 ± 1^{c}	0±0	39 ± 4^{c}	0±0
500 µg NP/mL	3	2 ± 2^{c}	0±0	40 ± 6^{c}	0±0

^{a,c} Different letters indicates significance at P<0.05

Table 2. *Effects NP and 17β-E_2 diluted in DMSO on the motility of bull sperm*

Tablo 2. *DMSO içerisinde çözdürülen NP ve 17β-E_2'nin boğa sperma motilitesi üzerine etkisi*

Treatment	n	Percent Motility (mean±SEM)			
		1 h	2 h	3 h	4 h
Control	3	$91\pm1^{a,*}$	$85\pm3^{a,*}$	$84\pm3^{a,*}$	$90\pm2^{a,*}$
DMSO	3	$92\pm1^{a,*}$	$83\pm3^{a,*}$	$80\pm4^{a,*}$	$77\pm7^{a,*}$
17β- Estradiol	3	$85\pm6^{a,*}$	$81\pm5^{a,*}$	$87\pm1^{a,*}$	$79\pm5^{a,*}$
1 µg NP/mL	3	$87\pm3^{a,*}$	$83\pm3^{a,*}$	$83\pm4^{a,*}$	$86\pm2^{a,*}$
10 µg NP/mL	3	$92\pm4^{a,*}$	$89\pm2^{a,*}$	$89\pm1^{a,*}$	$82\pm3^{a,*}$
100 µg NP/mL	3	$87\pm4^{a,*}$	$88\pm1^{a,*}$	$86\pm3^{a,*}$	$75\pm6^{a,*}$
250 µg NP/mL	3	$56\pm4^{a,**}$	$53\pm5^{a,**}$	$53\pm6^{a,**}$	$47\pm9^{a,**}$
500 µg NP/mL	3	$14\pm1^{a,***}$	$19\pm3^{a,***}$	$9\pm6^{a,***}$	$3\pm2^{a,***}$

*^{a,b} Different letters indicate significance in rows at (P<0.05); * Numbers indicate significance in columns at (P<0.05)*

Table 4. *Percent progressive motility and velocity values of bull sperm that were subjected to different NP concentrations dissolved in DMSO*

Tablo 4. *DMSO ile çözdürülen değişik konsantrasyonlardaki NP'nin, boğa sperması prograsiv motilite ve hızına etkisi*

Treatment	n	Progressive Motility (%) (means ± SEM)		Velocity (VAP) mic/sec (means ± SEM)	
		1 h	4 h	1 h	4 h
Control	3	65 ± 5^{a}	63 ± 5^{a}	108 ± 4^{a}	97 ± 1^{a}
DMSO	3	63 ± 8^{a}	53 ± 5^{ab}	105 ± 2^{a}	91 ± 2^{ab}
17β- Estradiol	3	58 ± 4^{ab}	50 ± 4^{ab}	96 ± 3^{ab}	99 ± 7^{ab}
1 µg NP/mL	3	54 ± 2^{ab}	61 ± 2^{a}	103 ± 1^{a}	96 ± 9^{a}
10 µg NP/mL	3	57 ± 5^{ab}	55 ± 6^{ab}	84 ± 9^{ab}	105 ± 2^{a}
100 µg NP/mL	3	60 ± 2^{ab}	53 ± 6^{ab}	104 ± 7^{a}	102 ± 5^{a}
250 µg NP/mL	3	46 ± 7^{abc}	29 ± 6^{bcd}	99 ± 7^{ab}	86 ± 9^{ab}
500 µg NP/mL	3	33 ± 15^{bcd}	25 ± 16^{bcd}	82 ± 10^{b}	41 ± 26^{c}

^{a,d} Different letters indicates significance at P<0.05

mL treatment at the end of all incubation time periods (*Table 1* and *Table 2*) in comparison to non-treated control. As shown in *Table 2*, bull sperm motility values was not significantly affected by 1, 10, and 100 µg NP/mL dissolved in DMSO (P>0.05), while it was significantly affected by 250 and 500 µg NP/mL dissolved in DMSO treatments (P<0.05). The progressive motility and velocity values in response to different concentrations are shown in *Table 3* and *Table 4*. Although there are gradual decreases in both progressive motility and velocity values in a dose and time dependent manner, significant decrease occur in concentrations over 100 µg NP/mL, especially in dissolved ETOH NP treated groups (P<0.05).

Treatment of sperm samples with 1 µg NP/mL dissolved in ETOH did not significantly affect the rate of acrosome defect *(Fig. 1)* in comparison to control (P>0.05), while 1 µg NP/mL dissolved in DMSO significantly increased the rate of acrosome defect in sperm samples *(Fig. 2)* compared to the control (P<0.05). Estradiol dissolved in both ETOH and DMSO as well as higher concentration of NP including 10, 100, 250, and 500 µg NP/mL dissolved either in ETOH or DMSO significantly induced bull sperm AR in comparison to both non-treated control and solvent control groups (P<0.05) (*Fig. 1* and *Fig. 2*).

Flow cytometric dot plots of bull sperm stained with JC-1 followed by exposure to different concentrations of NP dissolved in either ETOH or DMSO are shown in *Fig. 3*. This experiment was carried out for 1 or 4 h. MMP was significantly (*P*<0.05) reduced by incubation of bull sperm with 250 and 500 µg/mL NP dissolved in either ETOH *(Fig. 4)* or DMSO *(Fig. 5)*. Although it is not significant, the NP induced decrease in MMP appear to be higher in NP dissolved in ETOH group than NP dissolved in DMSO.

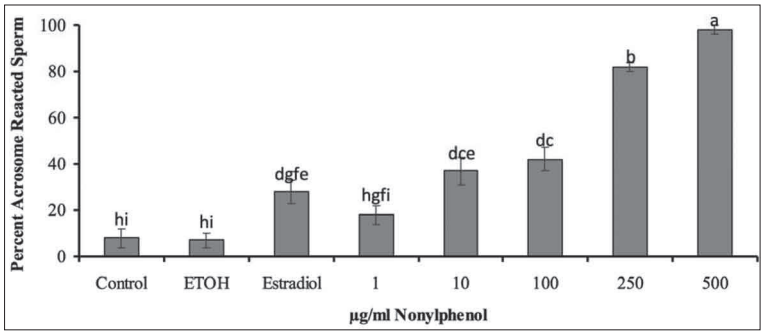
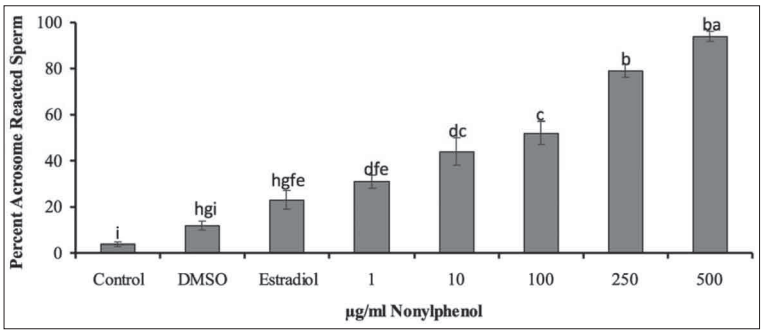


Fig 1. Effects of various concentrations of NP dissolved in ethanol on acrosomal integrity of epididymal bull sperm (P<0.05; different letters indicate significance, n =5)

Şekil 1. Ethanol içerisinde değişik konsantrasyonlarda hazırlanan NP'nin, boğa sperması akrozom bütünlüğüne etkisi (P<0.05; farklı harfler istatistiki farkı belirtir, n =5)

Fig 2. Effects of various concentrations of NP dissolved in DMSO on acrosomal integrity of epididymal bull sperm (P<0.05; different letters indicate significance, n =5)

Şekil 2. DMSO içerisinde değişik konsantrasyonlarda hazırlanan NP'nin, boğa sperması akrozom bütünlüğüne etkisi (P<0.05; farklı harfler istatistiki farkı belirtir, n =5)



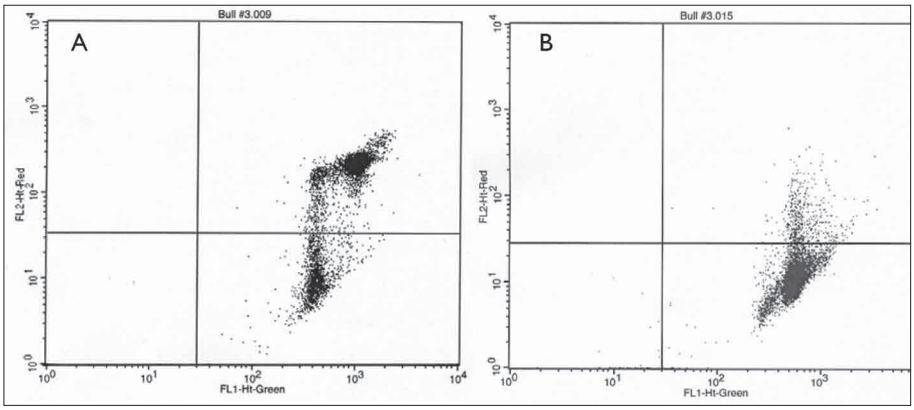


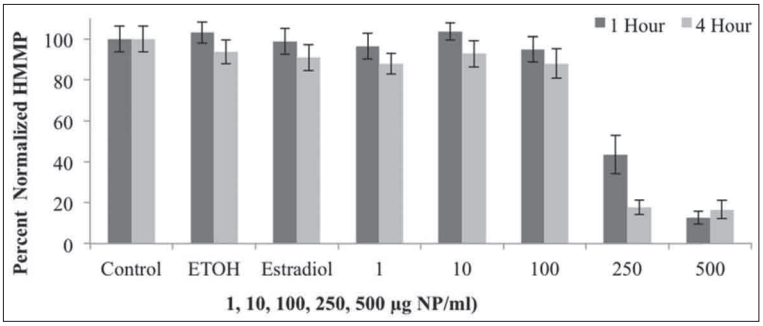
Fig 3. Representative flow cytometric dot plots to illustrate the effects of NP dissolved in ETOH or DMSO on bull sperm. Sperm cells in control or solvent control groups are shown in Panel A, while panel B shows the effects of 250 and 500 µg NP/ml dissolved in ETOH or DMSO on sperm cells

Şekil 3. ETOH ve DMSO içerisinde çözdürülen NP'nin, boğa sperması üzerine etkisinin flow sitometrik analiz ile gösterimi. Kontrol ve solvent kontrol grupları panel A'da gösterilmiştir. Panel B ise, ETOH veya DMSO içerisinde çözdürülen 250 ve 500 µg/ml oranlarındaki NP'nin, sperma üzerine etkisini göstermektedir

Fig 4. The effects of various concentrations of NP dissolved in ethanol on mitochondrial membrane potential of bull sperm (*P<0.05 vs. untreated control). HMMP: high mitochondrial membrane potential

1 h: 100% = 65.71, 4 h: 100% 63.38

Şekil 4. ETOH içerisinde değişik konsantrasyonlarda hazırlanan NP'nin, boğa sperması mitokondrial membran potansiyeline etkisi (*P<0.05, işlem uygulanmamış grup). HMMP: yüksek mitokondrial membran potansiyeli



In this experiment, we did not observe any NP induced impairment (P>0.05) of chromatin integrity of bull sperm regardless of NP concentration and duration of NP exposure based on AO staining and subsequent flow cytometric analysis (n=3).

DISCUSSION

Alkylphenolpolyethoxylates (APEs) are known estrogenic environmental endocrine disrupter. One of the derivatives of APEs called NP has been shown to be widely found in fisheries and livestock products [14,29]. It has also been reported to be present in drink water and in foods [4,6,27]. Mothers consuming fish three times had NP in their milk with an amount of 32 ng/mL [29]. Therefore, it has been a serious concern for the world that many living organisms from lower vertebrates to mammals are likely to be directly or indirectly exposed to

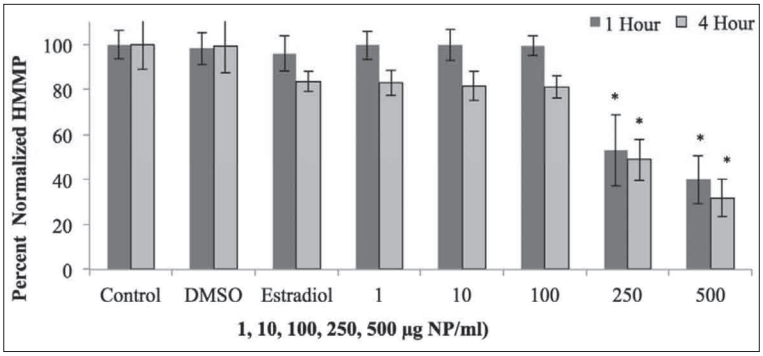


Fig 5. Effects of different concentrations of NP dissolved in DMSO on mitochondrial membrane potential of bull sperm (*P<0.05 vs. untreated control: n=3). HMMP: high mitochondrial membrane potential

1 h: 65.71, 4 h: 63.38

Şekil 5. DMSO içerisinde, değişik konsantrasyonlarda hazırlanan NP'nin, boğa sperması mitokondrial membran potansiyeline etkisi (*P<0.05, işlem uygulanmamış grup; n=3) HMMP: yüksek mitokondrial membran potansiyeli

various endocrine-disrupting substances present in the environment.

These contaminants could cause a number of reproductive health problems in exposed organisms including human, livestocks and aquatic species [1,2]. In vivo estrogenic activity of NP is much more potent than that of in vitro estrogenic activity of NP [37]. Exposure of the general population to NP is unavoidable because of the presence of the agent in the environment [17]. The effects of NP on livestocks have been described such as disruption in testicular development, decrease in sperm motility, viability, decrease in sperm count which eventually led to the decrease in male fertility [12-14,38]. Adverse effects of NP on mice [14,17], rat [13,18] and aquatic species [1,10,11,23,39] have also been reported.

Lukac et al.[16] reported that NP causes significant decrease in the viability and motility of bull sperm. Our results are in parallel to these findings. Indeed, motility analysis is most commonly used to evaluate semen quality. However, this assay alone is not enough to determine the fertilizing capacity of semen samples. Therefore, we examined the effects of NP on acrosome reaction and mitochondrial membrane potential in addition to sperm motion kinetics. It is possible to simultaneously measure multiple sperm parameters, including cell viability, acrosomal integrity and mitochondrial function. All these evaluation parameters help us to accurately describe the ability of spermatozoon fertilization capacity [40].

The present study showed that the motility of bull sperm was significantly (P<0.05) affected when they were exposed to ≥250 µg NP/mL solved in ETOH (*Table 1* and *Table 2*) or DMSO. As shown in *Table 3*, when sperm samples were exposed to NP in the presence of ETOH, the progressive motility and velocity of sperm were significantly decreased after treatment with 250 µg NP/mL (P<0.05). These results are in accordance with results obtained by Lukac et al.[16] in terms of NP mediated decrease in motility of bull sperm. However, we observed the NP mediated inhibition at a much higher concentration than used by Lukac et al.[16]. Inhibition of bull sperm motility by NP correlated with a decrease in the MMP (*Table 1, Table 2,* and *Fig. 3*).

Estrogens play an important role in the development and regulation of the male reproductive system. Non-nuclear receptors for estradiol are present on sperm plasma membrane [41]. It is well known that E2 and environmental estrogens can significantly stimulate mammalian sperm capacitation and acrosome reactions [37,41]. This research showed that NP-induced alterations in acrosomal status appeared to be one of the most sensitive indicators of NP exposure and significant acrosomal reaction of bull sperm started at NP exposures ≥1 µg NP/mL solved both in ETOH and DMSO. In addition, although motility parameters and mitochondrial integrity of sperm were affected at NP concentrations as low as 100 NP µg/mL and 250 µg NP/mL, respectively; acrosomal integrity was significantly reduced at even 1 µg NP/mL (*Fig. 1* and *Fig. 2*). These results support the sensitivity of acrosomal status as an indicator of toxic insult to sperm. This study on bull sperm is in agreement with previous studies which showed NP-induced sperm capacitation and subsequent acrosomal reaction in human and mouse sperm due to increase in intracellular level of cAMP [37,42]. Also, Adeoya-Osiguwa et al.[37] reported that E2 and environmental estrogens such as genistein (Gen), 8- prenylnaringenin (8-PN) and nonylphenol (NP) significantly affect the function of mature sperm by stimulating capacitation and fertilizing ability, compared with untreated controls. NP mediated capacitation or acrosome reaction is not considered an advantage for successful fertilization. On the contrary, it may be a disadvantage for sperm to fertilize an egg since it may cause premature acrosome reaction impairing sperm ability to reach the egg.

Sperm motility depends on mitochondrial function and thus sperm having adequate mitochondrial integrity (*i.e.,* high MMP) should have robust motility. Previous studies suggested that MMP of bovine sperm determined by JC-1 staining correlated well with their progressive motility [43]. Uguz et al.[13] reported that NP has adverse effects on progressive sperm motility and MMP in rats. In the present study, MMP of bull sperm responded to exposures to various concentrations of NP in a similar fashion as progressive motility. This may suggest that mitochondrial damage during chemical exposure could be one of the major reasons for reduced sperm motility. Bull sperm with normal mitochondrial integrity significantly declined

following exposure to 250 and 500 µg/NP. The amount of sperm with high MMP correlated well with the sperm having high percentages of progressive motility. This suggests that NP causes a decrease in motility by causing decrease in MMP in both rat and bull sperm. However, there are some differences in terms of NP-mediated damage between bull and rat sperm. Rat perm motility is more susceptible to NP exposure than that of bull sperm. For example, over 250 µg NP/mL concentrations have lethal effect on rat sperm [13]. The present study showed that 500 µg NP/mL concentrations were not lethal to bull sperm. This indicates that although NP has toxic effects on sperm, this effect is dose and time dependent in different species.

Although there was a dose-dependent adverse effect of NP on motility and mitochondrial and acrosomal integrity in bull, this study did not reveal any adverse effect of NP on sperm chromatin integrity of either species, up to 4 h of exposure in vitro. Han et al.[44] reported that the rats receiving NP by gavage at 250 mg/kg/day showed decrease in epididymal sperm density as well as the serum testosterone level. It has also been shown that the number of apoptotic cells in seminiferous tubules of treated rats was increased in a dose dependent manner. Similarly, Dobrzyńska [17] reported that eight-week exposure to NP and X-ray/NP combination reduced sperm counts while it increased the percent of abnormal spermatozoa and DNA damage in gametes in mice. Karadeniz et al.[45] reported that increasing concentration of NP dissolved in ethanol cause guanine oxidation in DNA determined by carbon nano-tube electrode. It has been suggested that base oxidation may lead to mutation in DNA [46]. However, Bennetts et al.[47] reported that 17β-estradiol, NP or Bisphenol A treated human spermatozoa did not show any DNA damage. It has also been shown that NP does not cause any damage in rat sperm DNA [13,48]. The results of the study confirm the findings of the above studies [13,47,48] that NP did not cause any damage in bull sperm DNA. Mammalian spermatogenesis is a dynamic process, during which the structure of sperm chromatin undergoes extensive alterations during the male germ cell development. These alterations are facilitated by a series of meiotic and mitotic events and the addition of protamines, ultimately leading to formation of tightly packed sperm chromatin [49]. Therefore, it is likely that mature sperm DNA are more resistant against mutagens than that of those early male germ cells (*i.e.* spermatogonia) [35].

NP is a lipophilic substance; therefore, it does not dissolve in water [24]. Dimethyl sulfoxide (DMSO) or ethanol (ETOH) is required to dissolve NP. In the present study, the solvents alone or in combination with NP were also tested for their ability to alter the effects of NP on sperm quality. NP appeared to be more detrimental when dissolved in ETOH than when dissolved in DMSO. For instance, bull sperm progressive motility was very low after exposure to 250 and 500 µg NP/mL in ETOH compared to DMSO (*Table 1* and *Table 2*). As expected, the corresponding velocity values also showed a similar pattern as determined for progressive motility (*Table 3* and *Table 4*). The velocity of sperm was significantly reduced after exposure to 500 µg NP/mL in DMSO, while the same effect on velocity was observed after exposure to 250 µg NP/mL solved in ETOH ($P<0.05$). The reduction in total motility was only significant after exposures to 250 and 500 µg NP/mL ($P<0.05$). The observed differences between NP dissolved in ETOH and that dissolved in DMSO may depend on solubility or bioavailability effects and ETOH-associated chemical alteration of NP into a more toxic compound. In conclusion, we have shown that NP alone is capable of inducing damage in sperm and that ETOH enhances the damaging effects of NP on bull sperm. However, the assessment of only one or two sperm parameters might not exactly predict the full effect of a xenobiotic on the fertility. For example, as mentioned above, although motility assessment is a powerful tool for evaluating fertility, chemical exposure can also cause impairment to other cellular elements, such as the acrosome or mitochondria which may result in fertilization failure or abnormal embryonic development. Although bull sperm is more resistant against NP mediated damage, the sperm parameters evaluated in this study showed that NP significantly impairs bull sperm function that may severely lower the fertilizing capacity of bull sperm.

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Kafkas Univ Vet Fak Derg
20 (4): 591-597, 2014
DOI: 10.9775/kvfd.2014.10583

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

The Effect of Silicone Tube and Silicone Tube + Hyaluronic Acid Application on Adhesion Formation in Experimental Peri- and Epi-neurorrhaphy in A Rat Model [1] [2]

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[1] *This study was supported by Research Fund of Kafkas University (Project No: KAÜ-BAP-2011-VF-13)*

[2] *This study was presented in "13th National Veterinary Surgery Congress (with international participation), June 27th- July 1st, 2012 in Sarıkamış, Kars - Turkey" as an oral presentation and in "Second International Neural Regeneration Symposium (INRS2012), 21st-25th September 2012, Shenyang - China" as a poster presentation*

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Makale Kodu (Article Code): KVFD-2014-10583

Summary

Following neurorrhaphy in Wistar albino rats with experimental sciatic nerve cut, the effectiveness of solely silicone tube (ST) and ST plus hyaluronic acid application on preventing fibrosis was clinically and histopathologically examined. After a total nerve cut is created in sciatic nerve, interfascicular and epineural anastomosis was used. While only anastomosis was applied for the first group (Control Group), for the second group (ST Group) anastomosis + silicone tube and for the third group (ST + HA Group) anastomosis + silicone tube + hyaluronic acid (HA) were applied. Animals in each group were divided into 2 sub-groups and macroscopic and histopathological examinations were conducted on the 30th and 60th postoperative day. On day 30 of the study all the animals had problematic walks. On the 60th day while animals in groups ST and ST + HA were walking normally, the problem was still going on for the control group. In the postmortem macroscopic examinations performed in the control group on the 30th and 60th days an irregular morphology and adhesion to surrounding in nerve tissue were seen. Whereas in group ST, in the anastomosis line within the tube, scar tissue which was clearer on the 60th day was observed, in group ST + HA it was seen that nerve anastomosis line was smooth on the 30th and 60th days. As a consequence, the reduction in myelin thickness and the increase in degenerated myelin for groups ST and ST + HA in 30 day show that HA does not create a positive effect on axon regeneration in the short run, on the other hand, the reduction in myelin degenerated along with the increase of axon myelin thickness and axon cross section areas in groups ST and ST + HA in day 60 has shown that silicone tube and HA application creates a positive impact on myelination in the long run.

Keywords: *Sciatic nerve, Neurorrhaphy, Hyaluronic acid, Silicone tube, Rat*

Deneysel Peri ve Epinöral Nörorafi Uygulanmış Rat Modellerinde Silikon Tüp ve Silikon Tüp + Hyaluronik Asit Uygulamasının Adezyon Formasyonuna Etkisi

Özet

Bu çalışmada deneysel siyatik sinir kesisi oluşturulan Wistar albino ratlarda nörorafiyi izleyerek, ilgili alana sadece silikon tüp (ST) ve ST ile birlikte hyaluronik asit (HA) uygulamasının fibrozisi engellemedeki etkinliği klinik ve histopatolojik olarak incelendi. Siyatik sinirde total kesi oluşturulduktan sonra interfasiküler ve epinöral anastomoz uygulandı. Birinci grupta (Kontrol Grubu) sadece anostomoz yapılırken, ikinci grubta (ST Grubu) anastomoz + silikon tüp, üçüncü grubta ise (ST + HA Grubu) anastomoz + silikon tüp + Hyaluronik asit (HA) uygulandı. Her bir gruptaki hayvanlar 2 alt gruba ayrılarak postoperatif 30 ve 60. günlerde makroskopik ve histopatolojik incelemeler yapıldı. Çalışmanın 30. gününde tüm hayvanların yürüyüşleri problemliydi. 60. günde ST ve ST + HA grubundaki hayvanlarda yürüyüş normal iken kontrol grubunda problem devam etmekteydi. Prostmortem makroskopik bakıda 30 ve 60. günlerde kontrol grubunda sinir dokuda düzensiz bir morfoloji ve çevreye yapışıklık mevcuttu. ST Grubunda 60. günde daha belirgin olmak üzere tüp içerisinde anastomoz hattında skar doku ile karşılaşılırken ST + HA grubunda 30 ve 60. günlerde sinir anastomoz hattının pürüzsüz bir görünümde olduğu saptandı. Sonuç olarak, 30 günde ST ve ST + HA gruplarında miyelin kalınlığının azalması ve miyelin dejenerasyonun artması HA'in kısa dönemde akson rejenerasyonu için pozitif bir etki oluşturmadığını, buna karşın 60. günde ST ve ST + HA gruplarında aksonların miyelin kalınlığı ve akson alanlarının arttırmasıyla birlikte miyelin dejenerasyonun azalması, silikon tüp ve HA'in uzun dönemde miyelinizasyon üzerinde pozitif bir etki oluşturduğunu göstermiştir.

Anahtar sözcükler: *Siyatik sinir, Nörorafi, Hyaluronik asit, Silikon tüp, Rat*

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INTRODUCTION

Peripheral nerve injury is a very common problem that medical doctors and veterinary physicians come across. These lesions develop as simple nerve rupture, ruptures that form with tissue loss and nerve laceration [1]. Common causes of nerve traumas can be listed as traffic accidents, falling down from great heights, compression, injuries by firearms, sharp object injuries, bite injuries, damages caused by fractured bone fragments, muscle tendon ruptures which appear depending upon overextension in extremities and simultaneous neurological disorders, iatrogenic reasons (false injections, intraoperative mistakes etc.) etc. [1,2].

Success rate in peripheral nerve regeneration depends on better understanding of factors that have an impact on recovery and elimination of negative factors. Regeneration rate for motor nerves is 80% and for mixed nerves 50-60% under suitable conditions. Age, diameter and length of nerve and material and technique practiced effect healing rate [3].

In ruptures where there is no neural tissue loss, by following trimmed of ruptured ends, epineural repair which is applied with solely epineural suture or interfascicular anastomosis is commonly used [1,4]. However, there are significant problems which appear during the recovery period such as intraneural fibrosis, perineural adhesion and neuroma formation that effect nerve healing in a negative way [1-5]. Extra and intraneural fibrosis forms compression on nerves along with vascular formations. Fibrosis and adhesion assume a role which induces new neurological disorders [1,3,6].

In order to make the desired recovery possible materials such as various carbohydrate polymer gels, antioxidant agents, antineoplastic preparations and corticosteroids were studied to determine their effectiveness in preventing adhesion. In addition, various organic and synthetic membranes or tube practices have been used so as to prevent adhesion to surrounding tissues and eliminate mechanical pressures and extensions on nerves [1-4,6-11].

HA is a non-sulfate glycosaminoglycan. Thanks to its viscoelastic characteristics, it has been used for many years primarily for the repair of muscle-tendon-ligament ruptures and in fields such as ophthalmology and oto-rhino-laryngology in order to prevent adhesion by eliminating fibrosis [6,12-14]. HA shows its viscoelastic characteristic acting as a barrier by forming a layer similar to film between tissues repaired and veining. Therefore this impact enables the repairing of main tissue cells directly without experiencing the inflammatory phase in which operated tissue is repaired by fibroblasts [14]. HA shows a long term impact on the tissue it is practiced by preserving its integrity for 4-5 weeks [12-14]. HA was used in combination with some gels [5] and in another study [2] by being injected into perineural area in the field of neurochirurgia.

The purpose of this study is to clinically, macroscopically and histopathologically examine the effects of only silicone tube and silicone tube plus hyaluronic acid-which is a non-sulfate glycosaminoglycan- (Na-Hyaluronate, HA) application on fibrosis following perineural and epineural neurorrhaphy in rats with experimental sciatic nerve cut.

MATERIAL and METHODS

Animals and Experimental Groups

This research was conducted after the approval of Kafkas University Animal Testing Local Ethics Council (Approval Number: KAÜ-HADYEK-2010/19-40).

Animal material of the study included 48 male adult Wistar albino rats, each weighing 250-300 g. Rats were kept under standard laboratory conditions, 12 h in darkness and 12 h in daylight, at average 20-22°C constant temperature. Animals were fed with standard rat feed and water was given ad libitum.

Rats were allocated into 3 groups containing 16 rats each.

Group I (Control Group): Only nerve cut and suture was applied.

Group II (Silicon Tube Group) (Group ST): Nerve cut, suture and silicone tube application which surrounded neurorrhaphy area were practiced.

Group III (Silicone Tube + Hyaluronic Acid Group) (Group ST + HA): Upon nerve cut and suture and intraneural silicone tube application which surrounded neurorrhaphy area approximately 0.3 ml perineural HA was applied.

Anesthesia and Experimental Procedure

For neurorrhaphy, 10/0 atraumatic polygylcolic acid suture, operation microscope, silicone tube, hyaluronic acid, routine soft tissue set; for histopathological cut and examinations routine equipments and consumables; for postoperative pain and infection control, suitable analgesic and antibiotics were used.

Upon following necessary preparations for surgical operations in all three groups, 5 minutes after intra-peritoneal injection of 5 mg/kg Xylazine HCl (Rompun® 2%, 50 ml, Bayer) and 40 mg/kg Ketamine HCl (Ketasol 10% enj, 10 ml, Richter Pharma) through anesthesia workstation (Veterinary Anesthesia WorkStation, Hallowell® EMC USA) and a suitable sized laryngeal mask with equivalent of 1 MAC isoflurane (1.4%, inspiration concentration) general anesthesia was started and went on by the end of operations. Sciatic nerve was exposed by routine surgical procedure and upon a total cut is made with bistoury

interfascicular and epineural anastomosis was practiced with 10/0 polygylcolic acid (PGA) (Ethicon; Johnson & Johnson, Somerville, NJ, USA).

In group I, surgical opening was bridged routinely and no process was applied other than this. In group II, after each nerve ending was suitably freed upon nerve cut, following neurorhapphy, silicone tube which was passed through one of the endings was placed by suture in a way that centers anastomosis line. In group III, with the same method, firstly silicone tube was passed through one of the nerve endings, secondly after neurorhapphy it was placed into the nerve tissue in stitching area and following neurorhapphy placed in a way to surround anastomosis line and then some (approximately 0.3 ml) HA (Hylartin® V, Pfizer) that could fill the tube was applied. In these groups, once again routine methods were used to close surgical opening.

Necessary processes were carried out for post operative pain and infection control and suitable care-nutrition conditions were provided for animals.

Animals in all groups were divided into 2 equal sub-groups (n: 8). Clinical assessments on postoperative 30th and 60th days were carried out and animals were decapitated under general anesthesia. Postmortem macroscopic and histomorphological examinations were conducted.

Histological Analysis

- Tissue Processing

On the 30th and 60th postoperative day, under general anesthesia, the distal blocks of sciatic nerves were removed from the nerves after electrophysiological assessment of the nerve. The nerves were stretched to *in situ* lengthwise by pinning them onto a card and then fixing them with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 4-6 h in 4°C. Following fixation, the tissues were rinsed in phosphate buffer (pH 7.4) two times. Specismens were postfixed in 1% osmium tetroxide for 2 h, dehydreted in ascending alcohol series and took into propylene oxside for 16 min. These prosedurs were completed by embeding the tissue 48 h in Epon embeding kit (Fluka Gmbh, Swithzerland). For embedding, we used a silicon embedding mold that has 21 consecutively numbered, bullet-shaped cavities with a depth of 5 mm each. Semi-thin sections of 1 µm thickness were cut by an ultra microtome (Super Nova Reichert- Yung, Austria) and stained with 1% toluidine blue.

- Stereological Analysis

Stereological analyses of sciatic nerves were conducted according to principles described previously [15,16]. A stereological workstation composed of a digital camera (mbf/Bioscience, Qimaging), otomatic controlled specimen stage, a light microscope (Leica, DM400B) and a software program (mbf/Bioscience, Stereo investigator, version 9) was used to count axons. To obtain an estimation of total myelinated axon number in an unbiased manner, the axon profiles in the nerve cross-section are sampled with equal probability regardless of shape, size, orientation and location that means each sampled item was selected with a systematic random manner [17]. For this aim, we used an area fraction approach. In the application, area of unbiased counting frame was 900 µm^2. Meander sampling of each sectioned nerve profiles was done in 70 µm x 70 µm step size in a systematic-random manner. This ensures that all locations within a nerve cross-section were equally represented and that all axon profiles were sampled with an equal probability regardless of shape, size, orientation and location [17-19].

The same stereological workstation was also used for stereological analyses of myelin thickness and axon cross-sectional area. A two-dimensional isotropic uniform random nucleator [20,21] was used for estimation of cross-sectional axon area and the thickness of myelin sheet using an oil objective (100x, NA 1.40). Meander sampling of each sectioned nerve profiles for axon cross-section area and myelin sheet thickness was done over successive, systemic-random steps of 70 µm-70 µm. Two dimensional nucleator at isotropic uniform random positions was used for estimation of axonal areas and the thickness of myelin sheet using an oil objective (100x, NA 1.40).

Statistical Analysis

Six rats were used for each experimental group. "n" which is used for statistical analysis is the number of animals. Non-parametric tests were used in statistical analysis. For the comparison of groups of two, Mann-Whitney-U test was used while Kruskal Wallis test was preferred for the comparison of groups of three. Statistical difference is important on $P<0.05$ level.

RESULTS

Clinical Findings

Animals' individual locomotion on a suitable platform before experiment ~~was~~ terminated was monitored in each group, findings were as follows: on 30th day, locomotion in group ST and ST + HA were problematic while there was no problem in groups ST and ST + HA on 60th day. The locomotion problem still existed in the control group.

Postmortem Macroscopic Findings

During postmortem macroscopic examinations on the 30th and 60th days, an irregular morphology and adhesion to surrounding were observed in the control group. When tissue growths similar to fibrosis mass in the anastomosis line within the tube, which was clearer on the 60th day in

groups ST, in group ST + HA it was observed that on the 30th day, HA in the tube preserved its transparent structure and it was still present on the 60th day. In this group it was observed that nerve anastomosis line was smooth and bright on the 30th and 60th days.

Histomorphological Findings

Upon histomorphological examination of nerve tissues which belonged to a 30 day period, reduction in myelin thickness though more apparent in groups ST and ST + HA and increase in myelin degenerated was observed. When data from 60 day subgroups for groups ST and ST + HA were compared to 30 day data, it was seen that there was an increase in axons myelin thickness and axon cross section areas and a decrease in myelin degenerated *(Fig. 1, 2, 3, 4* and *5)*.

DISCUSSION

Different types of biological and artificial grafts have been studied in comparison with autologous nerve grafts with regards to nerve regeneration and functional healing lately and important developments have been made. The tubulization is a good alternative for peripheral nerve reconstruction [22].

Three different tubulization methods which are biological, synthetic and combined are used for peripheral neurosurgery. In cases when nerve spaces are shorter than 30 mm, effectiveness of vein grafts was confirmed by experimental and clinical studies [18,23]. When nerve spaces are longer than 30 mm, in order for a successful nerve regeneration vein tubes and other tissues like nerve parts and skeletal muscle suture are used together in some studies and resulted in a better functional feedback [18,24,25]. In addition, use of silicone tubes for nerve spaces shorter than 5 mm has been reported to lead to a successful nerve regeneration [18,26]. In this study solely ST and ST plus HA which is a viscoelastic material were used with the purpose of a functional healing.

With silicone nerve guidance conduits (NGCs) which are used for nerve repair, silicone tube is filled with serum within the first couple of hours as blood vessels are cut. Inflammatory cells such as serum macrophages contain some cytokine, neuropathic factors and fibrin. Fibrin creates axon regeneration by forming two nerve ends on a longitudinal direction as a fibrin cable bridge. Fibrin bridge structure is a critical step towards healing [27]. Besides, Schwann cells and macrophages excrete mitogens which have an impact on neuropathic (growth) factors starting remyelination with axonal regeneration. Both cells express

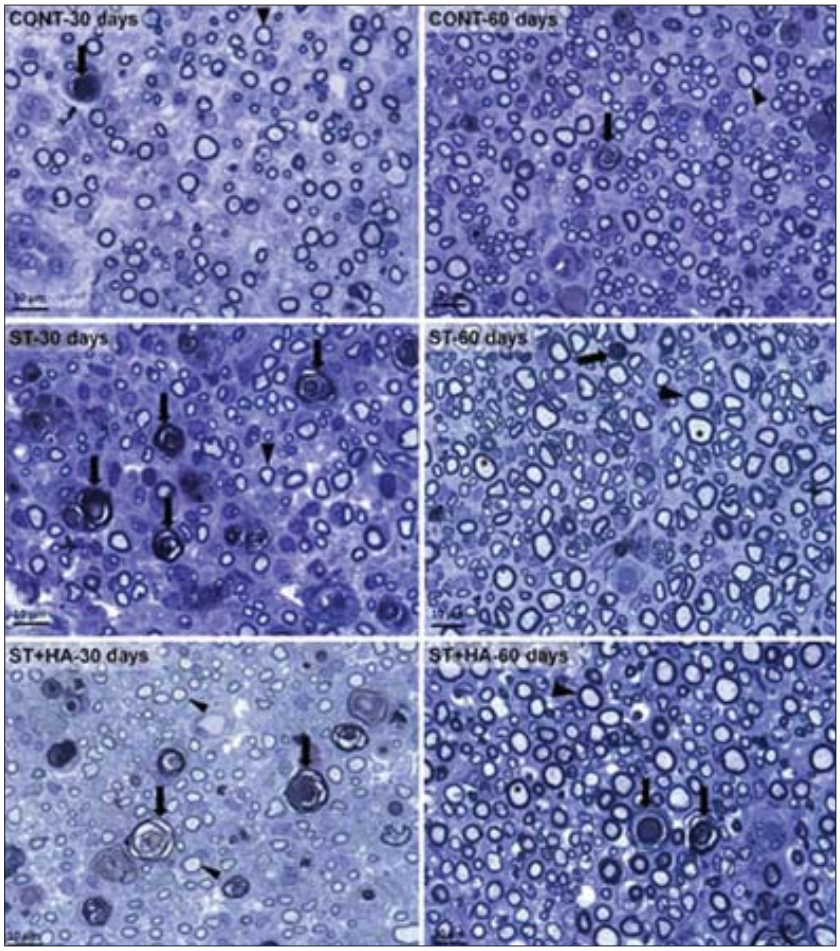


Fig 1. Light microscopic image of myelin degenerated, myelin thickness and axon cross section areas in Control group, ST and ST+HA groups (myelin degenerated in 30 day groups is more intense in groups ST and ST+HA compared to control group *(arrows)*. Myelin thickness is less in groups ST+HA than control group in the same period *(arrowheads)*. Myelin thickness and axon cross section areas are more in groups ST and ST+HA of 60 days than of 30 days (asterisk) and the number of myelin degenerated axons are less compared to 30 day animals (Scale bar: 10 µm)

Şekil 1. Kontrol, ST ve ST+HA gruplarında myelin dejenerasyonu, myelik kalınlıkları ve akson alanlarının ışık mikroskobik görünümü

(30 günlük gruplarda miyelin dejenerasyonu ST ve ST+HA gruplarında kontrol grubuna göre daha yoğundur *(Oklar)*. Aynı dönemde kontrol grubuna göre ST+HA grubunda miyelin kalınlıkları daha incedir *(Ok başları)*. 30 günlüklere göre 60 günlük hayvanların ST ve ST+HA gruplarında miyelin kalınlıkları ve akson alanları daha fazladır *(asteriks)* ve dejenere olmuş miyelinli aksonlar daha azdır) (Skala bar: 10 µm)

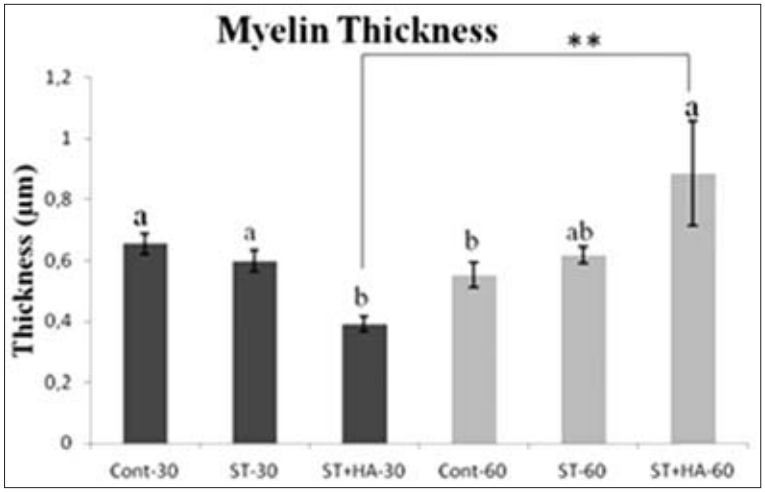


Fig 2. Myelin thickness

30 day groups (Cont-30, ST-30, ST+HA-30 ** P<0.01), 60 day groups (Cont-60, ST-60, ST+HA-60 * P<0.05), (Cont-30, Cont-60 ** P<0.01), (Average ± SEM)

Şekil 2. Miyelin kalınlığı

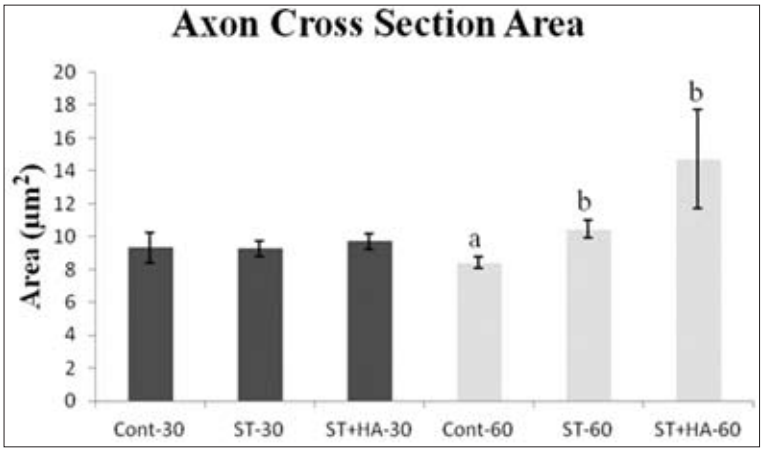
30 günlük gruplarda (Cont-30, ST-30, ST+HA-30 ** P<0.01), 60 günlük gruplarda (Cont-60, ST-60, ST+HA-60 * P<0.05), (Cont-30, Cont-60 **P<0.01), (Ortalama ± SEM)

Fig 3. Axon cross section area

30 day groups (Cont-30, ST-30, ST+HA-30, P>0.05), 60 day groups (Cont-60, ST-60, ST+HA-60, * P<0.05), (Average ± SEM)

Şekil 3. Akson alanı

30 günlük gruplarda (Cont-30, ST-30, ST+HA-30, P>0.05), 60 günlük gruplarda (Cont-60, ST-60, ST+HA-60, * P<0.05), (Ortalama ± SEM)



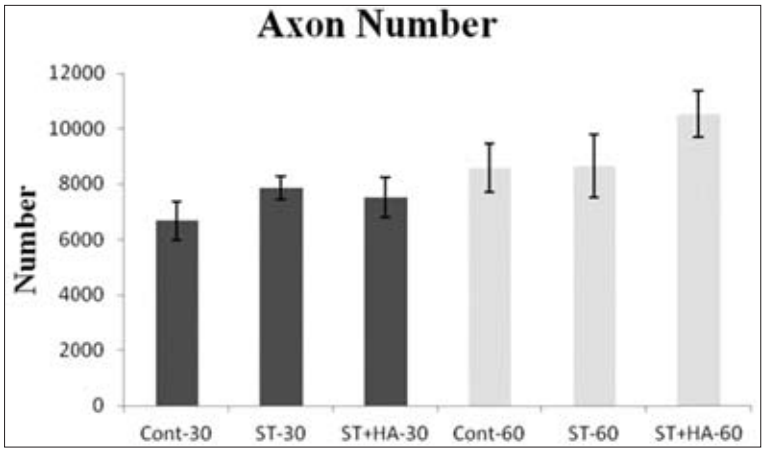


Fig 4. Axon number

30 day groups (Cont-30, ST-30, ST+HA-30, P>0.05) and 60 day groups (Cont-60, ST-60, ST+HA-60, * P<0.05) (Average ± SEM)

Şekil 4. Akson sayısı

30 günlük gruplarda (Cont-30, ST-30, ST+HA-30, P>0.05) ve 60 günlük gruplarda (Cont-60, ST-60, ST+HA-60, * P<0.05) (Ortalama ± SEM)

anti-inflammatoire cytokines such as interleukin (IL)-10 which represses the inflammatory process staring in Wallerian degeneration [28]. Schwann cells have shown to form a regeneration tube by forming long cell bands also known as Bungnerin Bands which make axon proceed along with axon growth. While Schwaan cells regenerate to form long cell bands, macrophages and monocytes migrate to degenerated nerve bruise to eliminate myelin and axon debris [28-30].

Substances that are not biodegradable stay where they are after nerve degeneration like foreign substances. Therefore, they cause a chronic foreign substance reaction with excessive scar tissue structure and in the end they are reported to prevent nerve function recovery [30,31]. After peripheral nerves are cut in a latitudinal way a set of molecular and cellular events named as Wallerian degeneration are triggered in the distal damaged zone [30,32].

In this study we observed that silicone tube applied after nerve damage reduced myelin thickness in the control group for 30 day animals, on the other hand, for

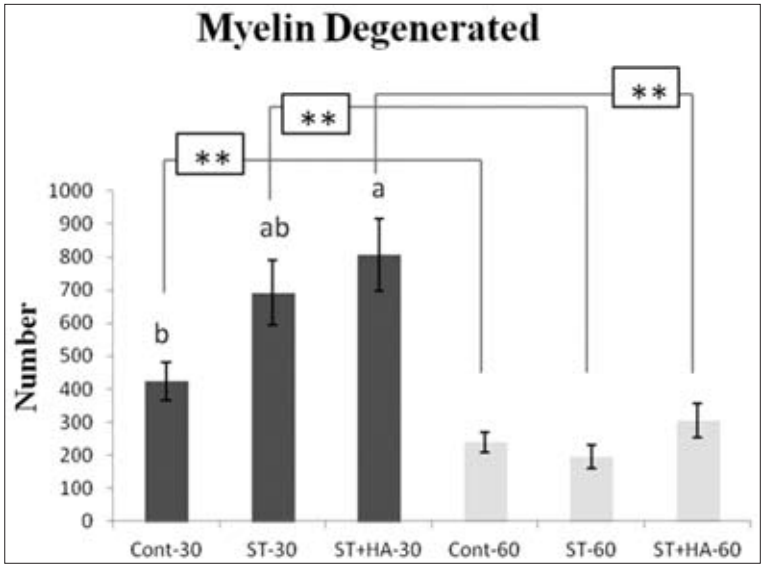


Fig 5. Myelin degenerated

30 day groups (Cont-30, ST-30, ST+HA-30 * P<0.05), 60 day groups (Cont-60, ST-60, ST+HA-60, P>0.05), (Cont-30, Cont-60, ** P<0.01), (ST-30, ST-60, ** P<0.01), (ST+HA-30, ST+HA-60, ** P<0.01). (Average ± SEM)

Şekil 5. Miyelin dejenerasyonu

30 günlük gruplarda (Cont-30, ST-30, ST+HA-30 * P<0.05), 60 günlük gruplarda (Cont-60, ST-60, ST+HA-60, P>0.05), (Cont-30, Cont-60, ** P<0.01), (ST-30, ST-60, ** P<0.01), (ST+HA-30, ST+HA-60, ** P<0.01). (Ortalama ± SEM)

60 day animals with an opposite impact, myelin thickness increased with the axon cross section area. This may suggest that negative impact of short term silicone tube application on recovery as mentioned in literature [30,31] may stem from foreign substance effect it creates on that area. High myelin degeneration in 30 day ST and ST + HA groups proves that there is Wallerian degeneration in distal zone as mentioned in literature [30,32].

Neuropathic factors and extracellular matrix molecules (ECM) are produced by Schwann cells for successful axon regeneration. With the help of these factors, it is stated that a regenerated unit surrounded by a basal lamina on the proximal part of nerves that are latitudally cut begin to sprout. Newly sprouting axons generally spread from nodes of Ranvier and become remyelinated by Schwann cells [30,33,34].

Hyaluronic acid is naturally composed of glucuronic acid and linear polysaccharides which are formed by recurring disaccharide units composed of N-acetylglucosamine and it plays an important role for tissue repair [30,35]. Superficial Hyaluronan gel application has been determined to prevent perineural scar structure and increase peripheral nerve regeneration [30,36]. Because of its characteristic that organize fibrin, HA may create axonal ingrowth and pathway for cells in the acellular fibrin matrix phase of peripheral nerve regeneration [32,37].

Certain studies [35,38] conducted by various materials combined with hyaluronic acid have shown a significant rise in axon number.

In this study, though silicone tube and ST+HA application upon nerve damage in 30 and 60 day groups did not cause a statistical difference compared to 30 day control group, the rise in numerical values showed that new axon sprouts developed as stated by certain studies [30,33,34]. The rate of increase stated by different researches [35,38] has not been detected in our study. However, our results are compatible with the results of various researches [30,32,35,36] which stated HA's positive impact on recovery.

In summary, the reduction in myelin thickness and the rise in myelin degenerated in silicone tube and hyaluronic acid groups of 30 day animals shows that hyaluronic acid does not have a positive impact on axon regeneration phases in the short run. In contrast, when silicone tube and hyaluronic acid groups of 60 day animals are compared to 30 day animals, the fact that axons increase myelin thickness and axon cross section areas and the decrease in myelin degenerated may shows that silicone tube and hyaluronic acid have a positive impact on myelination in the long run.

ST + HA application has contributed to early functional strength of extremities and created a proper walking. There has been a smooth recovery without fibrosis in the nerve tissue with macroscopic operation.

HA still preserved its presence in silicone tube in both groups (30 day and 60 day SA + HA groups), which was interpreted as a sign that as viscoelastic material it had very strong physical characteristics.

The decrease in myelin thickness and increase in myelin degenerated in ST and ST + HA groups of 30 day animals has shown that HA does not have a positive impact on axon regeneration in the short run, the rise in myelin thickness and axon cross section areas and the decrease in myelin degenerated in 60 day animals has shown that ST and HA have a positive impact on myelination in the long run.

However, it can be said that multi dimensional experimental studies which provide the following are required: a- formation of a model that makes it possible for HA to hold on to the area in larger volumes and that enables to repeat HA injection periodically, b- thus, examination of

clinical and microscopic table in future phases of recovery by keeping time of experiment longer, c- determination of the most suitable application method by detecting HA's effect of inhibiting scar formation in scored measurements, d- recovery table is pointed out by functional measurement (biomechanical and electrophysiological tests).

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Kafkas Univ Vet Fak Derg
20 (4): 599-604, 2014
DOI: 10.9775/kvfd.2014.10678

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effects of Single or Combined Dietary Supplementation of Boric acid and Plant Extract Mixture on Egg Production, Egg Quality and Blood Cholesterolemia in Laying Hens

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Makale Kodu (Article Code): KVFD-2014-10678

Summary

This experiment was conducted to determine the effects of boric acid (BA) (60 mg/kg of feed) and plant extract mixture (Fitococci, F) (0.75 g/kg of feed), individually or in combination, on egg production, egg quality parameters and blood cholesterol concentration of laying hens in 23 wk of age. After wk 7 of the trial the level of boric acid in feeds were increased to 180 mg/kg. The feeding period lasted 14 wk. One hundred ninety-two pullets (Hyline) were randomly divided into control and 3 treatment groups each containing 48 birds and 6 replicates (each with 8 birds). At the end of the experiment there were no significant effects of dietary boric acid and plant extract mixture inclusion on feed efficiency, egg weights and egg production, egg quality parameters (shell thickness, breaking strength, and shape index) compared with control. But feed intake of hens which were supplemented with plant extract mixture and boric acid were increased ($P<0.01$). It was also determined that there were no effects of treatments on blood cholesterol levels. As a result, since hens which were fed with basal diet plus plant extract mixture showed better feed conversion ratio at 9-10 wks, this combination of feed additives can be added to diets without any adverse effect on laying hens. But inclusion of 60 mg/kg boric acid is considered better than 180 mg/kg with regard to feed efficiency in laying hens.

Keywords: *Boron, plant extract mixture, Laying hen, Egg production, Egg quality, Cholesterolemia*

Yumurtacı Tavuk Rasyonlarına İlave Edilen Borik Asit ve/veya Bitki Ekstraktı Karışımının Yumurta Verimi ve Yumurta Kalitesi ile Kan Kolesterolü Üzerine Etkileri

Özet

Bu deneme borik asit (BA) (60 mg/kg yem) ve bitki ekstraktı karışımının (Fitococci, F) (0.75 g/kg yem) tek başlarına ya da birlikte kullanıldıkları zaman 23 haftalık yumurta tavuklarında yumurta verimi, yumurta kalite parametreleri ve kan kolesterol düzeylerine etkisini belirlemek amacıyla yapılmıştır. Denemenin 7. haftasının ardından yemlerdeki borik asit düzeyleri 180 mg/kg'a yükseltilmiştir. Yemleme dönemi 14 hafta sürmüştür. 192 adet yarka (Hyline) rastgele kontrol ve 3 deneme grubuna ayrılmış olup bu grupların her biri 6 tekrarlı 48 hayvandan oluşmaktadır (her bir tekrar 8 hayvandan oluşmaktadır). Denemenin sonunda rasyonlara borik asit ve bitki ekstraktı karışımı ilave edilen deneme gruplarında kontrol grubuna göre yemden yararlanma, yumurta ağırlık ve verimleri, yumurta kalite parametreleri (kabuk kalınlığı, kırılma direnci ve şekil indeksi) bakımından önemli etki görülmemiştir. Ancak bitki ekstraktı ve borik asit karışımıyla beslenen deneme grubunda yem tüketimi önemli düzeyde artmıştır ($P<0.01$). Ayrıca rasyona borik asit ve bitkisel ekstrakt karışımı ilavesinin kan kolesterol düzeylerine istatistiki önemde etki etmediği tespit edilmiştir. Sonuç olarak, bitki ekstraktı karışımıyla beslenen tavuklarda yemden yararlanma oranının 9-10. haftalarda daha iyi olmasından dolayı, bu yem katkı maddelerinin karışımı yumurta tavuğu rasyonlarına olumsuz etki göstermeksizin ilave edilebilir. Ancak yumurta tavuklarında 60 mg/kg borik asit ilavesi 180 mg/kg borik asit ilavesine oranla yemden yararlanma açısından daha iyi sonuç ortaya koymaktadır.

Anahtar sözcükler: *Bitki ekstraktı karışımı, Bor, Kolesterol, Yumurta kalitesi, Yumurtacı tavuk, Yumurta verimi*

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INTRODUCTION

Plant extracts and herbs have been used for thousands of years by many cultures to enhance the flavour and aroma of foods. Scientific experiments since the late 19th century have documented the anti-microbial properties of some spices, herbs and their components [1]. Supplementation of feed additive (0.75 g/kg Fitococci) had no significant effects about egg weights, egg quality parameters and serum cholesterol whereas it had negative effects (P<0.05) on egg production and feed efficiency compare with the control [2]. Even though usage of plant extract in laying hen diets like as feed additive is already known subject addition of boron/boric acids into rations is substantially new issue in this area.

Boron is primarily obtained from boron mines, located in arid regions of Turkey [3]. Boric acid based on biochemistry of boron. Kurtoglu et al.[4] showed that boron provided significant improvements on serum Ca levels and damaged egg ratio but no significant difference between the controls and the boron supplemented groups (50, 100, 150, 200 and 250 ppm) about feed consumption, feed conversion ratio, egg production, body weight and egg weight. Eren et al.[5] found that similar findings about B addition to laying hen diets at level of 400 ppm resulted negative values of live weight, feed consumption and egg productivity parameters.

This experiment was conducted to determine the effects of boric acid and plant extract mixture supplementation on egg production, egg quality, and serum cholesterol in laying hens.

Table 1. *Basal diet composition and analyzed results of feed ingredient for laying hens diets*
Tablo 1. *Bazal rasyonun bileşimi ve yem ham maddesi analiz sonuçları*

Ingredients	Percentage
Corn	40.00
Wheat	21.37
Soybean meal	15.20
Sun flower meal	5.00
Soybean, full fat	6.00
Oil	1.69
Limestone	8.28
Dicalcium phosphate	1.60
Common salt	0.35
DL-Methionine	0.24
DL-Treonine	0.06
Antioxidant	0.05
Vitamin and mineral premix*	0.16
Analyzed Results	
Crude protein (%)	16.60
ME (kcal/kg)**	2789
Calcium (%)	3.60
Phosphor (%)	0.50

** Supplied the following per kilogram/ton of diet:.Retinol (vit A), 12.000 IU; cholecalciferol, 8.000 IU; α-tocopherol acetate (vit E), 50 g; menadione (vit K_3), 10 g; thiamin (vit B_1), 4 g; riboflavin (vit B_2), 8 g; pyridoxine (vit B_6), 5 g; cyanocobalamin (vit B_{12}), 25 g; niacin, 50 g; cal. panthotenic acid, 20 g; folic acid, 20 g; biotin (vit H_2) 250 g; ascorbic acid (vit C) 75 g; choline cloride 175 g; Manganese (Mn), 100 g; zinc (Zn), 150 g; iron (Fe), 100 g; copper (Cu), 20 g; iodine (I)), 1.5 g; chobald (Co) 0.5 g; selenium (Se), 0.2 g; molybdenum (Mo), 1 g and magnesium (Mg), 50 g, ** The formula recommended by TSE (Turkish Standards Institute)* [6]

MATERIAL and METHODS

Layers, Experimental Design and Diet

This research was carried out on 23 wks old, totally 192 laying hens (Hyline White 98). The birds were randomly divided into 4 groups according to the dietary regimen and each group was constituted by 6 subgroups of 8 birds. The experimental protocol was approved by the local Ethics Committee of the Ankara University. In the control group, chickens were fed with basal standard diets based on corn and soybean meal, more specifically with a layer diets [16.5% crude proteins (CP) and 2750 kcal/kg metabolisable energy (ME)] for all experiment period (for 14 wks, *Table 1*). Birds from the groups 2 and 3 received standard diets supplemented with boric acid (BA) (60 ppm) or Fitococci (F) (0.75 g/kg) respectively whereas in the group 4, hens were supplemented with both of these additives (FBA) (60 ppm boric acid plus 0.75 g/kg humate). The dosage of boric acid from wk 8 to end of the trial was increased. Feeds were analyzed for nutrients *(Table 1)* according to the reference methods [7]. During the experiment feed and water were given to hens ad libitum. The birds were housed in battery cages with automatic drinker design during the 14 wks experimental period. The temperature maintained at 20-22°C according to normal management practice. Chicks were maintained on a 17 hours constant light schedule until the end of the experiment.

Plant extract mixture (Fitococci®) includes Origanum vulgare (thyme), Thymus vulgaris (oregano), thyme oil, garlic oil, anise oil and fennel oil. Active ingredients: 1,8-Cineole (0.24%), Allicine (0.24%), Alliine (0.12%), Alpha-Pinene (0.12%), Alpha-Terpineol (0.70%), Borneol (0.18%), Caffeic-Acid (2.28%), Camphene (0.08%), Carvacrol (4.48%), Eugenol (0.12%), Geraniol (1.04%), Limonene (0.56%), Linalool (0.96%), Myrcene (0.18%), P-Cymene (2.38%), Phenol (0.86%), Polyphenol (6.00%), Tannin (12.9%), Rosmarinic-Acid (7.60%), Terpinen-4-Ol (0.06%), Ursolic Acid (1.92%), Tymol (3.26%).

Performance Analysis

During the experimental period, the egg production of the hens was evaluated by daily recording whereas feed consumption recording weekly. Egg samples were collected, stored (at room temperature for one day) and

weighed biweekly. Feed conversion ratio was calculated as kilograms of feed consumed per kilogram of egg produced. An additional sample of 6 eggs was randomly collected from each experimental group every 14 days to assess egg quality parameters which were shape index, breaking strength and shell thickness.

Biochemical Analysis

At the end of the trial, blood samples (5 mL) were obtained from each one bird from every cage by the brachial vein for determine blood cholesterol level of hens. These samples were allowed to clot at room temperature for 6 hours and then they were centrifuged at 1500 g for 10 min at room temperature. Serum were carefully harvested and stored at -20°C until analysis. The serum cholesterol concentrations were analyzed using commercial kits (Teco Diagnostic, 1286 Anaheim, CA 92807).

Statistical Analysis

All data were analyzed by ANOVA using SPSS 11.50 program (Inc., Chicago, II, USA). Significant differences among treatment were determined using Duncan's multiple range tests [8] with a 5% level of probability.

RESULTS

Table 2 shows the result of dietary boric acid and plant extract mixture on feed consumption. Over the whole experimental period, feed consumption was increased (P<0.01) by addition of boric acid (BA) and combination of feed additives (FBA). Feed convertion ratio (FCR) was not affected by supplemented or not with boric acid and/or plant extract mixture *(Table 3)*. However at 9-10th wks of treatment, FCR for hens was significantly decreased in 1st trial group (F) while BA significant increased of it compare the control group (P<0.01). The egg yield and weight results were also summarized in *Table 4*. These parameters for performance were not significantly modified by the supplementation with boric acid and/or plant extract mixture combination over the whole experimental period. At the end of trial it was observed that there were no effects of dietary additions on egg quality parameters (shell thickness, breaking strength, and shape index) compared with control *(Table 5)*. In same way, blood cholesterol levels were not affected by boric acid and/or plant extract mixture *(Table 6)*.

Table 2. *Effects of plant extract mixture and boric acid on feed consumption of laying hens (mean ± standard error)*
Tablo 2. *Bitki ekstraktı karışımı ve borik asidin yem tüketimi üzerine etkileri (ortalama ± standart hata)*

Week	Control	F	BA	FBA	P
1-2	98.44±1.15	101.80±2.52	100.74±3.41	104.35±1.50	0.367
3-4	102.01±1.38 ^{b}	109.74±1.63 ^{a}	109.64±1.32 ^{a}	108.30±1.03 ^{a}	0.002**
5-6	106.61±1.75	109.78±1.82	102.48±4.32	112.97±1.52	0.058
7-8	107.73±1.23	106.18±1.88	108.09±1.54	112.05±1.46	0.078
9-10	109.22±0.90 ^{bc}	106.10±2.08 ^{c}	114.23±1.98 ^{ab}	119.64±3.37 ^{a}	0.002**
11-12	109.10±0.65	111.37±2.85	114.20±0.98	113.78±1.40	0.154
13-14	102.37±2.14 ^{a}	96.12±2.61 ^{b}	107.58±1.08 ^{a}	107.02±1.70 ^{a}	0.002**
1-14	105.07±1.01 ^{b}	105.87±1.44 ^{b}	108.14±0.91 ^{ab}	111.16±1.14 ^{a}	0.005**

a, b, c: *Means on the same line with different superscript differ significantly, ** P<0.01*

Table 3. *Effects of plant extract mixture and boric acid on FCR of laying hens (mean ± standard error)*
Tablo 3. *Bitki ekstraktı karışımı ve borik asidin yemden yararlanma oranı üzerine etkileri (ortalama ± standart hata)*

Week	Control	F	BA	FBA	P
1-2	2.32±0.12	2.45±0.12	2.36±0.15	2.26±0.14	0.767
3-4	1.92±0.04	2.13±0.05	1.81±0.33	2.03±0.04	0.593
5-6	1.96±0.03	2.08±0.08	1.90±0.09	2.10±±0.04	0.129
7-8	1.97±0.24	2.02±0.06	2.00±0.05	2.05±0.06	0.774
9-10	2.06±0.52 ^{ab}	1.94±0.03 ^{b}	2.15±0.05 ^{a}	2.19±0.06 ^{a}	0.009**
11-12	1.98±0.03	2.01±0.04	2.07±0.03	2.03±0.02	0.245
13-14	1.85±0.05	1.78±0.09	1.88±0.02	1.95±0.05	0.240
1-14	2.01±0.34	2.06±0.03	2.03±0.03	2.09±0.05	0.417

a, b: *Means on the same line with different superscript differ significantly, ** P<0.01*

Table 4. *Effects of plant extract mixture and boric acid on egg weight and egg yield of laying hens (mean ± standard error)*
Tablo 4. *Bitki ekstraktı karışımı ve borik asidin yumurta ağılığı ve yumurta verimi üzerine etkileri (ortalama ± standart hata)*

Week	Control	F	BA	FBA	P
Egg weight (g)					
1-2	53.88±0.35	54.28±0.36	54.92±0.77	55.74±1.85	0.626
3-4	55.27±0.55	55.20±0.55	54.98±0.54	54.96±0.56	0.972
5-6	57.06±0.46	56.99±0.68	57.15±0.40	56.94±0.59	0.994
7-8	57.95±0.80	57.81±0.61	57.47±0.49	57.60±0.59	0.951
9-10	58.50±0.68	59.61±0.40	58.24±0.43	58.29±0.76	0.340
11-12	60.03±0.74	60.30±0.44	59.96±0.43	60.27±0.29	0.949
13-14	61.29±0.79	60.98±0.47	60.49±0.68	60.03±0.56	0.533
1-14	57.71±0.57	57.88±0.36	57.60±0.42	57.69±0.46	0.977
Egg yield (%)					
1-2	79.76±3.94	77.23±3.68	78.87±4.92	84.23±3.55	0.658
3-4	96.43±0.40	93.75±2.29	91.96±1.93	97.17±0.78	0.100
5-6	95.39±0.84	93.30±2.66	94.49±1.95	94.49±0.54	0.863
7-8	94.35±1.38	91.37±3.10	94.05±1.43	95.24±0.95	0.540
9-10	90.92±1.62	91.96±2.69	91.52±1.36	93.75±1.49	0.737
11-12	91.96±1.11	91.82±2.14	92.27±0.88	93.15±1.14	0.907
13-14	90.63±0.82	89.29±2.25	94.49±1.21	91.67±1.23	0.114
1-14	91.35±0.72	89.82±1.87	91.09±1.00	92.81±0.96	0.405

Differences between treatment groups is not statistically significant (P>0.05)

DISCUSSION

The experiment has shown that after dose escalation of boric acid the feed consumption and feed conversion ratio of hens fed boric acid were significantly different from those of the control. Yesilbag and Eren [9] found that similar findings like as Olgun et al.[10] about supplementation of boric acid to the laying hens diet significantly increased the feed consumption while it did not affect the feed efficiency. These results are in agreement with the results of studies involving hens on supplementations of boric acid. At the same time there is a negative correlation with other study [11] which tried to determine different level of boron (25, 50, 75 ppm) effects on feed consumption and feed convertion ratio in laying hens. They did not find any significant differences about performance parameters of hens with supplementation of boric acid. Also, while Elkin et al.[12] reported that 1-sterilboronic acid had no effect on feed consumption and feed efficiency, Koksal et al.[13] reported that boric acid (90 ppm) supplementation to the hen diet decreased the feed convertion ratio. This differences was quantitatively accounted for by a difference level of the boron and different boron sources.

For the entire of experimental period, egg weight, egg yield and egg quality were similar for the present additives, results that agree with previous reports [9,14,15], which have shown that egg performance and egg quality were not affected by different level of boric acid in the diet. Moreover, Kurtoglu et al.[4] reported similar performance in laying hens when 50, 100, 200 and 250 ppm boric acid was added to the diet. Koksal and Kucukersan [2] indicated that plant extract mixture had no significant effect on egg production and egg quality in laying hens. Present study in a harmony with the other studies [16-22] that supplemented plant extract and essential oils to the diets. In contrast, Qin and Klandorf [15] reported that first two wks of spawning period, supplementation of 100 ppm boron, and the remaining three wks supplementation of 60 ppm boron decreased egg production in the level of boron addition of both.

In the current study, no significant variations in cholesterolemia were observed among groups. This result is positive correlation with the study [22] which determined the level of 60 ppm boric acid and Koksal and Kucukersan [2] which determined the level of 0.75 g/kg plant extract mixture had no effect on serum cholesterol concentration in broilers and laying hens, respectively. In addition, Sizmaz and Yildiz [23] reported similar result in broilers when 175 ppm boric acid added to the diet. Results that agree with earlier studies in rabbits [24], in broilers [25], which have shown that cholesterolemia were not affected by boric acid supplementation to the drinking water and to the diets, respectively. In contrast, previous studies [15,21,26] showed that plant extract reduced the serum cholesterolemia in layings. The differences about results between other

Table 5. *Effects of plant extract mixture and boric acid on egg quality parameters (mean ± standard error)*
Tablo 5. *Bitki ekstraktı karışımı ve borik asidin yumurta kalite parametreleri üzerine etkileri (ortalama ± standart hata)*

Week	Control	F	BA	FBA	P
Shell thickness					
1-2	39.36±0.45	38.64±0.45	39.89±0.44	39.14±0.63	0.372
3-4	38.11±1.35	35.88±1.67	36.69±1.26	35.75±1.19	0.606
5-6	34.14±0.83	35.89±0.68	35.67±0.86	35.25±0.77	0.413
7-8	35.28±0.78	32.56±1.06	33.33±0.73	35.19±0.86	0.074
9-10	38.25±0.36	37.61±0.47	38.06±0.60	38.03±0.31	0.783
11-12	36.71±0.49	37.13±0.59	35.74±0.55	36.14±0.56	0.301
13-14	36.97±0.41	36.28±0.53	36.56±0.34	36.59±0.43	0.733
Breaking strength					
1-2	1.89±0.25	1.94±0.17	2.14±0.21	1.76±0.18	0.605
3-4	1.88±0.17	1.85±0.21	1.71±0.18	1.84±0.17	0.917
5-6	1.96±0.10	2.06±0.12	1.87±0.15	1.99±0.14	0.779
7-8	2.38±0.18	1.97±0.14	2.06±0.15	2.40±0.16	0.132
9-10	2.31±0.10	2.03±0.12	2.31±0.13	2.15±0.09	0.244
11-12	1.81±0.14	2.03±0.17	1.91±0.13	1.75±0.13	0.562
13-14	2.04±0.07	1.98±0.09	2.00±0.06	2.00±0.04	0.935
Shape index					
1-2	77.27±0.52	77.23±0.59	78.17±0.57	77.50±0.46	0.591
3-4	77.83±0.67	78.83±0.62	77.58±0.73	78.08±0.43	0.508
5-6	78.58±0.55	78.46±0.45	79.08±0.73	77.33±0.72	0.252
7-8	77.38±0.46	77.96±0.69	77.58±0.79	76.79±0.66	0.654
9-10	78.32±0.69	77.46±1.15	78.90±0.77	77.86±0.36	0.613
11-12	77.50±0.53	78.42±0.52	77.33±0.66	77.58±0.65	0.576
13-14	77.81±0.27	78.06±0.34	78.11±0.29	77.53±0.27	0.487
Differences between treatment groups is not statistically significant (P>0.05)					

Table 6. *Effects of plant extract mixture and boric acid on blood cholesterol concentration (mean ± standard error)*
Tablo 6. *Bitki ekstraktı karışımı ve borik asidin kan kolesterol düzeyi üzerine etkileri (ortalama ± standart hata)*

Parameters	Control	F	BA	FBA	P
End of 7th wk	154.20±24.57	171.47±14.16	171.87±16.93	146.89±6.42	0.655
End of 14th wk	107.28±15.43	164.92±19.38	128.44±7.05	124.13±23.31	0.168
Differences between treatment groups is not statistically significant (P>0.05)					

studies and ours can be caused by using of different composition and different levels of plant extracts.

We conclude that boric acid and plant extract mixture can be used successfully in laying hen diets. The effects of supplementation of plant extract mixture and boric acid during the laying period on egg production and egg quality parameters were investigated, and their effectiveness was compared. Supplemental plant extract mixture and boric acid combination had linear effects on production parameters including increased feed intake (during the trial) and feed convertion ratio (9-10 wks). However, they had no specific effects on egg quality parameters.

Acknowledgements

We express our thanks to Farmavet for supplying the plant extract mixture (Fitococci) used in the experiments.

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DOI: 10.9775/kvfd.2014.10718

Journal Home-Page: http://vetdergi.kafkas.edu.tr
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RESEARCH ARTICLE

Evaluation of Prevalence of the Types of Thyroid Disorders Using Ultrasound and Pathology of One Humped Camel *(Camelus dromedarius)*

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Makale Kodu (Article Code): KVFD-2014-10718

Summary

The thyroid gland is the largest classic endocrine organ, that effects many organs of the body and plays a significant role in the process of metabolism in animals. The aim of this study was to investigate the prevalence of thyroid disorders diagnosed by ultrasound and microscopic lesions of the thyroid during the slaughter of apparently healthy One Humped Camels (*Camelus dromedarius*). Randomly, 400 male camels (with a mean age range of 4 to 8 years), were studied in 2012 to 2013. The Camels' thyroid glands were evaluated by ultrasound device using multi-frequency linear transducer 7-9 MHz caller doppler and in both longitudinal and transverse view. After stabilization of the sample in 10% buffered formalin and the process of preparation of paraffin sections in autotechnicon method, tissue sections of 5 microns in diameter were prepared and stained with H & E and finally examined by light microscopy. The results obtained in the pathology indicated the following: hyperplastic goiter (19%), degenerative changes (12%), follicular cysts (5%), follicular atrophy (4%), nodular hyperplasia (3%), adenoma (1%), carcinoma (1%) and simple goiter colloid (1%). Ultrasound evaluation of thyroid gland in adenoma and carcinoma showed enlargement of the gland, decreased echogenicity,and the heterogeneous thyroid parenchyma with irregular boundaries. Also, in follicular cysts were observed in the enlarged gland with no echo structures of different sizes and decreased echogenicity as a regional or general. In nodular hyperplasia, echogenicity and heterogeneous parenchymal were found to be increased. Comparison of the effects on the thyroid gland from different camels showed that there was no statistically significant difference (P>0.05). These findings suggest the use of Ultrasound as a screening test in the diagnosis of complications of thyroid disorders. Pathology also to be used for the diagnosis of thyroid problems and other side effects.

Keywords: *Thyroid gland, One Humped Camel, Ultrasound, Pathology*

Tek Hörgüçlü Develerde *(Camelus dromedarius)* Ultrasonografi ve Patolojik Muayene İle Tiroid Bozukluk Tiplerinin Preavalansının Değerlendirilmesi

Özet

Tiroid bezi vücuttaki pek çok organı etkileyen ve metablizma sürecinde önemli roller oynayan en büyük endokrin bezdir. Bu çalışmanın amacı kesim öncesi sağlıklı görünen Tek Hörgüçlü Develerde (*Camelus dromedarius*) ultrasonografi ve mikroskopik muayene ile tiroid bozukluk tiplerinin prevalansını araştırılmaktır. Çalışmada 2012 ile 2013 yılları arasında rastgele seçilen yaşları 4 ile 8 arasında değişen 400 adet erkek deve kullanıldı. Develerin tiroid bezleri longitudinal ve transversal doğrultularda olmak üzere multi-frekans lineer transducer 7-9 MHz doppler kullanılarak ultrason cihazı ile incelendi. Tiroid örnekleri %10'luk tamponlu formalin içerisinde tespit edildikten sonra parafin bloklar hazırlandı, 5 mikron kalınlığında kesildi ve H&E ile boyanarak ışık mikroskobunda inlendi. Patolojik incelemelerde hiperplastik guatr %19, dejeneratif değişiklikler %12, foliküler kistler %5, foliküler atrofi %4, nodüler hiperplazi %3, adenom %1, karsinom %1 ve basit guatr kolloid %1 olarak tespit edildi. Adenom ve karsinomlu tiroid bezlerinin ultrasonografik incelemeleri sonucunda bezin büyüdüğü, ekojinetisinin azaldığı ve düzensiz sınırlı heterojen tiroid parankimine sahip olduğu görüldü. Büyümüş bezlerde foliküler kistler de gözlendi. Nodüler hiperplazilerde, ekogenesiti ve heterojen parankimin arttığı gözlendi. Değişik develerdeki tiroid bezleri karşılaştırıldığında istatistiksel anlamda herhangi bir fark gözlenmedi (P>0.05). Bu bulgular ultrasonografinin tioid bozukluklarına ait komplikasyonları tarama amaçlı olarak kullanılabileceğini göstermektedir. Histopatolojik muayene ayrıca tiroid problemlerini ve diğer yan etkileri teşhis amaçlı kullanılabilir.

Anahtar sözcükler: *Tiroid bezi, Tek hörgüçlü deve, Ultrasonografi, Patoloji*

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INTRODUCTION

The thyroid gland is the largest classic endocrine organ which exude triiodothyronine, thyroglobulin and thyroxin hormones, that effects many organs of the body and plays a significant role in the process of metabolism in animals [1,2]. Thyroxin plays a significant role in metabolic activities of the animal body. The most outstanding characteristic of the vertebrate thyroid gland is its skill to concentrate large total of iodine for the synthesis of thyroxin and its disorders [2]. Thyroid hormones are involved in thermo-regulation of the body. In cooperation by other hormones, it raises the body temperature via oxidant of fat, CHO and protein and release heat [2-4].

Ultrasonography can be used as a screening test in the early evaluation of the patient, the results of other diagnostic procedures can be used to confirm its findings which could lead to more correct diagnosis [5].

The use of combined Ultrasonography and pathology of the thyroid can improve the diagnosis of patients with thyroid adenoma or carcinoma, parathyroid hyper-plasia and primary producer or neoplastic disorder. Ultra-sonography and pathology of the thyroid gland in animals such as sheep [6,7], goats and cows [8], cat [9], dogs [6] and horse [7] as well as in humans [10] have been studied.

Few studies have been done about histological and anatomical characteristics of camel thyroid glands in Iran [11], and no studies have been done about the use ultrasound and pathology for diagnosing thyroid disorders in One Humped Camel in Iran.

The present study was conducted to investigate the use of ultrasonography and pathology in the diagnosis of thyroid disorders of One Humped Camel *(Camelus dromedarius)* with the aim to evaluate the types of thyroid complications.

MATERIAL and METHODS

Sample Collection

This study was performed in the local abattoir of Najaf-Abbad, Esfahan province, central part of Iran from 2012 to 2013. In this abattoir, ruminants including sheep, goats, cattle and camels are slaughtered daily. The camels originated from east, south and south-east parts of Iran areas with warm, dry and windy weather. For this study, 400 thyroid glands of slaughtered one humped camel *(Camelus dromedarius)* (4 to 8 years old) were randomly inspected and specimens were taken for macroscopic and histo-pathological study.

Ultrasonography Investigation

The thyroid gland, in the view of the longitudinal and cross-section using an ultrasound device (EX8000 Medison ultrasound system) and multi linear transadiusr with frequency 7-9 MHz were studied.

Pathological Investigation

At the beginning of the thyroid tissue for longitudinal and transverse cross-section for different thyroid glands were examined macroscopically. The collected samples in 1 cm^3 thicknesses of the thyroid glands were fixed in 10% neutral buffered formalin (for period of 48 h) for histo-pathological examination. The samples were then de-hydrated in graded ethanol and embedded in paraffin wax. Sections of 5 µm in thickness were stained with hematoxylin and eosin (H&E) and then examined by an ordinary light microscopy.

Statistical Analysis

The data were entered, organized and grouped in the Microsoft Excel 2010. Data analysis was computed by using SPSS/PC-16.0 statistical software (SPSS Inc., Chicago, IL).

RESULTS

Ultrasonography Findings

Ultrasound of the normal thyroid parenchyma showed a uniform reflection and echogenic. In symptoms such as carcinoma and adenoma, enlargement of the gland often with reduced echogenicity and a heterogeneous range of irregular thyroid parenchyma were observed. Also thyroid enlargement in follicular cystic areas, without eco-structures with different sizes and decreased, reduced the echogenicity for regional or general, hyperplasia nudolar, increased echogenicity and being a heterogeneous parenchyma *(Fig. 1, 2, 3, 4,* and *5)*.

Pathological Findings

In thise study the major lesion of the thyroid gland was, the diffuse hyperplastic goiter with an abundance of 19%. Grossly, thyroid glands were enlarged uniform throughout the thyroid lobes. Follicles were irregular in size and shape and contained varying amounts of eosino-philic and vacuolated colloid. Some follicles were lack of colloid and collapsed. The follicles were covered by single or multiple layers of hyperplastic epithelial cells and formed papillary projections into the lumens *(Fig. 6)*. The frequency of nodular goiter was (3%). Affected thyroid showed multiple foci of hyperplastic follicular cells that were demarcated from the adjacent follicles. In microscopic study, hyperplastic nodules were composed of large, irregularly shaped follicles that lined by one or more layers of cuboidal cells. Papillary projections of follicular epithelium occurred into the lumen of some follicles *(Fig. 7)*. Other diagnosed lesion was colloid goiter

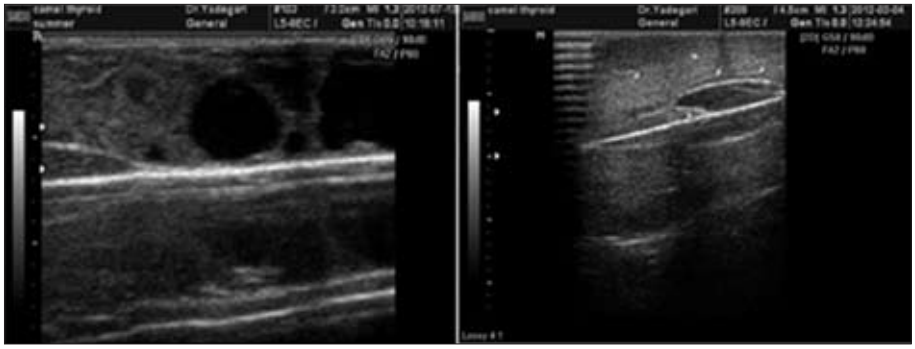


Fig 1. Decrease echogenicity for topical *(right)* and no reflection *(left)*, which is there with that expression cyst in multiple sizes

Şekil 1. Farklı boyutlarda kistle birlikte azalmış topikal ekojenite *(sağ)* ve yansıma yok *(sol)*

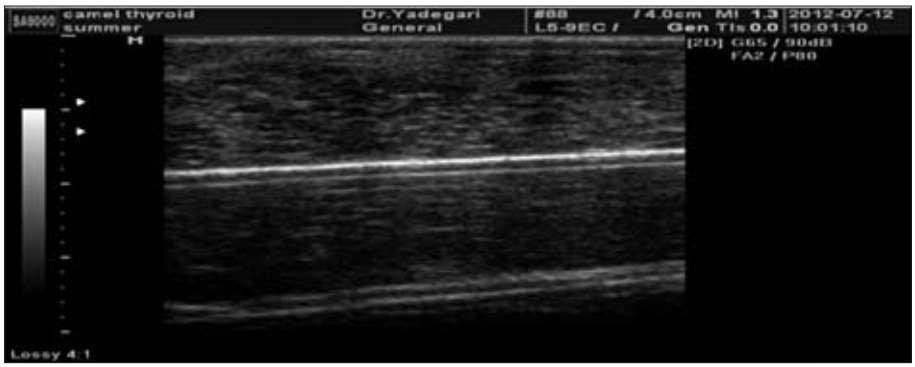


Fig 2. Diffuse increased echogenicity

Şekil 2. Diffuz artmış ekojenite

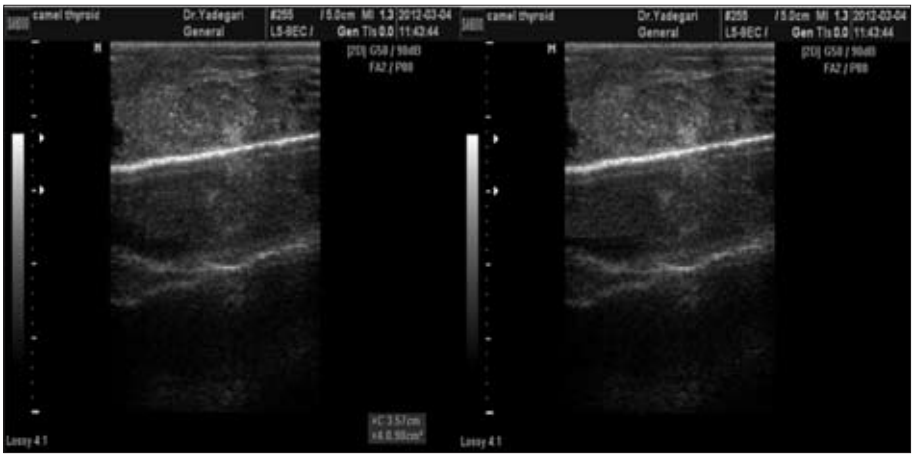


Fig 3. Nodules of the thyroid with hyperechoic and heterogeneous echogenicity

Şekil 3. Hiperekoik ve heterojen ekojeniteli tiroidte nodüller

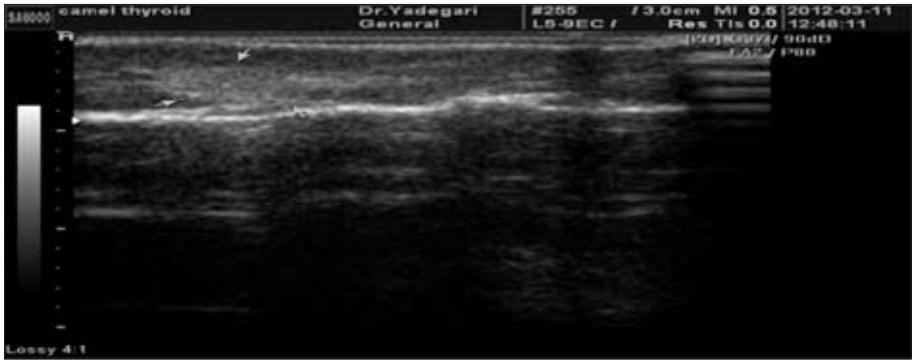


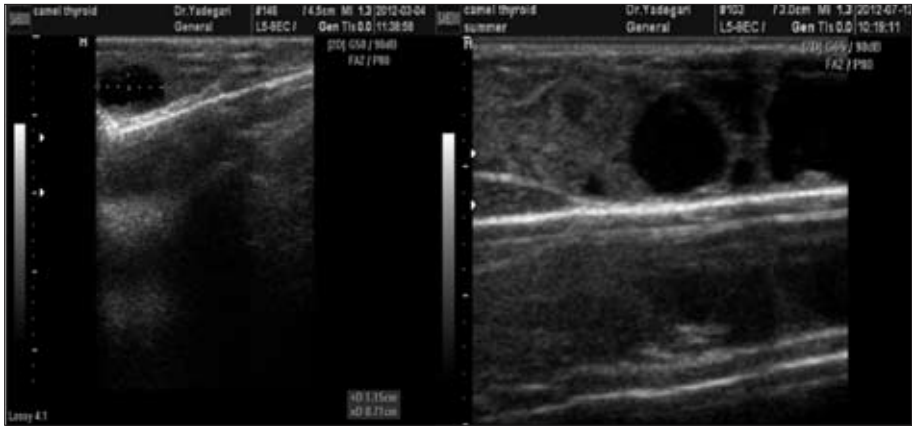
Fig 4. Local increased echogenicity

Şekil 4. Lokal artmış ekojenite

(1%). At the colloid goiter, follicles were dilated with deeply eosinophilic colloid. The macrofollicles were lined with flattened epithelial cells *(Fig. 8)*. Degenerative changes and atrophy was observed in (12%) and (4%) of thyroid samples respectively. The gland was smaller than normal. Follicles were shrinkage and contained little colloid. The epithelial cells of some follicles showed degenerative changes including eosinophilic cytoplasm and pyknotic nuclei. Follicular epithelium was desquamated into the space of follicles. Disruption of thyroid follicles was lead to

Fig 5. An echo areas and various sizes of cyst

Şekil 5. Bir eko alanı ve değişik boyda kistler



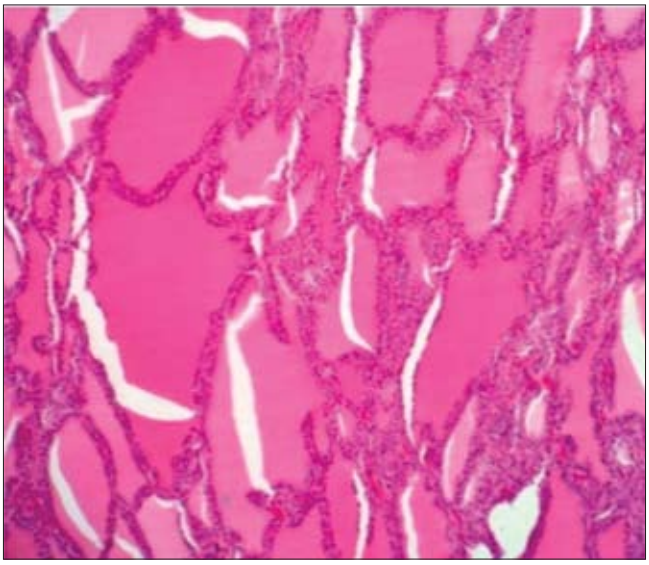


Fig 6. The follicles are irregular size and shape, and covered by single or multiple layers of hyperplastic epithelial cells. Papillary projections of hyperplastic epithelium are visible in the follicular space (H&E, ×100)

Şekil 6. Foliküller düzensiz boy ve şekillerde olup tek veya çok katlı hiperplastik epitelyum hücre ile örtülü. Foliküler boşluklarda hiperplastik epitelin papillar uzantıları görülmektedir (H&E, ×100)

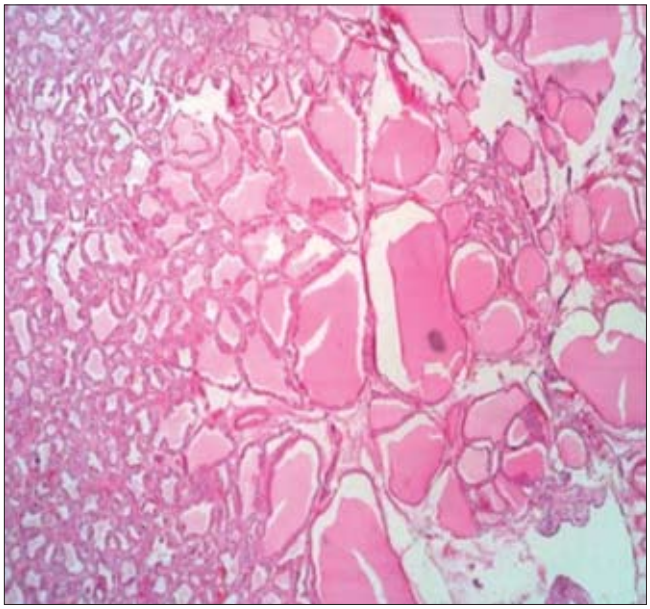


Fig 7. Nodular hyperplasia. Hyperplastic nodule *(right side of figure)* is composed of large and irregularly shaped follicles that lined by one or more layers of cuboidal cell (H&E, × 40)

Şekil 7. Nodüler hiperplazi. Hiperplastik nodül *(sağ tarafta)* bir veya çok katlı küboidal hücre ile çevrili büyük ve düzensiz şekilli foliküllerden oluşmuştur (H&E, × 40)

releasing of colloid into the interstitium and caused mild infiltration of lymphocytes. Also, fibrosis increased in the interstitial tissues *(Fig. 9)*. Follicular cysts were observed in (5%) of thyroid glands *(Fig. 10)*.

Follicular adenoma was observed in one case (1%). Grossly, thyroid was consisted of white-tan, small, solid nodules that were well demarcated from the surrounding thyroid parenchyma. The affected thyroid lobe was enlarged. Follicular adenoma was as solitary lesion in a normal gland and composed of microfollicular growth pattern *(Fig. 11)*. Papillary thyroid carcinoma was diagnosed in one sample (1%). Tumor cells had papillary pattern growth into cystic spaces of affected follicles. Papillary projection had fibrovascular stalks and lined with single or multiple layers of cuboidal cells. The nuclei were vesicular and pleomorphism and had prominent nucleoli *(Fig. 12)*.

DISCUSSION

The thyroid, the largest endocrine organ, influences the function of almost every organ in the body. The thyroid produces thyroxine (T4) and triiodothyronine (T3), which regulates the rate of metabolism and affect growth and rate of function of many other body systems [2,12]. Progressive basis of follicular epithelium loss and fat tissue inflammatory response [13]. Advanced follicular atrophy in dogs has been reported [13].

In the present study, thyroid pathological lesions observed included diffuse hyperplastic goiter, degenerative changes and follicular atrophy, diffuse lesions, follicular cysts, nodular hyperplasia, colloid simple goiter, adenocarcinoma, and adenoma follicular cells.

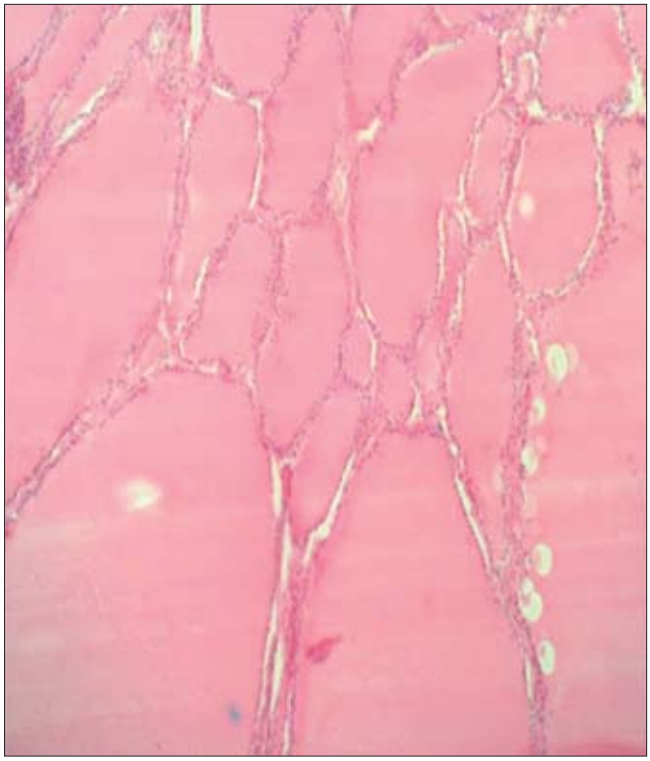


Fig 8. Goiter colloid. Follicles are dilated and filled with colloid (H&E, × 100)

Şekil 8. Kolloid guatr. Foliküller genişlemiş ve kolloid ile dolu (H&E, × 100)

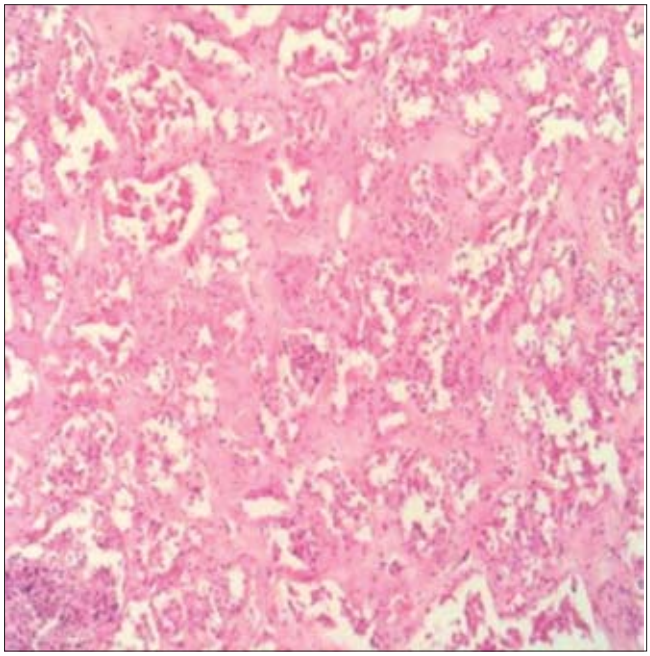


Fig 9. Follicular degeneration. Follicles are shrinkage and contained little colloid. Fibrosis increases in the interstitial tissues of follicles (H&E, × 40)

Şekil 9. Foliküler dejenerasyon. Foliküller büzüşmüş ve az miktarda kolloid içermektedir. Foliküllerin intersitisyel dokusunda artmış fibrozis (H&E, × 40)

Few studies have been done about thyroid glands of the camels. Though, Kausar and Shahid [12] reported, tissue

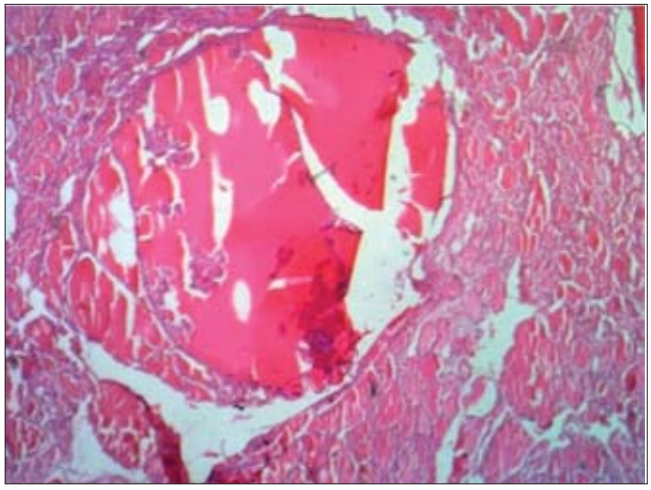


Fig 10. Follicular cysts (H&E, × 40)

Şekil 10. Foliküler kist (H&E, × 40)

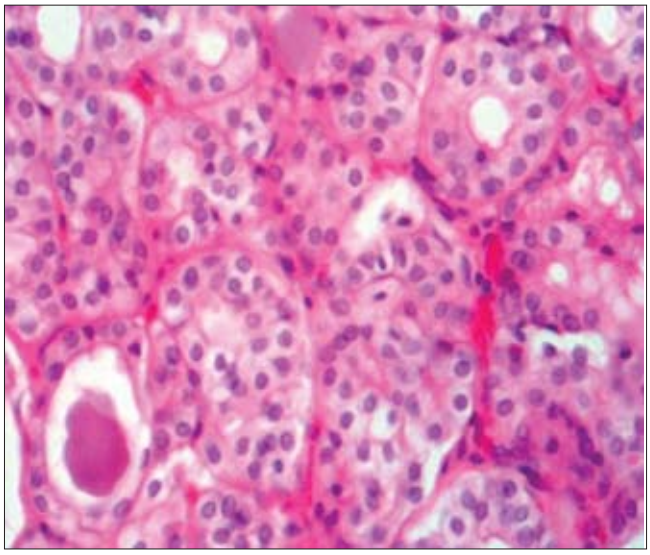


Fig 11. Follicular adenoma. It is compose of microfollicular growth pattern (H&E, × 400)

Şekil 11. Foliküler adenoma. Multifoliküler büyüme şekli (H&E, × 400)

samples of thyroid glands of 16 healthy camels *(Camelus dromedarius)* were investigated under two age groups i.e. group A (3-5 years) and group B (6-10 years) with equal number of animals, for their gross and microscopic anatomy.

In the present study nodular thyroid hyperplasia were observed. Nodular thyroid hyperplasia characterized by the formation of nonneoplastic nodules in the thyroid and has been reported in humans, horses, cats, dogs, and the rhesus monkey [14-17].

Follicular cyst was another prominent structure observed on the thyroid gland. Although the exact mechanism of this singularity is unknown, but the deficiency of vitamin A can be one of the possible causes that should be of interest to be used investigated [16].

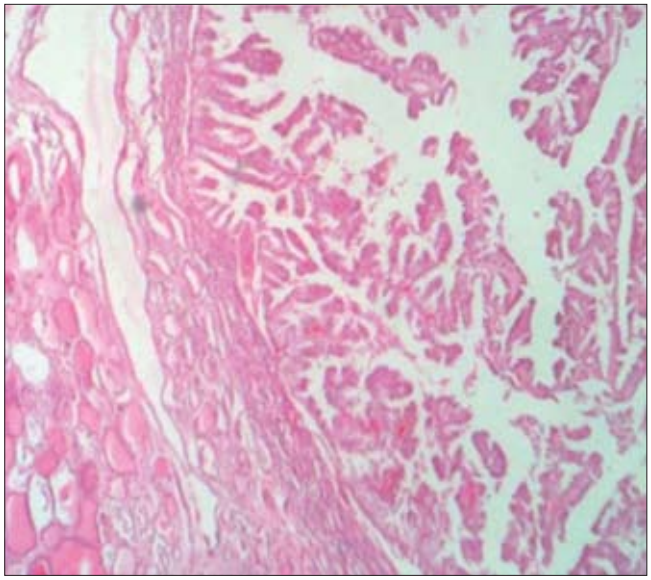


Fig 12. Papillary thyroid carcinoma. Tumor cells have papillary pattern growth into cystic spaces of affected follicles. Papillary projections have fibrovascular stalks and line with single or multiple layers of cuboidal cells (H&E, × 40)

Şekil 12. Papillar tiroid korsinomu. Tumör hücreleri etkilenmiş foliküllerin kistik boşlukları içine papillar uzantılar göstermektedir. Papillar uzantılar fibrovasküler bir sapa sahip olup tek veya çok katlı küboidal hücre ile çevrelenmiştir (H&E, × 40)

A Thyroid follicular adenoma findings in this study based on our understanding is the first report in camels in the world. A follicular adenoma is a benign encapsulated tumor of the thyroid gland. It is a firm or rubbery, homogeneous, round or oval tumor that is surrounded by a thin fibrous capsule. Adenoma of thyroid cystic adenoma (papillary, cystic) for the first time in cattle has been reported [13].

Mohajeri et al.[18], in their histopathological study on thyroid gland of goat in east Azerbaijan province of Iran examined a total of 386 thyroid specimens, where 71 cases had diffuse hyperplasia of thyroid follicular cells, 10 cases paranchymal cysts, 8 cases follicular atrophy, 5 cases colloid goiter, 4 cases thyroid fibrosis, 3 cases nodular hyperplasia and 1 case had C-cell adenoma and 1 case had C-cell carcinoma.

In a study by Alipourzamani et al.[19] in Tabriz of Iran thyroid glands of sheep were examined histopathologically. Of the total 100 thyroid examined, 33 showed various lesions on which 27% showed hyperplastic goiter, 4% showed colloid goiter and 2% showed lymphocytic (immuno-dediated) thyroiditis. Their findings were similar to our study by showing the highest frequency of the hyperplastic goiter.

Out of 800 pairs of thyroid glands in the study of Saber et al.[8], 15% had lesions in which histopathological changes were categorized as follicular atrophy (2.5%), focal hyperplastic goiter (0.88%), colloid goiter (3.39%), parenchymal cyst (1.38%), follicular cell hyperplasia (0.27%), thyroid fibrosis (0.635%), diffuse hyperplastic goiter and the parenchymal cyst (0.63%).

Other study by Nouri et al.[20] investigated 100 pairs of thyroid glands collected from ewes and their fetuses at the local municipal abattoir in Ahvaz city of Khuzestan province, multiple lesions were seen in 59% and 21% of the thyroid glands of ewes and fetuses, respectively. Histologically, ninety-nine lesions in the ewes and twenty-two lesions in the fetuses' thyroid glands were noticed. Histopathological changes for ewes were categorized as follicular hyperplasia (37%), bronchial cyst (31%), ultimobranchial cysts (14%), hyperemia and hemorrhagia (10%), follicular necrosis (4%), thyroiditis (1%), lymphocytic thyroiditis (1%), and trabecular adenoma (1%).

The pathologic conditions observed in thyroid glands of these animals (cow, sheep and goat) by Sayari et al.[7] included of simple colloid goiter: goat 12 (13.3%) cow 21 (11.66%) sheep 14 (15.5%), hyperplastic goiter: goat 43 (47.7%) sheep 30 (33.3%). parenchymal cysts: goat 3 (3.33%), cow 22 (12.2%), sheep 8 (8.9%), ultimobranchial nodules: goat 7 (7.7%) sheep 16 (17.8%) follicular atrophy: goat 10 (11.1%) cow 11 (6.66%) sheep 5 (5.5%), inflammatory and degenerative lesions: goat 4 (4.4%) cow 12 (6.6%) sheep 2 (2.2%). The number of animals without lesion included: goat 11 (12.2%) cow 114 (63%) and sheep 15 (16.6%).

There are several reports with pathological changes in the thyroid gland of various animals, including ruminants and dog [21,22]. The major pathological case of thyroid diseases is goiter [21-23], previous studies have shown that colloid goiter was most and thyroid lymphocytic inflammation was the lowest case of pathological lesions in buffalo of east Azerbaijan [24]. However, the hyperplastic goiter was the most and lymphocytic thyroiditis was the lowest thyroid pathology in carcasses of slaughtered sheep in East Azerbaijan [25]. The presence of lesion such as the types of goiter (colloidal and hyperplastic) in camels case study of deficiency iodine and deficiency vitamin A, calls for plans to prevent its occurrence. Perhaps the mode of feeding the camel is the main cause of this defficiency.

There seems to be some factors simultaneously that induce pathological changes in the thyroid glands of mothers and their fetuses. It is well known that among environmental factors two are more importantly to affect the thyroid glands: ambient temperature and feed intake [23,26,27].

The overall prevalence of thyroid problems can cause camel feeding and maintenance conditions of the animals.

Acknowledgements

The authors would like to express their sincere thanks to Dr. Augustino A. Chengula of Sokoine University of

Agriculture in Morogoro, Tanzania and the staffs of the Veterinary Department of the Shahrekord Veterinary Hospital.

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Kafkas Univ Vet Fak Derg
20 (4): 613-619, 2014
DOI: 10.9775/kvfd.2014.10727

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

The Comparison of Three Different Cryoprotectants in Cryopreservation of Angora Goat Semen [1][2]

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[1] *This study was supported by the Republic of Turkey, Ministry of Food, Agriculture and Livestock, General Directorate of Agricultural Research and Policy (GDAR) (Project number: 09/08/04/01)*

[2] *This study had been presented 4th International Congress on Cell Membranes and Oxidative Stres: Focus on Calcium Signaling and TRP Channels), 26-29 June 2012, Isparta -Turkey*

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Makale Kodu (Article Code): KVFD-2014-10727

Summary

The objective of this study was to evaluate glycerol (G), ethylene glycol (EG) and dimethylsulfoxide (DMSO) which were used two different doses on in vitro semen parameters, antioxidant enzymes activities and DNA damage after the freeze-thaw process in Angora goat semen. Semen samples from 5 mature Angora goats were used in this study. A total number of 40 ejaculates were collected twice a week from the goats using an artificial vagina and the semen pooled to minimize individual variation. Each pooled ejaculate was split into 6 equal aliquots and diluted with tris base extenders supplemented with two different doses of cryoprotectants (G 3%, 6%; EG 3%, 6%; DMSO 3%, 6%). G 3% and 6% was added as a cryoprotectant had better CASA motility ($P<0.01$) and progressive motility ($P<0.001$) values when compared to EG and DMSO groups. On the other hand, EG 6% showed the best values of preserved membrane integrity ($P<0.01$). The evaluation of CASA sperm motions parameters, adverse effects were procured in the groups with DMSO groups when compared to the other groups ($P<0.05$; $P<0.001$). G 6% group was the greatest VAP, VSL and VCL values than the other groups ($P<0.05$; $P<0.001$). DNA damage was not affected by supplemented different doses of cryoprotectants as well as antioxidant activity ($P>0.05$). In conclusion, no advantages were found in using EG or DMSO to replace G for freezing of Angora goat sperm.

Keywords: *Angora goat, Antioxidant activity, Cryoprotectans, DNA integrity, Sperm freezing*

Ankara Keçisi Teke Spermasının Dondurulmasında Üç Farklı Kryoprotektanın Karşılaştırılması

Özet

Bu çalışmanın konusu, iki farklı dozlarda kullanılan gliserol (G), etilen glikol (EG) ve dimethil sulfoksidin (DMSO) Ankara keçisi teke spermasının dondurma-çözdürme sonrasındaki in vitro sperma parametrelerinde, antioksidan enzim aktivitelerinde ve DNA hasarındaki etkilerini değerlendirmekti. Çalışmada 5 yetişkin Ankara keçisinden (3 ve 4 yaşlı) spermalar kullanıldı. Haftada iki kez suni vajen kullanılarak alınan 40 ejakülat kullanıldı ve bireysel farkları azaltmak için spermalar birleştirildi. Her birleştirilen sperma 6 eşit kısma bölündü ve içerisinde iki farklı dozda kryoprotektan (G %3, %6; EG %3, %6; DMSO %3, %6) katılmış tris sulandırıcısıyla sulandırıldı. Kriyoprotektan olarak G'ün %3 ve %6 oranında eklenmesi EG ve DMSO gruplarıyla kıyaslandığında en iyi CASA motilitesi ($P<0.01$) ve progressif motilite ($P<0.001$) değerleri verdi. Diğer taraftan, membran bütünlüğünün korunmasında EG %6 en iyi sonucu verdi ($P<0.01$). CASA sperma hareket parametrelerinin değerlendirilmesinde DMSO gruplarında diğer gruplara göre daha olumsuz etkiler gözlendi ($P<0.05$; $P<0.001$). G'ün %6'lık grubu VAP, VSL ve VCL değerlerinde digger gruplara gore en yüksek değer verdi ($P<0.05$; $P<0.001$). Antioksidan aktivitesinde olduğu gibi farklı dozda kriyoprotektanların eklenmesi DNA hasarının korunmasında etkili olmadı ($P>0.05$). Sonuçta, Ankara keçisi teke spermalarının dondurulmasında G yerine EG ve DMSO'nun kullanılmasının avantajı bulunmadı.

Anahtar sözcükler: *Ankara keçisi, Antioksidan aktivite, DNA bütünlüğü, Kriyoprotektanlar, Sperma dondurma*

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INTRODUCTION

The combinations of storage temperature, cooling rate, chemical composition of the extender, cryoprotectant concentration, reactive oxygen species (ROS), seminal plasma composition and hygienic control are the key factors that affect the fertilizing capacity of spermatozoa [1]. In general, a goat sperm cryopreservation medium includes a non-penetrating cryoprotectant (milk or egg yolk), a penetrating cryoprotectant [glycerol (G), ethylene glycol (EG), or dimethyl sulfoxide (DMSO)], a buffer (Tris or Test), one or more sugars (glucose, lactose, raffinose, saccharose, or trehalose) [2]. A cryoprotectant is included in a cryopreservation medium to minimize the physical and chemical stresses resulting from the cooling, freezing, and thawing of sperm cells. Since Polge et al.[3] reported that glycerol is beneficial to sperm preservation, it has been routinely inclueded in most cryopreservation protocols for many types of cells, including mammalian spermatozoa [4,5]. Addition of G can induce osmotic damage to spermatozoa, but the extent of the damage varies according to the species. However, goat spermatozoa are reasonably tolerant to these osmotic conditions and can withstand a rapid exposure to G. G (molecular weight: 92.10) is a penetrating cryoprotectant universally used for sperm freezing [6]. Similarly to G [7], EG is an alcohol-based cryoprotector with a low molecular weight (62.07) [8]. G causes membrane lipid and protein rearrangement, which results in increased membrane fluidity and permeability for ions and increase ATP consumption, greater dehydration at lower temperature and therefore an increased ability to survive cryopreservation [9]. Moleculer weight value of DMSO (78.13) ranks among G and EG values. Since Lovelock and Bishop [10] first reported DMSO was superior to G for protecting erythrocytes during freezing, it has been widely used as a cryoproservative agent alone or in combination with other cryoprotectants [11,12]. DMSO penetrates in cells more quickly [5] and therefore, will dehydrate the spermatozoa and minimize the intracellular ice formation [12]. DMSO has a beneficial effect for sperm cryopreservation of bovine, goat and rabbit, it cause a damaging effect during freezing-thawing process of buffalo semen [13].

The objective of this study was to compare the effects of type and concentration of cryoprotectants glycerol, ethylene glycol and dimethyl sulfoxide on the plasma membrane and DNA integrity as well as antioxidant activity of cryopreserved Angora goat sperm.

MATERIAL and METHODS

Animals, Semen Collection and Chemicals

Semen samples from 5 Angora goats (3 and 4 years of age), were used in this study. The bucks, belonging to the Livestock Central Research Institute were maintained under uniform breeding conditions. A total number of 40 ejaculates were collected twice a week intervals from the goats using an artificial vagina, during the breeding season and the semen mixed to minimize individual variation. Ejaculates which met the following criteria were evaluated: volume of 0.5-2 ml; minimum sperm concentration of 3×10^9 sperm/ml; motility of 80%. Immediately following collection, the ejaculates were placed in a water bath (35°C), until evaluation in the laboratory. Semen assessment was performed within approximately 5 min following collection. Each group was replicated eight times. The experimental procedures were approved by the Animal Care Committee of Lalahan Livestock Central Research Institute. All chemicals used in this study were obtained from Sigma-Aldrich Chemical Co. (Interlab Ltd., Ankara, Turkey).

Semen Processing

A Tris-based extender (Tris 254 mM, citric acid 78 mM, fructose 70 mM, egg yolk 15% (v/v), pH 6.8) was used as the base extender. After the extender is divided into 6 equal proportions. G, EG and DMSO cryoprotectants were added into each of them at 3% and 6% rates and extenders were prepared. Then the collected ejaculates were divided into 6 equal proportions and they were diluted 1:1 (v/v) with a basic extender that 3 different cryoprotectant were added at 2 different doses. After being stored at 35°C water bath for 10 minutes the final dilution was performed to be $200x10^6$ spermatozoa/ml in plastic centrifuge tubes. Actual sperm concentrations were calculated with the aid of an accuel photometer (IMV, France). Diluted samples were equilibrated at 5°C for a period of 4 h and then loaded in 0.25 ml French straws automatically and were frozen which was described as Taşdemir et al.[14]. After being stored for at least 24 h, straws were thawed individually (37°C), for 30 s in a water bath for sperm evaluation. Sperm evaluation was performed on all semen samples immediately after thawing.

Assessment of in vitro Sperm Quality

Progressive motility was assessed using a phase-contrast microscope (×100 magnification), fitted with a warm stage maintained at 37°C. Sperm motility estimations were performed in 3 different microscopic fields for each semen sample and the mean of the 3 successive estimations recorded as the final motility score. Besides recording the subjective sperm motility, a computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyze sperm motility, progressive sperm motility and sperm motion characteristics. The method described by Bucak et al.[15]. For the evaluation of sperm abnormalities and the hypo-osmotic swelling test (HOST) were performed in the semen samples. These tests were performed by Taşdemir et al.[14] as described.

Assessment of Biochemical Assays

Semen samples were thawed in 37°C water for 20 s and they were centrifuged at 4°C at 1.000 g for 15 min in order to separate spermatozoa. Pellet was washed 3 times with a 0.5 ml of PBS. This final solution was homogenized 5 times by sonication in cold for 15 s for the Lipid Peroxidation Analysis (LPO), 120 µl of homogenate was mixed with 10 µl 0.5 mM butyl hydroxyl toluene (BHT) and kept in –80°C until the analysis. The rest of the homogenate was centrifuged at 8.000 g for 15 min and the supernatant was separated and kept in –80°C for the other enzyme analysis. Enzyme levels were determined using commercial kits by spectrophotometry (Cintra 303-UV, GBC, Australia). Biochemical assay kits were obtained from Sigma-Aldrich Chemical (Interlab Ltd., Ankara, Turkey).

Determination of Sperm DNA Damage using Comet Assay

In our study, the most commonly used alkaline comet assay parameters have been used, which are; Tail intensity (percentage of DNA in the tail compared to the percentage in the 'head' or unfragmented DNA); Tail length (the length of the tail measured from the leading edge of the head) and Tail moment (percentage of DNA in the tail - tail DNA - times the distance between the means of the tail and head fluorescence measures). Each of these parameters describes endogenous DNA damage corresponding to DNA strand breakage and/or alkali-labile sites. Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay, which was performed at high alkaline conditions. The method described by Tuncer et al.[16].

Statistical Analysis

Data set is normally distributed using the Shapiro Wilk normality test. Homogeneity of variances with Levene's test groups was compared. The test revealed that the variances were homogeneous. After that, comparisons between the groups were made using analysis of variance with Duncan post hoc test. The results are expressed as means or proportions (±S.D.). P>0.05, not significant; P<0.05; P<0.01 and P<0.001.

RESULTS

As shown in *Table 1*, G 6% led the highest values CASA (P<0.01) and progressive (P<0.001) motility when compared to EG and DMSO groups. But, EG 6% showed the best values of preserved membrane integrity (P<0.01). Acrosome and total morphology were affected by the type of cryoprotectant used; DMSO (3 and 6%) resulted in greater damage than the other groups (P<0.001; P<0.01). Additionally in *Table 1*, at the evaluation of CASA sperm motions, adverse effects were procured in the groups with DMSO groups when compared to the other groups with cryoprotectants (P<0.05; P<0.001). G 6% group was the greatest values (VAP and VCL) than the other groups (P<0.05; P<0.001). Also G 3% and 6% had better VSL sperm motion parameter than the other groups (P<0.001).

As shown in *Table 1* and *Table 2*, DNA damage and antioxidant activities were not affected by supplemented different doses and different cryoprotectants as well as antioxidant activity (P>0.05).

Table 1. *Mean (±SEM) sperm parameters, CASA sperm motion parameters and chromatin damage values of detected by Comet Assay in frozen–thawed Angora goat semen*

Tablo 1. *Dondurulmuş çözdürülmüş Ankara keçisi spermasının ortalama spermatolojik parametreleri, CASA spermatozoon hareket parametreleri ve Comet testi ile tanımlanan kromatin hasarı*

Sperm Parameters	G 3%	G 6%	EG 3%	EG 6%	DMSO 3%	DMSO 6%	P
Subjective Mot. %	50.0±5.09c	58.8±2.27c	36.3±4.30b	31.3±6.03b	8.8±2.46a	15.0±4.23a	***
CASA Mot. %	25.4±4.87^{b}	36.9±4.62^{c}	27.0±2.78^{b}	30.4±4.03^{b}	11.0±2.43^{a}	14.6±1.64^{ab}	**
Prog. Motility %	5.9±1.27^{b}	9.3±1.63^{c}	5.3±1.29^{b}	5.4±0.91^{b}	0.9±0.23^{a}	2.0±0.46^{a}	***
VAP (µm/s)	86.4±3.57^{b}	92.2±2.38^{c}	77.9±2.81^{b}	80.6±1.79^{b}	51.9±2.21^{a}	57.3±1.26^{a}	*
VSL (µm/s)	66.3±2.68^{c}	70.4±2.57^{c}	59.5±2.57^{b}	58.2±1.50^{b}	36.5±2.02^{a}	41.1±0.70^{a}	***
VCL(µm/s)	178.9±7.98^{bc}	192.1±5.75^{d}	164.0±6.00^{b}	175.8±3.45^{bc}	118.1±5.12^{a}	128.7±3.20^{a}	***
ALH (µm)	8.9±0.28^{b}	9.4±0.27^{b}	8.8±0.29^{b}	8.6±0.28^{b}	7.2±0.50^{a}	7.3±0.29^{a}	***
STR	73.1±0.52^{c}	72.6±1.18^{bc}	72.1±0.72^{bc}	71.3±0.73^{bc}	66.1±1.48^{a}	69.0±0.71^{a}	***
LIN (%)	36.4±0.63^{c}	36.1±0.81^{c}	35.1±0.72^{c}	33.9±0.44^{ab}	30.3±0.84^{a}	31.3±0.53^{ab}	***
HOST %	48.0±4.39^{b}	49.9±3.22^{b}	50.1±3.61^{b}	56.0±2.77^{c}	34.3±2.89^{a}	41.9±2.14^{a}	**
Acrosome %	4.0±0.57^{b}	3.1±0.52^{ab}	2.8±0.37^{ab}	2.3±0.25^{a}	6.5±0.63^{c}	6.0±0.54^{c}	***
Total Morphology %	13.1±0.52^{b}	12.9±1.11^{b}	8.9±0.69^{a}	10.1±0.81^{a}	21.0±1.49^{c}	20.3±0.75^{c}	**
Tail Length (µm)	101.86±8.42	90.22±10.73	88.01±11.84	93.77±9.23	98.25±6.82	95.31±8.05	N.S
Tail Intensity (%)	19.14±2.56	16.34±2.65	16.95±3.85	17.43±3.05	19.36±3.99	15.79±1.81	N.S
Tail Moment (µm.%)	11.83±2.52	9.40±2.15	10.04±2.88	11.85±3.11	11.69±3.27	9.60±1.87	N.S

a-d: *Different superscripts within the same row demonstrate significant differences among groups, * P<0.05, ** P<0.01, *** P<0.001,* ***NS:*** *No significant difference (P>0.05)*

Table 2. *Mean (±SEM) glutathione peroxidase (GPx), lipid peroxidation (LPO), glutathione (GSH), catalase (CAT) and total antioxidant levels in frozen–thawed Angora goat semen*

Tablo 2. *Dondurulmuş çözdürülmüş Ankara keçisi spermasında ortalama glutatyon peroksidaz (GPx), lipid peroksidasyonon (LPO), glutatyon (GSH), katalaz (CAT) ve total antioksidan değerleri*

Antioxidant	G 3%	G 6%	EG 3%	EG 6%	DMSO 3%	DMSO 6%	P
GPx (mU/ml-10^9 cell/ml)	11.8±0.42	11.6±0.13	11.7±0.15	12.4±0.53	11.7±0.20	11.3±0.13	N.S
LPO (mU/ml-10^9 cell/ml)	4.1±1.48	2.1±0.45	1.8±0.43	1.9±0.47	1.9±0.63	2.0±0.53	
GSH (mU/ml-10^9 cell/ml)	37.2±3.97	46.7±8.78	45.1±4.61	40.9±5.92	43.4±4.44	38.2±4.33	
Catalase (mU/ml-10^9 cell/ml)	23.4±5.13	14.5±1.05	14.9±1.11	17.0±2.91	15.7±1.20	16.2±1.05	
Total Antioxidant (mmol/trılox/ml-10^9 cell/ml)	0.9±0.28	0.4±0.19	0.7±0.29	0.4±0.26	0.5±0.26	0.6±0.33	

NS: *No significant difference (P>0.05)*

DISCUSSION

The cryoprotectants are added to extenders to protect the sperm from damage during freezing process [17]. The level and type of cryoprotectants in semen diluent influence these events and their effects on the sperm cells during freezing [18]. G can cause great osmotic damage to spermatozoa because G passes through the sperm membrane much slower than other cryoprotectants [19,20]. However, the literature suggests that other cryoprotectants, such as DMSO has been shown to protect spermatozoa against cryodamage as well as G [10]. Other researchers believe that a low-molecular-weight cryoprotectant, such as EG, may cause less damage to spermatozoa than G [21]. EG can be used as a cryoprotectant agent as opposed to G in dogs [22] and in horse sperm [23]. Researchers addded G, EG, PG, and DMSO as cryoprotectants to the extender for freezing of Red deer epididymal sperm and DMSO showed the highest toxicity and G showed the lowest [24]. G, EG, and DMSO are generally used in a range of 1-8%, but the greatest recovery of sperm post-thawing has been achieved with G [11,17,25]. This study showed that using EG and DMSO to replace G as a cryoprotectant did not improve the sperm's motility after thawing. The highest subjective sperm motility rates are obtained at the groups that 3% (50.0±5.09%) and 6% (58.8±2.27%) G was added and the groups with 3% and 6% DMSO had the lowest values (8.8±2.46%, 15.0±4.23%; respectively). Similar with our result that researchers [11] determined the highest sperm motility (35%) in the extender which 6% G was added. In the same study diffferent from ours, they found DMSO was (21%) more successful when compared with EG (13%). There is a research [18] showed that post-thawing and freezability in buffalo semen extended with G or DMSO was significantly better than EG. Other researchers [17] showed that both motility and percentage of live goat bucks spermatozoa were improved with a combination of G + DMSO. Awad [26] suggested that the CASA values (VAP, VSL, VCL and LIN) were affected by the type and concentration of the cryoprotectant. In addition, he reported that the VAP and VCL values were higher in the test groups containing G and EG and that the VSL and LIN values were higher in the high concentration of G group. However, the CASA values did not differ among any of the groups with various concentrations of EG. In our study, the CASA values were different between each group. The greater values of VAP and VCL shown in the G 6% group and VSL shown in the G 3% and 6% groups. Freitas et al.[27] and Muino et al.[28] showed that semen with rapid and progressive sperm had the best post-thaw sperm longevity. Singh et al.[17] had used varying concentrations of G, DMSO, G+DMSO and G+lactose in different extenders for freezing sperm of different goat races. Both motility and the percentage of live spermatozoa were most affected by extenders containing only DMSO. Acrosomal and tail abnormalities tended to increase between post equilibration and post thawing stage, and were higher in extenders containing the higher levels of DMSO. Significantly lower percentages of abnormalities were recorded in the G+lactose extenders. Whereas in our study, the best protection against total morphological defects were found in the group that EG 3% and 6% were added, also acrosome defect was found in the groups 6% G, 3% and 6% EG were added and there was a lot of damage in the DMSO groups (3% ve 6%).

In a study [17], G and DMSO combined use have given better results for viability and motility in a goat sperm freezing study, 2% G and 4% DMSO combined use causes less damage in the acrosome structure after freezing thawing in a rabbit sperm [29], 3% EG used in stallion sperm [23] freezing has been shown to represent better cryoprotectant effect than other concentrations (6% and 9%) and also reported that EG has better results than G. EG (0.5M) was efficient for freezing ram sperm, allowing post-thaw motility similar to G (0.72M) but with a high number of intact acrosomes [6]. EG could provide similar or better results than those obtained with G during cryopreservation of ram semen [30]. G has osmotic and toxic effects on the plasma membrane and metabolism of cryopreserved cells. It is responsible for the disorganization of sperm plasma membrane and reducing motility and fertilizing ability. Higher concentrations of G lead to cell death [6]. EG has fewer detrimental effects on the viability and motility of spermatozoa [31], providing a better protective effect to the acrosome than G. In a ram sperm freezing study [32] reported that no difference was observed between EG and G for acrossome status and sperm motility. The sperm

cells that were preserved with EG showed more integrity of the plasmatic, nuclear and mitochondrial membranes. From the viewpoint of cell membrane integrity, it can be concluded that EG gives higher protection to the sperm cell than G. According to researchers [33], G enhances preservation of the acrosome and plasma membrane of ram spermatozoa, despite being toxic when used in high concentrations. Cooling and freeze–thawing produce physical, chemical and oxidative stress on the sperm membrane, which result in reduced sperm viability and fertilizing ability [2]. Similar to the findings of the literature, plasma membrane integrity and acrosomal defects were less deteriorated at the groups that EG 6% was added and less acrosomal defects were detected at both EG groups (3-6%) and at 6% G group.

Oxidative stress is a cellular condition generally characterized by an imbalance between the production of ROS and the scavenging capacity of the antioxidants. When the production of ROS exceeds the available antioxidant defense system, significant oxidative damage occurs to the sperm organelles through the damage of lipids, proteins and DNA [34]. Cryopreservation of spermatozoa enhances oxidative stress, which not only disrupts the motility and fertilizing ability of spermatozoa, but also increases DNA damage [35]. On the other hand, DNA integrity was not affected by type of cryoprotectant in our study. In other study [36] it is reported that, despite the temperature difference causes less change on the sperm morphology during the freezing and thawing process, it reduces the spermatozoa acrosome reaction and alters the DNA structure. In addition, 1.2-3% DNA damage is normal in high fertility characteristic bulls [37]. It is shown on various studies that, freezing and thawing process on ram [38] and bull [39] sperms cause permanent structural alterations on DNA and this causes fertility problems. According to Taşdemir et al.[40] the DNA integrity was also affected by the type of cryoprotectant used in bull. DMSO 6% and EG+DMSO 3% resulted in more sperm with damaged DNA than the other groups. In a study [41] reported that total abnormal spermatozoa frequencies are positively correlated with DNA damage. Spermatozoa plasma membrane damage is also correlated with DNA damage. In our study, in terms of the plasma membrane integrity, even though the 6% EG group has yielded statistically significant results but it could not gain an advantage over preventing DNA damage when compared with the other groups which 3 different cryoprotectants were added at different doses to the extender.

When cells are frozen, they are subjected to various stresses such as cold shock and oxidative stress that arise through ice crystallization and LPO due to membrane changes [42]. Ultrastructural damage of the plasma membrane increases the susceptibility to LPO when high production of ROS occurs during the freeze-thawing process. This was stated for ram [43], bull [44] and goat [45] sperm. Antioxidant mechanisms exist to maintain defense against oxidative stress-induced damages in semen [46,47]. However, the antioxidant capacity of sperm cells is insufficient in preventing oxidative stress during the freeze-thawing process [48]. In our study, there were no statistically significant difference in protecting GPx, LPO, GSH, CAT and total antioxidant levels between G, EG and DMSO which are added to basic Tris extender as cryoprotectants at 2 different doses ($P>0.05$). Taşdemir et al.[40] have founded the antioxidant activities of GPx, GSH, and CAT as well as the total antioxidant activity were affected by the type of cryoprotectant; notably, G+EG+DMSO 3% yielded the lowest activities when compared to the other groups ($P<0.001$) in Eastern Anatolian Red bull sperm. Aisen et al.[49] reported that the extender containing trehalose enhanced the level of GSH and decreased the oxidative stress provoked by the freeze-thaw process in ram semen. Atessahin et al.[50] found that an extender supplemented with trehalose increased the GSH-Px and CAT activity of frozen-thawed goat semen. Increasing the doses of trehalose resulted in greater activity of CAT and a marked improvement in bovine sperm motility [51]. Those reports were in contrast with our findings, the reason of this maybe using different male animals or using different species or the differences at composition of extenders.

Many membrane permeable cryoprotectants (G, EG and DMSO), and their combinations, have been tested with different goat breed sperm [11,17,25,52-54], but the most frequently used penetrating cryoprotectant is G, as our research.

While adding G to the extender at 3% or 6% as a cryprotectant, it had positive effect on sperm motility when compared with EG and DMSO, but also DMSO caused retardation in the sperm motion (VAP, VSL, VCL ve ALH) parameters compared with the other cryoprotectants. Highest protection was provided in the group which 6% EG was added in protecting membrane integrity. When total morphological evaluations were considered, fewer morphological defects were determined in the groups which EG were added at 3% and 6% rates. All three cryoprotectants didn't outmaneuver to one another both protecting DNA damage and protecting antioxidant activities. In conclusion, no advantages were found in using EG or DMSO to replace G for freezing of Angora goat sperm.

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Kafkas Univ Vet Fak Derg
20 (4): 621-624, 2014
DOI: 10.9775/kvfd.2013.10617

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Online Submission: http://vetdergikafkas.org

SHORT COMMUNICATION

Changes in the Activity of Aspartate- and Alanine Aminotransferase in Dogs with Experimentally Induced *Staphylococcus aureus* Infection

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Makale Kodu (Article Code): KVFD-2013-10617

Summary

The main purpose of this study was to evaluate the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) plasma concentrations in dogs with experimentally-induced *Staphylococcus aureus* infection. Correlations between AST, ALT and Respiratory Rate (RR), Pulse Rate (PR) and Internal Body Temperature (IBT) were also calculated. Bacterial suspension with density of $3.1x10^9$ cfu/mL was subcutaneously injected to 9 mongrel 2 years old male dogs whereas 6 other dogs served as negative controls. The concentrations were determined using commercial kits before application (0 h), 6, 24, 48, 72 h and 7, 14, 21 days after. The aminotransferase concentrations were higher in infected dogs than in the controls - AST peaked on days 7 and 14, and ALT - at the 72nd h. Strong positive correlations were recorded between ALT and AST concentrations and between RR and IBT. It was observed that the transaminases activities were slightly affected by the experimentally induced staphylococcal infection in dogs.

Keywords: *Staphylococcus aureus, AST, ALT, Clinical signs, Dogs*

Köpeklerde Deneysel Oluşturulan *Staphylococcus aureus* Enfeksiyonunda Aspartat ve Alanin Aminotransferaz Aktivitelerindeki Değişimler

Özet

Bu çalışmanın amacı köpeklerde deneysel *Staphylococcus aureus* enfeksiyonunda aspartat aminotransferaz (AST) ve alanin aminotransferaz (ALT) plazma knosantrasyonlarını değerlendirmektir. AST, ALT ve solunum oranı (RR), nabız oranı (PR) ve vücut iç sıcaklığı (IBT) arasındaki ilişki ayrıca değerlendirildi. Bakteri süspansiyonu (of $3.1x10^9$ cfu/mL) 9 adet melez 2 yaşlı erkek köpeğe subkutan yolla enjekte edilirken 6 adet diğer köpek negatif kontrol olarak kullanıldı. Konsantrasyonlar 0, 6, 24, 48 ve 72 saat ile 7, 14, 21. günlerde uygulamadan önce ticari kitler kullanılmak suretiyle belirlendi. Aminotrasferaz konsantrasyonları kontrole oranla enfekte köpeklerde daha yüksekti. AST 7. ve 14. günlerde, ALT ise 72. saatte en yüksek konsantrasyonlarda belirlendi. ALT ile AST konsantrasyonları arasında ve RR ile IBT arasında güçlü ilişki belirlendi. Transaminaz aktivitelerinin köpeklerde deneysel olarak oluşturulan Syafilokokal enfeksiyonu ile az derecede etkilendiği gözlemlendi.

Anahtar sözcükler: *Staphylococcus aureus, AST, ALT, Klinik Belirtiler, Köpek*

INTRODUCTION

Serum aminotransferases are enzymes that are often used to assist in the diagnosis of liver disease in domestic animals. Variable amounts of both ASA and ALT occur in the apo-enzyme form (inactive protein portion), which has no catalytic activity, and needs to be converted to an active holoenzyme form by the addition of pyridoxal 5-phosphate

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(P5P), the bio-active metabolite of dietary vitamin B_6 [1]. The hepatic function was evaluated by measuring AST and ALT enzymes, which reflect cell damage and more specifically are indicators of acute and chronic injury, respectively. The plasma half life of ALT in dogs is 4-72 h and the half-life of AST is 5 h [2].

Staphylococcus aureus (S. aureus) is often associated with suppurative infections and is recognized as an inherent member of the microflora of the skin of humans and dogs [3]. This infection was chosen because *S. aureus* is commonly found in various animal species including dogs, horses, cats and pigeons. Toxin-mediated diseases caused by *S. aureus* include range from cutaneous infections to infections of wounds, osteomyelitis, endocarditis, bacteremia with metastatic complications, toxic shock syndrome [4]. The bacterial components and secreted products that affect the pathogenesis of *S. aureus* infections are numerous and include surface-associated adhesins, exoenzymes, exotoxins [5]. According to some authors [6,7], the key elements in *S. aureus* are peptidoglycan (PepG) and lipoteichoic acid (LTA), which are a component of cell wall, synergize to cause shock and organ dysfunction. However, few studies on the biochemical changes in dogs infected with this bacteria exist. Thus, this study aimed to investigate some of the enzyme changes in dogs experimentally infected with *S. aureus*, during the acute phase of the infection. Therefore, we studied the clinical signs and changes in the AST and ALT concentrations in dogs during an experimental infection caused by subcutaneous application of *S. aureus*.

MATERIAL AND METHODS

Experimental Animals and Protocol Design

The experiment was approved by the Ethic Committee at the Faculty of Veterinary Medicine, Stara Zagora (Licence No 2/2009 issued by National Veterinary Medicine Office). The study was performed on 15 mongrel male dogs, 2 years old, weighing 12-15 kg, provided by the municipality of Stara Zagora. Prior to the experiment, the animals were vaccinated with vaccine Nobivac® (Intervet International B.V) and orally treated against internal parasites (Caniverm®, Bioveta, A. S. Czech Republic, 1 tablet/10 kg B.W.) and external parasites (Bolfo® Puder, Bayer, Germany). Dogs were housed in metal cages and exposed to a 12 hours light-dark cycle at room temperature (20-22°C). They were fed with a commercially available diet of dog pellet twice daily and had free access to water. Among them, 9 were inoculated in the lumbar region subcutaneously with a *S. aureus* ATCC 15564 suspension (5 mL, density $3.1x10^9$ cfu/mL) and constituted the experimental group, whereas the other dogs (n = 6) were injected with the same volume of saline solution and served as controls. Dynamics of internal body temperature (IBT) (°C), respiratory rate (RR) and pulse rate (PR) in control dogs and in dogs with *S. aureus* infection were also recorded.

Biochemical Analyses

Blood samples were collected from the puncture of the *v. cephalica antebrachii*. Blood samples were collected into heparinised tubes before inoculation (hour 0), then at 6, 24, 48, 72 h and 7, 14 and 21 days after *S. aureus* inoculation or saline injection. Heparinised blood samples were centrifuged (1500 x g, 10 min at room temperature) within 30 min after collection. Plasma was immediately separated and stored at -20°C until analysis. Plasma ALT and AST concentrations were determined with commercial kits (Human-GmbH, Germany).

Statistical Analysis

The statistical analysis was performed using one way analysis of variance (ANOVA). The results were processed with software Statistica v.6.1 (StatSoft Inc., 2002). All results are presented as mean and standard error of the mean (Mean ± SEM). The statistical significance of parameters was determined in the LSD test at $P<0.05$.

RESULTS

The changes in the AST and ALT concentrations after bacterial injection are shown in *Table 1* and *Table 2*. In the experimental and control groups, the activities were followed during a period of 21 days. The experimental staphylococcal infection in dogs was accompanied with swelling, painfulness and high temperature of the tissues at the site of the injection since the 1st day post-inoculation. At the site of bacteria injection, hair loss and tissue erosions occurred on days 14. Skin abscesses were evidenced at the 7th day in 5 inoculated dogs. A reduced appetite, impaired motor activity and enlargement of the

Table 1. *Plasma concentrations of aspartate aminotransferase (U/L) in healthy dogs (n = 6) and in dogs with experimental Staphylococcus aureus infection (n = 9) according to time after subcutaneous inoculation*

Tablo 1. *Subkutan inokulasyon sonrası zamana bağlı olarak sağlıklı (n= 6) ve Staphylococcus aureus ile enfekte (n = 9) köpeklerde plazma aspartat aminotransferaz (U/L) konsantrasyonları*

Time	Inoculated Group mean ± SEM	Control Group mean ± SEM
0 h	31.56±1.89	29.95±2.36
6 h	31.54±1.70	30.20±2.21
24 h	31.82±1.66	30.70±2.00
48 h	33.24±1.41	30.85±2.10
72 h	35.34±1.43	31.33±1.90
7 days	37.48±1.04**b	30.40±1.80
14 days	35.02±1.09*	29,86±1.92
21 days	31.70±1.42	30.16±1.88

*Superscript "b" indicate significant differences (P<0.01) according to time within a same group. For a given biochemical parameter: * (P<0.05) and ** (P<0.01) indicate significant differences between S. aureus inoculated and control dogs*

inguinal lymphatic nodes in the limp which was injected was also noted the 1st day post staphylococcal inoculation. Furthermore, fever and purulent conjunctivitis eye infection were also recorded in 9 and 1 infected dogs (after 48th h), respectively. One of the experimental dogs had oedema on the scrotum.

In the experimental group, initial levels (before inoculation) of AST were 31.56±1.89 U/L and of ALT-29.78±3.23 U/L. At 48 hours after this, AST levels began to rise and on the 7th day they peaked significantly (P<0.01) to 37.48±1.04 U/L compared to control values. At the same time, in the experimental group the values of AST were significantly higher (P<0.01) than the initial levels. The concentrations of ALT reached significant values at the 72nd h-38.44±2.11 U/L compared to the baselines (P<0.05). However, these values are in the normal reference range for the dogs. On the 21 days, the enzyme activities restored their initial levels. The results indicated that these enzymes are slightly influenced by the experimentally induced staphylococcal infection in dogs.

Table 2. *Plasma concentrations of alanine aminotransferase (U/L) in healthy dogs (n = 6) and in dogs with experimental Staphylococcus aureus infection (n = 9) according to time after subcutaneous inoculation*

Tablo 2. *Subkutan inokulasyon sonrası zamana bağlı olarak sağlıklı (n= 6) ve Staphylococcus aureus ile enfekte (n = 9) köpeklerde plazma alanin aminotransferaz (U/L) konsantrasyonları*

Time	Inoculated Group mean ± SEM	Control Group mean ±SEM
0 hour	29.78±3.23	35.25±4.62
6 hours	30.28±2.42	36.35±4.58
24 hours	31.26±2.12	36.21±4.87
48 hours	35.50±1.60	36.15±4.59
72 hours	38.44±2.11[a]	34.25±4.04
7 days	37.37±2.18	33.61±3.78
14 days	33.37±2.40	33.50±3.10
21 days	30.52±2.42	32.36±3,23

Superscript "a" indicate significant differences (P<0.05) according to time within a same group

Dynamics of internal body temperature (IBT), respiratory rate (RR) and pulse rate (PR) in healthy dogs and in artificially infected dogs are presented in *Table 3*. The results indicated that IBT is significantly increased from 24th h to 72nd h and RR from 48th to 72 nd h. It was also observed that PR enhanced at 24th h, 48th h and on day 7.

As shown in *Table 4*, strong positive associations were observed between ALT and AST concentrations (r = 0.86, P<0.05) and between RR and IBT (r = 0.84, P<0.05). In addition, ALT concentrations were moderate positively associated with RR and IBT (r = 0.5 and r=0.4 respectively, P<0.05), and weakly with PR (r = 0.2, P<0.05). The AST were also weakly positive coupled to the clinical parameters.

DISCUSSION

Infection accompanied by local and general systematic signs-enhanced fever, increase heart and respiratory rates at 24th h after inoculation of bacteria which are indicators for non-specific response and signs of inflammation *(Table 3)*. Similar clinical symptoms during infection were observed by Georgieva et al.[8].

Aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2) are enzymes found mainly in the liver, but also found in red blood cells, heart cells, muscle tissue and other organs, such as the pancreas and kidneys. The plasma activities of ALT and AST are useful indicators of hepatocellular injury. These markers are not specific for primary liver disease, because their enhancing can be induced by disease in other tissues, drugs, or liver injury secondary to another primary disease. The magnitude of their elevation may be proportional to the number of hepatocytes affected, so the absolute concentrations of the aminotransferases and their temporal elevation provide useful clinical clues to the cause of the liver disease. Increases in serum ALT activity are not liver specific, as increased serum ALT has been reported following muscle necrosis in dogs [9]. We

Table 3. *Dynamics of internal body temperature (IBT) (°C), respiratory rate (RR) and pulse rate (PR) in healthy dogs (n = 6) and in dogs (n = 9) with experimentally induced S. aureus infection. Results are expressed as means ± standard errors of the means (SEM)*

Tablo 3. *Subkutan inokulasyon sonrası zamana bağlı olarak sağlıklı (n= 6) ve S. aureus ile enfekte (n = 9) köpeklerde vücut iç sıcaklığı (IBT), solunum oranı (RR) ve nabız oranı (PR). Sonuçlar ortalama ± ortalamanın standart hatası (SEM) olarak verilmiştir*

Time After Treatment	IBT Control Dogs	IBT Inoculated Dogs	RR Control Dogs	RR Inoculated Dogs	PR Control Dogs	PR Inoculated Dogs
0 hour	38.63±0.16	38.95±0.17	28.66±2.81	37.33±2.496	87.33±5.35	118.22±6.11
6 hours	38.80±0.18	39.06±0.18[b]	35.00±2.35	38.77±2.66	86.33±5.14	122.88±6.10
24 hours	38.81±0.19	39.85±0.20[*b]	35.00±4.17	41.33±4.06	82.33±4.46	123.55±3.50[**]
48 hours	38.66±0.17	40.13±0.31[***c]	38.66±2.90	48.88±3.02[**b]	85.33±3.78	133.55±6.43[***]
72 hours	38.68±0.12	39.94±0.20[***c]	34.00±4.47	44.55±4.16[**a]	82.00±4.20	106.22±8.38
Day 7	38.60±0.17	39.03±0.18	34.33±3.87	38.88±2.58	81.16±3.22	132.22±10.10[***]
Day 14	38.76±0.18	39.34±0.29	35.50±4.42	35.11±2.94	78.50±5.25	105.77±7.15
Day 21	36.65±0.19	39.18±0.17	28.66±1.33	37.44±2.04	80.00±4.25	94.88±6.33

*For a given biochemical parameter: * (P<0.05), ** (P<0.01) and *** (P<0.001) indicate significant differences between infected and control dogs. Different superscripts a, b, c indicate significant difference (P<0.05 or more) according to time within the experimental group (S. aureus inoculated dogs)*

Table 4. *Correlations between aspartate- and alanine aminotransferase (AST, ALT) concentrations and respiratory rate (RR), pulse rate (PR) and internal body temperature (IBT) (ºC), in dogs with artificial S. aureus infection. Correlations were calculated from all time points (from 0 h to the 21st day) and significant associations (P<0.05) were in bold*

Tablo 4. *S. aureus ile enfekte köpeklerde aspartat ve alanin aminotrasferaz konsantrasyonları, solunum oranı (RR), nabız oranı (PR) ve vücut iç sıcaklıkları (IBT) (ºC) arasındaki ilişkiler. İlişkiler tüm zaman dilimlerinde (0. saatten 21. güne kadar) hesaplandı ve anlamlı ilişkiler (P<0.05) kalın yazılarak gösterildi*

	ALAT	ASAT	RR	PR	IBT
ALAT	-	**r=0.86 P<0.05**	r=0.51 NS	r=0.20 NS	r=0.46 NS
ASAT			r=0.06 NS	r=0.16 NS	r=0.05 NS
RR				r=0.44 NS	**r=0.84 P<0.05**
PR					r=0.14 NS
IBT					

NS: *not significant*

can conclude that low enhanced activity of ALT in the dogs in this study may be associated with skeletal muscle damage at the site of bacterial inoculation without hepatocellular injures. It has to be taken into account that in dogs increased activities on AST, especially accompanied by elevated levels of CK talking about muscle damage [10]. In this respect, in experimental group the creatine kinase activity (data not shown) were significantly higher compared to the control dogs [11]. These changes may be due to progression of inflammation caused by injection, destruction of fascia, as well as proteolytic enzymes of accumulated leukocytes at the site of injection.

No significantly changes in total activities of AST of experimental groups were observed and this shows that it is possible permeability of liver cells plasma membrane to slightly increased. During the study the alanine and aspartate aminotransferase concentrations are in normal reference range for the dogs. Costa et al.[12] observed that ALT levels were higher on day 20 after parasitical infection in dogs. According to them, increased ALT levels in dogs are often associated with hepatocellular injury as a result of infectious or toxic inflammatory processes, which promote enzyme release from the cytoplasm of hepatocytes, even though these levels did not exceed the reference values, they were 41% greater than on day 0. In our study the concentrations were 29.1% greater than on day 0.

The increase in AST levels in the infected group was more than 50%, compared with day 0 [12]. The increase in AST in this study was lower-18.7% than the baselines. Nevertheless, the reference values were not exceeded in both studies. In dogs normal values for AST and ALT are between 1-37 U/L and ALT-3-50 U/L [2,13] and according to Hines [14] - 5-55 U/L and 5-107 U/L, respectively. Ezeokonkwo et al.[15] show that infection (parasitic) in dogs caused a significant increase in the activities of AST and ALT on day 7, which coincides with our study. According to Quinn et al.[4], liver enzyme activities (AST, ALT) showed only mild to moderate increases during bacterial infection in dogs. In addition, the results displayed moderate positively correlation between ALT and RR and IBT, whereas AST associated weakly with the observed clinical parameters.

As a conclusion, these results indicate that AST and ALT activities are slightly influenced by *S. aureus* inoculation in dogs. In the course of this study the concentrations of AST (on day 7 and 14) and ALT (at the 72nd h) in the experimental group has little changed and could not be used as parameters with diagnostic value of experimental induced staphylococcal infection in dogs.

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Kafkas Univ Vet Fak Derg
20 (4): 625-627, 2014
DOI: 10.9775/kvfd.2013.10649

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

SHORT COMMUNICATION

Conjunctival Bacterial and Fungal Isolates in Clinically Healthy Working Horses in Iran

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Makale Kodu (Article Code): KVFD-2013-10649

Summary

This study was conducted to identify bacterial and fungal isolates of the normal eyes in working horses in Iran. Ninety swabs were taken from the conjunctival sac of 45 clinically healthy horses. Aerobic bacterial and fungal cultures were plated. A total of 9 different bacterial species (3 Gram-positive, 6 Gram-negative) and 7 different fungal species (6 molds, 1 yeast) were recovered. *Bacillus* spp., *Staphylococcus* spp. and *Klebsiella* spp. were the most frequently isolated bacteria. *Aspergillus* spp. and *Penicillium* spp. were the most frequently recovered fungi. The microbial species isolated are comparable with studies performed on horses in other areas.

Keywords: *Bacteria, Fungi, Conjunctiva, Working horse, Iran*

İran'da Klinik Olarak Sağlıklı Yük Atlarından İzole Edilen Konjunktival Bakteriyel ve Fungal Ajanlar

Özet

Bu çalışma, İran'da yaşayan sağlıklı yük atlarının gözünde bulunan bakteri ve mantar izolatlarının identifikasyonu amacıyla yapıldı. Klinik olarak sağlıklı görünen 45 atın konjunktival kesesinden 90 sürüntü örneği toplandı. Aerobik bakteri ve mantarlar yönünden ekimler yapıldı. 9 farklı bakteri (3 Gram-pozitif, 6 Gram-negatif) ve 7 farklı mantar türü (6 küf, 1 maya) izole edildi. En fazla izole edilen bakterinin *Bacillus* spp., *Staphylococcus* spp. ve *Klebsiella* spp. olduğu belirlendi. En yaygın izole edilen mantar türlerinin ise *Aspergillus* spp. ve *Penicillium* spp. olduğu tespit edildi. Bu mikroorganizmaların farklı ülkelerde yaşayan atlardan izole edilenler ile benzer olduğu anlaşıldı.

Anahtar sözcükler: *Bakteri, Mantar, Konjunktiva, Yük atı, Iran*

INTRODUCTION

Most reports of conjunctival bacterial flora in the healthy horses show predominance of Gram-positive organisms. The most commonly isolated bacteria include *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp. and *Corynebacterium* spp. [1-3]. From fungi genera of *Aspergillus*, *Cladosporium*, *Penicillium* and *Mucor* predominate in most studies [2-4]. When a corneal erosion/ulceration occurs, resident and transient ocular surface microbes penetrate the sub-epithelial tissue and result in infection [5]. Knowledge of normal conjunctival microflora is important to adopt proper treatment of corneal ulcers. This investigation was conducted to determine the fungal and aerobic bacterial flora in the conjunctival sac of healthy working horses in Iran.

MATERIAL and METHODS

Forty-five working horses, of different sexes (23 males and 22 females) and ages (1-20 years), without signs of ophthalmic abnormality, situated in rural areas of Urmia (northwest of Iran) were included in this study. During summer of 2012, samples from the conjunctival sac of both eyes of horses were obtained using a dry cotton swab and transported to the laboratory in tubes containing sterile transport medium. Swabs were plated in 5% ovine blood agar (Merck, Germany, catalog No. 940986), MacConkey agar (Merck, Germany, catalog No. 138521) and sabouraud dextrose agar (Merck, Germany, catalog No. 426638) and incubated at 37°C for 48 h for bacterial growth and at 25°C for 4 weeks for fungal growth. Colonies isolated from all plates were identified using standard microbiological and

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biochemical methods [6]. The study was approved by the ethics committee for animal experimentation by the Islamic Azad University-Urmia Branch (Serial No. 1714/2012).

RESULTS

The isolated organisms and the frequencies of isolation are shown in *Table 1* and *Table 2*.

Bacterial Isolates

Gram-positive organisms were the predominant bacteria, comprising 59.51% of isolates. *Bacillus* spp. (27.68%) was the most frequent isolate, followed by *Staphylococcus* spp. (24.22%). Gram-negative bacteria comprised 40.49% of all isolates, with *Klebsiella* spp. being the most prevalent (12.58%).

Table 1. *Isolated bacteria from normal conjunctiva of healthy working horses*
Tablo 1. *Sağlıklı iş atlarının normal konjunktivalarından izole edilen bakteriler*

Bacteria	Number of Isolates	Percent of Isolates
Gram-positive		
Bacillus cereus	80	27.68
Staphylococcus epidermidis	70	24.22
Beta-heamolytic streptococci	22	7.61
Gram-negative		
Klebsiella oxytoca	36	12.58
Escherichia coli	23	7.95
Providencia alcalifaciens	17	5.88
Enterobacter aerogenes	16	5.53
Citrobacter diversus	13	4.49
Proteus spp.	12	4.15
Total	289	100

Table 2. *Isolated fungi from normal conjunctiva of healthy working horses*
Tablo 2. *Sağlıklı iş atlarının normal konjunktivalarından izole edilen funguslar*

Fungi	Number of Isolates	Percent of Isolates
Mold		
Penicillium spp.	65	25.59
Aspergillus fumigatus	50	19.68
Aspergillus niger	44	17.32
Alternaria spp.	35	13.77
Aspergillus flavus	28	11.02
Mucor spp.	24	9.44
Yeast		
Candida spp.	8	3.14
Total	254	100

Fungal Isolates

Molds were the predominant fungi, comprising 96.85% of isolates. *Aspergillus* spp. (48.03%) was the most frequent isolate, followed by *Penicillium* spp. (25.59%). *Candida* spp. (3.14%) was the only isolated yeast species.

DISCUSSION

Bacillus spp. was the most frequently isolated bacterial organism in our study. This organism has been reported in several different studies as the most common isolate of conjunctival sac in healthy horses [1,3,7]. *Bacillus* spp. is usually considered as a non-pathogenic organism that isolate from healthy and diseased eyes [8].

Other Gram-positive isolates of this study especially Beta-heamolytic *streptococci* as potentially pathogens have been reported frequently from equine infectious keratitis [8,9].

Gram-negative bacteria are the most commonly isolated organisms from infectious keratitis of horses in various studies which include *Pseudomonas* spp., *Acinetobacter* spp., *Klebsiella* spp., *Escherichia coli*, *Moraxella* spp. and *Providencia* spp. [8,10]. Most of these potentially pathogenic organisms were isolated from eye of healthy horses in our study.

In various studies, molds vs. yeasts have been reported to be the predominant components of fungal flora in equine normal ocular surface by *Aspergillus* spp. having first rate [1,3,4]. In our study filamentous fungi with predominance of *Aspergillus* spp. were also the most frequently isolated fungi. Fungal genera isolated in this report are considered saprophytic; however, all of them had reported from equine keratomycosis [11,12].

The microbial species isolated in our study are comparable with studies performed on healthy horses in other countries. Most of these isolates are potentially pathogenic organisms.

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Kafkas Univ Vet Fak Derg
20 (4): 629-632, 2014
DOI: 10.9775/kvfd.2014.10692

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CASE REPORT

Simental Bir Buzağıda Görülen Çoklu Konjenital Anomaliler [1]

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[1] *25-28 Haziran 2013 tarihinde İstanbul'da düzenlenen VIII. Ulusal (Uluslararası katılımlı) Veteriner Anatomi Kongresi'nde poster bildiri olarak sunulmuştur*

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Makale Kodu (Article Code): KVFD-2014-10692

Özet

Bu olgu sunumunda; Simental bir buzağıda gözlenen çoklu konjenital anomaliler tanımlandı. Şekillenen anomalilerin çoğunlukla iskelet sisteminde yer aldığı saptandı. Buzağının ön ve arka bacak kemiklerinde çeşitli morfolojik bozukluklar ile birlikte bu kemiklere ait eklemlerin tamamında *arthrogryposis* şekillendiği gözlemlendi. İskelet sistemi dışındaki en önemli anomali; kalın bağırsaklarda *caecum*'dan önce, *caecum*'a benzer ve onunla aynı noktaya açılan bir bağırsak segmentinin daha mevcut olması idi. Kalpte ise musküler ventriküler septal defektin bulunduğu tespit edildi.

Anahtar sözcükler: *Caecum, Konjenital anomali, Simental buzağı, Ventriküler septal defekt*

Multiple Congenital Anomalies in a Simmental Calf

Summary

In this case presentation, multiple congenital anomalies observed in a Simmental calf were described. Most of the anomalies were recorded on the skeletal system. Various morphologic deformations were seen in the long bones of the front and hind limbs. *Arthrogryposis* was also detected on the joints of limbs. The most noteworthy anomaly outside the skeletal system was the presence of a *caecum*-like bowel segment beside the normal cecum, which both were open to the same location. In the heart, muscular ventricular septal defect was also noted.

Keywords: *Caecum, Congenital anomaly, Simmental calf, Ventricular septal defect*

GİRİŞ

Konjenital anomaliler (defekt, malformasyon) doğum esnasında mevcut olan yapısal ve fonksiyonel bozukluklardır [1]. Evcil hayvanlarda konjenital anomalilere sıklıkla rastlanmasına rağmen, multiple konjenital anomalilere oldukça az sayıda rastlanmaktadır [2,3]. Bu anomaliler çoğunlukla kas-iskelet, sindirim ve merkezi sinir sisteminde, az olarak da üro-genital sistem, göz ve deride meydana gelmektedir. Buzağılarda karşılaşılan başlıca konjenital anomaliler arasında *atresia recti, atresia ani, hernia umblicalis, bouleture, arqure, dermoid, rectovaginal fistül, arthrogryposis* ve *palatoschisis* sayılabilir [4,5].

Ruminantlarda görülen konjenital anomalilerin sebepleri tam olarak belirlenememekle birlikte, bu anomalilere mutasyon, kromozom anomalisi, viral ajanlar, çevresel faktörler ve gebeliğin erken dönemlerinde amniyon kesesine yapılan travmatik etkilerin neden olduğu bildirilmiştir [2,3,6].

Bu olgu sunumunda; Simental bir buzağıda şekillenen çoklu konjenital anomalilerin tanımlanması amaçlanmıştır.

OLGUNUN TANIMI

Olgu materyalini Kafkas Üniversitesi Veteriner Fakültesi Eğitim, Araştırma ve Uygulama Çiftliği'nde 2012 Nisan ayı içerisinde ölü doğan bir adet Simental ırkı erkek buzağı oluşturdu *(Şekil 1)*. Buzağının makroskobik değerlendirilmesi yapıldıktan sonra, gerekli görülen organlardan kesit alınarak histopatolojik muayene yapıldı.

Buzağıda, anomalilerin çoğunlukla iskelet sisteminde yer aldığı saptandı. Sağ-sol ön ve arka bacak uzun kemiklerinin hepsinde değişen derecelerde olmakla beraber kemiklerin orta kısımlarından veya orta kısmına yakın yerlerden kavis oluşturmak suretiyle yay şeklinde büküldüğü

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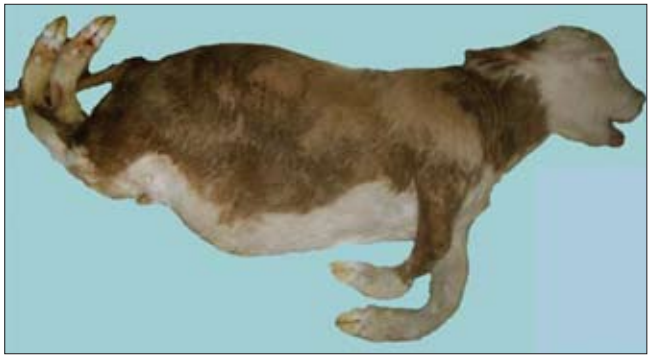
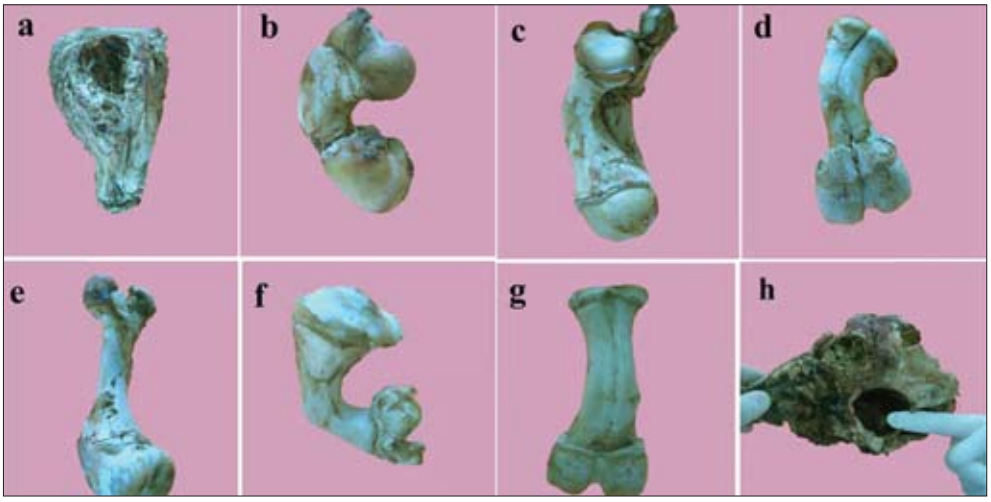
ve göreceli olarak daha kısa şekillendikleri dikkati çekti *(Şekil 2a-g)*. Bu kemiklerin eklem yüzeylerinin normale kıyasla genelde daha büyük şekillendiği ve anormal kemik yapısına paralel olarak morfolojik değişiklikler içerdiği gözlemlendi. *Corpus scapulae*'nın oldukça genişlemiş ve kemik doku içerisinde kanlı-seröz bir sıvının birikmiş olduğu görüldü *(Şekil 2a)*. *Corpus humeri*'nin cranial yönde aşırı bir dışbükeylik kazandığı belirlendi *(Şekil 2b)*. *Radius* ve *ulna*'ya bakıldığında ise her iki kemiğin *collum* ve *corpus* geçişinde dışbükeylik artışının olduğu saptandı *(Şekil 2c)*. *Metacarpus (III-IV)*'un *basis* ve *corpus ossis metacarpi* geçişinde *(Şekil 2d)*, *metatarsus (III-IV)*'un ise *corpus ossis metatarsi* seviyesinde medial yönlü bir eğikliğin olduğu gözlendi *(Şekil 2g)*. Arka bacak kemiklerinden *femur*'a bakıldığında *condylus ossis femoris*'in diğer kısımlara oranla oldukça genişlemiş olduğu tespit edildi *(Şekil 2e)*. Olguda *condylus tibiae*'nın diğer kısımlara göre belirgin derecede genişlediği ve *corpus tibiae*'daki bükülme nedeniyle *tibia*'nın 'C' harfi şeklini aldığı görüldü *(Şekil 2f)*. Ayrıca kemik morfolojisi bakımından anomalik olan eklemlerin tamamında da *arthrogryposis* şekillendiği gözlemlendi.



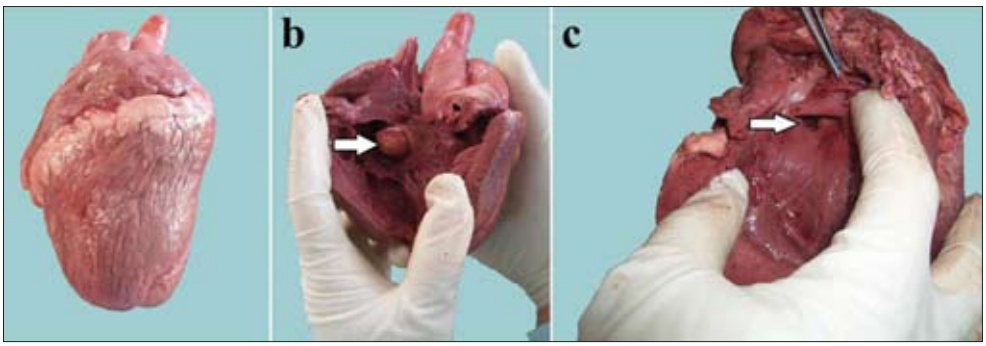
Şekil 1. Multiple konjenital anomalili buzağının genel görüntüsü
Fig 1. The calf with multiple congenital anomalia

Buzağının başı nasal ve frontal seviyede hafif derecede büyük olup hidrosefalik bir görünüm oluşturmaktaydı *(Şekil 1, Şekil 2h)*. Kafanın açılarak incelenmesinde bu hidrosefalik görüntünün beyinden değil, seröz karakterde sıvı ile dolu olan *frontal sinus*'ten kaynaklandığı saptandı *(Şekil 2h)*. Bu inceleme sırasında ayrıca *orbita*'nın arka duvarının kemik dokudan değil, yumuşak dokudan oluştuğu dikkati çekti *(Şekil 2h)*. Alt çenede *brachignatia* gözlemlendi. Apendiküler iskeletin dışında kalan iskelet bölümünde (*costa, vertebra, sternum*) yaygın bir ödemin varlığı tespit edildi. Kalpte, *apex cordis*'in genişlemiş olduğu görüldü *(Şekil 3a)*. Buzağılarda yaygın görülmesi nedeniyle anomali olarak kabul edilmemekle beraber *foramen ovale*'nin kısmen açık olduğu belirlenirken *(Şekil 3b)*, *ventriculus dexter et sinister* arasında *ostium aortae* seviyesinde düzgün sınırlı bir deliğin (musküler ventriküler septal defekt-VSD) olduğu belirlendi *(Şekil 3c)*. Sindirim sistemi ile ilgili olarak kalın bağırsaklarda iki *caecum* oluşumu tespit edildi *(Şekil 4)*. İkili olan *caecum*'dan büyük olanın *(Şekil 4a)* mikroskopik yapı olarak normal *caecum* morfolojisine sahip olduğu gözlendi. Burada organın mukoza ve submukoza katları ile musküler taba-



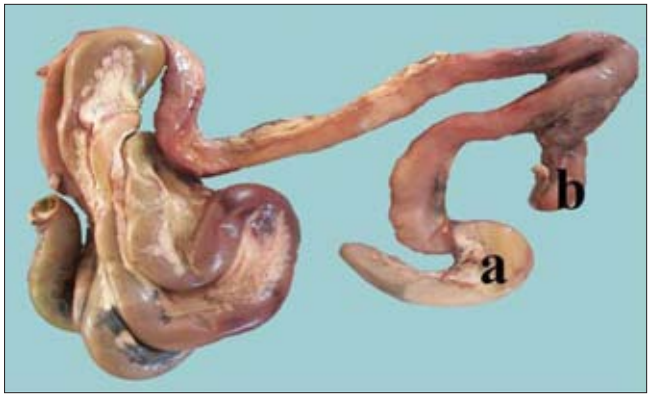
Şekil 2. Buzağı bacak kemiklerindeki yapısal bozukluklar, **a.** *scapula*, **b.** *humerus*, **c.** *antebrachium*, **d.** *metacarpus*, **e.** *femur*, **f.** *tibia*, **g.** *metatarsus*

Fig 2. Morphologic deformities in the limb bones of the calf. **a.** *scapula*, **b.** *humerus*, **c.** *antebrachium*, **d.** *metacarpus*, **e.** *femur*, **f.** *tibia*, **g.** *Metatarsus*



Şekil 3. Buzağı kalbinin görüntüsü, **a.** genişlemiş *apex cordis*, **b.** *foramen ovale*, **c.** ventriküler septal defekt

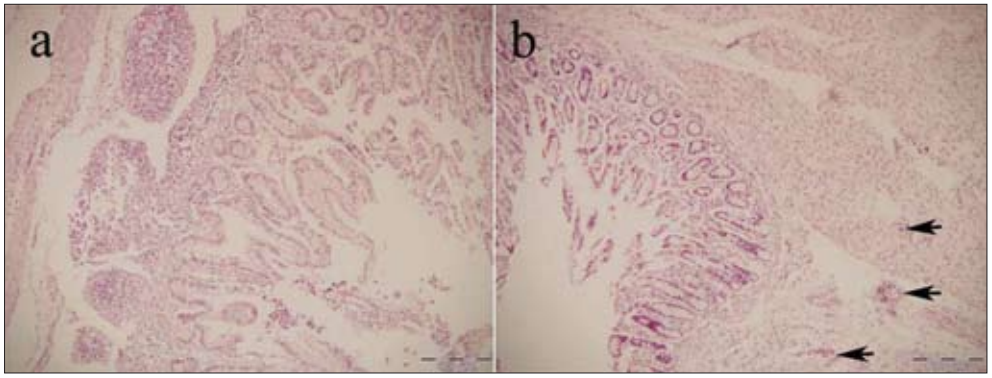
Fig 3. The heart of the calf. **a.** enlarged *apex cordis*, **b.** *foramen ovale*, **c.** ventricular septal defect



Şekil 4. Çift *caecum*, **a.** büyük *caecum*, **b.** küçük *caecum*
Fig 4. The double *caecum*, **a.** the big *caecum*, **b.** the small *caecum*

olarak gelişebilir [8]. Aynı zamanda primer miyopati [9] ve merkezi sinir sistemi gelişimindeki nörojenik aksaklıklar [10] da bu tür eklem bozukluklarına neden olmaktadır. Anomalileri sistematik olarak ele alınan bu buzağıda da apendiküler eklemlerin arthrogrypotik olduğu görülmüştür. Olguda sinir sisteminde makroskobik malformasyon gözlenmemiştir. Dolayısıyla olgudaki arthrogrypotik görünümün genetik veya beslenme bozukluklarına dayandığı söylenebilir.

Sindirim sistemi anomalileri konjenital anomaliler içerisinde görülme sıklığı bakımından ikinci sırada yer alır [11,12]. Bu olguda daha önce literatürde kayda geçmemiş bir bulgu olan *caecum*'un çift şekillenmesi ile karşılaşılmıştır. İki *caecum* arasında büyüklük farkı ile birlikte histolojik farklılıklara da rastlanmıştır.



Şekil 5. Çift caecum'un histomorfolojik görüntüsü. **a.** büyük *caecum*, **b.** küçük *caecum*. *Oklar:* küçük *caecum*'da submukozal mononuclear hücre odakları

Fig 5. The histomorphologic apperance of the double caecum. **a.** the big *caecum*, **b.** the small *caecum*. *The arrows:* submucosal mononuclear cellular foci in the small *caecum*

kalarının normal yapıda olduğu ve mukoza ile submukozada lenfoid odakların yer aldığı belirlendi *(Şekil 5a)*. Küçük *caecum*'da *(Şekil 4b)* ise mukoza tabakasında kriptlerin genel olarak diğer *caecum* yapısına benzerlik gösterdiği, ancak mukoza ve submukozada lenfoid odakların çoğu alanda mevcut olmadığı veya yer yer küçük hücre serpintileri şeklinde olduğu tespit edildi. İki *caecum* arasındaki diğer önemli bir fark ise eksternal musküler katın küçük *caecum*'da diğerine oranla oldukça kalın olarak şekillenmesiydi *(Şekil 5b)*. Her iki *caecum*'da kolumnar epitellerin yapı ve dizilimi ile Goblet hücrelerinin mevcudiyetinin birbiriyle uyumlu olduğu tespit edildi.

TARTIŞMA ve SONUÇ

Konjenital anomaliler, fertilitede azalma ve perinatal yavru kayıplarına neden olarak ekonomik zararlara yol açarlar. Bu ekonomik zararlar kimyasal, enfeksiyöz ve beslenme bozuklukları gibi nedenlerle oluşanlar kadar ciddi olmasa da bireysel yetiştiricilik için önemlidir [7]. Bireysel yetiştiricilik için ciddi seviyelerde kayıplara yol açan konjenital anomalilerin aslında nasıl ve neden şekillendiği kadar, ne derece etkilerinin olduğu da önemlidir. Bu bakımdan olgu sunumunda kullanılan hayvan materyalinin multiple konjenital anomalilerden etkilenme derecesi belirlenmiştir.

Arthrogryposis, genetik ve beslenme faktörlerine bağlı

Bu raporda Simental buzağı kalbinde, iki ventrikül arasında, *ostium aortae* seviyesinde muskuler ventriküler septal defektin olduğu görülmüştür. VSD kardiyak anomaliler içerisinde kalpte görülme sıklığı bakımından en çok görülen anomali çeşidi olarak kayda geçmiştir [13-15]. At, sığır ve insanlarda VSD' nin etiyolojisine dair çalışmalar devam etmektedir [16]. İnsanlarda bu duruma kromozom anomalilerinin sebep olabileceği (Trisomy 13 veya 5. kromozomun kısa uç anomalisi) belirtilmiştir [17]. VSD'nin oluşmasında otozomal dominant trait geninin mutasyonunun etkili olduğu da bildirilmiştir [18]. Sığırlarda VSD için Hereford ve Limousin ırklarında kalıtsal bir geçişin [13], Jersey ırkında da genetik predispozisyonun olabileceği öne sürülmüştür [19]. Bundan başka iki adet Holstein düve [20] ve bir adet Holstein buzağıda [21] VSD ile karşılaşılmasına rağmen, diğer ırklarla ilgili literatürde herhangi bir bilgi yoktur. Bu olguda da ilk kez bir Simental buzağıda VSD belirlenmiştir.

Yapılan bir çalışmada [16], on beş VSD'li hayvandan birinde musküler, diğerlerinde ise membranöz karakterli VSD tespit edilmiş, başka bir çalışmada [21] ise Holstein ırkı bir buzağıda membranöz VSD saptanmıştır. Bu vakada ise Simental ırkı bir buzağıda muskuler VSD ile karşılaşılmıştır.

Konjenital kardiyak anomaliler sığırlarda %0.17-2.7 görülme oranına sahiptir [15]. Bir kalpte VSD tek başına görülebildiği gibi, başka kardiyak anomaliler ile birlikte de görülebilir. Literatürde VSD'nin *aorta* dextropozisyonu,

patent ductus arteriosus [15,19], *persiste foramen ovale* [14,19] gibi kardiyak anomalilerle birlikte görüldüğü bildirilmiştir. Bu olguda ise VSD, membranöz *foramen ovale* ve *patent ductus arteriosus* ile birlikte görülmüştür.

Literatürde iskelet sistemi [22] ve bağırsak kanalı [23] ile ilgili anomalilerin sunulduğu çalışmalar mevcuttur. Bu olguda ise hareket sistemi, sindirim sistemi ve kardiyovasküler sistem anomalileri birlikte görülmüştür. Sistemlerin anomaliden bu kadar etkilenmesinin sebebi ise literatürde [24] de belirtilen, "çoklu konjenital anomalilerde vücudun bir kısmında oluşan anomali diğerlerine de öncülük eder" şeklindeki hipotez olarak düşünülmektedir.

Sonuç olarak, anatomik ve patolojik yönden değerlendirilen buzağıda kafatasının *frontal sinus*'lerden kaynaklı hidrosefalik görünümlü olduğu, gözün arka duvarının kemik dokudan değil bunun yerine yumuşak bir dokudan oluştuğu, bacak kemiklerinin normal şekillerde olmadığı, kalpte ventriküller arası deliğin (ventriküler septal defekt) bulunduğu ve *caecum*'un iki adet şekillendiği belirlenmiştir. Genel olarak ise anomalilerin iskelet sistemi, kardiyovasküler sistem ve sindirim sistemini etkilediği görülmüştür.

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Kafkas Univ Vet Fak Derg
20 (4): 633-639, 2014
DOI: 10.9775/kvfd.2013.10541

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

REVIEW

A Review on the Current Use of Alpha$_2$ Agonists in Small Ruminants

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Makale Kodu (Article Code): KVFD-2013-10541

Summary

In recent years, alpha$_2$ agonists are the most widely used as sedative and analgesic drugs in veterinary medicine because of several useful properties like fast onset, reversibility and analgesia. Alpha$_2$ agonists produce different actions by binding to their corresponding receptor subtypes located on various parts of the central nervous system. Ruminants are especially sensitive to alpha$_2$ agonists due to the distribution of the specific alpha$_2$ adrenoceptor subtypes, compared with other species. In ruminants, alpha$_2$ agonists are generally used as sedatives and analgesics for restraint, clinical diagnosis, and minor surgeries. Clinical experiments indicated that analgesia does not exist throughout the period of sedation, so these agents alone are not sufficient for painful or major surgical procedure. Epidural administration of alpha$_2$ agonists produced potent analgesia with minimal sedative or cardiovascular effects, and considered one of the most reliable techniques in ruminant. Alpha$_2$ agonists also used as a preanesthetic medication and have been obviously anesthetic sparing effects. They causes some unwanted effects, such as excessive saliva, bradycardia, depressed respiratory rate, decreased rectal temperature, hyperglycemia, uterine contractions and decreased ruminal and intestinal motility. These side effects are influenced by the doses and administration routes of alpha$_2$ agonists, and their selectivity to alpha$_2$ adrenoceptor subtypes. Attentions should be taken to avoid the use of these agents in sick patients, while careful monitoring of the patient condition is always mandatory after receiving these agents. Fortunately, the availability of specific antagonists assures the uses of alpha$_2$ agonists in ruminants. For the safety, an appropriate low dose of alpha$_2$ agonists is always recommended in ruminants.

Keywords: *Alpha$_2$ adrenergic agonists, Alpha$_2$ receptor, Sedation, Analgesia, Premedications, Anesthesia, Ruminant*

Alfa$_2$ Antagonistlerinin Küçük Ruminantlarda Kullanımı Üzerine Bir Derleme

Özet

Son yıllarda alfa$_2$ antagonistleri çabuk etki, geridönüşebilirlilik ve analji gibi bazı özellikleri nedeniyle veteriner hekimlikte sedatif ve analjezik olarak en yaygın kullanılan ilaçlardır. Alfa$_2$ antagonistleri merkezi sinir sisteminin çeşitli bölgelerinde yer alan ilişkili reseptörlere bağlanmak suretiyle değişik reaksiyonlara neden olur. Ruminantlar diğer türlerle karşılaştırıldığında spesifik alfa$_2$ adenoreseptör subtipleri nedeniyle alfa$_2$ antagonistlerine karşı özellikle sensitiflerdir. Ruminantlarda alfa$_2$ antagonistleri genel olarak zapturapt, klinik tanı ve küçük cerrahiler amacıyla sedatif ve analjezik olarak kullanılır. Klinik deneyler analjezinin sedasyonun süresinin tümü boyunca mevcut olmadığını göstermektedir. Bu nedenle bu maddeler acılı veya büyük cerrahi müdahalelerde tek başlarına yeterli değillerdir. Alfa$_2$ antagonistlerinin epidural uygulanması minimal sedatif veya kardiyovasküler etkiler ile birlikte muhtemel analjezi üretmektedir. Bu uygulama ayrıca ruminantlarda en güvenilir teknik olarak kabul edilmektedir. Alfa$_2$ antagonistleri ayrıca preanastetik uygulama olarak da kullanılmaktadır ve bariz olarak anestetik ajanın az kullanımının sağlayıcı etkileri vardır. Bu ilaçlar fazla salivasyon, bradikardi, azalmış respirasyon oranı, azalmış rektal derece, hiperglisemi, uterus kontraksiyonları ve azalmış rumen ve barsak motilitesi gibi istenmeyen etkilere neden olabilir. Bu yan etkiler alfa$_2$ antagonistlerinin dozuna ve uygulanma yoluna ve alfa$_2$ adrenoseptör subtiplerine karşı seçiciliği ile ilişkilidir. Bu ilaçların hasta hayvanlarda kullanılmasından kaçınma konusunda dikkatli davranılmalıdır ve bu ilaçların alınmasını takiben hastanın durumu daima kontrol edilmelidir. Spesifik antagonistlerin mevcudiyeti ruminantlarda alfa$_2$ antagonistlerin kullanılabilmesinde güvence oluşturur. Güvenlik amacıyla alfa$_2$ antagonistlerinin uygun düşük dozu daima ruminantlarda tavsiye edilmektedir.

Anahtar sözcükler: *Alpha$_2$ adrenerjik agonistleri, Alpha$_2$ reseptör, Sedasyon, Analjezi, Premedikayon, Anestezi, Ruminant*

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INTRODUCTION

General anesthesia (GA) is not commonly used in ruminants as its administration results several side effects such as ruminal tympany, regurgitation of reticuloruminal contents, aspiration of refluxed material or saliva, hypoventilation, hypotension and fluid and electrolyte imbalances. The course of anaesthesia is generally complicated and may even endanger the animal's life. Therefore, more caution should be taken while using GA in ruminants. So far, there are very few anesthetic drugs that are licensed for use in ruminants. Among them, alpha$_2$ agonists are one class of sedative and analgesic drugs widely used in veterinary practices because of several useful properties, including fast onset, reliability, analgesia and reversibility [1].

The alpha$_2$ agonists are non-irritant so it could be administered intravenously (IV), intramuscularly (IM) or sub-cutaneously (SC). Alpha$_2$ agonists facilitate the restraint of animals for minor surgical and diagnostic procedures as well as reduced the requirements of injectable and inhalant agents [2].

The sedative and analgesic effects exerted by alpha$_2$ agonists are due to binding to their corresponding receptors. With the development of the molecular biological techniques, numerous subtypes of alpha$_2$ adrenoreceptors (alpha$_{2A}$, alpha$_{2B}$, alpha$_{2C}$, and alpha$_{2D}$) have been found, which developed the knowledge for selective agonists and antagonists. Xylazine hydrochloride is an alpha$_2$-agonist firstly used in ruminant anesthesia. In recent years, detomidine, medetomidine and dexmedetomidine have been reported to use in ruminants for sedation and analgesia [3-5]. Because of variation in the distribution, subtypes of the alpha$_2$ adrenoceptor and their affinities to corresponding ligands, each alpha$_2$ agonists causes dissimilar physiologic effects, including sedation, analgesia, muscle relaxation and unwanted effects (excessive saliva, bradycardia, hypotension, depressed respiratory rate, decreased rectal temperature, hyperglycemia, uterine contractions, decreased ruminal and intestinal motility, and increased urination frequency [5]. In addition, its doses and routes are key factors influencing these effects [6]. Fortunately, the development of specific antagonists expands the safety margin of alpha$_2$ agonists and hence there is an increasing interest to the use of alpha$_2$ agonists in ruminants [7,8].

The purpose of the article is to provide an overview of the main physiological effects of alpha$_2$ agonists and to give a summary of the current scientific and clinical uses of these agents in ruminants.

ALPHA$_2$ ADRENOCEPTOR SUBTYPES AND THEIR AGONISTS

Alpha adrenoreceptors are found in both the central nerve system and peripheral tissues. According to nerve synaptic distribution, they are divided into two subtypes alpha$_1$ and alpha$_2$. Alpha$_1$ adrenoreceptors are found mostly postsynaptically. Alpha$_2$ adrenoreceptors, typically sited presynaptically and can also occur postsynaptically. Alpha adrenoreceptors are bound and activated by their corresponding ligands (alpha$_1$ and alpha$_2$ adrenoceptor agonists). In the central nervous system, alpha$_1$ adrenoreceptor agonists bind to alpha$_1$ receptors, and exert excitatory functions (arousal, restlessness, increased locomotors activity) [9], while alpha$_2$ adrenoreceptor agonists bind to alpha$_2$ receptors which mainly produce inhibitory functions. The beneficial physiological effects of alpha$_2$ adrenoreceptor agonists include sedation, analgesia and muscle relaxation [1].

The alpha$_2$ agonists like as xylazine, detomidine, medetomidine and dexmedetomidine are clinically licensed for use in animals while clonidine and romifidine are available only for human. Clinically, the degree of sedation and analgesia produced by alpha$_2$ agonists are related to the individual selectivity and affinity of alpha$_2$ agonists between the alpha$_1$ and alpha$_2$ receptor binding sites. The order of alpha$_1$ and alpha$_2$ selectivity for alpha agonists are medetomidine (1620:1), detomidine (260:1), clonidine (220:1), romifidine (340:1) and xylazine (160:1) [10]. Studies have demonstrated that central alpha$_1$ adrenoceptor stimulation antagonizes the hypnotic response to even potent alpha$_2$ agonists, such as dexmedetomidine, and that the alpha$_1$ adrenoceptor effects will predominate with increased or toxic doses of alpha$_2$ agonists. With the advent of the molecular biological techniques, numerous alpha$_2$ adrenoceptor subtypes are found. These include alpha$_{2A}$, alpha$_{2B}$, alpha$_{2C}$, and alpha$_{2D}$ [11,12]. Alpha$_{2A}$ receptors are most prevalent in the CNS, whereas alpha$_{2B}$ receptors are most prevalent peripherally. Alpha$_{2A}$, alpha$_{2B}$, and alpha$_{2C}$ receptors are thought to be involved in the analgesic actions of alpha$_2$ agonist drugs. Different species have different receptor subtypes, distributions and receptor densities [10].

In the CNS, alpha$_2$ agonists are responsible for different actions after binding to their corresponding receptor subtypes. The diversity in alpha$_2$ adrenoceptor subtypes and distribution has led to considerable differences in dose and overall effects of alpha$_2$ agonists in the various species. For example, alpha$_{2A}$ subtype predominated in canine and rat brainstems while the alpha$_{2D}$ subtype appears to predominate in the sheep brainstem [13,14]. Perhaps, this difference in subtypes gives an explanation why ruminants have greater sensitivity to alpha$_2$ agonists requiring less dose to produce the sedative effects as compared to other species [15].

USES OF ALPHA$_2$ ADRENERGIC AGONISTS

The use of alpha$_2$ agonists in veterinary practices

were first reported in late 1960 [16] and revolutionized for sedation and analgesia, particularly in large animals. Since then, alpha$_2$ agonists have been used for profound sedation and analgesia [5].

Sedative Effects

The interests in the use of alpha$_2$ agonists in veterinary practices are related to the ability of these drugs to produce reliable sedation and anxiolysis. These effects are mediated by the activation of the alpha$_{2A}$-adrenergic receptor subtype found in the locus coeruleus neurons in the pons and lower brainstem causing a decrease in norepinephrine release [17-19]. Centrally, norepinephrine is necessary for arousal. If the release of norepinephrine is blocked, the net result is sedation. Sometimes, failure to achieve deep sedation may be due to preexisting stress or pain and excitement, because all of these conditions increase endogenous catecholamine levels that interfere with the alpha$_2$-agonist-induced reduction of excitatory neurotransmitter release. In animals, the sedative effect is well achieved, when these agents are given in calm and quiet surroundings with minimal environmental stress [20].

The behavioral events are similar for goats administered any of the alpha$_2$ agonists, and include an initial appearance of nervousness followed by lowering head, protrusion of the nictitating membrane and tongue, partial drooping of the eyelids, and animals rapidly become ataxic [18]. There is generally a reduced awareness of the environment, although a response may occur to stimulation such as noise or touch. For all the drugs, the onset of sedation is slower following intramuscular administration [21-24].

Ruminants have a remarkable alpha$_{2D}$-adrenergic receptor, which makes them sensitive to the sedative effects. Several workers have investigated the effect of medetomidine in ruminants. The sedative effects were noted in 9 min and lasted for 90-120 min after administration of medetomidine (40 µg/kg, IM) in sheep [21]. The sedative effects of xylazine (0.1 mg/kg) and detomidine (50 µg/kg) in goats was observed with maximal effect within 5 to 12 min, and lasted for 77 to 85 min respectively [5].

Analgesic Effects

Alpha$_2$ agonists produce analgesia by stimulating receptors within the brain and spinal cord [22]. Studies showed that alpha$_2$ agonists bind with the receptors in the substantia gelatinosa of the dorsal horn of spinal cord [25] and in the brainstem, where modulations of nociceptive signals are likely to be started [26]. It is further indicated that presynaptic and postsynaptic inhibitory effect of alpha$_2$ agonists are responsible for antinociceptive action [27].

The modulation of pain is also involved in an interaction between alpha$_2$ adrenoreceptors and opiate receptors in brain and spinal cord [28,29]. Alpha$_2$ and opioid receptors are found in similar regions in the brain (even on same neurons), and inferred to share common molecular machinery beyond the receptors. Alpha$_2$ agonists or opioid agonists bind to their receptors, and activate signal transduction systems (membrane associated G proteins), which induces a chain of events that open potassium channels in the neuronal membrane. Activation of these channels in the postsynaptic neurons leads to hyperpolarization of the cells, which ultimately makes the cells unresponsive to the excitatory input and effectively blocks the pain pathway [9]. Consequently, the alpha$_2$ agonists and opioid agonists produce analgesia by similar mechanisms [9,23].

Clinical experiments indicate that analgesia does not exist throughout the entire period of sedation, and these agents alone are incompatible for painful or major surgical procedure. It is important to note that the analgesic effects last for half the duration of the sedation. Shah *et al.*[5] observed sedation after IV administrations of xylazine and detomidine in goats are 77 min and 85 min while skin analgesia remained only for 40 min and 33 min respectively. The analgesic effect produced by medetomidine is more profound and longer than that mediated by xylazine. The therapeutic response of alpha$_2$ agonists for the treatment of acute and chronic pain has been demonstrated as their usefulness by parenteral and epidural route [30,31].

Epidural administration of alpha$_2$ agonists produced potent analgesia by activation of alpha$_2$ adrenergic receptors ($\alpha_{2A/D}$, α_{2C}) in the substantia gelatinosa of the dorsal horn in spinal cord and inhibits the release of norepinephrine [32]. It has minimal sedative or cardiovascular effects [33] and considered as one of the most reliable techniques for regional analgesia in calves, sheep and goats for all surgical procedures caudal to the umbilicus [34,35].

Several alpha$_2$ agonists have been epidurally or sub-arachnoidly used for analgesia. It is reported that epidural administration of xylazine causes profound analgesia, relaxation of constrictor vulvae muscle, anal sphincter and urinary bladder [18,36]. Prado *et al.*[37] indicated that detomidine caused perineum and flank analgesia in cattle. Tranquilli *et al.*[38] used xylazine (0.05 mg/kg) or medetomidine (0.01 mg/kg) subarachnoidly in goats, and found the sensory blockage of nociceptive impulses for more than 2 h. However, their absorption from epidural space has apparently causes cardiopulmonary depression in the ruminants. Epidural administration of clonidine did not affect regional blood flow to the spinal cord in sheep [39]. Romifidine produced mild to moderate degrees of hindquarter analgesia within 5.2 min in goats when administered epidurally [40]. But this effect persisted shorter than intrathecal injection of xylazine (0.05 mg/kg) and medetomidine (10 µg/kg) in goats [41].

Epidural administration of alpha$_2$ agonists, alone, or in combination with local anesthetics causes bradycardia, respiratory acidosis, reduced ruminal motility, and increased urination frequency [6,42-44]. These side effects

are generally well tolerated by young, healthy animals while significant morbidity and mortality may occur in patients having poor cardiopulmonary functions [6]. However, at appropriate dose, epidural anesthesia-with local anesthetics produces minimal hemodynamic and respiratory changes in conscious animals [35].

Anesthetic Sparing Effects

Alpha$_2$ agonists as a preanesthetics medication have been obvious anesthetic sparing effects for injectable as well as inhalant agents. However, these effects are associated with the affinity of the drugs for the alpha$_2$ adrenoreceptors [45]. That is, more specific to the alpha$_2$ agonists, and betters the anesthetic sparing effect [46].

Xylazine as a preanesthetics can reduce the amount of anesthetics (such as thiopental) required by 25% to 50% of the original dosage. This decrease depends upon the dose of xylazine, species, and the speed with which the anesthetic is given. The dose level of 0.04-0.6 mg/kg IV of xylazine is usually given prior to 2.2 to 4.4 mg/kg of IV ketamine in sheep and goats. This has resulted in satisfactory induction of anesthesia persisting for 10-15 min [6,15]. The amount of halothane requirements for anesthesia (MAC) is reduced nearly by 40% and that of barbiturate reduced by one-third to be one-half of the estimated doses when xylazine is used as premedication [46]. One another study reported premedication of xylazine lead to 50% of a reduction in methohexital dose [47]. As premedication, romifidine (20 µg/kg) reduced the induction dose of propofol by 60% [48], and medetomidine (1 µg/kg/IV) reduced 38% of induction dose of propofol [49]. Although alpha$_2$ agonists and inhalant anesthetics do not share common receptors, as demonstrated by the inability of alpha$_2$ antagonists to reverse halothane anesthesia. However, a synergism may exist between these agents, since both increase potassium conductance and induce neuronal hyperpolarization in the brain [50].

SIDE EFFECTS OF ALPHA$_2$ AGONISTS

Cardiovascular Effects

The cardiovascular effects of all alpha$_2$ agonists include bradycardia and associated bradyarrhythmia (1st and 2nd degree atrioventricular heart block), a dramatic reduction in cardiac output by up to 50% (L blood/min), and an increase in systemic vascular resistance [51,52]. Several mechanisms contribute to induce bradycardia, including a decrease in sympathetic outflow from CNS, the direct depression of cardiac pacemaker and conduction, the inhibition of norepinephrine release from sympathetic nerve terminals, and an increase in the release of acetylcholine from parasympathetic nerves in heart [53,54]. Systemic vascular resistance is due to vasoconstriction in response to stimulation of alpha$_2$ receptors on the vascular smooth muscles. The decrease in cardiac output is initially concerned to baroreceptor reflex and central sympatholytic action leading decreased heart rate. Alpha$_2$ agonists also affect receptors exiting in the heart and blood vessels resulting significant cardiovascular side effects. These effects of alpha$_2$ agonists are mainly dose- and route-dependent, although IM administration of drug has less effect as compared to IV. The increases in arterial blood pressure, which is typically dose related particular with the higher dose, caused a more pronounced stimulation of peripheral adrenoreceptors and vasoconstriction [51,52]. The initial hypertension is greater when the alpha$_2$-agonist is administered IV and there is a 26% increase in MAP after IV administration of medetomidine or xylazine as compared to an 18% increase when the same doses were administered intramuscularly. The differences in the initial blood pressure response with different routes are likely related to variations in the speed of uptake and absorption of the drug on the overall effect at the peripheral adrenoreceptors [55]. Based on these findings, it is recommended that lowered doses of alpha$_2$-agonists when administered IM, avoid extremes of blood pressure [24,55].

Xylazine in sheep has minor cardiovascular side effects at the dose of 0.15 mg/kg IV, while at 0.5 mg/kg IV decreased heart rate by 25% and cardiac output by 37% and retained up to 1 h. Xylazine (0.05 mg/kg IM) caused only minor cardiovascular changes [56]. Jansen *et al.*[57] reported that xylazine (0.2 mg/kg IM) in pregnant ewes decrease maternal (30%) and fetal (20%) heart rate. The specific alpha$_2$ agonists, i.e. dexmedetomidine, indicate a beneficial effect of alpha$_2$ agonists in high-risk patients. In one clinical trial in human, it is confirmed that peri-operative administration of dexmedetomidine reduced a plasma catecholamine level by 90%, and thereby decreased intra-operative hypertension and tachycardia compared with placebo [58].

Premedication with atropine has been shown to prevent the 2nd degree heart block [59], however, the use is rather controversial, because atropine blocks transmission of vagal impulses to the heart, animals with a preexisting high vagal tone would show a relatively greater tachycardia than those with low vagal tone. Cardiac output tends to increase with atropine primarily because of the increase in heart rate. Large doses of atropine are directly depressant to the myocardium and also cause cutaneous dilation as a result of a direct vascular smooth muscle effect [15].

Alpha$_2$ agonists should not be used in animals with compromised cardiac output, such as preexisting heart disease, especially those with Brady arrhythmia, poor myocardial contractility, obstructive valvular disease, dehydration, hypovolemia, or sepsis. Attentions should be taken to avoid these agents in geriatric, pediatric, pregnant or diabetic patients. Careful monitoring of the patient's condition is always important after receiving these agents.

Fortunately, reversing agents are available to antagonize the adverse effects [60-63].

Respiratory Effects

Almost all alpha$_2$ agonists cause some respiratory side effects, due to secondary depression of CNS [51,60]. The respiratory effects are species specific [61,64]. In ruminants, alpha$_2$ agonists can cause bronchoconstriction and an increase in pulmonary vascular resistance, leading to pulmonary edema and impaired oxygenation of blood with resultant hypoxaemia. Care must be taken when used in ruminants, especially small ruminants [10]. In sheep, xylazine can cause rapid increases in respiratory rate, airway pressure, and pulmonary elastics with slight changes in $PaCO_2$. Hypoxemia is frequently observed, terminating in fulminant pulmonary edema and death. Within 3 min after administration, sheep have activation of pulmonary intravascular macrophages, and interstitial and alveolar edema [65].

Dexmedetomidine decreases respiratory rate in sheep and goat [66]. Several investigations have demonstrated a mild to severe decrease in arterial PO_2 [67]. The decrease in arterial oxygenation may be gradual and not recognized until the animal collapses. Alpha$_2$ agonists decrease the gastrointestinal motility, resulting in bloat, which further impair ventilation. Acute pulmonary edema, manifested as wheezy, labored breathing, has been reported in sheep 15 min after injection of xylazine [68].

The irregular breathing and reduced ventilation rate have been observed after epidural administration of a lower dose of alpha$_2$ agonists [36,41]. Researchers reported tachypnea, dyspnea and transient apnea followed by irregular and deep breathing in goats and other species. Besides these, there were a concurrent decrease in tidal volume in sheep and goats [20,69,70]. Administration of alpha$_2$ antagonists reversed the sedation however the respiratory effects were not completely abolished [60,62].

Other Side Effects of Alpha$_2$ Agonists

Alpha$_2$ agonists cause hyperglycemia, increase uterine contractions which may lead to premature abortion, and a decrease in intestinal motility. They inhibit sympathetic outflow and modulate the stress response to anesthesia and surgery. Alpha$_2$ agonists cause hyperglycemia by direct inhibition of insulin release from β-cells within the pancreas [71,72], increased production of glucose by the liver or a rise in adrenocortical hormones due to stress [73]. Alpha$_2$ agonists also increase growth hormone secretion, which may be contributed to the hyperglycemic effect. Polyuria is reported in goats within 20 min and continued till 70 min after xylazine administration (0.1 mg/kg) [74]. Mohammed and Yelwa, reported that polyuria is associated with hyperglycemia [70].

Alpha$_2$ agonists can cross the placenta, increase uterine blood flow and contraction by stimulating alpha$_2$ adrenoreceptors. Due to high lipid content, they may be partly trapped in the placenta. So there is a tiny amount in the fetal circulation. The effect of drugs to stimulate alpha$_2$ adrenoreceptors depend on estrogen which increase sensitivity of the alpha$_2$ adrenoreceptors; a high level of progesterone during pregnancy increases the sensitivity of alpha$_2$ adrenoreceptors and actually decreases the contractility of the uterus [75]. Xylazine like oxytocin causes contraction of the bovine uterus [32]. There has been a report of cows going into premature labor [76].

ANTAGONISM

Alpha$_2$ adrenergic receptor antagonists are used primarily in veterinary medicine to reverse the effects and expand the safety margin of alpha$_2$ agonists. Atipamezole, yohimbine, tolazoline and idazoxan are antagonists used commonly in ruminants [63,73].

The beneficial effects of atipamezole or yohimbine come from their specific alpha$_2$ adrenoceptor antagonist action in goats and sheep [77,78]. In sheep, yohimbine (0.2 and 0.4 mg/kg/IV) is effective to prevent bradycardia induced by xylazine [79]. Atipamezole a more potent having alpha$_2$ and alpha$_1$ selectivity with a ratio of 200:300 times higher than yohimbine requiring a lower dose [80]. Therefore, atipamezole is useful for antagonizing the CNS depressant effects of medetomidine in goats [81].

The combined uses of two kinds of alpha$_2$ adrenoceptor antagonists have better reversal effect. Atipamezole (0.2 mg/kg) and yohimbine (0.2 mg/kg) administration in goats significantly reduced recumbency period and stand within 1 min. Kinjavdekar *et al.*[82] reported that yohimbine (0.25 mg/kg IV) and atipamezole (0.005 mg/kg IV) antagonize the epidural effect of medetomidine (0.01 mg/kg) in goats. Tolazoline is an imidazole derivative (alpha$_1$ and alpha$_2$) adrenoreceptor antagonist and considered as a useful antagonist for sedation and side effects induced by xylazine in various species [23].

All alpha$_2$ antagonists cause CNS excitement, vasodilation and tachycardia. Therefore alpha$_2$ receptor antagonist should be given slowly to minimize adverse effects. Alternatively, a fractional dose may be given slowly IV and the rest through IM to minimize side effects [15].

CONCLUSION

Alpha$_2$ agonists are commonly used in veterinary practices for sedation, analgesia and muscle relaxation. The pharmacological activities of all alpha$_2$ agonists are almost similar, but some differences exist due to their selectivity for specific receptors. Alpha$_2$ agonists are routinely used in combination with other anaesthetic agents to reduce dose and side effects of the other agents.

Physiological alterations induced by alpha$_2$ agonists totally depend on its dose and route, and used alone or in combination with other sedatives or anesthetics. The lowest possible dose required to achieve the desired depth and duration of action for a given procedure should be used. When these factors are appropriately considered, the risks caused by alpha$_2$ agonists can be minimized. Alpha$_2$ antagonists produced reversal effects for sedation, CNS depression and cardiopulmonary changes induced by alpha$_2$ agonist. It is concluded that alpha$_2$ agonists are safe to use in ruminant in a field as well as hospital conditions.

Conflict of Interest Statement

The authors declare that they have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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YAZIM KURALLARI

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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.
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