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 : 3 MAYIS - HAZİRAN / MAY - JUNE
Yıl / Year : 2014

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

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

JOURNAL OF THE FACULTY OF VETERINARY MEDICINE, KAFKAS UNIVERSITY

(MAYIS - HAZİRAN)
(MAY - JUNE)

Cilt/Volume: 20 Sayı/Number: 3 Yıl/Year: 2014

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

- **Science Citation Index Expanded (also known as SciSearch®)**
- **Journal Citation Reports/Science Edition**

This journal is also indexed and abstracted in:

- **ELSEVIER**
- **CAB Abstracts**
- **TÜRKİYE ATIF DİZİNİ**
- **ULAKBİM-TÜBİTAK**
- **EBSCO**

YAZIŞMA ADRESİ (ADDRESS FOR CORRESPONDENCE)

Kafkas Üniversitesi Veteriner Fakültesi Dergisi Editörlüğü
36040 - Kars / TÜRKİYE
Phone: +90 474 2426807-2426836/5228
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

DANIŞMA KURULU
(Advisory Board)

Prof.Dr. Kemal AK	İstanbul Üniversitesi Veteriner Fakültesi
Prof.Dr. Harun AKSU	İstanbul Üniversitesi Veteriner Fakültesi
Prof.Dr. Belma ALABAY	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Mustafa ALİŞARLI	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
Prof.Dr. Feray ALKAN	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Çiğdem ALTINSAAT	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Kemal ALTUNATMAZ	İstanbul Üniversitesi Veteriner Fakültesi
Prof.Dr. Mustafa ARICAN	Selçuk Üniversitesi Veteriner Fakültesi
Prof.Dr. Mustafa ATASEVER	Atatürk Üniversitesi Veteriner Fakültesi
Prof.Dr. Sırrı AVKİ	Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
Prof.Dr. Les BAILLIE	Cardiff School of Pharmacy & Pharmaceutical Sciences
Prof.Dr. Metin BAYRAKTAR	Fırat Üniversitesi Veteriner Fakültesi
Prof.Dr. Burhan ÇETİNKAYA	Fırat Üniversitesi Veteriner Fakültesi
Prof.Dr. İbrahim DEMİRKAN	Afyon Kocatepe Üniversitesi Veteriner Fakültesi
Prof.Dr. Nazir DUMANLI	Fırat Üniversitesi Veteriner Fakültesi
Prof.Dr. Hasan Hüseyin DÖNMEZ	Selçuk Üniversitesi Veteriner Fakültesi
Prof.Dr. Emrullah EKEN	Selçuk Üniversitesi Veteriner Fakültesi
Prof.Dr. Hüdaverdi ERER	Selçuk Üniversitesi Veteriner Fakültesi
Prof.Dr. Ayhan FİLAZİ	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Aytekin GÜNLÜ	Selçuk Üniversitesi Veteriner Fakültesi
Prof.Dr. Ekrem GÜREL	Abant İzzet Baysal Üniversitesi Fen Edebiyat Fakültesi
Prof.Dr. Tolga GÜVENÇ	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
Prof.Dr. Armağan HAYIRLI	Atatürk Üniversitesi Veteriner Fakültesi
Prof.Dr. Ali İŞMEN	Çanakkale Onsekiz Mart Üniversitesi Su Ürünleri Fakültesi
Prof.Dr. Hakkı İZGÜR	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Zafer KARAER	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Arif KURTDEDE	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Erdoğan KÜÇÜKÖNER	Süleyman Demirel Üniversitesi Mühendislik Fakültesi
Prof.Dr. Mehmet MADEN	Selçuk Üniversitesi Veteriner Fakültesi
Prof.Dr. Kamil ÖCAL	Adnan Menderes Üniversitesi Veteriner Fakültesi
Prof.Dr. Metin PETEK	Uludağ Üniversitesi Veteriner Fakültesi
Prof.Dr. Sevim ROLLAS	Marmara Üniversitesi Eczacılık Fakültesi
Prof.Dr. Berrin SALMANOĞLU	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Sabine SCHÄFER-SOMI	University of Veterinary Medicine Vienna
Prof.Dr. Nesrin SULU	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Ayşe TOPAL	Uludağ Üniversitesi Veteriner Fakültesi
Prof.Dr. Ş. Doğan TUNCER	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Cevdet UĞUZ	Afyon Kocatepe Üniversitesi Veteriner Fakültesi
Prof.Dr. Zafer ULUTAŞ	Gaziosmanpaşa Üniversitesi Ziraat Fakültesi
Prof.Dr. Rıfat VURAL	Ankara Üniversitesi Veteriner Fakültesi
Prof.Dr. Halis YERLİKAYA	Fırat Üniversitesi Veteriner Fakültesi

İÇİNDEKİLER
(Contents)

Kafkas Univ Vet Fak Derg
20 (3): 331-336, 2014
DOI: 10.9775/kvfd.2013.9988

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

RESEARCH ARTICLE

Effects of *Saccharomyces cerevisiae* and *Spirulina platensis* on Growth Performances and Biochemical Parameters in Rabbits [1]

Nilay SEYİDOĞLU [1] Nurten GALİP [1]

[1] *This study was financed by the Research Fund of Uludag University in Bursa, Turkey (Project No: 2011/19)*

[1] Uludag University, Faculty of Veterinary, Department of Physiology, TR-16059 Campus, Bursa - TURKEY

Makale Kodu (Article Code): KVFD-2013-9988

Summary

A feeding trial was conducted to evaluate the effect of live yeast culture *Saccharomyces cerevisiae* (SC) and *Spirulina platensis* (SP) on the growth performance and serum biochemical parameters in rabbits. Forty, male New Zealand white rabbits, aged 5-6 weeks, were studied in 4 groups. The groups; I. Control (basal diet), II. SC (added 3 g/kg diet), III. SP (added 5% of the diet), IV. SC and SP (added 3 g/kg SC and 5% SP of the diet), respectively. The experiment lasted for 90 days. Blood samples were obtained by ear venipuncture on the 90th day. Also final body weight, total weight gain, total feed intake and feed conversion ratio were evaluated at the each month of the 90th day trial. There were no significant differences occurred in growth performances and biochemical parameters, but serum globulin value decreased and albumin globulin ratio increased in SP and SC+SP groups ($P<0.05$). More studies would be necessary to elucidate the effects of supplementing spirulina on growth and determine the optimum dietary concentration in animals.

Keywords: *Rabbits, Saccharomyces cerevisiae, Spirulina platensis, Growth performance, Serum biochemical parameters*

Tavşanlarda *Saccharomyces cerevisiae* ve *Spirulina platensis*'in Büyüme Performansı ve Serum Biyokimyasal Parametreleri Üzerine Etkileri

Özet

Bu araştırma canlı maya kültürü *Saccharomyces cerevisiae* (SC) ve *Spirulina platensis* (SP)'in tavşanlarda büyüme performansı ve serum biyokimyasal parametreleri üzerine etkilerini değerlendirmek için yapıldı. 40 tane, 5-6 haftalık beyaz Yeni Zelanda tavşanı 4 gruba ayrıldı. Gruplar sırasıyla; I. Kontrol (bazal diyet), II. SC (3 g/kg yeme ilave edildi), III. SP (%5 oranında yeme ilave edildi), IV. SC ve SP (SC 3 g/kg ve SP %5 oranında yeme ilave edildi) olarak belirlendi. Çalışma 90 gün sürdü. 90. gün sonunda kulak venasından kan örnekleri toplandı. Ayrıca, doksan günlük çalışmada her ay, son canlı ağırlık ile toplam canlı ağırlık artışı ve toplam yem tüketimi ile yemden yaralanma değerlendirildi. Büyüme parametreleri ve biyokimyasal parametrelerde önemli farklılık bulunamadı fakat SP ve SC+SP gruplarında serum globulin değeri azaldı ve albumin-globulin oranı ise arttı ($P<0.05$). Spirulina ilavesinin büyüme performansı üzerine olan etkilerini aydınlatmak ve hayvanlarda en uygun diyet konsantrasyonunu belirlemek için daha fazla çalışmaya gereksinim vardır.

Anahtar sözcükler: *Tavşan, Saccharomyces cerevisiae, Spirulina platensis, büyüme performansı, Serum biyokimyasal parametreleri*

INTRODUCTION

Single cell proteins like *Saccharomyces cerevisiae* (SC) and *Spirulina platensis* (SP) are considered as alternative protein sources in animal diets. These natural additives have been observed in studies of fish [1], cattle [2], pigs [3], chickens [4-6] and rabbits [7-9].

The yeast *S. cerevisiae* is a well-known probiotic having positive effects in the treatment and prevention of diseases [10]. The positive effect of probiotic can originate from either their direct nutritional effect or their health promoting effects such as acting as a bioregulator of the intestinal microflora and reinforcing the host's natural defenses [11]. *S. cerevisiae* contains biologically valuable proteins, vitamin B-complex, important trace minerals and several unique "plus" factors. First, Eckles and Williams [12] reported the use of *S. cerevisiae* as a growth promoter for

İletişim (Correspondence)
+90 224 2941272
nilays@uludag.edu.tr

ruminants. The inclusion of live yeast into animal feed has been shown to improve the digestibility, efficiency of feed utilization, and performance of animals [13,14]. The addition of Saccharomyces cerevisiae had a growth stimulating effect and generally, the responses were linearly related to the concentration of yeast [7]. Onifade et al.[7] studied with SC level at 1.5 g/kg and 3.0 g/kg, and they observed the positive effect on weight gain, feed intake and feed conversation ratio in rabbits. Eze and Ezema [15] suggested that SC in level of 0.12 g/kg of diet had a beneficial effect on growth and health status of rabbit. *S. cerevisiae*. is also called as single cell protein [11,16], has been shown to survive in the gastrointestinal tract while eliminating the potential pathogenic bacteria residing in channel [17]. Also, Kimsé et al.[18] was found that the survival rate of yeast in digestive tract was higher in rabbits. However, this addition did not affect the feed intake, feed efficiency and final body weight of rabbits.

It has been suggested that probiotics binds to bile acids which results in a reduced serum cholesterol value [19]. There are many conflicting studies about the effect of *S. cerevisiae* on serum lipid profile in animals. Although some of the studies showed reduction in cholesterol [5,16,20], the others demonstrated no benefits [11,21].

S. platensis is a microscopic filamentous alga, which contains several vitamins, especially vitamin B_{12} and pro-vitamin A (beta-carotene). Also, it is rich in polyunsaturated fatty acids, phycocyanin [22,23] and phenolic compounds [24]. Over the years, many dietary supplements of *S. platensis* have a widespread use. *S. platensis* has been approved as a health food by the World Health Organization (WHO) and it will become one of the most alternative treatments in the 21st century [25,26]. Researchers have reported the effects of *S. platensis* on blood protein and lipid content [23,27,28], and their antioxidant, antiviral and immunmodulator activities [24,29,30] in animals. According to some researchers, *S. platensis* and its extract may decrease the blood lipid values. Especially, phycocyanin and polyunsaturated fatty acids in *S. platensis* may play an important role in its hypocholesterolemic effect [28,29,31]. Nagaoka et al.[23] reported that cholesterol is lowered by inhibition of the cholesterol absorption from jejunum and bile acid resorption from ileum with phycocyanin in *S. platensis*. In addition, other researchers proposed that *Spirulina platensis* may have an effect on the increments in plasma total protein, albumin and globulin values [6,32] in animals. These researchers stated that the increased concentrations of plasma total protein, albumin and globulin may be related to the high protein contents in *S. platensis* (with values ranging from 55-65% and includes all of the essential amino acids).

Contradictory results are available in the literatures of dietary Spirulina effects on the growth performances in rabbits [33-35]. Dalle Zotte et al.[33] studied with female rabbits which reached a higher body weight due to high feed intake with *S. platensis* addition. The efficiency of *S. platensis* for rabbit growing has been tested by Peiretti and Meineri [34]. They showed that the final weight. weight gain and feed efficiency did not differ significantly among the dietary treatments, but *S. platensis* inclusion at a level of 10% gave the highest feed intake. Similary, Gerencsér et al.[35] had found no statistical differences for final weight and weight gain.

Although some studies have been performed in the performance of *S. cerevisiae* and *S. platensis* in animals [5,16,23], the combined effect of *S. cerevisiae* and *S. platensis* have not been addressed yet. This research was aimed to evaluate the combined effect of *S. cerevisiae* and *S. platensis* on growth performance and biochemical parameters. Also, there is not enough data about the effects of *S. platensis* in animals. So, this study contributes to an understanding of the literature about *S. platensis*.

MATERIAL and METHODS

Animals, Groups and Feeding

Forty male New Zealand white rabbits aged 5-6 weeks with 1000.9 g mean body weight were randomly allocated on a weight basis to four groups: I. Control, II. SC (added 3 g/kg diet), III. SP (added 5% of the diet), IV. Combination of SC and SP (added 3 g/kg SC and 5% SP of the diet), respectively. The rabbits were housed individually in metal cages and provided with separate facilities for feeding and watering. Feed and water were offered *ad libitum* to the rabbits throughout the 90 day trial. Basal diet (pelleted) was formulated to contain 2.500 kcal ME/kg metabolizable energy, 16% crude protein and was designed to meet maintenance requirements according to the NRC [36]. Chemical composition and ingredients of the diet are provided in *Table 1* and *Table 2*. Chemical analyses of diets were carried out according to AOAC [37]. Basal diet was supplemented with *S. cerevisiae* live yeast culture (Yea Sacc^{1026} Altech. Nicholasville: 1x10^{9} CFU g^{-1}) and/or *Spirulina platensis*.

The experimental protocols were approved by the Animal Care and Use Committee of Uludag University and are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The

Table 1. *Chemical composition of basal diet (%DM)*
Tablo 1. *Bazal diyetin kimyasal içeriği (%Kuru Madde)*

Chemical Analysis	Diet
Dry matter %	88.89
Crude protein %*	16.00
Ether extracts %*	3.52
Crude fiber %*	10.95
Ash	7.68

** Based on % dry matter*

Table 2. *Ratio of feed ingredients (%)*
Tablo 2. *Yem içeriği oranları (%)*

Ingredients	Usage Rate %
Barley	30.00
Corn14	17.61
Rice bran	10.00
Corn bran	3.60
Alfalfa meal	25.00
Soybean meal 46	10.83
Limestone	1.40
Dicalcium phosphate 18	0.28
Salt	0.80
Methionine	0.09
Anticoccidial	0.03
Vitamin premix*	0.25
Antioccidial	0.03
Total	100.00

** **Premix:** Vit A 4.800.000 IU, Vit D 800.000 IU, Vit E 14.000 mg, Biotin 18 mg, CH-CL 50.000 mg, Folic acid 400 mg, Niacin 8.000 mg, Pant. acid 4.000 mg, Riboflavin 2.800 mg, Thiamin 1.200 mg, Pyridoxine 2.000 mg, Vit K 1.600 mg, Zinc 24.000 mg, Iron 2.000 mg, Iodine 400 mg, Manganese 32.000 mg, Selenium 60 mg, Copper 24.000 mg*

study was carried out with the permission of Uludag University Animal Experimentation Local Ethics Committee (Approval No: 2010-09/01).

Measurements

Initial body weight, final body weight, total body weight gain, total feed intake and feed conversion ratio of each rabbit were determined for growth performance in the each month of the 90th day trial. Blood samples were collected for non-anticoagulant tubes by ear venipuncture on the 90th day from overnight-fasted rabbits. Serum concentrations of total protein, albumin, globulin, total lipid, triglyceride, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), glucose, creatinine, creatine kinase, amylase, lipase, urea, sodium, potassium, phosphorus, calcium, ferrous and activities of aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) were determined by "Clima MC15" auto analyzer (RAL, Barcelona, Spain).

Statistical Analysis

Statistical analyses were performed with SPSS [38] (Version 17.0; Chicago.IL). Data were tested for normality distribution and variance homogeneity assumptions. All the values were grouped and the means and standard errors were calculated. One-way ANOVA was applied to the all parameters to examine the difference between groups. Differences were considered significant at $P<0.05$. If the difference between groups was provided to be significant ($P<0.05$), differences evaluated by Tukey's test [39]. On the other hand, in non-homogenous groups, differences between means were analyzed by Kruskal Wallis and following Mann Whitney U test between groups one by one [40].

RESULTS

Performance characteristics of the control and experimental groups (SC, SP and SC+SP) are presented in *Table 3*. There were no significant changes in the growth performances of the groups, monthly or at the end of the experiment. Nevertheless there were no significant changes in the serum biochemical indices, as shown in *Table 4*; only the serum globulin value was significantly ($P<0.05$) lower and albumin-globulin ratio was significantly higher in the SP and SC+SP groups compared to the Control and SC groups. Also, data on serum cholesterols, enzymes and minerals are summarized in *Table 4*. There were no changes in these parameters ($P>0.05$).

DISCUSSIONS

S. cerevisiae and *S. platensis* have received attention as a good probiotic and prebiotic organism that can maintain growth performance characteristics, and also associated with health promoting effects. The present study is an attempt to identify natural effects of SP, SC and SP+SC combinations for rabbits. According to the results of this study, although not statistically significant, supplementing rabbit with a combination of *S. cerevisiae* and *S. platensis* had increased on mean body weight and body weight gain. The monthly body weight gain, feed intake and feed conversation ratio of rabbits were not significantly affected by SC, SP and combination of SC and SP. In addition to, the feed conversation ratio was affected positively in the group of feeding *S. platensis*.

The effect of *S. platensis* on growth performance values in animals was determined by some researchers [2,41,42]. Heidarpour et al.[2] studied with 3 levels of *S. platensis* (2, 6 and 25 g) in Holstein calves and they reported that there were no significant differences in weight gain, daily feed intake and feed conversion ratio. Also, Dernekbasi et al.[42] added 10%, 20%, 30% and 40% *S. platensis* into diet of fish and determined no effect on the growth parameters among groups. However, there are some reports indicating a significant increase in the body weight gain by dietary *S. platensis* supplementation [1,41,44]. In the present study, there were no significant differences in growth performances among the groups, but mean feed conversion ratio was slightly lower in the *S. platensis* group than the other groups. Similar to our results, Peiretti and Meineri [34] observed no significant differences in growth performances in rabbits fed with *S. platensis* supplementation. The mechanisms of growth promotion of yeast culture in rabbits, turkey poults and broiler chickens and the positive relationship between *S. cerevisiae* and animal performance characteristics have

Table 3. *Growth performances characteristics of rabbits in control and experimental groups (mean±standard error, n=40)*
Tablo 3. *Kontrol ve deney gruplarının büyüme performansı değerleri (ortalama±standart hata, n=40)*

Traits	Control n=10	SC n=10	SP n=10	SC+SP n=10
Initial body weight (g)	1009.20±73.94	1009.56±76.15	1009.20±75.45	1009.20±78.57
Final body weight (g)	2662.40±48.37	2717.78±83.42	2677.00±80.01	2718.80±100.50
Total weight gain (g)	1653.20±83.78	1680.22±69.89	1695.20±82.56	1709.60±77.93
Total feed intake (g)	7785.40±236.97	8431.33±143.90	7443.40±292.25	7815.80±317.52
Total feed conversion ratio	4.79±0.23	5.08±0.22	4.48±0.25	4.60±0.16
First Month (0-30 days)				
Live weight (g)	1771.60±44.61	1859.56±63.03	1847.60±63.52	1824.20±82.00
Body weight gain (g)	762.40±47.98	822.00±48.31	838.40±44.01	815.00±32.55
Feed intake (g)	2181.60±99.89	2400.00±56.95	2119.00±71.63	2179.80±137.88
Feed conversion ratio	2.99±0.27	2.99±0.18	2.60±0.18	2.67±0.10
Second Month (30-60 days)				
Live weight (g)	2353.60±38.17	2398.44±83.07	2386.60±70.12	2398.80±95.18
Body weight gain (g)	582.00±23.81	538.89±37.21	539.00±51.05	574.60±46.54
Feed intake (g)	2990.00±81.09	3377.78±116.04	2862.00±151.21	2954.20±173.37
Feed conversion ratio	5.19±0.20	6.66±0.77	5.63±0.45	5.40±0.43
Third Month (60-90 days)				
Live weight (g)	2662.40±48.37	2717.78±83.42	2677.00±80.01	2718.80±100.50
Body weight gain (g)	308.80±26.50	319.33±30.13	317.80±30.46	320.00±23.70
Feed intake (g)	2853.80±152.88	2986.89±142.84	2552.40±142.01	2861.80±99.18
Feed conversion ratio	9.63±0.66	10.21±1.23	8.68±0.91	9.30±0.62

Table 4. *Serum biochemical indices of rabbits in control and experimental groups. (mean±standard error, n=40)*
Tablo 4. *Kontrol ve deney gruplarının serum biyokimyasal değerleri (ortalama±standart hata, n=40)*

Biochemical Parameters	Control	SC	SP	SC+SP
Total protein (g/dl)	6.78±0.27	6.60±0.48	6.13±0.16	6.30±0.22
Albumin (g/dl)	3.56±0.20	3.90±0.18	3.91±0.14	4.13±0.92
Globulin (g/dl)	3.16±0.22^{a}	3.27±0.25^{a}	2.31±0.13^{b}	2.29±0.21^{b}
Albumin/Globulin	1.19±0.12^{a}	1.27±0.12^{a}	1.75±0.13^{b}	1.95±0.18^{b}
Total lipid (mg/dl)	378.50±7.90	385.78±3.55	364.70±7.31	375.70±7.11
Triglyceride (mg/dl)	119.40±8.24	114.89±10.71	113.40±10.71	107.10±7.97
Cholesterol (mg/dl)	53.60±7.87	62.67±8.12	63.00±10.86	77.00±8.97
HDL cholesterol (mg/dl)	31.60±4.40	30.11±3.18	38.40±5.65	37.90±5.78
LDL cholesterol (mg/dl)	15.25±1.43	17.37±328	16.92±2.34	21.02±5.36
LDL/HDL ratio	0.59±0.10	0.64±0.15	0.50±0.09	0.58±0.15
Urea (mg/dl)	40.90±2.77	37.89±4.30	38.70±2.19	34.90±1.72
Glucose (mg/dl)	138.80±6.23	167.67±15.69	133.9±8.23	130.70±6.94
Creatinine (mg/dl)	0.84±0.08	0.80±0.09	0.81±0.06	0.92±0.05
Creatinine kinase (U/L)	602.80±196.96	556.56±125.89	473.70±115.80	310.40±51.70
Amylase (U/L)	509.40±42.68	425.56±35.01	518.10±40.50	454.10±29.02
Lipase (U/L)	153.60±6.44	163.55±11.95	147.50±9.32	156.00±4.49
Phosporus (mg/dl)	6.23±0.24	5.69±0.41	6.34±0.37	5.85±0.32
Calcium (mg/dl)	12.52±0.61	10.73±0.58	10.47±0.74	10.38±0.62
Sodium (mmol/l)	159.30±4.14	167.44±9.18	151.80±8.88	171.50±3.72
Ferrous (mmol/l)	56.19±2.65	51.92±4.45	43.55±5.92	55.84±4.32
Potassium (mg/dl)	4.70±0.21	4.56±0.24	4.61±0.15	4.55±0.20
GOT (U/l)	33.30±6.45	40.67±5.62	36.10±7.03	45.90±8.21
GPT (ALT) (U/l)	56.80±11.20	61.56±6.29	52.00±8.70	66.70±10.82
ALP (U/l)	427.00±29.92	369.67±28.84	402.50±23.87	398.60±31.16

Different superscripts a, b, show differences (P<0.05) between groups

been reported [4,5,43,45]. Onifade and Babuntae [4] studied in broiler chicks with a diet containing 6.0 g/kg *S.cerevisiae* yeast. They interpreted that yeast may effect on improving feed quality. On the contrary, Lambertini et al.[45] and Chaudhary et al.[43] reported that yeast did not affect live weight, daily weight gain and feed intake in New Zealand rabbits. Also, in our study, growth parameters were not affected by the addition of the SC *(Table 3)*.

S. platensis and *S. cerevisiae* are used as a supplement because of their protein, vitamin and mineral content. In the present study, serum globulin value was significantly lower, and therefore albumin-globulin ratio was significantly higher in groups fed SP and SC+SP compared to the control and SC groups (*Table 4*, P<0.05). Globulin fractions were not determined in this study, so we do not know the source of the decline in the value of globulin. However,

addition of SP may inhibit the growth of harmful bacteria in intestine [46] and the lower value of serum globulin of rabbits fed at 5% *S. platensis* may be attributed to the inhibitory effect of *S. platensis* against harmful intestinal microflora. Because harmful enteric bacteria secretes inflammatory agents and lead to increase in globulin synthesis of liver or of other tissues such as lymphatic tissue or plasma cells. *S. cerevisiae* has also an inhibitory effect against harmful intestinal microflora [11], but the effects of *S. cerevisiae* and *S. platensis* on immune response may be different from each other. On the other hand, Heidarpour et al.[2] studied on albumin, globulin and their assigned ratio in calves feeding *S. platensis* in levels of 0, 2, 6 and 25 g/day, and found no significant effect on serum albumin and globulin levels among treatment groups. Also, Moreira et al.[41] found no significant effect of *S. platensis* on serum albumin and protein levels. However, Mariey et al.[6] stated that SP level at 0.2% had a significant increase in plasma total protein, albumin and globulin in laying hens. Bezeria et al.[32] determined the high serum protein value in lambs fed 0, 5 and 10 g SP. These researchers suggested that the high value of serum protein, globulin and albumin may be due to protein quality and quantity of *S. platensis*.

S. cerevisiae cell wall component, beta glucan, had a cholesterol lowering effect was documented by some researchers [16,19,20]. Paryad and Mahmoudi [16] reported reduction in plasma cholesterol and triglyceride value of chicks which was fed with yeast supplement. These researchers suggested that yeast may regulate the serum cholesterol concentrations by deconjunction of bile acids. However, Payandeh [47], Ozsoy et al.[48], and Yildiz et al.[49] observed that SC did not affect the serum lipid, triglyceride and cholesterol. Also in the present study, there were no changes in serum lipid, triglyceride and cholesterol values of rabbits fed with the *S. cerevisiae* supplement ($P>0.05$, *Table 4*).

S. platensis is rich in polyunsaturated fatty acids and phycocyanin [22-24]. Some researchers [31,50,51] reported that the polyunsaturated fatty acids in *S. platensis* help to reduce serum lipid profiles. The addition of 16% of *S. platensis* into the rat diet caused to significant inhibition of serum total cholesterol in the study accomplished by Kato et al.[50]. Also, Nagaoka et al.[23] reported that phycocyanin plays a crucial role in the hypocholesterolemic action of *S. platensis* concentrate in rats. These researchers suggested that phycocyanin may inhibit both jejunal cholesterol absorption and ileal bile acid reabsorption by binding bile acid. However, in the present study there were no changes in these parameters of rabbits fed with *S. platensis* supplementation.

Average values of aspartate aminotransferase (AST), alanin aminophosphatase (ALP) and alanine amino-transaminase (ALT) in serum of rabbits were revealed no statistically significant differences between control. SC, SP and SC+SP groups *(Table 4)*. These enzymes are located intracellularly in the body including liver, heart and kidney etc. Their level in the blood is increased when there is membrane damage in these cells. Hence, normal level of these enzymes in blood of rabbits fed diet containing 3 g/kg SC and 5% SP suggests that it has no adverse effect on the cells of vital organs. These results agree with results of Shrivastava and Jha [20], Saied et al.[52], Ibrahim et al.[53], Moreira et al.[41] and Sixabella et al.[54].

Administration of *S.cerevisiae* and *S. platensis* singly or in combination had no significant effect on serum biochemical parameters, except for serum globulin and albumin-globulin ratio. These results don't indicate that these supplements have any positive effects. However, there was a slightly positive effect of *S. platensis* on growth parameters, especially on feed conversion ratio. On the other hand, although not statistically significant, *S. platensis* and *S. cerevisiae* combination improved the body weight and body weight gain compared to control. So, *S. platensis* may be used as a growth promoter in animals. However, more studies would be necessary to elucidate the effects of supplementing spirulina on growth and to determine the optimum dietary concentration in animals.

REFERENCES

1. Promya J, Chitmanat C: The effects of *Spirulina platensis* and *Cladophora algae* on the growth performance, meat quality and immunity stimulating capacity of the African sharptooth catfish *(Clarias gariepinus)*. *Int J Agric Biol*, 13, 77-82, 2011.

2. Heidarpour A, Fourouzande H, Shahraki AD, Eghbalsaied S: Effects of *Spirulina platensis* on performance, digestibility and serum biochemical parameters of Holstein calves. *Afr J Agri Res,* 6(22): 5061-5065, 2011.

3. Veum TL, Bowman GL: *Saccharomyces cervisiae* yeast culture in diets for mechanically-fed neonatal piglets and early growing self-fed pigs. *J Anim Sci*, 37 (1): 67-71, 1973.

4. Onifade AA, Babatunde GM: Supplemental value of dried yeast in a high-fibre diet for broiler chicks. *Anim Feed Sci Tech*, 62, 91-96, 1996.

5. Onifade AA: Growth performance, carcass characteristics, organ measurements and haematology of broiler chickens fed a high fibre diet supplemented with antibiotics or dried yeast. *Die Nahrung*, 41, 370-374, 1997.

6. Mariey YA, Samak HR, Ibrahem MA: Effect of using *Spirulına platensis algae* as a feed additive for poultry diets: 1- Productive and reproductive performances of local laying hens., *Egypt Poult Sci*, 32 (1): 201-215, 2012.

7. Onifade AA, Obiyan RI, Onipede E, Adejumo DO, Abu OA, Babatunde GM: Assessment of the effects of supplementing rabbit diets with a culture of *Saccharomyces cerevisiae* using growth performance, blood composition and clinical enzyme activities. *Anim Feed Sci Tech*, 77, 25-32, 1999.

8. Sreemannarayana O, Raju KVS, Ramaraju G, Prasad JR: The use of Ulva fasciata, a marine alga as rabbit feed: Growth and conversion efficiency. *Indian Vet J,* 72, 989-999, 1995.

9. Peiretti PG, Meineri G: Effects of diets with increasing levels of Spirulina platensis on the performance and apparent digestibility in growing rabbits. *Livestock Sci*, 118, 173-177, 2008.

10. Zanello G, Meurens F, Berri M, Salmon H: *Saccharomyces boulardii* effects on gastrointestinal diseases. *Curr Issues Mol Biol*, 11, 47-58, 2009.

11. Hassanein MS, Soliman NK: Effect of probiotic *(Saccharomyces cerevisiae)* adding to diets on intestinal microflora and performance of

Hy-line layers hens. *J Am Sci,* 6 (11): 159-169, 2010.

12. Eckles CH, Williams VM: Yeast as a supplementary feed for lactating cows. *J Dairy Sci,* 8, 89-93, 1924.

13. Glade MJ, Sist MO: Dietary yeast culture supplementation enhances urea recycling in equine large intestine. *Nutr Rep Int*, 37,11-17, 1988.

14. Martin SA, Nisbel BJ, Dean RF: Influence of a commercial yeast supplement on the *in-vitro* ruminal fermentation. *Nutr Rep Int*, 40, 395-403, 1989.

15. Ezema C, Eze DC: Determination of the effect of probiotic *(Saccharomyces cerevisiae)* on growth performance and hematological parameters of rabbits. *Comp Clin Pathol*, 01, 2012. DOI:10.1007/s00580-010-1066-6

16. Paryad A, Mahmoudi M: Effect of different levels of supplemental yeast *(Saccharomyces cerevisiae)* on performance, blood constituents and carcass characteristics of broiler chicks. *Afr J Agri Res*, 3 (12): 835-842, 2008.

17. Bekatorou A, Psarianos C, Koutinas AA: Production of food grade yeasts. *Food Technol Biotechnol*, 44, 407-415, 2006.

18. Kimsé M, Bayourthe C, Monteils V, Gidenne T: Live yeast stability in the digestive tract of the rabbit: Relationship with digestion, growth and digestive health, nutrition and digestive physiology. *9th World Rabbit Congress,* June 10-13, Verona - Italy, 2008.

19. El-Arab AE, Foheid S, El-Said M: Effect of yeast and botanical β-glucan on serum lipid profile and cecum probiotic bacteria using rats fed cholesterol diet. *Pol J Food Nutr Sci*, 59 (2): 169-174, 2009.

20. Shrivastava AK, Jha RR: Effects of different chemical composition and probiotic on the haematobiochemical profile of rabbit. *EJEAFChe,* 9 (9): 1507-1513, 2010.

21. Ozsoy B, Yalçın S: The effects of dietary supplementation of yeast culture on performance, blood parameters and immune system in broiler turkeys. *Ankara Univ Vet Fak Derg*, 58, 117-122, 2011.

22. Estrada JEP, Bescós PB, Villar del Fresno AM: Antioxidant activity of different fractions of Spirulina platensis protean extract: II. *Farmaco,* 56, 497-500, 2001.

23. Nagaoka S, Shimizu K, Kaneko H, Shibayama F, Morikawa K, Kanamaru Y, Otsuka A, Hirahashi T, Kato T: A novel protein cphycocyanin plays a crucial role in the hypocholesterolemic action of *Spirulina platensis* concentrate in rats. *J Nutr*, 135, 2425-2430, 2005.

24. Miranda MS, Cintra RG, Barros SBM, Filho JM: Antioxidant activity of the microalga *Spirulina maxima*. *Brazil J Med Biol Res*, 31, 1075-1079, 1998.

25. Hasler C: Functional foods: Benefits, concerns and challenges - A position paper from the American Council on Science and Health. *J Nutr*, 132, 3772-3781, 2002.

26. Annapuna V, Shah N, Bhaskaram P: Bioavailability of *Spirulina carotenes* in pre-school children. *J Clin Biochem Nutr*, 10, 145-151, 1991.

27. Nakaya N, Honma Y, Goto Y: Cholesterol lowering effect of spirulina. *Nutrition Reports Int'l*, 37 (6): 1329-1337, 1988.

28. Riss J, Decorde K, Sutra T, Delage M, Baccou JC, Jouy N, Brune JP, Oreal H, Cristol JP, Roanet JM: Phyco-biliprotein C-phycocyanin from *Spirulina platensis* is powerfully responsible for induced by atherogenic diet in hamsters. *J Agri Food Chem*, 55, 7962-7967, 2007.

29. Henrikson R: Microalgae Spirulina, Superalimento del Futuro. *Ronore Enterprises*. 2th ed., Ediciones Urano, Barcelona, Espana, pp.222, 2010.

30. Belay A, Ota Y, Miyakawa K, Shimamatsu H: Current knowledge on potential health benefits of Spirulina. *J Appl Phycol*, 5, 235-241, 1993.

31. Iwata K, Inayama T, Kato T: Effects of *Spirulina platensis* on plasma lipoprotein lipase activity in fructose-induced hyperlipidemic rats. *J Nutr Sci Vitaminol*, 36, 165-171, 1990.

32. Bezerra LR, Azevedo Silva AM, Azevedo SA, Rodrigues OG, Azevedo PS, Sousa Mendes R: Serum concentrations of proteins and minerals in lambs artificially fed with *Spirulina platensis*-enriched milk. *Acta Vet Brasíl*, 3 (3): 132-137, 2009.

33. DalleZotte A, Sartori A, Bohatir P, Re´mignon H, Ricci R: Effect of dietary supplementation of Spirulina *(Arthrospira platensis)* and Thyme *(Thymus vulgaris)* on growth performance, apparent digestibility and health status of companion dwarf rabbits. *Livestock Sci*, 152, 182-191, 2013.

34. Peiretti PG, Meineri G: Effects of diets with increasing levels of *Spirulina platensis* on the performance and apparent digestibility in growing rabbits. *Livestock Sci,* 118, 173-177, 2008.

35. Gerencsér Z, Szendrő Z, Matics Z, Radnai I, Kovács M, Nagy I, Dal Bosco A, Dalle Zotte A: Dietary supplementation of Spirulina *(Arthrospira platensis)* and Thyme *(Thymus vulgaris* L.). Part 1: Effect on productive performance of growing rabbits. *World Rabbit Science Association Proceedings 10th World Rabbit Congress*, September 3-6, *Sharm El- Sheikh -Egypt*, 657-661, 2012.

36. National Research Council-NRC: Nutrient Requirements of Rabbits. 6th ed., National Academy Press, Washington, 1997.

37. AOAC, Official Methods of Analysis: MD Official Method 991.36, 17th ed., Gaithersburg, 2000.

38. SPSS: SPSS software package for Windows, Version 17.0, Chicago.

39. Dowdy S, Wearden S: Statistics for Research. pp.262-274, John Wiley&Sons Press, New York, 1981.

40. Dawson B, Trapp RG: Basic&Clinical Biostatistics. 3rd ed., Lange Medical Books/McGraw International Editions, New York, 2001.

41. Moreira LM, Rocha ASR, Ribeiro CLG, Rodrigues RS, Soares LS: Nutritional evaluation of single-cell protein produced by *Spirulina platensis*. *Afr J Food Sci*, 5 (15): 799-805, 2011.

42. Dernekbaşı S, Ünal H, Karayücel I, Aral O: Effect of dietary supplementation of different rates of Spirulina *(Spirulina platensis)* on growth and feed conversion in Guppy (*Poecilia reticulara* Peters, 1860). *J Anim Vet Adv*, 9 (9): 1395-1399, 2010.

43. Chaudhary LC, Singh R, Kamra DN, Pathak NN: Effect of oral administration of yeast *(Saccharomcyes cerevisiae)* on digestibility and growth performance of rabbits fed deist of different fibre content. *World Rabbit Sci*, 3 (1): 15-18, 1995.

44. Grinstead GS, Tokach MD, Dritz SS, Goodband RD, Nelssen JL: Effects of *Spirulina platensis* on growth performance of weanling pigs. *Anim Feed Sci Technol,* 83, 237-247, 2000.

45. Lambertini L, Vignola G, Beone GM, Zaghini G, Formigoni A: Effects of chromium yeast supplementation on growth performances and meat quality in rabbits. *World Rabbit Sci,* 12, 33-47, 2004.

46. Bhowmik D, Dubey J, Mehra S: Probiotic efficiency of *Spirulina platensis* - Stimulating growth of lactic acid bacteria. *World J Dairy & Food Sci,* 4 (2): 160-163, 2009

47. Payandeh S, Kafilzadeh F: The effect of yeast *(Saccharomyces cerevisiae)* on nutrient intake, digestibility and finishing performance of lambs fed a diet based on dried molasses sugar beet-pulp. *Pak J Biol Sci*, 10 (24): 4426-4431, 2007.

48. Ozsoy B, Yalçın S: The effects of dietary supplementation of yeast culture on performance, blood parameters and immune system in broiler turkeys. *Ankara Univ Vet Fak Derg*, 58, 117-122, 2011.

49. Yıldız, G, Köksal BH, Sızmaz O: Effects of dietary boric acid and yeast *(Saccharomyces cerevisiae)* supplementation on performance, carcass traits and some blood parameters of broilers. *Kafkas Univ Vet Fak. Derg,* 17 (3): 429-434, 2011.

50. Kato T, Takemoto K, Katayama H, Kuwabara Y: Effects of Spirulina *(Spirulina platensis)* on dietary hypercholesterolemia in rats. *J Jap Soc Nutr Food Sci*, 37, 323-332, 1984.

51. Colla LM, Muccillo-Baisch AL, Vieira Costa JA: *Spirulina platensis* effects on the levels of total cholesterol, HDL and triacylglycerols in rabbits fed with a hypercholesterolemic diet. *Brazil Arch Biol Technol*, 51, 405-411, 2008.

52. Saied JM, Al-Jabary QH, Thalij KM: Effect of dietary supplement yeast culture on production performance and hematological parameters in broiler chicks. *Int J Poult Sci*, 10 (5): 376-380, 2011.

53. Ibrahim DK, Al-Mashhadani EH, Al-Bandr LK: Effect of supplementing different levels of chromium yeast to diet on broiler chickens on some physiological traits. *Pakistan J Nutr*, 9 (10): 942-949, 2010.

54. Sixabela PSS, Chivandi E, Badenhorst M, Erlwanger KH: The effects of dietary supplementation with *Spirulina platensis* in growing rats. *JAVA,* 6 (6): 609-617, 2011.

Kafkas Univ Vet Fak Derg
20 (3): 337-343, 2014
DOI: 10.9775/kvfd.2013.10101

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

RESEARCH ARTICLE

The Treatment of Coxofemoral Luxation by Modified Synthetic Capsule Technique in Dogs: 6 Cases [1]

Ali BELGE [1] Zeynep BOZKAN [1] Murat SARIERLER [1] Rahime YAYGINGÜL [1]

[1] *Part of this research was presented as an oral presentation at the 11th National Congress of Veterinary Surgery, 26-29 June 2008, Kuşadası, Türkiye*

[1] Department of Surgery, Faculty of Veterinary Medicine, University of Adnan Menderes, TR-09016 Aydin - TURKEY

Makale Kodu (Article Code): KVFD-2013-10101

Summary

In this study, it was aimed to investigate that long-term clinical efficacy of modified synthetic capsule technique in treatment of coxofemoral luxation in dogs. As animal materials, six dogs which different breeds, sex and ages that detected coxofemoral luxation in clinical and radiological examination were subjected. As different from modified synthetic capsule technique, two cortical screws were inserted into the dorsal rim about 5 mm away from the acetabular edge at the 10- and 12-o'clock positions for the left hip and the 12- and 2-o'clock positions for the right hip. Also, the transverse hole was created in greater trochanter. Non-absorbable monofilament suture material was tied to the screw heads previously and then the suture ends were passed as crosswise through the transverse tunnel in the trochanter major. Subsequently, the suture material was tied on the greater trochanter by stretching following that the femoral head was placed into the acetabulum. It was seen that there was no complication related with the reduction or screws in clinical and radiological examinations of the dogs at fourth week. We determined that five dogs recovered "perfect" and one dog "good" end of sixth months.

Keywords: *Coxofemoral luxation, Synthetic capsule technique, Dog*

Köpeklerde Kalça Eklemi Çıkığının Modifiye Sentetik Kapsül Tekniği ile Sağaltımı: 6 Olgu

Özet

Bu çalışmada, köpeklerde coxofemoral lukzasyonun sağaltımında modifiye sentetik kapsül tekniğinin uzun dönem klinik etkinliğinin araştırılması amaçlandı. Materyali, klinik-radyolojik muayenesinde, kalça çıkığı saptanan değişik yaş ve ırkta 6 köpek oluşturdu. Tekniğin orijinalinden farklı olarak; sağ kalça için saat 12 ve 2, sol kalça için saat 10 ve 12'ye denk gelecek şekilde, asetabular kenardan 5 mm kadar uzağa, iki kortikal vida yerleştirildi ve trochanter majore transversal tünel açıldı. Emilmeyen monoflament iplik önce vida başlarına bağlandı, sonra çaprazlaştırılarak trohanter majora açılan tünel içerisinden geçirildi. Daha sonra femur başı asetabuluma yerleştirildi ve iplikler gergin şekilde trochanter major üzerinde düğümlendi. Dördüncü hafta yapılan klinik ve radyolojik muayenelerde köpeklerde redüksiyon veya yerleştirilen vidalara ilişkin herhangi bir komplikasyon yaşamadığı görüldü. Altıncı ayın sonunda 5 köpeğin mükemmel, 1 köpeğin ise iyi düzeyde basış sergilediği saptandı.

Anahtar sözcükler: *Koksofemoral çıkık, Sentetik kapsül tekniği, Köpek*

INTRODUCTION

In small animals, luxation of coxofemoral joint is the most common among all joint [1]. The coxofemoral luxation constitutes 39-90% of all luxations which occur in dogs have been reported by some investigators [2,3]. Due to strong pulling force of gluteal and iliopsoas muscles, direction of the coxofemoral luxations is often craniodorsal and rarely caudodorsal, ventral and medial [4-6].

The most common causes of the hip joint luxations are traffic accidents and falling from high. Traumatic luxations

İletişim (Correspondence)
+90 536 6381727
alibelge@hotmail.com

originate from the shifting of gravity center to outside of the hip in the cases which suddenly jumped or fell [7,8]. The joint capsule and also round ligament may completely or partially tear [3].

The basic principles of hip luxation treatment are to provide stability without damage to joint surfaces for regenerate the normal functioning of the joint and to restrict the animal movements for healing surrounding soft tissues as soon as possible [3]. Closed reduction is usually possible in normal joint luxation without any complication [4-6,8,9]. However, closed reduction can be ideally made in 48-72 hours after trauma [1,10]. As increase of elapsed time, closed reduction becomes more difficult, because of severity and width of inflammation, fibrosis and cartilage damage also increases [2,11]. Some methods have been used for supporting of closed reduction such as Ehmer sling stabilization, ischio-ilial pinning or dynamic transarticular pinning [1,2].

The luxation should be treated surgically; if there are complications such as hip dysplasia existence prior to trauma, femoral and/or acetabular avulsion fractures, intra articular fractures, arthrosis and conditions such as multiple orthopedic injuries or chronic luxation, closed reduction failure and extreme instability after reduction [5,8,12,13]. Techniques which are used for the surgical treatment of coxofemoral luxations can be categorized as extracapsular such as suture of a joint capsule (capsulorrhaphy) [3,5,12], synthetic capsule technique [2,3,6,14], transposition of the greater trochanter [2,5,6,12], triple pelvic osteotomy [2,8] and intra capsular techniques such as Modified Knowles Toggle pin fixation [3,6,8,15,16], Toggle rod stabilization [17,18], trans acetabular pinning [2,6,19] transposition of the sacrotuberous ligament [20,21]. Additionally, flexible external fixation [8], total hip replacement [22-24] and femoral head and neck osteotomy [2,25,26] can be also applied for the treatment of hip luxation. For surgery, there are different surgical exposures techniques (such as cranio-lateral or dorsal approach) which can be selected depending on the method of surgery, luxation direction, accompanying complications and physician's habits [8,13,27-29]. Postoperative immobilization should be provided with most of the surgical technique as with the closed reduction [3,19,28].

The joint capsule repair and tightening following the reduction is a technique that can be applied for luxations which occurred with simple tears [3,5,12]. Synthetic capsule technique is performed with eight shape suture between a transverse hole created in femoral neck (or a screw placed in the trochanteric fossa) and two bone screws which is inserted into the dorsal rim of the acetabulum at the 10- and 1-o'clock positions for the left hip and the 11- and 2-o'clock positions for the right hip. Care must be taken not to damage to the articular surface during screwing [1,3,8]. In intra articular techniques, mostly, caput femoris and acetabulum are connected to each other by various materials that mimic an intra-articular ligament. The major disadvantages of these methods are that they cause extra damage to the articular surface and may create predisposition to degenerative joint disease in long-term [8,30]. In the pinning techniques, complications such as pin tract infection or position changing of pin from where applied are possible [9,31]. The techniques of femoral head and neck osteotomy and total hip replacement are proposed for the dogs with hip dysplasia from mild to severe and for the animals with complications which restrain closed and open techniques [8].

In the present study, it was aimed to investigate and present that long-term clinical efficacy of the modification of synthetic capsule technique which is often preferred in case with wide joint capsule defect and considered to cause relatively fewer complications compared to other techniques for the treatment of coxofemoral luxation.

MATERIAL and METHODS

As materials, six dogs from various breeds and at different ages which were presented to Adnan Menderes University Veterinary Faculty Animal Hospital with complaints of severe lameness or inability to stand up and detected coxofemoral luxation at clinical and radiological examination were subjected *(Table 1)*.

Table 1. *The distribution of breed, age, gender, body weight, and clinical findings of cases*
Tablo 1. *Olguların ırk, yaş, cinsiyet, vücut ağırlığı ve klinik bulgularının dağılımı*

No	Breed	Age (month)	Gender	Weight	PTP (day)	Presentation	Diagnosis
1	Mix Breed	24	M	28 kg	4	Severe Lameness (Unilateral)	Unilateral Luxation (Right)
2	Pointer	72	M	22 kg	3	Constant Recumbency	Unilateral Luxation (Right), Femoral Fracture (Left)
3	German Shepherd	120	M	26 kg	4	Constant Recumbency	Bilateral Luxation, Greater Trochanter Fracture (Right)
4	Husky	48	M	25 kg	5	Severe Lameness (Unilateral)	Unilateral Luxation (Left)
5	Kangal	18	F	37 kg	3	Severe Lameness (Unilateral)	Unilateral Luxation (Left)
6	Mix Breed	72	M	18 kg	4	Severe Lameness (Unilateral)	Unilateral Luxation (Left)

M: *Male,* ***F:*** *Female;* ***PTP,*** *Posttraumatic Period*

According to the history; each of hip luxation resulted from traffic accident, 2 of dogs were not able to stand up and 4 of dogs could not use related legs. Posttraumatic periods were 3 days for 2 dogs, 4 days for 3 dogs and 5 days were 1 dog.

During clinical and radiological examination; coxofemoral luxations were determined as unilateral of 5 dogs, 3 at the left side and 2 at the right side, and bilateral of 1 dog. There was also femoral fracture in one dog with right sided hip luxation (case no. 2) and one dog with bilateral hip luxation (case no. 3) had also greater trochanter fracture. All of luxation was cranio-lateral direction, with one ventral direction exception (case no. 3, bilateral luxation). Treatment of the luxation in ventral direction of the dog which had bilateral luxation was not included the study. Furthermore, in study animals, it was determined that there were no other complications accompanying luxation and any stage of the hip dysplasia which exist prior to trauma by radiological examinations *(Table 1)*.

Dogs underwent surgery following 24 h starving period. Induction of anesthesia was performed with combination of atropine sulfate 0.04 mg/kg body weight, subcutaneously (Atropin®, Teknovet, Turkey), xylazine HCl 0.5 mg/kg body weight (Alfazyne®, Egevet, Turkey) and ketamine HCl 10 mg/kg body weight (Alfamine®, Egevet, Turkey), intramuscularly. Anesthesia was maintained with inhalation of Isoflurane (Forane®, Abbott, Latina, Italy) at a concentration of 2%.

The dog was placed in the lateral recumbence upon operating table. After disinfection of the region, the operation was started with skin incision extended up and down from front of the greater trochanter by using dorsal approach technique. After dissection of the subcutaneous tissues, the area where is in the triangle consisting of m. tensor fasciae latae and underlying rectus femoris at cranial, m. gluteus at dorsal and vastus lateralis at caudal was reached as a blunt. The joint capsule and the joint were exposed by external rotation. The remains of the round ligament and fibrous tissues were removed from the joint. Differently from original technique, two cortical bone screws (3.5Ø, 22-26 mm) were inserted into the dorsal acetabular rim about 5 mm away from the acetabular edge at the 10- and 12-o'clock positions for the left hip and the 12- and 2-o'clock positions for the right hip with a slope that provide not to enter into the joint *(Fig. 1/A, Fig. 2/A,B)*. Also, the transverse hole was created in greater trochanter instead of femoral neck (or a screw placed in the trochanteric fossa) *(Fig. 1/B)*. Non-absorbable monofilament suture material (USP:1, Ethilon, Ethicon, UK) was tied to the screw heads previously *(Fig. 2/C)*, then the suture ends were passed as crosswise through the transverse tunnel in the trochanter *(Fig. 1/C)*. Subsequently, the suture material was tied on the greater trochanter by stretching following that the femoral head was placed into the acetabulum (*Fig. 1/D* and *Fig. 2/D*). During above mentioned tying procedure, the femoral head was compressed into the acetabulum. The joint capsule was sutured within the possibilities. Skin and subcutaneous tissues were closed routinely.

Ehmer sling was applied to all dogs for one week after the surgery. Postoperative antibiotic, cefazolin sodium (20 mg/kg body weight, IM, Iespor®, I. E. Ulagay, Istanbul) and anti-inflammatory drug, carprofen (2 mg/kg body weight, PO, Rimadyl®, Pfizer, Zavantem, Belgium), were prescribed to the all of cases for 5 days. After a week, weight bearing of the related leg was allowed.

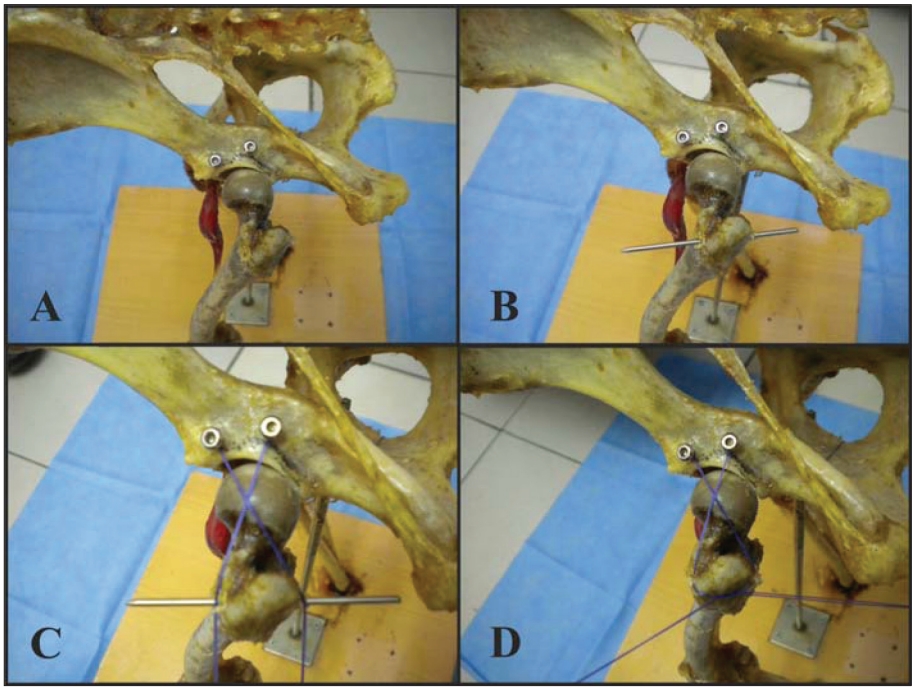


Fig 1. The modified synthetic capsule technique; **A-** insertion of the screws into the dorsal rim; **B-** the transverse tunnel drilled in the greater trochanter; **C-** tying of the suture material to the head of screws and passing of the suture as crosswise through the transverse tunnel in the greater trochanter; **D-** tying and stretching of the suture material on the greater trochanter

Şekil 1. Modifiye sentetik kapsül tekniği; **A-** dorsal kenara vidaların yerleştirilmesi; **B-** trochanter major'e açılan transversal kanal; **C-** vida başlarına dikiş materyalinin bağlanması ve ipin trochanter major'deki transversal tünelden çapraz şekilde geçirilmesi; **D-** dikiş materyalinin trochanter major üzerinde bağlanması ve gerilmesi

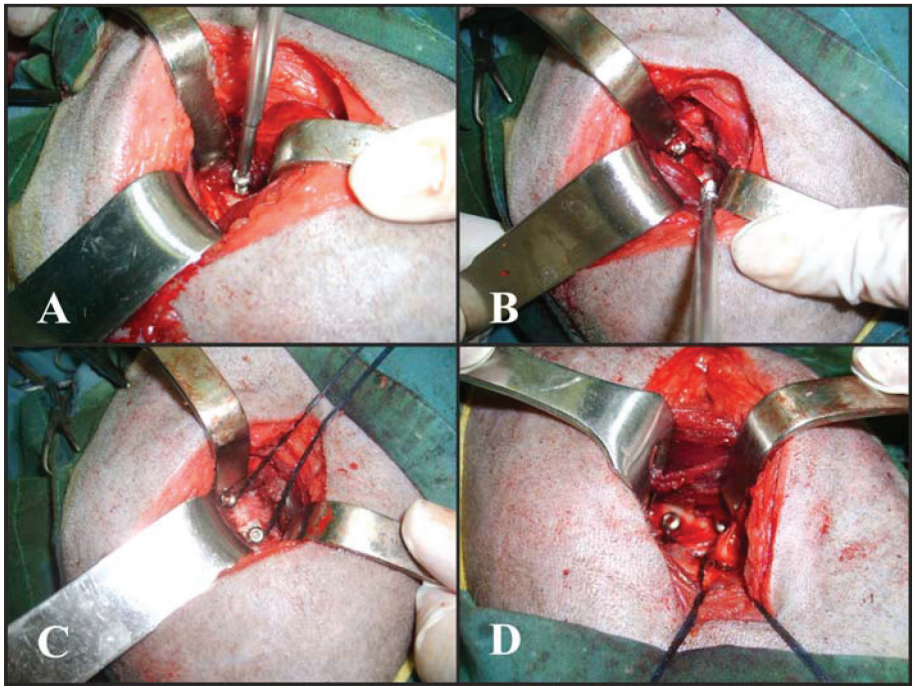


Fig 2. Implementation of the modified synthetic capsule technique during the operation; **A, B-** insertion of the screws into the dorsal acetabular rim; **C-** tying of the suture material to the head of screws; **D-** tying and stretching of the suture material between the screws and greater trochanter

Şekil 2. Operasyon sırasında modifiye sentetik kapsül tekniğinin uygulanışı; **A,B-** dorsal asetabular kenara vidaların yerleştirilmesi; **C-** vida başlarına dikiş materyalinin bağlanması; **D-** dikiş materyalinin vida başları ile trochanter major arasında bağlanması ve gerilmesi

Table 2. *At postoperative periods, start of weight bearing (post-operative day) and lameness scoring (at week 1, 2, 4) of dogs*
Tablo 2. *Köpeklerin operasyon sonrası dönemde ağırlık taşımaya başladıkları süre (postoperatif gün) ve topallık skorları (1, 2 ve 4. haftada)*

Case No	Start of Weight Bearing (post-op. day)	Lameness Scoring (Postoperative)		
		Week 1	Week 2	Week 4
1	9	Severe	Mild	Not Exist
2	12	Severe	Moderate	Not Exist
3	14	Severe	Moderate	Mild
4	10	Severe	Moderate	Not Exist
5	8	Severe	Mild	Not Exist
6	9	Severe	Mild	Not Exist

RESULTS

Clinical and radiological examinations were performed for all cases at weeks 1, 2 and 4 *(Fig. 3)*. For lameness evaluation, each of the dogs was observed by investigator while an assistant made the dog walk at least 10 m and lameness status was scored as; not exist, mild, moderate and severe *(Table 2)*.

At first week radiological examination, there was no problem in terms of the reduction in all of cases and then Ehmer slings were removed. None of the dogs were able to use the related legs. However, it was learned that one dog started to use the related leg on the day after, two dogs on 2 days after and one dog 3 days after removal of the Ehmer sling, with limping at different stages from the owners. Also, it was expressed that the dog with bilateral hip luxation were not able to stand up without support and the dog with right sided hip luxation plus left sided femoral fracture stood up with aid of the right leg but received support from bandage on left side while standing up.

Skin sutures were removed at second week controls. No infection-related complications were determined within this time period in all of cases. During clinical examination, 3 of dogs could use the related legs with frequently and one dog occasionally. The bandage on the dog's left sided femoral fracture, which applied after fracture repair, was removed and the dog started to use the right leg more. Besides, the dog with bilateral luxation was reluctant to stand up and showed rotational weight bearing between two rear legs, but more used the leg which performed synthetic capsule technique.

Clinical and radiological examination findings at fourth week were as follows; there was no complication related with reduction or screws in all of dogs, 3 of dogs were able to walk without limping and one dog with slightly limping, the dog with right sided hip luxation plus left sided femoral fracture could walk with the right side as completely healthy and with the left side as slightly limping and the dog with bilateral luxation (for treatment of right hip luxation with greater trochanter fracture, femoral head and neck osteotomy was performed) was not able to use

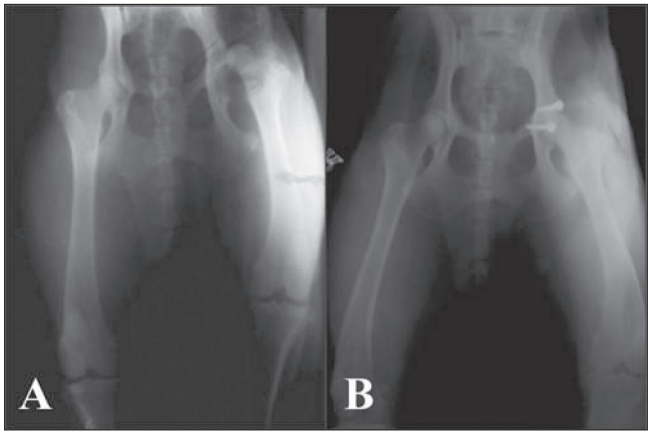


Fig 3. Preoperative (A) and postoperative 1st week (B) radiographs of the Case 6 (72 months old male mix breed dog)

Şekil 3. Altı numaralı vakanın (72 aylık, erkek melez köpek) preoperatif (A) ve postoperatif 1. hafta (B) radyografileri

both leg fully, but weight bearing mostly was performed with the leg which performed synthetic capsule technique.

The subsequent follow-up of the dogs were continued until 6th postoperative month for 3 of dogs and 12th postoperative month for others by phone call. At the end of these periods, it was learned that the 5 dogs which had "Not Exist" lameness score at week 4 were completely healthy and the one dog (case no. 3) could use both leg similarly but uncoordinatedly.

DISCUSSION

The mechanism of trauma-related hip dislocations; when the dog began to fall in the direction of impact force, the affected leg becomes adducted and the hip moves in ventrolaterally toward the ground. The adducted femoral head directs outward from the acetabulum to the extent permitted by the joint capsule and round ligament. When greater trochanter hits the ground, kinetic energy is transmitted to the caput femoris through the collum femoris. Caput femoris moves upward from acetabular rim, round ligament and joint capsule tear. Usually, caput femoris remains in craniodorsal position because of gluteal muscles contraction [7].

Based on this mechanism, it is understood that the restriction of the adduction of the leg is very important for prevention of reluxation in the postoperative period when joint capsule has not recovered yet. In our technique, distal screw was placed slightly cranially then the original synthetic capsule technique. Femoral connection was established by the transverse hole in the greater trochanter which is relatively more proximal instead of femoral neck (or a screw placed in the trochanteric fossa). Thus, it was hypothesized that the possibility of reluxation would reduce because of suture material tightening in this way would lead to more adduction and internal rotation of the leg. The original synthetic capsule technique has also restrictive effect on adduction and external rotation of the leg similar to Ehmer sling [8]. In our study, to improve of those effects of original technique was intended.

Closed reduction is possible for normal hip joint within 48-72 h after luxation. If the luxated hip joint waits longer time, the probability of pathological changes of femoral head and acetabulum will increase [1,2,8,10]. Small osteochondral fragments or hemorrhage may cause to closed reduction failure by joint movement restriction. The round ligament and inward folding of the joint capsule may prevent reduction of the femoral head [6].

According to history, posttraumatic period of the dogs included in the study was range 3-5 days. For this reason, it was thought that open reduction is a healthier option. Study was carried out on a series of 6 cases. The dogs which successful closed reduction could be performed and the dogs which the modified synthetic capsule technique was found unenforceable because of any stage of hip dysplasia or accompanying complications were not included in this study. For this reason, the number of cases remained limited.

There are numerous methods which perform successfully for open reduction of hip joint. For hip luxation treatment, the options which have minimal intraoperative and post-operative complications possibilities should be considered. The joint capsule repair and tightening following the reduction is a technique that can be applied for luxations which occurred with simple tears [3,5,12], that's why indication of this method is a relatively limited. The reduction with Toggle pin can be disrupted by suture breaking between femoral head and acetabulum [29,32]. In a study, traumatic craniodorsal coxofemoral luxations in cats and small dogs were treated successfully by using using a modified Knowles technique, but mean weight of included dogs in this study was 15 kg [16]. In another study which compared toggle rod and suture anchor, it was reported that toggle rod constructs failed primarily by breakage of the suture at the rod eyelet and suture anchor constructs failed when the anchors pulled through the medial acetabular wall [17]. In two different studies which used toggle rod with 62 dogs and 13 dogs, reluxation rates were declared as 11% [18] and 23% [33], respectively. Besides, intraarticular stabilization methods may cause articular damage and subsequent arthrosis [30]. The complications related with transarticular pinning or De Vita pinning such as pin migration, pin loosening, septic arthritis, sciatic nerve injury, subluxation, femoral head and neck osteonecrosis, penetration to colon and rectum has been reported [4,8]. There is limited information about flexible external fixator because of it has not been widely used. Possible complications of this technique are hemorrhage, sciatic nerve damage, pin loosening, pin track drainage and disruption of the flexible band [9,31]. Additionally, total hip replacement [22-24] and femoral head and neck osteotomy [2,25,26] can be also

applied for the treatment of hip luxation, however these techniques are usually preferred, if there is degenerative joint disease. In a multicenter internet based study on assessment of canine total hip replacement in 170 dogs, there were only 6 dogs which applied total hip replacement with coxofemoral luxation treatment indication [24].

Lower complication rate, higher clinical healing rate [8,13,30,34-36] and also 0 to 6% reluxation rate based on small number case series [14,34] has been reported on synthetic capsule technique. In our study, 4 dogs of 6 had (case no. 1, 4, 5, 6) only unilateral coxofemoral luxation without complications and Dog 2 had femoral fracture and Dog 3 had right hip luxation with greater trochanter fracture together with the opposite side hip luxation. Four dogs which have only coxofemoral luxation began to use their related leg within 7-9 days. The other two dogs needed more time (12 days for Dog 2 and 14 days for Dog 3) to start using their leg which applied modified synthetic capsule technique. The dog with bilateral luxation (for treatment of right hip luxation with greater trochanter fracture, femoral head and neck osteotomy was performed) was not able to use both leg fully, but weight bearing mostly was performed with the leg which performed synthetic capsule technique.

No sign of infection was observed in postoperative period. 5 of all dogs healed with almost excellent degree. According to information from Owner, one dog (case no. 3) could use both leg similarly but uncoordinatedly. No reluxation occurred in all of cases.

In a study on femoral head and neck osteotomy, it was indicated that weight is not as much effective as age on the outcome, when the dogs were grouped as under and above 10 kg body weight [26]. However, according to some sources, femoral head and neck osteotomy require the dog below 17 kg [1] or 22 kg [37] body weight. Acar et al.[33] reported that toggle pin technique is appropriate for the dog below 10 kg body weight based on the observation of reluxation in 3 of 4 dogs which weighing over 10 kg in their study. In our study, all of the dogs were weighed over 17 kg and only one dog was weighed below 22 kg, however 5 dogs healed completely, including even the dog which weighed 37 kg. Besides, it was thought that the uncoordinated walking of the one dog (case no. 3) might be resulted from spinal nerve injury which occurred during trauma. These results have led us to consider that the technique is effective regardless of the weight of the dog.

Smith et al.[7] has been indicated the hip luxation does not arise from individually rupture of the round ligament, joint capsule or dorsal acetabular rim. The hip luxations in small animals mostly result from simultaneous rupture of the round ligament and the joint capsule [10]. Based on this information, it was thought that the strong joint capsule can support position of femoral head within acetabulum.

The expectation of the synthetic capsule technique is the formation of organized scar tissue and remodeling of the joint capsule provide stabilization of reduction [3,38]. Some studies on hip luxation and subsequent joint capsule and ligament healing showed that fibrous reaction of the joint capsule and round ligament healing occurred at 14th day [39]. Also, using of Ehmer sling or other temporary stabilization materials for 2 weeks has been shown to be effective enough in ensuring the long term stability of the joint [6,13]. In our study, although Ehmer sling removed from all of dogs 1 week after surgery, no reluxation occurred. This results were attributed to modification of the original technique more limits the joint movement by providing more adduction and internal rotation.

As a result, postoperatively, infection signs, the hip joint laxity or reluxation were not determined in clinical and radiological examinations. Although a precise comment could not be made about contribution of the modification to the prevention of reluxation because of 7 days Ehmer sling application, the lower reluxation rate (0%) compared with reported reluxation rate of original technique (%6) seem to be advantage.

REFERENCES

1. Johnson AL, Dunning D: Atlas of Orthopedic Surgical Procedures of the Dog and Cat. 38-51, Elsevier Inc, USA, 2005.

2. Denny HR, Butterworth SJ: A Guide to Canine and Feline Orthopedic Surgery. 4th ed., 459-467, Blackwell Science, Oxford, 2000.

3. Piermattei DL, Flo GL, DeChamp C: Handbook of Small Animal Orthopedics and Fracture Repair. 4th ed., 461-475, Saunders, USA, 2006.

4. Duff SRI, Bennett D: Hip luxation in small animals: An evaluation of some methods of treatment. *Vet Rec*, 111, 140-143, 1982.

5. Basher AWP, Walter MC, Newton CD: Coxofemoral luxation in the dog and cat. *Vet Surg*, 15, 356-362, 1986.

6. Fox SM: Coxofemoral luxations in dogs. *Comp Cont Ed Pract Vet*, 13, 381-389, 1991.

7. Smith WS, Coleman CR, Olix ML, Slager RF: Etiology of congenital dislocation of the hip. *J Bone Joint Surg*, 45, 491-500, 1963.

8. Holsworth IG, De Camp CE: Canine hip dysplasia: Diagnosis and non surgical treatment. **In,** Slatter D (Ed): Textbook of Small Animal Surgery. 3rd ed., 2002-2008, Elsevier Sci, Philadelphia, 2003.

9. McLaughlin RM: Traumatic joint luxations in small animals. *Vet Clin North Am: Small Anim Pract*, 25, 1175, 1995.

10. Trostel CT, Peck JN, Haan JJ: Spontaneous bilateral coxofemoral luxation in four dogs. *J Am Anim Hosp Assoc*, 36, 268-276, 2000.

11. Harasen G: Coxofemoral luxations part 1: Diagnosis and closed reduction. *Can Vet J*, 46, 368-369, 2005.

12. Bone DL, Walker M, Cantwell HD: Traumatic coxofemoral luxation in dogs. Results of repair. *Vet Surg*, 13, 263-270, 1984.

13. Tangner SH: Managing cases of traumatic canine hip luxation. *Vet Med*, 4, 314-324, 1992.

14. Allen SW, Chambers JN: Extracapsular suture stabilization of canine coxofemoral luxation. *Comp Cont Ed*, 8, 457-463, 1986.

15. Cetinkaya MA, Olcay B: Modified Knowles toggle pin technique with nylon monofilament suture material for treatment of two caudoventral hip luxation cases. *Vet Comp Orthop Traumatol*, 23, 114-118, 2010.

16. Ash K, Rosselli D, Danielski A, Farrell M, Hamilton M, Fitzpatrick N: Correction of craniodorsal coxofemoral luxation in cats and small breed

dogs using a modified Knowles technique with the braided polyblend TightRope systems. *Vet Comp Orthop Traumatol*, 25, 54-60, 2012.

17. Spranklin D, Elder S, Boyle C, Mc Laughlin R: Comparison of a suture anchor and a toggle rod for use in toggle pin fixation of coxofemoral luxations. *J Am Anim Hosp Assoc*, 42, 121-126, 2006.

18. Demko JL, Sidaway BK, Thieman KM, Fox DB, Boyle CR, Mc Laughlin RM: Toggle rod stabilization for treatment of hip joint luxation in dogs: 62 cases (2000-2005). *J Am Vet Med Assoc*, 229, 984-989, 2006.

19. Hunt CA, Henry WB: Trans articular pinning for repair of hip dislocation in the dog: A retrospective study of 40 cases. *J Am Vet Med Assoc*, 187, 828-833, 1985.

20. Kılıç E, Özaydın I, Atalan G, Baran V: Transposition of the sacrotuberous ligament for the treatment of coxofemoral luxation in dogs. *J Small Anim Pract*, 43, 341-344, 2002.

21. Özaydın I, Kılıc E, Baran V, Demirkan I, Kamiloglu A, Vural S: Reduction and stabilization of hip luxation by the transposition of the ligamentum sacrotuberale in dogs: An *in-vivo* study. *Vet Surg*, 32, 46-51, 2003.

22. Dyce J, Wisner Er, Wang Q, Olmstead Ml: Evaluation of risk factors for luxation after total hip replacement in dogs. *Vet Surg*, 29, 524-532, 2000.

23. Hummel DW, Lanz OI, Werre SR: Complications of cementless total hip replacement: A retrospective study of 163 cases. *Vet Comp Orthop Traumatol,* 23, 424-432, 2010.

24. Forster KE, Wills A, Torrington AM, Moores AP, Thomson D, Arthurs G, Brown G, Denny HR, Scott HW, MacQueen I, Dunne J, Onyett J, Walker JD, Prior J,Owen MR, Burton N, Whitelock R, Girling S, Morrison S, Gilbert S, Langley-Hobbs SJ, Gemmill TJ, Innes JF: Complications and owner assessment of canine total hip replacement: A multicenter internet based survey. *Vet Surg,* 41, 545-550, 2012.

25. Harasen G: The femoral head and neck ostectomy. *Can Vet J*, 45, 163-164, 2004.

26. Fattahian H, Mohyeddin H, Hoseinzadeh A, Akbarein H, Moridpour R: Excision arthroplasty of the hip joint in dogs: The role of age, weight, degenerative joint disease on the outcome. *Kafkas Univ Vet Fak Derg*, 18, 431-436, 2012.

27. Piermattei DL: An Atlas of Surgical Approaches to the Bones of the Dog and Cat. 3rd ed., 230-251, Saunders, USA, 1993.

28. Leighton RL: A novel technique to manage coxofemoral luxations in dogs. *Vet Med*, 1, 53-58, 1985.

29. Beckham HP, Smith MM, Kern DA: Use of a modified toggle pin for repair of coxofemoral luxation in dogs with multiple orthopedic injuries: 14 cases (1986-1994). *J Am Vet Med Assoc*, 208, 81-84, 1996.

30. Meij BP, Hazewinkel HAW, Nap RC: Results of extra articular stabilization following open reduction of coxofemoral luxation in dogs and cats. *J Small Anim Pract*, 33, 320-326, 1992.

31. McLaughlin RM, Tillson DM: Flexible external fixation for cranio-dorsal coxofemoral luxation in dogs. *Vet Surg*, 23, 21-30, 1994.

32. Bennett D, Duff SR: Transarticular pinning as a treatment for hip luxation in the dog and cat. *J Small Anim Pract*, 21, 373-379, 1980.

33. Acar SE, Perk EC, Altunatmaz K: Köpeklerde luxation femoris olgularının toggle pin kullanılarak sağaltımı. *Vet Cerrahi Derg*, 2 (2): 26-30, 1996.

34. Johnson ME, Braden TD: A retrospective study of prosthetic capsule technique for the treatment of problem cases of dislocated hips. *Vet Surg*, 16, 346-351, 1987.

35. Mehl NB: A new method of surgical treatment of hip dislocation in dogs and cats. *J Small Anim Pract*, 29, 789-795, 1988.

36. Braden TD, Johnson ME: Technique and indications of a prosthetic capsule for repair of recurrent and chronic coxofemoral luxations. *Vet Comp Orthop Traumatol*, 1, 26-29, 1988.

37. Olmstead ML: Small Animal Orthopedics. 375-378, Mosby-Yearbook, St. Louis, 1995.

38. Tomlinson JL Jr: Reduction of coxofemoral luxations. **In,** Bojrab MJ (Ed): Current Techniques in Small Animal Surgery. 4th ed., 1178-1183, Williams &Wilkins, Baltimore, MD, 1998.

39. Martini FM, Simonazzi B, Del Bue M: Extra-articular absorbable suture stabilization of coxofemoral luxation in dogs. *Vet Surg*, 30, 468-475, 2001.

Kafkas Univ Vet Fak Derg
20 (3): 345-350, 2014
DOI: 10.9775/kvfd.2013.10105

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

RESEARCH ARTICLE

The Advantages of Autologus Adipose Derived Mesenchymal Stem Cells (AdMSCs) over the Non-steroidal Anti-inflammatory Drugs (NSAIDs) Application for Degenerative Elbow Joint Disease Treatment in Dogs - Twelve Cases

Jakub NICPOŃ [1] Krzysztof MARYCZ [2] Jakub GRZESIAK [2]
Agnieszka ŚMIESZEK [2] Nezir Yaşar TOKER [3]

[1] Wroclaw University of Environmental and Life Sciences, Department and Clinic of Veterinary Surgery, 51 Grunwaldzki Square, 50-366 Wroclaw - POLAND
[2] Wroclaw University of Environmental and Life Sciences, Electron Microscope Laboratory, Kożuchowska 5b, 51-631, Wroclaw - POLAND
[3] İstanbul Üniversitesi, Veteriner Fakültesi, Biyokimya Anabilim Dalı, TR-34320 Avcılar, İstanbul - TÜRKİYE

Makale Kodu (Article Code): KVFD-2013-10105

Summary

In the present study based on clinical case reports, twelve dogs (7 males, 5 females) between the ages of 5 and 11 years old, with clinically diagnosed osteoarthritis of the elbow joint were undertaken for comparison of the therapeutic effect derived from the standard non-steroidal antiinflammatory drug (NSAIDs) treatment with novel, autologous adipose-derived mesenchymal stem cell (AdMSCs) application. Animals were not under any treatment for 3 last months before the diagnosis. Individuals from experimental group were treated with autologous adipose-derived mesenchymal stem cells, while individuals from control group received Mavacoxib (Trocoxil). After 0, 60, 90 and 180 days of the treatment, all individuals were clinically evaluated. Additionally, examination of synovial fluid from all of the animals was performed after 0, 60, 90 and 180 days. The CT analysis were performed at day 0 and 180 days after the treatment. The AdMSCs therapy in the case of elbow joint degeneration (OA) decreased the discomfort and reduced accompanying clinical symptoms like pain, stiffness when walking and lameness. In contrast, though the animals from control group showed the improvement during first period of experiment, the OA symptoms reoccurred 90 days after the application of NSAIDs. This work is the first report that not only describes the picture of clinical examination, but is also based on synovial fluid analysis and CT evaluation. The injections of autologous stem cells did not exhibit any adverse reactions, which confirms the safety of this method. Our research confirmed the efficiency of AdMSC injections for the treatment of elbow joint osteoarthritis in dogs, which improve the clinical picture and the patients' condition, however without the regresses of osteofites.

Keywords: *Adipose-derived mesenchymal stem cells, Canine, Elbow joint diseases, Osteoarthritis*

Köpeklerde Dejeneratif Dirsek Eklemi Bozukluğu Tedavisinde Non-steroidal Antiinflamatuar Ajan (NSAIA) Uygulanmasından Sonra Adipoz Doku Kökenli Otolog Mezenkimal Kök Hücrelerin (ADkOMKH) Faydası - Oniki Olgu

Özet

Bu çalışma klinik olarak dirsek ekleminde osteoartritis tespit edilen, standart non-steroidal anti inflammatuar ilaçlardan (NSAIA) türetilmiş terapötik ilaçla tedavi edilen 5-11 yaş arasında, oniki (7 erkek, 5 dişi) köpekte, adipoz doku orijinli otolog mezenkimal kök hücrelerin (ADkOMKH) etkinliğini karşılaştırmak için yapılmıştır. Teşhisten önce hayvanlara 3 ay boyunca herhangi bir uygulama yapılmamıştır. Deneysel gruptaki bireyler adipoz kökenli otolog mezenkimal kök hücrelerle tedavi edildi, buna karşın kontrol grubun bireylerine Mavacoxib (Trocoxil) uygulandı. Uygulamanın 0, 60, 90 ve 180. günlerinde, her bir birey klinik olarak muayene edildi. Ayrıca, tüm hayvanlardan 0, 60, 90 ve 180. günlerde ilgili eklemlerden alınan siynovial sıvıların analizi yapıldı. Ayrıca, 0 ve 180. günlerde CT analizi yapıldı. Dirsek eklemi dejenerasyonunda (OA); ADkOMKH ile tedaviye alınan hayvanlarda; ağrı, yürürken oluşan sertlikler, topallıklar gibi klinik semptomlar hafifledi. Kontrol grubundaki hayvanlar da deneyin ilk peryodun da gelişme görülmesinin aksine, OA semptom'unun NSAIA uygulamasından 90 gün sonra tekrarladığı gözlendi. Bu çalışma, sadece klinik incelemenin görüntüsünü anlatan bir çalışma değil aynı zamanda siynovial sıvı analizi ve CT değerlendirilmesine dayanan ilk çalışmadır. Otolog mezenkimal kök hücrelerin uygulanması metodun güvenliğini onaylayarak, herhangi bir istenmeyen reaksiyon sergilemedi. Bu çalışma, AdMSC uygulamasının köpeklerde osteofitik gerileme olmaksızın; dirsek eklemi osteoartritisindeki verimliliği artırmış, klinik bulguları ve hasta kondüsyonunu geliştirmiştir.

Anahtar sözcükler: *Adipoz-kökenli mezenşimal kök hücreler, Köpek, Dirsek eklemi bozukluğu, Osteoartritis*

İletişim (Correspondence)
+90 532 3256478
nezirtoker@msn.com

INTRODUCTION

In recent years, an increasing percentage of degenerative joint diseases have occurred in the practice of small animal veterinary medicine. This is due to several reasons, the most important of which is the genetics factor. It often does not show any signs in the early stage of ontogenesis but symptoms appear in later periods of life [1,2]. One of the most often occurring locomotive system disorders in dogs is elbow joint degeneration (EJD). This disease results in movement disturbances and consequently, may lead to different stages of lameness and/or a stiff gait. These symptoms may increase with exercise, long periods of inactivity or cold weather. Also, a high degree of pain and inflammation usually accompanies this disease.

EJD treatment is based mainly on steroidal and non-steroidal anti-inflammatory drug application [3]. Unfortunately, this treatment does not support the regeneration of damaged articular cartilage but only relieves pain and reduces the inflammatory process. Moreover, prolonged usage of the above mentioned drugs may indirectly lead to clinical complications and bring unsatisfying treatment progress [4]. Therefore, alternative treatment strategies should be established.

Last decade resulted in the discovery of mesenchymal stem cells and their pro-regenerative abilities [5-7]. This population of adult stem cells is common in many tissues, including bone marrow and subcutaneous fat. Mesenchymal stem cells are characterized by a high capacity for self-renewal and plasticity for differentiation, thus giving hope of returning to full efficiency before the disease state. Mesenchymal stem cells (MSC) are a promising tool in current veterinary medicine, which is confirmed by an increasing number of clinical trials [8,9]. Experiments performed both on animal and human beings showed that low differentiated MSC are able to differentiate into specialized cells within the wound site, as well as having paracrine and endocrine abilities that support the regeneration of damaged tissues [10]. Additionally, MSC also show immunomodulatory properties since they are able to interact with T-cells and suppress their inflammatory reactions [11]. This feature results in a decrease in pain and inflammation during cellular therapy [12].

In our current research, we focused on the clinical effects of autologous mesenchymal stem cell therapy in dogs suffering from elbow joint degeneration, with regards to gold standard (NSAIDs). We confirmed the results of similar work showed by Black et al.[1], and additionally showed the data obtained from computer tomography observations and synovial fluid analysis. Our results confirmed the advantage of autologous AdMSCs intraarticular injections over the standard NSAIDS treatment in improving the clinical picture and the patients' comfort of life.

MATERIAL and METHODS

Ethical Approval

Ethical Approval The experiment was conducted with the approval of Bioethical Commission, as stated by the Second Local Bioethical Commission at the Department of Biology and Animal Breeding, at University of Environmental and Life Sciences in Wroclaw, Chełmonskiego 38C, Poland (dec. number 177/2010 from 11.15.2010).

Qualification of Patients

For the medical experiment, twelve dogs were used (7 males, 5 females), between the ages of 5 and 11 years old. The breeds of the dogs were as follows: German Shepherd (4 individuals), Labrador Retriever (2 individuals), Boxer (2 individuals) and 4 crossbreeds, all of whom suffered from elbow joint degeneration that occurred at least 5 months before the therapy. Patients' weight was determined to be between 25 and 50 kg. The animals' owners signed the approval for experimental therapy, while this research is based on results from clinical case reports obtained for last two years in Surgery Department of University of Environmental and Life Sciences in Wroclaw. Before the qualification to the therapy, blood tests were performed on all of the dogs for evaluation of their general condition and exclusion of any present comorbid diseases. In the clinical lameness evaluation, the lameness scale was applied, in which the 0 means no lameness and the 6 is the full walking disability. All animals undertaken to this study manifested walking disorders characteristic of osteoarthritis (OA), including lameness both in walking and trotting (2 and 3 from 6-ranged scale), limitation in range of movement and pain during manipulations. Computer tomography and synovial fluid analysis was performed in all of the qualified animals. According to the similarities in clinical symptoms, dogs were split into 2 groups with randomized individuals: the experimental group qualified for stem cell application consisted of 8 individuals, and the control group that undergo the NSAIDS treatment consisted of 4 individuals. Evaluations of synovial fluid were performed at day 0, 90 and 180 after treatment, while CT analyses were done at day 0 and 180. Clinical evaluations were performed at day 0, 60, 90 and 180. The control group consisted of one German Shepherd (6 years old, male), one Boxer (10 years old, female), one Labrador Retriever (5 years old, male) and one crossbred (8 years old, male), weighing between 25 and 50 kg. The experimental group consisted of the remaining 8 individuals (3 German Shepherds (5-10 years old, 1 male, 2 females), 1 Boxer (12 years old, female), 1 Labrador Retriever (5 years old, male) and 3 crossbreds (7-11 years old, 2 males, 1 female)), weighing also between 25 and 50 kg.

Computer Tomography

Prior to CT investigation, animals were sedated by a

combination of Medetomidine (Cepetor, 10-20 µg/kg bw) with Butorphanol (Butomidor, 0.1-0.2 µg/kg bw) using intravenous cannula. In specific situations, animals were introduced into primary sleep using Propofol (Scanofol, 1 mg/kg bw). The examinations were performed by means of 16-row computer tomography (Siemens Somatom Emotion). CT investigations were performed in dorsal-ventral position of all qualified patients.

Fat Tissue Collection

Dogs from the experimental group were sedated with Medetomidine (20 µg/kg,) intramuscularly and with Butorphanol (200 µg/kg) intramuscularly. The area of iliac crest was prepared according to the surgery rules, followed by infiltration anesthesia with Lignocaine (2%). Afterwards, a sample of about 5 grams of adipose tissue was collected from all of the experimental animals for isolation and multiplication of MSC *in vitro*. Tissue samples were processed in sterile conditions under the laminar hood. Biopsies were washed with Hank's Balanced Salt Solution (HBSS, Sigma Aldrich) for any blood traces. Afterwards, tissues were minced and placed in collagenase solution (5 mg/mL, Sigma Aldrich) for 40 min at 37°C. After the digestion process, samples were centrifuged at 1200 g for 10 min for separation of mononucleated cells from the released oil and undigested tissue remnants. Cell pellets were re-suspended in a primary culture medium (DMEM:F12/Ham's with 10% of FBS and 1% of penicillin/streptomycin/amphotericin b, Sigma Aldrich) and placed in 25 cm^2 tissue culture flasks at a concentration of $5x10^4$ cells/cm^2. Cultures were maintained in a humidified incubator with 37°C and 5% CO_2. After two days, the primary culture medium was changed to a secondary culture medium (DMEM with 4500 mg/L of glucose, 15% of FBS, 1% of penicillin/streptomycin/amphotericin b; Sigma Aldrich) and the cultures were propagated for the next five days. Before the application the cells were passaged twice. The time from adipose tissue collection to stem cell application was between 7 and 10 days. Cells were evaluated for proper morphology, phenotype ($CD29^+$, $CD44^+$, $CD45^-$, $CD105^+$) and behavior, in accordance with our previous research and the criteria determined by International Society for Cellular Therapy [12-14]. Microbiological tests were performed during the entire culture course for exclusion of any bacterial or fungal contamination. After multiplication, cells were collected in sterile 0.9% NaCl; counted and evaluated for viability, using the Thoma counting chamber and trypan blue exclusion methods. Afterwards, cell suspensions were collected into the syringe at a concentration of $1.5x10^6$ cells/mL and transported immediately to the clinic.

Treatments

In experimental group, every individual received single injection of prepared autologous stem cell solution intra-articularly (1 ml/joint) to the elbow joint, two weeks after the collection of adipose tissue. Prior to the injection, animals were sedated using Medetomidine (20 µg/kg bw.) intramuscularly and with Butorphanol (200 µg/kg) intramuscularly, and the joint sites were prepared aseptically. The patients' owners were counseled to continue on prescribed rehabilitation program or walk their dogs two times a day. In the control group, animals were only treated with NSAIDs, which was Mavacoxib (Trocoxil, 2 mg/kg bw.) applied for six months (at day 0, 14 and once every next 30 days), administered orally.

RESULTS

In a clinical examination on day 0, all 12 dogs manifested strong lameness of 2 and 3 degrees. In some cases, lameness was stronger during walking while others during a trot. At day 60, a clear improvement in the control group was noticed. Symptoms like stiffness in walk, pain and/or movement disabilities were prominently reduced. In dogs from the experimental group being treated with autologous stem cell applications, slight improvements were noticed at day 60, with decrease in lameness both in walk and in trot, and decrease in pain during manipulations. At day 90 in the control group, the results from clinical evaluation remained unchanged - the symptoms of movement disorders remained at the same level, while the pain was intensified, especially during manipulations. In experimental animals clinical results continued to improve - symptoms like walking stiffness and lameness or pain were found in only 3 of the 8 individuals from AdMSCs group. At day 180 in the experimental dogs, the clinical evaluation did not reveal any symptoms of walking disorders - lameness during walking and trotting and pain during manipulations were completely reduced. Only the range of movement in the joints remained at a constant, limited level. On the other hand, animals which received the NSAIDs manifested prominent lameness at the higher level than in day 0. The averaged results are shown in *Table 1*.

The results from synovial fluid analysis correlated with clinical pictures of the examined animals. At day 0 in all 12 dogs, characteristic signs of chronic joint

Table 1. *Mean results of lameness evaluation ± standard deviation at day 0, 60, 90 and 180*
Tablo 1. *Laminitisin gelişiminde ortalama sonuçların 0, 60, 90 ve 180. günlerdeki standart sapmasının ± değerlendirilmesi*

Group	Day 0	Day 60	Day 90	Day 180
AdMSCs	2.5±0.53	1.87±0.64	0.37±0.51	0.12±0.35
NSAIDs	2.3±0.57	1.5±0.57	1.5±0.5	3.75±0.5

degeneration were detected. The leukocyte level was determined below 6 x 10^3/L, neutrophil level between 5 to 12%, and mononuclear cells in the range between 86 and 98 (individual cells). The synovial fluid was evaluated from light-yellow to colorless, clear, and in 7 cases slightly turbid. At day 90 in dogs from the control group, these parameters remained unchanged. Synovial fluid was determined slight turbid, however leukocyte, neutrophil and mononuclear cell levels were decreased. Clinical improvement was observed in the experimental group treated with stem cell therapy. Only in 2 cases was the synovial fluid turbidity maintained, and only 1 of the 8 was viscosity still decreased. Remaining parameters were within the normal range, where the leukocyte number was determined below 4 x 10^3/L, and the percentage of neutrophils decreased below 5%. At day 180 in the control group, the results showed deterioration. The leukocyte, neutrophil and mononuclear cell levels were increased, and no increase in synovial fluid viscosity was noticed. In the experimental group, the synovial fluid analysis showed no signs of chronic inflammation. The number of leukocytes, as well as neutrophils and mononuclear cells was determined to be within normal range. In one case, persistent synovial colorless, with normal viscosity. The averaged results are shown in *Table 2*.

Computer tomography investigations in animals of both groups revealed osteoarthritis of elbow joint with prominently visible degenerative changes on medial epicondyle of humerus. The administration of AdMSCs in experimental group did not result in improvement of superficial bone changes.

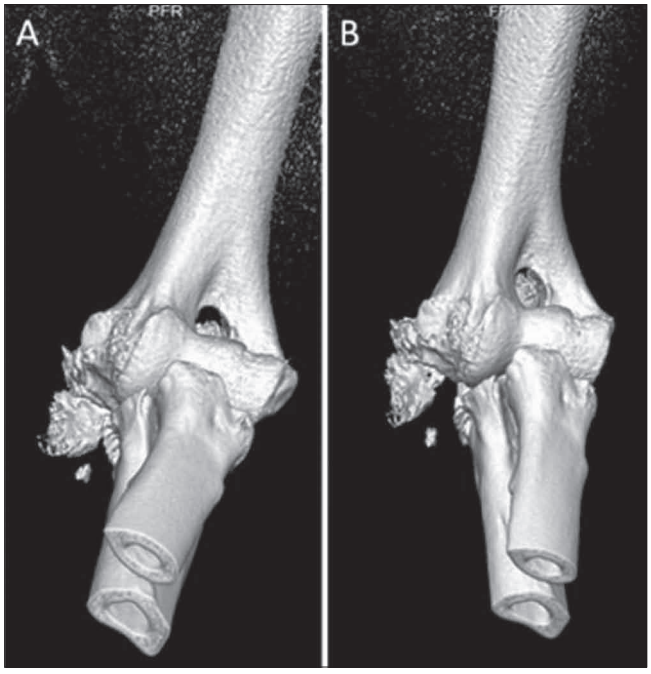


Fig 1. Computer tomography pictures showing changes in the elbow joint in dog from experimental group before (day 0, A) and 180 days after the application stem cells (B)

Şekil 1. Deney grubunda, 0. günde (A) ve 180. günde kök hücre uygulamasından sonra (B) köpek dirsek eklemineki değişimini gösteren bilgisayar tomografi resmi

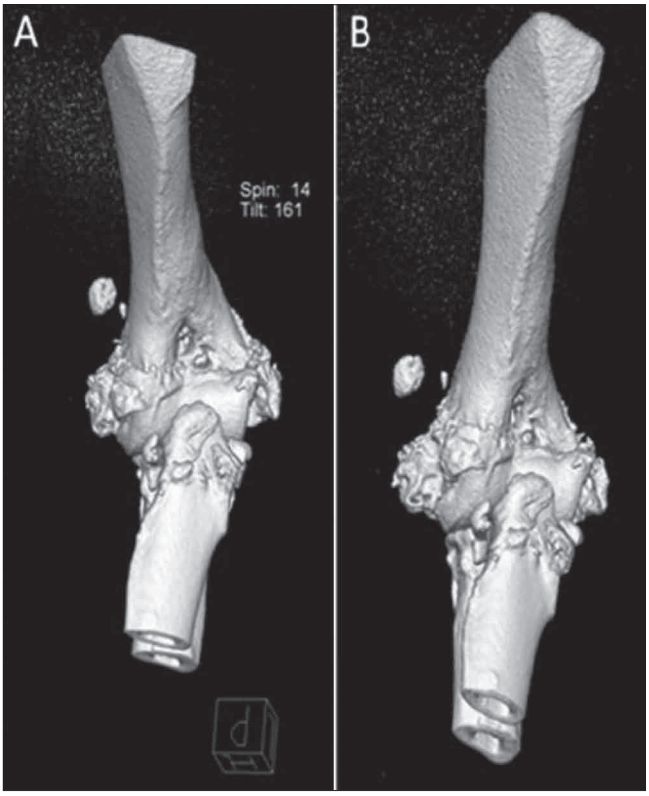


Fig 2. Computer tomography pictures showing changes in the elbow joint in dog from control group at day 0 (A) and day 180 of experiment (B)

Şekil 2. Kontrol grubunda deneyin 0. günde (A) ve 180. günde (B) köpek dirsek eklemi değişimini gösteren bilgisayar tomografi resmi

Table 2. *Mean results ± standard deviation of synovial fluid analysis at day 0, 90 and 180 in experimental and control group*
Tablo 2. *0, 90 ve 180. günlerde ki siynovial sıvı analiz bulguların standart sapması ±*

Group	Day 0		Day 90		Day 180	
	L/N%/M	SFCol/SFClar/SFV	L/N%/M	SFCol/SFClar/SFV	L/N%/M	SFCol/SFClar/SFV
EXP	4.12±0.83/9.1±2/ 94.5±2.4	Colorless/slight turbid/ decreased	2.37±0.91/3.1±1.6/ 93.4±1.6	Colorless/clear /normal	2.37±0.74/2.75±1.39 /90.5±2	Colorless/clear /normal
CTRL	4±1.41/10.2±3.3/ 94±2.8	Colorless/slight turbid/ decreased	3.75±2.06/5.7±3.9/ 93.7±2.1	Colorless/slight turbid/decreased	4.5±1.29/9±2.58 /97.2±0.95	Light yellow/slight turbid/decreased

L/N%/M - Leukocyte number (x10^3/l)/Neutrophil percentage/Mononuclear cell number; SFCol/SFClar/SFV - Synovial fluid color/Synovial fluid clarity/Synovial fluid viscosity

DISCUSSION

Presently, many dogs of different age and breed suffer from degenerative joint disorders (DJD). Since 1990, the Orthopedic Foundation for Animals has noticed that 78 breeds are predisposed to DJD, of which 1.2% to 47.9% suffer directly from elbow joint degeneration (EJD) [15]. This disorder results in various degrees of lameness and pain, but in some cases it shows no prominent clinical manifestation [16]. Among many locomotive system disorders in dogs, osteoarthritis (OA) of the elbow joint has no satisfactory treatment yet. Optimal therapy should guarantee the full recovery of physical activity and considerable pain reduction. The existing methods of OA treatment are based primarily on the application of steroidal and/or non-steroidal anti-inflammatory drugs, which act only temporarily [17]. One of the most promising treatment methods is the auto-transplantation of mesenchymal stem cells isolated from adipose tissue [18]. In current research, we decided to treat patients with elbow joint OA using this novel method for evaluation of its actual efficiency. Our previous studies [19,20] showed beneficial effects of AdMSCs applications in equine hoof fractures and tendon disorders. These results, also confirmed by other clinical groups, support the thesis that AdMSCs application may induce the tissue regeneration processes [21]. Our findings suggest that mesenchymal stem cells work multi-directionally. Because of their multipotent character and self-renewal capacity, AdMSCs might differentiate into chondrocytes under suitable circumstances. This leads to cartilage regeneration and thus recovery of physical activity. Most likely it may be caused by the paracrine effect of stem cells, which when introduced into an inflammatory environment, respond by shedding mesenchymal microvesicles (mMVs), as was reported by other groups [22]. An additional advantage of stem cell therapy is the immunomodulatory action of MSC [23], which leads to a decrease in local joint inflammation. Therefore, we can conclude that auto-transplantations of adipose-derived mesenchymal stem cells may also be utilized as anti-inflammatory agent, without causing adverse reactions seen after prolonged NSAIDS application. This thesis can be supported by our results from a cytological examination of synovial fluid, where the decrease in infiltrating inflammatory cells was noticed. Although the tendency is clear, the statistical analysis could not be performed because of the low number of individuals included in this research. Also no double blinded examination was performed, since this work is based on observations done from particular case reports.

The analysis of computer tomography pictures revealed that presence of significant osteophytes remained unchanged, both in experimental and control groups. It suggests that stem cell therapy treatment cannot decrease the quantity of osteophytes, although it brings the relief in pain and inflammation. According to other groups' findings [24], we assume that these beneficial effects strictly correlate with stem cell paracrine action.

After the application of stem cells, none of patients exhibited any adverse reaction, which confirmed the safety of this method. In the first evaluation period of the experimental group, symptoms like stiffness in walking and lameness were slightly reduced, while in the control group, they substantially intensified.

In conclusion, the therapy with autologous mesenchymal stem cells isolated from adipose tissue in cases of elbow joint degenerative disorders decreases discomfort by reducing accompanying clinical symptoms like pain, stiffness in walking and lameness. Therefore, we state that this method is a substantial advance over the NSAIDs treatment, which works only in short time periods and may bring many adverse reactions. However, there is still lack of information about long-term therapeutic effect of MSC application and about the quality of regenerated cartilage after stem cell applications, so further prolonged experiments are essential.

REFERENCES

1. Black LL, Gaynor J, Adams C, Dhupa S, Sams AE, Taylor R, Harman S, Gingerich DA, Harman R: Effect of intraarticular injection of autologous adipose- derived mesenchymal stem and regenerative cells on clinical signs of chronic osteoarthritis of the elbow joint in dogs. *Vet Ther,* 9 (3): 192-200, 2008.

2. Sandell LJ: Etiology of osteoarthritis: Genetics and synovial joint development. *Nat Rev Rheumatol,* 8 (2): 77-89, 2012.

3. Coleman CM, Curtin C, Barry FP, O'Flatharta C, Murphy JM: Mesenchymal stem cells and osteoarthritis: Remedy or accomplice? *Hum Gene Ther,* 21 (10): 1239-1250, 2010.

4. Hochberg MC, Altman RD, Brandt KD, Clark BM, Dieppe PA, Griffin MR, Moskowitz RW, Schnitzer TJ: Guidelines for the medical management of osteoarthritis. *Arthritis Rheum,* 38 (11): 1535-1540, 1995.

5. Del Bue M, Riccò S, Ramoni R, Conti V, Gnudi G, Grolli S: Equine adipose-tissue derived mesenchymal stem cells and platelet concentrates: Their association in vitro and *in vivo. Vet Res Commun,* 32 (1): 51-55, 2008.

6. Tuglu MI, Ozdal-Kurt F, Koca H, Sarac A, Barut T, Kazanc A: The contribution of differentiated bone marrow stromal cell-loaded biomaterial to treatment in critical size defect model in rats. *Kafkas Univ Vet Fak Derg,* 16 (5): 783-792, 2010.

7. Marycz K, Krzak-Roś J, Śmieszek A, Grzesiak J, Donesz-Sikorska A: Effect of oxide materials synthesized with sol-gel method on adhesion of mesenchymal stem cells. *Przem Chem,* 92 (6): 1097-1100, 2013.

8. Fortier LA, Smith RKW: Regenerative medicine for tendinous and ligamentous ınjuries of sport horses. *Vet Clin North Am: Equine Pract,* 24 (1): 191-201, 2008.

9. Qui Y, Feng G, Yan W: Mesenchymal stem cell-based treatment for cartilage defects in osteoarthritis. *Mol Biol Rep,* 39 (5): 5683-5689, 2012.

10. Hodgkiss-Geere HM, Argyle DJ, Corcoran BM, Whitelaw B, Milne E, Bennett D, Argyle SA: Characterisation and differentiation potential of bone marrow derived canine mesenchymal stem cells. *Vet J,* 194 (3): 361-368, 2012.

11. Lai RC, Chen TS, Lim SK: Mesenchymal stem cell exosome: A novel stem cell-based therapy for cardiovascular disease. *Regen Med,* 6 (4): 481-492, 2011.

12. Kang JW, Kang KS, Koo HC, Park JR, Choi EW, Park YH: Soluble factors-mediated immunomodulatory effects of canine adipose tissue-

derived mesenchymal stem cells. *Stem Cells Dev,* 17 (4): 681-693, 2008.

13. Grzesiak J, Marycz K, Wrzeszcz K, Czogała J: Isolation and morphological characterisation of ovine adipose-derived mesenchymal stem cells in culture. *Int J Stem Cells,* 4 (2): 99-104, 2011.

14. Grzesiak J, Marycz K, Czogala J, Wrzeszcz K, Nicpoń J: Comparison of behavior, morphology and morphometry of equine and canine adipose derived mesenchymal stem cells in culture. *Int J Morphol,* 29 (3): 1012-1017, 2011.

15. Olsson SE: Osteochondrosis in domestic animals. *ACTA Radiologic*, 358 (Suppl.): 299-305, 1978.

16. Grondalen J: Arthrosis in the elbow joint of young, rapidly growing dogs: Interrelation between clinical radiological and pathoanatomical findings. *Nordish Veterinarmedicin,* 34, 65-75, 1982.

17. Luna SPL, Basilio AC, Steagall PVM, Machado LP, Moutinho FQ, Takahira RK, Brandao CVS: Evaluation of adverse effects of long-term oral administration of carprofen, etodolac, flunixin meglumine, ketoprofen, and meloxicam in dogs. *Am J Vet Res,* 68 (3): 258-263, 2007.

18. Guercio A, Di Marco P, Casella S, Cannella V, Russotto L, Purpari G, Di Bella S, Piccione G: Production of canine mesenchymal stem cells from adipose tissue and their application in dogs with chronic osteoarthritis of the humeroradial joints. *Cell Biol Int,* 36 (2): 189-194, 2012.

19. Marycz K, Toker NY, Grzesiak J, Wrzeszcz K, Golonka P: The therapeutic effect of autogenic adipose derived stem cells combined with autogenic platelet rich plasma in tendons disorders in horses *in vitro* and *in vivo* research. *J Anim Vet Adv,* 11 (23): 4324-4331, 2012.

20. Marycz K, Grzesiak J, Wrzeszcz K, Golonka P: Adipose stem cell combined with plasma-based implant bone tissue differentiation in horse suffered phalanx digitalis distalis fracture and *in vitro*. *Vet Med Czech,* 57 (12): 610-617, 2012.

21. Black LL, Gaynor J, Gahring D, Adams C, Aron D, Harman S, Gingerich DA, Harman R: Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: A randomized, double-blinded, multicenter, controlled trial. *Vet Ther,* 8 (4): 272-284, 2007.

22. Ratajczak J, Kucia M, Mierzejewska K, Marlicz W, Pietrzkowski Z, Wojakowski W, Greco NJ, Tendera M, Ratajczak MZ: Paracrine proangiopoietic effects of human umbilical cord blood-derived purified CD133+ cells-implications for stem cell therapies in regenerative medicine. *Stem Cells Dev*, 22 (3): 422-430, 2013.

23. Nicpoń J, Marycz K, Grzesiak J: The therapeutic effect of adipose derived mesenchymal stem cells injection in horses suffering from bone spavin. *Polish J Vet Sci,* 16 (4): 753-754, 2013.

24. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ: Membrane-derived microvesicles: Important and under-appreciated mediators of cell-to-cell communication. *Leukemia,* 20 (9): 1487-1495, 2006.

Kafkas Univ Vet Fak Derg
20 (3): 351-356, 2014
DOI: 10.9775/kvfd.2013.10154

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

RESEARCH ARTICLE

Comparison of Classification Performance of Selected Algorithms Using Rural Development Investments Support Programme Data

Mehmet Ali ALAN [1] Cavit YEŞİLYURT [2] Saadettin AYDIN [3] Erol AYDIN [4]

[1] Cumhuriyet University, Faculty of Economics and Administrative Sciences, Department of Management Information Systems, TR-58140 Sivas - TURKEY
[2] Kafkas University, Faculty of Economics and Administrative Sciences, Department of Management, TR-36100 Kars - TURKEY
[3] Enstitute of Strategic Thinking, TR-06460 Ankara - TURKEY
[4] Kafkas University, Faculty of Veterinary Medicine, Department of Livestock Economics and Management, TR-36100 Kars - TURKEY

Makale Kodu (Article Code): KVFD-2013-10154

Summary

It is not always possible to solve a large size of data via traditional statistical techniques. In order to solve these kinds of data special tactics like data mining are needed. Data mining may meet these kinds of needs with both categorizing and piling tactic. In this study, we have used data mining by using Rural Development Investment Support Program (RDISP) data with various categorizing algorithms. The most prospering categorizing algorithm was tried to determine by using present data. At the end of analysis, it has been understood that MLP (multilayer perceptron), a nerve net model, is the best algorithm that makes the best categorizing.

Keywords: *Data mining, MLP, Nerve net model, RDISP, Rural development*

Kırsal Kalkınma Yatırımlarının Desteklenmesi Programı Verileri Kullanılarak Seçilen Algoritmalarının Sınıflandırma Performanslarının Karşılaştırılması

Özet

Kapsamlı verileri geleneksel istatistiksel teknikler yardımıyla değerlendirmek mümkün değildir. Bu tür kapsamlı verileri değerlendirmek için "Veri Madenciliği" gibi özel tekniklere ihtiyaç vardır. Veri madenciliği kapsamlı verileri hem sınıflandırarak hem de kümeleyerek değerlendirmeyi kolaylaştırmaktadır. Bu çalışmada, Kırsal Kalkınma Yatırım Destekleme Programı (KKYDP) verilerinde çeşitli kategorize algoritmaları yardımıyla veri madenciliği tekniği kullanılmıştır. Çalışmada en uygun kategorize algoritma mevcut veriler kullanarak belirlenmeye çalışılmıştır. Sonuç olarak; analizlerde en iyi kategorizasyon yapan algoritma modelinin Çok Katmanlı Algılayıcı (ÇKA) yapay sinir ağ modeli olduğu belirlenmiştir.

Anahtar sözcükler: *ÇKA, Kırsal kalkınma, KKYD, Sinir ağ modeli, Veri madenciliği*

INTRODUCTION

Databases are rich with hidden information that can be used for intelligent decision making. Classification and prediction are two forms of data analysis that can be used to extract models describing important data classes or to predict future data trends. Such analysis can help provide us with a better understanding of the data at large [1].

The different disciplines on database's data make statistical, mathematical, machine learning and visual analyses with different purposes. One of those analyses techniques is data mining. There are a lot of algorithms in data mining.

Data mining is an interdisciplinary field, the confluence of a set of disciplines, including database systems, statistics, machine learning, visualization, and information science [1].

Data mining, the science and technology of exploring data in order to discover previously unknown patterns, is a part of the overall process of knowledge discovery in

İletişim (Correspondence)
+90 542 7254725
alan@cumhuriyet.edu.tr

databases (KDD). In today's computer-driven world, these databases contain massive quantities of information. The accessibility and abundance of this information makes data mining a matter of considerable importance and necessity [2].

Data mining is known as knowledge discovery process of analyzing data from different point of views and to work out into useful information which can be applied in various application, including advertisement, bioinformatics, database marketing, fraud detection, e-commerce, health care, security, web, financial forecasting etc [3].

According to the Gartner Group, "Data mining is the process of discovering meaningful new correlations, patterns and trends by sifting through large amounts of data stored in repositories, using pattern recognition technologies as well as statistical and mathematical techniques [4].

Data mining is separated from the other statistical tactics in point of using the whole data. Instead of working with the small data that traditionally procured easier evaluation can be made and new independent data can be preferred [5].

Classification is a task that occurs very frequently in everyday life. Essentially it involves dividing up objects so that each is assigned to one of a number of mutually exhaustive and exclusive categories known as *classes*. The term "mutually exhaustive and exclusive"'simply means that each object must be assigned to precisely one class, i.e. never to more than one and never to no class at all.

Many practical decision-making tasks can be formulated as classification problems, i.e. assigning people or objects to one of a number of categories [6].

Classification is the operation of separating various entities into several classes. These classes can be defined by business rules, class boundaries, or some mathematical function. The classification operation may be based on a relationship between a known class assignment and characteristics of the entity to be classified. This type of classification is called supervised. If no known examples of a class are available, the classification is unsupervised. The most common unsupervised classification approach is clustering. The most common applications of clustering technology are in retail product affinity analysis (including market basket analysis) and fraud detection [7].

The concept of supervised classification in data mining is to learn a classification function or construct a classification model based on the known data, which is also called a classifier. This function or model maps the data in the data base to the target attribute, and can, therefore, be used to forecast the class of new data [8].

There are many data mining algorithms, such as association rules, clustering, decision trees, discriminant analysis, artificial neural networks, genetic algorithms, and so on. These algorithms are used to process data from various fields to retrieve information and discover knowledge that can drive an executive's decisions. Information is data associated with the past and the present. Knowledge provides a basis for the prediction of future trends based on original data and the necessary information extracted from the original data. Clearly, information and knowledge are communicated through data [9].

As a result of the data mining analysis, the algorithm of Multilayer Perceptron (MLP) has been the most successful classification algorithm. The algorithm of MLP is neural network model.

A neural network consists of a layered, feed-forward, completely connected network of artificial neurons, or nodes. Neural networks are used for classification or estimation [10].

Neural networks can be used for many purposes, notably descriptive and predictive data mining. They were originally developed in the field of machine learning to try to imitate the neurophysiology of the human brain through the combination of simple computational elements (neurons) in a highly interconnected system. They have become an important data mining method [11].

Representatives of machine learning methods are: artificial neural networks (ANN), self-organizing maps, Hopfield network, genetic algorithms, evolutionary algorithms, fuzzy systems, rough sets, rule-based systems, support vector machines, decision trees, Bayesian and probabilistic models [12].

Work on artificial neural networks (ANNs) has been motivated by the recognition that the human brain computes in an entirely different way from the conventional digital computer. It was a great challenge for many researchers in different disciplines to model the brain's computational processes. The brain is a highly complex, nonlinear, and parallel information-processing system. It has the capability to organize its components so as to perform certain computations with a higher quality and many times faster than the fastest computer in existence today. Examples of these processes are pattern recognition, perception, and motor control [13].

The backpropagation algorithm performs learning on a *multilayer feed-forward* neural network. It iteratively learns a set of weights for prediction of the class label of tuples. A multilayer feed-forward neural network consists of an *input layer*, one or more *hidden layers*, and an *output layer*. An example of a multilayer feed-forward network is shown by Han and Kamber [1]. Each layer is made up of units. The inputs to the network correspond to the attributes measured for each training tuple. The inputs are fed simultaneously into the units making up the input

layer. These inputs pass through the input layer and are then weighted and fed simultaneously to a second layer of "neuronlike" units, known as a hidden layer. The outputs of the hidden layer units can be input to another hidden layer, and so on. The number of hidden layers is arbitrary, although in practice, usually only one is used [1].

The weighted outputs of the last hidden layer are input to units making up the output layer, which emits the network's prediction for given tuples. The units in the input layer are called input units. The units in the hidden layers and output layer are sometimes referred to as neurodes, due to their symbolic biological basis, or as output units.

Multilayer feed-forward networks are one of the most important and most popular classes of ANNs in real-world applications. Typically, the network consists of a set of inputs that constitute the input layer of the network, one or more hidden layers of computational nodes, and finally an output layer of computational nodes. The processing is in a forward direction on a layer-by-layer basis [13].

A multiplayer perceptron has three distinctive characteristics:

1. The model of each neuron in the network includes usually a nonlinear activation function, sigmoidal or hyperbolic.
2. The network contains one or more layers of hidden neurons that are not a part of the input or output of the network. These hidden nodes enable the network to learn complex and highly nonlinear tasks by extracting progressively more meaningful features from the input patterns.
3. The network exhibits a high degree of connectivity from one layer to the next one.

The multilayer perceptron is the most commonly used architecture for predictive data mining. It is a feed-forward network, with possibly several hidden layers, one input layer and one output layer, totally interconnected. It can be considered as a highly non-linear generalization of the linear regression model when the output variables are quantitative, or of the logistic regression model when the output variables are qualitative [11].

In this study, the algorithms named as MultiLayer Perceptron, Ridor, DTNB, ADTree, LADTree, SPegasos, SMO, Dagging, IBk, FT, LMT, LBR, Voted Perceptron, OneR, IB1, VFI, Decorate, Bayes Net, RBF Network, Naïve Bayes were used to select the algorithm has the best classification performances by benefiting the Rural Development Investments Support Programme (RDISP) data. In order to determine the best algorithm, Correctly Classified Instances, Kappa statistic, Mean absolute error, Root mean squared error, Relative absolute error, Root relative squared error, TP Rate, FP Rate, F-Measure, classification timing values of the algorithms has taken into consideration.

MATERIAL and METHODS

In this study the data from Sivas Provincial Directorate of Agriculture were used in the frame of Rural Development Investment Support Program. There are 14859 data belong to 1143 appeal. Data have been taken into Excel format. Then necessary regulations, transformations are made by using Excel macros and the file saved in the name of "kkydp.arff". Both individual and corporate applications of RDISP are kept as B/K (I/C). Lands owned by applicants have been identified as "up to 30 decare", "between 31-40 decare" and "more than 40 decare" and applicant's loan land has been identified similarly as "reach up 30 decare", "between 31-40 decare" and "more than 40 decare". Applicant's request type is set as "M1/M2" (M1: local, M2: Imported). For city or county "i" value and for village "k" value was assigned as application location. Utilization statement was assigned as "twice", "once" or "never" from RDISP. Results were determined to be "Positive" if the application has been accepted and "Negative" if it has not. It was assigned whether there is no value belong to a variable or it's undefined "?" value.

Variable definitions in pre-processing step of prepared dataset are given below: @relation kkydp, @attribute BT {B,K}, @attribute S30 {E,H}, @attribute S3040 {Y,N}, @attribute S40 {Y,N}, @attribute K30 {Y,N}, @attribute K3040 {Y,N}, @attribute K40 {Y,N}, @attribute MType {M1,M2}, @attribute location {i,K}, @attribute twice {Y,N}, @attribute once {Y,N}, @attribute notbenefited {Y,N}, @attribute Class {Positive, Negative}, @DATA B,N,N,Y,N,N,Y,M1,K,N,N,Y, Positive; B,N,Y,N,N,N,N,M1,K,N,N,Y, Negative.

RESULTS

In this study, WEKA (Waikato Environment for Knowledge Analysis) program's 3.6.9 version that was developed in Waikato University was used [14]. WEKA program is open source code software. This program supports a lot of categorizing, piling and coupling rules algorithm. Instead of text based arff., arff.gz, names, data, csv, c45, libsvm, dat, bsi, xrff, xrff.gz file types WEKA supports databases and URL addresses that include data.

An Intel i5 model and 1.7 Ghz CPU, 6 Gb RAM and 64 bit Win 8 operating systemic laptop was used during the application.

There are different but in equal number of values for every defined variables as it is shown in *Fig. 1*. In addition, every variable has been take by using two different values as yes or no {Y,N} and these values represented as two different colors.

Results that come from post prepared data set used in WEKA program is given in the chart. As it can be understood from the *Table 1*, Multilayer Perception algorithm is the

best algorithm that makes the best categorizing with 992 correctly classified instances. This algorithm's cappa statistic is 0.7321, True Positive rate is 0.868, and False Positive rate is 0.124 and F-measure is 0.869. This algorithm's categorizing time is 1.99 seconds. Ridor algorithm follows this algorithm with 973 true categorizing numbers.

Fig 1. Variables Graphics
Şekil 1. Değişkenler grafiği

Table 1. *Performance ratings of selected algorithms in Decision Tree Analysis*
Tablo 1. *Karar Ağaçları Analizine ait bazı algoritmaların başarım dereceleri*

Algorithms	Correctly Classified Instances	Kappa Statistic	Mean Absolute Error	Root Mean Squared Error	Relative Absolute Error %	Root Relative Squared Error %	TP Rate	FP Rate	F-Measure	Time (in seconds)
MultiLayer Perceptron	992	0.7321	0.1465	0.2767	30.1889	56.1843	0.868	0.124	0.869	1.99
Ridor	973	0.6972	0.1487	0.3857	30.6551	78.3030	0.851	0.145	0.852	0.06
DTNB	965	0.6737	0.2085	0.3161	42.9768	64.1741	0.844	0.181	0.843	0.47
ADTree	965	0.6796	0.2562	0.3243	52.7993	65.8508	0.844	0.163	0.844	0.06
LADTree	962	0.6584	0.2294	0.3200	47.2783	64.9635	0.842	0.212	0.835	0.13
SPegasos	927	0.6036	0.1890	0.4347	38.9500	88.2634	0.811	0.218	0.809	0.11
SMO	927	0.6036	0.1890	0.4347	38.9500	88.2634	0.811	0.218	0.809	0.17
Dagging	926	0.5991	0.2203	0.4106	45.4056	83.3628	0.810	0.225	0.807	0.14
IBk	924	0.5807	0.2188	0.3597	45.0871	73.0424	0.808	0.261	0.797	0.01
FT	923	0.5952	0.2644	0.3889	54.5024	78.9647	0.808	0.224	0.805	0.36
LMT	921	0.5923	0.2809	0.3758	57.8871	76.2947	0.806	0.224	0.803	1.78
LBR	916	0.5682	0.2457	0.3819	50.6500	77.5334	0.801	0.263	0.791	0.02
Voted Perceptron	914	0.5788	0.2003	0.4476	41.2942	90.8754	0.800	0.232	0.797	0.02
OneR	904	0.5831	0.2091	0.4573	43.0974	92.8438	0.791	0.188	0.792	0.01
IB1	897	0.5270	0.2152	0.4639	44.3597	94.1936	0.785	0.291	0.770	0.01
VFI	882	0.5119	0.4655	0.4675	95.9414	94.9301	0.772	0.278	0.765	0.02
Decorate	870	0.5007	0.4171	0.4361	85.9614	88.5406	0.761	0.267	0.759	1.38
Bayes Net	842	0.4456	0.3048	0.4151	62.8232	84.2842	0.737	0.300	0.733	0.05
RBF Network	840	0.4409	0.3252	0.4086	67.0371	82.9662	0.735	0.304	0.731	0.31
Naïve Bayes	806	0.3801	0.3323	0.4529	68.4844	91.9612	0.705	0.705	0.701	0.01

Although this algorithm's categorizing time is shorter than MultiLayer Perceptron the other values are worse. Other algorithms follow them.

DISCUSSION

Nowadays, the amount of stored data extremely increases. Data storage is performed by not only private sector, but also by public enterprises such as provincial directorate of agriculture. While private enterprises are achieved in increasing customer commitment to the enterprise and customer satisfaction by using these data, especially public enterprises could not use these data effectively time to time. These data could include some beneficial hide patterns for both public and private sector. One of the most important methods used in producing beneficial information from these data is data mining. Data mining is to produce beneficial and useful data from large scale of data.

In data mining; different methods such as; statistical methods, decision trees, genetic algorithm, fuzzy logic and artificial neural networks could be used. Despite traditional methods, in data mining, inferences could be deduced oriented to results by using the entire data. In this technique, not only numerical data but also alpha-numerical data is used in analyses. A data warehouse is formed by changing both numeric and alpha numerical data to required form. This study is performed by subjecting current data to required change and a data warehouse is prepared in text format by pruning.

Both numerical results and visual results are used in data mining, In this study, there is a graphic *(Fig. 1)* which shows the place of each variable in the entire data besides determination of the most successful classification algorithm. There are a lot of studies as the current study and most of these are from different data sets about the subject. One of these is Palaniappan et al.[15], categorized decision tree, Naïve Bayes method and artificial nerve nets by using Heart Disease Prediction System (HDPS)'s data and they stated that these results may help nursing and medicine students. In addition; Frank et al.[16] introduced how to use WEKA software in Bioinformatics, emphasizing that it supports important categorizing and regression techniques like decision trees, rule masses, bayes sorters, SVM (Support Vector Machines), logistic and linear regression, MLP (Multi-Layer Perceptron) and the closest neighbour. Another study is Ngai et al.[17] categorized Customer Relations Method (CRM) and data mining articles by scanning and probed using data mining in customer relations. Kirkos et al.[18], presented that fraudulent statements can be determined by using decision trees, Artificial Nerve Nets and Bayes Nets from data mining algorithms. DIMIC et al.[19] collected student data by using Moodle e-learning materials and made analyses with categorizing, piling and coupling rules technique by those data. Hsieh [20] made analyses with artificial nerve nets and coupling rules by using the data from bank database and presented the data mining's contribution on behavioral methods of a credit card customer in a bank. Hung et al.[21] tried to guess customer transfers between mobile companies through both artificial nerve nets and decision trees data mining, using data from a telecom company in Taiwan.

Healthy estimation are very important in the studies. One of the most widely used techniques in data mining is the classification. Estimation techniques based on machine learning have been proved to be more successful than the traditional estimation techniques in parallel to developments in information technologies.

As a result of the study, MultiLayer Perceptron algorithm is the best classification algorithm which is an artificial neural networks model. In this study it has been introduced, artificial neural network classification performance in data mining algorithms is higher than that of the other algorithms.

REFERENCES

1. Han J, Kamber M: Data Mining: Concepts and Techniques. Second ed., Morgan Kaufmann Publications, San Francisco, 2006.

2. Rokach L, Maimon O: Data Mining With Decision Trees. World Scientific, New Jersey, 2008.

3. Jain YK, Yadav VK, Panday GS: An efficient association rule hiding algorithm for privacy preserving data mining. *IJERT*, 3 (7): 2792-2798, 2011.

4. Larose DT: Discovering Knowledge in Data. Wiley Publication, New Jersey, 2005.

5. Weiss SM, Zhang T: Performance Analysis and Evaluation. The Handbook of Data Mining. Arizona State University Press, Lawrence Erlbaum Associates, Mahwah, 425-440, 2003.

6. Bramer M: Principles of Data Mining. Springer, London, 2007.

7. Nisbet R, Elder J, Miner G: Handbook of Statistical Analysis and Data Mining Applications. Elsevier Inc, Burlington, 2009.

8. Dong-Peng Y, Li JL, Lun R, Chao Z: Applications of Data Mining Methods in the Evaluation of Client Credibility. **In.** Soares C, Peng Y,Meng J, Washio T, Zhou ZH (Ed): Applications of Data Mining in E-Business and Finance IOS Press, Amsterdam, 2008.

9. Wu T, Li X: Data- Storage and Management. **In,** Nong YE (Ed): The Handbook of Data Mining. New Jersey: Lawrence Erlbaum Associates, Inc., 393-407, 2003.

10. Larose DT: Data Mining Methods and Models, A John Wiley & Sons, Inc., Publication, New Jersey, 2006.

11. Giudici P, Figini S: Applied Data Mining For Business and Industry, Second ed., Wiley Publicition, West Sussex, 90-91, 2009.

12. Lappas G: Machine Learning and Web Mining: Methods and Applications in Societal Benefit Areas. **In,** Rahman H (Ed): Data Mining Applications for Empowering Knowledge Societies, 76-95, 2009.

13. Kantardzic M: Data Mining: Concepts, Models, Methods and Algorithms, John Wiley & Sons J. B. Speed Scientific School, University of Louisville IEEE Computer Society, 2003.

14. WEKA: Waikato Environment for Knowledge Analysis. http://www.cs.waikato.ac.nz/ml/weka/downloading.html, *Accessed:* 06.09.2013.

15. Palaniappan S, Awang R: Intelligent heart disease prediction system using data mining techniques. *IJCSNS*, 8 (8): 343-350, 2008.

16. Frank E, Hall M, Trigg L, Holmes G, Witten H: Data mining in bioinformatics using WEKA. *Bioinformatics*, 20 (15): 2479-2481, 2004.

17. Ngai EWT, Xiu L, Chau DCK: Application of data mining techniques in customer relationship management: A literature review and classification. *Expert Systems with Applications*, 36, 2592-2602, 2009.

18. Kirkos E, Spathis C, Manolopoulos Y: Data Mining techniques for the detection of fraudulent financial statements. *Expert Systems with Applications,* 32, 995-1003, 2007.

19. Dimić G, Kuk K, Ahorjanski M: Mining Student's data for analyze electronic learning materials available on the moodle course. *Metalurgia International*, 16 (12): 78-82, 2011.

20. Hsieh NC: An integrated data mining and behavioral scoring model for analyzing bank customers, *Expert Systems with Applications,* 27, 623–633, 2004.

21. Hung SY, Yen DC, Wang HY: Applying data mining to telecom churn management. *Expert Systems with Applications,* 31, 515-524, 2006.

Kafkas Univ Vet Fak Derg
20 (3): 357-362, 2014
DOI: 10.9775/kvfd.2013.10171

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

RESEARCH ARTICLE

Anadolu Mandası Malaklarında Büyüme Eğrisinin Çeşitli Doğrusal Olmayan Modeller Kullanılarak Karşılaştırılması

Aziz ŞAHİN [1] Zafer ULUTAŞ [2] Ufuk KARADAVUT [1] Arda YILDIRIM [2] Servet ARSLAN [2]

[1] Ahi Evran Üniversitesi, Ziraat Fakültesi, Zootekni Bölümü, TR-40100 Kırşehir - TÜRKİYE
[2] Gaziosmanpasa Üniversitesi, Ziraat Fakültesi, Zootekni Bölümü, TR-60250 Tokat - TÜRKİYE

Makale Kodu (Article Code): KVFD-2013-10171

Özet

Bu araştırmada, doğrusal olmayan modeller ile Anadolu mandalarının canlı ağırlıklarındaki değişimi açıklamak için en iyi matematiksel modelin belirlenmesi amaçlanmıştır. Bu hedefle, Tokat ili ve ilçelerinde 2011-2012 yılları arasında yetiştirilen 309 baş erkek ve 331 baş dişi olmak üzere toplam 640 baş Anadolu mandası malağına ait canlı ağırlık kayıtlarından yararlanılmıştır. Çalışmada, doğrusal olmayan Lojistik, Richards, Gompertz ve Brody modelleri kullanılmıştır. Büyümeyi en iyi tanımlayan modelin belirlenmesinde kriter olarak belirleme katsayısı (R^2) ve hata kareler ortalaması (HKO) kullanılmıştır. Belirleme katsayısı (R^2) yüksek ve hata kareler ortalaması (HKO) düşük olan model büyümeyi tanımlayan en iyi model olarak seçilmiştir. Lojistik, Brody, Gompertz ve Richards modellerde erkek malaklar için belirleme katsayıları 0.94, 0.93, 0.95 ve 0.97 olarak bulunurken, hata kareler ortalamaları (HKO) sırasıyla 637.48, 688.32, 598.12 ve 528.74 olarak saptanmıştır. Dişi malaklara ait belirleme katsayıları 0.96, 0.92, 0.96, 0.98 ve hata kareler ortalamaları 682.32, 703.51, 548.66 ve 498.63 olarak belirlenmiştir. Sonuç olarak, Richards modeli, Anadolu mandalarında büyümeyi en iyi tanımlayan model olmuştur. Ayrıca, Richards model kullanılarak, erkek ve dişi malaklarda eşeysel olgunluk yaşı, damızlıkta kullanma yaşı ve uygun kesim yaşı gibi bazı büyüme ve gelişme özellikleri tahmin edilebilecektir.

Anahtar sözcükler: *Büyüme eğrileri, Lojistik, Brody, Gompertz, Richards, Anadolu mandası*

Comparison of Growth Curve Using Some Nonlinear Models in Anatolian Buffaloe Calves

Summary

The aim of the research was to detect the best model to explain the variation of live weight of Anatolian buffaloes using the nonlinear models. For this purpose, in the production period of 2011-2012, live weight records of 640 heads Anatolian buffalo calves including 309 male and 331 female reared in different farm conditions of Tokat were used. To achieve the objective of the study, the non-linear models of Logistic, Richards, Gompertz and Brody function were used. To decide which one is the best model, the coefficient of determination (R^2) and the mean square error (MSE) statistics were used. The coefficient of determination (R^2) for Logistic, Brody, Gompertz and Richards models were found as 0.96, 0.92, 0.96 and 0.98 for female calves and 0.94, 0.93, 0.95 and 0.97 for male calves, respectively. And mean squared errors (MSE) were found as 682.32, 703.51, 548.66 and 498.63 for females and 637.48, 688.32, 598.12 and 528.74 for male Anatolian buffaloes, respectively. As a result, the best fitted model based on MSE and R^2 criterias was Richards model. Also, the growth and development traits such as sexual maturity age, breeding age, appropriate slaughter age in male and female Anatolian buffalo calves can be estimated using the Richards model.

Keywords: *Growth curves, Logistic, Brody, Gompertz, Richards, Anatolian buffalo*

GİRİŞ

Canlıların genetik yapısı ve bulundukları çevre koşullarının etkileşimi ile şekillenen, hücrelerin sayı ve boyutlarında hayvanın türü ile uyumlu olarak belirli zaman aralıklarında meydana gelen artışlar, büyüme olarak ifade edilmektedir. Gelişme ise canlılarda yeni biyolojik fonksiyonların işlevsel hale gelmesi ile oluşan fizyolojik ve morfolojik farklılaşmalar ile vücut kısımları oranlarında meydana gelen değişikliklerdir. Yumurtanın zigot tarafından döllenmesi ile başlayan büyüme, doğum öncesi ve doğum sonrası olmak üzere iki safhada incelenmektedir. Bir hayvan

İletişim (Correspondence)
+90 386 2804830
aziz.sahin@ahievran.edu.tr

doğumdan ergin hale gelene kadar değişik aşamalardan geçer. Büyüme özellikleri hayvanlarda karmaşık metabolik faaliyetler sonucunda ortaya çıkmaktadır. Bu yüzden, araştırıcılar karmaşık olan bu biyolojik olayı daha kolay ifade edebilmek için çeşitli matematik modeller geliştirerek sayısal olarak büyüme karakterini açıklamaya çalışmışlardır. Ergin yaşa ulaşan hayvanlardan beklenen performansın elde edilebilmesi, hayvanların büyüme ve gelişmeleri ile ilgilidir. Diğer bir ifade ile hayvan yetiştiriciliğinde ekonomik öneme sahip olan özellikler hayvanın cüssesi ve büyüme hızından doğrudan etkilenmektedir. Canlıların büyümesinde yaşa bağlı olarak oluşan değişim büyüme eğrisi olarak ifade edilmektedir [1-6]. Bu değişim, hücre büyüklüğü, hücre sayısı, bir organın ağırlığı ya da bireyin canlı ağırlığı şeklinde olabilmektedir [1,7,8]. Hayvanlarda büyümenin sona erdiği ergin canlı ağırlığa ulaşılana kadar geçen süreçte büyüme eğrisi modelleri ile, fizyolojik olarak büyümenin açıklanmasında önemli bir yeri olan biyolojik parametreler tahmin edilebilmektedir [9,10]. Büyüme eğrileri ile belirlenecek parametreler büyüme üzerinde etkili faktörlerin tespit edilmesi ve karmaşık bir yapıya sahip olan büyüme sürecinin tanımlanmasında kullanılmaktadır [11]. Hayvan yetiştiriciliğinde büyüme eğrilerinin kullanımı ilk defa Brody [12] tarafından çeşitli büyüme özelliklerinin, büyüme modeli kullanılarak tahmin edilmesi ile başlatılmış ve sonrasında Richards'ın [13] çalışmaları ile yaygınlaştırılmıştır. Gompertz, Lojistik, Brody ve Richards hayvan yetiştiriciliğinde büyüme özelliklerinin tahmin edilmesinde yaygın olarak kullanılan büyüme eğrisi modelleridir [1,14,15]. Büyüme eğrisi modelleri ile ortak olarak tahmin edilebilen parametreler ergin canlı ağırlık (A), erginleşme hızı (k) ve (B) parametreleridir. Bir hayvanın biyolojik büyüme süreci, büyüme eğrisi modelleri ile tespit edilen parametreler yardımı ile açıklanabilir. Canlılarda birim zamanda meydana gelen büyümenin ifade edilmesinde büyüme eğrisi modelleri kullanılmaktadır. Bu modeller ile hayvanların genel sağlık durumları, ileri dönemlerindeki büyümeleri, damızlığa ayrılma yaşı, optimum kesim yaşı ve bazı parametreler tahmin edilebilmektedir [1,5,16]. Bilgisayar teknolojisinde meydana gelen gelişmeler, model parametrelerinin tahmin edilmesini kolaylaştırmış ve büyüme eğrilerinin hayvan yetiştiriciliğinde kullanılmasının yaygınlaşmasına neden olmuştur. Büyüme eğrisi modelleri ile, hayvanlardan farklı yaş ve zamanda elde edilen ve yorumlanması zor olan veriler biyolojik olarak açıklanabilmektedir. Büyüme eğrilerini belirlemede kullanılacak model ile ilgili parametreler biyolojik olarak ifade edilebilir olmalıdır. Bu durum hayvanın üzerinde durulan özelliği ile ilgili genetik ve çevre etkileşiminin anlaşılabilir olmasına bağlıdır. Analizlerde kullanılacak verilerin yapısı ve analiz amacı büyüme eğrilerinin tahmin edilmesinde kullanılacak model veya modellerin tespit edilmesinde dikkate alınacak önemli kriterler arasında yer almaktadır. Diğer hayvan türlerinde olduğu gibi Anadolu mandalarında da yaş ve canlı ağırlık ilişkilerinin tespit edilebilmesi için fazla zaman ve işgücüne gereksinim bulunmaktadır. Büyümeyi en iyi tanımlayan modelin belirlenebilmesi, sonuçların seleksiyon kriteri olarak kullanılabilmesi ve güvenilir tahminler yapılarak yorumlanabilmesi açısından önemlidir. Son yıllarda çeşitli hayvan türlerinde belirli dönemlere ait büyüme eğrilerinden ıslah programlarında yararlanılmaya çalışılmaktadır [16-19]. Doğrusal olmayan büyüme eğrisi modelleri kullanılarak günümüze kadar çeşitli araştırıcılar tarafından bazı manda ırklarında [6,19-24] büyüme eğrilerinin modellenmesi ile ilgili araştırmalar yapılmış olmasına rağmen, Anadolu mandalarında bu konuda yapılmış bir çalışma bulgusuna rastlanılmamıştır.

Bu araştırmada, günümüzde hayvanlarda büyüme eğrilerinin tahmin edilmesinde yaygın olarak kullanılan, Lojistik, Richard, Brody ve Gompertz modelleri ile Tokat ili ve ilçelerinde yetiştirilen erkek ve dişi malaklarda yaşa bağlı olarak canlı ağırlıktaki değişimi açıklayabilecek en iyi büyüme eğrisi modelinin belirlenmesi amaçlanmıştır.

MATERYAL ve METOT

Materyal

Araştırma materyalini Tokat ili ve ilçelerinde 2011-2012 yıllarında doğan, 309 baş erkek, 331 baş dişi olmak üzere toplam 640 baş malağa ait canlı ağırlık kayıtları oluşturmuştur. Araştırmada kullanılan hayvanların canlı ağırlıkları Tarımsal Araştırmalar ve Politikalar Genel Müdürlüğü tarafından desteklenen Halk Elinde Manda Islahı Ülkesel Projesi kapsamında elde edilmiştir. Malaklar doğumu takip eden ilk 24 saat içerisinde tartılarak doğum ağırlıkları kayıt edilmiştir. Erkek ve dişi malaklar 360 günlük yaşa kadar, en az dört defa tartılmıştır [6,25]. Ağırlık ölçümleri 100 g hassasiyette ölçüm yapan 1000 kg kapasiteli baskül ile yapılmıştır. Doğum ağırlığı ve doğumdan 360 günlük yaşa kadar 3'er aylık periyotlar ile ağırlıkları alınan 309 baş erkek ve 331 baş dişi olmak üzere toplam 640 malağın ağırlık ve yaş verileri bu araştırmada değerlendirilmiştir. Malaklar doğumdan sütten kesime kadar anneleri ile birlikte barındırılarak yeterli kolostrum ve süt tüketmeleri sağlanmıştır. Yörede genellikle meraya dayalı bir manda yetiştiriciliği yapılmakta olup, mevsim şartlarının otlatma için uygun olduğu günlerde tüm mandalar sabah sağımından sonra meraya çıkartılmıştır. Mera döneminde mandalara genellikle ek yemleme yapıl-mamış, sadece kış ayları gibi mevsim şartlarının otlatma için uygun olmadığı durumlarda, işletmelerde bulunan yemler (saman, kuru yonca otu, silaj vb.) kullanılarak ek yemleme yapılmıştır.

Metot

Bu çalışmada kullanılan modellerin büyüme eğrilerine uyumlarının tahmininde Statistica 5.0. V [26] paket programı kullanılmıştır. Araştırmada kullanılan modeller *Tablo 1*'de gösterilmiştir.

Modellerde; A: Ağırlığın asimtotik limitidir. Asimtotik limit bazı çevresel faktörlerin etkisi, laktasyon ve gebelik

Tablo 1. *Büyüme eğrilerinin tahmininde kullanılan doğrusal olmayan modeller*
Table 1. *Used non linear models in the estimation of growth curves*

Büyüme Eğrisi Modelleri	Eşitlikler
Lojistik	$Yt = A(1 + B\exp(-kt))^{-1}$
Gompertz	$Yt = A\exp(-B\exp(-kt))$
Richards	$Yt = A(1 - B\exp(-kt))^{m}$
Brody	$Yt = A(1 - B\exp(-kt))$

Exp: *matematiksel üs fonksiyon*

gibi nedenler ile hayvanın canlı ağırlığındaki kısa dönem değişimlerinden bağımsız, hayvanın ulaşabileceği en yüksek ağırlıktır. Ergin canlı ağırlığı gösteren bu parametre bütün büyüme eğrisi modellerinde ortak olarak tahmin edilir ve ölçü birimi kg'dır. Bir hayvanın t aylık yaşta belirlenen ağırlığı, hiçbir zaman bu parametreden (A) fazla olamaz. B: doğum sonrası dönemde kazanılan ağırlığın ergin canlı ağırlığa oranını gösterir ve bu parametre ağırlık (Y_t) ve zamanın (t) başlangıç değeri kullanılarak tahmin edilir. Y_t: t aylık yaşta gözlenen ağırlığı, t: ağırlığın alındığı dönemlerde mandaların yaşını göstermektedir. k: erginleşme hızı olarak ifade edilir ve canlı ağırlığın (Y_t) hangi hızla ergin ağırlığa yaklaştığını göstermektedir. m: eğrinin şekli hakkında bilgi veren ve tahmin edilen büyüme hızındaki değişimin artıştan azalışa geçtiği durumda meydana gelen bükülme noktasını göstermektedir. Bu noktada ağırlıktaki değişim maksimumdur. Richards modeli çok geniş bir bükülme noktasına sahipken, Lojistik ve Gompertz modellerinde ise bu bükülme noktaları sabittir (m: -1, ∞). A, B, k ve m parametreleri, Statistica 5.0. V [26] istatistik programı kullanılarak genelleştirilmiş en küçük kareler metodu ve Levenberg Marquardt iterasyon işlemi sonucu tespit edilmiştir. İterasyon yapılırken, yakınsama kriteri olarak 1.0E-8 kullanılmıştır [5,27-29]. Modellerin karşılaştırılmasında, toplam varyasyonda modelin açıkladığı kısmı gösteren R^2 ve modele ait belirlenen büyüme eğrisi ile gerçek büyümeye ait noktalar arasındaki farkı gösteren HKO kullanılmıştır.

BULGULAR

Bu araştırmada Anadolu mandası malaklarına ait canlı ağırlıkların zamana bağlı olarak değişimi Lojistik, Richards, Brody ve Gompertz modelleri ile incelenmiş ve elde edilen parametreler, belirleme katsayıları (R^2) ve hata kareler ortalamaları (HKO) *Tablo 2* ve *Tablo 3*'te özetlenmiştir. Ayrıca, erkek ve dişi malaklarda dört farklı büyüme eğrisi modelinin kullanılması ile belirlenen tahmin eğrileri *Şekil 1* ve *Şekil 2*'de görülmektedir. Bu araştırmada

Lojistik, Richards, Brody ve Gompertz modellerde erkek malaklar için belirleme katsayılarının (R^2) sırası ile 0.94, 0.97, 0.93 ve 0.95 olduğu saptanmış ve hata kareler ortalamaları (HKO) aynı sıra ile 637.48, 528.74, 688.32 ve 598.12 olarak tahmin edilmiştir.

Tablo 2. *Erkek malaklarda Lojistik Gompertz, Richards ve Brody modelleri ile tahmin edilen parametreler ve standart hataları (Sx)*
Table 2. *Parameter estimates and their standard errors for Logistic, Gompertz, Richards and Brody models in male calves*

Modeller	Parametre	X	Sx	HKO	R^2
Lojistik	A	547.6	26.55	637.48	94.38
	B	2.682	0.309		
	k	0.360	0.054		
Gompertz	A	528.8	26.47	598.12	95.62
	B	2.093	0.406		
	k	0.218	0.039		
Richards	A	573.80	29.72	528.74	97.59
	B	1.057	0.108		
	k	0.0268	0.0189		
	m	1.26	0.0065		
Brody	A	528.82	29.64	688.32	93.24
	B	0.966	0.203		
	k	0.0324	0.0027		

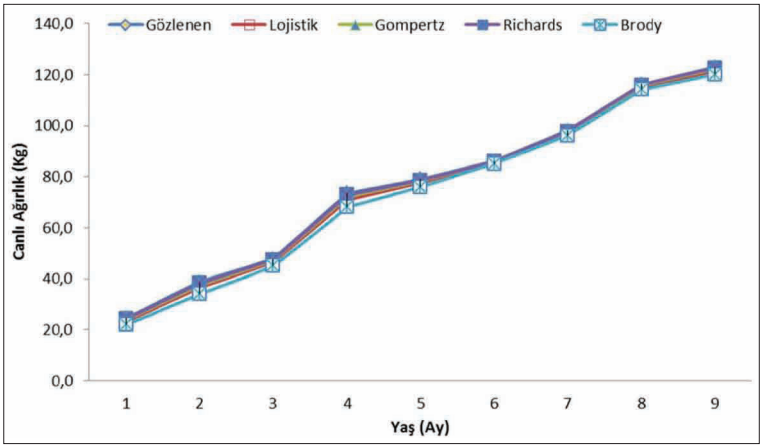
Tablo 3. *Dişi malaklarda Lojistik Gompertz, Richards ve Brody modelleri ile tahmin edilen parametreler ve standart hataları (Sx)*
Table 3. *Parameter estimates and their standard errors for Logistic, Gompertz, Richards and Brody models for the female calves*

Modeller	Parametre	X	Sx	HKO	R^2
Lojistik	A	516.2	25.47	682.32	96.14
	B	2.361	0.211		
	k	0.216	0.056		
Gompertz	A	496.8	23.68	548.66	96.87
	B	1.842	0.367		
	k	0.309	0.0477		
Richards	A	538.4	28.16	498.63	98.22
	B	0.966	0.096		
	k	0.0386	0.0217		
	m	1.18	0.006		
Brody	A	506.3	30.68	703.51	92.18
	B	1.068	0.106		
	k	0.0496	0.0018		

Yine bu çalışmada Lojistik, Richards, Brody ve Gompertz modellerde dişi malaklara ait belirleme katsayıları (R^2) 0.96, 0.98, 0.92 ve 0.96 olarak tespit edilmiştir. Bu modeller ile belirlenen hata kareler ortalamaları (HKO) ise 682.32, 498.63, 703.51 ve 548.66 olarak bulunmuştur.

TARTIŞMA ve SONUÇ

Büyüme modellerinin uyumları, belirleme katsayıları (R^2), hata kareler ortalamaları (HKO), parametrelerin standart hataları, biyolojik anlamlılıkları ve tahminlerdeki tutarlılık gibi birçok ölçüt hayvan yetiştiriciliğinde büyümeyi

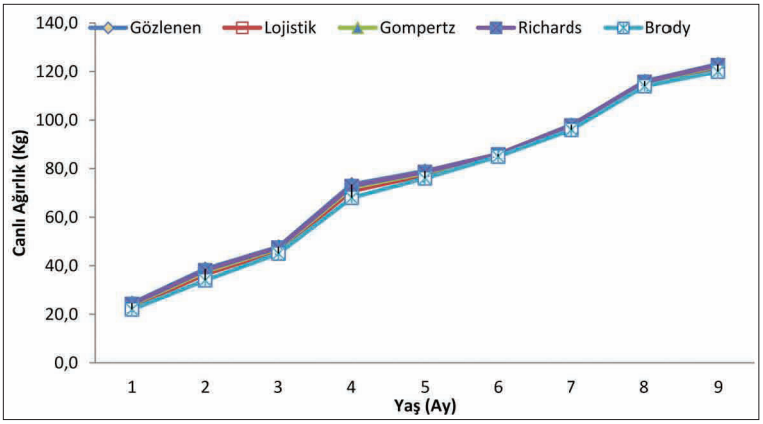


Şekil 1. Erkek mandalarda dört farklı büyüme eğrisi modelinin kullanılması ile belirlenen tahmin eğrileri

Fig 1. Prediction curves obtained from four different growth curve models in males

Şekil 2. Dişi malaklarda dört farklı büyüme eğrisi modelinin kullanılması ile belirlenen tahmin eğrileri

Fig 2. Prediction curves obtained from four different growth curve models in female calves



tanımlayan büyüme eğrisi modellerinin karşılaştırılmasında yaygın olarak kullanılabilmektedir [3,30,31]. Büyüme eğrisi modellerinin karşılaştırılmasında R^2 ve HKO değerlerinin birlikte değerlendirildiği araştırmalarda canlı ağırlığın yaşa göre değişimini en iyi, en yüksek R^2 ve en küçük HKO değerine sahip olan modelin açıkladığı birçok çalışmada bildirilmiştir [5,17,32]. Erkek ve dişi malaklarda büyümeyi en iyi tanımlayan Richards modeline göre R^2 değeri bakımından %4.35 ve %6.04 daha az uyumlu olan Brody modeli büyümeyi en az tanımlayan model olmuştur. Lojistik ve Gompertz modellerinin uyumları arasındaki fark erkek ve dişiler için sırası ile %1.24 ve %0.73 olarak belirlenmiştir. İlgili modellerin uyumları Richards modelinden düşük, Brody modelinden yüksek bulunmuştur. Büyümeyi en iyi tanımlayan Richards modeli ile Lojistik ve Gompertz modelleri arasındaki farklar sırası ile erkek malaklarda, %3.21, %1.97, dişilerde ise %2.08, %1.35 olarak tespit edilmiştir.

Büyüme eğrisi modelleri incelendiğinde modelleri karşılaştırmada kıstas olarak kullanılan R^2 erkek ve dişi malaklarda en düşük Brody modelinden elde edilmiştir. Her iki cinsiyette de en yüksek R^2 katsayısı Richards modelinden elde edilirken, bu modeli Gompertz ve Lojistik modeller takip etmiştir. Erkek ve dişi malaklarda HKO bakımından modeller karşılaştırıldığında ise, en yüksek değer Brody modeli kullanıldığında elde edilmiş ve bu modeli sırası ile Lojistik, Gompertz ve Richards modelleri izlemiştir. Hata kareler ortalaması ile ilgili en küçük değer her iki cinsiyette de Richards modeli ile tespit edilmiştir. Yani, her iki cinsiyette de canlı ağırlığın yaşa göre değişimini en iyi, en yüksek R^2 ve en küçük HKO değerine sahip olan dört parametreli (A, B, k, m) Richards modeli açıklamaktadır. Prestes ve ark.[6] Murrah ırkı mandalarda büyümeyi en iyi tanımlayan modelin Richards modeli olduğunu bildirmişlerdir. Araştırma bulgusu ve Prestes ve ark.'nın [6] bildirişinin aksine, Nehir mandalarında Lojistik modeli [20], Murrah ırkı mandalarda Gompertz modeli [32], melez mandalarda Brody ve Gompertz modelleri [18], Akdeniz mandalarında Brody modeli [21], Murrah ırkı mandalarda Lojistik ve Gompertz modelleri [19] büyümeyi en iyi tanımlayan model olarak belirlenmiştir. Erkek malaklarda ergin canlı ağırlık (A) en yüksek Richards, en düşük canlı ağırlık ise Gompertz ve Brody modelleri ile belirlenmiştir. Bu modeller arasındaki fark erkelerde 45 kg olarak tespit edilmiştir. Dişi malaklarda ise ergin canlı ağırlık (A) en yüksek Richards, en düşük canlı ağırlık ise Gompertz modeli ile tahmin edilmiştir. Bu modeller arasındaki fark dişilerde 32.1 kg olarak saptanmıştır. Bu araştırmada kullanılan bütün modellerde erkeklerin ergin canlı ağırlıkları dişilerden daha fazla bulunmuştur. Erkek malaklarda Richards modeli ile tahmin edilen canlı ağırlık, Gompertz ve Brody modelleri ile tahmin edilen canlı ağırlıktan, dişi malaklarda ise Lojistik ve Brody modelleri ile belirlenen canlı ağırlıktan yüksek bulunmuştur. Araştırma bulgusunun aksine, Murrah ırkı mandalarda yapılan bir çalışmada ergin canlı ağırlığı Brody modelinin yüksek,

lojistik modelin ise düşük tahmin ettiği bildirilmiştir [3].

Büyüme eğrisinin şekli hakkında bilgi veren bir parametre olan m, tahmin edilen büyüme hızındaki değişimin artıştan azalışa geçtiği durumlarda meydana gelen büküm noktasını göstermektedir. Erkek malaklar için tespit edilen m parametresi (1.26), dişi malaklardan (1.18) yüksek bulunmuştur. Bu sonuç, dişi malakların büküm noktasına daha erken ulaştıklarını ve bu noktada erkek malaklara göre daha düşük ağırlıkta olduklarını göstermektedir. Murrah ırkı mandalarda yapılan çalışmalarda [3,6] m parametresi sırası ile 1.14 ve 1.50 olarak tespitedilmiştir. Bu araştırmada erkek ve dişi malaklar için belirlenen m parametresi bu bildirişler ile uyumlu bulunmuştur.

Richards modeli ile A, B, k ve m parametreleri erkeklerde sırası ile 573.80 kg, 1.057, 0.0268 ve 1.26, dişilerde ise 538.4 kg, 0.966, 0.0386 ve 1.18 olarak tahmin edilmiştir. Erkek malaklarda Richards modeli ile tahmin edilen ergin canlı ağırlık, dişi malaklardan 35.4 kg kadar yüksek bulunmuştur. Bu araştırmada kullanılan büyüme eğrisi modellerinde ortak olarak tahmin edilen k parametresi, t. yaşta gözlenen canlı ağırlığın hangi hızla ergin canlı ağırlığa yaklaştığını ifade etmektedir. Erkek malaklarda büyüme hızı hakkında bilgi veren k parametresi ile ilgili en yüksek değer Lojistik (0.360) modelin kullanımı ile elde edilirken, bunu Gompertz (0.218), Brody (0.0324) ve Richards modelleri (0.0268) izlemiştir. Dişi malaklarda ise erginleşme hızı (k parametresi) ile ilgili en yüksek değer Gompertz (0.309) modeli kullanılarak saptanırken, bu modeli Lojistik (0.218), Brody (0.0496) ve Richards modelleri (0.0386) izlemiştir.

Murrah ırkı mandalarda yapılan bir çalışmada [3], Brody, Gompertz, Lojistik ve Richards modeller ile tahmin edilen k parametresi sırası ile 0.0011, 0.0022, 0.0026 ve 0.0015 olarak belirlenmiştir. Yine aynı ırk mandalara ait verilerin değerlendirildiği bir diğer çalışmada [19] ise Brody, Gompertz ve Lojistik modelleri ile erginleşme hızı (k parametresi) 0.0020, 0.0041 ve 0.0049 olarak tespit edilmiştir. Bu çalışmada erkek ve dişi Anadolu mandası malakları için aynı modeller ile tahmin edilen k parametresi Malhado ve ark.[3] ve Araújo ve ark.'nın [19] bulgularından yüksek bulunmuştur.

Araştırmada kullanılan modellerde ortak olarak tahmin edilen B parametresi doğum sonrasında kazanılan canlı ağırlığın ergin canlı ağırlığa oranını ifade etmektedir. Erkek malaklarda, en yüksek B parametresi Lojistik model kullanıldığında (2.682) elde edilmiş olup, bu modeli Gompertz, Richards ve Brody modelleri izlemiştir. Dişi malaklarda da B parametresi ile ilgili en yüksek değer, Lojistik model (2.361) kullanıldığında tespit edilmiş olup, bu modeli Gompertz, Brody ve Richards modelleri izlemiştir. Malhado ve ark.[3] tarafından Murrah ırkı mandalarda yapılan çalışmada B parametresi Brody, Gompertz ve Richards modelleri kullanıldığında sırası ile 0.98, 2.38 ve 0.86 olarak saptanmıştır. Murrah ırkı mandalarda yapılan bir çalışmada [3] B parametresi Brody, Gompertz ve Richards modeller ile sırası ile 0.98, 2.38 ve 0.86 olarak tespit edilmiştir. Aynı şekilde Murrah ırkı mandalarda yürütülen bir diğer araştırmada [19] B parametresi Brody ve Gompertz modeller kullanıldığında dişi malaklarda sırası ile 0.911, 1.866, erkek malaklarda ise 0.908 ve1.854 olarak belirlenmiştir.

Bu araştırmada erkek malaklarda Gompertz model ile tahmin edilen B parametresi Araújo ve ark.'nın [19] Murrah ırkı mandalarda aynı modeli kullanarak tespit ettiği değerden yüksek bulunmuştur. Brody modeli kullanılarak tespit edilen B parametresinin Araújo ve ark.'nın [19] bulgusu ile uyumlu olduğu belirlenmiştir. Dişi malaklarda Gompertz model ile tahmin edilen B parametresinin Araújo ve ark.'nın [19] Murrah ırkı mandalarda aynı modeli kullanarak tespit ettiği değerle uyumlu olduğu tespit edilmiştir. Brody model kullanılarak tespit edilen B parametresi Araújo ve ark.'nın [19] bulgusundan yüksek bulunmuştur.

Erkek malaklarda belirleme katsayıları Lojistik, Brody, Gompertz ve Richards modelleri kullanıldığında sırası ile 0.94, 0.93, 0.95 ve 0.97 olarak saptanmış ve hata kareler ortalamaları aynı sıra ile 637.48, 688.32, 598.12 ve 528.74 olarak tahmin edilmiştir. Belirleme katsayıları dişi malaklarda aynı modeller ile 0.96, 0.92, 0.96, 0.98 ve hata kareler ortalamaları ise 682.32, 703.51, 548.66 ve 498.63 olarak saptanmıştır.

Modellerin karşılaştırılmasında kriter olarak kullanılan R^2 ve HKO değerleri birlikte değerlendirildiğinde, erkek ve dişi malaklarda büyüme performansını en iyi Richards modelinin açıkladığı belirlenmiştir.

Büyüme süreci içerisinde bazı dönemlerde malakların büyüme ve gelişmelerinin izlenmesinin, sürü yönetimi, bakım ve beslemenin düzenlenmesi açısından işletmeye büyük yararı olacaktır. Ayrıca malakların büyümelerinin takip edilmesi ile büyümelerinde aksama tespit edilen malaklara erken müdahale imkanı sağlanabilecektir.

Özellikle doğum öncesinden doğuma, doğumdan sütten kesime, sütten kesimden ergin döneme kadar olan dönemlerde malakların bazı özelliklerinin (canlı ağırlığın) izlenmesinin yetiştiricilere yetiştirme amaçlarına (damızlık, kasaplık) uygun stratejik kararları (uygun kesim çağı, damızlıkta kullanma yaşı ve damızlık dışı bırakma vb. gibi) almalarında katkı sağlayacaktır. Araştırmanın yürütüldüğü işletmelerde Richards modeli kullanılarak, erkek ve dişi malakların genel gelişme ve büyüme durumları ile ilgili bilgi edinilebilecek, eşeysel olgunluk yaşı, damızlıkta kullanma yaşı ve uygun kesim yaşı gibi büyüme ve gelişme özellikleri tahmin edilebilecektir.

Teşekkür

Bu çalışmanın yürütülmesinde imkân sağlayan Gıda Tarım ve Hayvancılık Bakanlığı Tarımsal Araştırmalar ve Politikalar Genel Müdürlüğüne ve Tokat İli Damızlık Manda Yetiştiricileri Birliğine ve proje teknik elemanlarına katkılarından dolayı teşekkür ederiz.

KAYNAKLAR

1. Akbaş Y: Büyüme eğrisi modellerinin karşılaştırılması. *Hay Üret,* 36, 73-81, 1995.

2. Akbaş Y, Taşkın T, Demirören E: Farklı modellerin Kıvırcık ve Dağlıç erkek kuzularının büyüme eğrilerine uyumunun karşılaştırılması. *Turk J Vet Anim Sci,* 23 (Suppl 3): 537-544, 1999.

3. Malhado, CHM, Ramos AA, Carneiro PLS, Souza JC, Wechsler FS, Eler JP, Azevedoy DMMR, Sereno JRB: Non linear models to describe the growth of the buffaloes of murrah breed. *Arch Zootec,* 57 (220): 497-503, 2008.

4. Agudelo-Gómez D, Hurtado-Lugo N, Cerón-Muñoz MF: Growth curves and genetic parameters in colombian buffaloes (Bubalus bubalis Artiodactyla, Bovidae). *Rev Colomb Cienc Pecu,* 22, 178-188, 2009.

5. Bayram B, Akbulut Ö: Siyah Alaca sığırlarda büyüme eğrilerinin doğrusal ve doğrusal olmayan modellerle analizi. *Hay Üret,* 50 (2): 33-40, 2009.

6. Prestes AM, Garnero ADV, Marcondes CR, Damé MC, Janner EA, Rorato PRN: Estudo da curva de crescimento de bubalinos da raça Murrah criados no estado do Rio Grande do Sul. *IX Simpósio Brasileiro de Melhoramento Animal João Pessoa.* PB - 20 a 22 de junho de 2012 SBMA, 2012.

7. Kocabaş Z, Kesici T, Eliçin A: Akkaraman, İvesi x Akkaraman ve Malya x Akkaraman kuzularında büyüme eğrisi. *Turk J Vet Anim Sci,* 21, 267-275, 1997.

8. Bethard GL: A microcomputer simulation to evaluate management strategies for rearing dairy replacement. Doctor of philosophy in animal science (dairy), April 18, 1997, Blacksburg, Virginia, 1997.

9. Menchaca MA, Chase CC, Olson TA, Hammond AC: Evaluation of growth curves of Brahman cattle of various frame sizes. *J Anim Sci,* 74, 2140-2151, 1996.

10. Behr V, Hornick JL, Cabaraux JF, Alvarez A, Istasse L: Growth patterns of Belgian Blue replacement heifers and growing males in commercial farms. *Livest Prod Sci,* 71, 121-130, 2001.

11. Brown JE, Fitzhugh HA, Cartwright TC: A comparison of nonlinear models for describing weight-age relationships in cattle. *J Anim Sci,* 42 (4): 810-818, 1976.

12. Brody S: Bioenergetics and growth. Reinhhold Publication. New York. 1023, 1945.

13. Richards FJ: A flexible growth functions for empirical use. *J Exp Bot,* 10, 290-300, 1959.

14. Bathaei SS, Leroy PL: Genetic and phenotypic aspects of the growth curve characteristics in Mehraban Iranian fat-tailed sheep. *Small Rumin Res,* 29, 261-269, 1998.

15. Lopez S, France J, Gerrits WJ, Dhanoa MS, Humphries DJ, Dijkstra J: A generalized Michaelis-Menten equation for the analysis of growth. *J Anim Sci,* 78, 1816-1828, 2000.

16. Yakupoglu Ç, Atil H: Etlik piliçlerde büyüme eğrilerinin karşılaştırılması. *Yüksek Lisans Tezi,* Ege Üniv. Fen Bil. Enst., 1999.

17. Goonewardene LA, Berg RT, Hardin RT: A study growth of beef cattle. *Can J Anim Sci,* 61, 1041-1048, 1981.

18. Agudelo-Gómez D, Hurtado-Lugo N, Cerón-Muñoz MF: Growth curves and genetic parameters in colombian buffaloes (Bubalus bubalis Artiodactyla, Bovidae), *Rev Colomb Cienc Pecu,* 22, 178-188, 2009.

19. Araújo RO, Marcondes CR, Damé MCF, Garnero AV, Gunski RJ, Everling DM, Rorato PRN: Classical nonlinear models to describe the growth curve for Murrah buffalo breed. *Ciência Rural, Santa Maria,* 42 (3): 520-525, 2012.

20. Fundora O, Torres V, Gonzalez M. E, Noda A: Growth curve and live weight in a river buffalo herd. *Cuban J Agric Sci,* 40 (4): 401-405, 2006.

21. Nogueira JR, Lima, MLP, Gadını CH, Soares WVB: Estimativas De Parametros De Curvas De Crescimento De Bufalos Da Raca Mediterraneo. **In,** Reuniao Anual Da Sociedade Brasileira De Zootecnia, Botucatu. Anais Botucatu: Sociedade Brasileira De Zootecnia, (Cd-Rom), 374-376, 1998.

22. Sharma A, Basu SB: Genetic architecture of Nili buffaloes. 1. Growth. *Indian Vet J,* 61, 227-232, 1984.

23. Kirmani SMS, Rao GN, Sharma PLN: A note on the body weights from birth to two years of age in graded Murrah buffaloes. *Indian Vet J,* 62, 619-621, 1985.

24. Gupta JP, Sachdeva GK, Gandhi RS, Wakchaure RS, Chakravarty AK: Growth Based strategy formulation for selection of murrah buffalo at early ages. *J Adv Vet Res,* 1, 109-111, 2011.

25. Marinho KNS, Freitas AR,Falcão AJS, Dias FEF: Nonlinear models for fitting growth curves of Nellore cows reared in the Amazon Biome. *R Bras Zootec,* 42 (9): 645-650, 2013.

26. Statistica 5.0: Stat soft, Inc. 2325 East 13th Street, U.S.A, 1995.

27. Yıldız G, Soysal Mİ, Gürcan EK: Tekirdağ ilinde yetiştirilen Karacabey Merinosu x Kıvırcık melezi kuzularda büyüme eğrisinin farklı modellerle belirlenmesi. *Tekirdağ Zir Fak Derg,* 6 (1): 11-19, 2009.

28. Efe E: Büyüme eğrileri. *Doktora Tezi,* Çukurova Üniv. Fen Bil. Enst., 1990.

29. Akbaş Y, Akbulut Ö, Tüzemen N: Growth of Holstein cows in high altitude of Turkey. *Indian J Anim Sci,* 71 (5): 476-479, 2001.

30. Torres V, Ortiz J: Application of modelling and simulation to the production and feeding farm animals. *Cuban J Agri Sci,* 39, 385, 2005.

31. Torres V, Barbosa I, Meyer R, Noda A, Sarduy L: Criteria of goodness of fit test in the selection of non-linear models for the description of biological performances, *Cuban J Agric Sci,* 46 (4): 345-350, 2012.

32. Quirino CR, Bergmann JA, Vale Filho VR, Andrade VJ, Pereira JC: Evaluation of four mathematical functions to describe scrotal circumference maturation in Nellore bulls. *Theriogenology,* 52 (1): 25-34, 1999.

Kafkas Univ Vet Fak Derg
20 (3): 363-370, 2014
DOI: 10.9775/kvfd.2013.10173

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

RESEARCH ARTICLE

Effect of Kefir upon the Performance, Intestinal Microflora and Histopathology of Certain Organs in Laying Hens [1][2]

Güler YENİCE [1] Demet ÇELEBİ [2] Mehmet Akif YÖRÜK [1] Ömer UÇAR [3]
Yavuz Selim SAĞLAM [4] Muhammet Ali TUNÇ [5] Serdar ALTUN [4]

[1] *This project, originally entitled as "The effects of kefir as a probiotic on egg yield, quality and some parameters in laying hens", was funded by the Commission for Atatürk University Scientific Research Projects (BAP-Project no: 2010/123)*

[2] *This study has been presented as poster in the "45th Annual Conference of Physiology and Pathology of Reproduction, 37th Mutual Conference on Veterinary and Human Reproductive Medicine and 1st Joint German-Polish Conference on Reproductive Medicine". Berlin, 29 February - 2 March 2012 and published (as abstract) in Reprod Domest Anim, 47, (Suppl. 2): p.30 (Abstr. 68), 2012*

[1] Division of Animal Nutrition and Nutritional Disorders, Faculty of Veterinary Sciences, Atatürk University, TR-25240 Erzurum - TURKEY
[2] Division of Microbiology, Faculty of Veterinary Sciences, Atatürk University, TR-25240 Erzurum - TURKEY
[3] Division of Reproduction & A.I., Faculty of Veterinary Sciences, Atatürk University, TR-25240 Erzurum - TURKEY
[4] Division of Pathology, Faculty of Veterinary Sciences, Atatürk University, TR-25240 Erzurum - TURKEY
[5] Division of Laboratory and Veterinary Sciences, Narman Vocational Collage, Atatürk University, TR-25530 Erzurum - TURKEY

Makale Kodu (Article Code): KVFD-2013-10173

Summary

In the current study, the effect of kefir upon the performance, intestinal microflora and histopathology of certain organs in laying hens was investigated. Totally, 108 Lohmann Brown layers, aged 24 weeks, were allocated randomly into three groups, as; Group C (control, n=36): no treatment, Group A (n=36): 10 cc, and Group B (n=36): 7.5 cc kefir *per* litre of water. Animals were fed for 10 weeks with basal diets. Livers showed moderate level of hydropic degeneration, some lipidosis and focal haemorrhages with high amounts of kefir (Group A). Fewer active follicles in the ovarium were also observed in this group. The egg yield was significantly (P<0.01) lower in Group A (89.40±0.91) than in Group C (92.50±0.83) and Group B (92.86±0.87). For the pH of large intestines, unlike the small ones, it was significantly changed (P<0.01) from basic to acidic milieu in kefir-treated groups. The titres of coliform *(E. coli)*, aerobic *(Lactobacillus* spp.), and anaerobic bacteria *(Peptostreptococcus* spp.) were significantly decreased (P<0.05 to P<0.001) with increased intake for both intestinal tracts. We conclude that; i) high kefir intake could unfavourably impair the digestive organ structures, ii) the supplementations led to a marked decrease in the large intestinal pH and microbiological load of the intestines, and iii) high kefir level markedly decreased the egg yield, unlike the low concentration as leading to a considerable improvement from the 6th weeks onwards.

Keywords: *Kefir, Laying hen, Microbiology, Pathology, Probiotic*

Yumurtacı Tavuklarda Kefirin Performans, Barsak Mikroflorası ve Bazı Organların Histopatolojisi Üzerine Etkisi

Özet

Mevcut araştırmada, yumurtacı tavuklarda kefirin performans, barsak mikroflorası ve bazı organların histopatolojisi üzerine etkisi araştırıldı. 24 haftalık toplam 108 adet Lohmann Brown yumurtacı tavuk; Grup K (kontrol, n=36), Grup A (n=36): 10 cc ve Grup B (n=36): 7.5 cc kefir/L su olarak rastgele 3 gruba ayrıldı. Hayvanlar 10 hafta süreyle bazal rasyonla beslendi. Yüksek kefir miktarı (Grup A), karaciğerlerde orta düzey hidropik dejenerasyon, belli düzeyde lipidosis ve fokal hemorajiler oluşturdu. Ayrıca, bu grupta ovaryumdaki aktif follikül sayısının daha az olduğu gözlendi. Grup A'daki yumurta verimi (89.40±0.91), Grup C (92.50±0.83) ve Grup B'dekinden (92.86±0.87) önemli düzeyde (P<0.01) daha düşük bulundu. Kefir uygulanan gruplardaki kalın bağırsak pH'sı, ince bağırsakların aksine, bazikten asidik ortama doğru önemli düzeyde (P<0.01) değişti. Koliform *(E. coli)*, aerobik *(Lactobacillus* spp.), ve anaerobik bakteri *(Peptostreptococcus* spp.) titreleri artan probiyotik alımıyla birlikte önemli düzeyde (P<0.05 - P<0.001) azaldı. Sonuç olarak; i) yüksek kefir alımının sindirim organı yapılarını olumsuz yönde etkileyebildiği, ii) katkıların kalın bağırsak pH'sını ve bağırsakların mikrobiyolojik yükünü önemli düzeyde azalttığı, ve iii) yüksek kefir düzeyinin yumurta verimini önemli düzeyde azaltmasına karşın, düşük konsantrasyonun 6. haftadan sonra belli oranda artırdığı kanısına varıldı.

Anahtar sözcükler: *Kefir, Yumurtacı tavuk, Mikrobiyoloji, Patoloji, Probiyotik*

İletişim (Correspondence)
+90 442 2315560
gulerata@atauni.edu.tr

INTRODUCTION

Animal performance and feed efficiency are linked closely with the microbial load of digestive tract, structure of intestinal wall and immune system activity [1]. In recent years, supplements known as probiotics have gained considerable popularity.

Probiotics are live microorganisms and they improve the host health at adequate concentration [2]. The major microbes used as probiotics include *Lactobacillus, Saccharomyces, Streptococcus, Aspergillus spp*. and *Bacillus* [1,3]. The live bacteria in probiotics affect the host animal beneficially via improving the intestinal microbial balance [4]. Likewise, they inhibit the growth of pathogenic microorganisms by colonial formation [5]. Probiotics might enhance the permeability of epithelium, increase the phagocytosis and strengthen the non-specific immunity [6], and increase the feed efficiency by changing the intestinal microflora [7]. Several reports in poultry indicate valuable results obtained by various probiotics [3,8-10]. Of them, *Lactobacillus sporogenes* led to a greater egg yield [10]. Further, *BioPlus 2B* resulted in a higher egg yield but along with a decrease in egg yolk and serum cholesterol [11]. *B. subtilis* culture improved the egg production and eggshell thickness [12]. In broiler chickens, it was evidenced that high levels of intake may not always lead to the greatest performance [13]. Favourable effects of probiotics depend upon their abilities to tolerate heat, osmotic stress and oxygen stressors [14]. The probiotic bacteria have to survive in stomach (at low pH) and intestinal tract. *Lactobacillus* strains isolated from kefir have considerable probiotic properties at reasonable quantities [15]. Previously, we observed that kefir (7.5 ml/L) improved the feed conversion ratio [3], increased the live weights and reduced the total cholesterol and lipid levels of sera in broilers [9].

Kefir is a milk-based product and has long been used as soft drink in Northern Caucasia. It involves various bacteria and yeasts [16-18]. It ensures the enhancement and development of beneficial bacteria. These bacteria inhabit at the intestinal mucosa and provide an easier clearance of pathogen microorganisms [3,5,15,17,19].

Despite the numerous studies with probiotics in poultry, little research has been conducted on kefir to investigate their histopathological and microbiological effects in layers. Thus, we aimed to investigate the effect of kefir on the structures of digestive and reproductive organs, microbiological load of intestines and their consequences on the performance in laying hens.

MATERIAL and METHODS

Experimental Animals and Dietary Composition

One hundred and eight Lohmann Brown layers, aged 24-weeks-old were randomly allocated into three trial groups, as each group subdivided into 12 subgroups, comprising of 3 hens, as follows: Groups C (control, n=36): no treatment, A (n=36): 10 cc, and B (n=36): 7.5 cc kefir *per* litre of drinking water. All groups were fed with basal diets complying with the NRC [20] recommendations for 10 weeks. Water and feed were available *ad libitum*. Nutrient levels of diet given are illustrated in *Table 1*. Feed given were analysed according to the methods of AOAC [21]. For this study, a report of ethics has already been obtained from the Local Board of Ethics for Animal Experiments at Atatürk University (Decision No: 26.03.2010/8).

Sampling, Testing and Observations

Egg production and mortality (for calculation of egg yield *per* live animal) were recorded daily for 10 wks. Egg weights (by weekly calculation) and eggshell thickness (on the onset, during and at the end of experimental period) were determined.

Six birds selected randomly from each treatment group were sacrificed at the end of experiments to determine the organs weights (liver, heart, spleen, gizzard), histopathology of organ structures (liver, gizzard, intestine, ovarium), the populations of intestinal microflora and the pH.

For histopathological examinations, tissue samples were obtained from the visceral and genital organs and

Table 1. *The ingredients and chemical composition of basal diet*
Tablo 1. *Bazal rasyonun içeriği ve kimyasal bileşimi*

Ingredients	Amount %	Calculated Analysis	
Corn 8.53	44.5	ME	2.8 kcal g^{-1}
Soybean meal Brasil-46	17.0	Crude Protein	17.00
Wheat 10%	11.5	Calcium	3.37
Limestone	7.5	Available phosphate	0.38
Sunflower seed meal 36	7.5	Sodium	0.15
Soybean oil	5.0	Chloride	0.15
Corn Gluten-60	4.0	Linolenic acid	1.82
DCP 18	2.4	Lysine	0.79
Salt	0.26	Threonine	0.58
Min[1]-Vit[2] Premix	0.2	Tryptophan	0.19
DL Methionine 98%	0.09	Methionine+ Cysteine	0.73
L-Lysine	0.06		
Total[3]	100		

[1] Premix supplied per kg of diet: 10 mg Cu, 0.99 mg I, 50 mg Fe, 100 mg Mn, 0.08 mg Se, 100 mg Zn, [2] Premix supplied per kg of diet: 9.000 IU vitamin A, 1.78 mg vitamin B_1, 6.6 mg vitamin B_2, 30 mg niacin, 10 mg pantothenic acid, 3 mg vitamin B_6, 0.15 mg biotin, 1.500 mg choline, 0.015 mg vitamin B_{12}, 2.000 IU vitamin D, 18 IU vitamin E, 2 mg vitamin K, [3] All the values given were calculated from the NRC value [20]

fixed in 10% neutral buffered formalin solution. After the routine processing, tissue samples were embedded in paraffin wax and sectioned at 5 µ. The sections were stained with haematoxylin and eosin (H-E). The changes were semi-quantitatively assessed under the light microscope with an ocular grid and 4x, 10x, and 40x objectives, respectively. A total of 10 high-power fields were randomly chosen for evaluations. Changes in the histopathological parameters of different tissues were given in *Table 2*.

For microbiological examination, the entire intestinal tracts were removed aseptically from the body and sections of the duodenum, lower small intestine and both caeca were ligated with a nylon string. An approximately 1 g of intestinal content was mixed with 9 ml of pre-reduced sterile dilution blank solution [22] and homogenised (for 3 min) using a homogeniser (Hettich Rotina 380 R, UK). From the initial 10^{-1} dilution, subsequent 10-fold serial dilutions were made in a sterile pre-reduced dilution blank solution for anaerobic bacteria, while using 0.1% peptone for aerobics. The samples from duodenum, lower small intestine and caecum were diluted to 10^{-5}, 10^{-7} and 10^{-9}, respectively.

For each dilution, a volume of 0.1 ml was inoculated in agar roll-tube for anaerobes and on agar plate for aerobic ones. The medium (of 6 ml) roll-tube used for both culturing and counting the total anaerobes was FM 98-5 [23]. The plate media used were: MRS agar for *Lactobacilli* (Oxoid, England), Bifidobacteria agar for *Bifidobacteri* [24], Brain Heart Infusion agar (BHIA) for total aerobic bacterial count, MacConkay agar (BBL) for *Coliforms*, and KF Streptococcus agar (DIFCO, USA) for *Streptococci*. All the inoculated roll-tubes and plates were incubated at 39°C. The roll-tubes were incubated for 6 days to determine the total numbers of anaerobes, while the MRS and Bifidobacteria agar plates were incubated anaerobically for 2 days in a Gas-Pak container (Oxoid, England). The plates of total aerobes and *Coliforms* were incubated (aerobically) for 1 day, while those of *Streptococci* were incubated for 2 days.

For the pH values in the ileum and caecum, the caeca and 10 cm section of ileum (around the Meckel's diverticulum ±5 cm) were ligated and removed following decapitation of six birds. Intestinal contents were collected and their pH determined immediately using an electronic pH meter (WTV Inolab, Germany).

Probiotic kefir used was prepared daily as needed during the experimental period [3]. For culturing the kefir samples taken, Sabouraud's dextrose agar and enriched culture media were used. Following the incubation period for 18-72 h, both gram (-) and lactophenol cotton blue staining methods were employed. For microscopic evaluations, microorganism identifications of the samples were then made using the conventional methods routinely.

Statistical Analysis

Data were presented as mean ± SEM. The values of microbiological findings (*Coliform*, aerobic and anaerobic bacteria) and pH of both small and large intestines as well as those of egg yields (number, weight and eggshell thickness) from the experimental groups were subjected to one-way analysis of variance (ANOVA) and Duncan multiple comparison test [25]. Differences between the experimental groups were considered significant, using the least significant differences (when $P<0.05$).

Table 2. *Types of histopathological changes and the level of their severities in control and experimental groups using different doses of kefir*
Tablo 2. *Farklı dozlarda kefir uygulanan deneysel ve kontrol grubunda histopatolojik değişim tipleri ve şiddet dereceleri*

Lesions	Groups		
	A (10 ml/L) (n=6)	B (7.5 ml/L) (n=6)	Control (n=6)
Liver			
Haemorrhage	++	-	-
Hydropic degeneration	+++	+	-
Ovarium			
Follicle loss	+++	-	-
Intestine			
Villous atrophy	++	-	-
Gizzard			
Dilatation of glandules	++	-	-
Cellular infiltration	+	-	-

(-) No change, (+) mild change, (++) moderate change and (+++) severe change

RESULTS

Microbial counts of kefir samples used are given in *Table 3*. It can be seen clearly that the major microorganisms available were *Lactobacillus spp*.

The appearances of histopathological changes in the liver, intestine, gizzard and ovarium are given according to the groups (A, B and C) in *Fig. 1-3*, respectively. There were no apparent pathological lesions in the liver and ovaries

Table 3. *Microbial counts in kefir samples*
Tablo 3. *Kefir örneklerinin mikrobiyel bileşimi*

Microbial Group (n=6)	Microbial Counts (Log cfu ml^{-1})
Streptococcus spp.	3.62±0.29*
Lactobacillus spp.	6.50±0.50
Candida spp.	4.17±0.31

* *±SE*

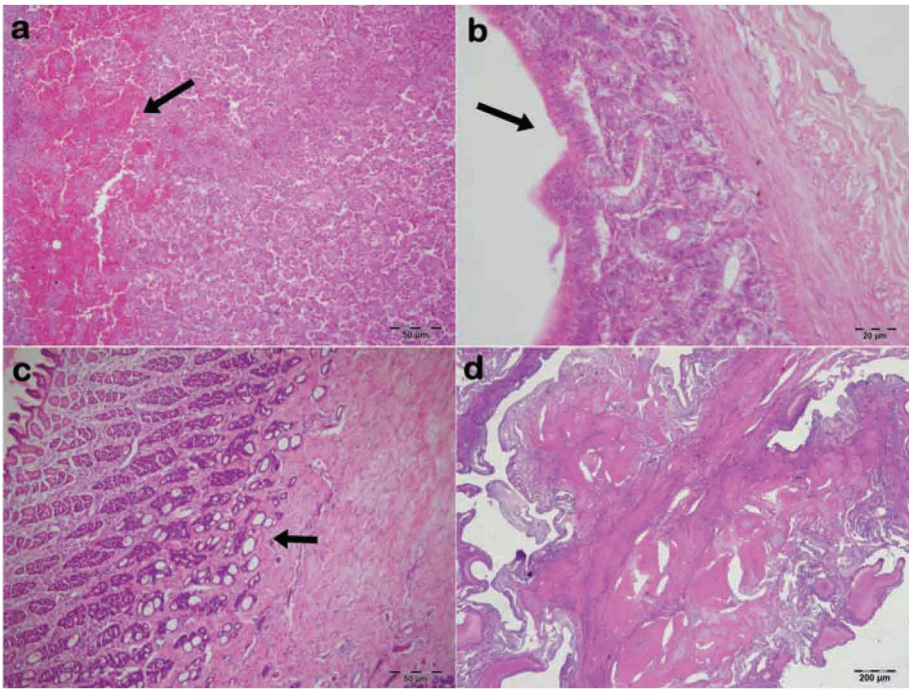


Fig 1. Histopathological findings of high concentration (10 cc/L) of kefir-treated group (Group A) in laying hens

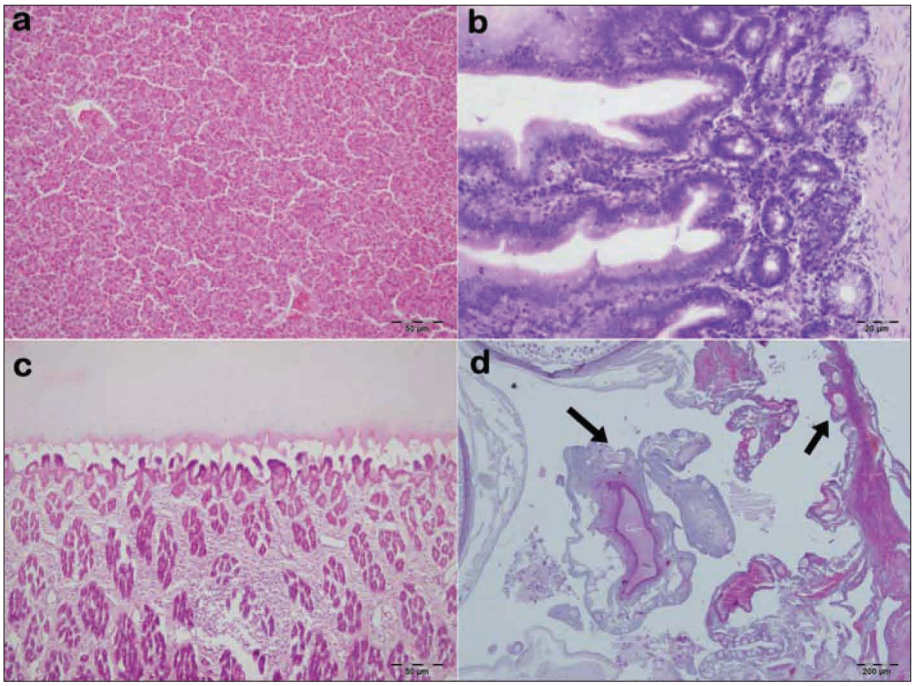
Group A; a) haemorrhagic foci in the liver tissue *(arrow),* x200, H-E, **b)** disappearances of villous architecture *(arrow)*, x400, H-E, **c)** dilatations in the glandules of gizzard *(arrow)*, x200, H-E and **d)** disappearances of normal follicular architecture in ovarium, x40, H-E

Şekil 1. Yüksek düzeyde (10 cc/L) kefir uygulanan gruptaki (Grup A) yumurtacı tavuklarda histopatolojik bulgular

Fig 2. Histopathological findings of low concentration (7.5 cc/L) of kefir-treated group (Group B) in laying hens

Group B; a) normal histological appearance of liver, x200, H-E, **b)** normal villous architecture and limited cellular infiltration of small intestine, x400, H-E, **c)** normal histological appearance of gizzard, x200, H-E and **d)** normal follicular appearance *(arrows)* of ovarium, x40, H-E

Şekil 2. Düşük düzeyde (7.5 cc/L) kefir uygulanan gruptaki (Grup B) yumurtacı tavuklarda histopatolojik bulgular



of the Groups C and B. Mild or moderate haemorrhage, hydropic degeneration in some hepatocytes were observed in liver tissues of Group A *(Fig. 1a)*. In intestinal sections, shortening intestinal villi or villous atrophy was detected *(Fig. 1b)*. Besides, an increase for glandules of gizzard *(Fig. 1c)* was detected and there was a lack of prominent follicles in ovarian tissues *(Fig. 1d)*.

The microbial loads and pH of small and large intestines with different levels of kefir are given in *Table 4*. The titres of *Coliform* (*E. coli*, for small intestines only), aerobic (*Lactobacillus spp*.), and anaerobic bacteria (*Peptostreptococcus spp.*) significantly (ranging from $P<0.05$ to 0.001) decreased as the amount of intake increased for both intestinal tracts.

For the pH of large intestines, unlike the small ones, it was significantly changed ($P<0.01$) from basic to acidic milieu in both kefir-treated groups.

The effects of kefir intake upon the parameters of egg yield in laying hens are given in *Table 5*. The weekly egg yields of groups concerned are illustrated in *Fig. 4*. For the egg yield, the decline observed during

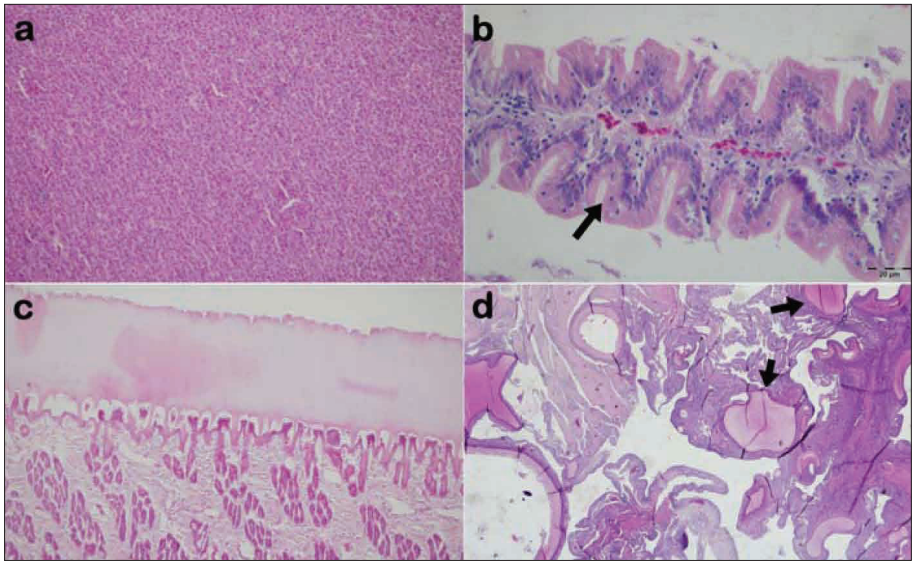


Fig 3. Histopathological findings of the control group (Group C) in laying hens

Group C; a) normal histological appearance of liver, x200, H-E, **b)** normal villous architecture *(arrow)* of small intestine, x400, H-E, **c)** normal histological appearance of gizzard, x200, H-E and **d)** normal follicular appearance *(arrows)* of ovarium, x40, H-E

Şekil 3. Kontrol grubundaki (Grup C) yumurtacı tavuklarda histopatolojik bulgular

Table 4. *The effects of kefir upon the microbial loads (Log cfu ml^{-1}) and pH of small and large intestines in laying hens*
Tablo 4. *Yumurtacı tavuklarda ince ve kalın bağırsakların mikrobiyel yükü (Log cfu ml^{-1}) ve pH'sı üzerine kefirin etkileri*

Parameters Studied		Groups			Statistics
		A (10 ml/L) (n=6)	B (7.5 ml/L) (n=6)	Control (n=6)	Significance
Small Intestine	*E. coli*	1.95±0.00^{b}	2.13±0.17^{ab}	2.67±0.33^{a}	*
	Enterobacter spp.	2.13±0.17	2.64±0.67	2.49±0.23	NS
	Lactobacillus spp.	2.46±0.51^{b}	3.15±0.41^{b}	5.00±0.52^{a}	**
	Peptostreptococcus spp.	1.95±0.00^{b}	1.95±0.00^{b}	3.99±0.52^{a}	***
	pH	5.81±0.32	6.47±0.13	6.19±0.37	NS
Large Intestine	*E.coli*	3.30±1.00	3.30±1.00	4.00±0.26	NS
	Enterobacter spp.	1.95±0.00	2.63±0.67	2.82±0.32	NS
	Lactobacillus spp.	1.95±0.00^{b}	3.64±1.09^{ab}	5.49±0.81^{a}	*
	Peptostreptococcus spp.	2.30±0.34^{b}	2.48±0.23^{b}	7.50±0.85^{a}	***
	pH	6.67±0.59^{b}	5.98±0.41^{b}	8.82±0.11^{a}	**

^{abc} Means (±SEM) within the same row having different superscripts are significantly different from each other, ***NS:*** *Non significant (P>0.05), * P<0.05, ** P<0.01, *** P<0.001*

Table 5. *The effects of kefir on production and egg quality parameters of hens*
Tablo 5. *Kefirin yumurtacı tavuklarda yumurta verimi ve kalitesi üzerine etkileri*

Parameters Studied	Groups			Statistics
	A (10 ml/L)	B (7.5 ml/L)	Control	Significance
Egg yield (%)	89.40±0.91^{b}	92.86±0.87^{a}	92.50±0.84^{a}	**
Egg weight (g)	63.14±0.27^{a}	61.93±0.29^{b}	63.04±0.28^{a}	**
Eggshell thickness (mm)	0.385±0.005^{a}	0.372±0.004^{b}	0.380±0.005^{ab}	*

^{ab} Means (±SEM) within the same row having different superscripts are significantly different from each other, ***NS:*** *Non significant (P>0.05), * P<0.05, ** P<0.01*

the 6th-8th weeks was minimised in Group B (7.5 ml/L) as compared to those in other groups. Moreover, the greatest values from the 6th weeks onwards were also obtained in this group.

The effects of kefir on some organ weights in laying hens are given in *Table 6*.

DISCUSSION

In the current study, the effects of kefir upon the relationships between digestive, microbiological and pathological traits, and their consequences on reproduction in laying hens were investigated.

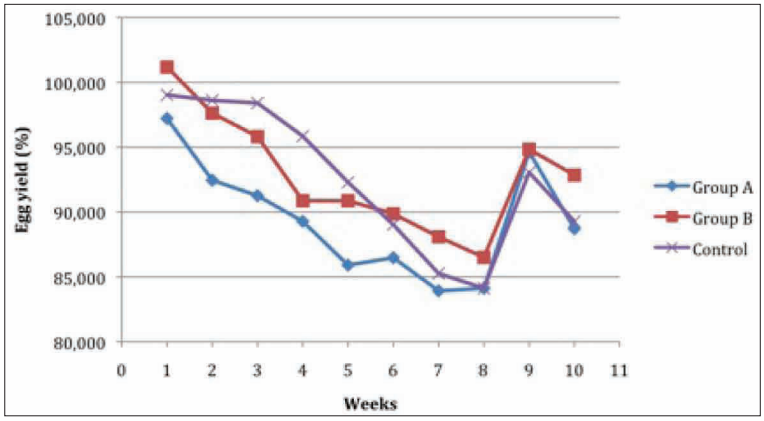


Fig 4. Weekly egg yields of kefir-treated groups* in laying hens

Groups; Group A: 10, **Group B:** 7.5, **Control:** zero ml/L kefir

Şekil 4. Kefir uygulanan gruplardaki* yumurtacı tavuklarda haftalık yumurta verimleri

Table 6. *The effects of kefir on some organs weights of hens*
Tablo 6. *Yumurtacı tavuklarda kefirin bazı organ ağırlıkları üzerine etkileri*

Organs Studied (g/100 g CA)	Groups			Statistics
	A (10 ml/L) (n=6)	B (7.5 ml/L) (n=6)	Control (n=6)	Significance
Liver	2.80±0.10^{a}	2.82±0.19^{a}	2.22±0.10^{b}	*
Heart	0.48±0.03	0.48±0.02	0.45±0.01	NS
Spleen	0.12±0.01	0.13±0.01	0.13±0.00	NS
Gizzard	2.88±0.15^{a}	2.54±0.97^{ab}	2.24±0.10^{b}	*

^{ab} Means (±SEM) within the same row having different superscripts are significantly different from each other, ***NS:*** *Non significant (P>0.05), * P<0.05*

Probiotics affect the host animal beneficially by both improving its intestinal balance and creating gut micro-ecological conditions that suppress harmful micro-organisms like *Campylobacters, Clostridium, Salmonella* and *Coliforms* [5,15,19,26], and by favouring the beneficial ones like *Lactobacillus* and *Bifidobacterium*. Kefir is claimed to act against the pathogens and to have some anti-inflammatory activities [18,19]. As with the previous findings, the increasing levels markedly lowered *Coliform* counts in small intestines. *Lactobacillus* can be classified as 'colonising' species [1], but its amount was markedly decreased in kefir-treated layers herein. The potential of probiotics to improve the beneficial bacteria while possibly suppressing the pathogenic ones in the intestines have been shown previously [8,17,26,27]. Further, an increase of beneficial micro-organisms in the intestine affected the performance favourably [28]. There was an obvious reduction in total bacterial count of ileum, while the number of *Lactobacilli* was increased with various yeast levels [29]. *Coliform* counts in the caecum of broilers receiving 0.05-0.10% *Lactobacillus* cultures were markedly lower than those of the controls [27]. Kefir, as *Lactobacilli*-yeast supplement (0.20% and 0.50%) markedly increased the numbers of *Lactobacilli,* while decreasing the numbers of total aerobic bacteria, *Coliforms*, *Enterobacteriaceae,* and *Enterococci* in faeces of goslings [30]. In our study, although the amount of intestinal microorganisms was decreased greatly, there was no increase in the population of beneficial ones. These observations may imply the apparent necessity of the optimisation of kefir concentration to be used.

The pH value is one of the main factors for flora competition and suppression of pathogenic bacteria [31]. Probiotics tend the cause favourable impact on nutrient absorption by reducing the pH [28]. Herein, we observed that kefir had no marked effect on the small intestinal pH, while it markedly decreased the pH of large intestine. The lowest pH value (5.98±0.41) was observed in Group B. Similarly, *Lactobacillus* culture in broiler ration had no effect on the pH of the small intestine, but it decreased the caecal one [8]. Also, the live yeast supplementation reduced the ileal pH in layers [29]. However, the probiotic had no effect on the caecal pH in quails [31]. These may presumably indicate the profound effects of the type of production and the species of birds.

Additionally, it was observed that although the supplementation of 7.5 ml/L kefir had no marked effect on the egg yield, but high concentration led to markedly lower yields. Besides, when weekly egg yield was considered *(Fig. 4)*, there was some increase (from the 6th week onwards) in 7.5 ml/L kefir group as compared to other groups. This was assumed to be the adaptation period for kefir intake during the earlier weeks. Our findings are somewhat similar to a previous study in that the 2.5 and 5.0 cfu.t^{-1} *Enterococcus lactiferm* both led to a relative decline in egg yield [32]. In another study, although the probiotic had no effect on the yield, but a marked decline was noted in egg weight [33]. Herein, the kefir at 7.5 ml/L did not affect the egg yield, while there was a marked decline in egg weight. The 100 mg/kg probiotic markedly improved daily egg yield and shell thickness [34]. Likewise, there were proportional

increases in the same parameters in layers fed with 0.4% and 0.8% live yeast [29]. Further, various levels of probiotic (10 and 20 g of probiotic/kg ration) led to a marked increase in egg weight, but with only a slight increase in egg yield [35]. It can bee seen clearly that the effects of probiotic on the egg yield, egg weight and eggshell thickness were variable in different studies. This may be due to differences of animal species and/or their age as well as the type/dose of probiotic used.

We observed no effect of kefir on heart and spleen weights, while a marked increase in liver and gizzard weights in kefir-treated groups. This may indicate that the increase of liver weight could be associated with the lipidosis occurred. By contrast, kefir was reported to have no marked effect on the organ weights in geese [36] and broilers [3].

In broilers, the probiotic led to a marked increase in the serum levels of LH, FSH and T_3 hormones [35]. So, we presume that these could collectively lead to an improvement in the egg weights and egg yields following the supplementation at optimal level. The T_3 is responsible for the gonadotropic hormone secretion. In poultry, the FSH is responsible mainly from the follicular growth, while the LH is responsible from the ovulation [37]. We observed broadly that the 10 ml/L kefir led to a small-size of follicles and low follicular numbers in the ovarium, while the 7.5 ml/L had no such adverse effects. Clearly, the ultimate results might vary due to both the type of supplement and its concentration used at rather narrow limits.

In Group A, common hyperplasic epithelial cells with desquamation, hyperplasia and dilatation in the intestinal lumens were observed. There were also hyperkeratotic areas in the lamina epithelialis and mononuclear cell infiltration in the intestinal propria. Further, the cell infiltration was observed in the gizzard propria. Likewise, the feeding with 1-2 kg/ton of *Bioplus 2B* caused a marked proliferation of lymphatic system in the lamina propria layer, along with hyperplasia in intestine of layers [38]. On the other hand, the probiotic had no deleterious effect on the morphology of gastrointestinal tract, liver and pancreas [39]. In the 10 ml/L kefir group, a shortening in the intestinal villi or villous atrophy was detected. By contrast, the higher levels of *B. subtilis LS 1-2* led to an increase in the villus height in duodenum and ileum [26]. Moreover, the yeast derivatives improved the numbers of intestinal goblet cells, while reducing those of enterocytes undergoing apoptosis in broilers [40].

Finally, the higher amounts of kefir led to hepatic haemorrhage, lipidosis and hydropic degeneration. The undesirable pathological organ changes were thought to affect, more or less, the yield unfavourably. Phospholipoproteins of the yolk are synthesised in the liver, so we may presume that the pathological changes might have led to a decrease in the egg yield.

Conclusively, we suggest that; i) the high amount of kefir intake *per se* impaired the organ structures of digestive system, large intestinal pH and microbiological load of the intestinal tracts, as collectively leading to markedly lower egg numbers only, ii) the lower amount of intake impaired the microbiological load of both intestinal tracts and resulted in marked change in the pH of large intestines, as collectively leading to markedly lower egg weight only. Nevertheless, the low concentration resulted in considerable improvement in egg yield from the 6th weeks onwards as compared to other groups. Furthermore, we could presume that the improvement in egg yield with 7.5 ml/L kefir may well enhance the yield, if the duration of intake would have been prolonged well beyond the 10th weeks (up to the entire laying period). However, further studies comprising various concentrations of kefir upon the layer performance during longer durations in different bird species are warranted in future to confirm the present findings.

Acknowledgements

We gratefully acknowledge Assoc.Prof. Ertan Oruç (DVM, PhD) of Atatürk University for his kind help with pathological assessment of samples.

REFERENCES

1. Huyghebaert G, Ducatelle R, Immerseel FV: An update on alternatives to antimicrobial growth promoters for broilers. *Vet J*, 187, 182-188, 2011.

2. FAO/WHO: Evaluation of health and nutritional properties of powder milk with live lactic acid bacteria. Report from FAO/WHO Expert Consultation, 1-4 Oct, Cordoba, Argentina, 2001.

3. Karademir G, Ünal Y: The use kefir as probiotic in broiler. *Lalahan Hay Araşt Enst Derg*, 49 (1): 47-54, 2008.

4. Otles S, Cagindi O: Kefir: A probiotic dairy-composition, nutritional and therapeutic aspects. *Pak J Nutr*, 2 (2): 54-59, 2003.

5. Santini C, Baffoni L, Gaggia F, Granata M, Gasbarri R, Di Gioia D, Biavati B: Characterization of probiotic strains: An application as feed additives in poultry against *Campylobacter jejuni*. *Int J Food Microbiol*, 141, 98-108, 2010.

6. Placha I, Simonova MP, Cobanova K, Laukova A, Faix S: Effect of *Enterococcus faecium* AL41 and *Thymus vulgaris* essential oil on small intestine integrity and antioxidative status of laying hens. *Res Vet Sci*, 89 (2): 257-261, 2010.

7. Alp M, Kahraman R: The use of probiotics in animal nutrition. *İstanbul Üniv Vet Fak Derg*, 22 (1): 1-8, 1996.

8. Jin LZ, Ho YW, Abdullah N, Ali, MA, Jalaludin S: Effects of adherent *Lactobacillus* cultures on growth, weight of organs and intestinal microflora and volatile fatty acids in broilers. *Anim Feed Sci Technol*, 70 (3): 197-209, 1998.

9. Cenesiz S, Yaman H, Ozcan A, Kart A, Karademir G: Effects of kefir as a probiotic on serum cholesterol, total lipid, aspartate amino transferase and alanine amino transferase activities in broiler chicks. *Medycyna Wet*, 64 (2): 168-170, 2008.

10. Panda AK, Rao SSR, Raju MVLN, Sharma SS: Effect of probiotic *(Lactobacillus sporogenes)* feeding on egg production and quality, yolk cholesterol and humoral immune response of White Leghorn layer breeders. *J Sci Food Agric*, 88 (1): 43-47, 2008.

11. Kurtoglu V, Kurtoglu F, Seker E, Coskun B, Balevi T, Polat ES: Effect of probiotic supplementation on laying hen diets on yield performance

and serum and egg yolk cholesterol. *Food Addit Contam*, 21 (9): 817-823, 2004.

12. Xu CL, Ji C, Ma Q, Hao K, Jin ZY, Li K: Effects of a dried *Bacillus subtilis* culture on egg quality. *Poult Sci*, 85, 364-368, 2006.

13. Alkhalf A, Alhaj M, Al-Homidan I: Influence of probiotic supplementation on blood parameters and growth performance in broiler chickens. *Saudi J Biol Sci*, 17, 219-225, 2010.

14. Ross RP, Desmond C, Fitzgerald GF, Stanton C: Overcoming the technological hurdles in the development of probiotic foods. *J Appl Microbiol*, 98 (6): 1410-1417, 2005.

15. Santos A, San Mauro M, Sanchez A, Torres JM, Marquina D: The antimicrobial properties of different strains of *Lactobacillus spp.* isolated from kefir. *Syst Appl Microbiol*, 26 (3): 434-437, 2003.

16. Abraham AG, De Antoni GL: Characterization of kefir grains grown in cows' milk and in soya milk. *J Dairy Res*, 66, 327-333, 1999.

17. Marquina D, Santos A, Corpas I, Muñoz J, Zazo J, Peinado JM: Dietary influence of kefir on microbial activities in the mouse bowel. *Lett Appl Microbiol*, 35, 136-140, 2002.

18. Rodrigues KL, Caputo LRG, Carvalho JCT, Evangelista J, Schneedorf JM: Antimicrobial and healing activity of kefir and kefiran extract. *Int J Antimicrob Agents*, 25 (5): 404-408, 2005.

19. Golowczyc MA, Mobili P, Garrote GL, Abraham AG, De Antoni GL: Protective action of *Lactobacillus* kefir carrying S-layer protein against *Salmonella enterica serovar enteritidis*. *Int J Food Microbiol*, 118, 264-273, 2007.

20. NRC: Nutrient Requirements of Poultry. 9th Revised ed., Natl Acad Press, Washington, DC, 1994.

21. AOAC: Official Methods of Analysis. 15th ed., Association of Official Analytical Chemists, Arlington, VA, 1990.

22. Bryant MP, Burkey LA: Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J Dairy Sci*, 36, 205-217, 1953.

23. Jin LZ, Abdullah N, Ho YW, Jalaludin S: Comparison of different media for isolation of anaerobic bacteria from the duodenum, jejunum and cecum of chicken. **In,** *Proc of the 17th Malaysian Microbiology Symposiom,* 1994, Penang, Malaysia, 87-90.

24. Sofos JN, Fagerberg DJ, Quarles CL: Effects of sorbic acid feed fungistat on the intestinal microflora of floor-reared broiler chickens. *Poult Sci*, 64 (5): 832-840, 1985.

25. SPSS 19 (for MacOS X): SPSS Inc, New York, USA.

26. Sen S, Ingale SL, Kim YW, Kim JS, Kim KH, Lohakare JD, Kim EK, Kim HS, Ryu MH, Kwon IK, Chae BJ: Effect of supplementation of *Bacillus subtilis LS 1-2* to broiler diets on growth performance, nutrient retention, caecal microbiology and small intestinal morphology. *Res Vet Sci*, 93 (1): 264-268, 2012.

27. Jin LZ, Ho YW, Abdullah N, Jalaludin S: Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing *Lactobacillus* cultures. *Poult Sci*, 77 (9): 1259-1265, 1998.

28. Chambers JR, Gong J: The intestinal microbiota and its modulation for *Salmonella* control in chickens. *Food Res Int*, 44, 3149-3159, 2011.

29. Saadia MH, Nagla KS: Effect of probiotic *(Saccharomyces cerevisiae)* adding to diets on intestinal microflora and performance of Hy-Line layers hens. *J Am Sci*, 6 (11): 159-169, 2010.

30. Yaman H, Ulukanli Z, Elmali M, Unal Y: The effect of a fermented probiotic, the kefir, on intestinal flora of poultry domesticated geese *(Anser anser). Revue Méd Vét*, 157 (7): 379-386, 2006.

31. Siriken B, Bayram I, Onol AG: Effects of probiotics: alone and in a mixture of Biosacc® plus Zinc Bacitracin on the caecal microflora of Japanese quail. *Res Vet Sci*, 75 (1): 9-14, 2003.

32. Horniakova E, Busta L, Flatnitzer F: Application of probiotic preparation IMB 52 in laying hens´ nutrition. *Slovak J Anim Sci, 39 (4): 191-196, 2006.*

33. Dizaji SB, Pirmohammadi R: Effect of *Saccharomyces cerevisiae* and Bioplus 2B on performance of laying hens. *Int J Agric Biol*, 11 (4): 495-497, 2009.

34. Panda AK, Reddy MR, Ramarao SV, Praharaj NK: Effect of dietary supplementation of probiotic on performance and immune response of layers in the decline phase of production. *Indian J Poult Sci*, 35 (1): 102-104, 2000.

35. Khalid HS, Saeb YAR: Effect of probiotic on some physiological parameters in broiler breeders. *Int J Poult Sci*, 10 (8): 626-628, 2011.

36. Sahin HE, Yardimci M: Effects of kefir as a probiotic on growth performance and carcass characteristics in geese *(Anser anser). J Anim Vet Adv*, 8 (3): 562-567, 2009.

37. Cebi C, Akcay E: Reproductive hormonal regulation in hens. *Lalahan Hay Araşt Enst Derg*, 50 (2): 103-115, 2010.

38. Mahdavi AH, Rahmani HR, Pourreza J: Effect of probiotic inclusion in different levels of barley substitution for corn diets on laying hen's histological changes of duodenum. *Proc 15th European Symp Poult Nutr,* Balatonfüred, Hungary, 25-29 Sept, 321-323, 2005.

39. Babińska I, Rotkiewicz T, Otrocka-Domagała I: The effect of *Lactobacillus acidophilus* and *Bifidobacterium spp.* administration on the morphology of the gastrointestinal tract, liver and pancreas in piglets. *Pol J Vet Sci*, 8 (1): 29-35, 2005.

40. Reisinger N, Ganner A, Masching S, Schatzmayr G, Applegate TJ: Efficacy of a yeast derivative on broiler performance, intestinal morphology and blood profile. *Livestock Sci*, 142 (2-3): 195-200, 2012.

Kafkas Univ Vet Fak Derg
20 (3): 371-374, 2014
DOI: 10.9775/kvfd.2013.10175

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

RESEARCH ARTICLE

Occurrence of *Clostridium difficile* in Raw Bovine, Ovine, Caprine, Camel and Buffalo Milk in Iran

Ebrahim RAHIMI [1] Hassan MOMTAZ [2] Maryam HEMMATI [3]

[1] Department of Food Hygiene, College of Veterinary Medicine, Islamic Azad University, Shahrekord Branch, Shahrekord - IRAN
[2] Gradated Student of Microbiology, College of Basice Science, Islamic Azad University, Shahrekord Branch, Shahrekord - IRAN
[3] Department of Microbiology, College of Veterinary Medicine, Islamic Azad University, Shahrekord Branch, Shahrekord - IRAN

Makale Kodu (Article Code): KVFD-2013-10175

Summary

This study was conducted to determine the prevalence of *Clostridium difficile* in raw milk in Iran. From January to August 2013, a total of 430 raw milk samples from bovine (n=135), ovine (n=100), caprine (n=80), buffalo (n=49) and camel (n=66) were purchased from randomly selected from 111 dairy farm in Iran and were evaluated for the presence of *C. difficile*. In this study, only 2 of 135 bovine milk samples (1.43%) were contaminated with *C. difficile*. One of the two *C. difficile* strains was positive for tcdA and tcdB toxin genes that was classified as ribotype 078. Susceptibilities of isolates were determined for 11 antimicrobial drugs using the disk diffusion assay. None of the isolates was resistant to vancomycin, metronidazole, chloramphenicol and tetracycline. To our knowledge, this study is the first report of direct identification of *C. difficile* in bulk milk samples from dairy herds in Iran and the first report of direct identification of *C. difficile* in bulk milk samples from dairy bovine herds.

Keywords: *Clostridium difficile, Raw milk, Camel, Buffalo, Bovine, Antimicrobial resistance*

İran'da Sığır, Koyun, Keçi, Deve ve Manda Çiğ Sütlerinde *Clostridium difficile*'nin Tespiti

Özet

Bu çalışma İran'da çiğ sütlerde *Clostridium difficile*'nin prevalansını belirlemek amacıyla yapılmıştır. Çalışmada, rastgele seçilmiş 111 süt çiftliğinden toplanmış sığır (n=135), koyun (n=100), keçi (n=80), manda (n=49) ve deve (n=66) toplam 430 çiğ süt örneği *C. difficile*'nin varlığını ortaya koymak maksadıyla incelendi. Çalışmada, sığır süt örneklerinden 135'inden sadece 2'sinde (1.43%) *C. difficile* kontaminasyonu tespit edildi. İki *C. difficile* suşundan birisi ribotip 078 olarak sınıflandırılan tcdA ve tcdB toksin genine pozitiflik gösterdi. Disk difüzyon testi kullanılarak 11 antimikrobial ilaca karşı izolatların hassasiyetlikleri belirlendi. İzolatların hiçbiri vankomisin, metronidazol, kloramfenikol ve tetrasikline karşı dayanıklı değildi. Bilgimiz dahilinde, bu çalışma İran'da sütçü sürülerden ve sığırlardan elde edilen ham süt örneklerinde *C. difficile*'nin direkt olarak tespit edildiği ilk çalışmadır.

Anahtar sözcükler: *Clostridium difficile, Çiğ süt, Deve, Manda, Sığır, Antimikrobiyal rezistans*

INTRODUCTION

Clostridium difficile is recognised as a nosocomial pathogen associated with antimicrobial drug-associated diarrhoea and pseudomembranous colitis in humans and the infection is believed to be acquired nosocomially [1-3]. The antimicrobial agents most frequently associated with *Clostridium difficile*-associated disease (CDAD) include clindamycin, cephalosporins and ampicillin but almost all antibiotics can cause the disease [1]. *C. difficile* has also been shown to be an important pathogen causing diarrhoea in humans in communities outside hospital environments [2,3].

The main virulence factors that are currently recognized are two large clostridial toxins, toxin A (*TcdA*, an enterotoxin) and toxin B (*TcdB*, a cytotoxin) [4]. A third, large, unrelated toxin, designated *C. difficile* binary toxin (CDT), can also be produced by some strains [4]. The role of binary toxin

İletişim (Correspondence)
+98 381 3361060
ebrahimrahimi55@yahoo.com

in disease is currently unclear [3,5], but there is information suggesting that this toxin may be clinically relevant [6].

C. difficile also appears to be an important cause of enteric disease in a wide variety of animal species [7-10], suggesting that animals and humans may share a common source [11,12]. In accordance herewith, recent reports show a remarkable overlap between isolates from animals and humans [13]. Food animals are an important source of human enteropathogenic micro-organisms and can be spread to humans through consumption of foods of animal origin. In recent studies *C. difficile* has been isolated from food animals such as poultry, sheep, pigs, goats and cattle [8,14,15].

Moreover, molecular typing of *C. difficile* isolates from calves and humans has shown similarities in PCR ribotypes from the two species including two PCR ribotypes associated with outbreaks of severe disease in humans [16], suggesting that cattle or other animals may be reservoirs of *C. difficile* for humans.

The epidemiology of CDI in Iran is essentially unknown, and to the authors' knowledge, the prevalence rate of *C. difficile* in foodstuff in Iran has never been reported. The aim of this study was to determine the occurrence of *C. difficile* in raw cow, ovine, caprine, buffalo and camel milk in Iran.

MATERIAL and METHODS

Sample Collection

Overall, 111 bovine, ovine, caprine, buffalo and camel herds were randomly selected in Isfahan, Chaharmahal va Bakhtiari, Khuzestan provinces, Iran. From January to August 2013, a total of 135 bovine bulk milk samples from 36 commercial dairy herds, 100 and 80 ovine and caprine bulk milk samples from 31 and 19 sheep and goat breeding farms, herds 49 buffalo bulk milk samples from 13 dairy buffalo herds and 66 camel bulk milk samples from 12 dairy camel were collected. The animals whose milk samples collected for this study were clinically healthy and the milk samples showed normal physical (color, pH, and density) characteristics. The samples were immediately transported to the laboratory in a cooler with ice packs and were processed within 12 h of collection.

Isolation and Identification of C. difficile

The samples were processed immediately upon arrival using aseptic techniques. The detection and isolation method used was based on the method described by Rodriguez-Palacios et al.[17] and de Boer et al.[18]. Briefly, 5 mL of each sample was transferred to 20 mL of *C. difficile* broth (CDB), containing *C. difficile* selective supplement (Oxoid SR0173) and 5% (v/v) defibrinated sheep blood. After incubation at 37°C for 10 to 15 days under anaerobic conditions 2 mL of the enrichment was added to 2 mL of 96% ethanol in a centrifuge tube and homogenized for 50 min on a shaker. After centrifugation (3800×g for 10 min), a loopful of material from the sediment was streaked onto *C. difficile* agar base (Oxoid CM0601) supplemented with an antibiotic supplement for the selective isolation of *C. difficile* (Oxoid SR0173) and 7% (v/v) defibrinated sheep blood and the plates were incubated for 48 h at 37°C, under anaerobic conditions. Three colonies per plate were subcultured onto tryptone soya agar (Oxoid CM0131) and tested by standard microbiological and biochemical procedures [8]. Crudely extracted DNA (boiling, 10 min) was used for PCR confirmation (housekeeping *tpi* gene detection), determination of toxin gene (*tcdA, tcdB* and *cdtB*), and PCR ribotyping of isolates as performed in previous studies [16,19].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the Kirby–Bauer disc diffusion method using Mueller–Hinton agar (HiMedia Laboratories, Mumbai, India) according to the Clinical Laboratory Standards Institute [20] as has been previously described [8,10]. The antimicrobial agents tested and their corresponding concentrations were as follows: nalidixic acid (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), tetracycline (30 µg), doxycycline (30 µg), gentamicin (10 µg), metronidazol (5 µg), ampicillin (10 µg), chloramphenicol (30 µg), vancomycin (30 µg), and clindamycin (2 µg). After incubating the inoculated plate for 48 h at 37°C, under anaerobic conditions, the susceptibility of the *C. difficile* to each antimicrobial agent was measured and the results were interpreted in accordance with interpretive criteria provided by CLSI [20].

RESULTS

In the present study, a total of 430 bulk milk samples from 111 dairy bovine, ovine, caprine, buffalo and camel herds in Isfahan, Chaharmahal va Bakhtiari and Khuzestan provinces of Iran were tested for *C. difficile*. In this study, 2 of 135 (1.48%) bovine milk samples were positive *(Table 1)*. The positive samples were from 1 of 36 (2.78%) commercial dairy herds *(Fig. 1)*. One of the *C. difficile* isolated was positive for *tcdA* and *tcdB* toxin genes and was classified as ribotype 078.

All 100 ovine bulk milk samples from 31 sheep breeding farms, 80 caprine bulk milk samples from 19 goat breeding farms, 49 buffalo bulk milk samples from 13 buffalo breeding farms and 66 camel bulk milk samples from 12 camel breeding farms were negative.

In this study, the antimicrobial resistance pattern of two *C. difficile* isolates was tested to 11 antimicrobial agents *(Table 2)*. All two isolates were resistant to clindomycin, gentamycin and nalidixic acid. No resistance to chloramphenicol, metronidazole, tetracycline and vancomycin was observed.

Table 1. *Prevalence of Clostridium difficile detected in bovine, ovine, caprine, camel and buffalo milk samples*
Tablo 1. Sığır, koyun, keçi, deve ve manda süt örneklerinde tespit edilen *Clostridium difficile prevalansı*

Meat Sample	No. of Samples	No. of *C. difficile* -Positive Samples	No. of Isolates Positive for Toxins			Ribotype 078
			tcdA	tcdB	cdtB	
Bovine	135	2 (1.48%)	1	1	-	1
Ovine	100	0 (0.0%)	-	-	-	-
Caprine	80	0 (0.0%)	-	-	-	-
Buffalo	49	0 (0.0%)	-	-	-	-
Camel	66	0 (0.0%)	-	-	-	-
Total	430	2 (0.47%)	1	1	-	1

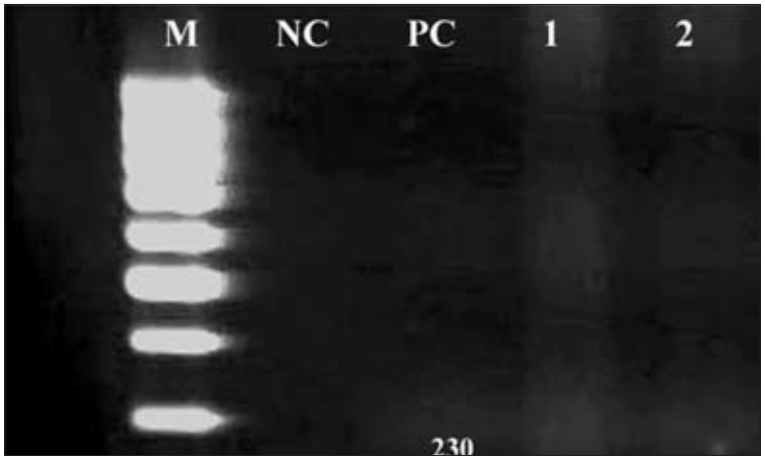


Fig 1. Electropherogram of the amplification products of the polymerase chain reaction (PCR) assay. M, 100 bp DNA ladder; lane 1, negative control (NC); lane 2, positive control (PC); lanes 3 and 4, *Clostridium difficile* positive milk samples

Şekil 1. Zincirleme Polimeraz Reaksiyonunda amplifikasyon ürünlerinin elektroferogramı. M, 100 bp DNA merdiveni; sütun 1, negatif kontrol (NC); sütun 2, pozitif kontrol (PC); sütun 3 ve 4, *Clostridium difficile* pozitif süt örnekleri

Table 2. *Antimicrobial resistance of two Clostridium difficile isolated from raw milk*
Tablo 2. *Çiğ sütlerden izole edilen 2 Clostridium difficile'nin antimikrobiyal rezistansı*

Antimicrobial agent	Sensitive	Intermediate	Resistant
Ampicillin	0 (0.0%)	1 (50.0%)	1 (50.0%)
Chloramphenicol	2 (0.0%)	0 (0.0%)	0 (0.0%)
Ciprofloxacin	1 (50.0%)	1 (50.0%)	0 (0.0%)
Clindamycin	0 (0.0%)	0 (0.0%)	2 (100%)
Doxycycline	1 (50.0%)	1 (50.0%)	0 (0.0%)
Erythromycin	1 (50.0%)	1 (50.0%)	0 (0.0%)
Gentamicin	0 (0.0%)	0 (0.0%)	2 (100%)
Metronidazole	2 (100%)	0 (0.0%)	0 (0.0%)
Nalidixic acid	0 (0.0%)	0 (0.0%)	2 (100%)
Tetracycline	2 (100%)	0 (0.0%)	0 (0.0%)
Vancomycin	2 (100%)	0 (0.0%)	0 (0.0%)

DISCUSSION

Recent reports indicate that a large proportion of CDAD are not linked to recent antibiotic therapy, older age, significant comorbidity or previous hospitalization. Possible community sources for CDAD include animals and food, and therefore a surveillance study on the prevalence of *C. difficile* in MILK was performed. In total, only 2 of 430 raw milk samples (0.47%) were found to be contaminated with *C. difficile*. The positive samples were from 2 of 135 (1.48%) bovine milk samples. This result is similar to a recent report in Austria that showed, all 50 raw bulk milk samples were negative for *C. difficile* [21]. The results of this study show that raw milk is not an important source for *C. difficile* infection. Nevertheless, it could also indicate that the extent of *C. difficile* contamination is below the detection limit of the method used. In a recently published Swedish study, the total spore count in milk varied between 10^2 and 2×10^2 spores per litre of raw milk [22]. Since the mass of spores originated from aerobic spore formers or clostridia species other than *C. difficile*, the negative results could also be explained by the very low number that was likely to be present, or even suboptimal culture conditions.

The source of *C. difficile* in food products is unclear. Contamination of milk might be due to *C. difficile* residing in the gastro-intestinal tract of animals, but could also originate from the hands of personnel during milking, milk processing equipment. The prolonged survival of *C. difficile* spores in the environment increases the possibilities for contamination of animals and foods.

One of the *C. difficile* isolated in this study that were positive for *tcdA* and *tcdB* toxin genes was classified as ribotype 078. Similar results were reported in other studies [7,23]. Ribotype 078 has been associated with hyper-virulent properties [13], a toxin regulatory gene, and which is

often associated with food animals [24]. It is also increasingly associated with community-onset *C. difficile* infection [25]. Contamination of food with this ribotype suggests a possible human health concern. Ribotype 078 has been reported to be the predominant type in food animals such as cattle and pigs [9,24], and other food sources [3,25].

None of the isolates was resistant to vancomycin, metronidazole, chloramphenicol and tetracycline. Vancomycin and metronidazole, are the most commonly used to treat *C. difficile* diarrhoea. The isolates were resistant or intermediately resistant to clindomycin, gentamycin, nalidixic acid ciprofloxacin, erythromycin, and ampicillin. These results are comparable to those reported by other investigators [15,21,26]. The results of antimicrobial resistance found in this study are correlated with antibiotics usage to treat infections in food animals in Iran.

To our knowledge, the present study is the first report of direct identification of *C. difficile* in bulk milk samples from dairy bovine in Iran. Although no extensive prevalence study was undertaken, the results of this study indicate that clinically healthy cow can be sources of *C. difficile* infection. The present results also suggest that the bulk tank milk, which is easy and inexpensive to collect, could be used to assess, on a larger scale at a low cost, the efficiency of control schemes aimed at controlling and/or preventing *C. difficile* shedding in dairy herds.

Acknowledgements

The authors would like to thank Dr. Mohammad Jalali, Dr. Amir Shakerian, Mohammad Jahanmard and Majid Riahi for the sincere help in performing technical parts of the project.

REFERENCES

1. Mylonakis E, Ryan ET, Calderwood SB: *Clostridium difficile*-associated diarrhea: A review. *Arch Intern Med*, 161, 525-533, 2001.

2. Kyne L, Merry C, O'Connell B, Keane C, O'Neill D: Community-acquired *Clostridium difficile* infection. *J Infect*, 36, 287-288, 1998.

3. Weese JS: *Clostridium difficile* in food-innocent bystander or serious threat? *Clin Microbiol Infect*, 16, 3-10, 2010.

4. Rupnik M, Dupuy B, Fairweather NF, Gerding DN, Johnson S, Just I, Lyerly DM, Popoff MR, Rood JI, Sonenshein AL, Thelestam M, Wren BW, Wilkins TD, von Eichel-Streiber C: Revised nomenclature of *Clostridium difficile* toxins and associated genes. *J Med Microbiol*, 54, 113-117, 2005.

5. Loo VG, Poirier L, Miller MA, Oughton M, Libman MD, Michaud S, Bourgault AM, Nguyen T, Frenette C, Kelly M, Vibien A, Brassard P, Fenn S, Dewar K, Hudson TJ, Horn R, René P, Monczak Y, Dascal A: A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Engl J Med*, 353, 2442-2449, 2005.

6. Barbut F, Decré D, Lalande V, Burghoffer B, Noussair L, Gigandon A, Espinasse F, Raskine L, Robert J, Mangeol A, Branger C, Petit JC: Clinical features of *Clostridium difficile*-associated diarrhoea due to binary toxin (actin-specific ADPribosyltransferase)-producing strains. *J Med Microbiol*, 54, 181-185, 2005.

7. French E, Rodriguez-Palacios A, LeJeune JT: Enteric bacterial pathogens with zoonotic potential isolated from farm-raised deer. *Foodborne Pathog Dis*, 7, 1031-1037, 2010.

8. Harvey RB, Norman KN, Andrews K, Hume ME, Scanlan CM, Callaway TR, Anderson RC, Nisbet DJ: *Clostridium difficile* in poultry and poultry meat. *Foodborne Pathog Dis*, 8, 1321-1323, 2011.

9. Keessen EC, Gaastra W, Lipman LJA: *Clostridium difficile* infection in humans and animals, differences and similarities. *Vet Microbiol*, 153, 205-217, 2011.

10. Norman KN, Harvey RB, Scott HM, Hume ME, Andrews K, Brawley AD: Varied prevalence of *Clostridium difficile* in an integrated swine operation. *Anaerobe*, 15, 256-260, 2009.

11. Rupnik M, Wilcox MH, Gerding DN: *Clostridium difficile* infection: New developments in epidemiology and pathogenesis. *Natur Rev Microbiol*, 7, 526-536, 2009.

12. Songer JG: *Clostridia* as agents of zoonotic disease. *Vet Microbiol*, 140, 399-404, 2010.

13. Goorhuis A, Debast SB, van Leengoed LA, Harmanus C, Notermans DW, Bergwerff AA, Kuijper EJ: *Clostridium difficile* PCR ribotype 078: An emerging strain in humans and in pigs? *J Clin Microbiol*, 46, 1157-1158, 2008.

14. Hoffer E, Haechler H, Frei R, Stephan R: Low occurrence of *Clostridium difficile* in fecal samples of healthy calves and pigs at slaughter and in minced meat in Switzerland. *J Food Prot*, 73, 973-975, 2010.

15. Simango C, Mwakurudza S: *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. *Int J Food Microbiol*, 124, 268-270, 2008.

16. Rodriguez-Palacios A, Stämpfli HR, Duffield T, Peregrine AS, Trotz-Williams LA, Arroyo LG, Brazier JS, Weese JS: *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg Infect Dis*, 12, 1730-1736, 2006.

17. Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS: *Clostridium difficile* in retail ground meat Canada. *Emerg Infect Dis*, 13, 485-487, 2007.

18. de Boer E, Zwartkruis-Nahuis A, Heuvelink AE, Harmanus C, Kuijper EJ: Prevalence of *Clostridium difficile* in retailed meat in The Netherlands. *Int J Food Microbiol*, 144, 561-564, 2011.

19. Rodriguez-Palacios A, Reid-Smith RJ, Staempfli HR, Daignault D, Janecko N, Avery BP, Martin H, Thompson AD, McDonald LC, Limbago B, Weese JS: Possibility of seasonality of *Clostridium difficile* in retail meat, Canada. *Emerg Infect Dis*, 15, 802-805, 2009.

20. Clinical and Laboratory Standards Institute: Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard, 7th ed., CLSI document M11-A7. Wayne (PA): The Institute, p.50, 2007

21. Jöbstl M, Heuberger S, Indra A, Nepf R, Köfer J, Wagner M: *Clostridium difficile* in raw products of animal origin. *Int J Food Microbiol*, 138, 172-175, 2010.

22. Svensson B, Ekelund K, Ogura H, Christiansson A: Characterisation of *Bacillus cereus* isolated from milk silo tanks at eight different dairy plants. *Int Dairy J*, 14, 17-27, 2004.

23. Curry SR, Marsh JW, Schlackman JL, Harrison LH: Prevalence of *Clostridium difficile* in uncooked ground meat products from Pittsburgh, Pennsylvania. *Appl Environ Microbiol*, 78, 4183-4186, 2012.

24. Keel K, Brazier JS, Post KW, Weese S, Songer JG: Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. *J Clin Microbiol*, 45, 1963-1964, 2007.

25. Metcalf D, Costa M, Dew W, Weese JS: *Clostridium difficile* in vegetables. *Lett Appl Microbiol*, 51, 600-602, 2010.

26. Indra A, Schmid D, Huhulescu S, Hell M, Gattringer R, Hasenberger P, Fiedler A, Wewalka G, Allerberger F: Characterization of clinical *Clostridium difficile* isolates by PCR ribotyping and detection of toxin genes in Austria, 2006-2007. *J Med Microbiol*, 57, 702-708, 2008.

Kafkas Univ Vet Fak Derg
20 (3): 375-382, 2014
DOI: 10.9775/kvfd.2013.10228

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

RESEARCH ARTICLE

The Effects of Black Tea Factory Waste Supplementation into Laying Hen Diets on Performance, Egg Quality, Yolk Peroxidation, and Blood Parameters

Hatice KAYA [1] Mehmet GÜL [2] Şaban ÇELEBİ [1] Adem KAYA [1]
Betül APAYDIN YILDIRIM [3] Muhlis MACİT [1]

[1] Department of Animal Sciences, Faculty of Agriculture, Atatürk University, TR-25240 Erzurum - TURKEY
[2] Department of Animal Nutrition and Nutritional Disorders, Faculty of Veterinary Medicine, Atatürk University, TR-25240 Erzurum - TURKEY
[3] Department of Biochemistry, Faculty of Veterinary Medicine, Atatürk University, TR-25240 Erzurum - TURKEY

Makale Kodu (Article Code): KVFD-2013-10228

Summary

The experiment was carried out to evaluate the effects of supplementing laying hen diets with different percentages of black tea factory waste (BTFW), evaluating performance, egg quality, yolk oxidation, and blood parameters. Twenty-four-week-old Lohmann layers (n=144) were divided into six dietary treatment groups (24 hens each), which were fed standard commercial diets supplemented with 0% (control), 2%, 4%, 6%, 8%, and 10% BTFW for 12 weeks. In this study, increasing BTFW levels were associated with impaired linearly feed consumption, final body weight, shell strength, shell weight, shell thickness, and increased cracked egg yield. The results showed a quadratic effect on albumen index, haugh unit score, and a cubic effect on feed conversion ratio, egg production, egg weight, yolk color due to the BTFW supplementation into layer diets. Whereas, shape index and yolk index were not affected by BTFW. In response to increasing BTFW percentage, yolk MDA values were decreased following storage for 14 and 28 days, but not 56 days. Plasma cholesterol, HDL, and aspartate aminotransferase (AST) were not influenced by BTFW. Increasing BTFW percentages led to linear increases in serum albumin and total protein, quadratic increases in triglyceride and alanine aminotransferase, and decreases in glucose (quadratic) and alkaline phosphatase (cubic). Results from present study showed that supplementing laying hen diets with 2% and 4% BTFW resulted in strong antioxidative activity without adverse effect on laying performance, quality characteristics, and blood parameters. In addition, more than 4% BTFW had deleterious effects on performance and egg quality traits, due to high tannic acid content.

Keywords: *Antioxidant, Black tea factory waste, Egg quality, Laying hen, Performance*

Yumurtacı Tavuk Rasyonlarına Siyah Çay Fabrika Atığı İlavesinin Performans, Yumurta Kalitesi, Yumurta Sarısında Lipid Peroksidasyonu ve Bazı Kan Parametreleri Üzerine Etkisi

Özet

Bu çalışma fabrika siyah çay atığının (SÇFA), farklı seviyelerde yumurta tavuğu yemlerine ilavesinin performans, yumurta kalitesi, yumurta sarısı oksidasyonu ve bazı kan parametreleri üzerine etkilerini incelemek amacıyla yapılmıştır. Bu amaçla, 24 haftalık yaşta 144 adet beyaz Lohmann yumurta tavuğu her grupta 24 hayvan olacak şekilde 6 gruba ayrılarak, ticari yumurta tavuğu yemine; %0 (Kontrol), %2, %4, %6, %8 ve %10 düzeylerinde fabrika siyah çay atığı ilave edilerek oluşturulan rasyonlarla 12 hafta süre ile beslenmişlerdir. Çalışmada, SÇFA'nın artan seviyesiyle birlikte, yem tüketimi, deneme sonu canlı ağırlığı, kabuk ağırlığı, kabuk kalınlığı, kırılma mukavemeti linear olarak azalmış, hasarlı yumurta oranı linear olarak artmıştır. Rasyona SÇFA ilavesinin haugh birimi ve ak indeksi üzerine kuadratik, yumurta verimi, yumurta ağırlığı ve yumurta sarı rengini üzerine kübik etkiye sahip olduğu belirlenmiştir. Buna karşın, şekil indeksi ve sarı indeksi değerleri ise rasyona SÇFA ilavesinden etkilenmemiştir. 14 ve 28 gün depolanan yumurtaların MDA değerleri rasyona artan seviyede SÇFA ilavesiyle birlikte azalırken, 56 gün depolanan yumurtaların MDA değerleri etkilenmemiştir. Diyetsel muamelenin plazma kolesterolü, HDL ve aspartat aminotransferaz (AST) üzerine etkisi önemsiz olmuştur. Artan SÇFA'nın seviyesiyle birlikte serum albumini ve toplam protein linear olarak artmış, trigliserid, alanine aminotransferaz (ALT) ve glukoz kuadratik, alkalin fosfataz (ALP) ise kübik olarak etkilenmiştir. Çalışmadan elde edilen sonuçlar yumurtacı tavuk rasyonlarına %2 ve %4 düzeylerinde SÇFA ilavesinin performans, yumurta kalite kriterleri ve kan parametreleri üzerine olumsuz bir etki yapmaksızın antioksidan aktiviteye sahip olduğunu göstermiştir. Ayrıca, SÇFA'nın yüksek tannik asit içeriğinden dolayı rasyona %4'ten daha fazla seviyede ilavesi performans ve kalite kriterlerini olumsuz etkilemiştir.

Anahtar sözcükler: *Antioksidan, Fabrika siyah çay atığı, Performans, Yumurta Kalitesi, Yumurtacı tavuk*

İletişim (Correspondence)
+90 442 2311397
hatice__karaca@hotmail.com

INTRODUCTION

Tea made from the plant *Camellia sinensis* is one of the cheapest and most popular beverages worldwide [1]. *C. sinensis* is grown in about 30 countries around the world, and freshly harvested tea leaves are processed differently in different parts of the world to produce oolong tea (2%), green tea (20%), or black tea (78%) [2]. Fresh tea leaves comprise 36% polyphenolic compounds, 25% carbohydrates, 15% protein, 6.5% lignin, 5% ash, 4% amino acids, 2% lipids, 1.5% organic acids, 0.5% chlorophyll, and less than 0.1% carotenoids and other volatile substances [3]. However, the exact chemical composition of tea leaves varies depending on several factors, including tea type, species, shrub or tree, age of leaves, collection season, climate, drying conditions and technological processing during tea production, soil condition, cultivation method, and environmental pollution [4]. Flavanols are important and characteristic tea polyphenols that predominantly include catechins, such as epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, and catechin [5]. Black teas also contain the flavonols kaempferol, quercetin, myricetin [6], and the two major pigments theaflavins and thearubigins . Many studies have already described the positive effects of tea, tea catechins, tea extract, and tea powder on animal performance and product quality [8-15]. However, many of these materials are too expensive to use in the livestock industry [16]. Green tea by-product obtained through tea beverage production is reasonably cheap and effective, and has therefore been used as a feed supplement [16]. Yang et al.[17] reported significantly lower TBA values in broiler meat of broilers that are fed diets containing 0.5% to 2.0% green tea by-product supplementation compared to those fed a diet containing antibiotics.

Livestock sector plays a significant role in Turkey and is essential for the food security of rural population. However, inadequacy of animal feed resources in both quantitatively and qualitatively is most often the limiting factor of the development of livestock production in Turkey [18]. Many agricultural and agro-industrial by product have the potential as animal feeds. Black tea factory waste (BTFW), being one of these by products, obtained from used tea leaves in factories. BTFW is typically disposed of as compost, dumped into landfills, or burned, which causes both economical and environmental problems [19]. In Turkey, the total tea plantation area is 70.000 hectare, and tea leaf production is about 1.2 billion tons [20]. The amount of factory tea waste produced depends on the manufacturing techniques and physical properties of the raw tea leaf; it varies from 7% to 15% of the total dried tea leaf amount, which is about 30.000 tons annually from state-owned companies [21]. When BTFW was used correctly in the least cost feed formulation diets, they will allow for replacement of wheat bran due to the same ingredient.

No published information is available on the quality of feed provided by BTFW or on how it could be evaluated as feed ingredient in laying hen diets. Therefore, the objectives of this study were to investigate the possibility of BTFW into the diets of laying hens and to determine the effects of different percentages of BTFW (2%, 4%, 6%, 8%, and 10%) on performance, egg quality, yolk oxidation degree, and some blood parameters.

MATERIAL and METHODS

Animals, Diet, and Management

This study was conducted by the researchers based on protocols by Atatürk University Ethical Commission Report (No: 2013 /4/111).

One hundred and fourty four 24-week-old Lohmann layers were blocked according to the location of the battery type cages (50 × 46 × 46 cm, width × depth × height), and then randomly assigned to receive one of six dietary treatments with 6 replicates of four hens in each replicate for 12 weeks. We obtained BTFW from a tea factory in Rize (northern Turkey), and substituted it for wheat bran in the diet at 2%, 4%, 6%, 8%, and 10% based on weight. The experimental diets were formulated to meet NRC recommendations [22] and analyzed using AOAC methods [23]; Neutral detergent fiber (NDF) and Acid detergent fiber (ADF) were determined according to Goering and van Soest [24], Metabolizable energy contents of the experimental diets were calculated from tabular values of feedstuffs reported for chickens [22]. *Table 1* lists their ingredients and analyzed compositions. *Table 2* presents the mineral and chemical composition of the BTFW used in this study, which was analyzed for tannin using the method of Kondo et al.[25]. Macro and micro elements content of BTFW were determined Inductively Couple Plasma spectrophotometer (Perkin-Elmer, Optima 2100 DV, ICP/OES, Shelton, CT 06484-4794, USA) [26]. From 24 to 36 weeks of age, the hens were fed either a standard commercial laying hen diet with no BTFW (Control) or the basal diet plus 2%, 4%, 6%, 8%, or 10% BTFW. Hens were fed *ad libitum* once daily at 08:30 hours, and water was available at all times. Lighting was 17 h light/7 h dark throughout the experimental period.

Sample Collection and Analytical Procedure

Feed intake and egg production were recorded daily, egg weight was measured bi-weekly using egg wights taken as daily from each treatment groups, and body weights were measured at the beginning and end of the experiment as to cage basis in all of the treatment groups. Feed conversion ratio (FCR) was recorded as kilogram of feed consumed per kilogram of eggs produced. Before determination of egg weight, 12 eggs from each experimental

Table 1. *Ingredients and chemical composition of the experimental basal diets*
Tablo 1. *Denemede rasyonlarının bileşimi ve kimyasal komposizyonu*

Ingredient (%)	Experimental Diets (% Black Tea Factory Waste)					
	0%*	2%	4%	6%	8%	10%
Corn	35	35	35	35	35	35
Soybean meal (48% CP)	20	20	20	20	20	20
Tea waste	-	2	4	6	8	10
Wheat bran	10	8	6	4	2	-
Wheat	15	15	15	15	15	15
Sunflower seed meal	5	5	5	5	5	5
Fish meal	2.5	2.5	2.5	2.5	2.5	2.5
Vegetable fat	2	2	2	2	2	2
Dicalcium phosphate [1]	2.5	2.5	2.5	2.5	2.5	2.5
Marble	7.3	7.3	7.3	7.3	7.3	7.3
Vitamin-mineral premix [2]	0.22	0.22	0.22	0.22	0.22	0.22
Salt	0.3	0.3	0.3	0.3	0.3	0.3
Methionine [3]	0.12	0.12	0.12	0.12	0.12	0.12
Lysine [4]	0.06	0.06	0.06	0.06	0.06	0.06
Chemical composition (analyzed on dry matter basis)						
Dry matter (%)	90.1	89.9	89.7	89.9	90.7	90.4
Crude protein (%)	17.3	17.4	17.6	17.5	17.8	17.8
Crude fiber (%)	3.6	4.5	4.6	5.3	5.6	6.3
Ether extract (%)	2.8	2.8	2.7	2.9	2.4	2.8
Crude ash (%)	11.4	11.6	11.0	11.8	11.3	11.6
Metabolizable energy (kcal/kg) [5]	2649	2626	2619	2610	2621	2606

*[1] Each kilogram contained: 24% Ca and 17.5% P, [2] The premix provided per 1 kg of diet: vitamin A, 15.000 IU; cholecalciferol, 1500 ICU; DL-α-tocopheryl acetate, 30 IU; menadione, 5.0 mg; thiamine, 3.0 mg; riboflavin, 6.0 mg; niacin, 20.0 mg; panthotenic acid, 8.0 mg; pyridoxine, 5.0 mg; folic acid, 1.0 mg; vitamin B_{12}, 15 µg; Mn, 80.0 mg; Zn, 60.0 mg; Fe, 30.0 mg; Cu, 5.0 mg; I, 2.0 mg; and Se, 0.15 mg, [3] DL-methionine, [4] L-lysine hydrochloride, [5] This value was found to with calculation from tabular values of feedstuffs reported for chickens NRC [22], * Basal diet contained: 4.11% Ca and 0.59% total P*

Table 2. *Mineral and chemical composition of black tea factory waste*
Tablo 2. *Siyah çay fabrika atığının mineral madde ve kimyasal komposizyonu*

Mineral	mg/kg	Chemical Composition	On A DM Basis (%)
Na	0.01	DM	92.1
K	2.10	Crude protein	18.2
Ca	0.59	Crude fiber	19.8
Mg	0.24	Ether extract	1.6
P	0.19	Crude ash	5.8
S	0.63	ADF	35.4
Fe	0.05	NDF	46.4
Zn	0.01	Metabolizable energy *	2442 (kcal/kg)
Al	0.20	Tannin	5.7
Mn	0.14		

** This value was found to with calculation, TSE [49]*

group were stored for 24 h at room temperature. Twelve eggs were randomly selected from each experimental group every month to evaluate egg quality parameters, including shape index, shell strength, shell thickness, albumen index, yolk index, yolk color (Yolk Colour Fan, the CIE standard colorimetric system, F. Hoffman-La Roche Ltd., Basel, Switzerland), and Haugh unit. Eggs were assessed according to the method of Kaya et al.[27].

At the end of the experiment, blood samples taken from the subcutaneous vena ulnaris were collected from two hens from each cage and stored in additive-free vacutainers. Serum was obtained following centrifugation at 3.000 g for 10 min at 20°C, and kept at -20°C until laboratory analyses. Serum parameters-albumin, triglyceride, cholesterol, high density lipoprotein (HDL-C), total protein, glucose, ALP, AST, and ALT were measured using commercial kits (DDS® Spectrophotometric Kits, Diasis Diagnostic Systems Co., İstanbul, Turkey) with an autoanalyzer Vitros 5.1FS.

The malondialdehyde (MDA) formed during refrigerated storage was evaluated as an index of lipid peroxidation[28]. To determine the MDA in yolk, 24 eggs were taken from each group at the end of the experiment, and were stored for 0, 14, 28, and 56 days at +4°C. Six egg samples from each group were then analyzed following the method of Placer et al.[29] using a Biotek ELISA Reader.

Statistical Analysis

Data from the present experiment were statistically analyzed with ANOVA using the GLM procedure of SPSS software [30]. Linear, quadratic, and cubic polynomial contrasts were used to evaluate treatment effects. The effects of the dietary treatments on response variables were considered significant at P<0.05.

RESULTS

Table 3 summarizes the effects of the experimental diets on laying performance parameters. Increasing levels of BTFW supplementation were associated with a linear decrease in feed intake (P<0.05). There was a cubic effect of BTFW levels on egg production values, with the 2% and 4% BTFW groups showing higher production than the control and 6%, 8%, and 10% BTFW groups; the lowest egg production was seen in the 8% BTFW group (P<0.001). BTFW levels had a similar cubic effect on the feed conversion ratio (P<0.001); with increasing levels of BTFW supplementation, FCR first decreased (2% and 4%), then increased (6% and 8%) and then decreased again (10%). Increasing BTFW supplementation was associated with linear increases in cracked egg yield (P<0.01) and linear decreases in egg weight (P<0.001).

Table 4 summarizes the effects of the experimental diets on egg quality parameters. BTFW supplementation did not affect the shape index or yolk index. Shell strength (P<0.001), shell thickness (P<0.001), and shell weight (P<0.01) decreased linearly with increases in BTFW supplementation level. There was a cubic effect of BTFW levels on yolk color (P<0.05), as increasing level of BTFW supplementation caused yolk color to first decrease (2%, 4%, and 6%), then increase (8%), and then again decrease (10%). Albumen index and Haugh unit increased quadratically with the increasing proportion of BTFW in the diet (P<0.01 and P<0.01, respectively).

Table 5 summarizes the yolk lipid oxidation effects of dietary treatments measured at different time periods. The extent of lipid oxidation was measured based on yolk MDA content, which did not differ among dietary treatments after 56 days of storage. However, increasing supplemental BTFW level was associated with quadratic decreases in MDA values in the yolks of eggs stored for 14 and 28 days (P<0.01). Compared to the control treatment, the egg yolk MDA values tended to be lower with increasing levels of BTFW.

Table 6 presents the effects of different levels of BTFW supplementation on some blood parameters. Plasma cholesterol, HDL, and AST (aspartate aminotransferase) were not influenced by the increasing levels of BTFW. Increasing proportions of BTFW in the diet were associated with linearly increased serum albumin and total protein concentrations, quadratically increased triglyceride and alanine aminotransferase (ALT) concentrations, and quadratically decreased glucose concentration. BTFW levels had a cubic effect on serum alkaline phosphatase (ALP) (P<0.01).

DISCUSSION

To our knowledge, no previous publication has used the same animals and type tea waste as in our study; therefore, the present findings have been mostly compared

Table 3. *Effects of black tea factory waste supplementation on laying performance parameters*
Tablo 3. *Siyah çay fabrika atığının performans parametreleri üzerine etkisi*

% BTFW	Perfromance Parameters							
	Feed Consumption (g/d)	Egg Production (%)	Cracked Egg Yield (%)	Egg Weight (g)	FCR[1]	Body weight (g)		
						Initial	Final	Gain
0	123.62	88.19	0.74	59.85	2.36	1500.3	1576.0	75.7
2	113.04	91.67	0.54	59.17	2.17	1512.3	1598.8	86.5
4	115.43	89.03	2.28	56.39	2.29	1465.8	1517.0	51.2
6	118.93	76.38	3.40	54.96	2.83	1500.3	1505.7	5.4
8	111.98	64.64	3.87	52.94	3.28	1474.0	1442.8	–31.2
10	110.88	72.21	2.67	53.23	2.89	1487.7	1413.3	–74.4
SEM	3.74	2.59	0.78	0.52	0.11	24.61	24.82	22.63
Probabilities								
Polynomial contrasts								
Linear	0.045	0.000	0.002	0.000	0.000	0.488	0.000	0.000
Quadratic	0.770	0.504	0.160	0.097	0.522	0.698	0.419	0.195
Cubic	0.164	0.000	0.86	0.021	0.000	0.840	0.338	0.403

[1] ***FCR:*** *feed conversion ratio (kg feed intake:kg egg yield)*

Table 4. *Effects of black tea factory waste supplementation on egg quality parameters*
Tablo 4. *Siyah çay fabrika atığının yumurta kalite kriterleri üzerine etkisi*

% BTFW	Parameter							
	Shape Index (%)	Shell Strength (kg/cm²)	Shell Thickness (mm × 10⁻²)	Shell Weight (g)	Yolk Color	Yolk Index (%)	Albumen Index (%)	Haugh Unit[1]
0	76.36	2.18	0.35	7.11	11.72	44.92	8.62	82.15
2	75.47	2.02	0.33	6.99	11.22	44.58	10.12	88.16
4	75.33	1.72	0.32	6.34	11.00	44.37	10.36	87.9
6	74.97	1.74	0.32	6.70	10.89	44.47	11.60	92.39
8	75.41	1.45	0.31	6.22	11.73	44.06	11.40	91.28
10	75.53	1.35	0.30	6.68	11.33	44.68	11.51	91.48
SEM	0.93	0.14	0.01	0.20	0.16	0.70	0.34	1.18
Probabilities								
Polynomial contrasts								
Linear	0.538	0.000	0.000	0.005	0.679	0.632	0.000	0.000
Quadratic	0.212	0.767	0.100	0.149	0.001	0.506	0.008	0.003
Cubic	0.454	0.876	0.453	0.226	0.014	0.816	0.898	0.650

[1] ***Haugh unit:*** *100 log (H + 7.57 – 1.7 W^0.37); **H:** albumen height in mm; **W:** egg weight in g*

Table 5. *Effects of black tea factory waste supplementation on MDA values (ng/g) of egg samples stored for 14, 28, and 56 days*
Tablo 5. *Siyah çay fabrika atığının 14, 28 ve 56 gün depolanan yumurta örneklerindeki MDA düzeylerine (ng/g) etkisi*

% BTFW	Days		
	14	28	56
0	1.81	2.25	2.53
2	1.74	1.89	2.21
4	1.72	1.84	2.38
6	1.77	1.89	2.26
8	1.21	1.82	2.23
10	1.16	1.78	2.14
SEM	0.07	0.07	0.15
Probabilities			
Polynomial contrasts			
Linear	0.000	0.000	0.125
Quadratic	0.002	0.013	0.801
Cubic	0.776	0.26	0.436

to the results of other studies conducted with hens fed green tea or green tea by-product. The present results showed that inclusion of different percentages of BTFW into the diet of laying hens affected all laying performance variables *(Table 3)*. Increasing levels of BTFW decreased feed consumption. In contrast, Uuganbayar et al.[12] previously reported increased feed intake following addition of green tea powder (0.5%, 1.0%, and 1.5%) into laying hen diets. The present study also showed that BTFW supplementation at 2% and 4% led to a trend of increasing egg production; however, inclusion of 6%, 8%, and 10% BTFW in the basal diet caused reduced egg production. The high egg production observed in groups fed 2% and 4% BTFW could occur due to absorption of the flavonoid and catechin contents of BTFW through the intestinal wall, which may positively affect both digestive function and the egg formation process of laying hens. However, high BTFW levels would substantially increase the fiber content of the diet, potentially preventing these benefits. These findings support the results of Kojima and Yoshida [31], which showed that egg production decreased when hen diets were supplemented with 5% and 10% green tea powder, but revealed no significant differences in egg production between two layer groups that were fed diets containing 1% green tea powder or control. Yang et al.[32] reported significantly increased egg production rates in hens fed diets containing 4.0% and 6.0% green tea by-product. The incorporation of 4% BTFW into the basal diet was associated with increases in cracked egg yield and FCR, and decreases in egg weight, final body weight, and body weight change *(Table 3)*. Azeke and Ekpo [33] reported that inclusion of 1% and 2% black tea into laying hen diets had no effect on egg weight. It has also been noted that egg weight was not significantly reduced when layers were fed diets containing 0.6% green tea supplementation [34]. Uuganbayar et al.[12] reported no significant differences in egg weight between two layer groups fed diets containing 1.0% or 1.5% green tea powder; however, egg weight was significantly decreased in the layers fed a 0.5% green tea diet compared to that of the control. Xu et al.[13] found that feed conversion ratio was positively affected by laying hen diets containing 0.5%, 1.5%, and 2.5% black tea powder. The differences between the present results and those of other studies may be due to differences in the varieties or levels of tea and tea waste, and in the types of animals studied. In general, the body weight gain tended to decrease with

Table 6. *Effects of black tea factory waste supplementation on metabolic profile in hens serum*
Tablo 6. *Siyah çay fabrika atığının serum metabolik profili üzerine etkisi*

% BTFW	Response Variables[1]								
	Alb	TG	Chol	HDL	TP	Glu	ALP	AST	ALT
0	1.58	786.4	116.5	26.3	4.2	206.0	218.3	173.3	16.5
2	1.56	852.5	109.3	26.0	4.5	192.0	288.5	165.5	16.0
4	1.90	847.0	104.8	26.5	4.1	178.5	189.0	164.0	18.5
6	2.00	969.0	111.0	25.5	5.0	177.0	181.5	171.0	17.0
8	1.98	808.3	112.0	28.3	4.9	179.5	108.0	176.5	33.0
10	1.90	839.5	112.0	26.5	5.2	203.0	139.0	182.0	40.0
SEM	0.13	40.86	17.64	2.35	0.20	6.33	22.45	9.79	1.89
Probabilities									
Polynomial contrasts									
Linear	0.015	0.464	0.379	0.726	0.000	0.322	0.000	0.320	0.000
Quadratic	0.170	0.048	0.688	0.945	0.505	0.000	0.659	0.307	0.000
Cubic	0.342	0.874	0.729	0.743	0.570	0.368	0.008	0.647	0.861

[1] ***Alb:*** *albumin (mg/dL),* ***TG:*** *triglyceride (mg/dL);* ***Chol:*** *cholesterol (mg/dL),* ***HDL:*** *high-density lipoprotein (mg/dL),* ***TP:*** *total protein (g/dL),* ***Glu:*** *glucose (mg/dL);* ***ALP:*** *alkaline phosphatase (unit/L),* ***AST:*** *aspartate aminotransferase (unit/L),* ***ALT:*** *alanine aminotransferase (unit/L)*

increasing levels of BTFW. This is probably due to the high fiber content of the BTFW used in our experiment, and the high tannin levels *(Table 2)* that may interfere with protein metabolism since the tannins can presumably form tannin-protein complexes. Excessive tannin consumption is known to result in depressed growth rate, poor feed efficiency, and decreased nutrient digestibility, with the maximum tannin tolerance reportedly 1% for chickens [35]. Kondo et al.[25] reported that tea leaves are rich in nitrogen compounds, amino acids, tannins, polyphenols, and vitamins, and they observed reduced feed intake and feed digestibility in animals fed tannin-rich diets. In the present study, although BTFW added to the basal diet at 2%, 4%, 6%, 8%, and 10% did not ($P>0.05$) affect feed consumption, this property decreased numerically with increasing levels of BTFW.

In present study, it was observed that supplementation of the basal diet with different levels BTFW adversely affected eggshell thickness, shell weight and strength. This finding was in agreement with data from the existing literature. Uuganbayar et al.[12] found that eggshell thickness was reduced significantly ($P<0.05$) in layer group fed diets containing green tea powder, regardless of the percentage (0.5%, 1.0%, 1.5%, or 2.0%). Similarly, Kojima and Yoshida [31] reported that weaker and thinner egg shells were produced in layers that were fed increasing levels of green tea powder (0%, 1%, 5%, and 10%). Yang et al.[32] also found that eggshell thickness was reduced when layers were fed diets containing 2% to 6% green tea by-product supplementation. The results regarding shell strength and shell thickness in the present study supported those of Zhang and Xu [14], who found that black tea powder (0.5%, 1.5%, and 2.5% of laying hen diets) reduced shell thickness and shell strength. Decreased shell thickness and strength may lead to lower nutrient retention and nutrient availability, especially of calcium through the intestines during shell formation due to the use of BTFW containing tannin. The differences between the present results and those of other studies may be due to differences in the varieties or levels of tea and tea waste.

Dietary BTFW had significant effect on yolk color in this study. In contrast, Kojima and Yoshida [31] reported no significant differences in the yolk color fan score among groups of hens fed diets with 0%, 1%, 5%, and 10% green tea powder. However, Abdo et al.[36] found that in Inshas hens fed diets containing 1%, 3%, and 5% green tea leaves the yolk color scores increased gradually with increasing levels of green tea leaves.

In accordance with the present findings, Biswas et al.[32] reported that the Haugh unit score was improved for eggs from layers fed a diet containing 0.6% green tea. However, Uuganbayar et al.[37] found that the Haugh unit scores did not differ between eggs from layers fed diets containing 1% and 2% green tea or control diets. The general nutrients in layer feed did not appear to have any beneficial effect on Haugh unit, but it has been suggested that certain natural antioxidants, such as vitamin C, vitamin E, and selenium, may be beneficial to albumen quality due to their antioxidant properties [38]. Farhoosh et al.[39] reported that BTFW has antioxidant activities and can be used as a potent natural antioxidative source.

In this experiment, it was found that the egg yolk lipid peroxidation (measured as MDA formation in yolk) was significantly altered by the inclusion of BTFW in the basal diet after 14 and 28 days of refrigerated storage, but not

after 56 days. This finding indicates that supplementation of the laying hen diets with BTFW retarded oxidation in egg yolks at least up until the 28th day of storage. It is possible that transfer of the antioxidant constituents (catechins and theaflavins) of BTFW into the hen through feeding might inhibit the chain reaction involved in oxidation of the consumed lipids, thus decreasing the oxidation products transferred into the yolk and reducing the MDA level in the yolk. Ishikawa et al.[40] reported that flavonoids from green and black tea, when added directly to isolated LDL, protect against the lipid peroxidation induced by free radicals, copper ions, and cells. These results are in accordance with those found by Biswas et al.[34] and Uuganbayar et al.[37], who reported that different levels of green tea addition to laying hen diets reduced the TBA values of egg yolk. Şahin et al.[41] also reported that 200 or 400 mg of epigallocatechin-3-gallate (EGCG), a polyphenol derived from green tea, exerted antioxidant effects and decreased the hepatic MDA level in quails.

The polyphenols found in black tea are also very strong antioxidants [3]. Leung et al.[42] reported that the theaflavins present in black tea possess at least the same antioxidant potency as catechins present in green tea, and that the conversion of catechins to theaflavins during fermentation while making black tea does not alter their free radical-scavenging activity.

Supplementation of the basal hen diet with BTFW had a significant effect on the investigated serum parameters in this study. Ahmad et al.[43] indicated that ALT, AST, and ALP in liver cells are liver function indicators, with functional and structural alterations of the liver leading to increased levels of these enzymes in circulation. All of these enzymes are intracellular and located in the mitochondria, cytoplasm, or both; when the cell's function is altered, damaged, or destroyed, the enzymes escape into the blood [44]. Tannin interferes with protein metabolism, and compromises starch digestibility and activities of pancreatic and intestinal enzymes, and consequently may adversely affect the metabolic profile [35]. Our present findings are in agreement with the results of El-Deek and Al-Harthi [45], who reported that addition of green tea at level of 0.5% into the broiler diet had no significant effect on plasma albumin, cholesterol, and AST. Similarly, Xu et al.[46] found that supplementation with fuzhuan tea (a Chinese dark tea produced by fermentation) did not affect levels of total serum cholesterol and high density lipoproteins (HDL-C) in laying hen serum. Yang et al.[17] found that addition of 0.5% and 2% green tea by-product to broiler diets tended to increase HDL levels, but not addition of 1% green tea by-product. In contrast to the present results, Abdo et al.[34] reported that inclusion of 3% and 5% green tea leaves or between 0.5 and 2.5 L/100 kg green tea extract into the diet of Inshas hens significantly decreased blood plasma cholesterol. Tea leaves destined to become black tea are rolled and allowed to ferment (oxidize), resulting in relatively high theaflavin and thearubigin concentrations and relatively low catechin concentrations [47]. Theaflavins and thearubigins account for 10% and 50-60% of total flavonoids, respectively, and the catechin content of black tea is only 20-30% [48]. The differences between the present results and those of other studies may be alter due to differences in flavonoids of the varieties of tea and tea waste used or the types of animals studied.

As a results of the present experiment supplementation of a laying hen diet with black tea factory waste at a percentage above 4% has deleterious effects on performance and egg quality traits, due to the high tannic acid content. However, supplementation of the laying hen diet with 2% and 4% black tea factory waste showed strong antioxidative activity, without having negative effects on laying performance, quality characteristics, or blood parameters. Further studies should be performed to investigate whether suitable, easy, and cheap methodologies, such as soaking, could be used to enable the incorporation of higher amounts of BTFW into laying hen diets without deleterious effects.

REFERENCES

1. Chacko SM, Thambi PT, Kuttan R, Nishigaki I: Beneficial effects of green tea: A literature review. *Chin Med,* 5 (13): 1-9, 2010.

2. Sharma VK, Bhattacharya A, Kumar A, Sharma HK: Health benefits of tea consumption. *Trop J Pharma Res*, 6, 785-792, 2007.

3. Akram S, Amir RM, Nadeem M, Sattar MU, Faiz F: Antioxidant potential of black tea (*Camellia sinensis* L.) - A review. *Pak J Food Sci,* 22, 128-132, 2012.

4. Gramza A, Khokhar S, Yoko S, Gliszczynska-Swiglo A, Hes M, Korczak J: Antioxidant activity of tea extracts in lipids and correlation with polyphenol content. *Eur Lipid Sci Technol,* 108, 355-362, 2006.

5. Chaturvedula VSP, Prakash I: The aroma, taste, color and bioactive constituents of tea. *J Med Plants Research,* 5, 2110-2124, 2011.

6. Hara Y, Luo SJ, Wickremashinghe RL, Yamanishi T: VI, Biochemistry of processing black tea. *Food Rev Int,* 11, 457-471, 1995.

7. Horzic D, Komes D, Belscak A, Ganic KK, Ivekovic D, Karlovic D: The composition of polyphenols and methylxanthines in teas and herbal infusions. *Food Chemist,* 115, 441-448, 2009.

8. Kono M, Furukawa K, Sagesaka YM, Nakagawa K, Fujimoto K: Effect of green tea grounds as dietary supplements on cultured yellowtail and ayu. *J Jpn Soc Food Sci,* 47, 932-937, 2000.

9. Ishihara N, Chu DC, Akachiand S, Juneja LR: Improvement of intestinal microflora balance and prevention of digestive and respiratory organ diseases in calves by green tea extracts. *Livest Prod Sci,* 68, 217-229, 2001.

10. Kaneko K, Yamasakil K, Tagawa Y, Tokunaga M, Tobisa M, Furuse M: Effects of dietary japanese green tea powder on growth, meat ingredient and lipid accumulation in broilers. *Jpn Poult Sci*, 38, 77-85, 2001.

11. Cao BH, Karasawa Y, Guo YM: Effects of green tea polyphenols and fructooligosaccharides in semi-purified diets on broiler's performance and caecal microflora and their metabolites. *Asian-Aust J Anim Sci,* 18, 85-89, 2005.

12. Uuganbayar D, Bae IH, Choi KS, Shin IS, Firman JD, Yang CJ: Effects of green tea powder on laying performance and egg quality in laying hens. *Asian-Aust J Anim Sci,* 18, 1769-1774, 2005.

13. Xu Y, Zhan K, Zhao Y, Zhao R, Li J, Zhou J, Zhen Q, Tang Y: Effects

of black tea powder on egg laying and reproductive performance of huainan spotted-yellow chicken. *Chin Agr Sci Bull,* 25, 19-21, 2009.

14. Zhang Z, Xu Y: Effects of black tea powder on performances and egg quality of laying hens. *J Anhui Agr Sci,* 24, 13113-13114, 2010.

15. Yosef TA, Al-Julaifi MZ, Kandeel M: The effects of green tea *(Camellia sinensis)* probiotics on broilers exposed to lead-induced oxidative stress. *J Am Sci,* 8, 499-506, 2012.

16. Ko SY, Bae IH, Yee ST, Lee SS, Uuganbayar D, Oh IJ, Yang CJ: Comparison of the effect of green tea by-product and green tea probiotics on the growth performance, meat quality, and immune response of finishing pigs. *Asian-Aust J Anim Sci,* 21, 1486-1494, 2008.

17. Yang CJ, Yang IY, Oh DH, Bae IH, Cho SG, Kong IG, Uuganbayar D, Nou IS, Choi KS: Effect of green tea by-product on performance and body composition in broiler chicks. *Asian-Aust J Anim Sci,* 16, 867-872, 2003.

18. Esenbuga N, Macit M, Karaoglu M, Aksakal V, Yoruk MA, Gül M, Aksu Mİ, Bilgin ÖC: A study on possibility of *Rosa canina* seed use as feed ingredient in diets of Morkaraman male lambs. *Trop Anim Health Prod,* 43, 1379-1384, 2011.

19. Hossain E, Ko SY, Yang CJ: Dietary supplementation of green tea by-products on growth performance, meat quality, blood parameters and immunity in finishing pigs. *J Med Plants Res,* 6, 2458-2467, 2012.

20. Anonymous: http://www.tuik.gov.tr/VeriBilgi.do?alt_id=45. *Accessed:* 30.01.2013.

21. Çavdar AD, Kalaycioglu H, Mengeloglu F: Tea mill waste fibers filled thermoplastic composites: The effects of plastic type and fiber loading. *J Reinforced Plastic Composit*, 30, 833-844, 2011.

22. National Research Council: Nutrient Requirements of Poultry. 9th Revised ed., National Academy Press, Washington, DC., 1994.

23. AOAC: Official Methods of Analysis. Vol. 1. 15th ed., Arlington, VA., 1990.

24. Goering HK, van Soest PJ: Forage Fiber Analyses (Apparatus, Reagents, Procedures, and Some Applications). Agricultural Hand Book No. 379, Agricultural Research Service, United States Department of Agriculture, Washington, DC, pp.12-15, 1970.

25. Kondo M, Kita K, Yokota H: Ensiled or oven-dried green tea by-product as protein feedstuffs: Effects of tannin on nutritive value in goats. *Asian-Aust J Anim Sci,* 20 (6): 880-886, 2007.

26. Güneş A, Ataoğlu N, Esringu A, Uzun O, Ata S, Turan M: Yield and chemical composition of corn *(Zea mays L.)* as affected by boron management. *Inter J Plant Anim Envir Sci*, 1, 42-53, 2011.

27. Kaya A, Kaya H, Macit M, Çelebi Ş, Esenbuğa N, Yörük MA, Karaoğlu M: Effects of dietary ınclusion of plant extract mixture and copper into layer diets on egg yield and quality, yolk cholesterol and fatty acid composition. *Kafkas Univ Vet Fak Derg*, 19 (4): 673-679, 2013. DOI: 10.9775/kvfd.2013.8644

28. Botsoglou N, Florou-Paneri P, Botsoglou E, Dotas V, Giannenas I, Koidis A, Mitrakos P: The effect of feeding rosemary, oregano, saffron and α-tocopheryl acetate on hen performance and oxidative stability of eggs. *South Afr J Anim Sci,* 35, 143-151, 2005.

29. Placer ZA, Cushman LL, Johnson BC: Estimation of product of lipid peroxidation (malonyldialdehyde) in biochemical systems. *Anal Biochem,* 16, 359-364, 1966.

30. SPSS: SPSS for Windows Release 10.0, SPSS Inc,1999.

31. Kojima S, Yoshida Y: Effects of green tea powder feed supplement on performance of hens in the late stage of laying. *Int J Poult Sci,* 7, 491-496, 2008.

32. Yang CJ, Jung YC, Uuganbayar D: Effect of feeding diets containing green tea by-products on laying performance and egg quality in hens. *Kor J Poult Sci,* 30, 183-189, 2003.

33. Azeke MA, Ekpo KE: Egg yolk cholesterol lowering effects of garlic and tea. *J Biol Sci,* 8, 456-460, 2008.

34. Biswas Md AH, Miyazaki Y, Nomura K, Wakita M: Influences of long-term feeding of Japanese green tea powder on laying performance and egg quality in hens. *Asian-Aust J Anim Sci,* 13, 980-985, 2000.

35. İmik H, Hayirli A, Turgut L, Laçin E, Çelebi Ş, Koç F, Yıldız L: Effects of additives on laying performance, metabolic profile, and egg quality of hens fed a high level of sorghum *(Sorghum vulgare)* during the peak laying period. *Asian-Aust J Anim Sci,* 19, 573-581, 2006.

36. Abdo ZMA, Hassan RA, El-Salam AA, Helmy SA: Effect of adding green tea and its aqueous extract as natural antıoxıdants to laying hen diet on productive, reproductive performance and egg quality during storage and its content of cholesterol. *Egyp Poult Sci J,* 30, 1121-1149, 2010.

37. Uuganbayar D, Shin IS, Yang CJ: Comparative performance of hens fed diets containing korean, japanese and chinese green tea. *Asian-Aust J Anim Sci,* 19, 1190-1196, 2006.

38. Lim KS, You SJ, An BK, Kang CW: Effects of dietary garlic powder and copper on cholesterol content and quality characteristics of chicken eggs. *Asian-Aust J Anim Sci,* 19, 582-586, 2006.

39. Farhoosh R, Gholam GA, Khodaparast MHH: Antioxidant activity of various extracts of old tea leaves and black tea wastes (*Camellia sinensis* L.). *Food Chem,* 100, 231-236, 2007.

40. Ishikawa T, Suzukawa M, Ito T, Yoshida H, Ayaori M, Nishiwaki M, Yonemura A, Hara Y, Nakamura H: Effect of tea flavonoid supplementation on the susceptibility of low-density lipoprotein to oxidative modification. *Am J Clin Nutr*, 66, 261-266, 1997.

41. Şahin K, Orhan C, Tuzcu M, Ali S, Şahin N, Hayirli A: Epigallocatechin-3-gallate prevents lipid peroxidation and enhances antioxidant defense system via modulating hepatic nuclear transcription factors in heat-stressed quails. *Poult Sci,* 89, 2251-2258, 2010.

42. Leung LK, Su Y, Chen R, Zhang Z, Huang Y, Chen ZY: Theaflavins in black tea and catechins in green tea are equally effective antioxidants. *J Nutr,* 131, 2248-2251, 2001.

43. Ahmad I, Qureshi TA, Khan FA, Mughal SAK, Sadique U, Shah Z, Khan S, Hassan ZU: Evaluation of biochemical effects of diclofenac sodium in goats. *The J Anim Plant Sci,* 22 (2 Suppl.): 1-4, 2012.

44. O'Connor N, Dargan PI, Jones AL: Hepatocellular damage from non-steroidal anti-inflammatory drugs. *Q J Med,* 96, 787-791, 2003.

45. El-Deek AA, Al-Harthi MA: Responses of modern broiler chicks to stocking density, green tea, commercial multi enzymes and their interactions on productive performance, carcass characteristics, liver composition and plasma constituents. *Int J Poult Sci,* 3, 635-645, 2004.

46. Xu X, Hu Y, Xiao W, Huang J, He X, Wu J, Ryan EP, Weir TL: Effects of fermented *Camilla sinensis*, Fuzhuan tea, on egg cholesterol and production performance in laying hens. *Herald J Agric Food Sci Res*, 1, 6-10, 2012.

47. Frei B, Higdon JV: Antioxidant activity of tea polyphenols *in vivo:* Evidence from animal studies. *J Nutr*, 133, 3275-3284, 2003.

48. Stangl V, Lorenz M, Stangl K: The role of tea and tea flavonoids in cardiovascular health. *Mol Nutr Food Res,* 50, 218-228, 2006.

49. TSE: Hayvan yemleri-metabolik (çevrilebilir) enerji tayini (kimyasal metot). TSE No: 9610. Türk Standartları Enstitüsü, Ankara, 1991.

Kafkas Univ Vet Fak Derg
20 (3): 383-392, 2014
DOI: 10.9775/kvfd.2013.10239

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

RESEARCH ARTICLE

Clinical Study on the Effects of Diacerein and Diacerein Combined with Chondroitin Sulfate on Canine Hip Osteoarthritis [1]

Korakot NGANVONGPANIT [1,2] Burin BOONSRI [3] Thatdanai SRIPRATAK [3]
Patsanan MARKMEE [3] Prachya KONGTAWELERT [4]

[1] *This research was financially supported by the National Research University Project under Thailand's Office of the Higher Education Commission*

[1] Animals Bone and Joint Research Laboratory, Department of Veterinary Biosciences and Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, THAILAND
[2] Materials Science Research Center, Faculty of Science, Chiang Mai University, Chiang Mai 50100, THAILAND
[3] Metta Pet Hospital, Chang Khlan, Chiang Mai 50000, THAILAND
[4] Thailand Excellence Center for Tissue Engineering, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50000, THAILAND

Makale Kodu (Article Code): KVFD-2013-10239

Summary

This study aimed to evaluate the effects of diacerein (DAR) and DAR combined with chondroitin sulfate (CS) for treatment of canine hip osteoarthritis (OA) in a 6-month clinical trial. Client-owned dogs included in the study consisted of 27 males and 25 females, aged 59.43±17.05 months old and weighing 17.63±5.19 kg. The dogs were randomly divided into five groups: DAR50 (administration of DAR 50 mg daily); DAR100 (DAR 100 mg daily); DAR50/CS (DAR 50 mg + CS 525 mg daily); DAR100/CS (DAR 100 mg + CS 525 mg daily); and CS (CS 525 mg daily). Dogs were re-examined monthly for 6 months after initiation of treatment. The assessment protocol included clinical scores and radiographic findings. Blood samples were collected three times (pre-treatment, and after 3 and 6 months) for evaluation of the serum biomarker, CS-WF6. Dogs treated with DAR showed statistically significant improvements ($P<0.05$) in lameness, joint mobility, pain on palpation, weight-bearing, and overall clinical score at 3, 6, 5, 4, and 4 months, respectively, after the start of treatment. Side effects, including diarrhea and dark-colored urine, were found in all groups receiving DAR. After the 3rd month, the level of serum CS-WF6 in the CS group was significantly elevated ($P<0.05$), while the other four groups showed a significant decrease ($P<0.05$). The results showed that DAR 50 or 100 mg had a similarly positive therapeutic effect on dogs with osteoarthritis. The use of DAR alone or in combination with CS resulted in decreased degradation of OA cartilage.

Keywords: *Condroitin sulfate, Chondroprotective drug, Diacerein, Dog, Osteoarthritis*

Diacerein ve Kondroitin Sülfat Eklenmiş Diacerein'in Köpek Kalça Osteoartritisindeki Etkileri Üzerine Klinik Bir Çalışma

Özet

Bu çalışma Diacerein (DAR) ve Kondroitin Sülfat (CS) Eklenmiş Diacerein 'in tedavi amaçlı olarak Canin Kalça Osteoartritisinde (OA) 6 aylık klinik denemedeki etkisini değerlendirmeyi amaçlamaktadır. Çalışmada yaşları 59.43±17.05 ay ve kiloları 17.63±5.19 kg arasında değişen 27 erkek ve 25 dişi sahipli kopek kullanıldı. Köpekler rastgele olarak 5 gruba ayrıldı; DAR50 (günlük 50 mg DAR uygulanan); DAR100 (günlük 100 mg DAR); DAR50/CS (günlük 50 mg DAR + 525 mg CS); DAR100/CS (günlük 100 mg DAR + 525 mg CS) ve CS (günlük 525 mg CS). Köpekler tedavinin başlangıcından itibaren aylık olarak 6 ay boyunca yeniden muayene edildi. Protokol değerlendirmesi klinik skoru ve radyografik bulguları içerdi. Kan örnekleri üç defa (tedavi öncesi, tedavinin 3. ve 6. aylarında) serum biyomarkırı CS-WF6'nın değerlendirilmesi amacıyla toplandı. DAR ile tedavi edilen köpeklerde topallık, eklem hareketliliği, dokunmaya karşı acı, ağırlık taşıma ve genel olarak sırasıyla 3, 6, 5, 4 ve 4 aylarda klinik skorda tedavinin başlangıcından sonra istatistiksel olarak önemli derecede iyileşmeler gösterdi. İshal ve koyu renkli idrar yan etki olarak DAR alan tüm gruplarda gözlendi. Üçüncü aydan sonra CS grubunda serum CS-WF6 düzeyi önemli ölçüde yükselirken ($P<0.05$) diğer dört grupta önemli oranda düşme gösterdi ($P<0.05$). Elde edilen sonuçlar osteoartritisde 50 veya 100 mg DAR uygulamasının benzer olarak pozitif tedavi edici etkiye sahip olduğunu göstermektedir. DAR'ın tek başına veya CS ile birlikte kullanılması OA kartilajın degredasyonunda azalma ile sonuçlanmıştır.

Anahtar sözcükler: *Kondroitin sülfat, Kondrokoruyucu ilaç, Diacerein, Köpek, Osteoartritis*

İletişim (Correspondence)
+66 5394 8046
korakot.n@cmu.ac.th

INTRODUCTION

In recent years, a variety of pharmacological agents have been introduced into veterinary medicine and human medicine as well for treatment of one of the classical musculoskeletal diseases, osteoarthritis (OA). However, no one agent has demonstrated a superior therapeutic effect over the others, based on several published meta-analysis studies [1-3]. To increase the efficiency of chondroprotective agents, using them in combination is one choice: for example, glucosamine combined with chondroitin sulfate or with other effective agents [4,5].

It is well documented that the catabolic effect of interleukin-1β (IL-1β) plays a major role in the pathophysiology of OA. This cytokine is stimulated to increase the synthesis of catabolic factors, and also decreases cartilage proteoglycan synthesis [6-8]. Diacerein (9,10-dihydro-4,5-bis(acetyloxy)-9,10-dihydro-9,10-dioxo-2-anthracene carboxylic acid) acts as an IL-1β blocker [9], and was introduced for use as a chondroprotective drug in OA patients. After entering the body, diacerein is converted entirely into rhein before reaching the systemic circulation. About 20% of rhein is eliminated from the body directly by renal function. The other 80% is conjugated in the liver into rhein glucuronide (60%) and rhein sulfate (20%); these metabolites are then eliminated via the renal system [9]. The potential OA-modifying properties of diacerein have been demonstrated both *in vitro* and *in vivo*. A recent *in vitro* study showed that diacerein inhibited IL-1β secretion [10]. Diacerein can reduce the production of matrix metalloproteinase 13 (MMP-13) in subchondral bone via inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38. Moreover, it can inhibit cathepsin K as well [11]. In a cartilage explant model, it was found that diacerein and rhein could reduce the levels of caspase-3 and iNOS [12]. Previous studies have reported that diacerein is safer than non-steroidal anti-inflammatory drugs [1,13,14]. Moreover, it can even be used in cases of renal and liver impairment [14]. No significant difference has been found in the pharmacokinetics of rhein between patients with liver and kidney impairment and healthy control subjects [15,16].

Diacerein has been widely investigated in human *in vivo* studies [1,3,10,13,17]; however, research has been limited in dogs [18]. In a human study that graded the efficacy of chondroprotective drugs, diacerein was classified as 'platinum', which indicated that there was good evidence for its effectiveness in the treatment of OA [19]. A report in 1999 used a canine cruciate-deficiency model of OA [20]. A daily diacerein dose of 40 mg/kg was administered for 32 weeks after surgery. Diacerein treatment significantly reduced the severity of morphological changes of OA, compared with a placebo. Based on a search of the PubMed database, no studies have been conducted on the clinical use of diacerein for OA in dogs since this report by Smith and colleagues in 1999 [20]. Therefore, it is still unclear whether orally administered diacerein improves the symptoms of OA. To clarify this issue, we designed a 6-month clinical study to compare the effects of diacerein, chondroitin sulfate, and a combination of diacerein plus chondroitin sulfate.

MATERIAL and METHODS

Animals

Of the 60 client-owned dogs originally included in the study, 52 completed the 6-month trial: 27 males and 25 females with an average age of 59.43±17.05 months and average weight of 17.63±5.19 kg. Informed owner consent was obtained, and the trial protocol was approved by the Faculty of Veterinary Medicine and the Ethics Committee of Chiang Mai University, Chiang Mai, Thailand, in 2011.

Inclusion/Exclusion Criteria

Dogs weighing less than 25 kg and showing clinical signs of chronic lameness, stiffness and joint pain, and radiological evidence of OA of the hip (grades 1–3) were considered eligible for the study. Animals were excluded if they were classified as OA grade 4, weighed more than 25 kg, were pregnant, receiving medication, or had hepatic, cardiovascular, gastrointestinal or neurological disease. Dogs with lameness due to lumbosacral instability, infection, immune disease or fractures, and dogs which had previously received drugs or dietary supplements for OA treatment were also excluded.

Treatment Protocol

Dogs were randomly assigned to five treatment groups. The first group (DAR50) received diacerein (Artrodar®; TRB Chemedica, Vouvry, Switzerland) 50 mg daily; the second group (DAR100) received diacerein 100 mg daily; the third group (DAR50/CS) received diacerein 50 mg plus chondroitin sulfate (Fortiflex®; Virbac, Carros, France) 525 mg daily; the fourth group (DAR100/CS) received diacerein 100 mg plus chondroitin sulfate 525 mg daily; and the fifth group (CS) served as a control group, and received chondroitin sulfate 525 mg daily. Animals were re-assessed monthly for clinical evaluation and blood collection, while radiographs were taken every 2 months. Treatment was stopped at the end of the 6th month.

Assessment Protocol

Three veterinarians (blinded to group classification) recorded the severity of clinical signs at each monthly visit using an ordinal scoring system *(Table 1)*. Radiographs of hip joints were taken every 2 months (three times per animal) and were interpreted by two veterinarians using a radiographic scoring system *(Table 2)*. Side effects of the medicine - diacerein alone, or diacerein plus chondroitin sulfate - included diarrhea, discoloration of urine, and

Table 1. *Clinical scoring system for assessing dogs with osteoarthritis*
Tablo 1. *Osteoartritisli köpeklerin değerlendirilmesi için klinik skorlama sistemi*

Criterion	Grade	Clinical Evaluation
Lameness	1	Walks normally
	2	Slightly lame when walking
	3	Moderately lame when walking
	4	Severely lame when walking
	5	Reluctant to rise and will not walk more than five paces
Joint mobility	1	Full range of motion
	2	Mild limitation (10-20%) in range of motion; no crepitus
	3	Mild limitation (10-20%) in range of motion; crepitus
	4	Moderate limitation (20-50%) in range of motion; ± crepitus
	5	Severe limitation (>50%) in range of motion; ± crepitus
Pain on palpation	1	None
	2	Mild signs; dog turns head in recognition
	3	Moderate signs; dog pulls limb away
	4	Severe signs; dog vocalizes or becomes aggressive
	5	Dog will not allow palpation
Weight-bearing	1	Equal on all limbs standing and walking
	2	Normal standing; favors affected limb when walking
	3	Partial weight-bearing standing and walking
	4	Partial weight-bearing standing; non-weight-bearing walking
	5	Non-weight-bearing standing and walking
Overall score of clinical condition	1	Not affected
	2	Mildly affected
	3	Moderately affected
	4	Severely affected
	5	Very severely affected

Table 2. *Radiographic scoring system for assessing dogs with osteoarthritis*
Tablo 2. *Osteoartritisli köpeklerin değerlendirilmesi için radyografik skorlama sistemi*

Grade		Radiographic Evaluation
0	Normal	Not affected
1	Mild	Doubtful narrowing of joint space and possible osteophytic lipping
2	Moderate	Definite osteophytes and possible narrowing of joint space
3	Severe	Moderate multiple osteophytes, definite narrowing of joint space, some sclerosis and possible deformity of bone contour
4	Very severe	Large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone contour

vomiting. Two ml of blood was collected from the cephalic vein monthly for determination of the level of the biomarker for OA, chondroitin sulfate epitope WF6.

Clinical Score

Efficacy of the treatment was determined by means of a clinical scoring system [21,22], which assessed the animal's lameness, joint mobility, pain on palpation, weight-bearing, and overall score. The dogs had to walk and trot 6 m three times for evaluation of lameness by three veterinarians; this was followed by palpation of the hip joint for joint mobility and pain evaluation. Palpation was performed by three veterinarians, 30 min apart.

Radiographs

Structural joint changes were assessed from serial radiographs performed according to standardized technique [21,23]. Radiographs were taken for each animal at enrollment and after 3 and 6 months of treatment by the same technician using a standard X-ray machine (KELEX; Kongsak X-Ray Medical Industry Co., Ltd., Bangkok, Thailand). Ventrodorsal radiographs were obtained of the dog's hip and the leg in full extension position. Repositioning of the dog for subsequent radiographs was guided by the original film, and the same radiographic settings (i.e. kV, mA and ms) were used. All radiographs in a set (three films) were evaluated by two veterinarians using the criteria in *Table 2*.

Competitive Immunoassay Using Monoclonal Antibody WF6

A mouse monoclonal antibody WF6 was raised against a shark cartilage aggrecan preparation. A quantitative ELISA method for recognition of the WF6 epitope by the monoclonal antibody was modified from previous studies [21,24,25]. The antibody was specific for intact chondroitin sulfate chains, and showed no interaction with other sulfated glycosaminoglycans, hyaluronan, or other polyanions such as DNA, RNA or dextran sulfate. The standard used in the assay was shark cartilage aggrecan (A1 fraction) at concentrations of 19-10.000 ng/ml in 6% bovine serum albumin (BSA) with TE buffer (0.1 M Tris HCl, pH 7.4, containing 0.15 M sodium chloride, 0.1% Tween 20 and 0.1% BSA). Diluted canine serum samples (1:5 in 6% BSA-TE) were added to 1.5 ml plastic tubes containing an equal volume of WF6 (cell culture supernatant, 1:200 dilution in TE buffer). They were incubated at 37°C for 1 h, and then added to the microtiter plate, which was pre-coated with shark aggrecan (A1 fraction). Non-specific protein binding was blocked with BSA. The plates were incubated at 37°C for 1 h; the wells were then washed, and peroxidase-conjugated anti-mouse IgM antibody (1:2.000) was added (100 µl/well in TE buffer). The bound conjugate was detected by adding ortho-phenylenediamine (o-PD) substrate (100 µl/well in 0.05 M citrate buffer, pH 5.0). The reaction was stopped after 10 min with 50 µl/well of 4 M sulfuric acid. Absorbance was determined using a microplate reader

at 492/690 nm. The concentration of the WF6 epitope in supernatant samples was calculated by reference to a standard curve.

Data Collection and Statistical Analysis

The results of CS-WF6 serum level were calculated based on relative change compared to pre-treatment (month 0). Clinical sign scores, radiographic scores, and relative change of the serum biomarker were presented as mean ± SD. A non-parametric two-sample Mann-Whitney procedure was used to test for differences before and after treatment. The side effects were report as percentages. Data analysis was performed using the SAS version 8.0 software package (SAS Institute, USA). A *P*-value of ≤0.05 was considered to be statistically significant.

RESULTS

Table 3 shows a summary of age, sex and body weight data of the 60 dogs that entered the trial. All dogs enrolled in the trial had hemogram and biochemical profile results within the reference range (data not shown). Comparison of pre-treatment disease scores found no significant difference (*P*>0.05) between the DAR and CS groups *(Table 4)*.

However, during the study 8 dogs were withdrawn (2 dogs in the DAR50 group, 1 dog in DAR100, 2 dogs in DAR50/CS and 3 dogs in DAR100/CS) due to various adverse responses, including failure to attend an assessment appointment, illness, and death from a car accident. Clinical evaluations and radiographic scores of those animals were excluded from the data analysis.

Lameness scores *(Table 5)* in the DAR50 and DAR100 groups were significantly improved (*P*<0.05) after 3 months of receiving the medicine; however, the scores of the DAR50/CS, DAR100/CS and CS groups showed earlier significant improvement (*P*<0.05), after only 2 months of treatment. When groups were compared for the same month, it was found that the lameness scores of the DAR50/CS, DAR100/CS and CS groups were significantly better (*P*<0.05) than those of the DAR50 and DAR100 groups from the 2nd through the 6th month of the study.

Joint mobility scores *(Table 6)* in the DAR50 and DAR100 groups were significantly improved (*P*<0.05) by the final month of the study; but the DAR50/CS, DAR100/CS and CS groups showed significant improvement (*P*<0.05) earlier, after 4 months. When groups were compared for the same month, it was found that after 5 months the joint mobility scores of the DAR50 and DAR100 groups were significantly worse (*P*<0.05) than those of the DAR50/CS, DAR100/CS and CS groups.

Pain on palpation scores *(Table 7)* in the DAR50/CS, DAR100/CS and CS groups showed the fastest significant improvement (*P*<0.05), after 2 months of receiving medicine. This was followed by the DAR100 group, which

Table 3. *Sex, age and body weight distribution for all 60 dogs*
Tablo 3. *Altmış köpeğin cinsiyet, yaş ve vücut ağırlığı dağılımı*

Group	Total	Gender		Age (months)	Weight (kg)
		Male	Female		
DAR50	12	5	7	57.33±18.62	18.58±5.66
DAR100	12	6	6	60.17±21.27	16.75±5.33
DAR50/CS	12	6	6	55.25±15.36	16.92±5.21
DAR100/CS	12	5	7	64.42±15.10	17.00±4.86
CS	12	5	7	60.00±5.60	18.92±6.26

Age and weight data are expressed as mean ± SD; neither were significantly different among the five groups (P>0.05)

Table 4. *Comparison of pre-treatment clinical and radiographic scores*
Tablo 4. *Tedavi öncesi klinik ve radyografik skorların karşılaştırılması*

Parameters	Groups				
	DAR50	DAR100	DAR50/CS	DAR100/CS	CS
Lameness	3.7±0.5	3.7±0.5	3.6±0.7	3.7±0.7	3.7±0.9
Joint mobility	3.1±0.6	3.0±0.6	3.1±0.6	3.1±0.6	3.0±0.6
Pain on palpation	2.3±0.5	2.3±0.5	2.5±0.5	2.6±0.5	2.3±0.5
Weight-bearing	3.6±0.5	3.5±0.5	3.4±0.7	3.2±0.7	3.5±0.5
Overall score	3.3±0.5	3.1±0.5	3.1±0.6	3.3±0.5	3.3±0.7
Radiography score	2.5±0.5	2.5±0.5	2.5±0.5	2.6±0.5	2.4±0.5

Data are expressed as mean ± SD. There were no significant differences among the 5 groups

showed significant improvement ($P<0.05$) after 4 months; while the DAR50 group was slowest to improve, beginning after the 5th month of the study. When group scores were compared for the same month, a significant difference ($P<0.05$) was found in months 2 and 3. The DAR50/CS, DAR100/CS and CS groups showed a significant ($P<0.05$) improvement in pain on palpation compared with the DAR50 and DAR100 groups.

Weight-bearing scores *(Table 8)* in the DAR100/CS and CS groups showed the fastest significant improvement ($P<0.05$), after 2 months of treatment. This was followed by the DAR100 and DAR50/CS groups, whose scores significantly improved ($P<0.05$) after the 3rd month; while the DAR50 group was slowest to improve, beginning after the 4th month of the study. When groups were compared for the same month, a significant difference in weight-

Table 5. *Comparison of lameness scores*
Tablo 5. *Topallık skorlarının karşılaştırılması*

Months	Groups				
	DAR50	DAR100	DAR50/CS	DAR100/CS	CS
Pre-treatment	3.7±0.5	3.7±0.5	3.6±0.7	3.7±0.7	3.7±0.9
1st month	3.4±0.5	3.5±0.5	3.1±0.7	2.9±0.9	2.7±0.7
2nd month	3.4±0.5[a]	3.4±0.5[a]	2.5±0.8*,[b]	2.3±1.0*,[b]	2.3±0.9*,[b]
3rd month	3.2±0.6*,[a]	2.6±0.5*,[a]	2.0±0.7*,[b]	1.7±0.7*,[b]	1.7±0.7*,[b]
4th month	2.8±0.8*,[a]	2.5±0.5*,[a]	1.5±0.5*,[b]	1.3±0.5*,[b]	1.3±0.5*,[b]
5th month	2.5±0.7*,[a]	2.4±0.5*,[a]	1.2±0.4*,[b]	1.1±0.3*,[b]	1.2±0.4*,[b]
6th month	2.2±0.6*,[a]	2.2±0.8*,[a]	1.2±0.4*,[b]	1.1±0.3*,[b]	1.2±0.4*,[b]

*Data are expressed as mean ± SD. *A significant difference (P<0.05) compared with pre-treatment in the same group. Superscripts [a,b] indicate a significant difference (P<0.05) between groups within the same time period*

Table 6. *Comparison of joint mobility scores*
Tablo 6. *Eklem hareketliliği skorlarının karşılaştırılması*

Months	Groups				
	DAR50	DAR100	DAR50/CS	DAR100/CS	CS
Pre-treatment	3.1±0.6	3.0±0.6	3.1±0.6	3.1±0.6	3.0±0.6
1st month	3.1±0.6	3.0±0.6	3.1±0.6	3.1±0.6	3.0±0.6
2nd month	3.1±0.6	3.0±0.6	3.0±0.7	2.9±0.6	2.8±0.6
3rd month	2.9±0.6	2.9±0.7	2.6±0.7	2.4±0.5	2.5±0.7
4th month	2.7±0.5	2.6±0.5	2.3±0.5*	2.3±0.5*	2.3±0.5*
5th month	2.7±0.5[a]	2.5±0.5[a,b]	2.1±0.6*,[b]	2.2±0.4*,[b]	2.1±0.5*,[b]
6th month	2.2±0.6*	2.1±0.5*	2.0±0.5*	2.2±0.4*	2.0±0.4*

*Data are expressed as mean ± SD. *A significant difference (P<0.05) compared with pre-treatment in the same group. Superscripts [a,b] indicate a significant difference (P<0.05) between groups within the same time period*

Table 7. *Comparison of pain on palpation scores*
Tablo 7. *Dokunmaya karşı acı skorlarının karşılaştırılması*

Months	Groups				
	DAR50	DAR100	DAR50/CS	DAR100/CS	CS
Pre-treatment	2.3±0.5	2.3±0.5	2.5±0.5	2.6±0.5	2.3±0.5
1st month	2.3±0.5	2.3±0.5	2.2±0.4	2.0±0.7	1.9±0.7
2nd month	2.2±0.6[a]	2.2±0.6[a]	1.6±0.5*,[b]	1.6±0.5*,[b]	1.3±0.5*,[b]
3rd month	2.2±0.4[a]	2.0±0.4[a]	1.5±0.5*,[b]	1.2±0.4*,[b]	1.2±0.4*,[b]
4th month	1.9±0.6	1.7±0.5*	1.3±0.5*	1.2±0.4*	1.2±0.4*
5th month	1.6±0.5*	1.5±0.5*	1.1±0.3*	1.1±0.3*	1.1±0.3*
6th month	1.4±0.5*	1.4±0.5*	1.1±0.3*	1.1±0.3*	1.1±0.3*

*Data are expressed as mean ± SD. *A significant difference (P<0.05) compared with pre-treatment in the same group. Superscripts [a,b] indicate a significant difference (P<0.05) between groups within the same time period*

bearing scores ($P<0.05$) was found in months 2-6. The DAR50/CS, DAR100/CS and CS groups showed significantly ($P<0.05$) improved weight-bearing scores compared with the DAR50 and DAR100 groups.

Overall scores *(Table 9)* were significantly improved ($P<0.05$) fastest in the DAR50/CS, DAR100/CS and CS groups, after 2 months of receiving medicine. The DAR100 group showed significant improvement ($P<0.05$) after 3 months, while the DAR50 group only showed significant improvement after 4 months. When comparing scores of groups within the same month, significant differences ($P<0.05$) were found in months 2-5. The DAR50/CS, DAR100/CS and CS groups had significantly ($P<0.05$) improved scores compared with the DAR50 and DAR100 groups after 2 and 3 months. But after the 4th and 5th months, the score in the DAR50 group was significantly higher ($P<0.05$) than those of the other groups.

Radiographic scores *(Table 10)* were found to significantly increase ($P<0.05$) in the DAR50 and DAR100 groups, while the other three groups showed no significant change. Most of the clinical scores improved after dogs had received diacerein for 5-6 months; there was no difference between 50 mg or 100 mg per day.

Table 8. *Comparison of weight-bearing scores*
Tablo 8. *Ağırlık taşıma skorlarının karşılaştırılması*

Months	Groups				
	DAR50	DAR100	DAR50/CS	DAR100/CS	CS
Pre-treatment	3.6±0.5	3.5±0.5	3.4±0.7	3.2±0.7	3.5±0.5
1st month	3.6±0.5	3.5±0.5	3.3±0.8	2.9±0.9	2.9±1.0
2nd month	3.5±0.5[a]	3.5±0.5[a]	2.7±0.5[b]	2.0±0.9*,[b]	2.3±0.9*,[b]
3rd month	3.1±0.6[a]	3.0±0.6*,[a]	2.2±0.8*,[b]	1.9±0.8*,[b]	2.0±0.9*,[b]
4th month	2.9±0.6*,[a]	2.8±0.6*,[a]	1.9±0.7*,[b]	1.6±0.7*,[b]	1.7±0.8*,[b]
5th month	2.8±0.6*,[a]	2.7±0.6*,[a]	1.5±0.5*,[b]	1.3±0.5*,[b]	1.3±0.5*,[b]
6th month	1.9±0.6*,[a]	1.8±0.6*,[a]	1.3±0.3*,[b]	1.1±0.3*,[b]	1.3±0.5*,[b]

*Data are expressed as mean ± SD. *A significant difference ($P<0.05$) compared with pre-treatment in the same group. Superscripts [a,b] indicate a significant difference ($P<0.05$) between groups within the same time period*

Table 9. *Comparison of overall scores*
Tablo 9. *Tüm skorların karşılaştırılması*

Months	Groups				
	DAR50	DAR100	DAR50/CS	DAR100/CS	CS
Pre-treatment	3.3±0.5	3.1±0.5	3.1±0.6	3.3±0.5	3.3±0.7
1st month	3.3±0.5	3.1±0.5	3.0±0.7	3.1±0.6	2.9±0.5
2nd month	3.3±0.5[a]	2.9±0.5[a]	2.6±0.5*,[b]	2.4±0.5*,[b]	2.6±0.5*,[b]
3rd month	2.9±0.3[a]	2.4±0.5*,[a]	2.2±0.4*,[b]	2.2±0.4*,[b]	2.4±0.5*,[b]
4th month	2.7±0.5*,[a]	2.3±0.5*,[b]	1.8±0.4*,[b]	1.8±0.4*,[b]	2.0±0.6*,[b]
5th month	2.4±0.5*,[a]	2.0±0.9*,[a,b]	1.5±0.5*,[b]	1.6±0.5*,[b]	1.7±0.5*,[b]
6th month	1.9±0.6*	1.6±0.7*	1.5±0.5*	1.6±0.5*	1.6±0.5*

*Data are expressed as mean ± SD. *A significant difference ($P<0.05$) compared with pre-treatment in the same group. Superscripts [a,b] indicate a significant difference ($P<0.05$) between groups within the same time period*

Table 10. *Comparison of radiographic scores*
Tablo 10. *Radyografik skorların karşılaştırılması*

Months	Groups				
	DAR50	DAR100	DAR50/CS	DAR100/CS	CS
Pre-treatment	2.5±0.5	2.5±0.5	2.5±0.5	2.6±0.5	2.4±0.5
3rd month	2.5±0.5	2.6±0.5	2.6±0.5	2.7±0.5	2.7±0.5
6th month	2.7±0.5*	3.0±0.4*	2.8±0.6	2.7±0.5	2.8±0.4

*Data are expressed as mean ± SD. *A significant difference ($P<0.05$) compared with pre-treatment in the same group*

Table 11. *Three side effects observed in all 5 groups (data expressed as mean ± SD)*
Tablo 11. *5 grupta gözlenen üç yan etki (veri, ortalama± standart sapma olarak verilmiştir)*

Weeks	DAR50	DAR100	DAR50/CS	DAR100/CS	CS
Diarrhea					
1	5/12 (41.67%)	7/12 (58.33%)	4/12 (33.33%)	6/12 (50.00%)	0/12 (0.00%)
2	1/12 (8.33%)	2/12 (16.67%)	1/12 (8.33%)	3/12 (25.00%)	0/12 (0.00%)
3	1/12 (8.33%)	2/12 (16.67%)	1/12 (8.33%)	2/12 (16.67%)	0/12 (0.00%)
4	1/11 (9.09%)	1/12 (8.33%)	1/12 (8.33%)	1/10 (10.00%)	0/12 (0.00%)
5	0/11 (0.00%)	1/12 (8.33%)	0/12 (0.00%)	0/10 (0.00%)	0/12 (0.00%)
6	0/11 (0.00%)	1/12 (8.33%)	0/12 (0.00%)	0/10 (0.00%)	0/12 (0.00%)
Discoloration of Urine					
1	10/12 (83.33%)	11/12 (91.67%)	10/12 (83.33%)	11/12 (91.67%)	0/12 (0.00%)
2	9/12 (75.00%)	11/12 (91.67%)	10/12 (83.33%)	10/12 (83.33%)	0/12 (0.00%)
3	9/12 (75.00%)	11/12 (91.67%)	10/12 (83.33%)	10/12 (83.33%)	0/12 (0.00%)
4	8/11 (72.73%)	11/12 (91.67%)	9/12 (75.00%)	9/10 (90.00%)	0/12 (0.00%)
5	8/11 (72.73%)	11/12 (91.67%)	9/10 (90.00%)	8/10 (80.00%)	0/12 (0.00%)
6	9/11 (81.82%)	10/12 (83.33%)	8/10 (80.00%)	8/10 (80.00%)	0/12 (0.00%)
Vomiting					
1	0/12 (0.00%)	1/12 (8.33%)	0/12 (0.00%)	2/12 (16.67%)	0/12 (0.00%)

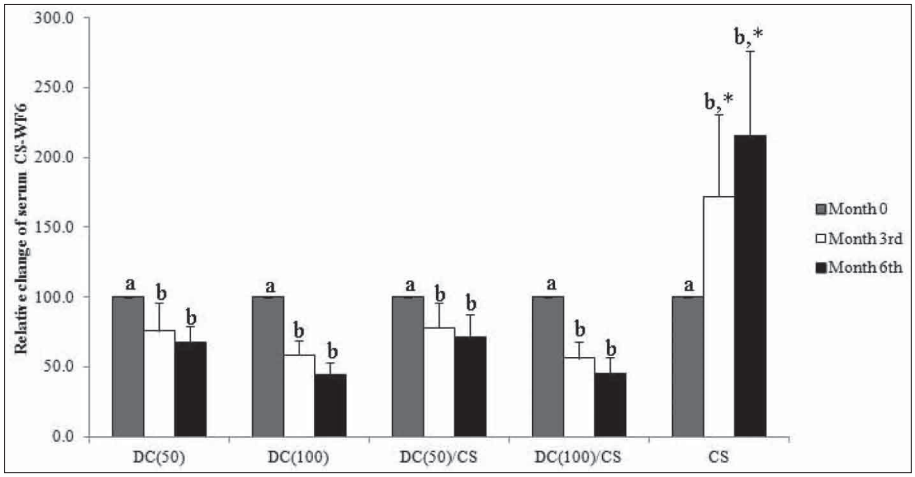


Fig 1. Mean (± SD) of relative change (%) of serum chondroitin sulfate (CS) epitope WF6. Gray bar = pre-treatment; white bar = 3 months after treatment; and black bar = 6 months after treatment. [a,b] Values were significantly different compared to month 0 within groups ($P<0.05$) **Values were significantly different between groups within a particular month ($P<0.05$)*

Şekil 1. Serum kondroitin sülfat (CS) epitop WF6'nın orantısal değişiminin (%) ortalaması (± standart sapma). Gri bar = tedavi öncesi; beyaz bar = tedaviden sonra 3 ay; ve siyah bar = tedaviden sonra 6 ay. [a,b] Değerler gruplar arasında 0. aya kıyasla önemli oranda farklılık göstermektedir ($P<0.05$)
** Değerler belli aylar arasında önemli oranda farklılık göstermektedir ($P<0.05$)*

The major side effects found in the study included soft stools, mild diarrhea, dark-colored urine, and vomiting *(Table 11)*. Dark urine was the major side effect after the 1st month of the study; the highest number was found in the DAR100 and DAR100/CS groups (91.67%), followed by the DAR50 and DAR50/CS groups (83.33%); however, no evidence of discoloration of urine was found in the CS group. Diarrhea or soft stools were also found in all groups that received diacerein. After the first month of the study, this side effect was found to be highest (58.33%) in the DAR100 group, followed by the DAR100/CS group (50.00%); the other two groups (DAR50 and DAR50/CS) were found to have 41.67 and 33.33%, respectively. Diarrhea slightly decreased after receiving medicine for a few months. Vomiting was found only in the 1st month of the study, in the DAR100/CS group (16.67%) and DAR100 group (8.33%).

The results of serum CS-WF6 are shown in *Fig. 1*. The level of CS-WF6 in the CS group was significantly higher ($P<0.05$), while the levels of this biomarker in the other four groups were significantly decreased ($P<0.05$) compared with pre-treatment. After 3 and 6 months of treatment, serum CS-WF6 in the CS group was significantly higher ($P<0.05$) than in the other four groups.

DISCUSSION

The results of the study showed that dogs with OA had significant ($P<0.05$) improvements in clinical evaluation scores after treatment with diacerein, chondroitin sulfate, and diacerein plus chondroitin sulfate. However, all of the effects appeared more slowly in the diacerein groups compared with groups treated

with chondroitin sulfate and diacerein plus chondroitin sulfate.

Previous *in vitro* studies have reported that diacerein inhibits the secretion of IL-1β in a dose-dependent manner [9,19]. But in clinical usage, there was no significant difference between 50 and 150 mg per day for pain score and clinical signs of OA [19]. The present study used 50 mg and 100 mg in the experimental design because previous human studies reported that the effective dose of diacerein was between 50–150 mg per day [13,19]. However, because the weights of all animals in this study were not more than 25 kg, we limited the dosage to 50 and 100 mg per day, while in human studies the highest dose was 150 mg for an average body weight of 60 kg. The results from this study showed that there was no significant difference in clinical sign scores for all five categories, regardless of whether 50 or 100 mg of diacerein was administered per day. But this study found that dogs receiving 100 mg per day had a higher percentage of side effects: 25% and 58% of dogs receiving diacerein 100 mg per day had diarrhea and discoloration of urine, respectively, while the corresponding percentages for dogs receiving diacerein 50 mg per day were 17% and 33%.

Human studies have found diacerein to be effective for treating OA [13,17,26]. In 2007, Louthrenoo and colleagues [26] reported on the efficacy, safety and carry-over effect of diacerein, in comparison to piroxicam, in the treatment of Thai patients with symptomatic knee osteoarthritis. Ninety percent of the patients treated with diacerein showed significantly reduced pain compared with 70% baseline. Diacerein was as effective as piroxicam in reducing pain and improving function, with longer carry-over effect and a better safety profile. Although the present study did not compare the effects of diacerein with NSAIDs, the effects were compared with another chondroprotective drug, chondroitin sulfate, as in a previous study [21]. Moreover, we studied the efficiency of diacerein when combined with chondroitin sulfate. Diacerein combined with chondroitin sulfate had a greater effect than diacerein alone from the 2nd through the 5th month of the experiment, while after 6 months there was no significant difference. Moreover, no significant difference was found when comparing the effects of 3 groups; diacerein 50 mg plus chondroitin sulfate, diacerein 100 mg plus chondroitin sulfate, and chondroitin sulfate alone. However, after 6 months of the experiment, no significant difference was found between all five groups. This study found that using chondroitin sulfate alone or in combination with diacerein could improve clinical signs faster than using diacerein alone.

The limitations of this clinical study included a lack of instruments to assess joint mobility (e.g. arthroscopy) and instruments to evaluate motion (e.g. faceplate analysis or motion analysis). However, to increase confidence in the subjective assessment, clinical scores and radiographic scores were evaluated by three and two blinded veterinarians, respectively. Another limit of the study was the lack of a negative control group receiving a placebo. The university's ethics committee did not permit the use of a negative control group, because all dogs enrolled in this study were pets of the owners.

This study found dogs that received only diacerein (both DAR50 and DAR100 groups) showed steady improvement of clinical signs compared to dogs that received chondroitin sulfate (CS group) or diacerein plus chondroitin sulfate (DAR50/CS and DAR100/CS groups). Administration of 100 mg per day was slightly more effective than 50 mg per day for pain on palpation, weight-bearing and overall score; there was no difference in the effect on lameness and joint mobility. Diacerein combined with chondroitin sulfate showed similar effects to the administration of chondroitin sulfate alone. However, when comparing the radiographic scores between pre-treatment, 3 and 6 months of treatment, dogs that received only diacerein (50 and 100 mg) showed significantly increased radiographic scores after 6 months of treatment. No significant increase in radiographic scores was found in the other three groups. The study results may indicate that diacerein alone (either 50 or 100 mg) does not prevent pathophysiological changes of OA in dogs weighing less than 25 kg. On the other hand, chondroitin sulfate and diacerein plus chondroitin sulfate both showed preventive effects against pathological change. To conclude that, the other study has to done in particular using biomarker for osteoarthritis to evaluate the micromolecular or biochemical changes while receiving these medicines [21,24,25].

Two previous studies [18,20] from the same research group reported conflicting results from using diacerein. In 1997 [18], Brandt and colleagues induced OA in 14 adult mongrel dogs; 7 dogs received diacerein (15-20 mg/kg) daily, while the other 7 dogs served as OA controls. At the end of the study (8 weeks), no statistical significance was found between the two groups. In a 1999 study [20], Smith and colleagues induced OA in 20 adult mongrel dogs by transection of the anterior cruciate ligament, and then provided a total daily dose of 40 mg/kg for 32 weeks. At the end of the study, a significant reduction in the severity of morphological changes of OA (under arthroscopic evaluation) was found for diacerein compared with the placebo group. This is accordance with the results of the present study, which found that there was a positive effect from administration of diacerein for at least 4 to 5 months, and that there was no significant difference between 50 and 100 mg per day. This study showed that the use of 50 mg diacerein had similar effects to 100 mg diacerein in dogs weighing not over 25 kg; this result was comparable to a previous study using doxycycline in canine hip OA [21], where the efficiency was similar but with a slower affect compared with chondroitin sulfate.

Because of the increased use of diacerein in small animal clinics, the side effects of this medicine must be

considered. In humans, the main side effects that have been reported are diarrhea/soft stools and dark urine [19]. Our study is the first to report on the side effects of diacerein in dogs. The main side effect was found to be dark urine (89-90%) which highest number during study. The second most common side effect was diarrhea, which was also found in higher numbers (30-50%) during the first few months, and then decreased. Vomiting was found in a few dogs receiving 100 mg diacerein, but this occurred only during the first month of the study. Although the pharmacokinetics of diacerein in dogs has not been established, the results of human studies may explain the causes of diarrhea and dark urine after receiving diacerein. The cause of diarrhea after receiving diacerein is not well understood; however it is believed that this may be due to the chemical structure of diacerein and rhein, which are anthraquinone derivatives [14]. Anthraquinone is a laxative agent, and for this reason diacerein has a laxative effect as well. Darkening of urine is caused by chemical reactions occurring in an acidic medium, and is directly linked to the antraquinonic structure of the molecule [14].

Previous studies have reported that in chronic OA, the level of CS-WF6 is higher than normal because the native CS chain in cartilage is degraded and released into the blood system [24,27]. The finding of changed levels of serum CS-WF6 after treatment reflects the alteration of cartilage metabolism. This study found that serum CS-WF6 was elevated after dogs received chondroitin sulfate alone; however, the level of this biomarker decreased after dogs received diacerein or diacerein plus chondroitin sulfate. It possible that diacerein blocked the action of IL-1β, causing downregulation of other degradation enzymes such as matrix metalloproteinases and the ADAMTS family [9]. Moreover, the suppression function of IL-1β resulted in nitric oxide (NO) synthesis [8]. For this reason, the levels of serum CS-WF6 in dogs receiving diacerein were decreased, with a significant decrease (25%) after 3 months of receiving medication. This effect also occurred in the groups receiving a combination of diacerein and chondroitin sulfate. However, when comparing administration of 50 and 100 mg per day of diacerein, no significant differences in serum CS-WF6 levels were observed.

In conclusion, this study showed similar effects for diacerein doses of 50 and 100 mg; dogs showed improvement in metabolism after 3 months and in clinical signs 4-5 months after the start of treatment. The clinical signs improved faster after receiving diacerein, when combined with chondroitin sulfate. Moreover, using chondroitin sulfate alone gave efficacy similar to that of diacerein plus chondroitin sulfate. However, 100 mg doses increased the side effects, including diarrhea, dark-colored urine, and vomiting. Pet owners should be informed in advance about the two main side effects, diarrhea and dark urine, to prevent any unnecessary feelings of fear or anxiety.

List of Abbreviations

BSA: bovine serum albumin
CS: chondroitin sulfate
DAR: diacerein
ELISA: enzyme-linked immunosorbent assay
ERK1/2: extracellular signal-regulated kinase 1/2
IL-1β: interleukin-1β
MMP: matrix metalloproteinase
NOS: nitric oxide syntheses
NSAIDs: non-steroidal anti-inflammation drugs
OA: osteoarthritis
o-PD: ortho-phenylenediamine
SD: standard derivation

REFERENCES

1. Rintelen B, Neumann K, Leeb BF: A meta-analysis of controlled clinical studies with diacerein in the treatment of osteoarthritis. *Arch Intern Med,* 166, 1899-1906, 2006.

2. Wandel S, Jüni P, Tendal B, Nüesch E, Villiger PM, Welton NJ, Reichenbach S, Trelle S: Effects of glucosamine, chondroitin, or placebo in patients with osteoarthritis of hip or knee: Network meta-analysis. *BMJ,* 341, c4675, 2010.

3. Bruyère O, Burlet N, Delmas PD, Rizzoli R, Cooper C, Reginster JY: Evaluation of symptomatic slow-acting drugs in osteoarthritis using the GRADE system. *BMC Musculoskelet Disord,* 9, 165, 2008.

4. Nakasone Y, Watabe K, Watanabe K, Tomonaga A, Nagaoka I, Yamamoto T, Yamaguchi H: Effect of a glucosamine-based combination supplement containing chondroitin sulfate and antioxidant micronutrients in subjects with symptomatic knee osteoarthritis: A pilot study. *Exp Ther Med,* 2, 893-899, 2011.

5. Sawitzke AD, Shi H, Finco MF, Dunlop DD, Harris CL, Singer NG, Bradley JD, Silver D, Jackson CG, Lane NE, Oddis CV, Wolfe F, Lisse J, Furst DE, Bingham CO, Reda DJ, Moskowitz RW, Williams HJ, Clegg DO: Clinical efficacy and safety of glucosamine, chondroitin sulphate, their combination, celecoxib or placebo taken to treat osteoarthritis of the knee: 2-year results from GAIT. *Ann Rheum Dis,* 69, 1459-1464, 2010.

6. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H: Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol,* 7, 33-42, 2011.

7. Daheshia M, Yao JQ: The interleukin 1β pathway in the pathogenesis of osteoarthritis. *J Rheumatol,* 35, 2306-2312, 2008.

8. Pelletier JP, Mineau F, Fernandes JC, Duval N, Martel-Pelletier J: Diacerhein and rhein reduce the interleukin 1 beta stimulated inducible nitric oxide synthesis level and activity while stimulating cyclooxygenase-2 synthesis in human osteoarthritic chondrocytes. *J Rheumatol,* 25, 2417-2424, 1998.

9. Martel-Pelletier J, Pelletier JP: Effects of diacerein at the molecular level in the osteoarthritis disease process. *Ther Adv Musculoskelet Dis,* 2, 95-104, 2010.

10. Martel-Pelletier J, Mineau F, Jolicoeur FC, Cloutier JM, Pelletier JP: *In vitro* effects of diacerhein and rhein on interleukin-1 and tumor necrosis factor-alpha systems in human osteoarthritic synovium and chondrocytes. *J Rheumatol,* 25, 753-762, 1998.

11. Boileau C, Tat SK, Pelletier JP, Cheng S, Martel-Pelletier J: Diacerein inhibits the synthesis of resorptive enzymes and reduces osteoclastic differentiation/survival in osteoarthritic subchondral bone: A possible mechanism for a protective effect against subchondral bone remodelling. *Arthritis Res Ther,* 10, R71, 2008.

12. Pelletier JP, Mineau F, Boileau C, Martel-Pelletier J: Diacerein

reduces the level of cartilage chondrocyte DNA fragmentation and death in experimental dog osteoarthritic cartilage at the same time that it inhibits caspase-3 and inducible nitric oxide synthase. *Clin Exp Rheumatol,* 21, 171-177, 2003.

13. Pelletier JP, Yaron M, Haraoui B, Cohen P, Nahir MA, Choquette D, Wigler I, Rosner IA, Beaulieu AD: Efficacy and safety of diacerein in osteoarthritis of the knee: A double-blind, placebo-controlled trial. The Diacerein Study Group. *Arthritis Rheum,* 43, 2339-2348, 2000.

14. Nicolas P, Tod M, Padoin C, Petitjean O: Clinical pharmacokinetics of diacerein. *Clin Pharmacokinet,* 35, 347-359, 1998.

15. Magnard O, Louchahi K, Tod M, Petitjean O, Molinier P, Berdah L, Perret G: Pharmacokinetics of diacerein in patients with liver cirrhosis. *Biopharm Drug Dispos,* 14, 401-408, 1993.

16. Debord P, Louchahi K, Tod M, Molinier P, Berdah L, Petitjean O: Influence of renal function on the pharmacokinetics of diacerein after a single oral dose. *Eur J Drug Metab Pharmacokinet,* 19, 13-19, 1994.

17. Dougados M, Nguyen M, Berdah L, Maziéres B, Vignon E, Lequesne M, ECHODIAH Investigators Study Group: Evaluation of the structure-modifying effects of diacerein in hip osteoarthritis: ECHODIAH, a three-year, placebo-controlled trial. Evaluation of the chondromodulating effect of diacerein in OA of the hip. *Arthritis Rheum,* 44, 2539-2547, 2001.

18. Brandt KD, Smith G, Kang SY, Myers S, O'Connor B, Albrecht M: Effects of diacerhein in an accelerated canine model of osteoarthritis. *Osteoarthritis Cartilage,* 5, 438-449, 1997.

19. Fidelix TS, Soares BG, Trevisani VF: Diacerein for osteoarthritis. *Cochrane Database Syst Rev,* 25, CD005117, 2006.

20. Smith GNJ, Myers SL, Brandt KD, Mickler EA, Albrecht ME: Diacerhein treatment reduces the severity of osteoarthritis in the canine cruciate-deficiency model of osteoarthritis. *Arthritis and Rheumatism,* 42, 545–554, 1999.

21. Nganvongpanit K, Pothacharoen P, Suwankong N, Ong-Chai S, Kongtawelert P: The effect of doxycycline on canine hip osteoarthritis: Design of a 6-months clinical trial. *J Vet Sci,* 10, 239-247, 2009.

22. McCarthy G, O'Donovan J, Jones B, McAllister H, Seed M, Mooney C: Randomised double-blind, positive-controlled trial to assess the efficacy of glucosamine/chondroitin sulfate for the treatment of dogs with osteoarthritis. *Vet J,* 174, 54-61, 2007.

23. Takahashi M, Naito K, Abe M, Sawada T, Nagano A: Relationship between radiographic grading of osteoarthritis and the biochemical markers for arthritis in knee osteoarthritis. *Arthritis Res Ther,* 6, R208-212, 2004.

24. Nganvongpanit K, Itthiarbha A, Ong-Chai S, Kongtawelert P: Evaluation of serum chondroitin sulfate and hyaluronan: Biomarkers for osteoarthritis in canine hip dysplasia. *J Vet Sci,* 9, 317-325, 2008.

25. Nganvongpanit K, Pothacharoen P, Chaochird P, Klunklin K, Warrit K, Settakorn J, Pattamapaspong N, Luevitoonvechkij S, Arpornchayanon O, Kongtawelert P, Pruksakorn D: Prospective evaluation of serum biomarker levels and cartilage repair by autologous chondrocyte transplantation and subchondral drilling in a canine model. *Arthritis Res Ther,* 11, R78, 2009.

26. Louthrenoo W, Nilganuwong S, Aksaranugraha S, Asavatanabodee P, Saengnipanthkul S (Thai Study Group): The efficacy, safety and carry-over effect of diacerein in the treatment of painful knee osteoarthritis: A randomised, double-blind, NSAID-controlled study. *Osteoarthritis Cartilage,* 15, 605-614, 2007.

27. Pothacharoen P, Teekachunhatean S, Louthrenoo W, Yingsung W, Ong-Chai S, Hardingham T, Kongtawelert P: Raised chondroitin sulfate epitopes and hyaluronan in serum from rheumatoid arthritis and osteoarthritis patients. *Osteoarthritis Cartilage,* 14, 299-301, 2006.

Kafkas Univ Vet Fak Derg
20 (3): 393-398, 2014
DOI: 10.9775/kvfd.2013.10248

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

RESEARCH ARTICLE

Effect of Egg Yolk and Soybean Lecithin on Tris-Based Extender in Post-Thaw Ram Semen Quality and *in vitro* Fertility [1]

Burcu ÜSTÜNER [1] Selim ALÇAY [1] Zekariya NUR [1]
Hakan SAĞIRKAYA [1] M. Kemal SOYLU [1]

[1] *This study presented 6th National Congress on Animal Reproduction and Artificial Insemination, 18-22 May 2011, p.86-87, Belek, Antalya - TURKEY*

[1] Uludag University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, TR-16059 Gorukle, Bursa - TURKEY

Makale Kodu (Article Code): KVFD-2013-10248

Summary

The aim of the current study was to evaluate the effect of egg yolk and different soybean lecithin concentrations on the efficiency of ram semen cryopreservation and to test the fertilizing ability of frozen-thawed ram semen. Ejaculates with a thick consistency, rapid wave motion (3-5), and >70% initial motility were pooled. Pooled semen were then divided into four groups and diluted at 1/5 (semen/extender) with 1%, 3%, 6% lecithin (L1, L3 and L6) or 20% egg yolk (EY20) using the two-step dilution method. As expected, the results of the current study showed that both motility and the rates of defective acrosomes in sperm were negatively affected by the cryopreservation procedure (P<0.001). The motility values of at 5°C and post-thawed semen in the EY20 group were significantly higher than those in the L1, L3 and L6 groups (P<0.05). There were no differences in motility rates among the lecithin groups at the dilution, cooling, equilibration or post thawing stages (P>0.05). The results of in vitro fertilization, as assessed by the rate of blastocyst formation, were more successful in the EY20 group than those noted in different lecithin groups. In conclusion, freezing ram semen with an extender containing egg yolk could yield better post-thaw sperm parameters and embryonic development compared to lecithin containing extenders.

Keywords: *Cryopreservation, Lecithin, Egg yolk, Ram semen, IVF*

Tris-Bazlı Sulandırıcılarda Yumurta Sarısı ve Soya Lesitininin Eritme Sonrası Koç Spermasının Kalitesi ve *in vitro* Fertilite Üzerine Etkisi

Özet

Bu çalışmanın amacı, yumurta sarısı ve farklı lesitin konsantrasyonlarının koç spermasının dondurulabilirliği üzerine etkisini değerlendirmek ve dondurup çözdürülen koç spermasının fertilizasyon yeteneğini tespit etmektir. Kitle hareketi (3-5) ve >%70 motiliteye sahip ejakülatlar birleştirildi (pooling). Pooling yapılan sperma dört gruba bölündü ve %1, %3, %6 (L1, L3 ve L6) lesitin veya %20 yumurta sarısı (EY20) içeren sulandırıcılar ile 1/5 (sperma/sulandırıcı) oranında iki aşamalı sulandırma yöntemi kullanılarak sulandırıldı. Bu çalışmanın sonucunda; motilite ve akrozomal bozukluk oranlarının dondurma prosedüründen olumsuz yönde etkilendiği tespit edildi (P<0.001). EY20 grubunun 5°C'de ve eritme sonrası motilite değerleri L1, L3 ve L6 gruplarına göre yüksek bulundu (P<0.05). Sulandırma, soğutma, ekilibrasyon ve eritme sonrası aşamalarda motilite oranları bakımından lesitin grupları arasında farklılık saptanmadı (P>0.05). Blastosist oranları bakımından değerlendirilen in vitro fertilizasyon sonuçlarına göre, EY20 grubunun farklı lesitin gruplarından daha başarılı olduğu tespit edildi. Sonuç olarak, yumurta sarısı içeren sulandırıcı ile dondurulan koç spermasının lesitin gruplarına göre eritme sonrası spermatolojik özellikler ve embriyonik gelişim bakımından daha üstün olduğu tespit edildi.

Anahtar sözcükler: *Dondurma, Lesitin, Yumurta sarısı, Koç sperması, IVF*

INTRODUCTION

Cryopreservation impacts lipid composition and the organization of the sperm plasma membrane [1]. In addition, sudden temperature changes, such as cold shocks, ice formation and dissolution during the freezing-

İletişim (Correspondence)
+90 224 2941245
bbaspinar@uludag.edu.tr

thawing process, affect the integrity and function of the acrosome, nucleus, mitochondria, axonema and plasma membrane [2-4].

Extenders with different cryoprotectants such as glycerol have been used to protect various cell compartments [5,6]. Semen extenders generally contain simple carbohydrates (such as glucose) as an energy source, a high-molecular-weight material to prevent cold shock (such as egg yolk, milk, or soybean lecithin), ionic or non-ionic substances to maintain a suitable osmotic pressure and pH, and antibiotics [7,8].

Egg yolk and skim milk are the most common additives of animal origin that are used for sperm cryopreservation. The egg yolk's main effective component is the lipoprotein fraction, e.g., lecithin, which protects the membrane's phospholipid integrity during cryopreservation [5,9,10]. However, various experts have recommended against the use of egg yolk due to the wide variability of its constituents [9,10]. In addition, some researchers have shown that products of animal origin could increase the risk of microbial contamination during the AI procedure of domestic animals [11] and that microbial contamination could result in the subsequent production of endotoxin, which could reduce the potential fertilizing capacity of spermatozoa [5]. Egg yolk has been shown to be antigenic and to induce antibodies in both systemic circulation and the reproductive tract [12]. Lecithin (or phosphatidylcholine) is a phospholipid that is distributed widely in plants and plays an important role in the regulation of an animal cells' bio-membrane [13].

This has resulted in the replacement of egg yolk with alternative cryoprotectants such as soybean-derived lecithin for both animal and human sperm cryopreservation. The aim of the current study was to evaluate the effects of egg yolk and three different soybean lecithin concentrations (1%, 3% or 6%) on the freezability and post-thaw fertilizing ability of ram semen.

MATERIAL and METHODS

Semen Extender Preparation

Tris based four different extenders were prepared by the addition of egg yolk and different concentrations of soybean lecithin (L-α-Phosphatidyl choline from Soybean Type II-S Sigma, P5638) [14]. Extenders (Extender A and B) were designated as follows: EY20, 20% (v/v) egg yolk, L1, 1% (w/v) lecithin, L3, 3% (w/v) lecithin and L6, 6% (w/v) lecithin. Each supernatant was obtained after centrifugation (1.000 g, 20 min). These supernatants were filtered through a 0.22 µm- filter (Millex-GV, 0.22 µm). While the Extender A did not contain glycerol, the freezing extender (Extender B) was composed of the cooling extenders (88%, v/v) and glycerol (12%, v/v).

Semen Collection, Evaluation and Dilution

Six Kivircik rams aged 3-5 yrs old and maintained at Uludag University, Faculty of Veterinary Medicine in Bursa, Turkey, were used as the material during a non-breeding season. Semen was collected by electrically stimulated ejaculation (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand) [2,15]. Ram semen was collected five times in every other day.

Collected semen was placed in a warm water bath (30°C) and immediately evaluated for consistency, wave motion (0-5 scale), and percentage of motile spermatozoa [15]. Ejaculates with a thick consistency, rapid wave motion (3-5 on a 0-5 scale), and >70% initial motility were pooled.

Briefly, pooled semen was divided into four groups. Each of the groups was diluted to a ratio of 1:1 (semen/extender) with extender A, which was one of four soybean-derived lecithin 1%, 3%, 6% (L1, L3 or L6) or 20% egg yolk (EY20) and cooled to 5°C within 1 h. The cooled sperm groups were then diluted to a ratio of 1/1 (semen/extender) with extender B (previously cooled at 5°C), which was one of four soybean-derived lecithin 1%, 3%, 6% (L1, L3 or L6) or 20% egg yolk (EY20) (6% glycerol). Extender B was added in five steps at 5 min intervals and equilibrated at 5°C for 2 h.

Semen Freezing and Thawing

Equilibrated semen was placed into 0.25 ml straws and frozen at 3°C/min from +5°C to -8°C and at 25°C/min from -8°C to -120°C in liquid nitrogen vapor using the Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France) [2]. The straws were then plunged into liquid nitrogen at -196°C where they were stored for at least one month. At least three straws from each group of pooled ejaculates were thawed at 37°C for 30 sec in a water bath to evaluate post-thaw semen characteristics.

Semen Evaluation

Motility and acrosomal and other morphological defects (OMD) were assessed at the following four time points: after dilution with extender A, at 5°C, after equilibration, and post-thaw. All semen samples studied were frozen by the same person, and each of the semen parameters was evaluated by the same person on each occasion throughout the study. Sperm motility was assessed subjectively using a phase-contrast microscope (Olympus BX 51) (400x) with a warm slide (38°C) [15]. Defected acrosome and OMDs (head, midpiece and tail defects) were assessed using the Giemsa staining method. At least 200 spermatozoa per smear were evaluated for morphological defects [14].

In vitro fertilization (IVF)

Oocyte in vitro maturation: All chemicals were purchased from Sigma. Ovaries were collected from slaughtered ewes (during the non-breeding season) and placed in normal

saline at approximately 30°C. The ovaries were transported to the laboratory within 2 to 3 h of collection. The conditions for maturation, fertilization and culture were a slightly modified version of those described by Gómez et al.[16]. After washing the ovaries with fresh normal saline, cumulus-oocyte complexes were recovered by slicing. Cumulus-oocyte complexes were collected into tissue culture medium 199, which was supplemented with Hepes (free acid) 15 mM, sodium Hepes 15 mM, 0.33 mg/ml sodium bicarbonate, 0.01 mg/ml heparin sodium salt, 0.075 mg/ml penicillin G-potassium salt, 0.05 mg/ml streptomycin sulfate, 0.08 mg/ml kanamycin monosulfate and 10% fetal bovine serum (FBS) (F9665). After rinsing 3 times in this medium, oocytes with a homogeneous ooplasm surrounded by several layers of cumulus cells were matured in multi-well dishes (Nunc™, 176740). Each well contained 50 cumulus-oocyte complexes (COC) and 500 µl of maturation medium-199 containing 10% v/v FBS, 10 µg/ml FSH and 10 µg/ml LH. The wells were covered with mineral oil (M8410), and the oocytes were cultured for 24 h in 5% CO_2 in humidified air at 39°C.

In vitro fertilization and culture: Matured COCs were denuded of their cumulus and corona cells by aspiration through a narrow hand-drawn pipette. The cumulus-free oocytes were washed and transferred into multi-well dishes. Each well contained 500 µl of bicarbonate-buffered synthetic oviduct fluid (BSOF) medium, supplemented with 2% sheep serum, 0.1 mg pyruvic acid, 0.15 mg/ml L-glutamine, 0.08 mg/ml kanamycin monosulfate, 0.075 mg/ml penicillin G- potassium salt and 0.05 mg/ml streptomycin sulfate. For insemination, three straws of frozen sperm were thawed (37°C 30 s) and pooled. Two layer Percoll gradients (90 to 45%) were prepared in 10 ml centrifuge tubes, 2 ml of 90% Percoll was placed at the bottom of the tube, and 2 ml of 45% Percoll was carefully layered on the top. Care was taken to avoid mixing the 2 layers. Then, 200 µl of the semen samples were placed at the top of the Percoll gradient. The samples were centrifuged at 3.000 g for 15 min. The top layer of Percoll was discarded, and the sperm pellet was resuspended in 3 ml of Hepes buffered modified SOF (HSOF) and centrifuged again at 600 *g* for 6 min. The supernatant was then discarded. Oocytes were co-incubated with 0.6-1.0x10^6 sperm/ml in groups of 50 in 500 µl of BSOF medium covered with mineral oil in 5% CO_2 in humidified air at 39°C for 24 h. *IVF* experiments were repeated four times with straws of different groups (L1, L3, L6 or EY20). Oocytes were washed in SOF medium to remove the spermatozoa and were then cultured in SOF medium without serum and glucose. On day 3 post-insemination (Day 0= day of insemination), embryos were transferred into SOF medium supplemented with FBS and glucose. Cleavage and blastocyst rates were assessed on Days 2 and 8 post-insemination, respectively.

Statistical Analysis

Sperm-related data were analyzed by analysis of variance (ANOVA) using the General Linear Model (GLM) procedure. When the ANOVA test showed statistical differences, the mean of the treatments were compared using the Tukey's test. Repeated measures ANOVA (using GLM procedures) were conducted to compare the results at different stages of the cryopreservation process. *In vitro* fertilization-related statistical computations were performed using Chi-square analysis (SPSS 10.0 for Windows; SPSS, Chicago, IL, USA). P-values less than 0.05 were considered to be statistically significant.

RESULTS

Percentages of motility, defected acrosomes and OMD (other morphological defects) of the diluted, cooled at 5°C, equilibrated and post thawed ram semen from different extenders presented in *Table 1*.

Sperm motility was progressively reduced by the cooling and freeze-thawing processes (P<0.001). The motility values of semen cooled at 5°C and post-thaw in the EY20 group were significantly higher than those in the L1, L3 and L6 groups (P<0.05). There were no differences in motility rates between lecithin groups at the stages of dilution, cooling, equilibration or post-thaw (P>0.05).

Acrosome integrity was negatively affected by the freeze-thawing process (P<0.001). The percentage of defective acrosome and OMD rates were not affected by the extender components (L1, L3, L6 and EY20) during the freeze-thawing stages (P>0.05).

The cleavage and blactocyst formation rates after insemination with frozen-thawed spermatozoa using L1, L3, L6 and EY20 presented in *Table 2*. The highest cleavage rates were observed with the EY20 treatment group (85.50%) as compared with the L1 (28.6%), L3 (41.76%) or L6 (37.4%) groups (P<0.05). In addition, there were significant differences with respect to the number of 8 cell embryos and morulae among the EY20 group and other lecithin extender groups (P<0.05).

The number of blastocysts in fertilized oocytes in the EY20 group was 5 (4.03%). However, no blastocyst was obtained in the L1, L3 and L6 groups.

DISCUSSION

This study compared the effectiveness of soybean lecithin and egg yolk containing Tris-based extenders on post-thaw sperm parameters and *in vitro* fertilizing ability. Most semen extenders contain egg yolk and skim milk as sources of lipoprotein that protects sperm cells from cold shock and other damage [9,10]. However, the possible disadvantages of using egg yolk, including its potential to be a cause of allergic reactions, the risk of bacterial contamination and its variable effect on semen have been

Table 1. *The mean percentage of motility and rates of defective acrosomes and other morphological defects (OMD) after dilution, at 5°C, equilibration and post-thawing using different extenders composed of different concentrations of lecithin (1%, 3% and 6%) and egg yolk (20%)*

Tablo 1. *Farklı lesitin konsantrasyonları (%1, %3 ve %6) ve yumurta sarısı (%20) kullanarak dondurulan sperma gruplarının, sulandırma sonrası, 5°C, ekilibrasyon ve eritme sonrası aşamalarda ortalama motilite, akrozomal ve diğer morfolojik bozukluk oranları*

Stages	Groups	n	Motility (%)	Defective Acrosome (%)	OMD (%)
After dilution	Lecithin 1%	5	72.0±1.2	13.5±3.0	1.9±0.4
	Lecithin 3%	5	72.0±1.2	10.5±2.5	1.5±0.7
	Lecithin 6%	5	71.0±1.0	8.6±2.2	1.4±0.6
	Eggyolk20%	5	75.0±2.7	7.5±1.6	1.7±0.4
	General Mean	20	72.5±1.5^{x}	10.0±2.3^{x}	1.6±0.6
At 5°C	Lecithin 1%	5	62.0±2.6^{b}	11.9±1.0	1.8±0.8
	Lecithin 3%	5	65.0±1.6^{b}	14.5±1.7	1.3±0.7
	Lecithin 6%	5	65.0±0.1^{b}	13.7±2.9	1.9±0.6
	Eggyolk20%	5	71.0±1.0^{a}	14.8±1.9	2.0±0.5
	General Mean	20	65.8±4.7^{y}	13.7±1.9^{y}	1.8±0.6
Equilibration	Lecithin 1%	5	50.0±5.2	22.1±2.6	1.5±0.5
	Lecithin 3%	5	53.0±4.6	19.2±1.6	1.4±0.6
	Lecithin 6%	5	53.0±3.4	20.9±2.4	1.6±0.2
	Eggyolk20%	5	61.0±1.9	18.6±1.4	2.5±0.7
	General Mean	20	54.3±9.2^{z}	20.2±2.0^{z}	1.7±0.5
Post-thaw	Lecithin 1%	15	31.0±1.8^{b}	31.7±2.6	1.9±0.4
	Lecithin 3%	15	28.7±1.6^{b}	36.2±4.1	2.3±0.6
	Lecithin 6%	15	30.0±1.4^{b}	36.3±4.3	1.5±0.3
	Eggyolk20%	15	45.7±1.4^{a}	27.4±1.6	2.5±0.4
	General Mean	60	33.8±1.5^{t}	32.9±3.1^{t}	2.1±0.4

a,b: *The mean values having different letters within the same column showed significant differences (P<0.05),* ***x,y,z,t:*** *The general mean values having different letters within same column for the different stages showed significant differences (P<0.001),* ***OMD:*** *Other morphological defects*

Table 2. *The number of in vitro-matured oocytes and their number of 2-cell, 8-cell, morulae and blactocyst rates after insemination with post frozen-thawed spermatozoa using different extenders*

Tablo 2. *Farklı sulandırıcılar kullanılarak dondurulan spermalarla yapılan fertilizasyon sonrası in vitro olgunlaştırılmış oosit sayıları ile 2-hücreli, 8-hücreli, morula ve blastosist oranları*

Groups	Number of Fertilized Oocytes	Number of 2-Cell Embryos (%)	Number of 8-Cell Embryos (%)	Number of Morulae (%)	Number of Blastocyst (%)
L1	140	40 (28.60)^{a}	16 (11.43)^{a}	5 (3.60)^{a}	0^{a}
L3	91	38 (41.76)^{b}	15 (16.50)^{a}	5 (5.50)^{a}	0^{a}
L6	123	46 (37.40)^{ab}	18 (14.63)^{a}	3 (2.44)^{a}	0^{a}
EY20	124	106 (85.50)^{c}	86 (69.35)^{b}	17 (14.01)^{b}	5 (4.03)^{b}

a-c: *The mean values having different letters within the same column showed significant differences (P<0.05)*

reported [7,9,11,17]. On the other hand, soybeans contain a high component of low-density lipoprotein, e.g., lecithin or egg yolk-like lecithin [5]. The use of animal-free culture medium (defined component of medium) is a popular choice in assisted reproductive technology.

Extender composition assists with the stabilization of cells during the freezing and thawing processes [2,14]. Extenders containing soybean lecithin could be an alternative to the conventional extenders that include egg yolk [5,18]. De Leeuw et al. has noted that bull sperm survive freezing more effectively in egg yolk-containing diluents than in soybean lecithin [19]. In this study, the freeze-thaw process negatively affected sperm motility and acrosome integrity (P<0.001). Post-thaw sperm recovery was significantly greater when sperm was frozen in egg yolk containing extenders as compared to lecithin-containing groups. The beneficial effect of egg yolk may be due to its cryoprotective abilities and nutritive properties [14].

High soybean lecithin concentrations have been noted to be toxic for sperm motility and viability [5]. The impact of lecithin on sperm motility results from the extender viscosity and the presence of particulate debris [20]. Herein, there were no significant differences between the L1, L3 and L6 groups in terms of post-thaw motility (P>0.05). The extenders were filtered in a 0.22 µm filter to remove debris. The similarity in the results obtained from the different lecithin concentrations was perhaps due to the filtration technique, with small micron membranes used.

The freeze-thawing process may have a detrimental effect on sperm morphology, specifically for acrosome integrity [2]. The post-thaw percentage of defective acrosomes was higher than those of diluted, cooled and equilibrated spermatozoa (P<0.001). It has been suggested that phospholipids from egg yolk or soybean lecithin might integrate with the sperm membrane to form a protective film against the formation of lethal intracellular

ice crystals and protect the sperm membrane from mechanical damage during the freeze-thawing process [4]. The percentage of post-thaw defective acrosomes and OMD rates were not affected by the extender groups used.

Although, several researchers have developed different extender compositions and protocols for freezing ram semen, in general, fertility results are not comparable to those obtained with fresh semen and natural mating. These reductions in fertilization capacity have typically been attributed to a reduced rate of sperm motility and freeze-thawing-induced morphological and genomic abnormalities [2,14,15]. Spermatozoa that are morphologically defective or have poor motility tend to have low success rate of oocyte fertilization. The decreased fertility rates of the L1, L3 and L6 groups compared to those observed in sperm that were frozen with egg yolk are consistent with findings from other authors [17,19-21]. Those authors have observed a deleterious effect or a reduction in the fertility of semen frozen in the presence of soybean lecithin.

This reduction may be the result of soybean lecithin adherence to the surface of the sperm plasma membrane [19], changes in the composition of the sperm membrane or a strong interaction between soybean lecithin and the lipids of the sperm membrane during freezing and thawing procedures [22]. In addition, the adherence of soybean lecithin to the sperm membrane results in the inhibition of sperm acrosome reaction [23]. However, these results are different from studies of semen frozen in diluents containing egg yolk or soybean lecithin, which were conducted by Aires et al.[7], Akhter et al.[24] and Forouzanfar et al.[5].

There are many reports concerning the presence of lecithin concentrations in freezing media. However, there are conflicting reports concerning the beneficial effect of lecithin [5,17-19,21]. Increased lecithin concentrations in freezing media positively affect bull [7,11,25] and stallion [21] sperm fertility rates. In the present study, reducing the soybean lecithin concentration from 6% to 1% did not negatively affect the survival rates of ram sperm following cryo preservation. In general, similar embryonic development was observed among the lecithin-containing groups, with the exception of the number of 2-cell embryos in the L1 group with the lowest number.

The process of fertilization involves complex bio-chemical and physiological events that cannot be explained only by semen or oocyte quality. For example, the media used during IVF and the breeding season during which oocytes are recovered both impact embryonic development [26]. In addition, herein, frozen ram semen was evaluated using *in vitro* fertilization. The cleavage rate in IVF studies using frozen ram semen varies between 13-88% [27,28]. Higher cleavage rates to 2-cell embryos were obtained in the EY20 group (85.50%) as compared to the L1, L3 and L6 groups (28.6%, 41.76%, 37.40%, respectively ($P<0.05$).

Poor oocyte quality is a common cause of infertility. The quality tends to be poor during the prepubertal stage and anestrous periods in seasonal breeders [29]. In our study, embryonic development related results, i.e., morulae and blastocyst rates, were lower than that noted in other studies [5,16,28]. This finding could be explained by the fact that the oocytes were collected during a non-breeding season.

In conclusion, freezing ram semen with an extender containing egg yolk could yield greater post-thaw sperm parameters and embryonic development compared to lecithin-containing groups. In addition, freeze-thawing processes had a detrimental effect on sperm motility and morphology. Post-thaw sperm quality and fertilizing ability were not affected by different lecithin concentrations. Further studies should be performed to determine the lecithin concentration and extender preparation technique that would properly improve post-thaw semen quality and fertilizing capability.

REFERENCES

1. Hinkovska-Galcheva V, Petkova D, Koumanov K: Changes in the phospholipid composition and phospholipid asymmetry of ram sperm plasma membranes after cryopreservation. *Cryobiology,* 26, 70-75, 1989.

2. Nur Z, Zik B, Ustuner B, Sagirkaya H, Ozguden CG: Effects of different cryoprotective agents on ram sperm morphology and DNA integrity. *Theriogenology,* 73, 1267-1275, 2010.

3. O'Connell M, Mcclure M, Lewis SEM: The effects of cryopreservation on sperm morphology, motility and mitochondrial function. *Hum Reprod,* 17, 704-779, 2002.

4. Quinn PJ, Chow P, White I: Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. *Reprod Fertil,* 60, 403-407, 1980.

5. Forouzanfar M, Sharafi M, Hosseini SM, Ostadhosseini S, Hajian M, Hosseini L, Abedi P, Nili N, Rahmani HR, Nasr-Esfahani MH: *In vitro* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. *Theriogenology,* 73, 480-487, 2010.

6. Polge C, Smith AU, Parkes AS: Revival of spermatozoa after vitrification and dehydration at low temperature. *Nature,* 164, 666-668, 1949.

7. Aires VA, Hinsch KD, Mueller-Schloesser F, Bogner K, Mueller-Schloesser S, Hinsch E: *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology,* 60, 269-279, 2003.

8. Viswanath R, Shannon P: Storage of bovine semen in liquid and frozen state. *Anim Reprod Sci,* 62, 23-53, 2000.

9. Amirat L, Tainturier D, Jeanneau L, Thorin C, Gerard O, Courtens JL, Anton M: Bull semen *in vitro* fertility after cryopreservation using egg yolk LDL: A comparison with Optidyl®, a commercial egg yolk extender. *Theriogenology,* 61, 895-907, 2004.

10. Moussa M, Matinet V, Trimeche A, Tainturier D, Anton M: Low density lipoproteins extracted from hen egg yolk by an easy method: Cryoprotective effect on frozen-thawed bull semen. *Theriogenology,* 57, 1695-1706, 2002.

11. Bousseau S, Brillard JP, Guienne BML, Guèrin B, Camus A, Lechat M: Comparison of bacteriological qualities of various egg yolk sources and the *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology,* 50, 699-706, 1998.

12. Edwards RG: Antigenicity of rabbit semen, bull semen and egg yolk after intravaginal or intra-muscular injections into female rabbits. *J Reprod Fertil,* 1, 385-401, 1960.

13. Voet DJ, Voet JG: Biochemistry. John Wiley&Sons, New York, 1995.

14. Hafez ESE: Reproduction in Farm Animals. 6th ed., 330-343, 405-424, Lea&Febiger, Philadelphia, 1993.

15. Soylu MK, Nur Z, Ustuner B, Dogan I, Sagirkaya H, Gunay U, Ak K: Effects of various cryoprotective agents and extender osmolality on post-thaw ram semen. *Bull Vet Inst Pulawy,* 51, 241-246, 2007.

16. Gómez MC, Catt JW, Evans G, Maxwell WMC: Cleavage, development and competence of sheep embryos fertilized by intracytoplasmic sperm injection and in vitro fertilization. *Theriogenology,* 49, 1143-1154, 1998.

17. Fukui Y, Kohno H, Togari T, Hiwasa M, Okabe K: Fertility after artificial insemination using a soybean-based extender in sheep. *J Reprod Dev,* 54, 286-289, 2008.

18. Zhang SS, Hu JH, Li QW, Jiang ZL, Zhang XY: The cryoprotective effects of soybean lecithin on boar spermatozoa quality. *Afr J Biotechnol,* 8, 6476-6480, 2009.

19. De Leeuw FE, De Leeuwa M, Den Daas JHG, Colenbrander B, Verkleij AJ: Effects of various cryoprotective agents and membrane stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology,* 30, 32-44, 1993.

20. Van Wagtendonk-De Leeuw AM, Haring RM, Kaal-Lansbergen LMTE, Den Daas JHG: Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. *Theriogenology,* 54, 57-67, 2000.

21. Papa FO, Felício GB, Melo-Oña CM, Alvarenga MA, Vita BD, Trinque C, Puoli-Filho JNP, Dell'aqua Jr JA: Replacing egg yolk with soybean lecithin in the cryopreservatoin of stallion semen. *Anim Reprod Sci,* 129, 73-77, 2011.

22. Ricker JV, Linfor JJ, Delfino WJ, Kysar P, Scholtz EL, Tablin F, Crowe JH, Ball BA, Meyers SA: Equine sperm membrane phase behavior: The effects of lipid-based cryoprotectants. *Biol Reprod,* 74, 359-365, 2006.

23. Zahn FS, Papa FO, Dell'Aqua JA: Cholesterol incorporation on equine sperm membrane: effects on post-thaw parameters and fertility. *Theriogenology,* 58, 237-240, 2002.

24. Akhter S, Ansari MS, Andrabi SMH, Rakha BA, Ullah N, Khalid M: Soya-lecithin in extender improves the freezability and fertility of buffalo *(Bubalus bubalis)* bull spermatozoa. *Reprod Dom Anim,* 47 (5): 815-819, 2012.

25. Thun R, Hurtado M, Janett F: Comparison of Biociphos-Plus® and Tris-egg yolk extender for cryopreservation of bull semen. *Theriogenology,* 57, 1087-1094, 2002.

26. Pérez LJ, Valcárcel A, De Las Heras MA, Moses D, Baldassarre H: Evidence that frozen/thawed ram spermatozoa show accelerated capacitation *in vitro* as assessed by chlortetracycline assay. *Theriogenology,* 46, 131-140, 1996.

27. Birler S, Pabuccuoglu S, Alkan S, Evecen M, Ileri IK: Effects of serum and hormone additives in maturation media and co-culture with sheep oviductal epithelial cells (SOEC) on *in vitro* fertilization of sheep oocytes. *Society for the Study of Fertility. Annual Conference, 4-7 July,* Aberystwyth-England, 1999.

28. Birler S, Pabuccuoglu S, Alkan S, Ozdas OB, Atalla H, Ileri IK: Development of in vitro derived sheep embryos to the blastocyst stage. *Turk J Vet Anim Sci,* 26, 891-894, 2002.

29. Squires EJ: Applied Animal Endocrinology. CABI Publishing, 234, London 2003.

Kafkas Univ Vet Fak Derg
20 (3): 399-404, 2014
DOI: 10.9775/kvfd.2013.10266

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

RESEARCH ARTICLE

Feeding Raw Garlic to Dairy Goats: Effects on Blood Metabolites and Lactation Performance [1]

Zahra ZAKERI [1] Rasoul PIRMOHAMMADI [2] Ehsan ANASSORI [3] Mortaza TAHMOUZI [1]

[1] *This study was financially supported from the Faculty of Agricultural Science (Urmia University - Iran)*

[1] Graduated from Faculty of Agriculture, Urmia University, Urmia - IRAN
[2] Department of Animal Science, Faculty of Agriculture, Urmia University, Urmia - IRAN
[3] Department of Clinical Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia - IRAN

Makale Kodu (Article Code): KVFD-2013-10266

Summary

The study were conducted to evaluating the effects of raw garlic (GAR; 0, 30, 50 and 70 g/kg of DM) on metabolic status and milk production of dairy goats. Eight dairy goats (59±1 kg initial live weight) were randomly assigned according to a repeated 4×4 Latin square design over an 8-week period. Each experimental period lasted 14 d with the first 12 days used for diet adaptation and 2 days for data collection. Results of this study showed that there were no differences in feed intake or body weight changes during the experimental period due to garlic supplementation. Also feeding garlic increased serum glucose concentration (P<0.05), however no effects of garlic supplementation on blood non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), total triglycerides, total protein, and albumin concentration were observed. In addition production of milk, milk protein, and lactose and fat corrected milk showed no treatment effects. However, milk fat content was decreased significantly (P<0.05) by garlic supplementation. The results of this study indicate that potency of garlic to increase serum glucose concentration can play a role in improvement the energy status of dairy goats.

Keywords: *Blood, Dairy, Garlic, Goat, Milk*

Sütçü Keçilerin Çiğ Sarımsak ile Beslenmesi: Kan Metabolitleri ve Laktasyon Performansına Etkileri

Özet

Bu çalışma çiğ sarımsağın (GAR; 0, 30, 50 ve 70 g/kg DM) sütçü keçilerde metabolizmaya ve süt üretimine etkisini araştırmak amacıyla yapılmıştır. Sekiz sütçü keçi (başlangıç ağırlıkları 59±1 kg) 8 haftalık tekrarlanan 4×4 Latin kare dizayna uygun olarak rastgele dağıtılmışlardır. Her bir deneysel periyot ilk 12 günü diyet adaptasyonu ve 2 günü veri toplama olmak üzere toplam 14 gün sürdü. Çalışma sonuçları sarımsak ile beslemenin yem tüketimi ve vücut ağılık artışı açısından fark oluşturmadığını gösterdi. Sarımsak ile besleme serum glikoz konsantrasyonunu artırırken kan esterlenmemiş yağ asitleri (NEFA), β-hidroksibütrat (BHB), total trigliserid, total protein ve albümin konsantrasyonlarında bir değişime neden olmadı. Sarımsak ile besleme uygulamasının ayrıca süt ve süt proteini üretimi ile laktoz ve yağ düzeltilmiş süte bir etkisinin olmadığı gözlemlendi. Ancak sarımsak uygulaması süt yağı miktarını önemli oranda düşürdü (P<0.05). Bu çalışmanın sonuçları serum glikoz konsantrasyonunu artırmak suretiyle sütçü keçilerde enerji düzeyini geliştirmek amacıyla sarımsağın kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Kan, Sütçü, Sarımsak, Keçi, Süt*

INTRODUCTION

During the transition between late pregnancy and early lacta tion, dairy goats, like dairy cows, are under metabolic stress [1]. Failure to counteract this stress compromises postpartum health and milk production. In recent years, aromatic plants and their extracts have received increased attention as potential alternatives to growth promoters. In this regard, effects of garlic and its bioactive components have been partly demonstrated on rumen manipulation

İletişim (Correspondence)
+90 441 3466761
r.pirmohammadi@urmia.ac.ir

(*e.g.* defaunation, decreased methane production, decreased ruminal degradation of dietary proteins, reducing the proportion of acetate and increasing that of propionate) and consequently on animal production and performances [2-5]. The hypothesis addressed in this paper was that dietary addition of raw garlic would lead to efficacy of food utilization and improving in energy related blood metabolites, which would be reflected by increases in milk production and changes in milk composition.

MATERIAL and METHODS

Experimental Design, Animal Management and Diets

The study was performed at the experimental dairy goat farm of the University of Urmia, Iran. Eight pregnant Mahabadi (a native breed) goats with age of 4 years and body weight of 59±1 kg were selected as far as possible, for kidding date, parity and were randomly assigned according to a repeated 4×4 Latin square design with 12 days of adaptation and 2 days of sampling periods. At day 120±1 of pregnancy, the goats were fed daily a diet designed to cover 2.16 Mcal of metabolizable energy and 11.5% of crude protein (CP) per kg of DM [6]. The animals were divided into four groups and acclimatized to individual pens (5 m^2) for at least 4 weeks before the start of the experiment. Does were given their respective diets throughout gestation and were switched to a standard lactation diet (15.5% CP and 2.35 Mcal of metabolizable energy per kg of DM), offered to the animals immediately after kidding. The composition of the lactation diets is shown in *Table 1*.

All animals were fed with alfalfa hay and corn silage without or with garlic (0, 30, 50 and 70 g per Kg of DM, respectively) on top of concentrate. Concentrate was offered in equal amounts twice daily (08:00 and 18:00 h) while alfalfa hay and corn silage was offered *ad libitum* as a roughage source. Meat grinder used for fine grinding the fresh garlic bulb. The homogenized raw garlic mixed thoroughly with concentrate just before feeding. The animals from the 30, 50 and 70 gram garlic treatments consumed, on average, 0.1, 0.15 and 0.2 g allicin respectively (assuming an average allicin content of 4.5 mg/g [7]. Diets were adjusted weekly to account for changes in BW and physiological status. Raw garlic bulb were from the Hamadan County and purchased at the peak of their maturity at the local market. This study was approved by the Institutional Animal Care and Use Committee of the University of Urmia, Iran.

Feed Intake and BW

Feed consumption was recorded daily by weighing feeds offered to and refused by the goats and data from day 10 to 14 were included in the statistical analysis. Body weight was determined at the beginning and the end of each experimental period after the a.m. milking on 2 consecutive sampling days.

Milk Production and Milk Composition

Goats were milked twice daily (07:00 and 17:00 h) and milk yield was recorded at each milking. During the last two days of each 14-d period, milk samples were taken from each animal at each milking, pooled on a yield basis, stored at +4°C with a preservative (bronopol-B2) and measured for fat, protein, lactose, and total solids within 48 h.

Sample Collection and Analytical Procedure

Analytical DM contents of basal diet and garlic were determined by oven-drying at 105°C for 48 h [8]. Ash contents of basal diet and garlic were determined by incineration at 550°C overnight, and the organic matter (OM) content was calculated as the difference between 100 and the percentage of ash [8]. Ether extract (EE), Kjeldahl N, calcium and phosphorous were determined according to AOAC [8]. Kjeldahl N was multiplied by 6.25 to estimate crud protein (CP). The concentrations of neutral detergent fiber (NDF) in basal diet and garlic were determined as described by Van Soest et al.[9] without the use of sodium sulfite. The acid detergent fiber (ADF) content of diet and garlic were determined according to AOAC [8].

Blood samples were collected from the jugular vein on day 13 and 14 of each experimental period at 3 h after morning feedings. Blood samples were left at room temperature until clotting was finished. Tubes were then centrifuged for 20 min at 2500 × g. Serum for determination of glucose, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), cholesterol, albumin, sodium, potassium, calcium and phosphorus were stored at −20°C until analyses. β-Hydroxybutyrate and NEFA were determined by a D-3-hydroxybutyrate kit and a NEFA Kit (Randox Laboratories Ltd, Ardmore, UK), respectively. The concentration of serum glucose, cholesterol, triglyceride, total protein, albumin, calcium and inorganic phosphorus were determined using an auto analyzer spectrophotometer (RA 1000, Unico, model S, serial number 2100, USA) using commercial kits (Parsazmoon, Tehran, Iran). Sodium and potassium concentrations were measured with flame photometery (Model PFP7, Serial number 12377, Genewey factory England).

Statistical Analysis

Data were analyzed using the PROC GLM of SAS [10], according to the following statistical model: $Y_{ijk} = \mu + Ti + Cj + Pk + eijk$, where, Yijk = observed variables; μ = general mean; T_i = effect of treatment i, (where i = 1, 2, 3, 4); C_j = effect of animal j, (where j = 1, 2...8); P_k = effect of period k, (where k = 1, 2, 3, 4); e_{ijk} = experimental error of the observations Y_{ijk}. Differences between treatments were declared significant at $P<0.05$ using the Tukey correction for multiple comparisons.

RESULTS

Chemical Composition of Basal Diet and Garlic

Table 1 and *Table 2* respectively presents the results obtained from analysis of the chemical composition of basal diet and garlic fed to experimental animals. Chemical analysis of garlic showed that crude fat content of garlic was 6.9 g kg^{-1} on a DM basis and 370 g kg^{-1} of DM, which could consist of active aromatic compound as previously reported by Saenthhaweesuk [11].

Table 1. *Daily ingredient allowance and chemical composition of the basal diet offered to dairy goats*
Tablo 1. *Sütçü keçilere verilen bazal diyetin günlük içeriği ve kimyasal kompozisyonu*

Item	Diet
Ingredient (%)	
Lucerne hay	34.70
Corn silage	28.90
Barley	23.20
Soybean meal	11.50
Vitamin and mineral mixture	1.70
Chemical analysis	
DM (%)	89.90
CP (%)	15.53
ME Mcal/kg of DM	2.35
EE (%)	3.88
NDF (%)	37.50
ADF (%)	23.10
Ca (%)	0.99
P (%)	0.48

***DM** = Dry matter, **CP** = Crude protein, **NDF** = Neutral detergent fiber, **ADF** = Acid detergent fiber, **EE** = Ether extract. **ME** = Metabolizable energy*

Table 2. *Chemical composition of the raw garlic*
Tablo 2. *Çiğ sarımsağın kimyasal kompozisyonu*

Composition	Raw Garlic %
DM	37.03
OM	95.74
Crude protein	9.62
Ether extract	0.69
NDF	6.70
ADF	5.21
Calcium	0.03
Phosphorus	0.02

***DM** = Dry matter, **OM** = Organic matter, **CP** = Crude protein, **NDF** = Neutral detergent fiber, **ADF** = Acid detergent fiber, **EE** = Ether extract*

Feed Intake and Body Weight Changes

In the current study, no significant differences observed in the dry matter intake (DMI) and body weight changes between treatments in the lactational period *(Table 3)*.

Milk Production and Milk Composition

Milk production ranged from 1.12 to 1.24 kg/d and was not different among the treatments *(Table 3)*. Overall, there were no treatment differences (P>0.05) in milk and fat corrected milk yield, milk protein, lactose and total solids. Even though feeding garlic showed the lower-milk fat percentage compared to animal not received the garlic in the diet *(Table 3)*.

Blood Metabolites

Blood metabolite concentrations are shown in *Table 4*. Administering garlic (in the two levels of 50 and 70 g/Kg DM) in dairy goats increased significantly (P<0.05) serum glucose concentration. No significant effects were found for BHB, NEFA, triglycerides, cholesterol, total protein, albumin, calcium, inorganic phosphorus, sodium and potassium concentration in lactating dairy goats *(Table 4)*.

DISCUSSION

The effects of feeding garlic or other garlic components have been reported to vary according to the dose and type of products. In contrast with Kholif et al.[5] who reported that supplementing goats with garlic oil increased DMI, garlic supplementation at the selected levels did not affect the feed consumption in this study, these findings were in agreement with previous reports with total mixed ration of lambs [12], sheep [4,13] and cows [14].

All the blood metabolites investigated were within the normal range for dairy goats [15,16]. Similar to those reported by Kholif et al.[5] who found an increased serum glucose in response to rumen propionate increment in garlic treated lactating Goats, in the current study serum glucose was improved significantly by garlic supplementation. Blood glucose levels could be considered as a reflection of gluconeogenesis. Bergman [17] estimated that 27% to 55% of the glucose metabolized by ruminants originates from propionate (as an energy precursor). Previous studies documented the lower acetate, the greater propionate and butyrate proportions and resulting lower acetate to propionate ratio in garlic supplemented diet suggested that garlic constituents might help to improve the efficiency of energy utilization in the rumen [3,4,18]. This may explain to some extent how garlic can improve serum glucose concentration. These results are contrast to the finding of Chaves el al.[12] who reported no difference in serum glucose concentration of growing lambs fed diets supplemented with garlic compared with control.

Table 3. *Body weight, feed intake, milk production and composition in dairy goats fed raw garlic*
Tablo 3. *Çiğ sarımsak ile beslenen sütçü keçilerin vücut ağırlığı, yem tüketimi, süt üretimi ve kompozisyonu*

Parameters	Treatments				SE	*P*- Value
	GAR0	GAR30	GAR50	GAR70		
Body weight (kg)	51.8	51.52	50.74	49.24	0.57	NS
Feed intake (kg DM/day)	1.63	1.65	1.76	1.78	0.04	NS
Milk production (kg/day)	1.14	1.24	1.22	1.12	0.05	NS
Fat Corrected Milk	1.26	1.31	1.22	1.28	0.09	NS
Milk composition (%)						
Fat	4.24^{a}	3.8^{b}	3.74^{b}	3.6^{b}	0.15	*
Protein	3.21	3.33	3.26	3.25	0.09	NS
Lactose	4.06	4.16	4.2	4.37	0.10	NS
Total solids	13.53	13.22	13.42	13.25	0.20	NS

GAR0 *= basal diet without garlic;* ***GAR30*** *= basal diet + raw garlic (30 g/kg DM);* ***GAR50*** *= basal diet + raw garlic (50 g/kg DM);* ***GAR70*** *= basal diet + raw garlic (70 g/kg DM);* ^{a,b} *Different letters indicate significant differences: P<0.05;* ***** *P<0.05;* ***NS*** *= Not significant*

Table 4. *Blood serum constituents of lactating goats fed on rations supplemented with raw garlic*
Tablo 4. *Çiğ sarımsak katılmış rasyon ile beslenen laktasyondaki keçilerin kan serum içerikleri*

Variables	Treatments				SE	*P*-value
	GAR0	GAR30	GAR50	GAR70		
Serum glucose (mmol/l)	3.10^{c}	3.11^{c}	3.60^{ab}	3.64^{a}	0.10	**
BHB (mmol/l)	0.59	0.49	0.55	0.61	0.12	NS
NEFA (mmol/l)	0.26	0.27	0.27	0.24	0.14	NS
Total triglycerides (mg/dl)	18.50	14.25	17.12	15.50	5.10	NS
Cholesterol (mg/dl)	59.37	56.25	57.37	56.87	1.58	NS
T. Protein (g/dl)	7.75	7.42	7.30	7.53	0.96	NS
Albumin (g/dl)	4.37	3.80	4.38	4.25	0.38	NS
Calcium (mg/dl)	9.11	8.40	8.24	7.80	1.44	NS
Phosphorous (mg/dl)	10.19	11.92	11.45	10.54	1.58	NS
Sodium (mEq/l)	94.87	97.87	95.12	97.62	9.05	NS
Potassium (mEq/l)	2.48	2.68	2.67	2.63	0.35	NS

GAR0 *= basal diet without garlic;* ***GAR30*** *= basal diet + raw garlic (30 g/kg DM);* ***GAR50*** *= basal diet + raw garlic (50 g/kg DM);* ***GAR70*** *= basal diet + raw garlic (70 g/kg DM);* ***BHB*** *= β-Hydroxy Butyrate,* ***NEFA*** *= Non esterified fatty acids;* ****** *P<0.01;* ***NS*** *= Not significant*

Negative energy balance (NEB) in dairy goats can be monitored by testing for BHBA or NEFA. In contrast to reports obtained in dairy goat [19], cows [20] and ewes [21], the blood concentration of BHB and NEFA did not change over entire experiments. Blood concentration of NEFA is considered an appropriate index of energy status in goats and concentrations of 0.20-0.21 mmol/l have been suggested for lactating does at zero energy balance [22]. When energy balance is negative, animals always mobilize the lipids stored in adipose tissues, mainly in the form of NEFA. In contrast to Zhu et al.[23] who observed lower concentration of NEFA in garlic oil supplemented dairy goats, in the present study, the concentration of NEFA did not change during the experimental period suggesting that the mobilization was not affected. Also BHB is one of the important energy status indicators during the peri-parturient period. Blood concentrations of BHB of 0.8 to 1.6 mmol/L are indicative of a NEB in ewes [24]. There are no data regarding the cutoff point for NEB in Mahabadi goats. As expected and similar to cows, BHB concentrations were higher postpartum than prepartum because of the high energy demands associated with the onset of lactation [25] and mobilization of adipose tissue resulting in variations of plasma glucose and BHB could occur when an increase of milk production is not supported by a proper energy intake. However in the current study change in plasma BHB concentrations did not differ significantly between groups. It has been proved that when animals are in negative energy balance (early lactation), the additional energy available due to the essential oil from medicinal supplementation was used to improve performance and reduce body reserve losses [26]. In this study, higher level

of serum glucose of treated goats without any changes in other plasma energy indicators (BHBA and NEFA) suggesting enough energy providing to support the milk production.

Most findings demonstrated lipid-lowering as well as hypochlosterolemic effects of garlic in human and animal [27-29]. However, the present study showed that garlic supplementation had no influences on the concentrations of total triglycerides and cholesterol in serum. These results were in agreement with the previous reports in sheep [12,21] and in dairy goats [23] supplemented with garlic oil. The discrepancies in the results of the studies can be attributed to differences in the experimental trials, composition and quantity of garlic supplemented to animal's diet.

Blood levels of minerals have a high diagnostic value in determining the nutritional status of animals due to their low variability in blood [30]. In the current study profiles of calcium, phosphorous, sodium and potassium concentrations in serum did not exhibit any changes. There is no information on effects of feeding garlic or its constituents on blood mineral concentration through the lactational period in dairy goats. In agreement with Amer et al.[31] who observed any changes in serum calcium and phosphorous levels during postpartum period in Saudi Ardy goats we also did not observe any significant effect of garlic supplementation on blood concentrations of calcium, inorganic phosphorus, sodium and potassium.

To our knowledge, little information is available on the effects of garlic supplementation on milk components. According to Kholif et al.[5] the present study showed that garlic supplementation did not affect milk components with the exception of decreasing milk fat content. This finding is in contrast with Yang et al.[14] who reported that garlic oil supplementation in dairy cattle tended to increase of fat milk content. Also Zhu et al.[23] observed that supplemented garlic oil in dairy goats' diets had no effect on milk concentrations of fat, protein and milk yields of fat and protein when compared with goats fed no garlic oil.

Based on the results of this investigation, garlic supplementation had no adverse effects upon efficacy of feed intake and milk production as well as on blood constituents known to be critical for the transition goats. Furthermore blood glucose indicated significantly, a positive energy balance for treatment goats. However, further research is warranted to investigate the underlying mechanism for better application of garlic and garlic component as a feed additives and to evaluate involvement of metabolic changes on the long-term health and productivity of dairy goats.

Acknowledgments

Financial support from the Faculty of Agricultural Science (Urmia University-Iran) is fully appreciated.

REFERENCES

1. Azab ME, Hussein A, Maksoud A: Changes in some haematological and biochemical parameters during prepartum and postpartum periods in female baladi goats. *Small Rumin Res*, 34, 77-85, 1999.

2. Cardozo PW, Calsamiglia S, Ferret A, Kamel C: Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. *J Anim Sci,* 82, 3230-3236, 2004.

3. Busquet M, Calsamiglia S, Ferret A, Carro MD, Kamel C: Effect of garlic oil and four of its compounds on rumen microbial fermentation. *J Dairy Sci*, 88, 4393-4404, 2005.

4. Anassori E, Dalir-Naghadeh B, Pirmohammadi R, Taghizadeh A, Asri-Rezaei S, Maham M, Farahmand-Azar S, Farhoomand P: Garlic: A potential alternative for monensin as a rumen modifier. *Livest Sci*, 142, 276-287, 2011.

5. Kholif SM, Morsy TA, Abdo MM, Matloup OH, Abu El-Ella AA: Effect of supplementing jactating goats rations with garlic, cinnamon or ginger oils on milk yield, milk composition and milk fatty ccids profile. *J Life Sci*, 4 (1): 27-34, 2012.

6. NRC: Nutrient requirements of small ruminants: Sheep goats cervids and New World camelids. National Research Council of the National Academies National Academies Press Washington, DC, 2007.

7. Sterling SJ, Eagling DR: Agronomics and allicin yield of Australian garlic *(Allium sativum). Int Symp Edible Alliaceae,* 555, 63-73, 1997.

8. AOAC: Association of Official Analytical Chemists Official methods of analysis. 15th ed., Arlington, VA, 1990.

9. Van Soest PJ, Robertson JB, Lewis BA: Methods for dietary fiber neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*, 74, 3583-3597, 1991.

10. SAS: User's Guide: Statistics, Version 8.01. SAS Inst., Inc., Cary, NC, USA, 2000.

11. Saenthaweesuk S: Effect of substituting garlic herbal powder for antibiotic growth promoters in ration on growth performance, carcass characteristics, meat quality, blood chemistry, plasma lipids and antioxidising responses of broilers. *PhD Thesis*, Graduate School, Khon Kaen Univ., Thailand, 2006.

12. Chaves A, Stanford K, Dugan M, Gibson L, McAllister T, Vanherk F, Benchaar C: Effects of cinnamaldehyde garlic and juniper berry essential oils on rumen fermentation blood metabolites growth performance and carcass characteristics of growing lambs. *Livest Sci,* 117, 215-224, 2008.

13. Horton GMJ, Blethen DB, Prasad BM: The effect of garlic *(Allium sativum)* on feed palatability of horses and feed consumption selected performance and blood parameters in sheep and swine. *Can J Anim Sci,* 71, 607-610, 1991.

14. Yang WZ, Benchaar C, Ametaj BN, Chaves AV, He ML, McAllister TA: Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. *J Dairy Sci*, 90, 5671-5681, 2007.

15. Bava L, Rapetti L, Provetto GM, Tamburini A, Sandrucci A, Galassi G, Succi G: Effects of nonforage diet on milk production, energy, and nitrogen metabolism in dairy goats throughout lactation. *J Dairy Sci*, 84, 2450-2459, 2001.

16. Stella AV, Paratte R, Valnegri L, Cigalino G, Soncini G, Chevaux E, Dell Orto V, Savoini G: Effect of administration of live *Saccharomyces cerevisiae* on milk production, milk composition, blood metabolites, and faecal flora in early lactating dairy goats. *Small Rumin Res*, 67, 7-13, 2007.

17. Bergman EN: Glucose metabolism in ruminants as related to hypoglycemia and ketosis. *Am J Physiol*, 215, 865-873, 1973.

18. Kongmun P, Wanapat M, Pakdee P, Navanukraw C: Effect of coconut oil and garlic powder on *in vitro* fermentation using gas production technique. *Livest Sci*, 127, 38-44, 2010.

19. Sadjadian R, Seifi HA, Mohri M, Naserian AA, Farzaneh N: Variations of energy biochemical metabolites in periparturient dairy Saanen goats. *Comp Clin Pathol*, 22 (3): 449-456, 2013.

20. Seifi HA, Gorji-Dooz M, Mohri M, Dalirnaghadeh B, Farzaneh N:

Variations of energy-related biochemical metabolites during transition period in dairy cows. *Comp Clin Pathol*, 16, 253-258, 2007.

21. Taghipour B, Seifi HA, Mohri M, Farzaneh N, Naserian AA: Variations of energy related biochemical metabolites during periparturition period in fat-tailed baloochi breed sheep. *Iran J Vet Sci Tech*, 2, 85-92, 2011.

22. Dunshea FR, Bell AW: Non-esterified fatty acid re-esterification and fat mobilization in goats during early lactation. *J Dairy Sci,* 70 (Suppl.), 10, 1987.

23. Zhu Z, Hang S, Zhu H, Zhong S, Mao S, Zhu W: Effects of garlic oil on milk fatty acid profile and lipogenesis-related gene expression in mammary gland of dairy goats. *J Sci Food Agric*, 93 (3): 560-567, 2013.

24. Navarre, CB, Pugh DG: Diseases of the gastrointestinal system. **In,** Pugh DG (Ed): Sheep and Goat Medicine. 97-99, Philadelphia, Saunders, 2002.

25. Herdt TH, Gerloff BJ: Ketosis. **In,** Anderson DE, Rings DM (Eds): Current Veterinary Therapy Food Animal Practice. 2nd ed., 141-144, Saunders, St Louis, 2009.

26. Tedeschi LO, Fox DG, Tylutki TP: Potential environmental benefits of ionophores in ruminant diets. *J Environ Qual*, 32, 1591-1602, 2003.

27. Chowdhury SR, Chowdhury SD, Smith TK: Effects of dietary garlic on cholesterol metabolism in laying hens. *Poult Sci*, 81, 1856-1862, 2002.

28. Mahmoodi M, Islami MR, Asadi Karam GR, Khaksari M, Sahebghadam Lotfi A, Hajizadeh MR, Mirzaee MR: Study of the effects of raw garlic consumption on the level of lipids and other blood biochemical factors in hyperlipidemic individuals. *Pakistan J Pharm Sci*, 19, 295-298, 2006.

29. Habibian Dehkordi S, Zamani Moghadam A, Maghsoudi N, Aali E, Gerami R, Dehsadeghi E: The effects of fresh garlic on the serum concentration of total cholesterol, total triglyceride and adipose tissues of broilers. *Comp Clin Pathol*, 19, 363-365, 2010.

30. Mahusoon MM, Perera ANF, Perera ERK, Perera KA: Effects of molybdenum supplementation on circulating mineral levels nematode infection and body weight gain in goats as related to season. *Peradeniya Trop Agric Res*, 16, 128-136, 2004.

31. Amer HA, Salem HAH, Al-Hozab AA: Biochemical changes in serum and milk constituents during postpartum period in Saudi Ardy goats. *Small Rumin Res*, 34, 167-173, 1999.

32. Yan L, Meng QW, Ao X, Zhou TX, Yoo JS, Kim HJ, Kim IH: Effects of fermented garlic powder supplementation on growth performance, blood characteristics and meat quality in finishing pigs fed low-nutrient-density diets. *Livest Sci*, 137 (1-3): 255-259, 2011.

33. Hurtaud C, Lemosquet S, Rulquin H: Effect of graded duodenal infusions of glucose on yield and composition of milk from dairy cows Part 2 Diets based on grass silage. *J Dairy Sci*, 83, 2952-2962, 2000.

34. Janicek BN, Kononoff PJ, Gehman AM, Karges K, Gibson ML: Effect of increasing levels of corn bran on milk yield and composition. *J Dairy Sci*, 90, 4313-4316, 2007.

35. Cant JP, Trout DR, Qiao F, Purdie NG: Milk synthetic response of the ovine mammary gland to an increase in the local concentration of arterial glucose. *J Dairy Sci*, 85, 494-503, 2002.

36. Hurtaud C, Rulquin H, Verite R: Effect of infused volatile fatty acids and caseinate on milk composition and coagulation in dairy cows. *J Dairy Sci*, 76, 3011-3020, 1993.

37. Harvatine KJ, Allen MS: The effect of production level on feed intake, milk yield, and endocrine responses to two fatty acid supplements in lactating cows. *J Dairy Sci*, 88, 4018-4027, 2005.

Kafkas Univ Vet Fak Derg
20 (3): 405-410, 2014
DOI: 10.9775/kvfd.2013.10282

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

RESEARCH ARTICLE

Using Phosphorylated Mannan Oligosaccharide and Fibrolytic Enzyme as Natural Feed Additive Substitutes for Growth-Enhancing Technologies in Sustainable Beef Production [1]

Songül ŞENTÜRKLÜ [1] Douglas LANDBLOM [2] Cheryl WACHENHEIM [3] Timothy PETRY [4]

[1] *Infrastructure for this study was provided by the North Dakota State University Agricultural Experiment Station, Fargo, North Dakota, USA 58108 and the North Dakota State University Dickinson Research Extension Center, Dickinson, North Dakota, USA 58601, under project number ND06253 and a USDA/CSREES Cool Season Food Legume Special Program Funding administered by the University of Idaho, USA, project number BAK463*

[1] Department of Animal Science, Canakkale Onsekiz Mart Universitesi, BMYO, TR-17200 Canakkale - TURKEY
[2] North Dakota State University - Dickinson Research Extension Center, Dickinson, North Dakota, 58601, USA
[3] North Dakota State University - Agribusiness and Applied Economics Department, Fargo, North Dakota, 58105, USA
[4] North Dakota State University - Extension Livestock Specialist, Fargo, North Dakota, 58108, USA

Makale Kodu (Article Code): KVFD-2013-10282

Summary

The study objective was to determine the effect on beef production sustainability when growth-enhancing technologies (GET) were substituted with the natural feed additives (NFA) phosphorylated mannan oligosaccharide (Bio-MOS®-(MOS)) and fibrolytic enzyme (Fibrozyme®-(FIB)). Angus x Hereford x Gelbvieh steers, after weaning (n=80; BW=279.6±3 kg), were used in an 84-day backgrounding study (4 treatments; 4 pen replicates/treatment) that was followed by a 122-day finishing study. A control (C) treatment with GET (Revelor-IS® and Rumensin®) was compared to NFA (10 mg/head/day): MOS, FIB, and MOS+FIB. Data were analyzed using mixed procedure of SAS. The backgrounding C steers end weight, weight gain, and average daily gain (ADG) were greater (P<0.01) compared to MOS, FIB, and MOS+FIB. Feed efficiency ratio did not differ (P=0.198). Feed cost/kg of gain was lower for the C treatment (P<0.01). The C treatment net return was 45.9% greater than the average of MOS, FIB, and MOS+FIB treatments. For finishing, the C treatment ADG was greater (P<0.05) compared to MOS, FIB, and MOS+FIB. In addition, the C treatment harvest weight and hot carcass weight were greater (P<0.01) and were harvested 5 days earlier. However, other carcass measurements did not differ (P>0.10). Ending net return was $54.22, -$33.62, -$20.65, and -48.69 for the C, MOS, FIB, and MOS+FIB, respectively. The NFA were less profitable during backgrounding, but not profitable for finishing.

Keywords: *Fibrolytic enzyme, Monensin sodium, Phosphorylated mannan oligosaccharide, Steroid implant, Sustainable beef production*

Sürdürülebilir Sığır Eti Üretiminde Büyümeyi Artırıcı Teknolojilere İkame Olarak Doğal Yem Katkı Maddesi Fosforile Mannan Oligosakkarit ve Fibrolitik Enzim Kullanımı

Özet

Bu çalışmanın amacı sürdürülebilir sığır eti üretiminde büyümeyi arttırıcı teknolojilere (GET) ikame olarak doğal yem katkı maddeleri (NFA) phosphorylated mannan oligosaccharide (Bio-MOS®-(MOS)) ve fibrolitik enzimin (Fibrozyme®-(FIB)) etkisini belirlemektir. 84 günlük büyütme ve bunu izleyen 122 günlük bitirme çalışmasında sütten kesilmiş Angus x Hereford x Gelbvieh melez kastre edilmiş tosunları (n=80; BW=279.6±3 kg) kullanılmıştır (4 grup; 4 tekrar/grup). GET içerikli (Revelor-IS® ve Rumensin®) kontrol (C) grubu NFA içerikli (10 mg/head/day): MOS, FIB ve MOS+FIB gruplar ile karşılaştırılmıştır. Veriler SAS istatistik programı kullanılarak analiz edilmiştir. Büyüme döneminde; C grubu tosunlarda son ağırlık, ağırlık artışı ve ortalama günlük artış (ADG) diğer MOS, FIB ve MOS+FIB gruplarına göre daha yüksektir (P<0.01). Yemden yararlanma oranı bakımından gruplar arasında farklılık bulunmamıştır (P=0.198). Birim ağırlık artışı için yem maliyeti C grubunda daha düşüktür (P<0.01). Büyüme dönemi net kazancı C grubunda diger MOS, FIB ve MOS+FIB grup ortalamalarından %45.9 daha yüksektir. Bitirme döneminde; C grubu ADG diğer MOS, FIB ve MOS+ FIB gruplardan daha yüksektir (P<0.05). Ayrıca, C grubu kesim ağırlığı ve sıcak karkas ağırlığıda diğer gruplardan daha yüksek bulunmuştur (P<0.01) ve C grubu tosunları 5 gün önce kesilmiştir. Ancak, diğer karkas parametrelerinde farklılık görülmemiştir (P>0.10). Araştırma sonunda C, MOS, FIB ve MOS+FIB gruplarında net kazanç sırasıyla 54.22$, -33.62$, -20.65$ ve -48.69$ olarak tespit edilmiştir. NFA büyüme döneminde az da olsa kârlı iken bitirme döneminde zarar etmiştir.

Anahtar sözcükler: *Fibrolitik enzim, Monensin sodyum, Fosforile mannan oligosakkarit, Steroid implant, Sürdürülebilir sığır eti üretimi*

İletişim (Correspondence)
+90 286 3162878
ssenturklu@comu.edu.tr; songuls2011@hotmail.com

INTRODUCTION

The USA cattle industry has experienced significant growth in "natural beef" as cattle producers respond to increasing consumer concerns over the use of growth promoting hormones, ionophores, and antibiotics in their meat; however, growth-promoting technologies improve animal performance and reduce environmental impact [1]. These compounds have the potential to be replaced with phosphorylated mannan oligosaccharide and fibrolytic enzymes that in separate research investigations have been shown to reduce stress, enhance immune response, inhibit intestinal binding, improve fiber digestion, increase feed intake, gain, and feed efficiency [2-4]. Enzyme preparations with cellulase and xylanase activity have been shown to improve fiber and dry matter digestion and growth performance in cattle [5-8].

The research objective of this field study was to determine beef production sustainability when using (NFA) mannan oligosaccharide and a fibrolytic enzyme as replacements for growth-enhancing technology (GET), during the backgrounding period, and to document the subsequent carryover effect on finishing feedlot performance, carcass traits, and economics.

MATERIAL and METHODS

This research was conducted in accordance with guidelines approved by the North Dakota State University Institutional Animal Care and Use Committee (Approval number A0610)

March-April born crossbred steers (Angus x Hereford x Gelbvieh; n=80; w=279.6 kg, age=7.4 months) were weaned the first week of November and fed in an 84-day backgrounding period study using a complete randomized design consisting of four treatments and four pen replicates with five steers per replicate (n=20 per treatment). The investigation was conducted using sixteen 9.75 m x 34.1 m pens at the Dickinson Research Extension Center feedlot located southwest of Manning, North Dakota, USA. Feedlot pens were affixed with steel fencing, anti-siphoning frost-free water fountains, slotted windbreak, with a three-row tree windbreak oriented northwest of the study area. The experimental treatments were:

1. Growth-Enhancing Technology (GET), Control (C) - (Revelor-IS® implant: Trenbolone acetate (80 mg) + estradiol benzoate (16 mg) + monensin sodium (30 g/ton) - (Rumensin®))

2. Natural Feed Additive (NFA), Fibrolytic Enzyme (FIB) (Fibrozyme®10 g/head/day)

3. Natural Feed Additive (NFA), Mannan Oligosaccharide (MOS) (Bio-MOS®10 g/head/day)

4. Natural Feed Additive (NFA), Bio-MOS® + Fibrozyme® (MOS+FIB) (10 g+10 g/head/day)

The experimental diets were formulated according to National Research Council specifications for steers estimated to gain 1.4-1.6 kg/head/day [9]. Two feed supplements were prepared that were top-dressed over medium quality alfalfa-bromegrass hay *(Medicago sativa*, *Bromus inermis:* CP=9.1%; ADF=35.0%; NDF=59.9%; TDN=57.4%; NEm Mcal/kg=1.37; NEg Mcal/kg=0.68). The natural feed additives MOS, FIB and MOS+FIB (Alltech Biotechnology Inc., Nicholasville, KY, USA) were blended with cracked corn, shredded beet pulp, corn oil, and molasses in meal form as a carrier *(Table 1)*, for the first supplement, and were fed at the rate of 454 g/head/day to provide 10 g/head/day of each feed additive. For the second supplement, a fortified protein-energy backgrounding feed was prepared as a pelleted complete feed *(Table 2)*. The C steers were

Table 1. *Feed additive supplement ingredient composition and analysis (DM)*
Tablo 1. *Yem katkısı içerikli karma yemin hammadde kompozisyonu ve analizi (DM)*

İngredient Composition	**C**	**MOS**	**FIB**	**FIB+MOS**
Cracked Corn, %	46.0	44.9	44.9	43.8
Shredded Beef Pulp, %	46.0	44.9	44.9	43.8
Corn Oil, %	3.0	3.0	3.0	3.0
Molasses, %	5.0	5.0	5.0	5.0
Bio-MOS, %	---	2.2	---	2.2
Fibrozyme, %	---	---	2.2	2.2
Analysis				
CP, %	9.34	10.2	9.33	10.2
TDN, %	85.3	85.1	85.4	85.1
Fat, %	5.46	5.42	5.5	5.5
Acid Detergent Fiber, %	14.3	13.9	14.3	13.9
NEm, Mcal/kg	2.15	2.15	2.15	2.15
NEg, Mcal/kg	1.38	1.38	1.38	1.38

implanted once with Revelor-IS® and fed Rumensin® (35.3 mg/kg/supplement) throughout the study. At the end of the 84-day backgrounding period, the steers were moved to the Decatur County Feed Yard, Oberlin, Kansas, USA for a feedlot finishing period and final harvest to determine the subsequent carryover effect of the 84-day backgrounding period treatment on finishing feedlot performance, carcass traits, and overall production economics. During the feedlot finishing period, GET and NFA were not used. Harvest end point for the steers was based on back fat depth and determined using MicroBeef Technologies' Electronic Cattle Management system in use at the Decatur County Feed Yard [10] and were slaughtered at Cargill Meat Solutions, Ft. Morgan, Colorado, and sold on the Angus America grid.

Table 2. *Protein-energy supplement ingredient composition and analysis (DM)*
Tablo 2. *Protein-enerji ilaveli karma yemin hammadde kompozisyonu ve analizi (DM)*

Ingredient Composition	C	MOS, FIB and MOS+FIB
Soybean Hull, %	30.753	30.80
Field Pea, %	20.00	20.00
Corn, %	15.00	15.00
Barley Malt Sprout, %	10.00	10.00
Wheat Middling, %	10.00	10.00
Distillers Dried Grain with Solubles, %	8.00	8.00
Decoquinate (6.0 %), %	0.027	-
Monensin (36.3 gm/kg), %	0.02	-
Other*, %	6.20	6.20
Analysis		
CP, %	15.10	15.1
TDN, %	70.20	70.25
Fat, %	2.65	2.65
Acid Detergent Fiber, %	18.03	18.05
NEm, Mcal/kg	1.73	1.73
NEg, Mcal/kg	1.16	1.16

** Beet Molasses, 5.0%; Calcium Carbonate, 0.50%; Salt, 0.50%; Dicalcium Phosphate 21%, 0.10%; Feedlot Trace Mineral Premix, 0.075%; Feedlot Vitamin Premix, 0.025%*

Backgrounding and finishing period data were analyzed using pen as the experimental unit for both growth and carcass closeout data. The mixed procedure of SAS was used to separate means [11]. In the model, diet served as the fixed effect and block served as a random effect. Differences between the treatments were considered significant at $P<0.05$ and a trend at $P<0.10$.

RESULTS

The 84-day backgrounding period performance, feed efficiency, and partial feeding economics are shown in *Table 3*. The control treatment steers, which were implanted with Revelor-IS® and fed diets containing Rumensin® medication, gained 0.31 kg faster ($P<0.01$) than the average gain of steers fed MOS, FIB, and MOS+FIB. This was an 18.9% improvement in average daily gain, or an average of 32.3 kg more per C treatment steer during the 84-day backgrounding period compared to the average gain of the treatments fed the NFA. However, daily feed intake ($P=0.85$), feed to gain ($P=0.20$), and daily feed cost per steer ($P=0.60$) did not differ. The C treatment steers consumed a numerically smaller amount of feed per kg of gain, but the difference was not significant ($P=0.198$). However, when feed cost/kg of gain was determined, the numerically lower quantity of feed consumed by the C steers contrasted with the significantly greater rate of gain

Table 3. *Backgrounding period performance*
Tablo 3. *Büyüme dönemi performansı*

Animal Performance	C	MOS	FIB	FIB+MOS	SEM	P-Value
Number of Steers	20	20	20	20		
Number of days Fed	84	84	84	84		
Start Backgrounding Wt, kg	284.2	278.8	277.8	278.0	3.16	0.43
End Backgrounding Wt., kg	423.4[a]	390.8[b]	393.1[b]	389.3[b]	4.63	<0.01
Gain, kg	139.1[a]	112.0[b]	115.3[b]	111.3[b]	3.44	<0.01
ADG, kg	1.66[a]	1.33[b]	1.38[b]	1.33[b]	0.041	<0.01
Dry Matter Intake/Head/Day, kg	10.03	9.49	9.41	9.45	0.57	0.85
Protein-Energy Suppl./Head/day, kg	4.74	4.77	4.77	4.77	0.57	0.43
Alfalfa-Brome Hay/Head/Day, kg	4.83	4.27	4.19	4.23	0.57	0.84
Feed Additive Suppl./Head/Day, kg	0.454	0.454	0.454	0.454		
Feed: Gain, kg/kg	6.04	7.14	6.86	7.11	0.371	0.20
Feed Cost/Head/Day, $	1.388	1.377	1.380	1.425	0.0275	0.60
Feed Cost/kg of Gain, $	0.8361[a]	1.035[b]	1.00[b]	1.071[b]	0.0088	<0.01

a-b: *Means with different superscripts within a line are significantly different (P<0.05)*

among the C steers resulted in a significantly lower feed cost/kg of gain among the C steers compared to the steers that were fed MOS, FIB, and MOS+FIB additives. When comparing estimated profitability potential between treatments at the end of the 84-day backgrounding period, all treatments were profitable, but treatments fed the natural feed additives were an average 45.9% less profitable *(Table 6)*. The research assumption was that any profitability realized among treatments at the end of the backgrounding period would carry over into the finishing period.

The subsequent carryover effect on finishing feedlot performance following the 84-day backgrounding period is shown in *Table 4*. The significant weight advantage that the C treatment steers gained during the backgrounding period carried over through the finishing feedlot period, which was not anticipated, because GET were removed for finishing. The C treatment steers continued to gain at a faster rate during the finishing feedlot period reducing the number of days on feed to final harvest by 5 days. There appeared to be a carryover effect from the use of GET in the C treatment during the 84-day backgrounding period, because the C treatment steers gained faster (P<0.05), consumed more feed (P<0.01), and live harvest and hot carcass weights were heavier (P<0.01) than steers previously backgrounded with MOS, FIB, and MOS+FIB *(Table 5)*. Except for hot carcass weight (P<0.01), all of the other carcass measurements did not differ: fat depth (P=0.54), ribeye area (P=0.53), yield grade (P=0.79), quality grade (P=0.21), and percent grading choice or greater (P=0.81). Total carcass value and marketing analysis *(Table 6)* of the treatment comparisons resulted in a profit of $54.22 per head for the C treatment steers compared to net losses of -$33.62, -$20.65, and -$48.69 per carcass for MOS, FIB,

Table 4. *Finishing feedlot period performance*
Tablo 4. *Bitirme Feedlot dönemi performansı*

Animal Performance	C	MOS	FIB	FIB+MOS	SEM	P-Value
Number of Days Fed	116.3	122.2	120.1	121.2		
Start Finish Weight, kg	410.3[a]	381.3[b]	383.6[b]	376.3[b]	18.50	<0.01
Harvest Weight, kg	615.1[a]	576.0[b]	583.7[b]	572.4[b]	15.67	<0.01
Gain, kg	204.8	194.7	200.1	196.1	5.80	0.32
ADG, kg	1.76[a]	1.59[b]	1.67[b]	1.62[b]	0.399	.022
Dry Matter Intake/Head/Day, kg	9.95[a]	9.64[b]	9.60[b]	9.55[b]	0.170	<0.01
Feed:Gain, kg	5.65	6.06	5.75	5.90	0.122	0.23

a-b: *Means with different superscripts within a lines significantly different (P<0.05)*

Table 5. *Carcass measurement*
Tablo 5. *Karkas parametreleri*

Carcass Measurement	C	MOS	FIB	FIB+MOS	SEM	P-Value
Hot Carcass Weight, kg	390.4[a]	361.8[b]	366.8[b]	362.2[b]	9.83	<0.01
Fat Depth, cm	1.32	1.32	1.32	1.27	1.04	0.54
Ribeye Area, cm^2	84.6	80.0	79.9	81.5	2.61	0.53
Yield Grade	2.95	2.80	3.05	2.80	0.2508	0.79
Quality Grade	4.35	3.4	4.8	5.05	0.8091	0.21
Percent Choice, %	75.0	70.0	65.0	58.8	12.26	0.81

a-b: *Means with different superscripts within a line are significantly different (P<0.05)*

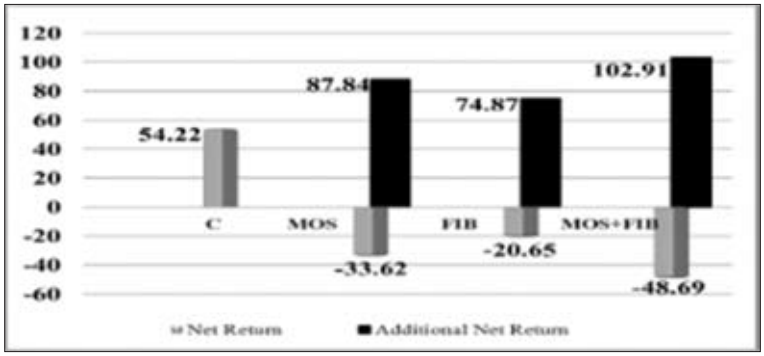


Fig 1. Additional net return needed from NFA to equal the control treatment

Şekil 1. Kontrol grubu kârına eşit olması için NFA grubuna gerekli ilave net kazanç

Table 6. *Beef production economic analysis*
Tablo 6. *Sığır eti üretiminde ekonomik analiz*

Economic Analysis	C	MOS	FIB	FIB+MOS	SEM	P-Value
84-Day Backgrounding Economics						
Weight sold with 3.0% shrink, kg	410.37	378.84	381.07	377.35		
Price/kg, $*	2.089	2.182	2.183	2.183		
Gross Return, $	857.20	826.65	831.70	823.58		
Feeder Calf Cost, $	680.77	667.73	665.55	665.55		
Feed Cost, $	116.65	115.69	115.93	119.71		
Yardage Cost, $	25.20	25.20	25.20	25.20		
84-Day Backgrounding Net Return, $	34.58	18.03	25.02	13.12		
Total Beef Production Economics						
Total Carcass Value, $**	1243.55[a]	1149.52[b]	1153.66[b]	1130.98[b]	3.58	<0.01
Feeder Calf Cost, $	680.77	667.73	665.55	665.55		
Backgrounding Feed and Yardage, $	141.85	140.89	141.13	144.91		
Feedlot Feed and Yardage Cost/Head, $	325.71	333.52	326.63	328.21	6.73	0.78
Transportation, $***	41.00	41.00	41.00	41.00		
Total Net Return, $	54.22	-33.62	-20.65	-48.69		

** Backgrounding economics prices are from Stockmen's Livestock Exchange, Dickinson, North Dakota, ** Total carcass value amount paid by Cargill Meat Solutions based on the Angus America value grid, *** Transportation from Dickinson, North Dakota to Oberlin, Kansas,* ***a-b:*** *Means with different superscripts within a line are significantly different, (P<0.05)*

and MOS+FIB additives, respectively. Although there was no difference between treatments in the percentage of carcasses grading choice, steers fed natural additives during backgrounding returned significantly less gross return/carcass that affected total net return.

DISCUSSION

Growth-enhancing technology (steroid hormones and feed antibiotics) is used extensively in the USA cattle feeding industry to increase muscle accretion, alter rumen volatile fatty acid production, and improve gain, and feed efficiency. However, the consuming public is becoming increasingly more concerned about the use of hormones and antibiotics, and buying habits are changing as evidenced by meat sale increases for natural and organically grown meat [12]. This consumer message must be taken seriously. The NFA, mannan oligosaccharides and fibrolytic enzymes (cellulase and xylanase activity) have been evaluated in separate investigations, but research comparing the two additives fed together as replacements for GET is limited. The research objective was to determine whether comparable animal response can be realized when feeding the NFA separately or in combination as alternatives to using GET.

Mannan oligosaccharides (MOS) used commercially are products containing a minimum of 28% glucomannoprotein from *S. cerevisiae* and have been mostly evaluated as dietary alternatives for antibiotics in simple stomach food producing animals [13-16]. Feeding supplemental MOS resulted in comparable performance when compared to feeding antibiotics. However, to a lesser extent in ruminant animals. In growing-finishing cattle, steers fed 85% concentrate diets with MOS were compared to steers fed a conventional diet regime that included the feed antibiotic Rumensin® [2]. No difference was measured in growth rate, days on feed, feed efficiency, fat thickness, ribeye area, yield grade, or quality grade, suggesting MOS was an effective replacement for feed grade antibiotic. In the current study that included a steroid implant and the feed additive Rumensin, our results show that feeding MOS alone or in combination with Fibrozyme® (FIB+MOS) did not differ from the C for total daily feed intake, feed required per kg of gain, and feed cost per steer per day. The results of the current study agree with the reported feed efficiency and carcass data [2], but do not agree with the reported growth performance [2], because using the steroid implant in this study significantly increased ADG.

Increasing fiber digestion is the main reason for feeding enzymatic products that have been shown to improve forage digestion, resulting in improved milk production and growth performance in beef cattle [5-7,10,17,18]. The effectiveness of enzyme additives can be variable due to the additive formulation and the enzymes present, forage variability, and feeding level [7]. Considering the results of others [5-7,10,17,18], steer response to NFA in the present study was encouraging in the fact that DMI, feed efficiency, and the resultant feed cost/steer/day during the 84-day backgrounding period was similar to the C treatment. In fact, had ADG among the NFA treatments not averaged 18.9% less/day, the bottom line feed cost/kg of gain would have been more favorable for the NFA treatments. The depression in ADG is certainly understandable, since

the mode of action for GET and the NFA is very different. Overall, since steer performance with FIB, MOS and MOS+FIB was the same, there is no production advantage for feeding MOS and FIB together.

Economics for NFA compared to employing GET in this study suggest that there is no economic advantage for feeding MOS or FIB individually or in combination during the 84-day backgrounding period and there was no economic carryover advantage during the feedlot finishing period. Combining the two additives added to the cost of production *(Fig. 1)*. Therefore, feedlot managers would be advised not to feed the two additives together. Using GET in the C treatment contributed to significantly improved growth performance and lowered feed cost per kg of gain resulting in improved overall productivity and a large net return advantage, which has also been documented by others [19-21].

Since the human population is socially responsible to reduce impact from resource inputs and waste outputs, deterministic models [22,23] have shown that reduced production efficiency increases the amount of feed, land, and water necessary to produce a kg of beef [23].

In conclusion, NFA were less profitable during backgrounding and unprofitable for finishing. Producers growing cattle for NFA markets without the use of GET will need to feed cattle longer, commit more feed, land and water resources, and obtain additional net return from natural markets to capture unrealized revenue.

REFERENCES

1. Basarab J, Baron V, Lopez-Campos O, Aalhus J, Haugen-Kozyra K, Okine E: Greenhouse gas emissions from calf- and yearling-fed beef production systems, with and without the use of growth promotants. *Animals,* 2 (2): 195-220, 2012.

2. Anderson VL, Schoonmaker J: Evaluation of performance and carcass quality of finishing beef with natural feeding practices in North Dakota. **In,** *NDSU Carrington Research Extension Center Crop and Livestock Review,* 45, 22-23, 2004.

3. Spring P, Wenk C, Dawson KA, Newman KE: The effects of dietary mannan oligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of Salmonella-challenged broiler chicks. *Poult Sci,* 79-205-211, 2000.

4. Newman K: Mannan-oligosaccharides natural polymers with significant impact on the gastrointestinal microflora and the immune system. **In,** Lyons TP, Jacques KA (Eds): Biotechnology in the Feed Industry: Proceeding from Alltech's 10th Annual Synposium, Nottingham Press University, Nottingham, UK, 167-174, 1994.

5. Beauchemin KA, Rode LM, Sewalt VJH: Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. *CJAS,* 75, 641-644, 1995.

6. Beauchemin KA, Jones SDM, Rode LM, Sewalt VJH: Effects of Fibrolytic enzymes in corn or barley diets on performance and carcass characteristics of feedlot cattle. *CJAS,* 77, 645-653, 1997.

7. Beauchemin KA, Colombatto D, Morgavi DP, Yang WZ: Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *J Anim Sci,* 81 (E. Suppl. 2): E37-E47, 2003.

8. Lewis GE, Hunt CW, Sanchez WK, Treacher R, Pritchard GT, Feng P: Effects of direct fed fibrolytic enzymes on the digestive characteristics of a forage-based diet fed to beef steers. *J Anim Sci,* 75, 3020, 1996.

9. NRC: Nutrient Requirements of Beef Cattle. 7th Rev ed., Natl Acad Sci, Washington, DC, 1996.

10. ACCU-TRAC: Electronic Cattle Management System, Micro Beef Technologies, Amarillo, Texas. http://www.microbeef.com/ *Accessed:* 09.03.2013.

11. SAS: SAS/STAT Inst Inc Cary, NC, 27513, 2008.

12. Loe ER, Melroe TA: Conventional, natural, and organic beef production and consumption. South Dakota State University Extension Extra ExEx2059, http://agbiopubs.sdstate.edu/articles/ExEx2059.pdf. *Accessed*: 10.09.2013.

13. Funderburke DW: Effects of dietary supplementation with mannan oligosaccharides on performance of commercial sows and their litters. **In,** Lyons TP, Jacques KA (Eds): Proceeding from Alltech's 18th Annual Symposium, Nottingham Press University, Nottighham, UK, 407, 2002.

14. Heinrichs AJ, Jones CM, Heinrichs BS: Effects of mannan oligosaccharide or antibiotics in neonatal diets on health and growth of dairy calves. *J Dairy Sci,* 86, 4064-4069, 2003.

15. Ferket PR, Parks CW, Grimes JL: Mannan oligosaccharides versus antibiotics for turkeys. **In,** Lyons TP, Jacques KA (Eds): Nutritional Biotechnology in the Feed and food Industries: Proceeding from Alltech's 18th Annual Synposium, Nottingham Press University, Nottingham, UK, pp.43, 2002.

16. LeMieux FM, Southern LL, Bidner TD: Effect of mannan oligosaccharides on growth performance of weanling pigs. *J Anim Sci,* 81, 2482-2487, 2003.

17. McAllister TA, Oosting SJ, Poop JD, Mir Z, Yanke LJ, Hristov AN, Treacher RJ, Cheng KJ: Effect of exogenous enzymes on digestibility of barley silage and growth performance of feedlot cattle. *CJAS,* 79, 353-360, 1999.

18. Romero JJ, Zarate MA, Querioz OCM, Han JH, Shin JH, Staples CR Brown WF, Adesogan AT: Fibrolytic enzyme and ammonia application effects on the nutritive value, intake, and digestion kinetics of bermudagrass hay in beef cattle. *J Anim Sci,* 91, 4345-4356, 2013.

19. Foutz CP, Dolezal HG, Gardner TL, Gill DR, Hensley JL, Morgan JB: Anabolic implant effects on steer performance, carcass traits, subprimal yields, and longissimus muscle properties. *J Anim Sci,* 75, 1256-1265, 1997.

20. Herschler RC, Olmsted AW, Edwards AJ, Hale RL Montgomery T, Preston RL, Bartle SJ, Sheldon JJ: Production responses to various doses and ratios of estradiol benzoate and trenbolone acetate implants in steers and heifers. *J Anim Sci,* 73, 2873-2881, 1995.

21. Berthiaume R, Mandell I, Faucitano L, Lafreniere C: Comparison of alternative beef production systems based on forage finishing or grain-forage diets with or without growth promotants: 1. Feedlot performance, carcass quality, and production costs. *J Anim Sci,* 84, 2168-2177, 2006.

22. Capper JL: The environmental impact of beef production in the United States: 1997 compared with 2007. *J Anim Sci,* 89, 4249-4261, 2001.

23. Capper JL, Hayes DJ: The environmental and economic impact of removing growth-enhancing technologies from U.S. beef production. *J Anim Sci,* 90, 3527-3537, 2012.

Kafkas Univ Vet Fak Derg
20 (3): 411-415, 2014
DOI: 10.9775/kvfd.2013.10305

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

RESEARCH ARTICLE

Single Nucleotide Polymorphism Analysis of the *rpoB* Gene Region for Genotyping of *Brucella melitensis* Strains Isolated from Field in Turkey [1]

Murat SAYAN [1] Sevil ERDENLİĞ GÜRBİLEK [2]

[1] *This study has been presented in XVI. Turkish Clinic Microbiology and Infection Diseases Congress, 13-17 March 2013, Antalya - TURKEY*

[1] Clinical Laboratory, PCR Unit, Faculty of Medicine, University of Kocaeli, TR-41000 Kocaeli - TURKEY
[2] Department of Microbiology, Faculty of Veterinary Medicine, University of Harran, TR-63200 Şanlıurfa - TURKEY

Makale Kodu (Article Code): KVFD-2013-10305

Summary

We previously described the first molecular characterization of *Brucella* isolates in Turkey that were examined by single nucleotide polymorphisms (SNPs) at the *rpoB* locus of *B. melitensis* strains isolated from adult and pediatric patients. However, the molecular typing of *B. melitensis* strains causing animal infections in Turkey has not been previously investigated. The aim of this study was to evaluate SNP analysis of *rpoB* gene of *B. melitensis* from field isolates in Turkey and to try to find out one of the most appropriate methods other than conventional method for long term evaluation of epidemiological studies. Thirty-two *B. melitensis* strains isolated from Marmara, Aegean, Mediterranean, Central Anatolia and Eastern Anatolia regions of Turkey were investigated together with 3 reference strains. According to *rpoB* sequencing results, three distinct genotypes (SNP type 1, type variant 2 and type 2) were recognized. SNP technique characterized the strains at the molecular levels independently from *B. melitensis* biovars. Our study showed that SNP analysis has a better discriminatory capability in identification of *B. melitensis* strains compared to classical method. In conclusion, it was suggested that SNP analysis could be useful as a molecular epidemiological method to determine relationships between *B. melitensis* isolates and might aid in effective surveillance and control method for brucellosis particularly in conjunction with a national databases.

Keywords: *Brucella melitensis, Molecular typing, Single nucleotide polymorphism, Sequence analysis*

Türkiye'de Sahadan İzole Edilmiş *Brucella melitensis* Suşlarının Genotiplendirilmesinde *rpoB* Gen Bölgesi Tek Nükleotit Polimorfizm Analizi

Özet

Ülkemizde ilk kez *Brucella* izolatlarının moleküler karakterini ortaya çıkardığımız önceki çalışmada erişkin ve çocuk hastalardan izole edilen *B. melitensis* izolatlarında *rpoB* geni tek nükleotit polimorfizm (SNP) analizi ile değerlendirilmişti. Ülkemizde daha once hayvan enfeksiyonlarından izole edilen *B. melitensis* suşlarında SNP analizi ile genotiplendirme değerlendirilmiş değildir. Bu çalışmada, *B. melitensis* saha suşlarının moleküler tiplendirmesinde *rpoB* geninin SNP ile analizinin değerlendirilmesi ve epidemiyolojik çalışmalarda kullanılabilecek uygun tiplendirme yönteminin tanımlanması amaçlanmıştır. Araştırmada referans suşlar ile birlikte Marmara, Ege, Akdeniz, Orta Anadolu ve Güneydoğu Anadolu bölgelerimizden izol edilmiş toplam 32 *B. melitensis* saha suşu çalışıldı. *rpoB* geni sekanslarının değerlendirilmesi sonrasında *B. melitensis* saha suşlarında üç moleküler tip tanımlandı; SNP tip 1, SNP tip 2 ve SNP variant tip 2. SNP analiz tekniği *B. melitensis* suşlarını biyovar özelliklerinden bağımsız bir şekilde moleküler olarak tiplendirmektedir. Çalışmamız, epidemiyolojik olarak SNP analizinin *B. melitensis* suşlarını tanımlamada klasik yönteme göre yüksek ayrım gücüne sahip olduğunu göstermektedir. Sonuç olarak SNP analizinin *B. melitensis* izolatları arasındaki ilişkiyi saptayacak faydalı bir moleküler epidemiyolojik metot olduğu ve bu yöntemin brusellozun kontrolü ve etkili bir surveyansına yönelik hazırlanacak ulusal bir brusella veritabanının oluşturulması halinde katkı sağlayacağı görüşüne varılmıştır.

Anahtar sözcükler: *Brucella melitensis, Moleküler tiplendirme, Tek nükleotit polimorfizmi, Sekans analizi*

İletişim (Correspondence)
+90 414 3183918
serdenlig@harran.edu.tr

INTRODUCTION

An important component for recognition and control disease outbreaks is identification of the reservoir and mode of transmission of the infectious agents involved. This often requires the establishment of relationship among the pathogens isolated during the outbreak. Because each species of microorganism comprises almost limitless number of strains, identification of an organism to the species level is not sufficient for most of molecular epidemiological works. Strain typing, which is the method mostly used in order to establish a relationship among organisms belonging to the same species, is generally required.

Genotypic characterization is important for patient management and may be used to trace sources of *Brucella* infection and to distinguish between relapse and re-infection [1-3]. If re-infection is observed, the patient should be further educated to avoid consumption of unpasteurized dairy products and contact with infected animals. If it relapses, treatment options may need to be reconsidered. Hence, control measures can be implemented very early and further spread of the disease may be prevented [4,5].

Rapid and accurate typing procedures are crucial for epidemiologic surveillance, investigation of outbreaks, and follow-up of a control program. Many molecular typing methods commonly used for the subtyping of isolates of other bacterial species are not appropriate for routine typing of *Brucella* strains, and none has proven to be fully satisfactory for epidemiological trace-back investigations of brucellosis [5,6]. Insertion sequence based typing and polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) are the examples of such methods [7,8]. In recent studies investigating reference and clinical *Brucella* isolates, the utility of the *rpoB* gene, encoding the DNA-dependent RNA polymerase β subunit (RNAP), for genotyping *Brucella* strains via a single nucleotide polymorphism (SNP) based method were examined [9-11].

Brucellosis is endemic and approximately 10.000 human brucellosis cases are reported annually in Turkey. The reported incidence is 150 cases per 1 million inhabitants [12]. Previous studies conducted in different regions of Turkey found that human brucellosis was almost exclusively caused by *B. melitensis,* accounting for 99% of the total cases, and *B. melitensis* biovar 3 was the biovar most frequently isolated in humans [13-16]. High resolution typing of *Brucella* isolates is important for epidemiological surveillance; investigation of outbreaks in regions of both low and high endemicity; and distinguishing cases of human reinfection from relapse; thereby influencing clinical therapeutic decisions [1]. In a recent publication, the MLVA-16_{UPSUD} assay was applied to investigate epidemiological relationships for the first time, among human brucellosis isolates collected from all regions of Turkey [17]. But, the molecular typing of *Brucella* strains isolated especially from animal infections has not been sufficiently investigated in Turkey. The aim of this study was to evaluate to SNP analysis of *rpoB* gene using field isolates of *B. melitensis* from animals and human beings from Turkey in order to assess the value of this analysis for epidemiological surveillance.

MATERIAL and METHODS

Brucella Strains

In this study, 32 *B. melitensis* field strains isolated various provinces from Marmara (Canakkale, Yalova, Kirklareli, Istanbul, Edirne, Bilecik, Kocaeli), Aegean (Afyon), Mediterranean (Adana), Central Anatolia (Ankara, Cankiri, Aksaray, Konya, Eskisehir, Kırsehir) and Eastern Anatolia (Erzurum) regions of Turkey were investigated together with 3 control reference strains. *B. melitensis* biovar 1 (16M; ATCC 23456), biovar 2 (63/9; ATCC 23457), and biovar 3 (Ether; ATCC 23458) were used as reference strains. All *Brucella* isolates were biotyped using the classical biotyping procedures described by Alton *et al*.[18], i.e. CO_2 requirement, H_2S production, urea hydrolysis, agglutination with monospecific antisera, dye sensitivity and phage typing.

Polymerase Chain Reaction

Bacterial nucleic acid was extracted from cultures by magnetic-particle technology on the BioRobot EZ1 (QIAGEN GmbH, Hilden, Germany) instrument. Two PCR tests were carried out to amplify two specific regions of the *rpoB* gene. The regions of the *B. melitensis* genome covering the biovar reference strain specific codons (Cd); Cd 629, Cd 985, Cd 1249, Cd 1309 were amplified using primers as described by Marianelli *et al*.[19]. Primers rBseq7 and - 4143rB gave a 1254 bp-long fragment and the primers +1418rB and rBseq5 gave a 738 bp-long fragment. PCR amplifications were carried out in a Mastercycler (Eppendorf AG, Hamburg, Germany) using Quantitect SYBR Green PCR mix (QIAGEN GmbH, Hilden, Germany). Amplifications were initiated by denaturing the sample for 15 min at 94°C, followed by 40 cycles at 94°C for 45 s, 60°C for 45 s and 72°C for 90 s. After the last cycle, samples were incubated for an additional 10 min at 72°C. Three micro liters of each reaction mixture were analyzed by electrophoresis through a 1% agarose gel.

rpoB Sequencing

All PCR products were purified using the High Pure PCR Products Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) and directly sequenced with the ABI PRISM 310 Genetic Analyzer equipment using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc, Piscataway, USA). Primers rBseq7, rBseq9, +1418rB [9] and an additional designed primer, rBseq8-AS:

(5'-TGGATCTTGCGCTTCACAG-3'), were used for sequencing. The electropherogram were assembled by Vector NTI v5.1 (InforMaxTM InvitrogenTM life science software, Frederick, MD 21704, USA) software based on the published *rpoB* sequence of *B. melitensis* 16M. All consensus sequences were generated, and then compared to the published *B. melitensis* 16M *rpoB* gene for detection of nucleotide diversity [19].

RESULTS

According to *rpoB* sequencing results 3 distinct genotypes (SNP type 1, type variant 2 and type 2) were recognized *(Table 1)*. Four strains (27, 30, 184 and 212) had no miss-sense mutations at Cd 629, Cd 985, Cd 1249 and Cd 1309 showing a genotype identical to that of the *B. melitensis* biovar 1 reference strain 16M. Except one strain(212), rest of the three isolates were classically typed as biovar 3 at the *Brucella* Laboratory of Pendik Veterinary Control Institute *(Table 1)*.

Twelve strains had miss-sense mutations at Cd 629, Cd 985 and Cd 1309 with a genotype identical to the *B. melitensis* biovar 2 reference strain 63/9. In the case of the remaining 16 strains, miss-sense mutations were found only at Cd 629 and Cd 1309, and no miss-sense mutation was found at Cd 985. Therefore these strains are different from any of the reference *Brucella* strains. However given their apparent closer relationship to the *B. melitensis* biovar 2 reference strain sequence with regard to the *rpoB* gene analysis they were named as variant 2 *(Table 1)*. Although most isolates were identified classically as biovar 3, no isolate was found to share the *rpoB* genotype of the biovar 3 reference strain Ether.

DISCUSSION

Phenotypic characteristics (e.g. biotyping, serotyping, antimicrobial susceptibility profiles) historically have been used to type strains, However, these methods often have disadvantages because of their inability to consistently discriminate between different strains, labor intensity, or lack of reproducibility. In contrast, certain molecular methods do not have these limitations and have increased strain typing capabilities.

The distribution of biovars may vary between localities or even within a locality and this can provide useful epidemiological information. However, in many instances a single biovar predominates and this makes the tracing of sources of infection difficult. There have been some limited studies examining the biovars associated with Turkish *B. melitensis* isolates. Almost all *B. melitensis* strains are reported to be biovar 3 or 1 as not 2. From January 1996 to May 2002, 243 brucellosis patients were admitted to Dokuzoguz and colleague's clinic. *Brucella spp.* were isolated from blood cultures of 54 patients out of 243 (22%). Eighty-three percent of the isolates were speciated as *B. melitensis,* and 17% as *B. abortus.* Among *B. melitensis* species, 35 (78%) were identified as biovar 3, and 10 (22%) as biovar 1 [14]. In the studies between the years 2002-2005, 41 out of 50 *B. melitensis* isolates from Central Turkey were demonstrated as biotype 3, predominantly [13,15]. In a study, 162 human brucella isolates collected from different parts of Turkey during an 8-year period (from 2001 to 2008) were evaluated by bacteriological, epidemiological, and molecular typing (MLVA-16) characteristics. A total of 162 *Brucella* isolates were identified as *B. melitensis* biovar 3 (161 isolates) and *B. abortus* biovar 3 (one isolate) [17]. In our recently published reports, 94 human *Brucella* isolates collected also in an 8-year period from the beginning of 2002 to the end of 2009 throughout Turkey were investigated. The isolates were identified at species and biovar levels by conventional methods. Except one isolate, all were identified as *B. melitensis* biovar 3 [16]. These findings indicated that *B. melitensis* biovar 3 is predominant biovar responsible for human brucellosis in Turkey.

In our previous study, we described the first molecular characterization of *Brucella* isolates in Turkey examining mutations by using SNP analysis of the *rpoB* gene region to type *B. melitensis* strains isolated from our adult and pediatric patients. Sixty two *B. melitensis strains of human and animal origin* isolated from various regions of Turkey were used in this study. It was found that 52 *B. melitensis* isolates represented biovar 3 and 10 isolates represented biovar 1 by using conventional biotyping procedures. Eight strains, which had no miss-sense mutations at Cd 629, Cd 985, Cd 1249 and Cd 1309 were identified as genotype 1 (shared with the biovar 1 reference strain). Six strains that had miss-sense mutations at Cd 629, Cd 985 and Cd 1309 were identified as genotype 2 (shared with the biovar 2 reference strain). In the other 48 strains, miss-sense mutations were found only at Cd 629 and Cd 1309, and no miss-sense mutation was found at Cd 985. Therefore, those strains were identified as variants of genotype 2 [10]. In the present study, SNP molecular method and conventional biotyping procedures were applied to 28 animal and 4 human isolates of *B. melitensis* obtained from five regions of Turkey. However, there were still same inconsistency between results of SNP and classical biotyping methods.

SNP analysis for *Brucella* genotyping was found to be promising for a couple of reasons: if one has the right potential molecular marker for genotyping, SNP analysis is easy to perform. Actually, SNP technique is characterized the strains at the molecular levels independently from *B. melitensis* biovars. The results of *rpoB* sequencing by SNP analysis were seemed to be very useful while comparing to biovar analysis by conventional methods. Because of the uncontrolled animal movements in borders of Turkey, it is always possible some exogeneous *B. melitensis* strains might enter into country. In these circumstances, SNP

Table 1. *rpoB sequencing results of B. melitensis strains according to single nucleotide polymorphism method in the study group*
Tablo 1. *Çalışma grubunda tek nükleotit polimorfizm yöntemine gore B.melitensis suşlarının rpoB dizileme sonuçları*

Strain	Provience*	District	Village	Origin	Biovar	SNP type	Codon Residue										
							495-628	629	630-712	965-984	985	986-1062	1217-1248	1249	1250-1308	1309	1310-1364
							•	GCG	•	•	GCC	•	•	ATG	•	CTG	•
16M	-	-	-	Reference	1	1	•	•	•	•	•	•	•	•	•	•	•
63/9	-	-	-	Reference	2	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
Ether	-	-	-	Reference	3	3	•	•	•	•	•	•	•	ATA	•	•	•
184	Canakkale	Gelibolu	Findikli	Goat	3	1	•	•	•	•	•	•	•	•	•	•	•
283	Yalova	Termal	Akkoy	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
281	Kirklareli	Kofcar	Y.Kanara	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
212	Kirklareli	Pinarhisar	Y.Kasabasi	Goat	1	1	•	•	•	•	•	•	•	•	•	•	•
234	Erzurum	Askale	Gorkaynak	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
293	Istanbul	Silivri	Beyciler	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
227	Erzurum	Askale	Kukurtlu	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
236	Erzurum	Cat	Kaplica	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
229	Erzurum	Cat	Tuzlatasi	Cattle	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
285	Adana	Yumurtalik	Yahsiler	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
235	Erzurum	Olur	Kaledibi	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
230	Erzurum	Merkez	Tepekoy	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
248	Ankara	Ayas	Hacimemmi	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
260	Edirne	Merkez	Tayakadin	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
259	Canakkale	Gelibolu	Namaztepe	Sheep	1	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
280	Edirne	Havsa	Kabagac	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
F7	Istanbul	Hadımkoy	-	Human	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
282	Bilecik	Bozuyuk	Kandilli	Lamb	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
38	Aksaray	Merkez	Sultanhani	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
F9	Istanbul	Omerli	-	Human	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
162	Aksaray	Merkez	-	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
6	Ankara	Bala	Merkez	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
296	Cankiri	Eldivan	Saritarla	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
61	Afyon	Goynuk	B.Cobanlar	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
255	Kirsehir	Merkez	M.Uzunali	Sheep	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
30	Konya	Merkez	Saricalar	Sheep	3	1	•	•	•	•	•	•	•	•	•	•	•
240	Eskisehir	Merkez	Satilmisoglu	Sheep	1	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
298	Cankiri	Cerkes	Orenli	Sheep	1	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
284	Bilecik	Osmaneli	Yesilcimen	Sheep	3	variant 2	•	GTG	•	•	•	•	•	•	•	CTA	•
27	Istanbul	Catalca	Incegiz	Sheep	3	1	•	•	•	•	•	•	•	•	•	•	•
4988	Kocaeli	Golcuk	-	Human	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•
6130	Istanbul	Kavakli	-	Human	3	2	•	GTG	•	•	GTC	•	•	•	•	CTA	•

** Provience from Marmara (Canakkale, Yalova, Kirklareli, Istanbul, Edirne, Bilecik, Kocaeli), Aegean (Afyon), Mediterranean (Adana), Central Anatolia (Ankara, Cankiri, Aksaray, Konya, Eskisehir, Kırsehir) and Eastern Anatolia (Erzurum) regions of Turkey*

technique may be used as a epidemiological tool in the light of national database in Turkey. Moreover, the SNP pattern of a *B. melitensis* strain proved to be stable by comparing both *Brucella* strains isolated from different patients within the same outbreak, and strains from the same patient before first-line therapy and after relapse despite antibiotic treatment [1,5].

SNP typing method is not congruent with phenotypic observations. Upon re-analysing the whole sample set one would expect to see *rpoB* SNP typing forming its own subgroups/genetic variants, that would not be homogenous with observed phenotypes [20]. As molecular typing is much more robust and it is not subject to interpretation when comparing to classical typing, outputs are repeatable and transferable across many laboratories. This is one of the major driving factors behind making molecular typing a confirmatory tool used alongside phenotypic typing.

In conclusion, our results provide proof of the different characteristics of SNP in genotyping of *B. melitensis* isolates that could not be differentiated by conventional microbiological methods. *rpoB* SNP typing can be used as a molecular epidemiological tool to determine relationships for *B. melitensis* isolates and might provide effective surveillance and control mechanisms in brucellosis in Turkey.

REFERENCES

1. Al Dahouk S, Hagen RM, Nockler K, Tomaso H, Wittig M, Scholz HC: Failure of a short-term antibiotic therapy for human brucellosis using ciprofloxacin. A study on *in vitro* susceptibility of *Brucella* strains. *Chemotherapy,* 51, 352-356, 2005.

2. Kattar MM, Jaafar RF, Araj GF, Le Fleche P, Matar GM, Rached RA: Evaluation of a multilocus variable-number tandem-repeat analysis scheme for typing Human *Brucella* isolates in a region of brucellosis endemicity. *J Clin Microbiol*, 46, 3935-3940, 2008.

3. Marianelli C, Petruccab A, Pasquali P, Ciuchini F, Papadopouloub S, Cipriani P: Use of MLVA-16 typing to trace the source of a laboratory-acquired *Brucella* infection. *J Hosp Infect*, 68, 274-276, 2008.

4. Al Dahouk S, Nöckler K, Hensel A, Tomaso H, Scholz HC, Hagen RM: Human brucellosis in a nonendemic country: A report from Germany, 2002 and 2003. *Eur J Clin Microbiol Infect Dis*, 24, 450-456, 2005.

5. Al Dahouk S, Le Flèche P, Nöckler K, Jacques I, Grayon M, Scholz HC: Evaluation of *Brucella* MLVA typing for human brucellosis. *J Microbiol Meth*, 69, 137-145, 2007.

6. Whatmore AM, Shankster SJ, Perrett LL, Murphy TJ, Brew SD, Thirlwall RE: Identification and characterization of variable-number tandem-repeat markers for biotyping of *Brucella* spp. *J Clin Microbiol*, 44, 1982-1893, 2006.

7. Bricker BJ, Ewalt DR, MacMillan AP, Foster G, Brew S: Molecular characterization of *Brucella* strains isolated from marine mammals. *J Clin Microbiol,* 38, 1258-1262, 2000.

8. Al Dahouk S, Tomaso H, Berninghoff PE, Splettstoesser WD, Scholz HC, Neubauer H: Identification of *Brucella* species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP). *Crit Rev Microbiol*, 31, 191-196, 2005.

9. Marianelli C, Ciuchini F, Tarantino M, Pasquali P, Adone R: Molecular characterization of the *rpoB* gene in *Brucella* species: new potential molecular markers for genotyping. *Microbes Infect*, 8, 860-865, 2006.

10. Sayan M. Yumuk Z, Bilenoğlu O, Erdenliğ S, Willke A: Genotyping of *B. melitensis* by *rpoB* gene analysis and reevaluation of conventional serotyping method. *Jpn J Infect Dis*, 62, 160-163, 2009.

11. Sayan M: *Brucella melitensis* biovar 2 can not be detected by serological method in Turkey. Letter to Editor. *Turk Klin Tip Bilim Derg*, 29, 1351-1352, 2009.

12. Turkish Statistical Institute: Address-based population register system, December 31, 2008 census. Turkish Statistical Institute, Ankara, Turkey. www.turkstat.gov.tr, May, 2012.

13. Bodur H, Balaban N, Aksaray S, Yetener V, Akıncı E, Colpan A: Biotypes and antimicrobial susceptibilities of *Brucella* isolates. *Scand J Infect Dis*, 35, 337-338, 2003.

14. Dokuzoguz B, Ergönül O, Baykam N, Esener H, Kiliç S, Celikbas A: Characteristics of *B. melitensis* versus *B. abortus* bacteraemias. *J Infect,* 50, 41-45, 2005.

15. Tanyel E, Coban AY, Koruk ST, Simsek H, Hepsert S, Cirit OS: Actual antibiotic resistance pattern of *Brucella melitensis* in central Anatolia. An update from an endemic region. *Saudi Med J*, 28, 1239-1242, 2007.

16. Sayan M, Kılıc S, Uyanık MH: Epidemiological survey of rifampicin resistance in clinic isolates of *Brucella melitensis* obtained from all regions of Turkey. *J Infect Chemother*, 18, 41-46, 2012.

17. Kilic S, Ivanov IN, Durmaz R, Bayraktar MR, Ayaslioglu E, Uyanik H: MLVA genotyping of human *Brucella* isolates from Turkey. *J Clin Microbiol*, 49, 3276-3283, 2011.

18. Alton GG, Jones LM, Angus RD, Verger JM: Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris,1988.

19. Marianelli C, Ciuchini F, Tarantino M, Pasquali P, Adone R: Genetic bases of the rifampicin resistance phenotype in *Brucella* spp. *J Clin Microbiol*, 42, 5439-5443, 2004.

20. Whatmore AM, Perret LL, MacMillan A: Characterization of the genetic diversity of *Brucella* by multilocus sequencing. *BMC Microbiol,* 7, 34, 2007. DOI: 10.1186/1471-2180-7-34

Kafkas Univ Vet Fak Derg
20 (3): 417-419, 2014
DOI: 10.9775/kvfd.2013.10306

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

RESEARCH ARTICLE

Bazı Sinek (Dizi: *Diptera*) Türlerinde *Wolbachia* spp'nin PZR ile Araştırılması

Cem Ecmel ŞAKİ [1] Sami ŞİMŞEK [1]

[1] Fırat Üniversitesi, Veteriner Fakültesi, Parazitoloji Anabilim Dalı, TR-23119 Elazığ - TÜRKİYE

Makale Kodu (Article Code): KVFD-2013-10306

Özet

Wolbachia spp., *Riketsia* soyundan gram negatif hücre içi bir bakteri olup, arthropodlarda yatay ve dikey olarak taşınmakta, üreme ile ilgili dokularında da bulunmaktadırlar. Bu çalışmanın amacı farklı tür erkek ve dişi sineklerde *Wolbachia* spp.'nin PZR ile aranmasıdır. Bu amaçla 33 dişi ve 15 erkek *Musca* spp., 30 dişi ve 7 erkek *Lucilia sericata*, 16 dişi ve 4 erkek *Calliphora vicina,* 1 dişi ve 4 erkek *Sarcophaga haemorrhoidalis* ile 27 dişi ve 10 erkek *Chrysomia albiceps* kullanılmıştır. Genomik DNA izolasyonundan sonra *Wolbachia* wsp (*Wolbachia* surface protein) genini çoğaltan spesifik primerler ile PZR yapılmıştır. Çalışma neticesinde pozitif kontrol örneğinde 630 bp'lik band gözlenirken, negatif kontrol ve test edilen hiçbir örnekte pozitiflik belirlenememiştir. Sonuç olarak bu çalışma ile Türkiye'de ilk kez bazı Diptera populasyonlarında *Wolbachia* spp. moleküler olarak araştırılmıştır.

Anahtar sözcükler: *Musca, Lucilia, Chrysomia, Calliphora, Sarcophaga, Wolbachia, PZR*

Investigation of *Wolbachia* spp in Some Flies Species (Order: *Diptera*) by PCR

Summary

Wolbachia is a gram negative intracellular parasite from *Rickettsia* family which is located into reproductive organs of arthropods and transmitted by horizontally and vertically. The aim of this study was to investigate of *Wolbachia* spp. in different species of flies by PCR. For this aim, 33 females and 15 males of *Musca* spp., 30 females and 7 males of *Lucilia sericata*, 16 females and 4 males of *Calliphora vicina,* 1 female and 4 males of *Sarcophaga haemorrhoidalis* and 27 females and 10 males of *Chrysomia albiceps* adult flies were used. After the isolation of genomic DNA, the specific primers were used and PCR was performed for the amplification of *Wolbachia* wsp (*Wolbachia* surface protein) gene. As a result, the positive control sample yielded 630 bp band while negative control and none of the tested samples yielded band. In conclusion, *Wolbachia* spp has been molecularly investigated in some Diptera populations for the first time in Turkey.

Keywords: *Musca, Lucilia, Chrysomia, Calliphora, Sarcophaga, Wolbachia, PCR*

GİRİŞ

Diptera dizisinde myiasise sebep olan sineklerin oluşturdukları hastalıkların yanı sıra bazı patojenleri de taşıdıkları bilinmektedir [1-4].

Wolbachia, *ricketsia* soyundan hücre içi bir bakteri olup, omurgasızların üreme ve diğer dokularında bulunmaktadır. *Wolbachia*, insektlerde en yaygın olarak gözlenen endosimbiotik bakterilerden biri olup maternal olarak nakledilen bir alfaproteobakteridir. Bu bakteri çok farklı insekt türleri ve filarial nematodlardan bildirilmiş olup, insekt türlerinin %15-20'sinde bulunduğu saptanmıştır. Bu bakteri hücre içi bir döngüye sahip olup, enfeksiyon insekt türlerinin somatik ve üreme dokularında geçmektedir. *Wolbachia*'nın konağında birtakım reprodüktif değişikliklere neden olduğu bilinmektedir. Bunlar arasında, geniş bir insekt grubunda sitoplasmik uyumsuzluk, *Hymenoptera*'larda partenogenezis, *Isopod*'ların erkeklerinde genetik feminizasyon ve erkeklerde ölüm bulunmaktadır. Bu nedenlerden dolayı *Wolbachia*'nın medikal, veteriner ve tarımsal önemi olan arthropodların biyolojik kontrolünde kullanılabileceği düşünülmektedir [5].

İletişim (Correspondence)
+90 532 5431309
cesaki@yahoo.com

Mekanizması henüz tam olarak bilinmemesine rağmen, *Wolbachia*'nın insekt soyları arasında horizontal naklinin şekillendiği bilinmektedir. Bu nedenlerle adı geçen bakteri insekt türleri arasında oldukça yaygındır. West ve ark.[6], İngiltere'deki insektlerin %22'sinin *Wolbachia* ile enfekte olduğunu bildirmişlerdir. *Wolbachia*'nın gerçek prevalansının halen tam olarak tahmin edilemediği ve bütün insekt türlerindeki yaygınlığının %76'lara kadar çıkabileceği bildirilmektedir [7].

Bu çalışmanın amacı, Elazığ ilinden elde edilen farklı Diptera türlerinde *Wolbachia*'nın yaygınlığını PZR ile belirlemektir.

MATERYAL ve METOT

Çalışmada kullanılmak üzere toplanması planlanan sinekler için Elazığ ili merkez kesimhaneleri civarında sinek populasyonunun yoğun olduğu günün sıcak saatlerinde kokuşmuş materyal ve insektisitten oluşmuş tuzaklar kullanılmıştır. Tuzaklardan toplanan sinekler laboratuvara getirilerek temizlenmiş ve %70'lik alkol bulunan şişelerde muhafaza edilmiştir. Daha sonra ilgili literatürler [8,9] ışığında tür ve cinsiyetleri belirlenmiştir.

Çalışmada kullanılan sinek türleri, sayısı ve cinsiyetleri *Tablo 1*'de verilmiştir.

Genomik DNA izolasyonundan önce, her türün erkek ve dişilerinden ayrı ayrı sinek havuzları oluşturulmuş, %70'lik etanolden çıkarılan sinekler 1X PBS ile en az 5 kez yıkandıktan sonra steril tüplerde parçalanıp eppendorf tüplere alınmış ve üzerlerine Fermentas genomik DNA izolasyon kit içeriğinde bulunan digestion solüsyonundan 180 µl ve 25 µl proteinaz-K eklenerek 56°C'de bir gece inkübasyona bırakılmıştır. Ertesi gün kit protokolü takip edilerek gDNA izolasyonu tamamlanmış ve PZR kurulluncaya kadar -20°C'de saklanmıştır.

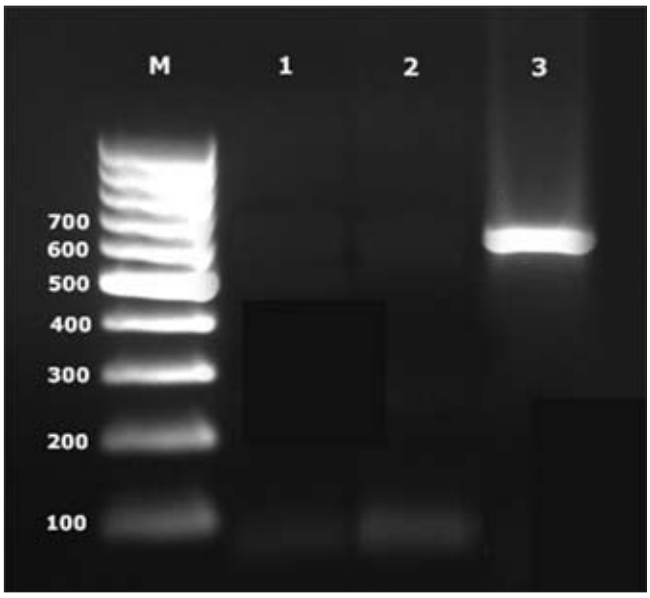
Tablo 1. *Çalışmada kullanılan sinek türlerinin sayısı ve cinsiyetleri*
Table 1. *Number and genders of fly species used in the study*

Tür	Cinsiyet	Adet (n)
Musca spp.	Dişi	33
Musca spp.	Erkek	15
Lucilia sericata	Dişi	30
L. sericata	Erkek	7
Chrysomia albiceps	Dişi	27
Ch. albiceps	Erkek	10
Calliphora vicina	Dişi	16
C. vicina	Erkek	4
Sarcophaga haemorrhoidalis	Dişi	1
S. haemorrhoidalis	Erkek	4
Toplam		147

Eldeki genomik DNA örneklerinde *Wolbachia* spp. DNA'sının aranması amacıyla *Wolbachia* wsp (*Wolbachia* surface protein) genini çoğaltan spesifik primerler ile (Forward 5'-TGGTCCAATAAGTGATGAAGAAACTAGCTA-3', reverse 5'-AAAATTAAACGCTACTCCAGCTTCTGCAC-3') PZR yapılmıştır. Bu amaçla; 5 µl 10X PZR buffer, 5 µl $MgCl_2$, 125 µM dNTP, her primer çiftinden 20 pmol, 0.2 µl Taq DNA Polymerase (5 IU) ve 5 µl genomik DNA içeren PZR karışımı hazırlanmış ve 94°C'de 3 dak.; 94°C'de 1 dak., 52°C'de 1 dak. ve 72°C'de 1 dak. (40 siklus) ve 72°C'de 5 dak. son uzama aşamalarından oluşan PZR şartlarına tabi tutulmuştur. Takiben PZR ürünleri %1.4'lük agaroz jelde yürütülüp ethidium bromide ile boyandıktan sonra UV transilluminatörde görüntülenmiştir. Bu işlem Zhou ve ark.'nın [10] bildirdiği şekilde uygulanmıştır. Çalışmada pozitif kontrol olarak, Lethbridge Research Center'da (Alberta, Kanada) Prof. Dr. Kevin Floate'dan temin edilen enfekte bir *Haematobia* spp sineğinden gDNA izolasyonu ile elde edilen *Wolbachia* DNA'sı kullanılmıştır.

BULGULAR

Çalışma neticesinde pozitif kontrol örneğinde 630 bp'lik band gözlenirken, negatif kontrol ve test edilen hiçbir örnekte pozitiflik belirlenememiştir *(Şekil 1)*.



Şekil 1. *Wolbachia* wsp primerleriyle çoğaltılmış PZR ürünlerinin görünümü. M: Moleküler ağırlık belirleyicisi (100 bp); 1: Negatif Kontrol; 2: Örnek; 3: Pozitif Kontrol (630 bp)

Fig 1. PCR products of amplified with *Wolbachia* wsp primers. M: Molecular weight marker (100 bp); 1: Negative control; 2: Sample; 3: Positive control (630 bp)

TARTIŞMA ve SONUÇ

Wolbachia, konaklarıyla mutual bir ilişkiye sahip olduğu için bu bakterinin konaktan ayrılması ile parazit tedavisi daha etkili sonuç verebilmektedir. Çünkü *Wolbachia* dişi

eklem bacaklılarda partenogenezisi uyarmakta, dolayısıyla *Wolbachia* tedavisiyle insekt populasyonu azaltılabilmektedir [3]. Nitekim *Wolbachia* enfeksiyonunun bazı *Crustacea*'larda ve *Lepidoptera* türlerinde genotipik erkekleri, fenotipik fonksiyonel dişilere çevirdiği belirlenmiştir [11,12].

İran'da yapılan bir çalışmada [3], 770 arthropod (22 soya ait) ve 41 nematod (6 soya ait) *Wolbachia* yönünden araştırılmış ve %14.08'lik bir pozitiflik bulunmuş ve bu oranın daha yüksek olabileceği belirtilmiştir. Bahsi geçen çalışmada incelenen 7 cinse ait artropodların 167'si pozitif bulunmuştur. İncelenen örnekler içerisinde pozitif olarak tespit edilen *Diptera* takımında yer alan sineklerden en fazla *Drosphila melanogaster* en az olarak da *Musca domestica* tespit edilmiştir. *Sarcophaga haemorrhoidalis* örnekleri ise negatif bulunmuş ve sineklerde tespit edilen *Wolbachia*'nın A tipi *Wolbachia* olduğu belirlenmiştir.

Werren ve Windsor [13], aynı PZR metodunu kullanarak, Kuzey Amerika'daki insektlerin %19.3'ünün *Wolbachia* ile enfekte olduğunu bildirmişlerdir. Araştırıcılar, insekt takımları arasında B tip *Wolbachia* yerine A tipi ile enfeksiyon oluşması ile ilgili kesin bir farklılığın olduğunu belirtmişlerdir. Özellikle, *Hymenoptera*'lar A tip *Wolbachia* ile daha yüksek enfeksiyon oranı gösterirken, *Lepidoptera*'larda B tip daha yüksek gözlenmiştir. Bu sonuçlar, A ve B tip *Wolbachia*'ların farklı takım insektleri enfekte edebilme yeteneklerinin olabileceği şeklinde yorumlanabilir.

Bizim çalışmamızda hiçbir örnekte *Wolbachia* pozitifliği elde edilemediği için tiplendirme de yapılamamıştır. Çalışmada incelenen sineklerin hiçbirinde *Wolbachia* spp. tespit edilememesinin nedeni, bu sineklerin *Filaria* grubundaki nematodlara arakonakçılık yapmaması olabilir. Çünkü *Wolbachia* spp. *Filarial* nematodlar tarafından taşınmakta ve arthropodları da bu yolla enfekte etmektedir. Türkiye'de ilk kez yapılan bu araştırma ileride yapılacak daha kapsamlı çalışmalara kaynak teşkil edebilecektir.

KAYNAKLAR

1. Baumgartner DL: Review of *Chrysomya rufifacies* (*Diptera*: *Calliphoridae*). *J Med Entomol*, 30 (2): 338-352, 1993.

2. Fischer O, Matlova L, Dvorska L, Sïvastova P, Bartl J, Melicharek I, Weston RT, Pavliâk I: *Diptera* as vectors of mycobacterial infections in cattle and pigs. *Med Vet Entomol*, 15, 208-211, 2001.

3. Pourali P, Roayaei Ardakani M, Jolodar A, Razi Jalali MH: PCR screening of the *Wolbachia* in some arthropods and nematodes in Khuzestan province. *Iranian J Vet Res*, 10 (3): 216-222, 2009.

4. Tachibana SI, Numata H: Seasonal prevalence of blowflies and flesh flies in Osaka City. *Entomol Sci*, 9, 341-345, 2006.

5. Dobson S, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, O'Neill SL: *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect Biochem Mol Biol*, 29, 153-160, 1999.

6. West SA, Cook JM, Werren JH, Godfray HCJ: *Wolbachia* in two insect host parasitoid communities. *Mol Ecol*, 7, 1457-1465, 1998.

7. Jeyaprakash A, Hoy MA: Long PCR improves *Wolbachia* DNA amplification: Wsp sequences found in 76% of sixty three arthropod species. *Insect Mol Biol*, 9, 393-405, 2000

8. Bei-Bienko GY: Keys to the Insects European Part of the USSR. Part 2. Smithsonian Institution Libraries and the National Science Foundation. Washington, D.C. 1988

9. Zumpt F: Myiasis in Man and Animals in the Old World, Butterwoths & Co. ltd. London, 1965.

10. Zhou W, Rousset F, O'neill S: Phylogeny and PCR-based classification of *Wolbachia* strains using Wsp gene sequences. *Proc R Soc Lond B,* 265, 509-515, 1998.

11. Juchault P, Frelon M, Bouchon D, Rigaud T: New evidence for feminizing bacteria in terrestrial isopods. *C R Acad Sci Paris*, 317, 225-230, 1994.

12. Kageyama D, Nishimura G, Hoshizaki S, Ishikawa Y: Feminizing *Wolbachia* in an insect, *Ostrinia furnacalis* (Lepidoptera: Crambidae). *Heredity*, 88, 444-449, 2002.

13. Werren JH, Windsor DM: *Wolbachia* infection frequency in insects: Evidence of a global equilibrium? *Proc R Soc London*, *B Biol Sci*, 267, 1277-1285, 2000.

Kafkas Univ Vet Fak Derg
20 (3): 421-427, 2014
DOI: 10.9775/kvfd.2013.10311

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

RESEARCH ARTICLE

Merada Otlayan Danalarda Alkan İndikatör Tekniği Kullanılarak Yem Tüketimi ve Sindirilebilirlik Tahmini [1]

Yücel ÜNAL [1] İsmail KAYA [2] Tarkan ŞAHİN [3]
Ahmet ÖNCÜER [3] Dilek A. ELMALI [4] Güler YENİCE [5]

[1] *Bu çalışma TOVAG-104 O 433 proje numarasıyla TÜBİTAK tarafından desteklenmiştir*

[1] Ağrı İbrahim Çeçen Üniversitesi, Fen Edebiyat Fakültesi, Zooloji Anabilim Dalı, TR-04100 Ağrı - TÜRKİYE
[2] Ondokuz Mayıs Üniversitesi, Veteriner Fakültesi, Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı, TR-55200 Atakum, Samsun - TÜRKİYE
[3] Kafkas Üniversitesi, Veteriner Fakültesi, Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı, TR-36300 Kars - TÜRKİYE
[4] Mustafa Kemal Üniversitesi, Veteriner Fakültesi, Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı, TR-31034 Antakya - TÜRKİYE
[5] Atatürk Üniversitesi, Veteriner Fakültesi, Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı, TR-25240 Yakutiye, Erzurum - TÜRKİYE

Makale Kodu (Article Code): KVFD-2013-10311

Özet

Bu çalışmada, merada otlayan danalarda alkan indikatör metodu ile Kuru Madde Tüketimi (KMTt) ve Kuru Madde Sindirilebilirliği (KMSt) tahmini ve Kars şartlarında hayvanların sezonun değişik dönemlerinde meradan ne kadar yararlanabildiklerinin araştırılması amaçlanmıştır. Çalışma iki deneme halinde yürütüldü, ilk denemede 5 adet erkek dana (ort. CA 244.5±2.3 kg) kullanılmış olup deneme 20 gün sürmüştür. İlk denemede kapalı mekanda kontrollü yemleme ile belirlenen Kuru Madde Tüketimi (KMT) ve Sindirilebilirliğinin (KMS), alkan indikatörler kullanılarak yapılan KMTt ve KMSt ile karşılaştırılması yapılmış ve alkan indikatör tekniği ile yapılan tahminlerin doğruluk ve güvenilebilirliği belirlenmiştir. İkinci deneme ise merada otlayan hayvanlar kullanılarak 20'şer günlük 5 dönem halinde yürütülmüştür. Bu denemede toplam 10 adet erkek dana (ort. CA 164.9±1.5 kg) 2 gruba ayrılarak her bir dönemde farklı grup hayvan kullanılmıştır. Ahır denemesinde en iyi KMTt karma (24 saat boyunca toplanan) numunelerde alkan C_{33}:C_{32} çiftiyle yapılan hesaplamalarla elde edilmiştir (R^2: 0.86). Alkan C_{32} ve C_{33} kullanılarak elde edilen KMSt'leri KMS'ne benzer bulunurken, alkan C_{36} kullanılarak elde edilen KMSt'leri düşük bulunmuştur ($P<0.05$). Ahır denemesinde elde edilen sonuçlar alkan indikatör kapsüllerin alkan indikatör metodunda dozlama amacıyla başarıyla kullanılabileceğini göstermiştir. Mera çalışması sonuçlarına göre hayvanların Ağustos ayı sonlarından itibaren meradan kuru madde ihtiyaçlarını yeterince karşılayamadıkları belirlenmiştir.

Anahtar sözcükler: *Alkan indikatör, Kuru madde tüketimi, Sindirilebilirlik, Dana, Mera*

Estimation of Feed Intake and Digestibility in Grazing Cattle Using Alkane Indicator Technique

Summary

This study examined the estimation of the Dry-Matter Intake (DMIe) and Digestibility (DMDe) and the utilization of the pasture by grazing cattle in different seasons of the Kars region by using alkane indicator technique. Two experiments were carried out, and in the first experiment, 5 male cattle (mean LW. 244.5±2.3 kg) were used for 20 days. In first experiment, the precision and reliability of alkane indicator technique were determined by comparing the real Dry Matter Intake (DMI) and Dry Matter Digestibility (DMD) which was obtained by the controlled indoor study with DMIe and DMDe estimated by using alkane indicators. The second experiment was conducted with the pasturing cattle in meadow in 5 terms each lasted 20 days. Ten male cattle (mean LW. 164.9±1.5 kg) were divided in two groups, and different animals were used in each term. The best result for DMIe was obtained by using the alkanes C_{33}:C_{32} couples in mixed (collected in 24 h) samples in indoor study (R^2: 0.86). DMDe's calculated by using alkanes C_{32} and C_{33} were similar to the real DMD, whereas the DMDe's with alkane C_{36} were underestimated ($P<0.05$). The results obtained with indoor study showed that alkane indicator capsules could be used successfully as a dosing method in alkane indicator method. According to the second experiment results, it was determined that the animals, grazing in the pasture in Kars conditions, were not able to get required nutrient from the pasture towards the end of August.

Keywords: *Alkane indicator, Dry matter intake, Digestibility, Cattle, Pasture*

İletişim (Correspondence)
+90 472 2159994
yucel.unal@hotmail.com

GİRİŞ

Ruminantlardan maksimum verim dengeli bir rasyon uygulaması ile sağlanabilir. Besin maddesi tüketimi ve özellikle enerji alımı, Yem Kuru Madde Tüketimi (KMT) ve Sindirilebilirliği (KMS) tarafından öncelikle etkilendiği için KMT ve KMS hayvan beslemede en yaygın olarak kullanılan iki parametredir.

KMT ve KMS tahmininde (KMTt ve KMSt) kullanılan geleneksel metotlarda toplam dışkı toplama söz konusu olduğu için bu işlemler zaman ve iş gücü gerektirmektedir. Bu nedenle KMTt'de daha çok İndikatör metotları kullanılmaktadır [1,2].

Son yıllarda geliştirilen Alkan İndikatör Metodu uygulama ve hesaplama bakımından geleneksel indikatör tekniklerine göre daha pratik bir yöntemdir [3,4]. Alkanlar kaba yemlerin kütikular yapısında bulunan hidrokarbonlar olup sindirilmediği için internal indikatör olarak kullanılabilen maddelerdir. Alkan İndikatör Metodu bir çift indikatör tekniği olup geleneksel indikatör metotlarından farklı bir temeli vardır. Toplam dışkı miktarı ya da sindirilebilirlik kullanılmasına gerek kalmadan tekniğin kendi özel formülü ile KMTt yapılabilmektedir. Bu metotta prensip dışkıdaki tek zincirli alkanların çift zincirli alkanlara oranıdır [5]. Tekniğin en büyük avantajı ise bireysel KMTt ve KMSt'ne olanak vermesidir [6,7].

Tek numaralı alkanlar (C_{29}, C_{31}, C_{33} gibi) bitkilerde yoğun olarak bulunurken, çift numaralı alkanlar (C_{32}, C_{34}, C_{36} gibi) düşük düzeyde bulunmaktadır. Dolayısıyla yapılan çalışmalarda genellikle alkan C_{33} dahili indikatör (Dİ) olarak kullanılırken, alkan C_{32} ya da C_{36} harici indikatör (Hİ) olarak her gün dışarıdan verilmektedir. Alkan C_{34} miktarı da bitkilerde çok düşük olduğu için standart olarak kullanılmaktadır [8]. Alkan İndikatör Metodu kullanarak KMTt yapmak için öncelikle hayvanın Hİ ile dozlanması gerekmektedir. Doz olarak kullanılan ve sentetik olan Hİ; filtre [9], kapsül [10], jelatin kapsül [11], doğrudan yeme katma [12] şekillerinde kullanılabilmektedir. Son yıllarda yeni bir teknik olarak rumen içine yerleştirilen ve hemen hemen sabit bir düzeyde (CV %4.07) Hİ bırakan ve Alkan İndikatör Kapsül (AİK) adı verilen düzenek kullanılabilmektedir [13].

Literatürde rastlanılan alkan indikatör çalışmaları daha çok küçük ruminantlarda ve ahırda kontrollü yemleme ile yapılmıştır. Büyük ruminantlarla ve özellikle merada otlayan hayvanlarla yapılan çalışmalar oldukça az sayıdadır. Bu çalışmanın amacı, alkan indikatör kapsülleri kullanarak alkan indikatör metodunun mera şartlarında büyük ruminantlarda kullanılabilirliğini ve saat 10:00 ve 16:00'da alınacak numunelerle doğru KMTt ve KMSt yapılabilirliğini tespit etmek ve Kars şartlarında merada otlayan hayvanların sezonun değişik dönemlerinde meradan ne kadar kuru madde tüketebildiklerini alkan indikatör metodu kullanarak araştırmaktır.

MATERYAL ve METOT

Çalışma iki deneme halinde yürütülmüştür. Her iki deneme öncesi denemelerde kullanılan hayvanların gerekli aşılamaları ve parazit ilaçlamaları yapılmıştır. Birinci denemede 5 adet erkek dana (ort. CA 244.5±2.3 kg) patozdan geçirilmiş kuru ot ile günlük eşit 2 öğün halinde saat 09:00 ve 18:00'da olmak üzere ahırda beslenmiştir. Danalara ortalama canlı ağırlığa göre KM ihtiyacı + %10 olacak şekilde kuru ot verilmiştir. Hayvanlara verilen, artan kuru ot ile üretilen dışkı miktarı günlük kayıt edilerek klasik dışkı toplama yöntemi ile gerçek KMT ve KMS belirlenmiştir. Deneme, 7 gün alıştırma dönemi sonrası hayvanlara AİK'in yutturulması ile başlamış ve 20 gün devam etmiştir. Denemenin 8-14. günleri arasında her gün saat 10:00 ve 16:00 civarında üretilen dışkılardan numune alınmıştır. Üretilen dışkılar darası alınmış büyük plastik bidonlara biriktirilmiş ve ertesi sabah saat 09:00'da günlük dışkı miktarı belirlenmiştir. Böylelikle toplam dışkı toplama yöntemi ile gerçek KMS'i belirlenmiştir. Toplanan dışkılar kürekle iyice karıştırıldıktan sonra 100-150 g civarında karma numune alınmış ve -20°C'de saklanmıştır. Her 3 dışkı numunesi de kullanılarak ayrı ayrı KMTt ve KMSt'leri yapılmıştır.

Hİ olarak kullanılan alkan C_{32} ve C_{36}'nın dozlanması için AİK'ler kullanılmıştır. Kapsüllerde alkan C_{32} ve C_{36} karışık ve preslenmiş olarak yer almaktadır. Kapsül özel sondası ile hayvana yutturulmakta ve rumen içine yerleştirildikten sonra 18-22 gün süreyle alkan C_{32} ve C_{36} salınımı yapmaktadır. Denemede kullanılan kapsüllerin salınım raporu ahır denemesinde kullanılan kapsüllerde alkan C_{32} ve C_{36} için sırasıyla 200.0 mg/gün ve 200.5 mg/gün olarak ve mera denemesinde kullanılan kapsüllerde alkan C_{32} ve C_{36} için sırasıyla 199.3 mg/gün ve 198.4 mg/gün olarak bildirilmiştir [13].

İkinci deneme ise merada otlayan hayvanlar kullanılarak 20'şer günlük aşağıda verilen tarihlerde 5 dönem halinde yürütülmüştür:

1. dönem: 01 Haziran - 20 Haziran 2006
2. dönem: 21 Haziran - 10 Temmuz 2006
3. dönem: 11 Temmuz - 30 Temmuz 2006
4. dönem: 31 Temmuz - 19 Ağustos 2006
5. dönem: 20 Ağustos - 09 Eylül 2006

Her dönemin ilk günü hayvanlara indikatör kapsüller yutturulmuş ve 8-14. günler arasında saat 10:00 ve 16:00 civarında hayvanların otlama esnasında ürettikleri dışkılardan dışkı numunesi alınmıştır. Bu denemede toplam 10 adet erkek dana (ort. CA 164.9±1.5 kg) canlı ağırlıkları eşit olacak şekilde eşit 2 gruba ayrılmış ve her bir dönemde farklı grup hayvan kullanılmıştır. Böylece bir dönemde kullanılan hayvanlar takip eden dönemde kullanılmamış daha sonraki dönemde kullanılmış ve aradan geçen sürede hayvanların dışkısında önceki Hİ'ün kalıntısı kalmamıştır.

Taze halde 100-150 g civarında alınan dışkı numuneleri -20°C'de derin dondurucuda saklanmış ve daha sonra etüvde 60°C'de ağırlıkları sabit oluncaya kadar kurutularak 0.5 mm elekli değirmende öğütülmüştür.

Ahır denemesinde kullanılan kuru ot numuneleri ile her bir mera döneminde 8-14. günlerde merada otlayan hayvanlar takip edilerek hayvanların otladığı yerlerden henüz hayvanların koparmadığı otlardan alınan taze ot numunelerinin besin madde analizleri AOAC'de [14] belirtilen yöntemlere göre yapılmıştır. Mera döneminde yukarıda açıklanan örnek alımından başka, yeni bir uygulama olarak merada otlayan hayvanlar otlama esnasında yakalanarak yerden kopardıkları ve henüz yutmadıkları ağızdaki otlardan da numune alınarak bitki alkan konsantrasyonu analizi için kullanılmıştır. Ayrıca hayvanlar gözlenerek yerden koparttıkları ottan yerde kalanlardan da numuneler alınmıştır.

Alkan analizleri Gaz Kromatografi (GK) cihazında (Agilent 6890 N, Agilent Tech., USA), Unal ve Garnsworthy'nin [15] belirttiği metoda göre yapılmış olup, analizlerde C_{34} standart olarak kullanılmıştır.

Alkan İndikatör Metodu kullanılarak (C_{33}:C_{32} ya da C_{33}:C_{36} çifti ile ayrı ayrı) yapılan KMTt'leri aşağıdaki formüle göre hesaplanmıştır:

$$KMTt = \frac{\frac{\text{Dışkı } C_{33}}{\text{Dışkı } C_S} * \text{Doz}_S}{\text{Ot } C_{33} - \frac{\text{Dışkı } C_{33}}{\text{Dışkı } C_S} * \text{Ot } C_S}$$

Alkan indikatör metodunda KMTt için kullanılan formül,

KMTt: Kuru madde tüketimi tahmini (kg KM/gün)

$_S$: Çift zincirli alkan C_{32} ya da C_{36}

Ot C_{33} ve Dışkı C_{33}: Alkan C_{33}'ün kaba yem ve dışkıdaki miktarları (mg/kg KM)

Ot C_S ve Dışkı C_S: Alkan C_{32} ya da Alkan C_{36}'nın kaba yem ve dışkıdaki miktarları (mg/kg KM)

Doz$_S$: Dışardan doz olarak verilen alkan C_{32} ya da C_{36} miktarı (yaklaşık 0.200 g/gün)

KMS ve KMSt ile İndikatör geri alınabilirliğini (GA) hesaplamada kullanılan formüller aşağıda verilmiştir:

$$KMS = \frac{\text{Kuru madde tüketimi - toplam dışkı KM'si}}{\text{Kuru madde tüketimi}}$$

$$KMSt = \frac{\text{g indikatör/kg dışkı - g indikatör/kg yem}}{\text{g indikatör/kg dışkı}}$$

$$GA = \frac{(\text{g indikatör/kg dışkı} * \text{toplam dışkı KM'si})}{(\text{g indikatör/kg yem} * \text{toplam KMT}) + \text{Doz}}$$

Toplam dışkı toplama yöntemi ile bulunan gerçek KMS'i sadece ahır denemesinde hesap edilebilmiştir. Mera çalışmasında toplam dışkı toplanmadığı için KMS'i hesap edilememiş ve indikatörle KMSt'i yapılmıştır.

Verilerin istatistik analizleri için SPSS 19 (for MacOS X) programı kullanıldı [16].

BULGULAR

Ahır ve merada hayvanlar tarafından tüketilen otların besin madde ve alkan içerikleri *Tablo 1*'de verilmiştir. Yem numunelerinin analizinde alkan C_{36} tespit edilememiştir.

Ahır denemesindeki gerçek KMT ile saat 10:00 ve 16:00'da alınan dışkı numuneleri ve karma numuneler kullanılarak hesap edilen KMTt ortalamaları *Tablo 2*'de, toplam dışkı toplama yöntemi ile belirlenen gerçek KMS ile alkan C_{32}, C_{33} ve C_{36} kullanılarak hesap edilen KMSt ortalamaları *Tablo 3*'te, hesap edilen alkan C_{32}, C_{33} ve C_{36} geri alınabilirlikleri *Tablo 4*'te verilmiştir.

Mera denemesinde saat 10:00 ve 16:00'da alınan dışkı numunelerindeki alkan C_{33}:C_{32} ve C_{33}:C_{36} çiftleri kullanılarak hesap edilen KMTt ortalamaları *Tablo 5*'te, alkan C_{32}, C_{33} ve C_{36} kullanılarak hesap edilen KMSt ortalamaları *Tablo 6*'da verilmiştir.

Mera denemesinde kullanılan hayvanlara ait canlı ağırlık ortalamaları ve canlı ağırlık artışı ortalamasına ait sonuçlar *Tablo 7*'de verilmiştir.

Tablo1. *Denemelerde kullanılan otların besin madde (% KM) ve alkan (mg/kg KM) içerikleri*
Table1.*Chemical composition (% DM) and alkane content(mg/kg DM)of forage used in the experiments*

Deneme	KM	HP	HY	HS	HK	C_{32}	C_{33}	C_{36}
Ahır	88.24	9.55	2.88	30.65	8.01	4.66	15.99	-
Mera 1	26.48	17.72	1.78	26.95	9.15	4.78	35.95	-
Mera 2	25.87	16.24	1.83	29.08	9.38	4.81	36.28	-
Mera 3	27.72	13.48	2.11	31.85	9.04	4.97	36.71	-
Mera 4	34.79	11.61	2.28	32.49	9.67	5.34	37.45	-
Mera 5	35.73	9.77	2.21	35.05	8.95	5.25	36.82	-

KM: *Kuru madde,* ***HP:*** *Ham protein,* ***HY:*** *Ham yağ,* ***HS:*** *Ham selüloz,* ***HK:*** *Ham kül*

Tablo 2. *Ahır denemesinde gerçek KMT ile hesap edilen KMTt'i sonuçları (kg/gün, X±Sx)*
Table 2. *Actual DMI and calculated DMIe results in indoor experiment(kg/day, X±Sx)*

İstatistik	KMT	KMTt $C_{33:32}$			KMTt $C_{33:36}$		
		10:00	16:00	Karma	10:00	16:00	Karma
NS	6.862±0.030	6.792±0.088	6.806±0.064	6.827±0.062	6.773±0.063	6.816±0.080	6.814±0.071
Fark		-0.070	-0.056	-0.035	-0.089	-0.046	-0.048
R^2	-	0.66 [1]	0.73 [2]	0.86 [3]	0.80 [4]	0.42 [5]	0.63 [6]

KMT: *Kuru Madde Tüketimi,* ***KMTt:*** *Kuru Madde Tüketimi Tahmini,* [1] ***KMT*** *= 4.97 + 0.279 KMTt32,* [2] ***KMT*** *= 4.13 + 0.401 KMTt32,* [3] ***KMT*** *= 3.79 + 0.451 KMTt32,* [4] ***KMT*** *= 3.95 + 0.430 KMTt36,* [5] ***KMT*** *= 5.20 + 0.243 KMTt36,* [6] *KMT 4.59 + 0.334 KMTt36,* ***NS:*** *Değerler arasında istatistiki açıdan önem yoktur*

Tablo 3. *Ahır denemesinde gerçek KMS ile hesap edilen KMSt sonuçları (%, X±Sx)*
Table 3.*Actual DMD and calculated DMDe results in indoor experiment(%, X±Sx)*

KMS (X±Sx)	KMSt32 (X±Sx)			KMSt33 (X±Sx)			KMSt36 (X±Sx)		
	10:00	16:00	Karma	10:00	16:00	Karma	10:00	16:00	Karma
0.62±0.003 [bc]	0.65±0.006 [a 1]	0.60±0.009 [cde 2]	0.62±0.006 [bc 3]	0.62±0.004 [b 4]	0.64±0.003 [a 5]	0.61±0.008 [bcd 6]	0.59±0.004 [f 7]	0.59±0.005 [ef 8]	0.60±0.006 [def 9]

KMS: *Kuru Madde Sindirilebilirligi,* ***KMSt:*** *Kuru Madde Sindirilebilirligi Tahmini,* [1] ***KMS*** *= 0.524 + 0.147 KMSt32,* [2] ***KMS*** *= 0.620 - 0.000 KMSt32,* [3] *KMS = 0.449 + 0.278 KMSt32,* [4] ***KMS*** *= 0.425 + 0.312 KMSt33,* [5] ***KMS*** *= - 0.0200 + 1.00 KMSt33,* [6] ***KMS*** *= 0.489 + 0.214 KMSt33,* [7] ***KMS*** *= 0.200 + 0.714 KMSt36,* [8] ***KMS*** *= 0.522 + 0.167 KMSt36,* [9] ***KMS*** *= 0.620 - 0.000 KMSt36,* [a,b,c,...] *Aynı satırda farklı harf taşıyan değerler arasında istatistiki olarak fark önemlidir (P<0.05)*

Tablo 4. *Ahır denemesinde alkan C_{32}, C_{33} ve C_{36}'nın geri alınabilirlikleri (GA, %, X±Sx)*
Table 4.*Alkane C_{32}, C_{33} and C_{36} recoveries in indoor experiment(R, %, X±Sx)*

GA_{32}	GA_{33}	GA_{36}
0.88±0.009	0.88±0.010	0.89±0.011

TARTIŞMA ve SONUÇ

Mera denemesinde, otlardaki alkan konsantrasyonlarının belirlenmesi amacıyla alınan 3 çeşit numunedeki (hayvanların otladığı alanlarda henüz hayvanlar tarafından koparılmamış olan ot, hayvanların otlama esnasında

Tablo 5. *Mera denemesinde hesap edilen KMTt sonuçları (kg/gün, X±Sx)*
Table 5. *Calculated DMIe results in pasture experiment(kg/day,X±Sx)*

Deneme	Saat 10:00 Numunesi		Saat 16:00 Numunesi	
	$KMTt_{33:32}$	$KMTt_{33:36}$	$KMTt_{33:32}$	$KMTt_{33:36}$
1. dönem	5.256±0.383[a]	5.191±0.353[b]	5.170±0.383[a]	5.187±0.399[a]
2. dönem	4.150±0.092[b]	4.176±0.101[c]	4.033±0.120[b]	4.061±0.139[b]
3. dönem	5.806±0.228[a]	5.859±0.227[a]	5.819±0.249[a]	5.751±0.224[a]
4. dönem	5.580±0.222[a]	5.608±0.240[ab]	5.563±0.272[a]	5.591±0.308[a]
5. dönem	4.321±0.263[b]	4.319±0.284[c]	4.274±0.271[b]	4.259±0.228[b]

[a,b,c,...] *Aynı sütunda farklı harf taşıyan değerler arasında istatistiki olarak fark önemlidir (P<0.001)*

Tablo 6. *Mera denemesinde hesap edilen KMSt sonuçları (%, X±Sx)*
Table 6. *Calculated DMDe results in pasture experiment(%, X±Sx)*

Deneme	Saat 10:00 Numunesi			Saat 16:00 Numunesi		
	$KMSt_{32}$	$KMSt_{33}$	$KMSt_{36}$	$KMSt_{32}$	$KMSt_{33}$	$KMSt_{36}$
1. dönem	0.79±0.003[aA]	0.77±0.003[abA]	0.76±0.004[abA]	0.76±0.006[abA]	0.75±0.014[bA]	0.74±0.016[bA]
2. dönem	0.69±0.016[C]	0.67±0.016[B]	0.67±0.015[C]	0.69±0.006[C]	0.66±0.010[B]	0.68±0.012[B]
3. dönem	0.78±0.016[A]	0.75±0.014[A]	0.74±0.017[AB]	0.77±0.015[A]	0.74±0.018[A]	0.74±0.017[A]
4. dönem	0.74±0.021[aB]	0.70±0.017[abB]	0.72±0.018[abB]	0.74±0.010[aB]	0.69±0.010[bB]	0.71±0.013[abAB]
5. dönem	0.50±0.029[abD]	0.48±0.025[abC]	0.45±0.012[bD]	0.52±0.013[aD]	0.50±0.016[abC]	0.47±0.035[bC]

[a,b,c,...] *Aynı satırda farklı harf taşıyan değerler arasında istatistiki olarak fark önemlidir (P<0.05),* [A,B,C,...] *Aynı sütunda farklı harf taşıyan değerler arasında istatistiki olarak fark önemlidir (P<0.001)*

Tablo 7. *Merada otlayan hayvanların canlı ağırlık (CA) ve canlı ağırlık artışı (CAA) ortalamaları (X±Sx)*
Table 7. *Mean live weight (LW) and live weight gain (LWG) of grazing animals (X±Sx)*

Parametre	Tarih				
	01 Haziran	21 Haziran	11 Temmuz	31 Temmuz	20 Ağustos
CA (kg)	164.88±1.52	171.38±3.01	188.63±3.32	201.00±3.25	216.00±5.82
CAA (kg/gün)	0.64±0.07				

yakalanarak yerden kopardıkları ve henüz yutmadıkları ağızdaki ot ve merada otlayan hayvanlar gözlenerek hayvanların yerden koparttıkları ottan yerde kalan ot) alkan konsantrasyonlarında önemli bir fark bulunmamıştır. Çalışmada en yüksek alkan konsantrasyonu, hem ahır denemesinde kullanılan kuru otta (15.99 mg/kg KM) hem de mera otlarında (35.95-37.45 mg/kg KM) C_{33} için ölçülmüştür. Bu sonuç, bitkilerde C_{33}'ün yüksek konsantrasyonda olduğunu bildiren yayınlarla uyumludur [17-19]. Benzer bir çalışmada C_{33} alkanı kaba yemde 44 mg/kg KM olarak bulunurken C_{36}'nın tespit edilemediği bildirilmiştir [18]. Benzer şekilde çalışmamızda yem numunelerinde C_{36} tespiti yapılamamıştır. Alkan C_{36} bitkilerde çok düşük düzeyde olup genellikle analizlerde tespit edilememektedir. Bununla birlikte, bitkilerde alkan C_{36} konsantrasyonu bildiren çalışmalarda da mevcuttur [17,20,21].

Alkan C_{33}'ün konsantrasyonu ahır denemesinde kullanılan kuru otta, meradaki taze otlara nazaran daha düşük bulunmuştur. Çeşitli araştırmalarda bitkilerdeki alkan içeriğinin bitki türüne [8,19,22], bölümüne [23-25] ve vejetasyon dönemine göre [26] değişkenlik gösterdiği bildirilmektedir. Çalışmamızda da alkan C_{32} konsantrasyonu hem kuru otta, hem taze çayır otunda aynı olmakla birlikte, alkan C_{32} ve C_{33} konsantrasyonlarının vejetasyon dönemi sonuna doğru artış gösterdiği tespit edilmiştir.

Bazı araştırıcılar tarafından [20] rasyon C_{33} alkan konsantrasyonunun 10 mg/kg KM düzeyinin altında olması durumunda, geri alınabilirlik hesaplamalarının dolayısıyla KMTt'lerinin iyi olmayacağı bildirilmektedir. Casson ve ark.[27] ise güvenilir tahminler için bitkideki alkan C_{33} konsantrasyonunun 50 mg/kg düzeyinin üzerinde olması gerektiğini bildirmektedirler. Çalışmamızda alkan C_{33} konsantrasyonu 50 mg/kg düzeyinin altında olmasına rağmen (ahırda denemesinde 15.99, mera otlarında 35.95-37.45 mg/kg KM) elde edilen KMTt'leri oldukça başarılıdır.

Ahır denemesinde öncelikle AİK'lerin alkan indikatör metodunda dozlama için kullanılabilirliğini tespit etmek amaçlanmıştır. AİK'ler dışındaki dozlama yöntemlerinde dozlama her gün ve günde 1 ya da 2 kez yapılmaktadır. Bu her gün fazlaca iş gücü gerektirmesinin yanında indikatörün dışkıdaki konsantrasyonunda gün içi dalgalanmalara da yol açmakta ve sağlıklı sonuç almayı engellemektedir [28]. AİK'lerin bu dezavantajları ortadan kaldırdığı son zamanlarda yapılan çalışmalarda ortaya konmuştur [18,20,29]. Oliveira ve ark.[25] AİK kapsüllerdeki alkan içeriğinin ve günlük salınım düzeyinin doğru ölçülmesi durumunda AİK kapsüller kullanılarak başarılı KMTt'leri yapılabileceğini bildirmektedir. Çalışmada elde edilen başarılı sonuçlar da bunu doğrulamaktadır.

Her iki denemede KMTt'leri gerçek KMT'ne yakın hesap edilmiş olup, KMT ve KMTt'leri arasında istatistiki olarak fark gözlenmemiştir. Bu sonuçlar, AİK'lerin dozlama amacıyla kullanılması durumunda alkan indikatörlerin dışkıdaki konsantrasyonlarındaki gün içi dalgalanmaları ortadan kaldırdığını ya da oldukça düşürdüğünü, her gün aynı saatte olmak şartıyla sabah ya da öğleden sonra alınacak dışkı numuneleri ile başarılı KMTt'lerinin yapılabileceğini göstermektedir. Çalışmamızla benzer şekilde Berry ve ark.[18] da saat 06:30, 13:00 ve 20:30'da numune alımının sonuçları etkilemediğini bildirmiştir. Hendricksen ve ark.[20] ise AİK'lerden salınan alkan C_{36}'nın dışkıdaki değişik saatlerdeki konsantrasyonunun çok az farklılık gösterdiğini bildirmiştir. Bu konuda kesin bir kanıya varmak için, gün içinde daha fazla numune alımının yapıldığı yeni çalışmalara ihtiyaç vardır.

Alkan indikatör metodu ile yapılan çalışmalarda genellikle alkan C_{33}:C_{32} çifti ile daha başarılı sonuçlar alındığı bildirilmektedir [17,18,29]. Ancak bu çalışmanın sonuçları alkan C_{33}:C_{36} çifti ile de başarılı sonuçlar alınabileceğini göstermiştir. Bununla birlikte Olivan ve ark.[21] alkan C_{23}:C_{24} çifti ile daha başarılı sonuç aldıklarını bildirmişlerdir.

Alkanların geri alınabilirliklerinin hesaplanabilmesi için toplam dışkı KM'sinin bilinmesi gerekmektedir. Çalışmamızda sadece ahır denemesinde toplam dışkı toplama yöntemi uygulandığı için alkanların geri alınabilirlikleri (GA) hesap edilebilmiştir. Tüm indikatör çalışmalarında GA önemli bir problemdir. Alkan metodunun bir çift indikatör yöntemi olması nedeniyle, KMTt'de kullanılan alkan çiftinin geri alınabilirliklerinin birbirine yakın olması yapılan tahminlerin doğruluk oranını yükseltmektedir [6,7,30]. Berry ve ark.[18] çalışmalarında alkan C_{31}'in GA'nin C_{32}'den düşük olduğunu bu nedenle KMTt'lerinin gerçek KMT'nin altında hesap edildiğini, buna karşın C_{32} ve C_{33}'ün GA'lerinin birbirine yakın olduğunu dolayısıyla daha başarılı KMTt yapıldığını bildirmiştir. Çalışmamızda da alkanların GA'leri birbirine oldukça yakın bulunmuştur.

Ahır denemesinde KMS'ne en yakın KMSt'i sonuçları kullanılan alkana ve numune alım zamanına göre değişkenlik göstermiştir. En yakın KMSt karma numunede C_{32} alkanı ile ve sabah numunesinde C_{33} alkanı ile yapılan hesaplamalardan elde edilmiştir. Bununla birlikte karma

numunelerde yapılan KMSt ortalamaları C_{33} ve C_{36} alkanı için sırasıyla 0.61 ve 0.60 olarak hesaplanmış olup KMS ne yakın bulunmuştur. Tüm numunelerde alkan C_{36} ile yapılan KMSt lerinin gerçek KMS değerinin altında olduğu gözlenmiştir. Berry ve ark.[18] C_{33} alkanı kullanarak yaptıkları sindirilebilirlik hesaplamalarında oldukça başarılı sonuçlar elde etmişlerdir. Hendricksen ve ark.[20] sığırlarda yaptıkları beslenme çalışmasında gerçek KMS oranını %49.1 ve KMSt oranını %52.6 olarak belirlemiş olup, en iyi KMSt'nin C_{33} alkanı ile yapılan hesaplamalarla elde edildiğini bildirmişlerdir. Çalışmamızda gerçek KMS'inden (0.62) en farklı sonuç 0.65 ile sabah numunesinde alkan C_{32} ile yapılan KMSt hesaplamalarından alınmıştır. KMS ve KMSt arasındaki fark (0.03) bahsi geçen çalışmanın sonuçları ile karşılaştırıldığında elde edilen KMSt gerçek KMS'e daha yakın bir sonuçtur. Bu sonuç alkan C_{32} ile de başarılı KMSt yapılabileceğini göstermektedir.

Alkan indikatör metodunda KMTt için hayvanın tükettiği kaba yemdeki alkan konsantrasyonları hesaplamalar için çok önemli olup, bu numunelerin tüketilen kaba yemi en iyi şekilde temsil etmesi gerekmektedir. Kapalı mekânda kontrollü yemleme yapıldığı için söz konusu numunenin alımı kolay olmaktadır. Mera çalışmalarında ise hayvanlar bitkiyi seçerek tüketmektedirler. Dolayısıyla rastgele el ile meradan alınacak numunelerin hayvanın tükettiği otları tam olarak temsil edememe ihtimali vardır. Bu da hesaplamalarda yanlışlığa yol açabilmektedir. Geçmişte geleneksel indikatör yöntemlerinde (lignin, silika, ADF gibi) özafagus fistüllü hayvanlar kullanılmıştır. Ancak bu yöntemin sadece homojen meralarda kullanılabileceği ve günlük alınması gereken numune sayısının fazla olması gerektiği bildirilmiştir [8,31]. Bu çalışmada ise bir yenilik olarak el ile alınan numuneye ek olarak, hayvanların otlama esnasında yakalanarak yerden kopardığı ve henüz yutmadığı ağızdaki otlardan numuneler alınmış ve bitki alkan konsantrasyonu analizi için kullanılmıştır. Yerden alınan numuneler ise otlayan hayvanlar gözlemlenerek hayvanın kopardığı otun yerde kalan bölümlerinden alınmıştır. Bu numunelerde alkan konsantrasyonları bir farklılık göstermemiştir.

Beş dönem halinde yürütülen mera çalışmasında hayvanların ortalama olarak (4 KMTt'i ortalamaları) dönemlere göre sırasıyla 5.201, 4.105, 5.809, 5.585 ve 4.293 KM kg/gün düzeyinde taze mera otu tükettikleri hesap edilmiştir. KMT'nin 2. ve 5. dönemlerde ani bir düşüş gösterdiği tespit edilmiştir. Paralel şekilde KMS'liğinde de aynı dönemlerde düşüş olduğu gözlenmiştir. Mera 2 döneminde tüketimin az olmasının muhtemel sebebi, yüksek seyreden hava sıcaklıkları olabilir. Bu döneme rastlayan tarihlerde hayvanlar, saat 11:00-15:00 arasında meraya salınmayıp gölgeliği bulunan padoklarda tutulmuşlardır. Yine son dönemde, meranın yeşilliğini kaybetmesi ve bitkinin lignifikasyonunun artması sonucu ot tüketiminin ve sindirilebilirliğinin azaldığı düşünülmektedir.

Mera denemesinde toplam dışkı toplama yöntemi uygulanamadığı için gerçek KMS tespiti yapılamamıştır. KMSt'leri dönemlere göre ortalama olarak sırasıyla 0.76, 0.68, 0.75, 0.72 ve 0.49 olarak hesap edilmiştir. İkinci ve üçüncü dönemlerde tüm numunelerle yapılan KMSt'lerinin birbirine yakın olduğu ve aralarında istatistiki fark olmadığı gözlenmiştir. Yine tüm dönemlerde aynı alkanla yapılan KMSt'lerinin sabah ve akşam numuneleri arasında istatistiki olarak anlamlı bir fark olmadığı tespit edilmiştir. Bu sonuç numune alım saatinin KMSt'ni etkilemediğini göstermekle birlikte, kesin karar verebilmek için gün içerisinde değişik saatlerde daha fazla numune alınarak yeni çalışmalar yapılması gerekmektedir. Tüm dönemlerde Alkan C_{33} ve C_{36} ile yapılan KMSt'lerinin birbirine yakın olduğu gözlenmiştir. Alkan indikatör çalışmalarında KMSt'lerinde, bitkide hazır olması ve genellikle tüm bitkilerde de konsantrasyonunun yüksek olması nedeniyle alkan C_{33} daha çok kullanılmıştır [8,18,20]. Bunun bir diğer nedeni de alkan C_{33}'ün GA'nin yüksek olmasıdır. Mayes ve ark.[6] ve Dove ve Mayes [8] de KMSt'i için en doğru sonuçların alkan C_{33} ile alındığını bildirmişlerdir. Ayrıca alkan C_{36} ile daha başarılı sonuçlar alındığını bildiren çalışmalar da literatürde mevcuttur [17]. Çalışmamızda C_{33} ve C_{36} alkanlarıyla yapılan KMSt lerinin birbirine yakın olması bahsi geçen araştırmaları destekler niteliktedir.

Haziran ve Ağustos aylarında 3 ay boyunca elde edilen KMTt'leri hayvanların kuru madde ihtiyaçlarını meradan karşılayabildiğini göstermektedir. Eylül ayından sonra mera kalitesinin azalmasıyla *(Tablo 1)* ve denemenin son döneminde KMSt'lerinde gözlenen ani düşüş *(Tablo 6)* bu dönemde hayvanların meradan yeterince faydalanamadığını göstermektedir. Görsel olarak da Ağustos sonu ve Eylül ayından sonra meraların yeşil rengini kaybedip sararması bunu doğrular niteliktedir. Halbuki, Kars ve çevresinde hayvan yetiştiricilerinin genel olarak 15 Mayıs-30 Kasım tarihlerini içerecek şekilde hayvanlarını merada tuttuğu gözlenmektedir. Haziran ayından önce hayvanların meraya salınması otlar yeterince uzamadığı için mera kalitesini düşürmektedir ve ayrıca hayvanlar merada yeterince ot zaten bulamamaktadır. Eylül ayından sonra da merada tutulan hayvanlara ise yem takviyesi yapılması gerektiğini bu çalışma sonuçları ortaya koymaktadır. Çünkü çalışmanın yapıldığı son döneme rastlayan 20 Ağustos - 09 Eylül tarihlerinde, dönem başında hayvanların 216 kg olduğu ve hayvanların yaşama payı besin madde ihtiyaçlarını karşılayabilmek için KMT'lerinin canlı ağırlıklarının %2.5'i olması gerektiği düşünülürse günlük 5.4 kg/gün KM tüketmeleri gerekmektedir. Ancak bu dönemde hayvanların ortalama 4.293 kg/gün KM tüketmişlerdir.

Mera çalışmasında hayvanların deneme başı ortalama canlı ağırlıkları 164.88±1.52 kg ve deneme sonu canlı ağırlıkları 216.00±5.82 kg olarak tespit edilmiştir. Tüm deneme boyunca hayvanlar günlük 0.64±0.07 kg/gün canlı ağırlık artışı sağlamışlardır. KMTt'leri tüm dönemlerin ortalaması 4.999 kg/gün olarak hesap edilmiştir. Deneme başı ve sonu canlı ağırlıklarının ortalaması 190.4 kg ve 1 yaşlı danalar için ortalama KMT canlı ağırlıklarının %2.5'i

alınırsa hayvanların normal bir tüketim gösterdiği görülmektedir. Hendricksen ve ark.[20] ise 215 kg ağırlığında 1.5 yaşlı erkek dana ve kuru ot ile ahırda yapmış oldukları iki çalışmada, gerçek KMT'lerini 3.45 ve 3.24 kg/gün olarak bildirmişlerdir. Çalışmamızda elde edilen KMTt'lerinin bahsi geçen çalışmada elde edilen sonuçlara göre oldukça yüksek olduğu görülmektedir. Bu durum bahsi geçen çalışmalarda hayvanların kuru ot ile beslenmesine bağlanabileceği gibi hayvanların otlamayıp kapalı mekânda beslenmesine de bağlanabilir.

Sonuç olarak ahır denemesinde elde edilen veriler AİK'lerin alkan indikatör metodunda dozlama amacıyla başarıyla kullanılabileceğini göstermektedir. Mera çalışmasında hayvanların Haziran, Temmuz ve Ağustos aylarında meradan kuru madde ihtiyaçlarını karşılayabildikleri, ancak Eylül ayı başından sonra ihtiyaçların tam olarak karşılanamadığı ortaya çıkmaktadır. Bu konuda kesin karar verebilmek için Eylül, Ekim hatta Kasım ayını da içine alacak yeni çalışmalara ihtiyaç vardır. Bu hipotez doğrulanırsa Eylül-Kasım aylarında merada tutulacak hayvanlara yarı entansif besi şeklinde yem takviyesi gerekecektir. Böylece bölgede hayvansal üretim daha verimli bir şekilde yapılabilecektir.

KAYNAKLAR

1. Ordakowski AL, Kronfeld DS, Holland JL, Hargreaves BJ, Gay LS, Harris PA, Dove H, Sklan D: Alkanes as internal markers to estimate digestibility of hay or hay plus concentrate diets in horses. *J Anim Sci*, 79, 1516-1522, 2001.

2. Lippke H: Estimation of forage intake by ruminants on pasture. *Crop Sci*, 42, 869-872, 2002.

3. Gudmundsson O, Thorhallsdottir AG: Evaluation of n-alkanes for intake and digestibility determination in horses. Techniques for investigating intake and ingestive behaviour by farm animals. *IXth European Intake Workshop,* 18-20 November, Devon-UK, 1-4, 1998.

4. Hatt JM, Gisler R, Mayes RW, Lechner-Doll M, Clauss M, Liesegang A, Wanner M: The use of dosed and herbage n-alkanes as markers for the determination of intake, digestibility, mean retention time and diet selection in galapagos tortoises *(Geochelone nigra). Herpetol J*, 12, 45-54, 2002.

5. Ünal Y: Alkan indikatör tekniği kullanilarak yem tüketimi ve sindirilebilirlik tahmini. *Lalahan Hay Araşt Enst Derg*, 44 (2): 53-59, 2004.

6. Mayes RW, Lamb CS, Colgrove PM: The use of dosed and herbage n-alkanes as markers for the determination of herbage intake. *J Agr Sci, Cambridge*, 107, 161-170, 1986.

7. Dillon P, Stakelum G: The use of n-alkanes and chromic oxide as markers for determining feed intake, faecal output and digestibility in dairy cows. **In,** *Proceedings of the 12th General Meeting of the European Grassland Federation,* Madrid-Spain, 154-158, 1988.

8. Dove H, Mayes RW: The use of plant wax alkanes as markers substances in studies of the nutrition of herbivores. A review. *Austr J Agr Res*, 42, 913-952, 1991.

9. Unal Y, Garnsworthy PC, Gorton P: The use of n-alkanes for prediction of intake in dairy cows. **In,** *Proceedings of the British Society of Animal Science,* March 1997, Scarborough-UK, 137, 1997.

10. Mayes RW, Lamp CS, Colgrove PM: Determination of herbage intake of sucking lambs using long-chain n-alkanes as markers. *Anim Prod*, 42, 457, 1986.

11. Dove H, Freer M, Foot JZ: Alkane capsules for measuring pasture intake. *Proc Nutr Soc Aust*, 13, 131, 1988.

12. Unal Y, Garnsworthy PC: An alternative method of administering alkane markers for estimating intake in dairy cows. **In,** *Proceedings of the British Society of Animal Science,* March 1998, Scarborough-UK, 91, 1999.

13. CAPTEC: http://www.captec.info/web/captecinfo/captecinfohp.nsf/web/index.html, *Accessed:* 28.07.2007.

14. AOAC: Official Methods of Analysis. 15th ed., Association of Official Analytical Chemists, Arlington, VA, 1990.

15. Unal Y, Garnsworthy PC: Estimating of intake and digestibility of forage-based diets in group-fed dairy cows using alkanes as markers. *J Agr Sci*, 133 (4): 419-426, 1999.

16. SPSS 19 (for MacOS X): SPSS Inc, New York, USA.

17. Gedir JV, Hudson RJ: Estimating dry matter digestibility and intake in wapiti *(Cervus elaphus canadensis)* using the double n-alkane ratio technique. *Small Rumin Res*, 36, 57-62, 2000.

18. Berry NR, Scheeder MRL, Sutter F, Kröber TF, Kreuzer M: The accuracy of intake estimation based on the use of alkane controlled-release capsules and faeces grab sampling in cows. *Ann Zootech*, 49, 3-13, 2000.

19. Ferreira LMM, Garcia U, Rodrigues MAM, Celaya R, Dias-da-Silva A, Osoro K: Estimation of feed intake and apparent digestibility of equines and cattle grazing on heathland vegetation communities using the n-alkane markers. *Livest Sci*, 110, 46-56, 2007.

20. Hendricksen RE, Reich MM, Roberton RF, Reid DJ, Gazzola C, Rideout JA, Hill RA: Estimating the voluntary intake and digestibility of buffel-grass and lucerne hays offered to Brahman-cross cattle using n-alkanes. *Anim Sci*, 74, 567-577, 2002.

21. Olivan M, Ferreira LMM, Celaya R, Osoro K: Accuracy of the n-alkane technique for intake estimates in beef cattle using different sampling procedures and feeding levels. *Livest Sci*, 106, 28-40, 2007.

22. Malossini F, Piasentier E, Bovolenta S: N-alkane content of some forages. *J Sci Food Agr*, 53, 405-409, 1990.

23. Dove H, Mayes RW: Plant wax components: A new approach to estimating intake and diet composition in herbivores. *J Nutr,* 126, 13-26, 1996.

24. Genro TGM, Thiago LRL De S, Prates ER, Herrero M, Sabatel V: Determination of n-alkanes at different stratum heights in a pasture of *Panicum maximum* cv. Mombaca, **In,** *Proc 19th Int Grassl Congr,* February 2001, Piracicaba-Brazil (Abstr.), 401, 2001.

25. Oliveira DE, Manella MQ, Tedeschi LO, Silva SC, Lanna DPD: N-Alkanes to estimate voluntary forage intake of cattle using controlled-release capsules. *Sci Agric (Piracicaba, Braz)*, 65 (3): 230-238, 2008.

26. Vulich SA, Hanrahan JP, O'Riordan EG: Pasture sampling for the estimation of herbage intake using n-alkanes: Evaluation of alternative sampling procedure. *Irish J Agr Food Res*, 32, 1-11, 1993.

27. Casson T, Rowe JB, Thorn CW, Harris D: The use of natural n-alkanes in medic and clover as indigestible markers. *Proc Aust Soc Anim Prod,* 18, 462 (Abstr.), 1990.

28. Vulich SA, Hanrahan JP: Sources of variation associated with the n-alkane technique for estimating herbage intake. **In,** *Proceedings of the 18th Annual Research Meeting.* April 1992, April, Irish Grassland and Animal Production Association, 89-90, 1992.

29. Smit HJ, Taweel HZ, Tas BM, Tamminga S, Elgersma A: Comparison of techniques for estimating herbage intake of grazing dairy cows. *J Dairy Sci*, 88, 1827-1836, 2005.

30. Vulich SA, Hanrahan JP: Estimation of intake by the grazing animal with special reference to the n-alkane technique. **In,** *Proceedings of the 43rd Annual Meeting of the Europan Association of Animal Production.* September 1992, Madrid, Spain, Paper S3.1, 1992.

31. Langlands JP: Techniques for estimating nutrient intake and its utilization by the grazing ruminant. **In,** McDonald IW, Warner ACI (Eds): Digestion and Metabolism in the Ruminant. 320-332, University of New England Publishing Unit, Armidale, 1975.

Kafkas Univ Vet Fak Derg
20 (3): 429-434, 2014
DOI: 10.9775/kvfd.2013.10321

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

RESEARCH ARTICLE

Leiomyomas of Oviduct and Its Ventral Ligament of Hens: Immunohistochemical Evaluation and the Plasma Concentration Levels of 17 β-Oestradiol and Progesterone [1]

Recai TUNCA [1] İ. Ayhan ÖZKUL [2] Berrin SALMANOĞLU [3]
Neslihan Aydoğdu ÖZNUR [4] Yılmaz AYDIN [2] Zafer ÖZYILDIZ [5]

[1] *This study was funded by Research Fund of the Ankara University (2000-08-009)*

[1] Adnan Menderes University, Faculty of Veterinary Medicine, Department of Pathology, TR-09010 Aydın - TURKEY
[2] Ankara University, Faculty of Veterinary Medicine, Department of Pathology, TR-06100 Ankara - TURKEY
[3] Ankara University, Faculty of Veterinary Medicine, Department of Biochemistry, TR-06100 Ankara - TURKEY
[4] Turkish Republic of Northern Cyprus, Ministry of Food, Agriculture and Energy, Veterinary Office, 99010 Lefkoşa - NORTHERN CYPRUS (KKTC)
[5] Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Pathology, TR-15030 Burdur - TURKEY

Makale Kodu (Article Code): KVFD-2013-10321

Summary

Leiomyoma's of the oviduct and its ventral ligament (VL) are common tumours of the domestic fowl which develop after the end of the first laying season. In the present study, macroscopic, microscopic, and immunohistochemical features of the leiomyomas of the reproductive tract and their prevalence were investigated in commercial laying hens. In 60-90 weeks old commercial laying hens, toward the end of the first laying period, the incidence of genital tract leiomyomas were determined as 10.43%. Of these, 95.81% were developed from oviduct VL and 4.19% were oviduct leiomyomas. These tumours were firm, round to oval and microscopically well-circumscribed, consisting of monomorphic spindle cells. In immunohistochemical examination with α-smooth muscle actin, desmin and vimentin, all tumours were found positive with these markers. To investigate the aetiological importance for tumourogenesis, the plasma concentrations of 17 β-oestradiol and progesterone levels were determined. Concentrations of 17 β-oestradiol and progesterone were higher in hens with tumours than that of non-tumour control animals. The results suggested that there was an association between the levels of 17 β-oestradiol and progesterone and the leiomyomas of genital tract in hens.

Keywords: *Leiomyomas of hens' oviduct, Incidence, Immunohistochemical evaluation, 17 β-oestradiol and Progesteron levels*

Yumurtacı Tavuklarda Ovidukt Ventral Ligamenti Leiomyomları: İmmunohistokimyasal İnceleme ve Plazma 17 β-Östradiol ve Progesteron Düzeyleri

Özet

Ovidukt ve ovidukt ventral ligamenti (VL)nin leiomiyomları, ilk yumurtlama periyodunu tamamlayan yumurtacı tavuklarda oldukça sık karşılaşılan tümörlerdir. Çalışmada, ticari olarak yetiştirilen yumurtacı tavukların reprodüktif sistemlerinde gelişen leiomiyomların sıklığı ve bu tümörlerin makroskobik, mikroskobik ve immunohistokimyasal özellikleri değerlendirildi. İlk yumurtalama periyodunu tamamlamış, 60-90 haftalık yaşta, ticari yumurtacı tavuklarda, reprodüktif sistem leiomiyomlarının insidensi %10.43 olarak saptandı. Saptanan leiomiyomların %95.81'inin oviducttun ventral ligamentinde, %4.19'un oviduktta yerleştiği belirlendi. Makroskobik olarak sert kıvamlı ve yuvarlak-oval şekilli olan bu tümörlerin mikroskobik incelemesinde iyi sınırlandırılmış mekik şeklindeki hücrelerden oluştuğu belirlendi. İmmunohistokimyasal incelemede, tüm tümörlerin α-smooth muscle actin, desmin ve vimentin primer antikorları ile pozitif reaksiyon verdikleri belirlendi. 17 β-östradiol ve progesteronun tümörogenezisteki etiyolojik öneminin değerlendirilmesi amacıyla plazma konsantrasyonları belirlendi. Tümör tespit edilen yumurtacı tavukların plazma 17 β-östradiol ve progesteron düzeyleri kontrol gruplarındaki yumurtacı tavuklardan anlamlı derecede yüksekti. Elde edilen bu sonuçlar ile, yumurtacı tavukların reprodüktif sistemlerinde sıklıkla ortaya çıkan leiomiyomların gelişiminin, plazma 17 β-östradiol ve progesteron düzeyleri ile ilişkili olduğu görüldü.

Anahtar sözcükler: *Yumurtacı tavuk, Ovidukt, Leiomiyom, İnsidens, İmmunohistokimyasal inceleme, 17 β-östradiol ve progesteron düzeyleri*

İletişim (Correspondence)
+90 256 2470700
rtunca26@hotmail.com

INTRODUCTION

The frequently observed spontaneous tumours of the unknown aetiology in domestic fowls are leiomyoma of the ventral ligament (VL) of the oviduct [1,2]. These tumours are capsulated, round, solid masses and grow as solitary within VL of the oviduct [3,4]. They are benign and consist of smooth muscle fibers located in a fibrous stroma [1]. Leiomyomas of the oviduct VL commonly arise after the end of a laying period [5]. They can reach considerable dimensions without impairing production in layer hens [6]. It has been suggested that there is an association between the number of eggs produced in a laying period and the incidence of leiomyomas [4]. The prevalence of these tumours varied from 0% to 60% [1]. Although most genital organ leiomyomas in laying hens develop from the oviduct VL, occasional oviduct leiomyomas can also be seen [2]. In humans, uterine leiomyomas are the most common type of reproductive tract tumours in women and leiomyomas of the oviduct in sexually mature domestic hens share important features with human uterine leiomyomas [7-9]. The high prevalence and ability to induce leiomyomas of the oviduct VL in hens means that this species could be of interest as a model for the study of similar tumours in mammals as well as women because the remarkable similarities between the ovulatory cyles of women and hens [3,7-11].

Oestrogen and progesterone hormones were suggested to have etiological importance on the tumourogenesis of the oviduct magnum region adenomas, oviduct adenocarcinomas and leiomyomas of the oviduct VL [1,5]. However, no such tumours could experimentally be induced in chicks by administration of only oestrogen or progesterone [1]. On the other hand, concurrent i.m. administration of oestrogen and progesterone to three weeks old chicks repeated for five weeks was shown to cause leiomyomas of the oviduct VL [1]. Fredrickson et al.[8] reported that there were no changes in the plasma concentrations of oestrogen and progesterone in oviduct gland tumours. In addition, such studies on the plasma concentrations of oestrogen and progesterone in leiomyomas of the oviduct VL are limited [5]. However, there are no studies investigating the association between these hormones and the leiomyomas of oviduct.

This study was undertaken to investigate the immunohistochemical profile of the leiomyomas of the oviduct and its VL and to better characterize the relationship between etiological importance of 17 β-oestradiol and progesteron on the tumourogenesis of these tumours.

MATERIAL and METHODS

Animals and Tissue Processing

The study materials were 60-90 weeks old hens which were at the end of a laying period. A total of 1600 commercial laying hens slaughtered in a privately owned company in Ankara were investigated. Each hen was given with a wing number. Then, blood samples were collected; sera samples were obtained within 4 hours and stored at −18ºC until analysis. Following the slaughter, internal organs, were examined for tumours lesions. When a tumour was observed it was grossly evaluated and fixed in 10% buffered formalin solution for microscopical investigation. Then, using routine techniques, 5-6 micron sections were cut from paraffin blocks and stained with haematoxylin and eosin for histopathological examination. To demonstrate the tumour components, Masson's Trichrome and Van Gieson were used.

Biochemical Analysis

Plasma concentration levels of 17 β-oestradiol and progesterone were determined according to the standards laid out by the USA Center for Disease Control/National Institute of Health Manual and Biosafety in Microbiological Laboratories, 1984. For the determination of 17 β-oestradiol concentration, an immunoassay kit (Boehringer-Manheim 1776002) was used [12,13]. In this assay, 50 microliters of serum sample from each of the 167 cases of leiomyomas and for the control 60 cases with no tumours were incubated with peroxide labelled 17 β-oestradiol and progesterone. Rabbit anti-17 β-oestradiol or rabbit anti-progesterone antibodies were then added, depending on which hormone was tested, and unbound labelled 17 β-oestradiol or progesterone was removed by washing. Hormone found in the serum and bound to the antibody of interest was determined by a colorimetric mean using hydrogen peroxide/TMB. Staining density showing the hormone concentration level was read in a spectrophotometry at 450 nm. Students't test was used to compare plasma concentrations of 17 β-oestradiol and progesterone between the groups of hens with and without tumour.

Immunohistochemistry

For each sample 3 µm sections were cut and immunohistochemically stained for vimentin (V9, DAKO, Carpinteria, CA, USA, M0725, 1:25), desmin (DE-R-11, DAKO, Carpinteria, CA, USA, M724, 1:50) and α-smooth muscle actin (1A4, DAKO, Carpinteria, CA, USA, M0635, 1:50) (Inter-Species Code N° 10 145). Antigen retrieval was performed by microwave treatment in citrate buffer (pH 6.0). After blocking the endogenous peroxidase activity with 0.3% H_2O_2, non-specific antibody binding was blocked by non-immune goat serum. Further, the sections were incubated with primary antibodies for 30 min at room temperature. Then, each section was stained by a modified labelled streptavidin-biotin (LSAB) method using a standard reagent kit (LSAB2 code K0675, Dako Corp., Carpinteria, CA). Antigen-antibody binding was visualised by 3,3-diaminobenzidine. The sections were counterstained with Mayer's haematoxylin. For each of the three methods, the internal

positive control was represented by normal cells or structures, found close to the tumors. In the above-mentioned conditions, we considered that an external positive control was not necessary. Negative controls were achieved by replacing the primary antibody with normal mouse serum.

RESULTS

In 167 of 1600 hens (~10.43%) which were toward the end of the first laying period, genital organ leiomyomas were determined. These tumours were only localized in the oviduct and its VL. Of these leiomyomas, 160 (95.81%) had localized in the oviduct VL and 7 (4.19%) in the oviduct *(Fig. 1)*. All of the leiomyomas of the oviduct VL was located in the centre of the ligament *(Fig. 1A* and *B)*. The size of these tumours varied from 0.5 x 0.6 x 0.5 cm to 7 x 6.8 x 6.4 cm. These tumours were solid, round to oval, firm and in cut surface, they were white yellowish and lobulated. The outer surface of the tumours contained many prominent blood vessels. Some of these leiomyomas showed whitish whorled cut surfaces *(Fig. 1D)*. In three cases, the cut surfaces were red or pinkish with necrosis. Leiomyomas developed from oviduct had similar appearances of the leiomyomas developed from the VL. The number of these tumours in any given case varied from one to seven. These leiomyomas were round, firm, white-yellowish and 0.5 to 1.5 cm in diameter *(Fig. 1C)*. These tumours were sharply circumscribed, with a line of cleavage in the surrounding myometrium that results in easy shelling out of these lesions. In five cases, with multiple leiomyomas, some were attached to the oviduct serosa with a stalk.

Microscopically, well-circumscribed leiomyomas of the VL and oviduct were observed to consist of monomorphic spindle cells that were arranged in interweaving fascicles surrounded by a thin layer of fibrous tissue *(Fig. 2A, B* and *C)*. The muscle fibers were stretched in various directions and crossing each other. These tumours were highly hyperaemic and had fibrous tissue and hyalinised collagen fibers that divided the tumour into nodules in their structure. Typical smooth muscle cells were uniform, elongated, with abundant eosinophilic cytoplasm and had cigar-shaped nuclei *(Fig. 2A)*. The muscle fibers were located parallel to each other. The nuclei of smooth-muscle cells were bland ended in longitudinal sections, sometimes the nuclei palisade in a pattern. The nuclei appear round in transverse sections. These leiomyomas showed no cytological atypia and had the mitotic index of < 8 at high-power fields. Only in three cases, coagulative tumour cell necrosis was observed in the centre of the tumours. Invasion was not observed in any case. In 125 cases (74.8%), inflammatory reaction was observed in leiomyomas especially around the vessels *(Fig. 2B)*. This inflammatory reaction was moderate to severe infiltration of small lymphocytes and a few activated large lymphoid cells accompanied by some plasma cells and histiocytes. The inflammatory reaction

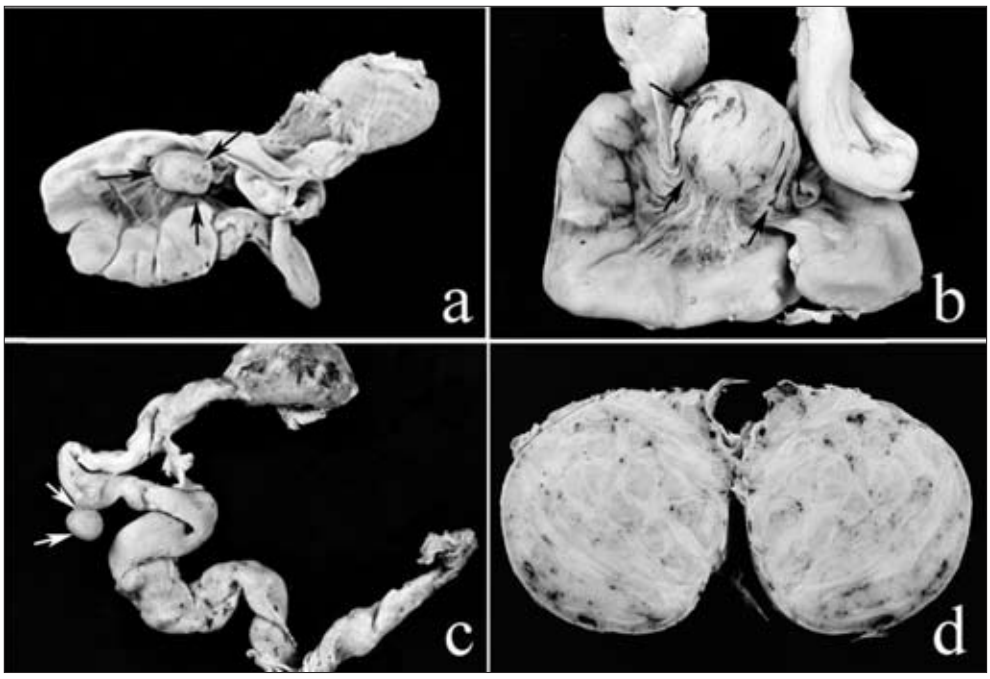


Fig 1. Macroscopic features of leiomyomas in hens. **A-** Solid round leiomyoma *(arrows)* within the centre of the VL, **B-** Large leiomyoma with prominent vascularization *(arrows)*, **C-** Leiomyoma *(arrows)* developed from oviduct serosa, **D-** Cut surfaces of the leiomyoma shows a well-demarcated whitish and whorled mass

Şekil 1. Yumurtacı tavuklarda leiomiyomların makroskobik özellikleri. **A-** Ventral ligamentin merkezinde sert kıvamlı ve yuvarlak şekilli leiomiyom *(oklar)*, **B-** Vaskülarizasyonun *(oklar)* belirgin olduğu leiomiyom, **C-** Ovidukt serozasından gelişen *(oklar)* leiomiyom, **D-** Çevresinden iyi sınırlandırılmış, kesit yüzü beyazımtırak renkte belirgin girdap-vari yapıların seçildiği leiomiyom

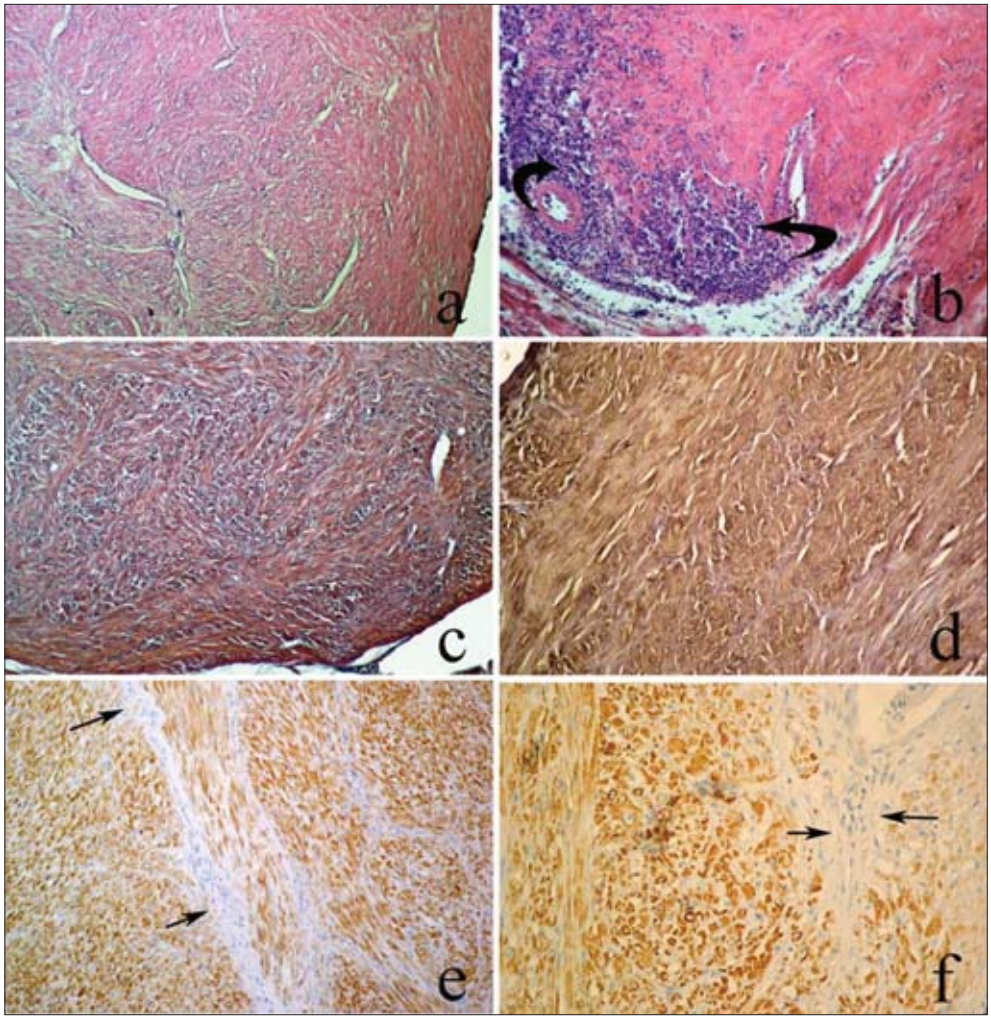


Fig 2. Microscopic (A, B and C) and immunohistochemical (D, E and F) feature of leiomyomas. **A-** The well developed leiomyoma of the oviduct ventral ligament, fascicles of smooth muscle fibers without necrosis HxE, x 80, **B-** The histological section show interlacing spindle-shaped smooth muscle cells, which infiltrated lymphoid cells *(arrows)* HxE, x 80, **C-** Typical smooth muscle cell are uniform, elongated, with abundant eosinophilic cytoplasm and had cigar-shaped nuclei. Spindle tumour cells located in various direction. Masson's Trichrome Stain, x 160, **D-** Diffuse and intense immunopositivity for vimentin antibody avidin biotin peroxidase complex (ABC) x 80, **E-** Immunohistochemical staining for desmin antibody showing strong positivity while the stromal tissue negative immunoreactivity *(arrows)* for desmin antibody, ABC x 80, **F-** Tumour cells showed strong cytoplasmic immunopositivity and stromal tissue negative immunoreactivity *(arrows)* for α-SMA antibody, ABC x 160

Şekil 2. Leiomiyomların mikroskobik (A, B ve C) ve immunohistokimyasal (D, E ve F) özellikleri. **A-** Ovidukt ventral ligamenttinden gelişen düz kas demetlerinden oluşan leiomiyom, HxE, x80, **B-** Birbirinin içine geçen mekik şekilli düz kas hücreleri arasına infiltre lenfoid hücreler *(oklar)* HxE, x80, **C-** Yoğun eozinofilik sitoplazmalı ve çekirdekleri sigara şeklinde, uzun, uniform düz kas hücreleri. Mekik şekilli tümör hücreleri farklı yönlerde uzanmakta. Masson'un Trichrome Boyası, x160, **D-** Vimentin primer antikoru ile diffuz ve yoğun boyanma. Avidin biotin peroksidaz kompleks (ABC) x80, **E-** Tümör hücreleri desmin primer antikoru ile pozitif olarak boyanırken çevredeki bağ doku hücrelerinin reaksiyon vermediği *(oklar)* görülmekte, ABC x80, **F-** Tümör hücreleri α-SMA antikoru ile immunpozitif olarak boyanırken stromal dokuda negatif immunreaksiyon *(oklar)*, ABC x160

was confined to the leiomyoma and did not extend in the surrounding non-neoplastic tissue. Within the tumour mass, the blood vessels were extremely enlarged and in some cases the tumourous tissue pressured through the vessel lumen in a finger-like appearance that gave the impression of papillary hyperplasia to the blood vessel.

Distinction of the muscle cells and the fibrous tissue elements were made with special stains. Masson's Trichrome and Van Gieson stain revealed collagen bundles, which gave a lobular appearance to the tumourous tissue *(Fig. 2C)*. Immunohistochemically, all samples had diffuse and intense expression of vimentin *(Fig. 2D)*. The positive reactivity was also detected in the cytoplasm of the spindle cells with desmin and α-SMA *(Fig. 2E* and *F)*. In general, reactivity for the muscle markers desmin and α-SMA was

Table 1. *Plasma concentration of 17 β-oestradiol and progesterone levels in laying hens with and without leiomyomas*
Tablo 1. *Leiomyomlu ve kontrol grubundaki yumurtacı tavuklarda plazma 17 β-östradiol ve progesteron seviyeleri*

Parameter	Groups	N	X ± SE	t
Progesterone	Control	60	0.588±0.11	2.208*
	Leiomyomas	167	1.054±0.11	
17 β-oestradiol	Control	60	132.78±26.39	2.423*
	Leiomyomas	167	234.00±32.39	

** P<0.05*

strong and diffuse. Because the stromas of the leiomyomas were not stained with desmin and α-SMA, the positive reactions were multi lobular in appearance.

In biochemical analysis, plasma concentrations of 17 β-oestradiol and progesterone in hens with leiomyomas were significantly ($P<0.05$) higher than that of control animals. Plasma concentrations of 17 β-oestradiol and progesterone of 60 controls and 167 leiomyoma observed hens were summarized in *Table 1*. Plasma concentrations of 17 β-oestradiol and progesterone in hens with leiomyoma were significantly higher than that of controls.

DISCUSSION

The most common tumours of genital tract in poultry are adenomas and adenocarcinomas [4]. The other common tumours of the genital tract are leiomyomas of the oviduct and its VL [2]. Studies on the prevalence of these tumours are limited. Although it is previously speculated that the incidence of these tumours varied from 0 to 60%, in post-mortem examination of hens. Reece [14] reported the prevalence of oviduct VL leiomyomas as 0.11%. In other studies, the prevalence was reported to vary from 1.36% to 7.28% [2,15]. Only in one report, the incidence of oviduct leiomyomas was reported as 0.02% [2]. In the present investigation, the prevalence of oviduct and its VL were found as 4.19% and 10.43%, respectively. Therefore, our results regarding to the incidences of these tumours were relatively higher compared to those of the previous reports. It has been suggested that a difference in the prevalence of the leiomyomas can occur depending on the breeds and lines [1]. Therefore, hereditary influence might the reason for higher findings in this study.

Leiomyomas consist of complex interlacing fascicles of smooth muscle fibers, with little or no mitotic activity [1,4]. These tumours are rarely accompanied by a focal to diffuse lymphocytic infiltration in humans [16]. Some unknown factors and pathogenesis were suggested for underlying causes of lymphoid infiltration. However, only in one report, in the majority of laying hens, moderate lymphocytic infiltration was reported [2]. Similarly, in 74.8% of leiomyomas of the present study, moderate to severe lymphocytic infiltration was found. Although the cause is not clear, this inflammatory infiltration is suggested to be an immunological reaction against the tumorous tissue as indicates a direct cytotoxic effect by an autoimmune mechanism.

Leiomyomas of oviduct and its VL in poultry have been described only by histomorphology. However a final diagnostic decision cannot be made depending on one criteria only. Therefore, specific markers might be necessary to show that the tumour is originated from smooth muscle tissue. Leiomyomas have been commonly described to be immunohictochemically positive for vimentin and α- Smooth Muscle Actin (α-SMA). These tumours could also be stained with desmin [7]. These markers are used in human and veterinary pathology as indicators of smooth muscle tumours [3,7,17,18]. Vimentin is an intermediate filament and expressed in most mesenchymal cells. Vimentin is considered a nonspecific marker mommonly expressed in less differentiated tumours and its usually associated with expression of other markers [19]. Desmin is a cytoskeletal intermediate filament that is expressed in skeletal, cardiac, and smooth muscle [10]. α-SMA is a cytosolic intermediate filament that is involved in the mechanism of contraction and that is specific to smooth muscle cells [10,19]. α-SMA and desmin are conventional smooth muscle markers and the use of both antibodies is recommented in immunohistochemical studies as poorly differentiated smooth muscle tumours may react with antibodies to either desmin or α-SMA [3,19]. The tumours in this series showed immunohistochemical staining for vimentin, desmin and α-SMA. The marked and diffuse positivity to these antibodies confirm that the tumours of oviduct and its VL were leiomyomas.

Development and function of genital canal is regulated by steroidal sex hormones [17]. Therefore, such hormones were thought to play important roles in the development of genital organ tumours [5,20,21]. The dorsal oviductal ligament suspends the oviduct and continues ventrally as the fanlike oviduct VL the free edge of which is reinforced by smooth muscle. It is known that oviduct VL is involved in oviduct peristaltics and egg drop [22]. The administration of dietilstillbesterol (DES) via skin implant was shown to increase the VL size and diameter. Hyperplasia of smooth muscles at the free border of the oviduct VL was reported in DES administered 22 weeks old laying hens [1]. The relationship between the genital tract tumours and the plasma concentrations of oestrogen and progesterone

were investigated previously. Fredrickson et al.[11] has found no correlation between the plasma concentration of these hormones and the magnum glandular tumours. On the other hand, Anjum and Payne [5] reported that plasma concentration of oestrogen but not progesterone was higher in hens with oviduct tumours than in non-tumourous hens. In the present study, plasma concentration levels of both 17 β-oestradiol and progesterone were found higher in laying hens with leiomyomas of oviduct and it's VL. Their levels were found approximately twice the levels of non-tumourous control animals. Therefore, our results partially correlate with the previous study regarding the high levels of 17 β-oestradiol in tumourous hens, and differ from it by high level of progesterone. While administration of oestrogen or progesterone alone was reported not to cause tumours, concurrent administration of these hormones were shown to induce leiomyoma of the VL [1]. Although many organs bear smooth muscles, higher incidences of leiomyomas in the oviduct might be explained by the presence of receptors for oestrogen and progesterone on the smooth muscle cells of this organ. These cells are known to be under the effect of these hormones during a laying season [20,23]. Therefore, longer hormone effects on oviduct might be thought to increase the incidence of tumour. In this respect, steroid sex hormones were thought to be effective on leiomyomas of VL in poultry animals.

Leiomyomas of oviduct and it's VL in hens share several histologic and biological features with human uterine leiomyomas [8-10]. Our histochemical and immunohistochemical findings confirm that tumours found on the oviduct and it's VL of hens are derived from smooth muscle cells and there are association between tumorigenesis and ovarian sex steroid hormones.

In conclusion, leiomyomas of the oviduct and its VL in hens were investigated both by histological and immunohistochemical means. In addition, a probable association between the plasma concentration levels of 17 β-oestradiol and progesterone was discussed and high levels of these hormones were thought to play a role in the tumourogenesis of these tumours. Therefore, if the aetiology of these tumours in hens were better understood, that would help in explaining similar tumours in other animals and humans. In this respect, more research is still needed to explain why these tumours arise in such high incidences.

REFERENCES

1. Anjum AD, Payne LN: Spontaneous occurrence and experimental induction of leiomyoma of the ventral ligament of the oviduct of the hen. *Res Vet Sci,* 45, 341-348, 1988.

2. Sonmez G, Ozyigit MO, Kahraman MM: The incidence and pathology of reproductive organ tumours in chickens. *Turk J Vet Anim Sci*, 26, 27-33, 2002.

3. Manarolla G, Caserio S, Sironi G, Rampin T: Morphological and Immunohistochemical Observations on leiomyoma of the ventral ligament of the oviduct of the hen. *J Comp Pathol,* 144, 180-186, 2011.

4. Reece RL: Neoplastic Diseases **In,** Saif YM (Ed): Diseases of Poultry. 7th ed., 541-564, Iowa State University Press, USA, 2003.

5. Anjum AD, **Payne LN:** Concentration of steroid sex hormones in the plasma of hens in relation to oviduct tumours. *Br Poult Sci*, 29, 729-734, 1988.

6. Fredrickson NT: Ovarian tumors of the hen. *EHP,* 12, 35-51, 1987.

7. Wilcox LS, Koonin LM, Pokra R, Strauss LT, Xia Z, Peterson HB: Hysterectomy in United States, 1988-1990. *Obstet Gynecol,* 55, 20-24,1994.

8. Machado SA, Bahr JM, Hales DB, Braundmeier AG, Quande BJ, Nowak RA: Validation of the aging hen *(Gallus gallus domesticus)* as an animal model for uterine leiomyomas. *Biol Reprod,* 2012. DOI: 10.1095/biolreprod.112.101188

9. Hakim AA, Barry CP, Barnes HJ, Anderson KE, Petitte J, Whitaker R, Lancaster JM, Wenham RM, Carver DK, Turbov J, Berchuck A, Kopelovich L, Rodriguez GC: Ovarian adenocarcinomas in the laying hen and women share similar alterations in p53, ras, and HER-2/neu. *Cancer Prev Res,* 2, 114-121, 2009.

10. Calnec BW: Chicken neoplasia - A model for cancer research. *Br Poult Sci,* 33, 3-16, 1992.

11. Fredrickson TN, Oklicz WC, Fournier DJ, Esber H: Spontaneous genital cancers in hens. A study of incidence, morphogenesis, hormonal background and steroid receptors. *PAACR*, 20, 243, 1979.

12. Passing H, Bablok W: A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry. Part I. *J Clin Chem Clin Biochem*, 21, 709-720, 1983.

13. Passing H, Bablok W: Comparison of several regression procedures for method comparison studies and determination of sample sizes. Application of linear regression procedures for method comparison studies in clinical chemistry. Part II. *J Clin Chem Clin Biochem*, 22, 431-445, 1984.

14. Reece RL: Some observations on naturally occurring neoplasms of domestic fowls in the state of Victoria, Australia (1977-87). *Avian Pathol,* 25, 407-447, 1996.

15. Sokkar SM, Mohammed MA, Zubaidy AJ, Mutalib A: Study of some non-leukotic avian neoplasms. *Avian Pathol,* 8, 69-75, 1979.

16. Burton JL, Wells M: Tumours of myometrium. **In,** Fletcher CDM (Ed): Diagnostic Histopathology of Tumours. 676-684, Harcourt, Londra, 2000.

17. Al-Nafussi A: Uterine smooth-muscle tumours: Practical approach to diagnosis. *Curr Diagn Pathol*, 10, 140-156, 2004.

18. Rizeq MN, Rijn MV, Hendrickson MR, Rouse RV: A comparative immunohistochemical study of uterine smooth muscle neoplasms with emphasis on the epitheloid variant. *Hum Pathol,* 25, 671-677, 1994.

19. Coopper BJ, Valentine BA: Tumors of muscle. **In,** Meuten DJ (Ed): Tumors in Domestic Animals. 4th ed., 319-337, Blackwell Publishing, Oxford, 2002.

20. Oklicz WC, Fournier DJ, Esber H, Fredrickson TN: Relationship of oestrogen and progesterone and their oviductal receptors in laying and non-laying 5-year old hens. *J Endocrinol*, 106, 343-348, 1985.

21. Anjum AD, Payne LN, Appleby EC: Oestrogen and progesterone receptors and their relationship to histological grades of epithelial tumours of the magnum region of the oviduct of the domestic fowl. *J Comp Pathol,* 100, 275-286, 1989.

22. King AS: Apparatus urogenitalis. **In,** Baumel JJ (Ed): Nomina Anatomica Avium. 2nd ed., 329-397, Massachusetts, Cambridge, 1993.

23. Bonifer C, Hecht A, Peters CWB, Sippel AE: Rat antibodies as probes for the characterization of progesteron receptor A and B proteins from laying hen oviduct cytosol. *BBA,* 968, 96-108, 1988.

Kafkas Univ Vet Fak Derg
20 (3): 435-438, 2014
DOI: 10.9775/kvfd.2013.10364

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

RESEARCH ARTICLE

Methicillin Resistance in *Staphylococcus pseudintermedius* Isolated from Shelter Dogs in Turkey

Barış SAREYYÜPOĞLU [1] H. Kaan MÜŞTAK [1] Zafer CANTEKIN [2] K. Serdar DİKER [1]

[1] Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, TR-06110 Diskapi, Ankara - TURKEY
[2] Department of Microbiology, Faculty of Veterinary Medicine, Mustafa Kemal University, TR-31000 Serinyol, Antakya - TURKEY

Makale Kodu (Article Code): KVFD-2013-10364

Summary

Staphylococcus pseudintermedius is a recently described species of staphylococci that is frequently isolated from dogs. The increase in antimicrobial resistance in staphylococci and transfer of *S. pseudintermedius* from infected pets to humans threaten the public health worldwide. In this study, it was aimed to determine the prevalence of *S. pseudintermedius* from skin infections of dogs and to determine the prevalence of methicillin-resistance in *S. pseudintermedius* isolates by phenotypic and genotypic methods. A total of 41 staphylococci were isolated from 53 dogs. Thirty three (80.4%) staphylococci were identified as *S. pseudintermedius* by PCR-RFLP analysis of *pta* gene. Methicillin resistance was also identified in 11 (33.3%) of these isolates by inoculation on chromogenic chromID MRSA agar, oxacillin disc diffusion method and the determination of *mec*A gene by PCR. This is the sole report in Turkey that described the situation of methicillin resistant *S. pseudintermedius*.

Keywords: *Dog, Staphylococcus pseudintermedius, Methicillin resistance*

Türkiye'deki Barınak Köpeklerinden İzole Edilen *Staphylococcus pseudintermedius*'larda Metisilin Direnci

Özet

Staphylococcus pseudintermedius, stafilokoklar içerisinde yeni tanımlanmış bir tür olup köpeklerden sıklıkla izole edilmektedir. Stafilokoklarda artan antimikrobiyal direnç ve *S. pseudintermedius*'un enfekte petlerden insanlara geçmesi, halk sağlığını tehdit etmektedir. Bu çalışmada, köpek deri enfeksiyonlarından izole edilen *S. pseudintermedius*'ların ve metisilin direncinin prevalansının fenotipik ve genotipik metotlar ile belirlenmesi amaçlanmıştır. Toplam 53 köpekten 41 stafilokok izole edildi. Stafilokok izolatlarının 33'ü (%80.4) *pta* geninin PCR-RFLP ile analiziyle *S. pseudintermedius* olarak belirlendi. Metisilin direnci kromogenik chromID MRSA agar, oxacillin disk difüzyon metodu ve *mec*A geninin belirlendiği PCR yöntemi ile bu izolatların 11'inde (%33.3) tespit edildi. Bu çalışma, Türkiye'de metisilin dirençli *S. pseudintermedius*'ların durumunu gösteren ilk bilimsel rapordur.

Anahtar sözcükler: *Köpek, Staphylococcus pseudintermedius, Metisilin direnci*

INTRODUCTION

Isolates phenotypically identified as *Staphylococcus intermedius* consist of three distinct species, including *S. intermedius*, *S. pseudintermedius*, and *S. delphini*, which together represent the *S. intermedius* group (SIG). *Staphylococcus pseudintermedius* rather than *S. intermedius* is the opportunistic species of the SIG that colonizes and causes infections in dogs and cats frequently. Beside this, *S. pseudintermedius* could be transferred from infected and/or colonized pets to humans, which causes an increasing public health concern [1]. *Staphylococcus pseudintermedius* is occasionally isolated from serious human infections, particularly from people who are in close contact with pets, such as small animal veterinarians and pet owners [2-5].

Recently, methicillin-resistant *S. pseudintermedius* (MRSP) has emerged worldwide and spread of strains has been reported increasingly in Europe [6-10]. Methicillin resistance determinant, *mec*A gene, carried on a mobile genetic element called Staphylococcal Cassette Chromosome (SCC), also exists on *S. pseudintermedius* similarly to *S. aureus* [11].

İletişim (Correspondence)
+90 312 3170315
kmustak@ankara.edu.tr

This gene encoding the penicillin-binding protein 2a which mediates methicillin resistance in *S. aureus* and coagulase negative staphylococci by lowering affinity to all β-lactam antimicrobials [2].

In order to detect methicillin resistant staphylococci (MRS), oxacillin is used and oxacillin resistant strains are referred as methicillin resistant. Besides this, any staphylococci resistant to oxacillin or methicillin should be considered resistant to other β-lactam antimicrobials such as amoxillin-clavulanic acid, cephalosporins, etc. Clinical Laboratory Standards Institute (CLSI) asked from laboratories to report *mec*A positive MRS as resistant to all other β-lactam antimicrobials regardless of antibiotic susceptibility tests [12]. In 2008, CLSI published a new document M31-A3, which determines the *in vitro* antimicrobial susceptibility of MRSP. According to this guideline, new interpretation criterion for MRSP isolates for oxacillin is ≥4 mg/L for agar and broth dilution, and ≤10 mm for disc diffusion tests similar to *S. aureus* clinical susceptibility breakpoints [13,14].

In Turkey, there are few reports on methicillin resistance in coagulase negative staphylococci isolated from companion animals and pets [15,16]. Nevertheless, the prevalence of MRSP is not known in particular. The aim of this study was to determine the prevalence of *S. pseudintermedius* among staphylococcal isolates from skin infections of dogs and to determine the prevalence of methicillin-resistance in *S. pseudintermedius* isolates.

MATERIAL and METHODS

Bacteriological Identification

A total of 41 isolates from 53 shelter dogs with dermatitis in Ankara region were used in the study. These isolates were obtained by inoculation of swap samples taken from skins of dogs on blood agar containing 5-7% ovine blood and incubated aerobically at 37°C for 24 h. After incubation, suspected colonies were Gram stained and treated with catalase, tube coagulase and DNAse tests. Further phenotypic identification was performed with the Microbact™ Staph 12S system (Staphylococcal 12S Identification System, MB1561, Oxoid).

Molecular Identification of Staphylococcus pseudintermedius

Staphylococci identified as *S. intermedius* by Microbact™ Staph 12S system were molecularly investigated for *S. pseudintermedius* by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the *pta* gene encoding the enzyme phosphoacetyltransferase [17]. *Mbo*I enzyme was used in RFLP to detect *S. pseudintermedius* isolates harboring this restriction site. Briefly; 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA Polymerase (Fermentas, Lithuania), 200 µM each dNTPs, 5 µl PCR reaction buffer (1×), 4 µl template DNA and 0.2 µM of each primer (pta_f1, AAA GAC AAA CTT TCA GGT AA, and pta_r1, GCA TAA ACA AGC ATT GTA CCG) were used to amplify a 320 bp fragment of *pta* gene. Thermal cycling conditions were as follows: initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min with final extension at 72°C for 7 min. Two fragments (213 bp and 107 bp) of *pta* gene confirming *S. pseudintermedius* identification were detected after the digestion of amplified products with 5 U *Mbo*I for 2h at 37°C. A *S. pseudintermedius* strain obtained from Associate Professor Dr. Ken Kikuchi (Department of Infection Control Science, Faculty of Medicine Juntendo University, Tokyo, Japan) was served as positive control in all phenotypic and genotypic tests.

Antimicrobial Susceptibility

All phenotypically and genotypically identified *S. pseudintermedius* strains were inoculated on chromogenic chromID MRSA agar (bioMérieux) for investigating methicillin resistance. After incubation at 37°C for 24 h, blue-green colonies were picked up and assigned as MRSP. Methicillin resistance was also investigated by disk diffusion method with oxacillin standard disks according to the guidelines of CLSI [14]. The zone diameter ≤10 mm for oxacillin is referred as resistance threshold. *Staphylococcus aureus* ATCC29213 was served as quality control strain in all tests.

Molecular Detection of Methicillin Resistance

For all isolates identified as *S. pseudintermedius*, methicillin resistance was investigated by a modified PCR method of Choi et al.[18] for detection of *mec*A gene. Primers (F: CCT AGT AAA GCT CCG GAA; and R: CTA GTC CAT TCG GTC CA) were used to amplify a 314 bp fragment of *mec*A gene in a total volume of 25 µl PCR mixture consists of 0.2 µM each primer, 200 µM each dNTPs, 5 µl PCR reaction buffer, 1.5 mM $MgCl_2$, 0.5 U Taq DNA Polymerase and 2 µl template DNA. The thermal cycling conditions were as follows: 1 min of preliminary denaturation at 95°C followed by 30 cycles of 2 min denaturation at 95°C, 1 min of annealing at 54°C, 1 min of extension at 72°C and then a final extension at 72°C for 7 min. *Staphylococcus aureus* SR27 strain was served as the positive control for the *mec*A gene.

RESULTS

Bacterial Identification

Out of 41 Staphylococcal isolates from skin infections of dogs, 35 (85.4%) isolates were phenotypically identified as *S. intermedius,* 4 (9.8%) as *S. aureus* and 2 (4.8%) as different species (*S. chromogenes* and *S. capitis*). According to the PCR-RFLP results 33/35 (94.2%) *S. intermedius* strains were molecularly identified as *S. pseudintermedius (Table 1).*

Table 1. *Phenotypic and genotypic identification test results*
Tablo 1. *Fenotipik ve genotipik testlere ait identifikasyon sonuçları*

Staphylococci	Microbact Staph 12S Identification	PCR-RFLP	MRSA Agar	*mec*A PCR
S. intermedius	35/41 (85.4%)	0/41	N	N
S. pseudintermedius	0/41	33/41 (80.4%)	11/33 (33.3%)	11/33 (33.3%)
S. aureus	4/41 (9.8%)	4/41*	N	N
S. capitis	1/41 (2.4%)	0/41	N	N
S. chromogenes	1/41 (2.4%)	0/41	N	N

** S. aureus isolates contained a unique MboI site, resulting in restriction fragments of 156 bp and 164 bp that appeared as a single band after agarose electrophoresis; this band was readily distinguishable from the S. pseudintermedius restriction fragment profile [17], **N:** not applicated*

Identification of Methicillin Resistant Staphylococcus pseudintermedius

Methicillin resistance was observed in 11/33 (33.3%) *S. pseudintermedius* isolates after incubation on chromogenic chromID MRSA agar. According to disc diffusion method, 11 (33.3%) *S. pseudintermedius* isolates were found to be resistant to oxacillin. The *mec*A gene was also detected in 11/33 (33.3%) *S. pseudintermedius* isolates *(Table 1)*.

DISCUSSION

Staphylococcal isolates from dogs with pyoderma identified conventionally as *S. intermedius* should be re-investigated with appropriate methods, since they could in fact be *S. pseudintermedius*. Results of the present study confirmed that *S. pseudintermedius* 33/41 (80.4%) is the predominant pathogenic *Staphylococcus* species isolated from dermatitis cases in dogs.

The prevalence and occurrence of MRSP has been described in companion animals [19,20]. Former studies revealed the prevalence rates of MRSP in dogs approximately between 0%-7% [21-24]. Unfortunately, there are several recent reports of MRSP isolates that exhibited high rates of prevalence in FarEastern parts of the world. In China, 69 of 144 (47.9%) *S. pseudintermedius* were identified as MRSP [25]. In Japan, 27 MRSP characterized by the growth on chromID MRSA agar and confirmed by the presence of *mec*A gene, in 200 cats and dogs, most of which were isolated from dogs (n: 25) [26]. Another study from Japan revealed 66.5% (113/170) as the prevalence of MRSP isolated from dogs based on the detection of *mec*A gene [27]. The prevalence of MRSP in Europe seems to be low as reported in Spain 4.6% (9/196) [6], in Germany 7.4% (60/814) [7] and in Italy 2% (10/590) [8]. In the present study, high prevalence of MRSP (33.3%) was detected in dogs with dermatitis. This prevalence was found to be higher when compared to the results of studies in Europe and similar to the results of studies in Far Eastern countries. Nevertheless, high prevalence could have been resulted from transmission of a resistant strain or strains between dogs held in close contact in shelter conditions.

In Turkey, there is limited data on MRS. Öztürk et al.[16], investigated 96 dogs with otitis externa, skin wounds and pyoderma for the presence of MRS. Of all isolated 54 coagulase-positive staphylococci, 4/33 (12.1%) and 1/21 (4.8%) were found to be methicillin resistant *S. aureus* and *S. pseudintermedius* respectively. Beside this, Aslantaş et al.[15], reported the prevalence of MRS in dogs as 15.4% (25/162) all of which were coagulase negative. However, none of the isolates were found to be MRSA or MRSP [15]. Authors concluded their report as methicillin resistant coagulase negative staphylococci were common in dogs in Turkey. In comparison with these studies in this report the isolation rate was found to be 33/41 (80.4%) that indicates the *S. pseudintermedius* is the leading agent among staphylococci that infects and/or colonizes the skins of dogs in Turkey. The prevalence of MRSP (33.3%) also exhibits that MRSP is an emerging problem that threatens the dog population and public health in Turkey. Although rare MRSP infections in humans have been described, MRSP has implications for public health as it can spread between people and pets via direct and indirect contact [13,28]. Methicillin-resistant staphylococci are known as important zoonotic pathogens that cause serious public health problems by increasing both the failure rate of antibiotic therapy and mortality rates among human beings and animals.

This is the first report that demonstrates the situation of MRSP isolated from dogs with dermatitis in Turkey. In conclusion, the high rate (i.e. one third of the isolates) of methicillin resistance of *S. pseudintermedius* isolates from dogs constitutes a significant risk for public health considering the pathogen's zoonotic potential and the risk of transmission from these companion animals to their owners. More and comprehensive studies should be performed to reveal the epidemiology of MRSP infections in companion animals in Turkey, and their role in transmission of antibiotic resistant bacteria between different species.

REFERENCES

1. vanDuijkeren E, Houwers DJ, Schoormans A, Broekhuizen-Stins MJ, Ikawaty R, Fluit AC, Wagenaar JA: Transmission of methicillin-resistant *Staphylococcus intermedius* between humans and animals. *Vet Microbiol*, 128, 213-215,2008.

2. Goodacre R, Harvey R, Howell SA, Greenham LW, Noble WC: An epidemiological study of *Staphylococcus intermedius* strains from dogs,

their owners and veterinary surgeons. *J Anal Appl Pyrolysis*, 44, 49-64, 1997.

3. Guardabassi L, Loeber M, Jacobson A: Transmission of multiple antimicrobial-resistant *Staphylococcus intermedius* between dogs affected by deep pyoderma and their owners. *Vet Microbiol*, 98, 23-27, 2004.

4. Talan DA, Staatz D, Staatz A, Overturf GD: Frequency of *Staphylococcus intermedius* as human nasopharyngeal flora. *J ClinMicrobiol*, 27, 2393, 1989.

5. Paul NC, Moodley A, Ghibaudo G, Guardabassi L: Carriage of methicillin-resistant *staphylococcus pseudintermedius* in small animal veterinarians: Indirect evidence of zoonotic transmission. *Zoonoses Public Health,* 58, 533-539, 2011.

6. Gómez-Sanz E, Torresa C, Lozano C, Sáenz Y, Zarazaga M: Detection and characterization of methicillin-resistant *Staphylococcus pseudintermedius* in healthy dogs in La Rioja, Spain. *Comp Immun Microbiol Infect Dis*, 34, 447-453, 2011.

7. Nienhoff U, Kadlec K, Chaberny IF, Verspohl J, Gerlach GF, Kreienbrock L, Schwarz S, Simon D, Nolte I: Methicillin-resistant *Staphylococcus pseudintermedius* among dogs admitted to a small animal hospital. *Vet Microbiol*, 150, 191-197, 2011.

8. De Lucia M, Moodley A, Latronico F, Giordano A, Caldin M, Fondati A, Guardabassi L: Prevalence of canine methicillin resistant *Staphylococcus pseudintermedius* in a veterinary diagnostic laboratory in Italy. *Res Vet Sci*, 91, 346-348, 2011.

9. Loeffler A, Linek M, Moodley A, Guardabassi L, Sung JM, Winkler M, Weiss R, Lloyd DH: First report of multi resistant, mecA-positive *Staphylococcus intermedius* in Europe: 12 cases from a veterinary dermatology referral clinic in Germany. *Vet Dermatol*, 18, 412-421, 2007.

10. Schwarz S, Kadlec K, Strommenger B: Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* detected in the BfT-GermVet monitoring programme 2004-2006 in Germany. *J Antimicrob Chemother*, 61, 282-285, 2008.

11. Weese JS, van Duijkeren E: Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. *Vet Microbiol*, 140, 418-429, 2010.

12. Papich MG: Selection of antibiotics for meticillin-resistant *Staphylococcus pseudintermedius*: Time to revisit some old drugs? *Vet Dermatol,* 23, 352-e64, 2012.

13. van Duijkeren E, Catry B, Greko C, Moreno MA, Constanca Pomba M, Pyorala S, Ruzauskas M, Sanders P, Threlfall EJ, Torren-Edo J, Torneke K: Review on methicillin-resistant *Staphylococcus pseudintermedius*. *Antimicrob Chemother*, 66, 2705-2714, 2011.

14. Clinical and Laboratory Standard Institute (CLSI): Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. M31-A3. CLSI Standards and Guidelines. Wayne, PA, 2008.

15. Aslantaş Ö, Türkyılmaz S, Yılmaz MA, Yılmaz EŞ: Prevalence of methicillin-resistant Staphylococci in dogs. *Kafkas Univ Vet Fak Derg*, 19 (1): 37-42, 2013. DOI: 10.9775/kvfd.2012.7073

16. Öztürk D, Avki S, Türütoğlu H, Yiğitarslan K, Sağnak S: Methicilli resistance among coagulase-positive Staphylococci isolated from dogs with otitis externa, skin wounds and pyoderma. *Kafkas Univ Vet Fak Derg*, 16 (4): 651-656, 2010.

17. Bannoehr J, Franco A, Iurescia M, Battisti A, Fitzgerald JR: Molecular diagnostic identification of *Staphylococcus pseudintermedius*. *J Clin Microbiol*, 47, 469-471, 2009.

18. Choi SM, Kim S, Kim H, Lee D, Choi J, Yoo J, Kang J, Shin W, Kang M: Multiplex PCR for the detection of genes encoding amimoglycoside modifying enzymes and methicillin resistance among *Staphylococcus* species. *J Korean Med Sci*, 18, 631-636, 2003.

19. Weese JS, van Duijkeren E: Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. *Vet Microbiol*, 140, 418-29, 2010.

20. Ruscher C, Lübke-Becker A, Semmler T, Wleklinski CG, Paasch A, Soba A, Stamm I, Kopp P, Wieler LH, Walther B: Widespread rapid emergence of a distinct methicillin- and multidrug-resistant *Staphylococcus pseudintermedius* (MRSP) genetic lineage in Europe. *Vet Microbiol,* 144, 340-346,2010.

21. Griffeth GC, Morris DO, Abraham JL, Shofer FS, Rankin SC: Screening for skin carriage of methicillin-resistant coagulase-positive staphylococci and *Staphylococcus schleiferi* in dogs with healthy and inflamed skin. *Vet Dermatol*, 19, 142-149, 2008.

22. Hanselman BA, Kruth SA, Rousseau J, Weese JS: Coagulase positive staphylococcal colonization of humans and their household pets. *Can Vet J*, 50, 954-548, 2009.

23. Hanselman BA, Kruth S, Weese JS: Methicillin-resistant staphylococcal colonization in dogs entering a veterinary teaching hospital. *Vet Microbiol*, 126, 277-281, 2008.

24. Vengust M, Anderson ME, Rousseau J, Weese JS: Methicillin-resistant staphylococcal colonization in clinically normal dogs and horses in the community. *Lett Appl Microbiol,* 43, 602-606, 2006.

25. Feng Y, Tian W, Lin D, Luo Q, Zhou Y, Yang T, Deng Y, Liu YH, Liu JH: Prevalence and characterization of methicillin-resistant *Staphylococcus pseudintermedius* in pets from South China. *Vet Microbiol*, 160, 517-524, 2012.

26. Bardiau M, Yamazaki K, Ote I, Misawa N, Mainil JG: Characterization of methicillin-resistant *Staphylococcus pseudintermedius* isolated from dogs and cats. *Microbiol Immunol*, 57, 496-501, 2013.

27. Kawakami T, Shibata S, Murayama N, Nagata M, Nishifuji K, Iwasaki T, Fukata T: Antimicrobial susceptibility and methicillin resistance in *Staphylococcus pseudintermedius* and *Staphylococcus schleiferi* subsp. *coagulans* isolated from dogs with pyoderma in Japan. *J Vet Med Sci*, 72, 1615-1619, 2010.

28. Guardabassi L, Larsen J, Weese JS, Butaye P, Battisti A, Kluytmans J, Lloyd DH, Skov RL: Public health impact and antimicrobial selection of meticillin-resistant staphylococci in animals. *J Global Antimicob Resist*, 1, 55-62, 2013.

Kafkas Univ Vet Fak Derg
20 (3): 439-444, 2014
DOI: 10.9775/kvfd.2013.10365

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

RESEARCH ARTICLE

Determination of Erythromycin, Spiramycin, Tilmicosin and Tylosin in Animal Feedingstuffs by Liquid Chromatography-Tandem Mass Spectrometry

Artun YIBAR [1] Ali ÖZCAN [2] Mehmet Yılmaz KARACA [2]

[1] Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, University of Uludag, TR-16059 Gorukle Campus, Bursa - TURKEY
[2] Central Research Institute of Food and Feed Control, Animal Originated Foodstuffs Department, TR-16036 Hürriyet, Bursa - TURKEY

Makale Kodu (Article Code): KVFD-2013-10365

Summary

Detection of macrolides residues in animal feedingstuffs to ensure food safety is very important issue. For this purpose, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated for the determination of residues of 4 macrolides (erythromycin, spiramycin, tilmicosin and tylosin) in animal feedingstuffs. Roxithromycin (ROX), a macrolide not used in veterinary medicine, was used as internal standard. The mass spectral acquisition was done in the positive-ion mode applying multiple reaction monitoring with the following ions (mass-to-charge ratio, *m/z*): *m/z* 837.4→679.2; *m/z* 734.4→576.2; *m/z* 422.4→174.1; *m/z* 869.1→696.1 and *m/z* 916.4→772.4 for roxithromycin (ROX), erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), and tylosin (TYL), respectively. Good repeatibility, reproducibility and recovery values was obtained. Average recoveries ranged from 98.9% (TIL) to 101% (ERY) and an overall mean of 99.9%. Method limits of detection (LODs) of ERY, SPI, TIL and TYL 5.1, 6.6, 6.7 and 7.5 µg kg-1 were achieved respectively. Method limits of quantification (LOQs) were 8.5, 11.1, 11.2 and 12.4 µg kg-1 for the same drugs, respectively. Satisfactory decision limits (CCα) and detection capabilities (CCβ) were also attained. The method is simple, rapid, sensitive and suitable for the simultaneous determination of macrolide antibiotics in animal feedingstuffs.

Keywords: *Macrolides, Feedingstuffs, Validation, LC-MS/MS*

Hayvan Yemlerinde Eritromisin, Spiramisin, Tilmikosin ve Tilosinin LC-MS/MS İle Tespiti

Özet

Makrolid grubu antibiyotiklerin hayvan yemlerinde tespit edilmesi gıda güvenliğinin sağlanması açısından çok önemli bir konudur. Bu amaçla, hayvan yemlerinde kullanılan 4 adet makrolid (eritromisin, spiramisin, tilmikosin ve tilosin)'in tespiti için sıvı kromatografi-tandem kütle spektrometrisi (LC-MS/MS) metodu valide edilerek kullanılmıştır. Roksitromisin (ROX), veteriner hekimlik alanında kullanılmayan bir makroliddir ve bu yüzden çalışmada iç standart olarak kullanılmıştır. Kütle spektrası uygulaması pozitif-iyon modunda ve çoklu reaksiyon izleme modunda gerçekleştirilerek, (kütle/şarj oranı, *m/z*) roksitromisin (ROX) için *m/z* 837.4→679.2; eritromisin (ERY) için *m/z* 734.4→576.2; spiramisin (SPI) için *m/z* 422.4→174.1; tilmikosin (TIL) için *m/z* 869.1→696.1 ve tilosin (TYL) için *m/z* 916.4→772.4 taranmıştır. İyi bir tekrarlanabilirlik, üretilebilirlik ve geri kazanım değerleri elde edildi. Hesaplanan ortalama geri kazanım değerleri %98.9 (TIL)'den %101'e (ERY) kadar değişmekle beraber, toplam ortalama %99.9 olarak gerçekleşti. Metodun tespit limitleri (LOD) ERY, SPI, TIL ve TYL için sırasıyla, 5.1, 6.6, 6.7 and 7.5 µg kg-1 olarak hesaplandı. Metodun sayı verilebilir limitleri aynı sırayla 8.5, 11.1, 11.2 and 12.4 µg kg-1 olarak gerçekleşti. Validasyon sonucunda belirtilen bu dört makrolid için memnun edici karar limitleri (CCα) ve karar verebilme kapasitelerine (CCβ) de ulaşılabildi. Validasyon sonuçlarına bakıldığında basit, hızlı, duyarlı ve uygun olan bu metot yukarıda belirtilen dört adet makrolid grubu antibiyotiğin hayvan yemlerinde aranmasında kullanılabilmektedir.

Anahtar sözcükler: *Makrolid, Hayvan Yemi, Validasyon, LC-MS/MS*

INTRODUCTION

Macrolides are characterised by a macrocyclic lactone ring containing 12 to 16 atoms with sugars linked via glycosidic bonds [1]. They active against gram positive and some gram-negative bacteria *(Pasteurella* and *Haemophilus)*,

İletişim (Correspondence)
+90 224 2941338
artunyibar@uludag.edu.tr

mycoplasms, and members of the chlamidia group [2,3]. The macrolides reversibly bind to the 50S ribosomal unit and inhibit bacterial protein biosynthesis [4].

They are widely used in veterinary medicine and in the husbandry of livestock to prevent and treat respiratory diseases, necrotic enteritis and to promote growth in food-producing animals when incorporated at subtherapeutic level into feedingstuffs or drinking water. Antibiotics in animal feedingstuff, in general, are used regularly to induce growth promotion and feed conversion ratio and to increase weight gain than to combat specific diseases [3,5]. Overuse and incorrect use of these antibiotics in animal feedingstuffs can cause harmful residue problems in the human food chain [5,6].

Development of resistant strains of bacteria *(Campylobacter* spp.) has been the major concern regarding these drugs use in food-producing animals, as pointed out by the World Health Organization (WHO) [7]. Consequently, the use of macrolides such as for their growth promotion properties is banned in the EU and in Turkey [8-10]. In this respect, sensitive and suitable analysis and control of banned antibacterial growth promoters in animal feedingstuffs plays a key role to ensure the safety of food for consumers.

A number of methods for the detection of macrolides in various of animal tissues have been proposed [1,3,6,11,12]. The aim of this study was to determine the feasibility, sensitivity, rapidity, simplicity and suitability of LC-MS/MS for the direct detection and quantitation of erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), and tylosin (TYL) in animal feedingstuffs. The following analytical parameters of the method were validated according to the guidelines laid down by Commission Decision 2002/657 [13] using an in-house validation linearity of the standard response in matrix extracts, specificity, recovery, repeatability, decision limit (CCα), detection capability (CCβ) and accuracy.

MATERIAL and METHODS

Chemicals and Reagents

All chemicals (extraction reagents and mobile phase A and B) of analytical grade were from ZIVAK® Technologies (Kocaeli, TURKEY). Ultra pure water was provided by a Milli-Q system (Millipore, Bedford, MA, USA). Roxithromycin (ROX), erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), and tylosin (TYL) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Standard Solutions

All the individual and composite stock standard solutions (10 µg mL^{-1}) were prepared by dissolution in methanol. Working standard solutions were made by diluting stock solution with methanol. Stock standard solutions for the internal standards were also prepared in a similar way. The stock standard solutions were stored at -80°C in Eppendorf micro tubes in the dark, until use. Warmed up to room temperature before use. The working standard solutions (1 µg mL^{-1} for all standards) were stored at +4°C in the dark.

Animal Feedingstuff Samples Used for the Validation

Animal feedingstuffs (pelleted cattle feeds) samples were collected from markets and farms. The absence of the target antibiotics was checked by LC-MS/MS analysis. Blank animal feedingstuffs were used for method development experiments. Since they proved to be blank samples in previous analysis they were used for all validation experiments.

In-house Validation of the Analytical Method

The method was validated according to the criteria laid down in the European Commission Decision 2002/657/EC for confirmatory purposes in LC technique using ROX as internal standard [3,13]. The validation included the determination of selectivity/specificity, linearity, recovery, repeatability, reproductibility, the decision limit (CCα), detection capability (CCβ), the limits of detection (LOD) and quantification (LOQ).

Method linearity was verified by matrix calibration curves constructed at the five concentration levels plus zero (blank). The procedure was validated at the following spiking levels: 62.5, 125, 250 and 500 µg kg^{-1} for ERY and SPI; 15.625, 31.25, 62.50 and 125 µg kg^{-1} for TIL; 31.25, 62.50, 125 and 250 µg kg^{-1} for TYL. Matrix effects were evaluated with matrix-matched calibration curves (both including zero). All samples were also fortified with an amount of internal standard (100 µL of a 10 µg mL^{-1} working solution of ROX). The calibration curves were constructed analyte/internal standard peak area ratio versus concentration of analyte.

Recoveries were assessed for all studied concentration levels, using six replicates for each spiking level. Repeatability and within-laboratory reproducibility where also estimated for all concentrations (n=6, each spiking level), with the whole procedure being repeated on two other days by the same technicians (repeatability) and on more two other different days by different technicians (within-laboratory reproducibility).

The method limits of detection (LODs) and limits of quantification (LOQs) were determined using the samples fortified at the lower validation level in within-laboratory reproducibility experiments. LODs and LOQs were calculated considering a signal-to-noise ratio equal to 3 and 10, respectively, for the first confirmation transition (the second most intense transition) by S-to-N using peak-to-peak script available in Tandem Gold Workstation® Version 6.9.1 software.

The decision limits (CCα) and the detection capabilities (CCβ) were calculated by overall calibration curve procedure from the same within-laboratory reproducibility data, applying weighted regression analysis.

Sample Extraction and Clean-up

Firstly, animal feedingstuffs (pelleted cattle feeds) were blended and homogenizated throughly with an ultra turrax homogenizer (T25 basic, IKA Labortechnik, Staufen, Germany). A total of 5 g of homogenised sample was weighed into 50 mL centrifuge tubes. 100 µL of a 10 µg mL-1 working solution of ROX (I.S.) was added. Moreover, the extraction and clean-up procedure were continued and performed according to the manufacturer's instructions [14]. Multi Speed Vortex (Biosan, MSV-3500), centrifuge (Sigma, 3K15) and nitrogen evoporator (Pierce, Rockford, IL, USA) were used for shaking, centrifugation and evoporating to dryness, respectively. At the end of the extraction procedure, the eluted solution was sonicated for 5 min using ultrasonic bath and then filtered through a 0.45 µm membrane filter. A 20 µL aliquot of the filtrate was injected into the LC-MS/MS system.

Instrumentation

LC-MS-MS analysis was acquired with Tandem Gold Triple Qudrople MS-MS system (ZIVAK, Kocaeli, TURKEY). Chromotography was performed on a 150 x 2 mm, 5 µm C_{18} column (ZV-1015-02C1). The flow rate was 0.2 mL min^{-1}, with a linear gradient at the following conditions: 0 to 6 min with 80% A, 6 to 10 min with 0% A, 10:01 to 14 with 80% A. The column temperature was maintained at 23°C and the injection volume was 20 µL.

Nitrogen was used for the gas nebuliser. The ions were monitored by Multiple Reaction Monitoring (MRM). The source block temperature was set at 300°C and the electrospray capillary voltage was 5000 V. The MS/MS conditions are presented in *Table 1* and the mass transitions of the analytes for the macrolides are given in *Table 2*. The mass spectrometer was operated in the positive electrosprey ionisation (ESI+) mode.

Table 1. *MS/MS conditions*
Tablo 1. *MS/MS şartları*

Ionisation Mode	ESI+
API nebulising gas pressure	55 psi
Drying gas temperature	300°C
Drying gas pressure	34 psi
Scan time	0.5 sec
SIM width	1.0 amu
Needle	+5000V
Shield	+600V
Capillary	30V
Detector	+1800V
CID Gas Pressure	2.00 m Torr
Spray Champer Temperature	50°C
Mass peak width in amu	1.5
Ouad 1	1.5
Ouad 3	1.5

Table 2. *Transition reactions monitored by LC-MS/MS*
Tablo 2. *LC-MS/MS ile taranan geçiş reaksiyonları*

Compound	Transition Reactions (m/z)	Collision Energy (eV)	Dwell (ms)
ROX	837.4 → 679.2	20	0.1
ERY	734.4 → 576.2	20	0.1
SPI	422.4 → 174.1	26	0.1
TIL	869.1 → 696.1	40	0.1
TYL	916.4 → 772.4	28	0.1

RESULTS

The limits of detection (LOD=3.3xSD/*m*) and quantification (LOQ=10xSD/*m*) were determined by analysing the animal feedingstuff samples spiked with standard solutions of the macrolides. The LODs were 5.1, 6.6, 6.7, 7.5 µg kg^{-1}, the LOQs were 8.5, 11.1, 11.2 and 12.5 µg kg^{-1} ERY, SPI, TIL and TYL, respectively. Response linearity was evaluated by calibration curves. The calibration curves were linear. The R^2 values for the system results were all >0.995 for the linear reggression equations in the concentration ranges tested. Data of the calibration curves (equation and regression coefficient) performed with the standards were respectively: erythromycin: y=0.0051x-0.0547, R^2 0.9983; spiramycin: y=0.0047x-0.0671, R^2 0.9994; tilmicosin: y= 0.0151x-0.0193, R^2 0.9982 and tylosin: y=0.0062x-0.0622, R^2 0.9951. Measurement uncertainties (U(C0)) 0.94, 0.31, 0.82 and 0.31 were achieved respectively. The values for the decision limit (CCα) and the detection capability (CCβ) were calculated using the calibration curve procedure described in Commission Decision 2002/657/EC *(Table 3)*.

Representetive chromotograms of a feedingstuff sample spiked with macrolides at concentrations equivalent to the following spiking levels: 250 µg kg^{-1} for ERY and SPI; 62.5 µg kg^{-1} for TIL; 125 µg kg^{-1} for TYL, and to 200 µg kg^{-1} for ROX *(Fig. 1)*.

The precision (repeatibility), recovery and accuracy of the method were obtained by analysing six replicates for each of the 0.5 and 1 MRL of tested fortification levels (125 and 250 µg kg^{-1} for ERY and SPI; 62.5 and 125 µg kg^{-1} for TYL; 31.25 and 62.5 µg kg^{-1} for TIL) on each of 3 days for the animal feedingstuff samples under investigation *(Fig. 2, Fig. 3, Fig. 4* and *Fig. 5)*. Precision was determined by calculating the relative standard deviation (RSD, %) for the replicated measurements and the accuracy (relative

Table 3. *Data summary showing the LC-MS/MS method performance results in animal feedingstuffs*
Tablo 3. *Hayvan yemlerinde LC-MS/MS metodu performans sonuçlarını gösteren veri özeti*

Compound	CCα (µg kg^{-1})	CCβ (µg kg^{-1})	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Measurement Uncertainty U(C0)	R^2
ERY	255.6	261.2	5.1	8.5	0.94	0.9983
SPI	256.1	262.3	6.6	11.1	0.31	0.9994
TIL	67	71.4	6.7	11.2	0.82	0.9982
TYL	130.6	136.2	7.5	12.5	0.31	0.9951

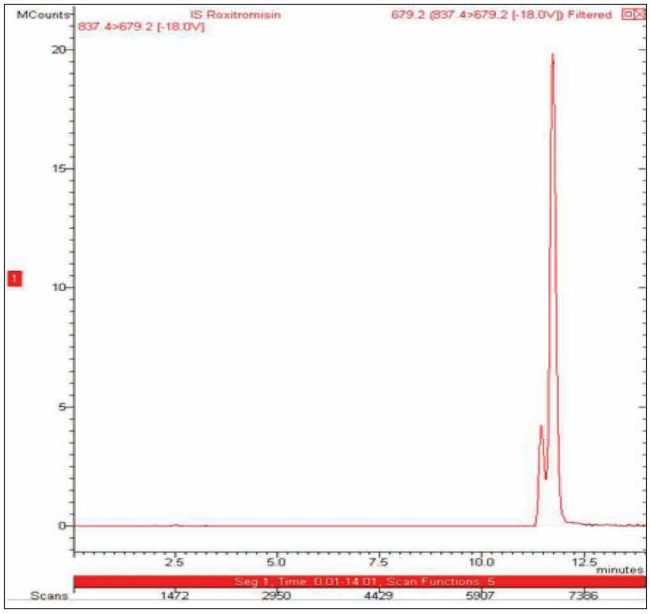


Fig 1. LC-MS/MS chromatogram of a spiked animal feedingstuff sample with roxithromycin (I.S.) (200 µg kg^{-1})
Şekil 1. Roksitromisin (I.S.) ile yükleme yapılmış hayvan yeminin LC-MS/MS kromotogramı (200 µg kg^{-1})

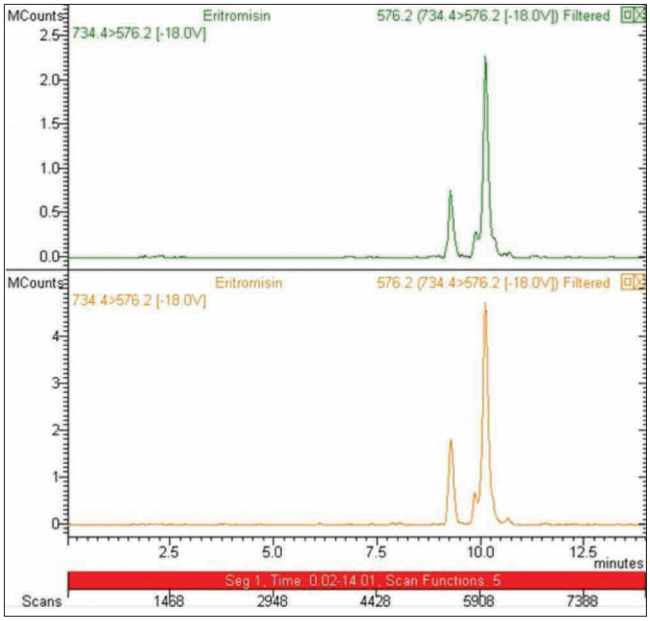


Fig 2. LC-MS/MS chromatogram of a spiked animal feedingstuff sample with erythromycin (125 and 250 µg kg^{-1})
Şekil 2. Eritromisin ile yükleme yapılmış hayvan yeminin LC-MS/MS kromotogramı (125 ve 250 µg kg^{-1})

Table 4. *Data summary showing the recovery mean results (%) in animal feedingstuffs*
Tablo 4. *Hayvan yemlerinde ortalama geri kazanım (%) sonuçlarını gösteren veri özeti*

Compound	Spiked level (µg kg^{-1})	Recovery (%)	RSD (%)
ERY	125	101	1.6
	250	100.5	1.6
SPI	125	99.9	2.2
	250	100.9	1.5
TIL	31.25	99.6	5.1
	62.5	98.9	2.5
TYL	62.5	99.8	1.6
	125	99.5	1.7

error, RE%) was calculated by the aggrement between the measured and the nominal concentrations for the fortified samples. The average recoveries of the four drugs from the feedingstuffs spiked at concentrations equivalent to the 0.5 and 1 MRL levels were between 98.9% and 101% with the relative standard deviations (RSD) were between 1.5% and 5.1% *(Table 4)*.

Selectivity/specificity of the method was demonstrated by analysing of 20 different blank samples in order to investigate possible interferens eluting on ERY, SPI, TIL and TYL retention time. No interference was observed around the macrolides retention times in the matrices with the monitored MS reactions (data not shown). Our results indicate that the method has adequate precision.

DISCUSSION

The goal of this study was to develop a specific, sensitive and reliable LC-MS/MS method for the identify and quantify macrolides (ERY, SPI, TIL and TYL) in animal feedingstuffs. ROX, a macrolide structurally related to the analyte under investigation and not used in veterinary medicine, was used as internal standard [3].

Lower LOD values for these drugs in animal feedingstuffs were also observed by De la Huebra et al.[15]. The CCα (130.6 µg kg^{-1}) and CCβ (136.2 µg kg^{-1}) values for tylosin obtained in animal feedingsuffs were better than those reported by Van Poucke et al.[16]. These calculated CCα and CCβ values were also below the minimum

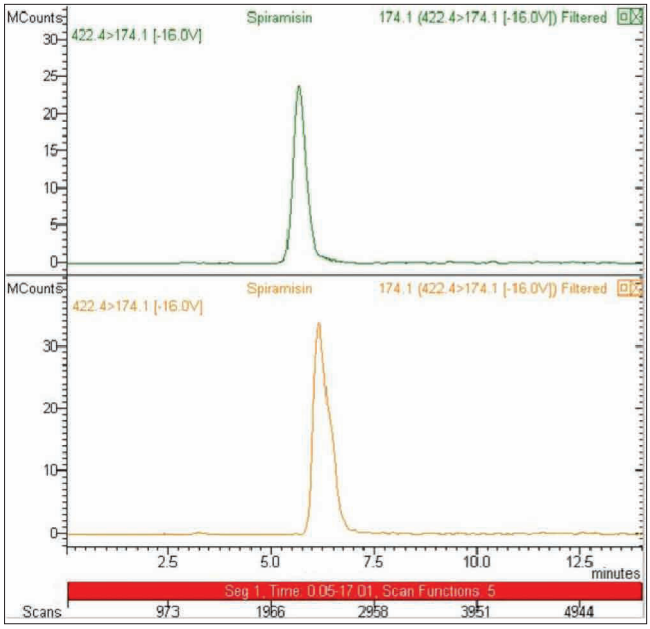


Fig 3. LC-MS/MS chromatogram of a spiked animal feedingstuff sample with spiramycin (125 and 250 µg kg^{-1})

Şekil 3. Spiramisin ile yükleme yapılmış hayvan yeminin LC-MS/MS kromotogramı (125 ve 250 µg kg^{-1})

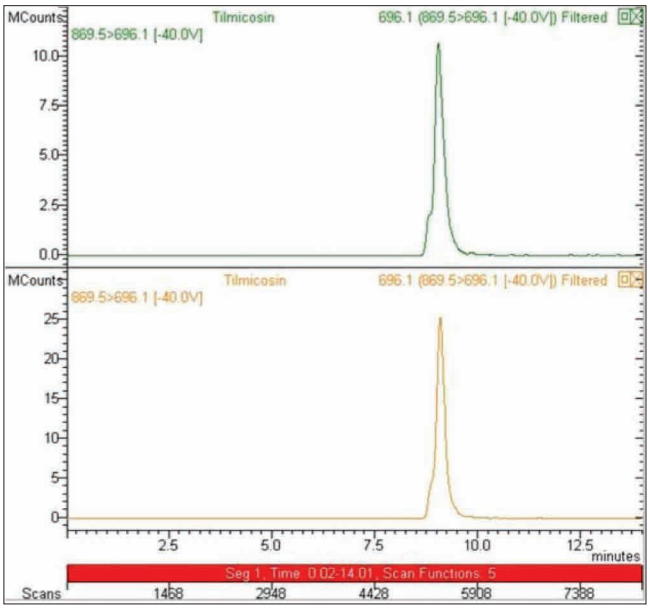


Fig 4. LC-MS/MS chromatogram of a spiked animal feedingstuff sample with tilmicosin (31.25 and 62.5 µg kg^{-1})

Şekil 4. Tilmicosin ile yükleme yapılmış hayvan yeminin LC-MS/MS kromotogramı (31.25 ve 62.5 µg kg^{-1})

required performance limit (MRPL) established by the EU, which is 1 mg kg^{-1} (ppm). Thus, these values appeared very satisfying. Precision values were all below 15.7% (RSD) and the overall accuracy ≤ 3.7% (2.7-3.7) were acceptable. These values could be considered satisfactory, on account of the complexity of the biological matrices. Higher-than-average recoveries for ERY, SPI, TIL and TYL [15]; for ERY and TYL [17] have also been reported

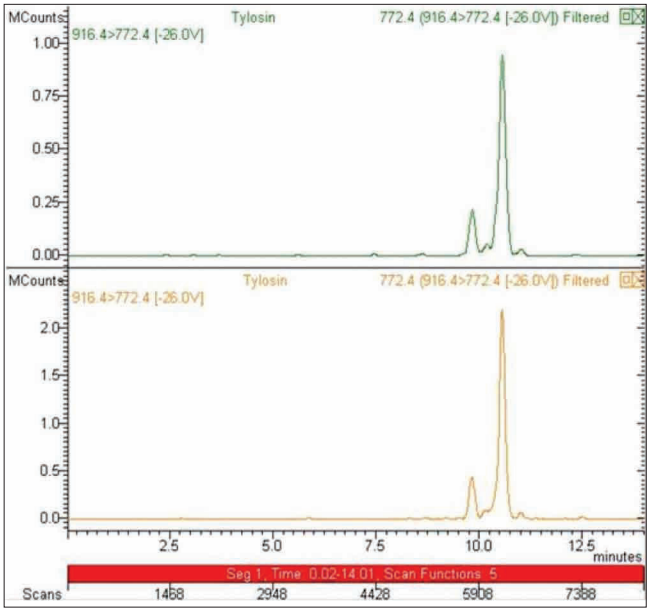


Fig 5. LC-MS/MS chromatogram of a spiked animal feedingstuff sample with tylosin (62.5 and 125 µg kg^{-1})

Şekil 5. Tilosin ile yükleme yapılmış hayvan yeminin LC-MS/MS kromotogramı (62.5 ve 125 µg kg^{-1})

for animal feedingstuffs. However, lower-than-average recoveries for TIL in pig feed samples were also observed by Kaklamanos et al.[18].

The linearities were good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients greater than 0.995 for all curves. However, the linearity of tylosin (0.9951) obtained in this study were lower than that (0.9995) reported by Civitareale et al.[19]. In addition, different types of blank feedingstuffs such as pig finisher, broiler breeder and poultry feeds [16] and macrolides such as rosamicin, clarithromycin, and lincomysin could have been used for the validation procedure [18].

In recent years, residues in animal originated foods arising from the use of zootechnical feed additives including macrolides. Furthermore, there has been increasing regulators, clinicians and the general public concern over the possible links between drug residues in edible tissues (muscle, liver and kidney) and milk the perception of widely use of antimicrobial compounds in animal feedingstuffs and the increasing number of resistant pathogenic bacteria and the spread of resistance genes from food-producing animals to humans as a result of veterinary and zoo-technical use in food-producing animals [8,20-22]. Therefore, analysis and detection of macrolides residues in animal feedingstuffs by accurate methods to maintain an efficient food safety management system across the human food chain is very important issue.

As a final result, the proposed method was successfully used for detection and quantification of these drugs in animal feedingstuffs. The method is simple, rapid, sensitive

and suitable for the simultaneous determination of ERY, SPI, TIL and TYL drugs in animal feedingstuffs.

REFERENCES

1. Dubois M, Fluchard D, Sior E, Delahaut P: Identification and quantification of five macrolide antibiotics in several tissues, eggs and milk by liquid chromotography-electrospray tandem mass spectrometry. *J Chromatogr B*, 753, 189-202, 2001.

2. Brisson-Noël A, Trieu-Cuot P, Courvalin P: Mechanism of action of spiramycin and other macrolides. *J Antimicrob Chemother*, 22 (Suppl B): 13-23, 1988.

3. Draisci R, Palleschi L, Ferretti E, Achene L, Cecilia A: Confirmatory method for macrolide residues in bovine tissues by micro-liquid chromatography-tandem mass spectrometry. *J Chromatogr A*, 926, 97-104, 2001.

4. Hamscher G, Limsuwan S, Tansakul N, Kietzmann M: Quantitative analysis of tylosin in eggs by high performance liquid chromatography with electrospray ionization tandem mass spectrometry: Residue depletion kinetics after administration via feed and drinking water in laying hens. *J Agric Food Chem*, 54 (24): 9017-9023, 2006.

5. Van Poucke C, Dumoulin F, De Keyser K, Elliott C, Van Peteghem C: Tylosin detection in animal feed by liquid chromatography-tandem mass spectrometry with enzymatic hydrolysis of the tylosin urea adduct. *J Agric Food Chem*, 52, 2803-2806, 2004.

6. Spisso BF, Ferreira RG, Pereira MU, Monteiro MA, Cruz TÁ, Da Costa RP, Lima AMB, Nóbrega AWDA: Simultaneous determination of polyether ionophores, macrolides and lincosamides in hen eggs by liquid chromatography-electrospray ionization tandem mass spectrometry using a simple solvent extraction. *Anal Chim Acta*, 682, 82-92, 2010.

7. World Health Organization (WHO): Critically important antimicrobials for human medicine: Categorization for the development of risk management strategies to contain antimicrobial resistance due to non-human antimicrobial use. *Report of the Second WHO Expert Meeting, Copenhagen,* 29-31 May 2007, http://www.who.int/foodborne disease/ resistance/ antimicrobials human.pdf, *Accessed:* 19.07.2013.

8. European Commission: Council Regulation (EC), 2821/98 of 17 December 1998 amending, as regards withdrawal of the authorisation of certain antibiotics, Directive 70/524/EEC concerning additives in feedingstuffs. *Off J Eur Commun*, L351, 4-8, 1998.

9. The European Medicines Agency (EMEA): Committee for Medicinal Products for Veterinary Use (CMVP). Reflection paper on the use of macrolides, lincosamides and streptogramins (MLS) in food-producing animals in the European Union: development of resistance and impact on human and animal health Adoption by CVMP on 12 October 2011. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/11/WC500118230.pdf, *Accessed:* 22.07.2013.

10. Turkish Food Codex: Regulation on the placing on the market and use of feedingstuffs, Offcial Gazette No. 28155, 27.12.2011. http://mevzuat.basbakanlik.gov.tr/Metin.Aspx?MevzuatKod=7.5.15666&MevzuatIliski=0&sourceXmlSearch=yemlerin, *Accessed:* 22.07.2013.

11. Berrada H, Borrull F, Font G, Marcé RM: Determination of macrolide antibiotics in meat and fish using pressurized liquid extraction and liquid chromatography-mass spectrometry. *J Chromatogr A*, 1208, 83-89, 2008.

12. Leal C, Codony R, Ramon C, Merce`G, Dolors PM: Determination of macrolide antibiotics by liquid chromatography. *J Chromatogr A*, 910, 285-290, 2001.

13. European Commission: Commission Decision 2002/657/EC of 12 August 2002 implementing Council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off J Eur Commun*, L221, 8, 2002.

14. Zivak Technologies: Operator's Manual. ZV-1020-0200-50. http://zivak.com/files/06598cd0-ef60-41f8-9d2f-5f84d388ac0a.pdf, *Accessed:* 22.07.2013.

15. De la Huebra MJG, Vincent U, Von Holst C: Sample preparation strategy for the simultaneous determination of macrolide antibiotics in animal feedingstuffs by liquid chromatography with electrochemical detection (HPLC-ECD). *J Pharmaceut Biomed Anal*, 43, 1628-1637, 2007.

16. Van Poucke C, De Keyser K, Baltusnikiene A, Mc Evoy JDG, Van Peteghem C: Liquid chromatographic-tandem mass spectrometric detection of banned antibacterial growth promoters in animal feed. *Anal Chim Acta*, 483, 99-109, 2003.

17. Yan L, Zhang F, Fang E, Guo Y, Zhou Y, Lin L, Chu X: Simultaneous determination of macrolide and lincosamide antibiotics in animal feeds by ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry. *Se Pu*, 28 (11): 1038-1042, 2010.

18. Kaklamanos G, Vincent U, von Holst C: Analysis of antimicrobial agents in pig feed by liquid chromatography coupled to orbitrap mass spectrometry. *J Chromatogr A*, 1293, 60-74, 2013.

19. Civitareale C, Fiori M, Ballerini A, Brambilla G: Identification and quantification method of spiramycin and tylosin in feedingstuffs with HPLC-UV/DAD at 1 ppm level. *J Pharm Biomed Anal*, 36 (2): 317-325, 2004.

20. Botsoglou NA, Fletouris DJ: Drug Residues in Foods, Marcel Dekker, New York, 2001.

21. European Commission: Opinion of the Scientific Committee on Animal Nutrition on the Criteria for Assessing the Safety of Micro-organisms Resistant to Antibiotics of Human and Veterinary Importance, July 3, 2001.

22. Güner A, Atasever M, Atasever AM: New emerging and re-emerging bacterial foodborne pathogens. *Kafkas Univ Vet Fak Derg,* 18 (5): 889-898, 2012.

Kafkas Univ Vet Fak Derg
20 (3): 445-449, 2014
DOI: 10.9775/kvfd.2013.10383

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

RESEARCH ARTICLE

Effect of Maturity Stages on Potential Nutritive Value, Methane Production and Condensed Tannin Content of *Sanguisorba minor* Hay

Mahmut KAPLAN [1] Adem KAMALAK [2] Ali Amer KASRA [2] İnan GÜVEN [2]

[1] University of Erciyes, Faculty of Seyrani Agriculture, Department of Field Crops, TR-38090 Kayseri - TURKEY
[2] Department of Animal Science, Faculty of Agriculture, Sutcu Imam University, TR-46100 Kahramanmaras - TURKEY

Makale Kodu (Article Code): KVFD-2013-10383

Summary

The current trial was conducted to study the effect of maturity on the potential nutritive value, methane production and condensed tannin of *Sanguisorba minor* hay. *Sanguisorba minor* hay harvested at three different maturity stages (pre- flowering, flowering and seeding stages). *Sanguisorba minor* hay was shade dried and analyzed for chemical composition. Gas and methane productions of *Sanguisorba minor* hay were determined at 24 h incubation time. Maturity had a significant effect (P<0.05) on the chemical composition, gas production, methane production metabolisable energy (ME) and organic matter digestibility (OMD). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) of *Sanguisorba minor* hay increased with advancing maturity whereas crude protein (CP) and ash content decreased. The CP contents of *Sanguisorba minor* hay varied between 6.7 and 20.7%. The NDF and ADF contents of *Sanguisorba minor* hay ranged from 36.2 to 54.5 and 17.4 to 36.2 % respectively. The condensed tannin content of *Sanguisorba minor* hay varied with maturity between 0.4 and 1.6 % and decreased (P<0.05) with increasing maturity. The gas and methane production at 24 h incubation ranged from 32.2 to 43.5 ml and 4.6 to 6.5 ml respectively and decreased (P<0.05) after flowering. The ME and OMD of *Sanguisorba minor* ranged from 7.0 and 9.3 MJ/kg DM and 46.9 to 63.2% respectively and decreased (P<0.05) with each increment of the maturity. In conclusion, maturity had a significant effect on the nutritive value of *Sanguisorba minor* hay. The nutritive value of *Sanguisorba minor* decreased with increased maturity. It can be suggested that *Sanguisorba minor* should be grazed or harvested at pre-flowering and flowering stage since these stage provides hay with high ME and CP for ruminant.

Keywords: *Sanguisorba minor hay, Nutritive value, Condensed tannin, In vitro gas production, Methane production*

Olgunlaşma Döneminin Çayır Düğmesi Otunun Potansiyel Besin Değerine, Metan Üretimine ve Kondense Tanen İçeriğine Etkisi

Özet

Yürütülen bu çalışmanın amacı, olgunlaşma döneminin çayır düğmesi otunun potansiyel besleme değerine, metan üretimine ve kondense tanen içeriğine olan etkisini araştırmaktır. Çayır düğmesi otu üç faklı olgunlaşma döneminde (çiçeklenme öncesi, çiçeklenme ve tohum bağlama) hasat edilip, gölgede kurutularak kimyasal kompozyonu için analizler yapılmıştır. Yirmi dört saatlik ünkibasyon sonunda çayır düğmesi otunun gaz ve metan üretimi belirlenmiştir. Olgunlaşma dönemi, çayır düğmesi otunun kompozisyonuna, gaz üretimine, metan üretimine, metabolik enerji ve organik madde sindirim derecesine önemli derecede (P<0.05) etki etmiştir. Olgunlaşma dönemin ilerlemesiyle nötral deterjan fiber ve asit deterjan fiber oranı artarken ham protein ve kül içeriği azalmıştır. Çayır düğmesi otunun ham protein içeriği %6.7 ile 20.7 arasında değişmiştir. Çayır düğmesi otunun nötral deterjan fiber ve asit deterjan fiber içeriği sırasıyla %36.2 ile 54.5 ve %17.4 ile 36.2 arasında değişmiştir. Çayır düğmesi otunun kondense tanen içeriği ise %0.4 ile 1.6 arasında değişmiş olup, olgunlaşma döneminin ilerlemesiyle birlikte azalmıştır. Çayır düğmesi otunun olgunlaşma döneminin ilerlemesiyle birlikte yirmi dört saatlik gaz ve metan üretimi azalmıştır. Yirmi dört saatlik gaz ve metan üretimi sırasıyla 32.2 ile 43.5 ml ve 4.6 ile 6.5 ml arasında değişmiştir. Çayır düğmesi otunun metabolik enerji ve organik madde sindirim derecesi sırasıyla 7.0 ile 9.3 MJ/kg kuru madde ve %46.9 ile 63.2 arasında değişmiştir. Sonuç olarak, olgunlaşma dönemi, çayır düğmesinin besleme değerini önemli derecede etkilemiştir. Besleme değeri olgunlaşma döneminin ilerlemesiyle birlikte düşmüştür. Ham protein ve metabolik enerji içeriği yüksek olmasından dolayı çayır düğmesi otunun çiçeklenme öncesi ve çiçeklenme döneminde otlatılması veya hasat edilmesi önerilebilir.

Anahtar sözcükler: *Çayır düğmesi otu, Besin değeri, Kimyasal kompozisyon, Kondense tanen, İn vitro gaz üretimi, Metan üretimi, Sindirim derecesi*

İletişim (Correspondence)
+90 344 2191620
akamalak@ksu.edu.tr

INTRODUCTION

There are several factors affecting the nutritive value of forages. The maturity stage at harvesting is one of important factors affecting the nutritive value of forages. The pastures provide important forage for ruminant. Rangeland is commonly grazed goat and sheep to meet some nutrient requirement depending on quality of rageland as well as provide fibre to ruminant for chewing and rumination in Turkey [1]. *Sanguisorba minor* a perennial plant of the family Rosaceous grow up to a length of 50-70 cm and is widely distributed the natural pasture of Turkey providing early grazing forage [2]. Although the nutritive value of many types of forages in the pasture harvested at different maturity is well established [3-6], the information about the nutritive value of *Sanguisorba minor* hay with maturity is scarce. Accurate prediction of nutritive value of forages at different maturity stages allows nutritionist to meet specific animal requirements [7].

Recently *in vitro* gas production technique with chemical composition have been widely used to evaluate the potential nutritive value of previously uninvestigated forages since *in vitro* gas production technique is quick, cheap, less time consuming [3-6]. In addition, *in vitro* gas production technique was used to screen the feedstuffs in terms of their methane reduction potential [8-11]. Methane production during rumen fermentation is one of important contributors to global warming [12].

The current trial was conducted to study the effect of maturity stage on the potential nutritive value, methane production and condensed tannin of *Sanguisorba minor* hay.

MATERIAL and METHODS

The experiment was carried out in University of Kahramanmaras Sutcu Imam, Faculty of Agriculture, and Department of Animal Science. The experimental protocols were approved by the Animal Experimentation Ethics Committee of University of Kahramanmaras Sutcu Imam, Faculty of Agriculture (Protocol No: 2013/03-3).

Sanguisorba minor plants were hand harvested at three maturity stages (pre-flowering (13.03.2013), flowering (15.04.2013) and seeding stages (07.05.2013) from three plots that established in completely randomized block design in 10x2 m plots in the experimental field in 2013 in Kahramanmaras, Turkey. Plant samples were shade dried and representative dry samples from each plot was taken to laboratory and milled in a hammer mill through a 1 mm sieve for subsequent analysis.

Dry matter (DM) content of *Sanguisorba minor* hay was analyzed by oven drying at 105°C 24 h. Ash content was determined by igniting the samples of *Sanguisorba minor* hay in muffle furnace at 525°C for 8 h. Nitrogen (N) content of *Sanguisorba minor* hay was measured by the Kjeldahl method [13]. Crude protein of *Sanguisorba minor* hay was calculated as N X 6.25. Neutral detergent fiber (NDF) of *Sanguisorba minor* hay was determined according to Van Soest and Wine [14] and ADF content of *Sanguisorba minor* hay were determined by the method of Van Soest [15]. Condensed tannin was determined by butanol-HCl method as described by Makkar *et al.*[16]. All chemical analyses were carried out in triplicate.

Sanguisorba minor hay samples that were also milled through a 1 mm sieve were incubated *in vitro* rumen fluid in 100 ml calibrated glass syringes following the procedures of Menke *et al*.[17]. Rumen fluid was obtained from three fistulated Awassi sheep fed twice daily with a diet containing alfalfa hay (60%) and concentrate (40%) with a free access to water and mineral block. Rumen fluid was collected before morning feeding and filtered through four layers of cheesecloth under flushing with CO_2. The rumen fluid was combined with buffered solution in the ratio of 1:2 respectively. Approximately 0.200 gram air dried samples of *Sanguisorba minor* hay samples was weighed into calibrated glass syringes which were prewarmed at 39°C. Then 30 mL rumen fluid-buffer mixture was transferred into each syringe. The glass syringes containing samples and rumen fluid-buffer mixture were placed in a water bath at 39°C. Gas production was measured at 24 h after incubation and corrected for blank and hay standard (University of Hohenheim, Germany).

ME (MJ/kg DM) content of *Sanguisorba minor* hay samples was calculated using equation of Menke *et al*.[17] as follows:

ME (MJ/kg DM) = 2.20 + 0.136 GP + 0.057 CP, where GP = 24 h net gas production (ml/200 mg); CP = Crude protein

Organic matter digestibility (%) of *Sanguisorba minor* hay samples was calculated using equation of Menke *et al*.[17] as follows:

OMD (%) = 14.88 + 0.889GP + 0.45CP +0.0651 XA, where XA: ash content (%)

Methane gas content of total gas produced at 24 h fermentation was measured using an infrared methane analyzer (Sensor Europe GmbH, Erkrath, Germany) [18]. After measuring gas produced at 24 h incubation, gas samples was transferred into inlet of the infrared methane analyzer using the plastics syringe. The infrared methane analyzer displays methane as percent of total gas. Methane production (mL) was calculated as follows:

Methane production (mL) = Total gas production (mL) X Percentage of Methane (%)

All data obtained were subjected to analysis of variance (ANOVA) using the randomized completed block design.

Significance between individual means was identified using the Tukey's multiple range tests. Mean differences were considered significant at P<0.05.

RESULTS

As shown in *Table 1* the maturity stage had a significant effect on the chemical composition of *Sanguisorba minor* hay. The NDF and ADF contents of *Sanguisorba minor* hay increased (P<0.05) whereas CP, ash and CT contents were decreased (P<0.05) with each increment of maturity stage. The DM content was similar (P>0.05) at fre-flowering and flowering stage, but was higher (P<0.05) at seeding stage. The DM, NDF and ADF contents varied between 22.6 and 33.7%, 36.2 and 54.5% and 17.4 and 36.2% respectively. On the other hand, CP, ash and CT contents varied between 6.7 and 20.7 and 0.5 and 1.6 % respectively.

As shown in *Table 2* the maturity stage had a significant effect on the gas production, methane production, ME and OMD of *Sanguisorba minor* hay. The gas and methane production at 24 h incubation ranged from 32.2 to 43.5 ml and 4.6 to 6.5 ml respectively and decreased (P<0.05) after flowering. The ME and OMD of *Sanguisorba minor* ranged from 7.0 and 9.3 MJ/kg DM and 46.9 to 63.2% respectively and decreased (P<0.05) with each increment of the maturity.

DISCUSSION

The marked decrease in CP and increase in DM, NDF and ADF with advancing maturity was in accordance with the findings of others studies with various forage spices. Similar changes with maturity were also observed by Kamalak *et al.*[3] in *Gundelia tournefortii* hay, Kamalak *et al.*[4] in *Sinapsis arvensis* hay, Kamalak and Canbolat [6] in *Trifolium angustifolium* hay, Kamalak *et al.*[19] in *Trigonella kotschi* hay, Canbolat [1] in *Convolvulus arvensis* hay. Decrease in CP content of hays due to advancing maturity is possibly due to a combination effect of decrease of CP in leaves and increase of stem content at the expense of leaves of hay samples advancing maturity. The protein content of stem is lower than that of leaves [20]. Daily reduction in CP was calculated by the difference between CP of hay obtained at pre-flowering and seeding stages, divided by the time (days) required to reach from pre-flowering to seeding stage. In the current study the reduction in CP content of *Sanguisorba minor* hay was approximately 2.54 g/kg/day. On the other hand the increase in NDF and ADF contents of *Sanguisorba minor* hay were 3.32 and 3.41 g/kg/day respectively.

CP content of *Sanguisorba minor* hay obtained in the current study was comparable with finding of Asaadi and Yazdi [21] who reported that CP ranged from

Table 1. *The effect of maturity stage on the chemical composition of Sanguisorba minor hay*
Tablo 1. *Hasat zamanının küçük çayır düğmesi otunun kompozisyonuna etkisi*

Nutrients (%)	Maturity Stages			SEM	Significance
	Pre-flowering	Flowering	Seeding		
DM	22.6^{b}	24.9^{b}	33.7^{a}	1.06	***
CP	20.7^{a}	13.7^{b}	6.7^{c}	0.37	***
Ash	8.7^{a}	7.5^{b}	6.5^{c}	0.15	***
NDF	36.2^{c}	49.2^{b}	54.5^{a}	0.93	***
ADF	17.4^{c}	29.8^{b}	36.2^{a}	0.82	***
CT	1.6^{a}	0.9^{b}	0.4^{c}	0.03	***

*$^{a, b, c}$ Row means with common superscripts do not differ (P<0.05); **S.E.M.:** standard error mean; **DM:** Dry matter %, **CP:** Crude protein, **NDF:** Neutral detergent fiber, **ADF:** Acid detergent fiber, **CT:** Condensed tannin, ******* P<0.001*

Table 2. *The effect of maturity stage on the gas production kinetics, metabolisable energy and organic matter digestibility of Sanguisorba minor hay*
Table 2. *Hasat zamanın Sanguisorba minor otunun gaz üretim parametrelerine, metabolik enerji ve organik madde sindirim derecesine etkisi*

Estimate Parameters	Maturity Stages			SEM	Significance
	Pre-flowering	Flowering	Seeding		
Total Gas (*mL*)	43.5^{a}	43.2^{a}	32.2^{b}	0.80	***
CH4 (*mL*)	6.5^{a}	6.37^{a}	4.6^{b}	0.05	***
CH4 % of Total Gas	14.9	14.8	14.4	0.26	NS
ME (*MJ /Kg DM*)	9.3^{a}	8.8^{b}	7.0^{c}	0.11	***
OMD (%)	63.2^{a}	59.8^{b}	46.9^{c}	0.71	***

*$^{a, b, c}$ Row means with common superscripts do not differ (P>0.05); **S.E.M.:** standard error mean; **NS:** Non-significant, **c:** gas production rate (%); **A:** potential gas production (mL), **ME:** Metabolisable energy (MJ/kg DM); **OMD:** Organic matter digestibility %, *** P<0.001*

5.21 to 17.04% and decreased with maturity.

In the current study the reduction in CP of *Sanguisorba minor* hay estimated was considerably higher than those obtained by Minson [22] and Kamalak and Canbolat [6] who indicated that the average reduction in CP of several forages due to maturity ranged from 0.82 and 1 g/kg/day. On the hand, the reduction in the current experiment was in accordance with the findings of Kamalak *et al*.[19] who reported that decline in CP due to maturity was 2.34 g/kg/day.

Condensed tannin had an important role in forages depending on the amount. Low level tannin (2-3% of DM) may have beneficial effect since the level tannin in diets prevent the CP from extensive degradation through formation of protein-tannin complexes [23]. On the other hand, high tannin level (5% of DM) in diets may result in the increased indigested CP due to excessive formation of tannin-protein complexes [24].

As can be seen from *Table 1*, the observed condensed tannin levels of *Sanguisorba minor* hay harvested at three maturity stages were low magnitude. Therefore, low condensed tannin of *Sanguisorba minor* hay seems to have a potential for beneficial effect when included into ruminant diets as it can increase rumen undegradable crude protein without decreasing digestibility.

The marked decreases in gas production, methane production, ME and OMD due to maturity were closely associated with increase in less digestible cell contents (NDF and ADF) and decrease in CP of *Sanguisorba minor* hay with maturity. As can be seen from the equation suggested by Menke *et al*.[17] OMD and ME values were estimated using the gas production and CP. The gas production is closely associated with the amount of fermented substrate in diets [25]. Therefore, decrease in fermentable fraction in forage with maturity due to increased cell wall contents that consist of less digestible carbohydrates resulted in less gas production. As a result, the decrease in gas production with maturity was inevitable. Similar observation with maturity were also obtained by Kamalak *et al*.[3] in *Gundelia tournefortii* hay, Kamalak *et al*.[4] in *Sinapsis arvensis* hay, Kamalak and Canbolat [6] in *Trifolium angustifolium* hay, Kamalak *et al*.[19] in *Trigonella kotschi* hay Canbolat [1], in *Convolvulus arvensis* hay.

Metabolisable energy content of *Sanguisorba minor* hay obtained in the current study was comparable with finding of Asaadi and Yazdi [21] who reported that ME content ranged from 5.54 to 9.96 MJ/kg DM and decreased with maturity.

Lopez *et al*.[26] suggested that the methane reduction potential of any feedstuffs can be estimated from the percentage of methane of *in vitro* gas production and the feedstuffs can be arbitrarily divided in three groups, low potential (% methane in gas between >11% and ≤14%), moderate potential (% methane in gas between >6% and <11%), high potential (% methane in gas between >0% and <6%). Therefore *Sanguisorba minor* hay had no methane reduction potential since the percentage of methane for all three maturity stages is higher than %14.

In conclusion, maturity had a significant effect on the nutritive value of the forage of *Sanguisorba minor*. The nutritive value of *Sanguisorba minor* decreased with increased maturity. *Sanguisorba minor* should be grazed or harvested at pre-flowering and flowering stage since these stage provides hay with high ME and CP for ruminant.

REFERENCES

1. Canbolat O: Potential nutritive value of Field Bindweed *(Convolvulus arvensis* L.) hay harvested at three different maturity stages. *Kafkas Univ Vet Fak Derg,* 18 (2): 331-335, 2012.

2. İpek A, Sevimay CS: Effects of nitrogenous fertilization on forage yield components of garden burnet (*Sanguisorba minor* scop). *J Agric Sci*, 8 (4): 274-279, 2002

3. Kamalak A, Canbolat O, Gurbuz Y, Erol A, Ozay O: Effect of maturity stage on chemical composition, *in vitro* and *in situ* dry matter degradation of tumbleweed hay *(Gundelia tournefortii* L). *Small Rumin Res*, 58 (2): 149-156, 2005.

4. Kamalak A, Canbolat O, Gurbuz Y, Ozkan CO, Kizilsimsek M: Determination of nutritive value of wild mustard, *Sinapsis arvensis* harvested at different maturity stages using *in situ* and *in vitro* measurements. *Asian-Austral J Anim Sci*, 18 (9): 1249-1254, 2005.

5. Kamalak A: Determination of potential nutritive value of *Polygonum aviculare* hay harvested at three maturity stages. *J Appl Anim Sci*, 38 (1): 69-71, 2010.

6. Kamalak A, Canbolat O: Determination of nutritive value of wild narrow-leaved clover *(Trifolium angustifolium)* hay harvested at three maturity stages using chemical composition and *in vitro* gas production. *Trop Grassland*, 44 (2): 128-133, 2010.

7. Valente ME, Borreani G, Peiretti PG, Tobacco E: Codified morphological stage for predicting digestibility of Italian ryegrass during the spring cycle. *Agron J*, 92, 967-973, 2000.

8. Jayanegara A, Togtokhbayar N, Makkar HPS, Becker K: Tannins determined by various methods as predictors of methane production reduction rumen potential of plants by an *in vitro* rumen fermentation system. *Anim Feed Sci Technol*, 150 (3-4): 230-237, 2009.

9. Jayanegara A, Wina E, Soliva CR, Marquardt Kreuzer M, Leiber F: Dependence of forage quality and methanogenic potential of tropical plants on their phenolic fractions as determined by principal component analysis. *Anim Feed Sci Technol*, 163 (2-4): 231-243, 2011.

10. Thang CM, Winding S, Hang LT: Effects of different foliages and drying methods on mitigation methane production based on cassava root meal using *in vitro* gas production. *J Anim Sci Technol,* 34, 40-50, 2012.

11. Lin B, Wang JH, Lu Y, Liang Q, Liu JX: *In vitro* rumen fermentation and methane production are influenced by active components of essential oils combined with fumarate. *Anim Physiol Anim Nutr*, 97 (1): 1-9, 2013.

12. Lassey KR: Livestock methane emissions from the individual grazing animal through national inventories to the global methane cycle. *Agr Forest Meteorol*, 142 (2-4): 120-132, 2007.

13. AOAC: Official Method of Analysis. 15th ed., pp.66-88. Association of Official Analytical Chemists, Washington, DC, USA, 1990.

14. Van Soest PJ, Wine RH: The use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell wall constituents. *J Assn Offic Anal Chem,* 50, 50-55, 1967.

15. Van Soest: The use of detergents in the analysis of fibrous feeds. II. A

rapid method for the determination of fiber and lignin. *J Assn Offic Anal Chem*, 46, 829-835, 1963.

16. Makkar HPS, Blummel M, Becker K: Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and their implication in gas production and true digestibility *in vitro* techniques. *Brit J Nutr*, 73 (6): 897-913, 1995.

17. Menke KH, Raab L, Salewski A, Steingass H, Fritz D, Schneider W: The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. *J Agric Sci Camb*, 93 (1): 217-222, 1979.

18. Goel G, Makkar HPS, Becker K: Effect of Sesbania sesban and *Carduus pycnocephalus* leaves and Fenugreek *(Trigonella foenum-graecum* L) seeds and their extract on partitioning of nutrients from roughage- and concentrate-based feeds to methane. *Anim Feed Sci Technol*, 147 (1-3): 72-89, 2008.

19. Kamalak A, Atalay AI, Ozkan CO, Kaya E, Tatlıyer A: Determination of nutritive value of *Trigonella kotschi* Fenz hay harvested at three different maturity stages. *Kafkas Univ Vet Fak Derg,* 17 (4): 635-640, 2011.

20. Buxton DR: Quality related characteristics of forages as influenced by plant environment and agronomic factors. *Anim Feed Sci Tech,* 59 (1-3): 37-49,1996.

21. Asaadi AM, **Yazdi AK:** Phonological stage effects on forage quality of four forbs species. *J Food Agric Envir*, 9 (2): 380-384. 2011.

22. Minson DJ: Forage in Ruminant Nutrition. 1-483, Academic Press, New York, 1990.

23. Barry TN: Secondary compounds of forages. **In,** Hacker JB, Ternouth JH (Eds): Nutrition of Herbivores. 91-120, Academic Press, Sydney, 1987.

24. Kumar R, Singh M: Tannins: Their adverse role in ruminant nutrition. *J Agric Food Chem*, 32 (3): 447-453, 1984.

25. Blummel M, Orskov ER: Comparison of an *in vitro* gas production and nylon bag degradability of roughages in predicting feed intake in cattle. *Anim Feed Sci Tech*, 40 (2-8): 109-119, 1993.

26. Lopez S, Makkar HPS, Soliva CR: Screening plants and plant products for methane inhibitors. **In,** Vercoe PE, Makkar HPS, Schlink A (Eds): *In vitro* Screening of Plant Resources for Extra-nutritional Attributes in Ruminants: Nuclear and Related Methodologies. pp.191-231, London, New York, 2010.

Kafkas Univ Vet Fak Derg
20 (3): 451-455, 2014
DOI: 10.9775/kvfd.2013.10427

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

RESEARCH ARTICLE

Leptospirozlu Sığırlarda Plazma Nitrik Oksit (NO) ve Tümör Nekrozis Faktör-α (TNF-α) Düzeyleri ile Adenozin Deaminaz (ADA), Gama Glutamil Transferaz (GGT) Aktiviteleri ve Perifer Kan Lökositlerinde Alfa Naftil Asetat Esteraz (ANAE) Yöntemiyle Lenfosit Oranlarının Belirlenmesi [1]

Emine ATAKİŞİ [1] Ali Haydar KIRMIZIGÜL [2] Onur ATAKİŞİ [3] Ebru KARADAĞ SARI [4]
Metin ÖĞÜN [1] Şaban MARAŞLI [1] Ayla ÖZCAN [1] Mahmut KARAPEHLİVAN [1]
Fatih BÜYÜK [5] Özgür ÇELEBİ [5]

[1] *This study was supported by Kafkas University Scientific Research Projects Commission (Project No: 2008-VF-20)*

[1] Department of Biochemistry, Faculty of Veterinary Medicine, Kafkas University, TR-36100 Kars - TURKEY
[2] Department of Internal Medicine, Faculty of Veterinary Medicine, Kafkas University TR-36100 Kars - TURKEY
[3] Department of Biochemistry, Faculty of Arts and Sciences, Kafkas University, TR-36100 Kars - TURKEY
[4] Department of Histology and Embryology, Faculty of Veterinary Medicine, Kafkas University, TR-36100 Kars - TURKEY
[5] Department of Microbiology, Faculty of Veterinary Medicine, Kafkas University, TR-36100 Kars - TURKEY

Makale Kodu (Article Code): KVFD-2013-10427

Özet

Yapılan bu çalışmada plazmada nitrik oksit (NO), Tümör Nekrozis Faktör-α (TNF-α) düzeyleri, adenozin deaminaz (ADA), gama glutamil transferaz (GGT) aktiviteleri ve alfa naftil asetat esteraz (ANAE) yöntemi ile lenfosit oranları belirlenerek, sığır leptospirozunun immunpatogenezinin ve biyokimyasal mekanizmalarının araştırılması amaçlanmıştır. Kars civarındaki köylerde yetiştirilen 20 baş leptospirozlu ve 20 baş sağlıklı danaların vena jugularislerinden heparinli tüplere kan örnekleri alındı ve histolojik analizler için frotiler alındıktan sonra, 3.000 rpm'de 15 dak. santrifüj edilerek plazmaları elde edildi. Plazmada NO düzeyi ile ADA aktivitesi kimyasal yöntemle, TNF-α düzeyi ile GGT aktivitesi ticari test kitleri kullanılarak kolorimetrik yöntemle analiz edildi. T/B lenfosit oranları ise perifer kan lökositlerinde ANAE yöntemiyle belirlendi. Leptospirozlu hayvanların plazma TNF-α ve NO düzeyleri kontrol grubuna göre sırasıyla P<0.001 ve P<0.05, ADA ve GGT aktiviteleri P<0.05 düzeyinde yüksek tespit edildi. ANAE pozitif (T) lenfosit oranı ise %43 olarak belirlendi. Sonuç olarak enfeksiyonun erken aşamasında saptanabilen bir sitokin olan TNF-α ve buna bağlı olarak NO düzeylerinin belirgin bir şekilde yükselmesi, ADA ve GGT aktivitelerinin artması ile B lenfosit yüzdesinin fazla olduğunun tespitinden yola çıkılarak, leptospirozun patogenezisinde bu parametrelerden yararlanılabileceği düşünülmektedir. Ayrıca doğal enfekte sığırlarda leptospirozun biyokimyasal mekanizmaları üzerine daha fazla çalışmaların yapılması gerektiği söylenebilir.

Anahtar sözcükler: *Leptospiroz, Dana, TNF-α, ADA, GGT, NO*

Plasma Nitric Oxide (NO) and Tumor Necrosis Factor-α (TNF-α) Levels, Adenosine Deaminase (ADA), Gamma Glutamyl Transferase (GGT) Activities and to Determine the Rate of Lymphocytes in the Peripheral Blood Leukocytes Alpha Naphthyl Acetate Esterase (ANAE) in Cattle with Leptospirosis

Summary

In this study, by determining in plasma the nitric oxide (NO), tumour necrosis factor-α (TNF-α) levels, adenosine deaminase (ADA), gamma glutamyl transferase (GGT) activities and percentage of T lymphocytes with alpha naphthyl acetate esterase method (ANAE), it was aimed to investigate the immunopathogenesis and biochemical mechanism of bovine leptospirosis. Blood samples of twenty healthy calves and twenty calves with leptospirosis which grown in the villages around Kars were collected into heparinised tubes from jugular vein. After taking the blood smear, blood samples were centrifuged at 3.000 rpm for 15 min. Plasma NO levels and ADA activities were determined with chemical method. TNF-α levels and GGT activities were determined with commercial kits. T/B lymphocyte ratios were determined at peripheral blood leukocytes with ANAE method. Plasma TNF-α and NO levels, ADA and GGT activities in animals with leptospirosis were found to be high P<0.001, P<0.05, P<0.05, P<0.05 respectively compared to healthy animals. ANAE positive T lymphocytes ratio were determined as 43%. As a result, TNF-α as a cytokine can be determined in early stage of infection and, accordingly, significantly increased levels of NO, ADA and GGT activities and, on the basis of the increased in the percentage of B lymphocytes, it is proposed to these parameters could be used for determining in explain the pathogenesis of the disease. It is tought that, it must be done more studies on biochemical mechanism of leptospirosis besides native infected cattle.

Keywords: *Leptospirosis, Calf, TNF-α, ADA, GGT, NO*

İletişim (Correspondence)
+90 474 2426800/5144
et_tasci@hotmail.com

GİRİŞ

Leptospiroz, *Leptospira* cinsi spiroketlerin sebep olduğu dünyaca yaygın bir zoonozdur. Hem endüstriyel ülkelerde hem de gelişmekte olan ülkelerde rastlanmaktadır. Enfekte hayvanların idrarıyla direkt temasla ya da kontamine suyla indirekt temasla bulaşır [1]. *Leptospira* vücuda girdiği zaman konakçının hedef organlarına ulaşır ve proksimal renal tubul gibi immun sistemin çok daha az etkili olduğu bölgelerde çoğalır. Avirulent leptospiralar vücuda girdikten birkaç saniye sonra retikuloendotelyal sistem hücreleri tarafından hızla fagosite edilerek ortadan kaldırılır. Virulent olanlar ise, fagositozisden kurtularak yaşamaya devam ederler ve hastalığın ilk haftalarında kandan izole edilebilirler [2]. Adenozin Deaminaz (ADA)'nın en önemli fizyolojik rolü lenfositlerin farklılaşması ve çoğalmasıyla ilgilidir. ADA aktivitesi lenfositlerde eritrositlere oranla 10 kat daha fazla, T lenfositlerde ise B lenfositlere göre daha yüksek oranlarda bulunmaktadır. T hücrelerinin farklılaşması esnasında özellikle olgunlaşmamış ve farklılaşmamış hücrelerde belirgin artış olmaktadır [3]. Leptospiroz olgularında ADA aktivitesinin belirlendiği çalışma sayısı çok azdır. Ancak özellikle kan hücrelerinin ve damar duvarının hasarına, dolayısıyla hemolize sebep olan leptospirozda ADA aktivitesinde değişim olması beklenmektedir. Plazma gama glutamil transpeptidaz (GGT; EC 2.3.2.2) aktivitesi safra kesesi tıkanıklığına bağlı karaciğer hasarının olduğu kadar hücre immunitesinin de belirteçlerinden biridir [4].

Nitrik oksit (NO) renksiz, küçük moleküllü, yağda çözünen, yarı ömrü oldukça kısa olan ve hücre zarlarından kolaylıkla geçebilen reaksiyon yeteneği oldukça yüksek olan toksik etkili nörotransmitter bir maddedir [5]. İmmun sistemde sitokinler (IL-1, TNF, IFN-γ) ve endotoksinler tarafından oluşturulan uyarılar sonucu birkaç saat içerisinde başlayan ve günler boyu süren nanomol düzeyde NO sentezi gerçekleştirilmektedir [6]. Tümör nekrosis faktör-alfa (TNF-α), yangı, hücre yaşamının devamı ve hücre ölümü süreçlerinde yer alan önemli bir sitokindir. Bu molekül makrofajların farlıklaşmasını ve uyarılabilir nitrik oksit sentetazı (iNOS) aktive ederek NO üretimini arttırabilmektedir [7]. iNOS normalde hücre içinde yapısal olarak sentezlenmez, uygun bir immun uyarı aracılığı ile sentezi başlar. Diğer NO sentetaz izoformlarından farklı olarak ortamda bulunduğu sürece NO sentezler. Sitotoksik ve zararlı etkilere karşı iNOS tarafından sentezlenen NO miktarı yüksektir. Özellikle bakteri, interferon-g veya yüksek miktarda lipopolisakkarit uyarıları ile makrofajlar tarafından aşırı miktarda üretilen NO'un, bakteri, parazit ve tümör gibi yabancı hücrelerde sitostatik veya sitotoksik etki meydana getirdiği gösterilmiştir [6]. Sığır babesiozisinde yapılan bir çalışmada parazit tarafından uyarılan makrofajların TNF-α düzeylerini ve bu molekülün de NO salınımını arttırdığı, NO' in sitotoksik etkisi nedeniyle bu mekanizmaların konakçı organizmanın parazite karşı geliştirdiği savunma mekanizması sonucu meydana geldiği ileri sürülmektedir [8].

Biyokimyasal parametrelerin histolojik olarak destelenmesi amacıyla incelenecek olan alfa naftil asetat esteraz (ANAE) lizozomal bir enzimdir [9]. Pratikte, gerek dokularda ve gerekse de perifer kan frotilerinde T-lenfosit, B-lenfosit ve monositlerin birbirinden ayırt edilmesinde yararlanılan bu enzimin T-lenfosit olgunlaşmasının ilk aşamalarında kazanıldığı bildirilmiştir [10]. Nonspesifik esterazlardan olan ANAE'nın T lenfositlerde bulunduğu, B lenfositlerde bulunmadığı belirtilmektedir [9].

Yapılan bu çalışmada plazmada NO, TNF-α düzeyleri, ADA, GGT aktiviteleri ve ANAE yöntemi ile lenfosit oranları belirlenerek, sığır leptospirozunun immunpatogenezinin ve biyokimyasal mekanizmalarının araştırılması amaçlanmıştır.

MATERYAL ve METOT

Hayvan Materyali ve Numunelerin Toplanması

Kars civarındaki köylerde yetiştirilen ve klinik belirtilerden yola çıkılarak toplanan idrar numunelerinde karanlık saha taramasıyla belirlenen leptospirozlu (20 baş) ve klinik olarak sağlıklı (20 baş) dana hayvan materyali olarak kullanıldı. Danaların vena jugularislerinden heparinli tüplere kan örnekleri alındı. Histolojik analizler için frotiler alındıktan sonra, 3.000 rpm'de 15 dak. santrifüj edilerek elde edilen plazmalar biyokimyasal analizler yapılıncaya kadar -20°C'de (derin dondurucu) saklandı.

Biyokimyasal Analizler

Plazmada ADA aktivitesi Giusti ve Galanti'nin [11] bildirdiği yönteme göre yapıldı. Bu yöntemde substrat olarak kullanılan adenozin, numune ile 37°C'de 1 saat inkübe edildiğinde amonyak meydana gelmekte, oluşan amonyak da alkali ortamda sodyum hipoklorit ve fenol ile kuvvetli olarak mavi renkli indofenol oluşturmaktadır. Amonyak derişimi indofenolün absorbansıyla doğru orantılı olup, sodyum nitroprussid katalizör etkisi göstermektedir.

Nitrik oksit düzeyleri, Miranda ve ark.'nın [12] bildirdikleri yöntemle tayin edildi. Bu yöntemde nitrat, vanadyum (III) klorür ile nitrite dönüştürülür. Nitritle sülfanilamidin asidik ortamda N-(1-Naftil) etilendiamin dihidroklorür ile reaksiyonu sonucu kompleks diazonyum bileşiği oluşur. Oluşan bu renkli kompleksin absorbansı 540 nm'de ölçüldü. Standart grafiğinden yararlanılarak NO düzeyleri tespit edildi.

TNF-α (R&D Systems; Bovine TNF-alpha DuoSet, 15 Plate) ve GGT (DDS; Diasis Diagnostic System, Holzheim, Germany) düzeyleri ticari test kitleri kullanılarak tayin edildi. TNF-α test kiti antikor kaplanmamış olarak alındı ve mikro-plaklar ölçümden önce antikorla kaplandı.

ANAE Yöntemi ile T/B Lenfosit Oranlarının Bulunması

Leptospirozlu ve sağlıklı sığırlardan alınan heparinize

kan örneklerinden frotiler hazırlanarak oda ısısında kurutuldu. Gluteraldehid-aseton tespit sıvısında -10°C'de 3 dak. tespit edilip, distile suda yıkandıktan sonra oda ısısında kurumaya bırakıldı. ANAE enzim aktivitesinin belirlenmesi için, 0.067 M fosfat tamponunun (pH 5.0) 40 ml'sine 2.4 ml hekzazotize edilen pararozanilin (Sigma P3750) çözeltisi ve 0.4 ml asetonda çözülen 10 mg alpha napthyl acetate (Sigma N8505) eklenerek hazırlanan inkübasyon çözeltisi kullanıldı [10]. İnkubasyon çözeltileri 2N NaOH ile pH 5.8'e ayarlandı. Üç saatlik inkubasyonu takiben metilen mavisi ile 10 dak. çekirdek boyası yapıldı. Işık mikroskopta (Olympus BX51, JAPAN) her frotide 3 değişik alanda toplam 300 lenfosit sayılarak ANAE pozitif lenfosit oranı belirlendi (10x40 büyütme).

İstatistik Analizler

İstatistiksel analizler SPSS Windows 16.0 paket programı ile yapıldı. Gruplar arası (sağlıklı Grup I ile leptospirozlu hayvanlardan oluşan Grup II) önemliliğin belirlenmesinde tek yönlü varyans analizi (ANOVA) uygulandı [13]. Tüm veriler ortalama±standart hata (x±Sx) olarak gösterildi ve P<0.05 değeri istatistik olarak anlamlı kabul edildi.

BULGULAR

Klinik Bulgular

Yüksek vücut sıcaklığına sahip leptospirozlu olgularda anoreksi, eksitasyon, ishal, anemi, ikterohemoglobinüri, konjuktiva ve mukoza membranlarda sarılık ve koyu kırmızı idrar görüldü. Klinik belirtiler leptospiroz enfeksiyonu için belirleyiciydi.

Mikrobiyolojik Bulgular

Klinik olarak şüpheli olguların idrarlarında karanlık saha mikroskopisi ile incelerek spiroketlerin varlığı tespit edildi.

Biyokimyasal Bulgular

Klinik olarak sağlıklı ve leptospirozlu danaların plazma TNF-α ve NO düzeyleri ile GGT ve ADA aktiviteleri *Tablo 1*'de verildi.

Leptospirozlu danaların plazma TNF-α düzeyleri sağlıklılara göre P<0.001, diğer parametreler ise P<0.05 düzeyinde yüksek bulundu.

Histolojik Bulgular

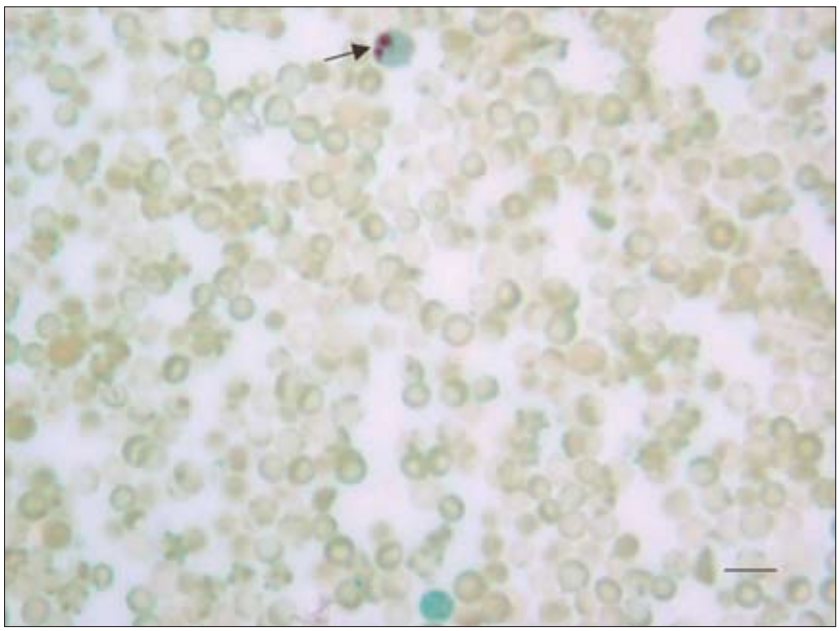
ANAE pozitif lenfositlerin çoğunda 1-2 adet kırmızı-kahverengi granüller görüldü (*Şekil 1* ve *Şekil 2*). Granüllerin görüldüğü hücreler T, granüllerin görülmediği hücreler B lenfosit olarak belirlendi. Sağlıklı sığırların perifer kanında ANAE pozitif (T) lenfosit oranı %61, leptospirozlu sığırların ANAE pozitif (T) lenfosit oranı ise %43 olarak belirlendi.

Tablo 1. *Klinik olarak sağlıklı ve Leptospirozlu hayvanlarda biyokimyasal parametrelerin ortalama ve standart hataları (X±SEM)*

Table 1*. Mean and standart errors of biochemical parameters in clinically healthy and animals with Leptospirosis*

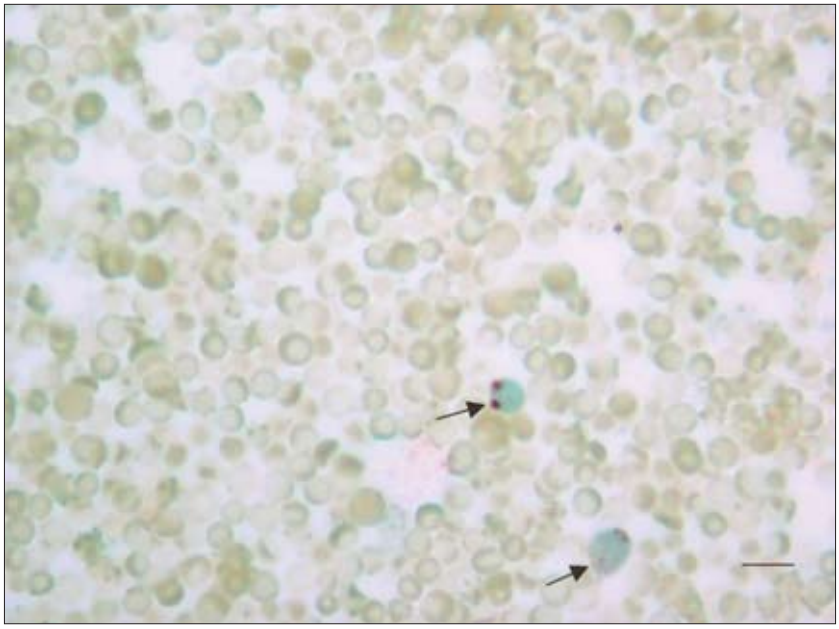
Parametre	Sağlıklı Danalar	Leptospirozlu Danalar	P
TNF-α (pg/ml)	408.72±53.08	3098.66±300.92*	0.001
NO (nmol/L)	8.15±0.72	12.85±1.32*	0.05
GGT (U/L)	12.10±0.64	25.48±4.08*	0.05
ADA (U/L)	5.94±0.80	17.99±5.4*	0.05

**Aynı satırdaki değerler arasındaki fark önemlidir (P<0.001 ve P<0.05)*



Şekil 1. Leptospirozlu sığır. *Ok:* ANAE pozitif (T) lenfosit, ANAE, (bar: 20 µm)

Fig 1. Bovine with leptospirosis. *Arrow:* ANAE Positive (T) lymphocyte, ANAE, (bar: 20 µm)



Şekil 2. Leptospirozlu sığır. *Oklar:* ANAE pozitif (T) lenfosit, ANAE, (bar: 20 µm)

Fig 2. Bovine with leptospirosis. *Arrow:* ANAE positive (T) lymphocyte, ANAE, (bar: 20 µm)

TARTIŞMA ve SONUÇ

Leptospiroz ateş, böbrek ve karaciğer yetmezliği ile pulmoner belirtiler ve reprodüktif başarısızlıkla karakterize, insan ve başlıca köpek, sığır, domuz gibi evcil hayvanların sistemik bir hastalığıdır. Klinik belirtiler hayvan türleri arasında oldukça çeşitlilik gösterir. Sığır ve domuzlarda hastalığın belirtileri reprodüktif yetmezlik, abort, ölü doğum, fötal mumyalaşma, zayıf buzağı doğumu ve laktasyonun kesilmesidir [14]. Bu çalışmada leptospirozlu sığırlarda tespit edilen klinik bulgular diğer çalışmalardakiyle [15,16] benzerlik göstermektedir. Çalışmadaki klinik belirtiler, mikrobiyolojik olarak idrarda spiroketlerin karanlık saha taramasıyla [17] desteklendi.

Leptospira sitoplazmik membran ve peptidoglikan hücre duvarından oluşan tipik çift membranlı yapıya sahip bir bakteridir. Dış membranda yer alan lipopolisakkaritler bakterinin temel antijenik etkisinden sorumludur [16]. Bu bakteriyel lipopolisakkaritler lökositleri aktive eder ve böylece TNF-α gibi pro-inflamatuar sitokinlerin salınımına sebep olur [18]. TNF-α, makrofajlar ve diğer proinflamatuar hücrelerin aktivasyonunu takiben kanda en erken saptanan sitokinlerden birisidir [19]. Yapılan bu çalışmada TNF-α düzeyleri sağlıklı gruba göre doğal olarak enfekte leptospirozlu hayvanlarda önemli derecede yüksek tespit edildi. Birçok çalışmada [20,21] TNF-α'nın laboratuvar hayvanlarında deneysel oluşturulan leptospirozda arttığının ortaya konulmasına rağmen, doğal olarak enfekte sığır leptospirozunda TNF-α ile ilgili bir çalışmaya rastlanılamadı. Leptospirozun türlere göre çeşitliliği ve belirtilerinin her türde farklı seyretmesi nedeniyle sunulan bu çalışmada ilk defa bu sitokinin düzeylerinin belirlenmesi önemlidir.

Sitokinler iNOS'u uyararak NO üretimine sebep olur. Bu çalışmada leptospirozlu grupta tespit edilen yüksek NO düzeyleri diğer çalışmalarla [16,22] benzerlik göstermektedir. Proinflamatuar veya antiinflamatuar etkileriyle akut ve kronik yangıda önemli bir role sahip olan NO oldukça reaktif bir moleküldür ve peroksinitrit oluşumu yoluyla oksidan etki gösterir. Bu oksidan özelliği nedeniyle bakterilere karşı sitotoksiktir ve savunma sisteminin bir parçası olarak görev yapar [22]. Yangısal hastalıklarda savunma sisteminin normal kapasitenin üstüne çıkması hastalığın seyrinin kötüleşmesine de yol açabilmektedir. Örneğin, artritisli hastalarda yapılan çalışmalarda [23,24] iNOS inhibitörlerinin sürekli bir şekilde verilmesi hastalığın kilinik belirtilerini azaltmış veya durdurmuştur. Bu nedenle hastalığın tedavisinde Non steroidal antiinflamatuar ilaçların yanında iNOS inhibitörlerinin de verilmesi iyileşmeyi hızlandırabilir.

Nonspesifik esterazlardan olan ANAE enziminin T lenfositlerde bulunduğu fakat B lenfositlerde bulunmadığı bildirilmektedir [10]. T lenfositler ve B lenfositler sağlıklı canlının perifer kanında belirli oranlarda bulunurken, bazı proliferatif hastalıklarda bu oranlarda önemli değişiklikler görülmektedir [25]. Lenfosit sistemine ait düzensizliklerde [26], sistemik lupus eritamatozis gibi bağ doku hastalıklarında ve aynı zamanda lenfositik ve monositik lösemiler gibi bazı proliferatif hastalıklarda da [27] basit sitomorfolojik bir metot olan "ANAE demonstrasyonu" metodundan yararlanılmaktadır. Leptospirozun patogenezinde erken ve ilerlemiş olmak üzere 2 safha vardır. Erken safhada humoral immunite söz konusu iken, ilerlemiş safhada ise hücresel immunite söz konusudur [28]. Yapılan bu çalışmada, sağlıklı hayvanların T lenfosit oranları %61 iken, leptospirozluların %43 olarak belirlenmiştir. Bu oranlar, leptospirozda T lenfosit oranı azalırken, B lenfosit oranının arttığını yani humoral immunitenin baskın olduğunu göstermektedir. Lökositlerin farklı tiplerinin miktarlarının flow sitometri yöntemiyle sayıldığı başka bir çalışmada [28] da bulgularımıza

benzer olarak toplam T lenfosit, T-helper, T baskılayıcı ve T-helper/T baskılayıcı indeksi sayısında azalma tespit edildiği, bunun yanında aktif T lenfosit sayısında, doğal öldürücü (NK) hücrelerinin alt türlerinde ve B lenfosit sayısında ise artış olduğu bildirilmiştir. İmmun sistemle ilgili biyokimyasal parametreler olan ADA ve GGT aktiviteri de leptospirozlu hayvanlarda sağlıklılara göre yüksek tespit edildi. ADA aktivitesi tüm hücre tiplerinde bulunmakla birlikte, lenfoid dokular, timus ve periferal lenfositlerde daha fazladır. Bu enzim, lenfosit fonksiyonu ve T lenfositlerinin normal büyüme, farklılaşma ve çoğalması için esansiyeldir [3]. GGT özellikle bellek T hücreleri olmak üzere T hücrelerinin aktivasyonunda artmaktadır [4]. Çalışmamızda ADA aktivitesi leptospirozlu hayvanlarda yüksek düzeyde tespit edilirken, Tonin ve ark.'nın [29] ratlarda oluşturdukları deneysel leptospiroz modelinde ise hem erken hem de geç dönemde düşük tespit edilmiştir. Doğal enfekte sığırlarda ise ADA ve GGT aktivitesinin ölçüldüğü herhangi bir çalışmaya rastlanılamamıştır.

Sonuç olarak, enfeksiyonun erken aşamasında saptanabilen bir sitokin olan TNF-α ve buna bağlı olarak NO düzeylerinin belirgin bir şekilde yükselmesi, ADA ve GGT aktivitelerinin artması ile B lenfosit yüzdesinin fazla olduğunun tespitinden yola çıkılarak, leptospiroz patogenezisinde bu parametrelerden yararlanılabileceği düşünülmektedir. Ayrıca özellikle doğal enfekte sığırlarda biyokimyasal mekanizmalar üzerine daha fazla çalışmaların yapılması gerektiği söylenebilir.

KAYNAKLAR

1. Lowanitchapat A, Payungporn S, Sereemaspun A, Ekpo P, Phulsuksombati D, Poovorawan Y, Chirathaworn C: Expression of TNF-α, TGF-β, IP- 10 and IL-10 mRNA in kidneys of hamsters infected with pathogenic Leptospira. *Comp Immunol Microbiol Infect Dis,* 33, 423-434, 2010.

2. Merien F, Baranton G, Perolat P: Invasion of vero cells and induction of apoptosis in macrophages by pathogenic *Leptospira interrogans* are correlated with virulence. *Infect Immun,* 65 (2): 729-738, 1997.

3. Baganha MF, Pego A, Lima MA, Gaspar EU, Pharma B: Serum and pleural adenosine deaminase correlation with lymphocytic populations. *Chest,* 97 (3): 605-610, 1990.

4. Henson SE, Nichols, TC Holers, VM Karp DR: The ectoenzyme γ-glutamyl transpeptidase regulates antiproliferative effects of *S*-nitrosoglutathione on human T and B lymphocytes. *J Immunol,* 163, 1845-1852, 1999.

5. Garthwaite J: Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci*, 14 (2): 60-67, 1991.

6. Stuehr DJ: Mammalian nitric oxide syntheses. *Biochim Biophy Acta,* 1411, 217-230, 1999.

7. Tangpong J, Sompol P, Vore M, St Clair W, Butterfield DA, St Clair DK: Tumor necrosis factor alpha–mediated nitric oxide production enhances manganese superoxide dismutase nitration and mitochondrial dysfunction in primary neurons: An insight into the role of glial cells. *Neuroscience,* 151, 622-629, 2008.

8. Kontas T, Salmanoglu B: Tumour necrosis factor-α, adenosine deaminase and nitric oxide levels in cattle babesiosis before and after treatment. *Bull Vet Inst Pulawy,* 50 (4): 485-48, 2006.

9. Knowles DM, Hoffman T, Ferrarini M, Kunkel HG: The demonstration of acid alpha naphthyl acetate esterase activity in human lymphocytes usefulness as a T cell marker. *Cell Immun,* 35, 112-123, 1978.

10. Mueller J, Brun del Re G, Buerki H, Keller HU, Hess MW, Cottier H: Nonspesific acid esterase activity: A criterion for differentiation of T and B lymphocytes in mouse lymph nodes. *Eur J Immunol,* 5, 270-274, 1975.

11. Giusti G, Galanti B: Methods of Enzymatic Analysis, s. 315-323, Verlac Chemie, Weinheim, 1984.

12. Miranda KM, Espey MG, Wink DA: A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide,* 5 (1): 62-71, 2001.

13. SPSS: SPSS for windows release 16.0.2, SPSS Inc., Chicago, April, 2008.

14. Adler B, Moctezuma AP: Leptospira and Leptospirosis. *Vet Microbiol,* 140, 287-296, 2010.

15. Rodostits OM, Blood DC, Gay CC: Veterinary Medicine: A Text Book of the Diseases of Cattle, Horses, Sheep, Pigs and Goats. 8th ed., 884-908, Baillaire Tindall, London, 1994.

16. Erdogan HM, Karapehlivan M, Citil M, Atakisi O, Uzlu E, Unver A: Serum sialic acid and oxidative stres parameters changes in cattle with leptospirosis. *Vet Res Com,* 32, 333-339, 2008.

17. Quin PJ, Carter ME, Markey B, Carter GR: Clinical Veterinary Microbiology. 1st ed., 292-298, Wolfe Publishing, London, 1994.

18. Urbanik-Sypniewska T, Choma A, Kutkowska J, Kaminska T, Kandefer-Szerszen M, Russa R, Dolecka J: Cytokine inducing activities of rhizobial and mesorhizobial lipopolysaccharides of different lethal toxicity. *Immunobiology,* 202, 408-420, 2000.

19. Ergün E, Ergün L, Özen A, Aştı RN: Determination of alpha naphtyl acetate esterase activity in the peripheral blood leukocytes of ostrich *(Struthio camelus masaicus). Revue Med Vet,* 81, 147-150, 2004.

20. Marinho M, Silva C, Lima VMF, Machado GF, Peiro JR, Perri SHV: Production of TNF-α and IL-6, antibody response, and bacterial recovery, during leptospirosis infection. *Vet Immunol Immunopathol,* 128, 211-347, 2009.

21. Marinho M, Silva C, Lima VMF, Peiró JR, Perri SHV: Cytokine and antibody production during murine Leptospirosis. *J Venom Anim Toxins incl Trop Dis,* 12 (4): 595-603, 2006.

22. Elves AP Maciel, Daniel A Athanazio, Eliana AG Reis, Fernando Q Cunha, Adriano Queiroz, Deusdelia Almeida, Alan JA McBride, Albert I Ko, Mitermayer G Reis: High serum nitric oxide levels in patiens with severe leptospirosis. *Acta Tropica,* 100, 256-260, 2006.

23. Lalenti A, Moncada S, Di-Rosa M: Modulation of adjuvant arthritis by endogenous nitric oxide. *Br J Pharmacol,* 110, 701-706, 1993.

24. Stefanovic-Racic M, Meyers K, Meschter C, Coffey JW, Hoffman RA, Evans CH: N-Monomethyl arginine. An inhibitor of nitric oxide synthase. Suppresses the devolopment of adjuvant arthritis in rats. *Arthritis Reum,* 37 (7): 1062-1069, 1994.

25. Kajikawa DVM, Koyama H, Yushikawa T, Tsubaki S: Use of alpha-naphthyl acetate esterase staining to identify T lymphocytes in cattle. *Am J Vet Res,* 44, 1549-1552, 1983.

26. Fehr J, Scherer O: Nonspecific esterase as a marker for human T-lymphocytes sequential studies during states of transient blood lymphocyte redistributions. *Ann Hematol,* 44, 201-109, 1981.

27. Kulenkampff J, Janossy G, Greaves JR: Acid esterase in human lymphoid cells and leukaemic blasts: A marker for T lymphocytes. *Br J Haematol,* 36, 231-240, 1977.

28. Gancheva G, Atanasova M, Lukanov T, Ilieva P: Flow cytometry in leptospirosis. *JIMAB,* 1, 3-97, 2008.

29. Tonin AA, Pimentel VC, Da Silva AS, De Azevedo MI, Souza VC, Wolkmer P, Rezer JF, Badke MR, Leal DB, Schetinger MR, Monteiro SG, Lopes ST: Adenosine deaminase activity in *serum, erthrocytes and lymphocytes of rats infected with Leptospira* icterohaemorrhagiae. *Res Vet Sci*, 92 (2): 197-201, 2012.

Kafkas Univ Vet Fak Derg
20 (3): 457-460, 2014
DOI: 10.9775/kvfd.2013.10198

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

SHORT COMMUNICATION

Gebeleme ve Hamur Olum Döneminde Hasat Edilen Buğdaygil Hasıllarının Protein Fraksiyonları ve Ham Protein Üretimleri [1]

Behiç COŞKUN [1] Gürhan KELEŞ [2] Fatma İNAL [1]
M. Selçuk ALATAŞ [1] Cahit ÖZCAN [1] Serkan ATEŞ [3]

[1] *Bu çalışma Selçuk Üniversitesi Bilimsel Araştırma Projeleri Koordinatörlüğü tarafından desteklenmiştir (Proje No: 11401094)*

[1] Selçuk Üniversitesi Veteriner Fakültesi, Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı, TR-42151 Konya - TÜRKİYE
[2] Adnan Menderes Üniversitesi, Ziraat Fakültesi, Zootekni Bölümü, TR-09100 Aydın - TÜRKİYE
[3] International Centre for Agricultural Research in the Dry Areas (ICARDA), Amman - JORDAN

Makale Kodu (Article Code): KVFD-2013-10198

Özet

Bu çalışmada gebeleme ve hamur olum döneminde hasat edilen arpa, buğday, çavdar, tritikale ve yulaf hasıllarının birim alana ürettikleri ham protein ve sindirilebilir ham protein verimleri (kg/da) ile Cornell Net Karbonhidrat ve Protein Sistemine göre protein fraksiyonları belirlenmiştir. Hamur olum döneminde hasılların ham protein içeriği gebeleme dönemine kıyasla ortalama olarak %42 daha düşükken (P<0.05), ham protein verimi %10 daha düşük (P<0.05) belirlenmiştir. Buna karşın sindirilebilir ham protein verimi her iki dönemde de benzer (P>0.05) bulunmuştur. Gebeleme döneminde buğday ve yulafın ham protein kalitesi diğer hasıllardan daha yüksek (P<0.05) belirlenmiştir. Araştırma sonucunda hasat zamanının istenilen protein özelliğine sahip hasıl üretimi amacıyla kullanılabileceği değerlendirilmiştir.

Anahtar sözcükler: *Gelişme dönemi, Protein kalitesi, Ruminant, Tahıl hasılı*

Protein Fractions and Crude Protein Yield of Cereal Forages Harvested at Booting and Dough Stage of Maturity

Summary

In this study, crude protein and digestible crude protein production (kg/da) of barley, wheat, rye, triticale and oat were measured at booting and dough stages of maturity and their protein fractions were determined according to Cornell Net Carbohydrate and Protein System. The average crude protein contents of cereal forages were 42% lower (P<0.05) at dough stage than booting stage while crude protein production decreased (P<0.05) by only 10% from dough stage to booting stage of maturity. However, digestible crude protein production was similar (P>0.05) in both periods. The crude protein quality of wheat and oat was higher than other cereal forages at the booting stage. These results suggest that time of harvest can be arranged according to cereal forages with desired protein properties that change with maturity stage.

Keywords: *Stage of maturity, Protein quality, Ruminant, Cereal forage*

GİRİŞ

Tahıl hasıllarının kuru ot üretimi yada silolama amacıyla gebeleme (başaklanmanın hemen öncesi) yada hamur olum dönemlerinde hasat edilmeleri tavsiye edilmektedir. Hamur olum döneminde yapılan hasatta gebeleme dönemine kıyasla birim alandan alınacak kuru madde (KM) verimi daha yüksek buna karşın üretilen kaba yemin besin değeri daha düşük olmaktadır [1-3]. Gebeleme ve hamur olum döneminde hasat edilmiş buğdaygil türleri arasında besin değeri bakımından farklılıklar bulunmaktadır [4]. Bitki gelişimine bağlı olarak buğdaygil hasıllarının ham protein (HP) içeriklerindeki düşüş enerji değerlerinden daha belirgin olmaktadır [1].

İletişim (Correspondence)
+90 256 7727024
gurhankeles@msn.com

Ruminantlar için protein kalitesi, proteinin rumende parçalanma hızı ve sindirilebilirliği ile yakından ilgilidir. Yemlerdeki protein tabiatında olmayan azotlu bileşikler (NPN), rumende amonyağa parçalanan oligopeptitler, serbest aminoasitler ve diğer Azot (N) içeren düşük moleküllü bileşiklerden oluşmaktadır. Yemlerdeki rumende parçalanmayan protein (RUP) ve rumende parçalanan proteinin (RDP) HP içerisindeki oranı yemin içerdiği HP'nin niteliğine bağlı olarak büyük farklılıklar gösterebilmektedir [5]. Rumende HP parçalanması hızlı olduğunda rumen mikroorganizmaları açığa çıkan aminoasit ve amonyağın tamamını kullanamamakta sonuçta sentezlenenden daha fazla HP parçalanmaktadır. Bu durum yem maliyetini artırdığı gibi çevre kirliliğine sebep olmakta ve ayrıca süt sığırlarının üreme performansını olumsuz etkilemektedir [5].

Buğdaygil hasıllarının gebeleme ve hamur olum dönemi arasında HP değerlerindeki büyük düşüş, hasılların ruminant beslemede daha etkili kullanımları için içerdikleri HP'nin niteliğinin ortaya konulmasını gerektirmektedir. Bu çalışmada, arpa, buğday, çavdar, tritikale ve yulaf hasıllarının sindirilebilir ham protein verimleri ile Cornell Net Karbonhidrat ve Protein Sistemi'ne göre protein fraksiyonları ve RUP düzeylerinin gebeleme ve hamur olum döneminde belirlenmesi amaçlanmıştır.

MATERYAL ve METOT

Bahri Dağdaş Uluslararası Tarımsal Araştırma Enstitüsünde (Konya) 12x78 m ebatlarında 3 tekerrürlü olarak ekimi yapılmış Buğday (Karahan), Tritikale (Tatlıcak), Çavdar (Aslım), Arpa (Beyşehir) ve Yulaf (Faikbey) bitkileri gebeleme ve hamur olum döneminde her tekerrürden 3 adet 0.25x 0.25 m'lik çemberlerle örneklenmiştir. Çemberlerden elde edilen otlar tartılmış ve KM düzeyleri belirlenerek hasılların KM verimleri tespit edilmiştir. Daha sonra elde edilen örnekler 1 mm'lik elekten geçecek şekilde öğütüldükten sonra besin maddesi analizlerinde kullanılmıştır.

Hasılların KM düzeyleri, 60ºC'de 48 saat süre ile ağırlık sabitleninceye kadar kurutularak tespit edilmiştir. Hasılların HP ile NDF ve ADF analizinden çıkan numunelerin nötr deterjanda çözünmeyen N (NDIN) ve asit deterjanda çözünmeyen N (ADIN) içerikleri AOAC'ye göre [6] tespit edilmiştir. Sindirilebilir HP değerleri ise *in vitro* gerçek KM sindirilebilirlikleri Daisy[II] inkübatör (Ankom, USA) ile yapılmış numunelerin HP içeriklerinin belirlenmesi [6] ile tespit edilmiştir. Çözülebilir HP (ÇP) ve gerçek protein (GP) Krishinamorthy ve ark.[7] tarafından bildirilen metoda göre belirlenmiştir.

Protein fraksiyonları Sniffen ve ark.'na göre [8] toplam N içerisinde hesaplanmıştır. Rumende parçalanmayan protein, belirlenen kimyasal kompozisyondan (HP, NDIN, ADIN, ÇP ve GP) hesaplanan protein fraksiyonları ve NDF değerleri kullanılarak, canlı ağırlığın %4'ü düzeyinde 633 g/kg kaba yem tüketimi varsayımıyla NRC'ye göre [5] hesaplanmıştır.

Cornell Net Karbonhidrat ve Protein Sisteminde, yemlerdeki HP üç kısma ayrılmaktadır. Özetle, Fraksiyon A NPN, Fraksiyon B potansiyel parçalanabilir gerçek protein, Fraksiyon C (ADIN) ise parçalanmayan ve kullanılamayan gerçek proteini ifade etmektedir. Fraksiyon B rumen parçalanabilirliğinin tahmini için ayrıca üç kısma ayrılmaktadır. Fraksiyon B1, B2 ve B3'ün rumende parçalanma hızları sırasıyla, hızlı, orta ve yavaş olarak kabul edilmektedir.

Çalışmadan elde edilen verilere tesadüf blokları bölünmüş parseller deneme desenine [9] göre SPSS [10] paket programında varyans analizi uygulanmıştır. Hasıllar ana parselleri, hasat zamanı ise alt parselleri oluşturmuştur. Ortalamalar arasındaki farklılıklar LSD testi ile belirlenmiştir.

BULGULAR

Gebeleme ve hamur olum döneminde hasat edilmiş arpa, buğday, çavdar, tritikale ve yulaf hasıllarının kimyasal kompozisyonları, protein fraksiyonları, RUP ve HP sindirilebilirlikleri *Tablo 1*'de verilmiştir.

Hasılların HP içerikleri gebeleme dönemine kıyasla hamur olum döneminde ortalama olarak %42 düşmüştür. Bu düşüş %54 ile çavdarda en yüksek, %24 ile yulafta en düşük olmuştur (P<0.05).

Olgunlaşmaya bağlı olarak arpa hariç diğer hasılların ADIN içerikleri artmıştır (P<0.05). Çavdar ve tritikalenin NDIN içerikleri gebeleme döneminde en düşük (P>0.05) olmasına rağmen, bitki gelişimine paralel olarak sadece bu iki bitkinin NDIN içeriği artmıştır (P<0.05). Bu iki hasıl türünün ayrıca olgunlaşma ile HP sindirilebilirlikleride en düşük (P<0.05) belirlenmiştir.

Buğday ve yulaf hasıllarının gebeleme dönemindeki A fraksiyonları diğer hasıllardan düşük (P<0.05) buna karşın, toplam gerçek protein (B) ve RUP düzeyleri yüksek (P<0.05) belirlenmiştir.

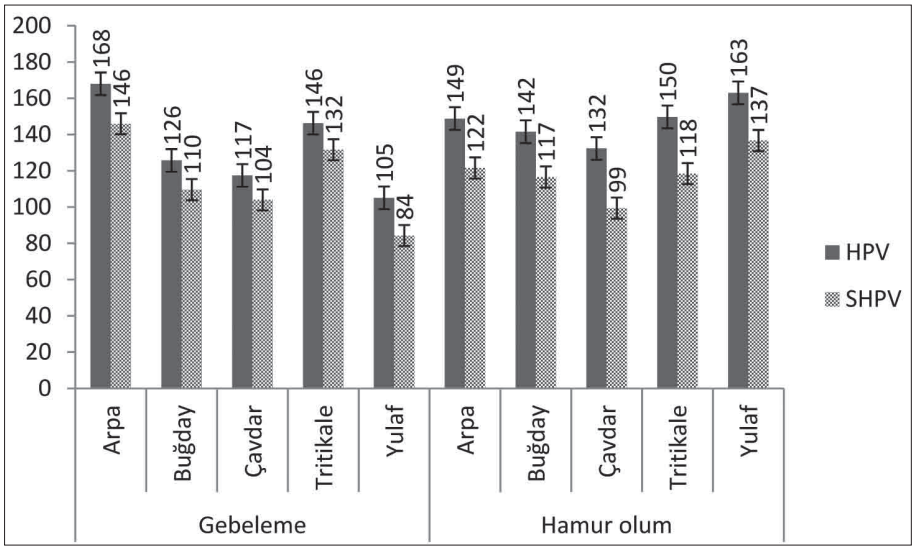
Hamur olum döneminde NDIN içerikleri diğer hasıllardan yüksek belirlenen çavdar ve tritikalenin B2 Fraksiyonu diğer hasıllardan düşük (P<0.05) belirlenmiştir.

Genel olarak tahıl hasıllarının gebeleme dönemindeki ortalama HP verimi hamur olum döneminden %10 daha düşükken (P<0.05), sindirilebilir HP verimi ise her iki dönemde de benzer (P>0.05) bulunmuştur *(Şekil 1)*. Hasadın hamur olum dönemine geciktirilmesi ile arpadan birim alana üretilecek HP (%11) ve sindirilebilir HP (%16) ile tritikaleden üretilecek sindirilebilir HP (%11) azalmıştır (P<0.05). Buna karşın yulafın HP ve sindirilebilir HP verimi hamur olum döneminde gebeleme döneminden sırasıyla %36 ve 39 daha yüksek (P<0.05) belirlenmiştir. Hasadın geciktirilmesi ile buğday ve çavdarın HP verimleri de %11 artarken (P<0.05), bu iki hasıl türünün sindirilebilir HP verimleri değişmemiştir (P<0.05).

Tablo 1. *Hasılların kimyasal kompozisyonu, protein fraksiyonları, RUP ve sindirilebilir HP içerikleri* [1]
Table1. *Chemical composition, protein fractions, RUP and digestible CP content of cereal forages*

Özellikler[2]		HP, g/kg KM	Kimyasal Kompozisyon				Protein Fraksiyonları, g/kg HP					RUP, g/kg HP	HPS, g/kg HP
			NDIN	ADIN	NPN	ÇP	A	B1	B2	B3	B		
Hasat													
G		152a	189b	97b	689a	498a	345a	153a	313b	92a	558b	318b	866a
HO		88b	199a	139a	550b	271b	148b	123b	531a	60b	713a	419a	804b
Hasıl													
A		135a	194a	133	660a	397b	270ab	127ab	409b	61	597c	364b	843
B		117c	200a	112	639ab	342c	224c	117b	458a	88	663a	388a	848
Ç		118bc	196a	111	602bc	411ab	259b	152a	394bc	84	630b	359b	818
T		123b	182b	105	629ab	438a	291a	147ab	380c	78	604c	339c	845
Y		108b	197a	128	567c	335c	189d	146ab	468a	69	683a	392a	821
Hasat	**Hasıl**												
G	A	169a	195c	147b	717a	536b	384b	152ab	269e	49de	469f	309c	869abc
G	B	142b	226a	98de	711a	427c	304c	123bcd	347cd	128a	598d	365b	873abc
G	Ç	162a	170e	78e	718a	509b	365b	144bc	321d	92b	556e	302c	884ab
G	T	161a	143f	77e	731a	593a	434a	159ab	264e	66cde	489f	253d	900a
G	Y	123c	210b	83e	567bc	425c	239d	186 a	364c	127a	678c	359b	803de
HO	A	100d	193c	120cd	604b	257ef	156e	102de	550a	73bcd	724a	420a	816cd
HO	B	91e	174de	126bc	566bc	256ef	145e	111cd	570a	48e	729a	410a	823bcd
HO	Ç	74f	221ab	144bc	485d	312d	152e	161ab	466b	77bc	704abc	415a	751e
HO	T	85e	222ab	132bc	526cd	283de	149e	134bcd	495b	90b	719ab	425a	790de
HO	Y	93de	184cd	173a	567bc	244f	138e	106cd	572a	10f	688bc	424 a	839abcd
	S.H.		4.5	8.7	23.9	12.5	10.3	13.1	10.7	8.4	11.3	7.0	21.1

[1] *Aynı sütunda farklı harflerle gösterilen ortalamalar arasındaki farklılıklar önemlidir (P<0.05),* [2] ***HP:*** *ham protein;* ***NDIN:*** *nötr deterjanda çözünmeyen HP;* ***ADIN:*** *asit deterjanda çözünmeyen HP;* ***NPN:*** *protein olmayan N;* ***ÇP:*** *çözünebilir protein;* ***RUP:*** *rumende parçalanmayan protein;* ***HPS:*** *HP sindirilebilirliği;* ***G:*** *gebeleme;* ***HO:*** *hamur olum;* ***A:*** *arpa;* ***B:*** *buğday;* ***Ç:*** *çavdar;* ***T:*** *tritikale;* ***Y:*** *yulaf*



Şekil 1. Hasılların ham protein verimi (HPV) ve sindirilebilir HP verimleri (SHP), kg/da, n=3, SH=3.0

Fig 1. Crude protein and digestible crude protein yield of cereal forages

TARTIŞMA ve SONUÇ

Hasılların olgunlaşmaya bağlı olarak HP değerindeki büyük düşüşler Khorosani ve ark.[1] tarafından arpa, yulaf ve tritikale için bildirilmiştir. Ayrıca aynı araştırıcılar tahıl hasıllarının içermiş oldukları HP değerindeki bu düşüşün yoncaya kıyasla çok daha belirgin olduğunu da belirlemişlerdir. Bu nedenle hasat zamanın tahıl hasıllarının

besin değerine etkisi baklagillerden çok daha önem arz etmektedir.

Çavdar ve tritikalenin NDIN içeriklerinin olgunlaşmaya bağlı olarak artması ve hamur olum döneminde bu iki hasıl türünün en düşük HP sindirilebilirlik değerlerine sahip olması, bu iki hasıl türünde protein kalitesindeki düşüşün diğer hasıl türlerine kıyasla daha belirgin olacağını düşündürmektedir. Nitekim, benzer şekilde Helsel ve Thomas [4] çavdarın besin değerinin başaklanmadan hemen sonra arpa, buğday ve yulafa kıyasla hızla düştüğünü, McCartney ve Vaage [11] ise hamur olum döneminde hasat edilen tritikalede N sindirilebilirliğinin arpa ve yulaftan daha düşük olduğunu bildirmişlerdir.

Buğday ve yulaf hasıllarının gebeleme dönemindeki A fraksiyonlarının düşük buna karşın, gerçek protein miktarlarının yüksek olması bu hasılların RUP düzeylerini artmıştır. Bu durum gebeleme döneminde buğday ve yulaf hasıllarının protein kalitesi açısından diğer hasıllardan daha üstün olacağını göstermektedir.

Hasılların tamamının hamur olum döneminde benzer RUP değerlerine sahip olmasından dolayı hasıllardan birim alandan üretilecek HP ve sindirilebilir HP'nin buğdaygil türünün seçiminde bu dönemde diğer protein özelliklerinden daha önemli olduğunu ortaya koymaktadır.

Sonuç olarak, sindirilebilir HP verimleri dikkate alındığında arpa ve tritikalenin başaklanma öncesi, yulafın ise hamur olum döneminde hasadının uygun olduğu değerlendirilmiştir. Ayrıca, yulaf hariç sindirilebilir HP veriminin gebeleme döneminde arpa ve tritikale hasıllarında daha yüksek, buğday ve çavdar hasıllarında ise her iki dönemde de benzer olması, tahıl hasılları için gebeleme döneminde yapılacak hasadın önemini ortaya koymaktadır.

KAYNAKLAR

1. Khorasani GR, Jedel PE, Helm JH, Kennelly JJ: Influence of stage of maturity on yield components and chemical composition of cereal grain silages. *Can J Anim Sci*, 77, 259-267, 1997.

2. Coşkun B, Keleş G, İnal F, Ateş S, Alataş MS: Başaklanma öncesi ve hamur olum döneminde kimyasal ya da biyolojik katkı maddesi ile silolanmış tahıl hasıllarının fermantasyon özellikleri ve besin madde içerikleri, Selçuk Üniversitesi BAP 11401094 nolu proje sonuç raporu, 2013.

3. Keleş G, Coşkun B, Işık Ş: Tahıl hasıllarının farklı gelişme dönemlerindeki agronomik özellikleri, besleme değerleri ve kuzu performansı üzerine etkileri. TÜBİTAK 111O009 nolu proje sonuç raporu, 2012.

4. Helsel ZR, Thomas JW: Small grains for forage. *J Dairy Sci,* 70, 2330-2338, 1987.

5. NRC: National Research Council, Nutrients requirements of dairy cattle, The national academic press, Washington DC, 2001.

6. AOAC: Official Methods of Analysis of AOAC International. 17th ed., 2nd Rev., Gaithersburg, MD, USA, Association of Analytical Communities, 2003.

7. Krishinamoorthy U, Muscato TV, Sniffen CJ, Van Soest PJ: Nitrogen fractions in selected feedstuffs. *J Dairy Sci*, 65, 217-225, 1982.

8. Sniffen CJ, O'Connor JD, Van Soest PJ, Fox DG, Russell JB: A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. *J Anim Sci*, 70, 3562-3577, 1992.

9. Düzgüneş O, Kesici T, Kavuncu O, Gürbüz F: Araştırma ve Deneme Metotları (İstatistik Metotları II). Ankara Üniv. Zir. Fak.Yay.: 1021, Ankara, 1987.

10. SPSS: SPSS for Windows, Version 17. SPSS Inc. Chicago, 2010.

11. McCartney DH, Vaage AS: Comparative yield and feeding value of barley, oat and triticale silages. *Can J Anim Sci,* 74, 91-96, 1994.

Kafkas Univ Vet Fak Derg
20 (3): 461-464, 2014
DOI: 10.9775/kvfd.2013.10261

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

SHORT COMMUNICATION

Effects of Nisin and Temperature on Behavior of Enterotoxigenic *Staphylococcus aureus* in Model Cheeses

Khosrow MOHAMMADI [1] Hossein JODEIRI [2]

[1] Department of Food Hygiene, Faculty of Veterinary Science, Tabriz Branch, Islamic Azad University, Tabriz - IRAN
[2] Research and Development Center, Pegah Dairy Industry, Tabriz, East Azerbaijan, IRAN

Makale Kodu (Article Code): KVFD-2013-10261

Summary

The growth and enterotoxin synthesis of *S. aureus* as affected by different concentration of nisin (0, 1 and 2 µg/g) and storage temperatures (8 and 25°C) were studied in model cheeses manufactured with ultra-filtered milk. Microbiological analysis of the cheese samples were performed at 0, 1, 8, 15, 30, 45 and 60 days. Detection of the enterotoxins was done by an indirect double-sandwich ELISA technique using anti-enterotoxin monoclonal antibodies. Nisin concentration as low as 1 µg/g was found to have an inhibitory effect on the growth and enterotoxin production of *S. aureus*, besides the effect was more pronounced at 8°C than at 25°C.

Keywords: *S. aureus, Enterotoxin, Nisin, ELISA, Cheese*

Model Peynirler İçinde Enterotoksijenik *Staphylococcus aureus* Davranış Üzerine Nisin ve Sıcaklığın Etkisi

Özet

Staphylococcus aureus'un büyüme ve enterotoksin sentezi farklı nisin konsantrasyon düzeylerinden (0, 1 ve 2 µg/g) ve saklama sıcaklıklarından (8 ve 25°C) etkilendiği için ultra-süzülmüş süt ile üretilen model peynirler üzerinde çalışılmıştır. Peynir örneklerinin mikrobiyolojik analizi 0, 1, 8, 15, 30, 45 ve 60 gün üzerinden gerçekleştirilmiştir. Enterotoksin tespiti anti-enterotoksin monoklonal antikorlar kullanarak dolaylı çift-sandviç ELISA tekniği ile yapılmıştır. 1 µg/g gibi düşük bir nisin konsantrasyonunda *S. aureus*'un büyüme ve enterotoksin üretimi üzerinde bir inhibitör etkisi görülmüş olup, söz konusu etki 8°C'de 25°C'ye kıyasla daha belirgin olmuştur.

Anahtar sözcükler: *S. aureus, Enterotoksin, Nisin, ELISA, Peynir*

INTRODUCTION

Staphylococcal food poisoning (SFP) caused by ingestion of one or more preformed toxins are one of the most common causes of reported food-borne illnesses [1]. In the last few decades SFP has been economically one of the most important diseases in the world [2]. The annual number of SFP cases is 185.000 with about 1750 hospitalizations in the United States [3].

Staphylococcus aureus is normal flora on the skin and mucosae of animal and it is frequently associated to subclinical mastitis leading to contamination of milk and predispose for the public health to risk due to food poisoning [3,4]. The origin of contamination by *S. aureus* can be mastitic milk, the processing plant environment or from personel activity during food processing [5].

The European regulation set the upper limit for *S. aureus* in cheeses at a count of 10^5 colony-forming units (CFU) per gram. Above this limit, there is an obligation to determine whether enterotoxins are present (European Community Regulation No. 852-853/2004). Therefore, it is important to control *S. aureus* growth and enterotoxin production throughout the cheese-making process [6].

Nisin is a bacteriocin widely used as a food preservative particularly in cheese and generally regarded as safe (GRAS) [7]. It is produced by some strains of

İletişim (Correspondence)
+98 914 4166372
mohammadi@iaut.ac.ir

Lactococcus lactis and is active against most Gram-positive bacteria [8,9].

The objective of this study was to determine whether different concentrations of nisin (0, 1 and 2 µg/g) as well as storage temperatures (8 and 25°C) could alter the growth and enterotoxin production of *S. aureus* in model cheeses made from ultra-filtered milk.

MATERIAL and METHODS

Preparation and Enumeration of the Bacterial Inocula

Enterotoxigenic *S. aureus* ATCC 13565 (American Type Culture Collection, Rockville, USA) was used for the inoculation. The inoculum preparation and enumeration of the inocula were performed as previously described by Alomar et al.[6], and FDA/BAM [10]. Briefly, *S. aureus* was grown on a brain-heart infusion (BHI) agar (Merck) at 35°C for 24 h. One to two well-grown colonies on the plate were transferred to 5 ml of sterile BHI broth (Merck) and incubated at 35°C for 24 h. The bacterial suspension was precipitated by centrifugation at 6200 ×g for 15 min. The supernatant was discarded and the resultant pellet was washed with two consecutive steps using Ringer solution containing 0.05% (w/v) Tween 80 (Merck). The pellet was resuspended in Ringer solution and was diluted to obtain the final load of 5 log cfu/g of UF-retentate.

Nisin

Nisin containing 2.5% active nisin was obtained from Sigma-Aldrich Inc. (United Kingdom, EC 215-807-5). Nisin stock solution was prepared with 0.02 mol l^{-1} HCl (pH 1.6) and was filter sterilized through a 0.45 µm sterile, disposal and non-pyrogenic syringe filter.

Manufacture of the Model Cheeses

Model cheeses were prepared in the pilot-plant of the Tabriz Pegah Dairy Processing Plant (Tabriz, Iran). The ultrafiltered milk with volume concentration factor of 5.1 kg milk to 1.0 kg UF-retentate was inoculated with approximately 5 log cfu/g of *S. aureus*. Nisin concentrations of 0, 1 and 2 µg/g was added and mixed thoroughly with the UF-retentate. Rennet 0.002% (w/w) was mixed with water and with 10 ppm anti-foaming and 15 ppm anti-sticking agents (Danapak, Elteknik Landbrugsvej, Denmark) were added to each cheese container and homogeneously mixed. In the sealing machine, 3% (w/w) salt was added onto the parchment paper on the top of cheese and then by using aluminum foil, the container was sealed. The samples were kept for 1 day at 37°C for pre-ripening (day 1) and 2 weeks at 8°C for further ripening (days 8 and 15). Then cheese samples were stored for 45 days at 8 and 25°C (days 30, 45 and 60). Experiments at two different temperatures were designed to determine retention of viability and enterotoxin production of *S. aureus*.

Enterotoxin Assay

Staphylococcal enterotoxin detection was performed by the RIDASCREEN SET kit (R-Biopharm, Darmstadt, Germany), an indirect double-sandwich ELISA technique using antienterotoxin monoclonal antibodies, with a minimum detectable limit of 0.50 to 0.75 ng toxins per gram sample. Enterotoxin assay was performed according to the kit manufacturing. The Ridascreen Kit results of the OD colored enzymatic reactions was determined at 450 nm using a microplate reader (Tecan, Sunrise, Durham, NC, USA).

Statistical Analysis

The experiment was repeated in triplicate and analysis of the variance (ANOVA) by mean of Duncan's test was performed on the microbial counts as well as quantity of enterotoxin.

RESULTS

Effect of Nisin on S. aureus Growth in Model Cheeses Stored at 8°C

Here, it was revealed that growth of *S. aureus* influenced by different concentrations of nisin (0,1 and 2 µg/g) in the model cheeses stored at 8°C *(Fig. 1)*. During the pre-ripening of control samples (no nisin) at 37°C, *S. aureus* increased significantly ($P<0.05$) exceeding the degree at inoculation time. After the growth period of *S. aureus*, we observed a stabilization of the number and then a slight decrease during the storage period (from 15 days to 60 days).

According to the results nisin had a significant ($P<0.01$) inhibitory effect on the *S. aureus*. In the model cheeses containing 1 µg of nisin/g cheese the number of *S. aureus* decreased within the first day of pre-ripening. For *S. aureus*, by the increase of nisin concentration to 2 µg/g, the growth of organism at 8°C was significantly affected and the $\log_{10}$ cfu/g of the organism reached $<10^1$ cfu/g.

Effect of Nisin on S. aureus Growth in Model Cheeses Stored at 25°C

In this study, the different concentration of nisin did not affect the growth of *S. aureus* at 25°C *(Fig. 2)*. The number of the organism almost reached the same as that was at the first day of the study. However, at the first day of fermentation, there was a significant difference ($P<0.01$) between in the model cheese with and without nisin for the number of *S. aureus*.

Enterotoxin Production

The production of enterotoxin affected by nisin and temperature in model cheeses was determined. These factors had a significant effect ($P<0.01$) on staphylococcal

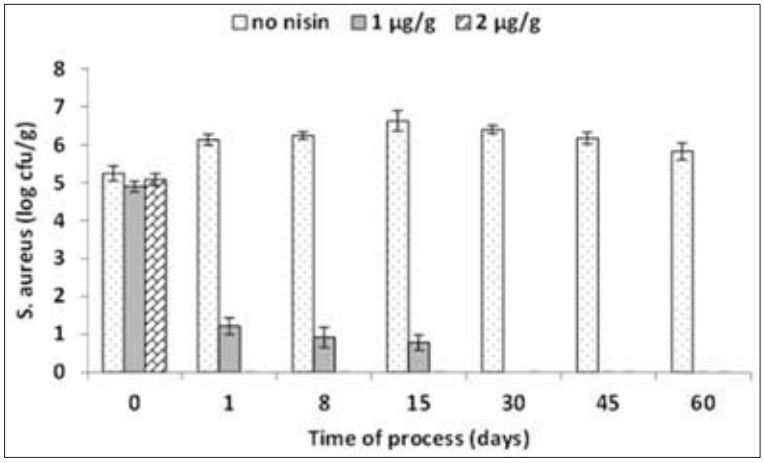
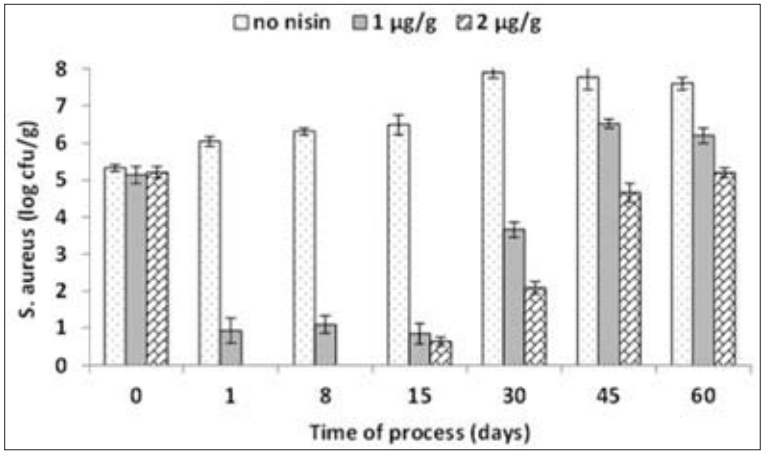


Fig 1. Effect of different concentrations of nisin on the growth of *S. aureus* during 60 days of storage at 8°C in model cheeses

Şekil 1. 60 gün boyunca 8°C'de muhafaza edilen model peynirlerde farklı konsantrasyonlardaki nisinin *S. aureus*'un gelişimi üzerine etkisi

Fig 2. Effect of different concentrations of nisin on the growth of *S. aureus* during 60 days of storage at 25°C in model cheeses

Şekil 2. 60 gün boyunca 25°C'de muhafaza edilen model peynirlerde farklı konsantrasyonlardaki nisinin *S. aureus*'un gelişimi üzerine etkisi



enterotoxin production throughout the ripening and storage. Staphylococcal enterotoxin was not detected in any of the cheese samples treated with nisin and or stored at 8°C. Enterotoxin A was detected in the nisin-free samples in quantities of 1.23, 1.28 and 1.11 ng/g during storage at 25°C in the 30, 45 and 60 days, respectively.

DISCUSSION

The model cheeses were produced based on a critical population density of *S. aureus*. Enterotoxin A was detected in model cheeses when counts of *S. aureus* were 7.9 log cfu/g. This result is in agreement with previous data [11], indicating that positive enterotoxin Camembert-type cheese made with artificially inoculated raw goats' milk had *staphylococci* counts higher than 10^6 cfu *S. aureus*/g.

According to the results, enterotoxin synthesis was inhibited by 1 µg of nisin per gram of cheese. However, during storage at 25°C, nisin degradation and *S. aureus* resuscitation occurred, there was no detectable enterotoxin in nisin treated samples (1 or 2 µg/g). In a previous work, diarrheal enterotoxin production of vegetative cells and spores of Bacillus cereus strain F3802A/84 in gravy was inhibited by adding 1 and 5 µg of nisin per ml at 15°C, respectively [12].

Decrease of nisin levels during storage was found to be temperature dependent. Nisin level remain relatively stable during refrigerated storage, but loss is faster at ambient temperatures [13]. Higher nisin levels are needed for products stored at high ambient temperatures for longer periods. Nisin may be degraded during storage by proteolytic enzymes derived from microbial, plant, or animal cells within the food [14].

The results obtained from this study indicates that the growth of *S. aureus* at 25°C is followed by enterotoxin production which yields a higher risk of food poisoning due to consumption of the cheeses. Those can be prevented by adding 1 µg of nisin per gram cheese.

ACKNOWLEDGMENT

This study was financially supported by the vice chancellor for research affairs of the Islamic Azad University of Tabriz. The authors would like to thank Eastern-Azerbaijan Pegah dairy processing establishment for their cooperation.

REFERENCES

1. Garthright, WE, Archer DL, Kvenberg JE: Estimates of and costs of intestinal infectious diseases in the United States. *Public Health Rep*, 103 (2): 107-115, 1988.

2. Çepoğlu H, Vatansever L, Oral NB: Isolation of *staphylococci* from food handlers and investigation of their enterotoxigenicity and susceptibility to some antibiotics. *Kafkas Univ Vet Fak Derg*, 16 (Suppl-A): S1-S5, 2010.

3. Normanno G, Salandra GL, Dambrosio A, Quaglia NC, Corrente M, Parisi A: Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. *Int J Food Microbiol*, 115, 290-296, 2007.

4. Tel OY, Aslantaş Ö, Keskin O, Yilmaz EŞ, Demir C: Investigation of toxin genes of *Staphylococcus aureus* strains isolated from gangrenous mastitis in ewes. *Kafkas Univ Vet Fak Derg*, 17 (5): 869-871, 2011.

5. Charlier C, Cretenet M, Even S, Le Loir Y: Interactions between *Staphylococcus aureus* and lactic acid bacteria: An old story with new perspectives. *Int J Food Microbiol*, 131, 30-39, 2009.

6. Alomar J, Lebert A, Montel MC: Effect of temperature and pH on growth of *Staphylococcus aureus* in co-culture with *Lactococcus garvieae*. *Curr Microbiol*, 56, 408-412, 2008.

7. Delves-Broughton J: Nisin as a food preservative. *Food Australia*, 57 (12): 525-527, 2005.

8. Aly S, Floury J, Piot M, Lortal S, Jeanson S: The efficacy of nisin can drastically vary when produced *in situ* in model cheeses. *Food Microbiol*, 32, 185-190, 2012.

9. Sezer Ç, Güven A: Investigation of bacteriocin production capability of lactic acid bacteria ısolated from foods. *Kafkas Univ Vet Fak Derg*, 15 (1): 45-50, 2009.

10. FDA/BAM: *Staphylococcus aureus*. January Bacteriological Analytical Manual, Chapter 12: *Staphylococcus aureus*, 2001.

11. Meyrand A, Loei SB, Gueniot SR, Mazuy C, Gaspard CE, Jaubert G, Perrin G, Lapeyre C, Rozand CV: Growth and enterotoxin production of *Staphylococcus aureus* during the manufacture and ripening of Camembert-type cheeses from raw goats' milk. *J Appl Microbiol*, 85, 537-544, 1998.

12. Beuchat LR, Clavero MRS, Jaquette CB: Effects of nisin and temperature on survival, growth, and enterotoxin production characteristics of psychrotrophic *Bacillus cereus* in beef gravy. *Appl Environ Microbiol*, 63 (5): 1953-1958, 1997.

13. Delves-Broughton J: Nisin and its uses as a food preservative. *Food Technol*, 44 (11): 104, 1990.

14. Thomas LV, Delves-Broughton J: Nisin. **In,** Davidson PM, Sofos JN, Branen AL (Eds): Antimicrobials in Food, 3rd ed., pp.247, CRC Press, Taylor & Francis Group, New York, 2005.

Kafkas Univ Vet Fak Derg
20 (3): 465-468, 2014
DOI: 10.9775/kvfd.2013.10373

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

SHORT COMMUNICATION

Immune Response and Production Perfomance in Piglets Vaccinated at 15 and 21 Days Old Against Circovirus Infection

Ognjen STEVANČEVIĆ [1] Nenad STOJANAC [1] Aleksandar POTKONJAK [1]
Milovan GAGRČIN [1] Božidar SAVIĆ [2] Ivan STANČIĆ [1] Vuk VRAČAR [1]

[1] Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovica 8, 21000 Novi Sad, SERBIA
[2] Institute of Veterinary Medicine Belgrade, Serbia, Vojvode Toze 14, 11000 Belgrade, REPUBLIC of SERBIA

Makale Kodu (Article Code): KVFD-2013-10373

Summary

The aim of this research was to determine the effect of vaccination on the amount of antibody titers specific for PCV2, and to determine the effect of vaccination on characteristics of pig production. The first group (A) was vaccinated at 15 days old, the second (B) at 21 days old while the third (C) was the control group. Group B piglets attained the best results, so the vaccination of piglets at 21 days old would have an advantage compared to vaccination at 15 days old, although we note that at 15 days old, there is a far greater influence of maternal antibodies on the creation and development of immune responses in the piglets after vaccination.

Keywords: *PCV2, Immunity, Antibodies, Piglets, Vaccine*

Domuzlarda Circovirus Enfeksiyonuna Karşı 15 ve 21 Günlükken Aşı Olan Domuz Yavrularının Bağışıklık Yanıtı ve Üretim Performansı

Özet

Bu incelemenin amacı, PCV2 Virüsüne özel antikor titresi seviyesine ve domuzların üretim özelliklerine aşılamanın etkisini belirlemektir. Birinci, A grubu, 15 günlükken aşı olmuştur, ikinci (B), 21 günlükken, üçüncü, C grubu ise, kontrol grubuydu. B grubu domuz yavrularında çok daha iyi sonuçlar alınmıştır. Dolayısıyla, domuz yavrularının 21 günlükken aşılanmasının, 15 günlükken yapılan aşılamadan daha başarılı olduğu anlaşılmıştır. Ancak, belirtmek gerekir ki maternal antikorların, 15 günlük olan domuz yavrularının kendi bağışıklık yanıtının gelişmesi ve oluşması üzerine etkisi çok daha büyüktür.

Anahtar sözcükler: *PCV2, Bağışıklık, Antikorlar, Domuz yavrusu, Aşı*

INTRODUCTION

Porcine circovirus type 2 (PCV2) is a widespread virus of domestic and wild pigs and is the primary cause of this pig disease group [1]. Increasing interest in circovirus infections began after the onset of the Post Weaning multisystemic wasting syndrome (PMWS) in Canada in 1991, and retrospective studies have demonstrated their presence in the late 1960s [2]. The group of circovirus diseases, in addition to PMWS, encompasses reproduction disorders, Porcine Dermatitis Nephropathy Syndrome (PDNS), and also a respiratory and enteric form of this disease [3]. The introduction of PCV2 vaccine significantly changed the impact of circovirus on pig production at the global level [4,5]. Vaccination of sows and piglets increases PCV2 antibody titers in serum and colostrums and protects piglets from PMWS development [6,7]. High titers generally provide solid protection against PCV2 infection, whereas lower titers do not provide protection against these infections. Time of vaccination is often problematic, as the large number of papers indicating the possibility of interference of colostral and vaccination antibodies indicates, and which is supported by the large difference in the time of the vaccine application suggested by the pharmaceutical companies [8].

İletişim (Correspondence)
+90 381 21 4853515
ognjen.stevancevic@gmail.com

The aim of this study was to examine the importance of piglet vaccination through following the antibody titers in the serum of vaccinated and non-vaccinated piglets and the production performance of pigs. The second objective was to determine the optimal piglet vaccination time.

MATERIAL and METHODS

Experimental Animals

The research was performed on a pig farm, capacity of 2500 sows, with intensive, enclosed growth conditions, in which the presence of PCV2 infection was demonstrated. The experiment was conducted on 900 piglets divided into 3 groups of 300 randomly chosen piglets. The first group (A) was vaccinated at 15 days old, the second group (B) at 21 days old, and the third group (C) was the control, unvaccinated group. All experimental animals were clinically healthy and in good shape. The piglets were selected by random sampling method. After weaning, all piglets were housed in one building and were separated into boxes of 10 piglets each.

Vaccine

Vaccination of pigs against circovirus infections used the commercial vaccine, licensed for 2 week old piglets and older. Commercial vaccine, is a recombinant ORF2 sub-unit vaccine containing PCV2 antigen. Vaccine (1 ml) was administered intramuscularly in the neck of experimental piglets in group A (on the 15th day) and group B (on the 21st day).

Blood Sampling

Blood samples were taken from 90 piglets (30 piglets from each experimental group) to obtain blood serum samples for the determination of antibody titers. Sampling was first performed in piglets on the day of vaccination (on 15th day of age for group A, 21st day for group B, and 21st day for group C), and then on the 7th, 14th, 21st, 28th, 35th, 60th, 90th and 120th days after the first sampling. Blood was taken by puncture of the brachiocephalic plexus of animals. Samples were collected in approximately 9 ml volumes in vacutainers with coagulation activator and were delivered in a cool-box to the laboratory. Serums were separated after coagulation and centrifugation, and were stored at -20°C until testing.

Determining the Antibody Titers Specific for PCV2

Detection of antibodies specific for PCV2 was performed by an indirect ELISA method - INGEZIM CIRCO IgG (Ingenasa, Spain) according to the manufacturer's instructions. The titer of each sample was calculated according to the formula (Titer = 53 (e 3.2x)), where e is an irrational constant and represents the base of the natural logarithm (2.718), and x is the S/P value of the sample. During statistical processing of the obtained results, the values of antibody titers specific for PCV2 were normalized using $\log_2$ values.

Determining the Effect of Vaccination Against PCV2 on Pig Production Characteristics

The following parameters were analyzed: mortality, average daily weight gain (ADWG), the percentage of culls, feed conversion rate (FCR), weight at slaughter. Mortality was calculated by the ratio of the number of dead pigs and the number of pigs tested and is expressed as a percentage. Pigs marked as rejects were those pigs which had a 20% lower body weight than the average weight of pigs at slaughter. All parameters were analyzed from the moment of vaccination to 175th day of life.

Statistical Analysis

One-way ANOVA test and Turkey`s test were used for mean comparison of the normally distributed variables between groups. Statistical significance of differences between means was determined at the level of $P<0.05$.

RESULTS

By observing the studied population of pigs in whole, from the beginning to the end of the study, it was noted that: the lowest average values of antibody titers in vaccinated groups were determined on the 35th day while the maximum values occurred on the 90th day after vaccination. In the control group, from the beginning of the trial, the average titer decreased continuously until the 60th day, after which the antibody titer specific for PCV2 tended to rise. There were no significant differences ($P>0.05$) in the average level of antibodies in piglet serum measured from the start of observations until the 28th day between the vaccinated groups (A and B) and the unvaccinated control piglets *(Fig. 1)*.

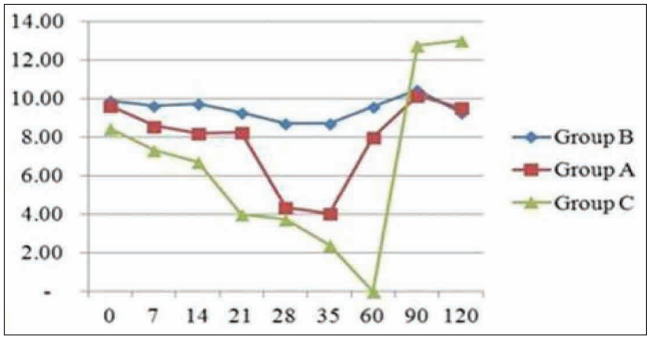


Fig 1. Comparative view of the average values of PCV2 specific antibody titers in the blood serum of piglets from the beginning of the study to 120th day after vaccination

Şekil 1. Deneyin başlamasından aşılamadan sonra 120. gününe kadar domuz yavrularının kan serumunda bulunan PCV2 Virüsüne karşı spesifik antikorların titresinin ortalama değerlerinin karşılaştırmalı görüntüleri

Table 1. *Comparative view of the production performance of pigs in all groups*

Tablo 1. *İncelenen tüm gruplarda domuzların üretim özelliklerinin karılaştırmalı verileri*

Item	Group A	Group B	Group C
ADWG (g/day)	746	750	690
FCR (kg)	3	3.01	3.09
Weight at slaughter (kg)	101.3	102	97
Mortality (%)	7.33	6.33	9
Cull (%)	11.66	11.33	17.33
Tested number	300	300	300

The data obtained on production characteristics of pigs vaccinated at 15 or 21 days old, and from the control group are shown in *Table 1*.

DISCUSSION

In our study, all piglets showed the presence of antibodies specific to PCV2, on the day of vaccination (titers ranged from 8.47 $\log_2$ in the control group to 9.91 $\log_2$ in Group B; *Fig. 1*). Seven days after vaccination, a slight decrease in specific antibodies in the blood serum of piglets was noticed *(Fig. 1)*, but complete absence of antibodies was not found in any piglet. This can certainly be explained, on one hand, by the antigen stimulation of applied vaccine, and, on the other hand, by immunosuppressive action of existing colostral or already created postinfectious antibodies in the blood serum of the piglets examined, which is in full accordance with the results of other authors [9,10]. The highest antibody titer (9.63 $\log_2$) was found in piglets 7 days after vaccination in group B (vaccinated at 21 days old), which may be related to the fact that this group had the highest average titer even before vaccination (9.91 $\log_2$; *Fig. 1*). The biggest decrease of immunoglobulin was observed in group C (1.14 $\log_2$). In group A, we measured a decrease of 1.04 $\log_2$, while in group B the decrease was only 0.28 $\log_2$. The above-mentioned decrease of specific antibodies lasted until the 90th day after vaccination, after which there was a jump in average serum titer levels to 10.46 $\log_2$ in Group B piglets. Levels again declined to 9.27 $\log_2$by day 120 *(Fig. 1)*. These phenomena are certainly attributable to postinfection antibody levels, which occured somewhere between the 35th and 60th days after vaccination *(Fig. 1)*. The most drastic changes occured in the blood sera of unvaccinated pigs (Group C), in which, on the 60th day after vaccination, no specific antibodies were found *(Fig. 1)*, which clearly indicates that there was a complete catabolism of colostral antibodies and the disappearance of passive immunity. In the same group, the average antibody titer on the 90th day suddenly jumped to 12.78 $\log_2$. Possible reasons for this sudden increase in titers of antibodies against PCV2 in blood serum of piglets are the widespread presence of this infection on the study farm, as well as the fact that our pig population initially lacked any immunity to the PCV2 infection. Group A piglets generally followed the same pattern of immunoglobulin changes, as was found in Group B piglets, although the changes were much less pronounced *(Fig. 1)*. The reasons for this are due to the fact that on the 15th day of age (when Group A piglets were vaccinated), large amounts of colostral immunoglobulins were still present, and these followed a similar pattern of changes to those we observed in the control group. This could explain the fact that in the sera of Group A piglets, even 35 days after vaccination, it was not possible to identify a single piglet with antibody titers above 9 $\log_2$, which undoubtedly corresponds to complete catabolism of colostrum antibodies and the lack of creating their own immunoglobulin. In this sense, vaccination of pigs at 21 days old would be advantageous compared to vaccination at 15 days old, although the period of 21 days is quite long, which could lead to infection of pigs with negative consequences in terms of health and achieved economic parameters (total weight gain, food consumption efficiency, etc.). The results we obtained are compliant with those of Krakowka et al.[9], and Meerts et al.[11], who stated that the conversion occurs 10 to 28 days after inoculation. Some other authors [12,13] suggest that the conversion of antibodies at 21 days after infection is lower than in subclinical infected pigs. This could be explained only by the level of colostral antibodies at the time of inoculation. On the other hand, Forth et al.[14], have shown that the anti-PCV2 IgG occurs between 7th and 14th day after inoculation of antigen, reaches its peak on the 21st day after inoculation and rises until the 69th day after inoculation. This is not consistent with our results, which clearly show that low levels of antibodies had a positive effect, and that the negative effect is observed only in the presence of high levels of maternal antibodies [10], although Ritzman et al.[15] in a study carried out on 1519 piglets demonstrated that the vaccine was effective regardless of the level of maternal antibodies.

Results to date have also shown a different number of pigs lagging in the growth. In the current study, 17.33% of pigs in the control group had insufficient weight gain. Overall, this resulted in 32.8% and 34.6% more unvaccinated piglets which did not thrive compared to vaccinated Group A or Group B piglets, respectively *(Table 1)*. Differences in the number of rejects caused quite different levels of mortality *(Table 1)*. In the control group of piglets, the mortality level was 9%, while that of group A was 7.33%, and that of group B was 6.33%. The differences found could be linked to differences in serological response, or the number of reject piglets in tested groups. Generally, however, during the course of our study, positive effects of the applied vaccine were noted. They are largely based on preventing larger oscillations in the level of antibodies specific for PCV2. The identified differences are likely the result of the presence of maternal antibodies in the blood serum of tested piglets at the time of the vaccination.

By preventing major fluctuations, we can prevent the possibility that a certain number of piglets, at any given moment, are without immunological protection and thus create the possibility of infection.

Overall, our study has shown that, with the vaccine studied, much better results are attained by vaccination of piglets at 21 days old, compared to 15 days old, there is a far greater influence of maternal antibodies on the creation and development of each piglet's own immune responses after vaccination.

REFERENCES

1. Llorenç Grau-Romaa, Frailea L, Segalésa J: Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. *Vet J,* 187 (1): 23-32, 2011.

2. Allan GM, McNeilly F, Cassidy JP, Reilly GAC, Adair B, Ellis WA, McNulty MS: Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. *Vet Microbiol,* 44 (1): 49-64, 1995.

3. Segalés J: Porcine circovirus type 2 (PCV2) infections: Clinical signs, pathology and laboratory diagnosis. *Virus Res,* 164 (1-2): 10-19, 2012.

4. Lyoo K, Joo H, Caldwell B, Kim H, Davies PR, Torrison J: Comparative efficacy of three commercial PCV2 vaccines in conventionally reared pigs. *Vet J,* 189 (1): 58-62, 2011.

5. Nathan MB, Xiang-Jin M: Efficacy and future prospects of commercially available and experimental vaccines against porcine circovirus type 2 (PCV2). *Virus Res,* 164 (1-2): 33-42, 2012.

6. Martelli P, Ferrari L, Morganti M, De Angelis E, Bonilauri P, Guazzetti S, Caleffi A, Borghetti P: One dose of a porcine circovirus 2 subunit vaccine induces humoral and cell-mediated immunity and protects against porcine circovirus-associated disease under field conditions. *Vet Microbiol,* 149 (3-4): 339-351, 2011.

7. Opriessnig T, Patterson AR, Madson DM, Pal N, Halbur PG: Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2-3-months post vaccination. *Vaccine,* 27 (7): 1002-1007, 2009.

8. Fort M, Fernandes LT, Nofrarias M, Diaz I, Sibila M, Pujols J, Mateu E, Segales J: Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets. *Vet Immunol Immunopathol*, 129 (1-2): 101-107, 2009.

9. Krakowka S, Ellis JA, McNeilly F, Ringler S, Rings DM, Allan G: Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). *Vet Pathol,* 38 (1): 31-42, 2001.

10. Opriessnig T, Xiang-Jin M, Halbur P: Porcine Circovirus type 2-associated disease: Update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *J Vet Diagn Invest,* 19 (6): 591-615, 2007.

11. Meerts P, Van GS, Cox E, Vandebosch A, Nauwynck HJ: Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. *Viral Immunol,* 18 (2): 333-341, 2005.

12. Bolin SR, Stoffregen WC, Nayar GPS, Hamel AL: Postweaning multisystemic wasting syndrome induced after experimental inoculation of cesarean-derived, colostrum-deprived piglets with type 2 porcine circovirus. *J Vet Diagn Invest,* 13 (3): 85-194, 2001.

13. Rovira A, Balasch M, Segales J, Garcia L, Plana-Duran J, Rosell C, Ellerbrok H, Mankertz A, Domingo M: Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. *J Virol,* 76 (7): 3232-3239, 2002.

14. Fort M, Olvera A, Sibila M, Segalés J, Mateu E: Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. *Vet Microbiol,* 125 (3-4): 244-255, 2007.

15. Ritzmann M, Palzer A, Eddicks M, Elicker S, Heinritzi K: Lack of interference with maternal immunity and reduction of viremia in PCV2 vaccinated pigs. *20th International Pig Veterinary Society Congress.* June 22-26, Durban South Africa, p.95, 2008.

Kafkas Univ Vet Fak Derg
20 (3): 469-472, 2014
DOI: 10.9775/kvfd.2013.10254

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

CASE REPORT

Ectopia Cordis Cervicalis and Its Surgical Treatment in A Holstein Calf [1]

Kürşat ÖZER [1] Didar AYDIN [1] Özlem GÜZEL [1] Dilek OLĞUN ERDİKMEN [1]
Ebru ERAVCI [1] Ümit UĞURLU [1] Lora KOENHEMSI [2] Tamer DODURKA [2]
Kıvılcım SÖNMEZ [3] Aydın GÜREL [3]

[1] *Presented at13th National Congress of Veterinary Surgery -participitation in international-, 27 July-01 June 2012, Sarıkamış-Kars/Turkey with a title of "Ectopia cordis and its surgical treatment in a six-day-old calf"*

[1] Department of Surgery, Faculty of Veterinary Medicine, University of Istanbul, TR-34320 Istanbul - TURKEY
[2] Department of Internal Medicine, Faculty of Veterinary Medicine, University of Istanbul, TR-34320 Istanbul - TURKEY
[3] Department of Pathology, Faculty of Veterinary Medicine, University of Istanbul, TR-34320 Istanbul - TURKEY

Makale Kodu (Article Code): KVFD-2013-10254

Summary

Ectopia cordis is a rare congenital anomaly characterized by partial or complete displacement of the heart, outside the thoracic cavity. Ectopia cordis is usually associated with other multiple anomalies and intra-cardiac defects due to developmental problems in the ventral body wall. In this report, a 6 day-old, 55 kg male Holstein calf with a case of ectopia cordis cervicalis and its surgical treatment has been presented. Following examinations (clinical, haematological, ECG, x-ray, USG and CT) a single staged surgery was performed to correct localisation of the heart in the thoracic cavity. The case was followed up 10 days postoperatively. However, the calf died at the end of post-operative day 10 due to septic shock.

Keywords: *Calf, Ectopia cordis cervicalis, Congenital abnormality*

Holstein Irkı Bir Buzağıda Ectopia Cordis Cervicalis Olgusu ve Operatif Sağaltımı

Özet

Ectopia Cordis, kalbin göğüs kafesi dışında, parsiyal ya da tam olarak anormal yerleşimiyle karakterize, seyrek rastlanan kongenital bir anomalidir. Ectopia cordis, ventral karın duvarının gelişimindeki problemlere bağlı olarak genellikle intrakardiyak defektlerle ya da diğer çoklu anomalilerle ilişkili olabilir. Bu makalede, 6 günlük, erkek, 55 kg ağırlığında, Holstein bir buzağıda görülen ectopia cordis cervicalis olgusu ve operatif sağaltımı anlatılmaktadır. Yapılan muayeneler (klinik, hematolojik, EKG, radyografik, ultrasonografik, BT) sonucunda hastaya, kalbin göğüs kafesi içine alınmasına yönelik olarak tek seansta, cerrahi sağaltımda bulunuldu. Hastanın, 10 gün süreyle postoperatif takibi yapıldı fakat buzağı 10. günün sonunda septik şok nedeniyle öldü.

Anahtar sözcükler: *Buzağı, Ectopia cordis cervicalis, Konjenital anomali*

INTRODUCTION

Ectopia cordis (EC) is the partial or complete displacement of the heart in an abnormal position outside the thoracic cavity [1-3]. While partial or complete ectopia cordis may occur depending on the volume of heart outside the thoracic cavity [4,5], three further different types may be present depending on localisation: cervical (upper and lower cervical ectopia cordis), pectoral and abdominal ectopia cordis [6]. Most frequently seen is the cervical type (82%). Rates at which pectoral and abdominal types are seen are 14% and 3%, respectively [2,3,6]. Observed in 1 in 5.2-7.2 million humans [7]; in animals, this anomaly is most frequently seen in calves [8]. Occurring less frequently than in humans, this anomaly has also been observed in animal species such as horses, goats and pigs [9-11]. Life expectancy in animals with ectopia cordis may range from 3 minutes to several years [5]. Cases where the displacement is either

İletişim (Correspondence)
+90 212 4737070/17077
didaraydin@hotmail.com

through the sternum or the costae usually end with neonatal death. In other types of displacement, animals may live for longer periods [8].

In this report, a case of ectopia cordis in a six-day old calf, in addition, the operation technique and necropsy findings of this condition are described.

CASE HISTORY

This case consisted of a 6-day old Holstein calf weighing 55 kg, with ectopia cordis cervicalis, referred to the Istanbul University Surgery Department. Anamnesis revealed that the calf had been born naturally and on inspection it was observed that it had completed its growth, the skin was complete and the coat was normal. However, during physical examination of the patient, right torticollis and a distinct swelling on the caudal aspect of the left cervical region was identified. The calf had a body temperature of 38.2°C and the lymph nodes and mucosae appeared normal. As well as the evident tachypnoae and tachycardia, moist rales were also audible on lung auscultation. Haemogram findings were assessed to reveal thrombocytopenia and leucopenia. Blood biochemistry revealed that ALP and iron binding values were increased, while LDH, cholesterol, total protein and Ca values were decreased. When electrocardiac findings were examined, heart frequency was found to be 250/min. Also, despite the absence of the P wave in all derivations, the R-R interval was seen to be constant. Supraventricular tachycardia was identified in the patient. Radiodiagnostic examination showed that, as a result of serial radiography and spiral BT assessment directed at the thorax, the base of the heart was at the level of the tracheal bifurcation, higher than normal and that it was located at the level of the jugulum (*Fig 1-A, 1-B* and *1-C*).

Following determination of the patient's clinical, radiographic and electrocardiographic findings, surgical intervention was decided in order to relocate the heart, which was positioned in the neck, to within the thoracic cavity. After the usual pre-operation preparations, the patient was placed in dorsal recumbency and general anaesthesia was induced via mask induction using Isoflurane at a concentration of 4%. The maintenance dose for anaesthesia was 2% Isoflurane (Forane, Abbout®, UK). Balanced fluid electrolyte solution (Lactated Ringer 10 ml/kg/h) was administered to the patient intravenously throughout the operation. Prophilactically, Cephazolin sodium (Sefazol, Mustafa Nevzat® Turkey) was given at a dose of 25 mg/kg IV. The incision was made for a median sternotomy, continued across the region where the heart was positioned and extended towards the ventral aspect of the neck (*Fig 2-A* and *2-B*). Thoracal retractors were placed in the site. It was not possible to separate the heart, which was located immediately under the skin, from adhesions and muscles, therefore pericardiectomy was performed *(Fig 3)*. In order to allow the heart to find its normal anatomic

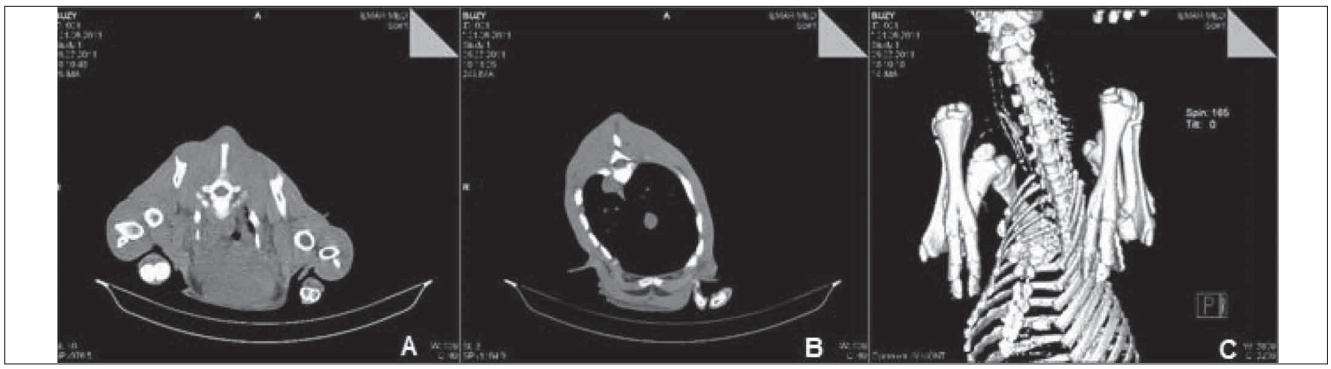


Fig 1. The base of the heart was at the level of the tracheal bifurcation **(A)**, the heart is not seen in thorax cavity **(B, C)**
Şekil 1. Kalp tabanı tracheal bifurkasyonu düzeyindedir **(A)**, göğüs boşluğunda kalp izlenmemektedir **(B, C)**

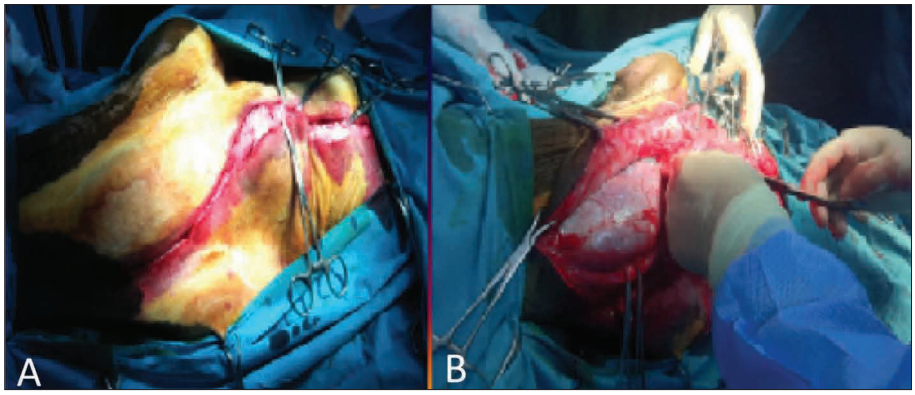


Fig 2. In dorsal recumbency, the incision was made on the ventral aspect of the neck and median sternotomy was performed **(A)**, the heart was located under the skin within adhesions and muscles **(B)**

Şekil 2. Sırtüstü yatış pozisyonunda boynun verntralinden yapılan ensizyonu takiben median sternotomi uygulanmış **(A)** ve kalp açığa çıkarılmıştır **(B)**

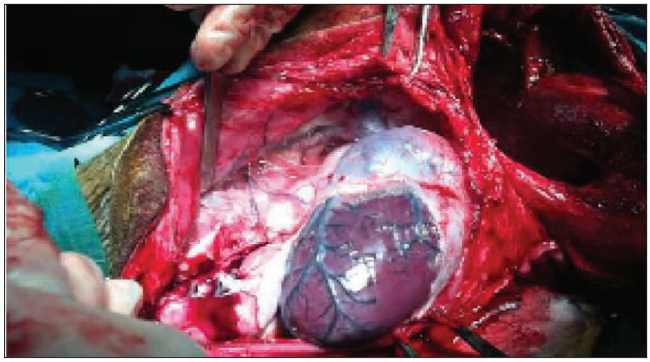


Fig 3. Pericardiectomy site
Şekil 3. Perikardiyektomi bölgesi

location, lobectomy was performed on the cranial lobe of the right lung *(Fig 4-A)*. The heart was then placed in the direction of the lobectomy site *(Fig 4-B)*. The pericardium was not sutured. Sternebrae were brought closer together using cerclage wire and the operation site was closed surgically. During this time, care was taken to intensively ventilate the lungs.

The patient regained consciousness shortly after the operation and was hospitalized. Approximately 24 h after the operation the patient was given food orally. There was no abnormality in the postoperative *(Fig. 7)* clinical findings up until the end of day 8. However, in the following days it was seen that the patient's appetite had declined, body

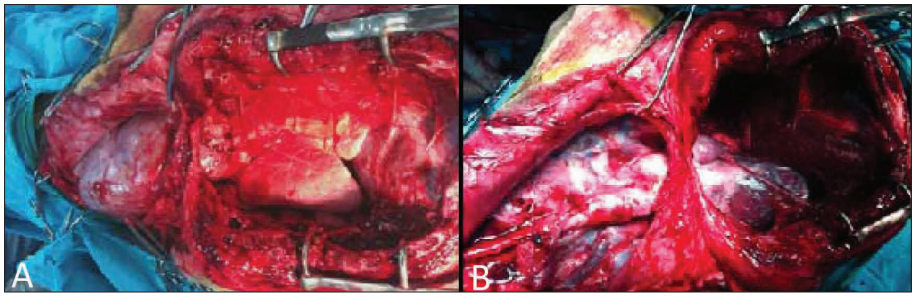


Fig 4. The position of the heart before the right cranial lobectomy **(A)**, the heart's new location after the lobectomy **(B)**

Şekil 4. Kraniyal lobektomi öncesinde kalbin pozisyonu **(A)**, lobektomi sonrasında kalbin yeni konumu **(B)**

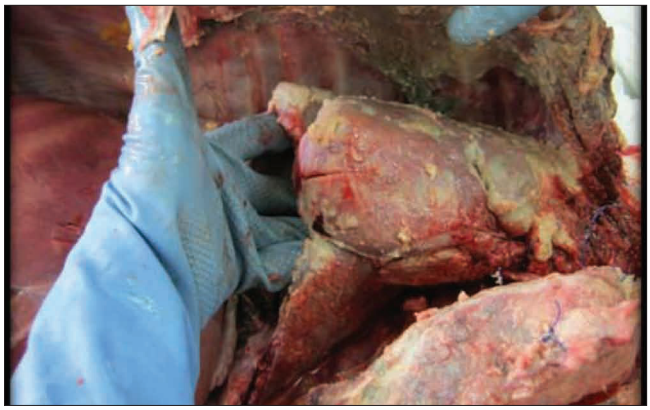


Fig 5. Fibrinous and purulent pleuritis in necropsy findings
Şekil 5. Nekropside fibrinöz ve purulent plöritis bulguları

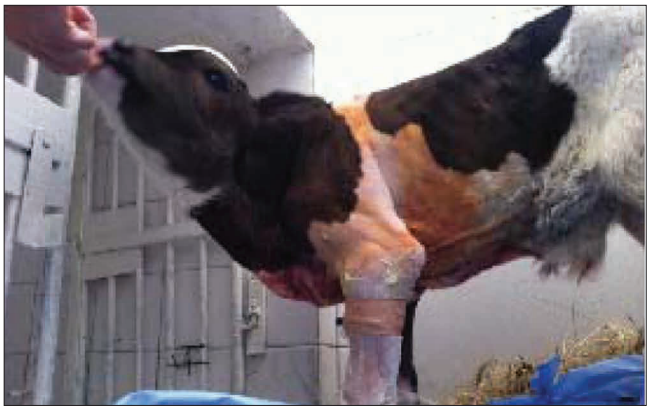


Fig 7. The calf's postoperative period
Şekil 7. Buzağının postoperatif dönemi

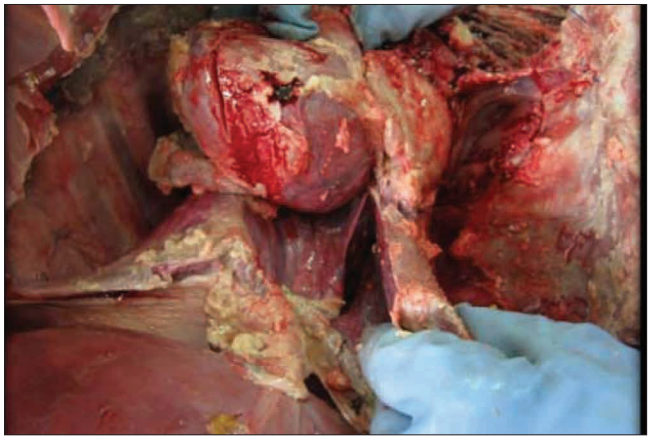


Fig 6. Branching type similarity to pig cattle model
Şekil 6. Domuz-köpek modeline benzer dallanma şekli

temperature had risen to 41°C and leucocytosis was present in haemogram findings. Meanwhile, a sticky seromucous discharge was seen to be present at the incision site. The antibiotic drug Cefazolin HCL given at a dose of 25 mg/kg postoperatively was changed to Ceftiofur hydrochloride (Excenel, Pfizer®) at a dose of 2 mg/kg. However, despite all interventions, the patient died of septicaemia at the end of day 10.

As a result of necropsy findings, the reason of death was found to be fibrinous and purulent pleuritis *(Fig. 6)* and septicaemia.

DISCUSSION

Abnormalities seen in a body structure or function at

birth are called congenital abnormalities [12]. Congenital abnormalities are multidisciplinary cases. These abnormalities may affect a single organ system or structure or all systems or even some systems (cardiovascular or integumentary system) [3,13].

Many factors, such as chemical teratogens, genetic or chromosomal abnormalities, environmental radiation and infectious agents, play a role in the development of congenital defects in calves [3,14]. The conceptus may encounter harmful factors at different developmental stages such as the preimplantation, embryonic or foetal stages [3].

Delayed descension of the heart in the embryonic period is thought to be the mechanism responsible for cervical ectopia cordis [4,5]. It is thought that the factor causing ectopia cordis affects the foetus before day 36 of the pregnancy [4]. While the etiology of this anomaly is not completely understood, it has been reported that this condition is encountered more often in humans and three theories have been suggested for its pathogenesis. These theories are: 1) A primary fault in the process of the lateral body curves meeting at the midline and descending, 2) A fault in midline closure in relation to premature separation of the chorion or the egg, 3) Amniotic band syndrome [5].

Any type of ectopia cordis may be complicated with other malformations either cardiac or non-cardiac [2]. In humans, ectopia cordis may be related to the quintet syndrome; abdominal wall, sternum, diaphragm, pericardium and heart defect, mostly described as Cantrell's pentalogy [7,15,16]. Unusual defects together with ectopia cordis may be also observed in calves. The presence of ectopic lungs and extremities together with ectopia cordis has been reported in a calf with multiple congenital anomalies [17]. In another calf monitored for a long time, ectopia cordis together with tricuspid valve dysplasia was identified and it was thought that this could be related to the Ebstein anomaly however it has been reported that this calf with ectopia cordis cervicalis completed its normal development, became pregnant and calved naturally [18].

Other anatomical defects frequently encountered are; double apex, double vena cava, double vena azygous and sternum abnormalities. In veterinary sources, while branching of the heart along the arcus aorta has been recorded, branching in pigs, dogs and cattle have been reported in three models [4].

Although branching of the blood vessels in calves with ectopia cordis cervicalis is in the type of branching mostly seen in dogs or similar to hybrid branching seen in pig and dog combination, the branching type in the necropsy findings of our case shows similarity to the pig-cattle model *(Fig. 5)*. Among characteristic findings in calves with cervical ectopia cordis are listed; a shortened and widened manubrium, xyphoid cartilage hypoplasia, double appearance sternebrae and presence of multiple sternebrae [4].

In calves with ectopia cordis cervicalis, as a result of surgical treatment to move the heart into the chest cavity, no literature has been found stating that the patient had survived for more than several hours.

The patient in this case report tolerated the operation well. The reason for septic shock development in this case was thought to be due to the pre-existing leucopenia and immune system deficiency. The authors decided to present to colleagues their experience in this case, due to the presence of a rare heart abnormality and the operation technique for the treatment of this condition.

REFERENCES

1. Aslanbey D, Gavuzoğlu MK, Taşbaş M, Berkin Ş, Yılmaz Y: Bir danada ectopia cordis cervicalis olgusu. *Ankara Univ Vet Fak Derg,* 27, 613-631, 1980 (article in Turkish with an abstract French).

2. Eröksüz H, Metin N, Eröksüz Y: Total pectoral ectopia cordis and other congenital malformations in a calf. *Vet Rec*, 142, 437, 1998.

3. Hamali H, Ashrafihelan J: A report on bovine fetal ectopia cordis cervicalis associated with two cervical sacs. *Iran J Vet Res*, 11 (3): 287-290, 2010.

4. Hiraga T, Abe M, Iwasa K, Takehana K, Tanigaki A: Cervico-pectoral ectopia cordis in two Holstein calves. *Vet Pathol,* 30, 529-539, 1993.

5. Shirian S, Oryan A, Samadian MR: Ectopia cordis in a male Holstein Friesian calf. *Open Anatomy J,* 2, 34-36, 2010.

6. Herzog A, Wiedeking J: Ectopiacordis congenital beim rind. *Giessener Beitr Erbpathol Zuchthyg*, 1-2, 1970.

7. Morales MJ, Patel SG, Duff JA, Villareal RL, Simpson JW: Ectopia cordis and other midline defects. *Ann Thorac Surg,* 70 (1): 111-114, 2000.

8. Cynthia MK: Circulatory System. Ectopic Heart. p.75. The Merck Veterinary Manual. Merck & Co., Inc., Whitehouse Station, NJ, USA, 2005.

9. Freeman LE, McGovern PT: Ectopia cordis thoracoabdominalis in a piglet. *Vet Rec,* 115, 431-433, 1994.

10. Reppas GP Canfield PJ, Hoffmann KI, Hartley WJ, Hutchkins D: Multiple congenital cardiac anomalies and idiopathic thoracic aortitis in a horse. *Vet Rec*, 138 (1): 14-16, 1996.

11. Upadhye SY, Dhoot VM : A case of ectopia cordis in a goat. *Indian Vet J*, 78 (11): 1028-1029, 2001.

12. Moura E, Pimpão TC: Veterinary Dysmorphology. **In,** Perez-Marin CC (Ed): A Bird's-Eye View of Veterinary Medicine. ISBN: 978-953-51-0031-7, 2012. InTech, Available from: http://www.intechopen.com/books/a-bird-s-eye-view-of-veterinary-medicine/veterinary-dysmorphology. *Accessed:* 05.03.2012.

13. Koç Y, Alkan F: Bir kuzuda ekstraumbilikal ektopia hepatika olgusu. *Vet Bil Derg,* 17 (4): 109-111, 2001.

14. Azizi S, Shahrzad A, Bakhtiary KM, Goodarzi M: Congenital sternal foramen in a stillborn Holstein calf. *Asian Pac J Trop Biomed*, 2 (1): 83-84, 2012.

15. Gao Z, Duan Q, Zhang Z, Ying L, Ma L: Pentalogy of cantrell associated with thoracoabdominal ectopia cordis. *Circulation,* 119 (15): 483-485, 2009.

16. Kumar B, Sharma C, Sinha D, Sumanlata D: Ectopia cordis associated with Cantrell's pentalogy. *Ann Thorac Med*, 3 (4): 152-153, 2008.

17. Islam MR, Roh YS, Cho A, Park H, Heo SY, Lee K, Lee HK, Lim CW, Kim B: Multiple congenital anomalies in a Korean native calf *(Bos taurus coreanae). Korean J Vet Res,* 51, 55-59, 2011.

18. Onda K, Sugiyama M, Niho K, Sato R, Arai S, Kaneko K, Ito S, Muto M, Saganuma T, Wakao Y, Wada Y: Long term survival of a cow with cervical ectopia cordis. *Can Vet J,* 52, 667-669, 2011.

Kafkas Univ Vet Fak Derg
20 (3): 473-480, 2014
DOI: 10.9775/kvfd.2013.10402

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

REVIEW

Surgical Interventions of Common Congenital Heart Defects in Dogs: A Comprehensive Review

Shi-Min YUAN [1]

[1] Department of Cardiothoracic Surgery, The First Hospital of Putian, Teaching Hospital, Fujian Medical University, Putian 351100, Fujian Province, CHINA

Makale Kodu (Article Code): KVFD-2013-10402

Summary

The most common congenital heart defects (CHDs) in the dog include aortic stenosis, patent ductus arteriosus, pulmonic stenosis, ventricular septal defects, mitral valve dysplasia, tricuspid valve dysplasia, tetralogy of Fallot, and endocardial fibroelastosis. They can occur as a result of genetic, environmental, infectious, or poisoning conditions, or malnutritional or maternal medical influences. Some authors believe that CHDs show gender and breed heritable predilections in dogs. Most dogs with severe CHDs may show congestive heart failure, and lead to an early death. The diagnoses of CHDs rely on electrocardiogram, chest radiograph, echocardiogram, and sometimes cardiac catheterization. Earlier accurate diagnosis with further proper treatments ensures better outcomes. Surgical interventions that are employed in humans can be applied in dogs as well. Open heart surgery may be necessary for the repair of the defects, but it costs much. Some CHDs may be curable to minimally invasive or hybrid procedures. Early diagnosis also allows owners to avoid continuing genetic defects in breeding lines. Attentions to the dog breeds with a known predisposition of an inheritable heart disease would merit a long-run veterinary significance.

Keywords: *Cardiac surgical procedure, Cardiopulmonary bypass, Septal occluder device, Veterinary clinics*

Köpeklerde Yaygın Konjenital Kalp Hatalarında Cerrahi Müdahaleler

Özet

Köpeklerde en yaygın konjenital kalp hataları (CHD) aort stenozu, açık duktus arteriozus, pulmoner darlık, ventriküler septal defekt, mitral kapak displazisi, trikuspital kapak displazisi, Fallot tetralojisi ve endokardiyal fibroelastozistir. Bu hatalar genetik, çevresel, enfeksiyöz veya zehirlenme durumları ile maternal malnutrisyon veya tıbbi etkiler sonucu oluşabilir. Bazı yazarlar CHD'lerin cinsiyet ve tür bazlı kalıtsal eğilime sahip olduğuna inanmakadır. CHD'li çoğu köpek konjestive kalp yetmezliği gösterir ve bunlar erken ölüme neden olur. CHD'nin teşhisi elektrokardiyogram, göğüs radyografisi, ekokardiyogram ve bazen kardiyak kateterizasyona dayanmaktadır. Erken doğru teşhis ve uygun tedavi daha iyi sonuçlar için gereklidir. İnsanlarda uygulanan cerrahi müdahaleler köpeklerde de uygulanabilir. Açık kalp ameliyatı hataların düzeltilmesi için gerekli olabilir ancak bu müdalae oldukça pahalıdır. Bazı CHD'ler invaziv ve hibrid yöntemler kullanılarak tedavi edilebilir. Erken teşhis hayvan sahiplerine sürülerinde genetik hatanın devam etmesini engellemede yardımcı olabilir. Bilinen kalıtsal bir kalp hastalığına yatkınlığı olduğu tespit edilen köpeklerin dikkatli takibi veterinerlik açısından uzun vadede önem kazanmaktadır.

Anahtar sözcükler: *Kardiyak cerrahi müdahale, Kardiyopulnomer bypass, Septal oklüzyon cihazı, Veteriner klinikleri*

INTRODUCTION

Congenital heart disease (CHD) represents a broad spectrum of heart defects at birth, single or combined, including those of the heart valves, cardiac chambers, great vessels, or abnormal connections between cardiac chambers [1], which may develop as genetics being the major causative etiology over environmental, infectious, or poisoning conditions, or maternal malnutritional or medical influences [2]. It is believed that CHDs show gender and breed heritable predilections in dogs [1]. In a study of a total of 1,132 heart defects in dogs, the incidence of CHD was 21.7%, with single defects accounting for 85%, 2 concurrent defects 14%, and 3 concurrent defects 1% [3]. Epidemiologic studies revealed that the most common CHDs were pulmonic stenosis, subaortic stenosis, and patent ductus arteriosus (PDA), followed by ventricular septal defect (VSD), valvular aortic stenosis, and tricuspid dysplasia [3,4]. Of them, subaortic stenosis, pulmonic stenosis, and VSD are frequently associated with other defects [3].

İletişim (Correspondence)
+86 594 6923117
shi_min_yuan@yahoo.com

Dogs with CHDs show varying symptoms with severity of the defects. Most dogs with severe CHDs show acute congestive heart failure, and lead to an early death [5], while dogs with mild-to-moderate CHDs may exhibit exercise intolerance, fainting episodes, and retarded growth, or even asymptomatic with only audible heart murmur on physical examination. They are easily managed and often have a good prognosis [6]. The diagnoses of CHDs rely on electrocardiogram, chest X-rays, echocardiogram and sometimes cardiac catheterization. Echocardiography has largely replaced cardiac catheterization as a routine diagnosis of CHDs in dogs due to accuracy and non-invasiveness [6]. With the speedy development of therapeutic strategies for CHDs, in particular, the development of minimally invasive surgical techniques in humans, CHDs in animals present more challenging management problems than before. The purpose of this article is to present a comprehensive literature review with regard to updated surgical interventions of the common CHDs in dogs of the current era.

PATENT DUCTUS ARTERIOSUS

PDA is the most common CHD in dogs, usually representing a left-to-right shunt [7]. It is more often seen in female than in male dogs, and in purebred than in mixed breeds [8]. The severity of the symptoms is closely related to the ductal size and aortopulmonary shunting [6]. Some animals remain asymptomatic for a long time and the cardiac murmurs are overlooked; others can manifest congestive heart failure as a result of pulmonary overperfusion. In right and left parasternal short-axis views of echocardiography at the level of the major vessels, the PDA presents as a hypoechoic space between the pulmonary arterial trunk and the aorta [9]. The angiographic studies on 43 dogs revealed minimal PDA diameter was 3.72±1.59 mm, and the diameter of the PDA ampulla was 8.46±3.01 mm. The frequency of PDA visualization was 78%, and the measurement deviation was 1-2 mm [10]. Good visualization of PDA by two-dimensional echocardiography may be achieved in 96% of the dogs [9]. Atrial fibrillation, mitral valvular incompetence, papillary muscle displacement and mitral valve prolapse are recognized complications of PDA [10]. If the PDA is left untreated, only 36% of dogs survive to one year of age. Thus, permanent occlusion at an early age is the current treatment strategy for PDA. Techniques for PDA repair include surgical ligation via thoracotomy and catheter-based occlusion. Surgical correction involves a left lateral thoracotomy and careful dissection around the PDA with circumferential ligatures. Thoracotomy ligation is an effective procedure with a success rate of up to 95% and a mortality rate of less than 2%. Complications such as fatal hemorrhage might occur due to increased ductus friability in particular in the older dog [11]. Catheter-based occlusion techniques are based on the use of coils, an Amplatzer plug, or an Amplatzer duct occluder. Preoperative angiography and transesophageal echocardiography are important means for the determination of the size and anatomy of the PDA [12]. The deployment of a detachable coil is usually under fluoroscopic guidance for locating correct placement for coils and the potential effectiveness of the occlusion [13]. The Amplatzer vascular plug is a self-expandable, cylindrical device attached to a delivery cable, and is usually delivered transvenously [14]. The choice of the Amplatz Canine Duct Occluder (ACDO) device size is 1.5-2 times of minimum ductal diameter based on echocardiographic or angiographic measurements. The procedures are comprised of advance of sheath and the device across the PDA into the main pulmonary artery, detachment of the device and subsequent retraction of the delivery cable from the descending aorta [15]. Tanaka et al.[16] informed that residual shunt was observed three months postoperatively and then a supplemental coil was inserted. Signh et al.[17] applied 4 kinds of devices ACDO (transarterial), Gianturco or MReye Flipper Detachable Embolization (Flipper) coil (transarterial), Amplatzer Vascular Plug (AVP) (transarterial), and Flipper coil (transvenous) for the management of PDA in dogs, and found ACDO had significantly fewer complications comparing with the other three. Saunders et al.[18] reported that the incidence of device migration was 3%. The ACDO mismatch can sometimes occur [19].

SUBAORTIC STENOSIS

Subaortic stenosis is a cardiac disorder with a narrowing of the descending aorta below the left ventricular outflow tract. The dog breeds such as Newfoundland are with more common subaortic stenosis which usually leads to an early death [20]. Subaortic stenosis has been genetically evidenced as an autosomal inheritance in Newfoundland dog [20]. A study on 195 untreated dogs with subaortic stenosis showed that sudden death was associated with severe subaortic stenosis 16 times more than moderate or mild, which often developed within the first three years of life [21]. Echocardiographic findings of 32 dogs with PDA were characterized by left ventricular hypertrophy in 37.5% (12/32), aortic insufficiency 62.5% (20/32) and subvalvular ridge 62.5% (20/32) [22]. The technique of cutting balloon valvuloplasty combined with high pressure valvuloplasty for dogs with severe subaortic stenosis has recently been reported to be a safe and feasible therapeutic option [23].

PULMONIC STENOSIS

Pulmonic stenosis is the third most common CHD in dogs. In pulmonic stenosis, the right ventricular outflow tract is narrowed at valve, supra-valve, or sub-valve levels, with the pulmonary valve stenosis being the most common

form. Minors et al.[24] studied infundibular pulmonic stenosis and further classified it into 3 subtypes: a fibrous diaphragm, fibromuscular, and muscular obstruction. Some animals with pulmonic stenosis manifest fatigue, fainting spells, ascites, exertional cyanosis, and even sudden death. Echocardiographic examinations reveal right ventricular concentric hypertrophy correlating to the severity of the pressure load imposed on the proximal right ventricular chamber by the stenosis. Performing balloon valvuloplasty reduces the risk of sudden death and improves quality of life as well. In the early years, balloon valvuloplasty was performed in 2 dogs, and immediate hemodynamic improvement was achieved with significant decrease of peak systolic pressure gradient across the pulmonic valve [25]. It was then evidenced that balloon valvuloplasty caused sustained right ventricular pressure reduction until 3 months after the intervention [26]. Minors et al.[24] performed surgical dilation of infundabular pulmonic stenosis without the need of cardiopulmonary bypass and systemic venous inflow occlusion. The dilated infundibular chamber was incised through a pursestring by a pediatric valve dilator across the stenosis for stricture relief. The peak pressure gradient was remarkably reduced 24 h after surgery than preoperation.

ATRIAL SEPTAL DEFECT

Atrial septal defect (ASD) represents 0.7% of the CHDs in dogs. The incidence of ASDs in dogs is rather high representing the second most commonly diagnosed CHD after mitral valve dysplasia [27]. Common signs associated with ASD include exercise intolerance, syncope, dyspnea, cough, heart murmur, cyanosis, and ascites if right heart failure develops. Medical treatment consists of systemic arterial vasodilation to reduce the shunt flow. Diuretics, angiotensin converting enzyme inhibitors and positive inotropic drugs are necessary for the animals with congestive heart failure, often resulting from severe aortic insufficiency [26]. Surgical repair of ASDs may be necessary in selected cases, but it costs much. A patch closure under cardiopulmonary bypass is a definitive treatment of ASD with large left-to-right shunting [28]. An alternative surgical operation is pulmonary artery banding for enhancing pulmonary artery resistance [6]. For some secundum-type ASDs, an Amplatzer device can be an alternative to close the defect. Amplatzer devices can be deployed by a right jugular or a transatrial approach through a right lateral thoracotomy [29]. Transcatheter ASD closure was successful in 10/13 dogs. After ASD closure, transthoracic color Doppler echocardiography indicated complete occlusion in 5 (50%) dogs, trivial to mild residual shunting in 4 (40%) dogs, and moderate residual shunting in 1 (10%) dog. Accidental right atrial release and embolization might occur in a few dogs. The mean event-free survival of the dogs with successful transcatheter ASD closure was 22.2±10.2 months.

VENTRICULAR SEPTAL DEFECT

Most VSDs in small animals are small and restrictive [30], moderate-sized VSDs are only partially restrictive with high right ventricle pressure, and large VSDs are nonrestrictive with a right ventricular pressure as high as systemic blood pressure [31]. Moderate and large defects impose an increased pressure load upon the right ventricle. Some animals can be asymptomatic [32]; others can be symptomatic with dyspnea, exercise intolerance, fainting, and cough. In severe cases, dogs may show congestive heart failure [33]. Diagnostic imaging can be helpful for the diagnosis of a VSD. Large shunting VSDs can be surgically repaired under cardiopulmonary bypass, and moderate or large shunting VSDs may also undergo pulmonary artery banding as a palliative procedure. Alternatively, the heart is approached by a right thoracotomy through the fifth intercostal space. By a ventricular incision, the VSD is closed primarily by an interrupted mattress suture with 6-0 pledgeted polypropylene. Simple closure of the VSD can effectively relieve of the associated aortic regurgitation, especially in case of the highly-located VSD. Early VSD closure may restrain the progression of the associated aortic valve regurgitation and minimize the risk of bacterial endocarditis [34]. In a study, a dog underwent a VSD repair with continuous sutures under cardiopulmonary bypass via a median sternotomy approach [35]. It was illustrated that right atrial incision was superior to right ventricular incision for the surgical repair of VSD under cardiopulmonary bypass in dogs, in particular, with better outcomes during postoperative recovery because of a shorter recovery period [36]. Besides, a secondary Gerbode defect due to infective endocarditis of the aortic valve was once reported in a 6-year-old intact male Great Pyrenees dog. Color Doppler revealed turbulent flow originating from the left ventricular outflow tract entering into the right atrium and right ventricle. Due to the severity of lesions and poor condition, the owner elected humane euthanasia and consented to necropsy without performing an operation [37]. In a dog with concurrent pulmonic stenosis, an Amplatzer occluder was used to successfully close the muscular type VSD [38].

ENDOCARDIAL CUSHION DEFECT (COMPLETE FORM)

The endocardial cushion defect (complete form) is composed of 6 leaflets: 3 left leaflets (left superior, left lateral, and left inferior) and 3 right leaflets (right superior, right lateral, and right inferior). Rastelli et al. classified complete endocardial cushion defects into types A, B, and C based on the morphology of the left superior leaflet and chordal attachment. Type A occurs most frequently, where the left superior leaflet is located above the left ventricle and attached to the crest of the VSD. Type B is

rare, where the left superior leaflet chordae are attached to anomalous papillary muscles from the inlet VSD in the right ventricle. Type C is characterized by marked bridging of the inlet VSD to the right ventricle by the left superior leaflet [39]. The complete form progresses more frequently into pulmonary hypertension compared with other CHDs. In children with complete endocardial cushion defect, irreversible pulmonary vascular damage occurs after 6 months of age, and surgical repair should be performed at 3-6 months of age. In dogs, timing of surgical intervention can be referred to that of the human. Two techniques are commonly used in people for repair of complete endocardial cushion defect: one-patch and two-patch techniques. In comparison to one-patch technique, two-patch technique in dogs is more effective in improvements of tricuspid regurgitation by avoiding postoperative residual shunting, and reducing operative mortality, incidence of arrhythmia, and re-intervention rate. A dog with endocardial cushion defect survived free of cardiac symptoms for 6 years and 5 months after two-patch technique repair under cardiopulmonary bypass [40]. The modified "Australian" technique [41] and no patch technique [42] require shorter aortic crossclamp and cardiopulmonary bypass times. Both are novel techniques for the surgical repair of complete endocardial cushion defect in human with good clinical results.

Two-patch technique [43]: A Gore-Tex patch is used to close the ventricular component of the defect and the atrioventricular valves are suspended to the top of the Gore-Tex patch. Another pericardial patch is used to close the atrial component and is sutured to the confluence of the atrioventricular valve and the Gore-Tex VSD patch. The mitral valve is sandwiched between the ventricular Gore-Tex patch and the atrial pericardial patch so that mitral valve laceration and possible dehiscence can be avoided.

Traditional one-patch technique [41]: After testing the competence of the common atrioventricular valve, a single approximating suture is placed over the septal crest between the superior and inferior leaflets. A Dacron patch is sutured to the middle of the septal crest by 5-0 pledgeted polypropylene sutures. The atrioventricular valve leaflets are anchored onto the Dacron patch. The mitral valve cleft is closed by interrupted polypropylene sutures. The atrial component of the defect is closed by a continuous suture placed in the remaining part of the patch, leaving the coronary sinus draining into the right atrium.

"Australian" one-patch technique [41]: This technique is different from the traditional one-patch technique in details of the surgical maneuver. After testing the competence of the common atrioventricular valve, the valve cleft is repaired using polypropylene sutures. The ventricular component of the defect is closed using pledgeted polypropylene sutures on the right ventricular aspect of the septal crest. Sutures are placed below the septal crest to avoid conduction tissue damage. The sutures are passed through the superior and inferior bridging leaflets making a partition boundary between the atrial outlets, and then passing through the Dacron patch. When tying the sutures, the ventricular component of the defect is obliterated. The Dacron patch was taken to close the atrial component of the defect by continuous sutures, leaving the coronary sinus draining into the right atrium.

No patch technique [42]: This technique is mainly pledgeted interrupted sutures anchoring on the right side of the VSD crest, passing through the midportion of the bridging atrioventricular leaflet. The left atrioventricular valve cleft is closed using interrupted sutures, and the ASD is closed by passing the VSD-closing interrupted sutures through the superior rim of the ostium primum defect, obliterating the VSD and ASD components by pulling the atrioventricular valve leaflets and atrial septum down to the VSD crest. The defects are closed without the use of a patch. A sliding plasty of the right atrioventricular valve septal leaflet is performed to improve the competence of the valve. In children, no-patch technique for repair of complete endocardial cushion defect resulted in no early deaths. This technique can surely be applied in animals with the advantages of shortened operation time.

TETRALOGY OF FALLOT

The incidence of tetralogy of Fallot is rarer ranging from 0.6% to 7% [44]. Diagnostic imaging may show a dextro-positioned and over-riding aorta, pulmonary valvular stenosis, ventricular and atrial septal defects, and right ventricular hypertrophy [45]. Conservative treatment includes diuretics, ß-blockers, and angiotensin-converting-enzyme inhibitor for relieving clinical symptoms. Oxygen therapy, puncture, and removal of fluids from the pleural and abdominal cavities may be necessary for deteriorated cases [44]. Surgical operations are an effective treatment. A complete correction of tetralogy of Fallot can be performed using a transatrial approach with limited ventriculotomy under cardiopulmonary bypass. The surgical procedures consist of hypertrophied infundibulum resection, primary closure of the VSD and right ventricular outflow tract reconstruction with a transannular pericardial patch. Transpulmonic pressure gradients were remarkably reduced at a 4-month follow-up [46]. Successful surgical repair can completely relieve the clinical signs [47]. A "side-by-side" type or modified Blalock-Taussig shunt between the subclavian artery to the pulmonary artery may increase pulmonary flow by systemic-pulmonary anastomoses and obliterating the animal's symptoms [44]. Surgical techniques of modified Blalock-Taussig shunt in human and animals have been described [48]. The balloon catheter dilation of the pulmonic stenotic lesion is a palliative procedure for animals with tetralogy of Fallot that are intolerable to anesthesia and open heart surgery. The balloon catheter is introduced into the

stenotic lesion under fluoroscopy for rapid and repeated inflations of the pulmonic infundibular stenosis. Post-dilation hemodynamic and angiographic evaluations revealed relief of pulmonic infundibular stenosis and increased pulmonary blood flow with no complications secondary to thoracotomy or long-term hospitalization [49].

COR TRIATRIATUM

Cor triatriatum dexter is a rare CHD in dogs, in which the right atrium is devided into two parts by a fibromuscular structure [50]. Affected dogs show signs of congestive heart failure including increasing exercise intolerance [51], severe cyanosis, dyspnea [52], tachypnea [53], progressive ascites [51], hepatomegaly [6], and growth retardation [54]. However, the diagnosis of cor triatriatum dexter is often delayed until the animal presents signs of right heart failure or a cardiac murmur. Nowadays, echocardiography is the diagnostic method of choice that has facilitated the definite diagnosis of cor triatriatum dexter. Cardiac catheterization can be helpful for the definitive diagnosis and hemodynamic evaluation. Medical management with enalaprild, furosemidee, and digoxin may transiently relieve congestive heart failure. Surgical correction under cardiopulmonary bypass is a reliable method for the treatment of cor triatriatum dexter [55]. Surgical repair of cor triatriatum dexter by a right lateral thoracotomy through the fifth intercostal space has been described [55]. The partitioning membrane was excised using Potts scissors and the inflow tract was expanded by a right atriotomy approach. Many animals experience an uneventful post-operative recovery [54,55], but surgical operation was declined in some animals due to associated complex abnormalities [50]. Balloon dilation has been applied in dogs with cor triatriatum dexter, and lead to improved clinical signs [51]. However, the partitioning membrane is often too fibrous to be easily ruptured by a balloon. Cutting balloon dilation was accordingly developed and successful relief of clinical signs including ascites in dogs has been reported [56]. Several balloon dilation procedures may be required to achieve permanent reduction of the obstructive pressure gradient across the membrane and resolution of clinical symptoms [57]. Therefore, despite non-invasiveness and effectiveness in some cases, balloon dilation is not always suitable for dogs and seems to be less reliable than surgical correction under cardiopulmonary bypass [55].

EBSTEIN'S ANOMALY

Ebstein's anomaly is a rare CHD of dog. This anomaly may be associated with ASD. Dogs may present exercise intolerance, dyspnea, cardiac murmur, and even ascites [58]. Echocardiogragraphy is helpful in reaching a definite diagnosis by illustration of right atrial enlargement and displacement and insufficiency of the tricuspid valve [59]. Oral administration of digoxin, vasodilators and diuretics partially improves the clinical symptoms, but sometimes cardiovascular deterioration and even sudden death may occur [58,60]. Recent veterinary literature has not shown successful surgical treatment for Ebstein's anomaly. However, it is believed that veterinarians may draw on the experience of relevant surgical techniques applied in human. Dogs with Ebstein's anomaly have poor prognosis. A dog suddenly died shortly after the diagnosis of Ebstein's anomaly was made, and another dog recovered better at the beginning, however, sudden death occurred later. Postmortem examinations showed right atrioventricular enlargement with thickened tricuspid leaflets [59].

MITRAL VALVE DYSPLASIA

Mitral valve dysplasia is a congenital malformation of the mitral valve complex representing 8% of the CHDs in dogs [4]. Canine breeds predisposing to develop mitral valve dysplasia are Bull Terriers, German Shepherds, and Great Danes [61]. Mitral valve dysplasia results in mitral valve regurgitation. Any component of the mitral valve complex (valve leaflets, chordae tendineae, and papillary muscles) may be malformed, and often more than one component is affected [62]. In addition, mitral valve dysplasia can be present as a member of a trilogy of defects (ASD, mitral valve dysplasia, and subaortic stenosis), which was once reported in a 4-year-old male castrated English bulldog who manifested exercise intolerance, multiple episodes of syncope, and a grade IV/VI heart murmur [27]. Dogs with mitral valve dysplasia often manifest congestive heart failure and a systolic cardiac murmur at the apex. Echocardiography discloses anterior mitral leaflet cleft resulting in left ventricular outflow tract obstruction [63]. The cleft divides the anterior leaflet into two parts, both of which extend across the subvalvular left ventricular outflow tract, and attachs to the subaortic interventricular septum [63]. White et al.[64] reported a dysplastic mitral valve dog case presenting with a history of collapse on exercise. Surgical intervention by open resection of the dysplastic mitral valve replaced with a bioprosthetic valve through a median sternotomy under cardiopulmonary bypass. The dog had a full recovery postoperatively. Behl et al.[65] reported successful mitral valve replacement in a beating heart, with resolved mitral regurgitation and improved cardiac performances.

TRICUSPID DYSPLASIA

Tricuspid dysplasia results in mostly tricuspid insufficiency and occasional tricuspid stenosis, which accounts for approximately 7% of all CHDs in dogs [4]. Tricuspid valve dysplasia has been reported in numerous dog breeds including old English sheepdogs, great Danes, German shepherds, and Irish setters [66]. Loud systolic heart murmurs can be a predomiant clinical manifestation [67]. Elongated and redundant tricuspid valve leaflets, thick,

shortened or even absent chordae tendineae, and severely dilated and malpositioned right atrium are common [68]. Associations with mitral valve dysplasia, septal defects, subaortic stenosis, pulmonic stenosis, situs inversus totalis [30] or Ebstein's anomaly [56] are rare, but can be present. Echocardiography demonstrates tricuspid valve malformations with severe dilations of the right atrium and ventricle, and Doppler echocardiography demonstrates severe tricuspid regurgitation [66]. Periodic thoracocentesis and/or abdominocentesis may be necessary [66]. Diuretics, vasodilators, digoxin, and an angiotensin converting enzyme inhibitor may also be indicated. Arai et al.[69] reported good intermediate-term outcomes in dogs with tricuspid vavle dysplagia undergoing tricuspid valve replacement with a bioprosthesis under cardiopulmonary bypass. Ten of the 12 (83.3%) dogs survived surgery and were discharged. Prosthesis-related complications include inflammatory pannus, thrombosis and endocarditis. Prognosis of tricuspid dysplasia can be poor if with associated cardiac abnormalities, and sometimes sudden death of the animals can occur [70].

COMMENTS

There are incidence and predilection discrepancies of CHDs between humans and animals. For example, tetralogy of Fallot occurs in 3/10.000 live births [71], which is much lower than the animal [44]. Animals with CHDs are likely to die prematurely. Valve dysplasias and large septal defects have a poor prognosis regardless of the method of treatment; whereas in humans, congenital valve dysplasia is not as predominant as in the animals, and the clinical outcome would be better. Therefore, affected dogs are often at a risk of congestive heart failure and sudden death. The diagnosis can usually be supported by echocardiography in addition to thoracic radiographic findings, etc. Early accurate diagnosis with further proper treatments may ensure the best outcomes. Interventional and/or surgical techniques that have been applied in humans are of considerable benefits to the animals with CHDs. Open heart surgery may be necessary to repair the defect, but it costs much. For CHDs, such as secundum-type ASDs, pulmonic stenosis, subaortic stenosis, and cor triatriatum dexter, etc., minimally invasive or hybrid procedures appear to be viable treatment options [70]. Early diagnosis also allows owners to avoid continuing genetic defects in breeding lines. Attentions to the dog breeds with a known predisposition of an inheritable heart disease would merit a long-run veterinary significance.

CONCLUSION

The occurrence of CHD in dogs shows breed and gender predilections. Definitive diagnosis of CHDs in dogs can be made by radiography, echocardiography and cardiac catheterization. CHDs in dogs may be curable by interventional or surgical treatment. Innovative surgical techniques especially minimally invasive procedures applied in humans may largely benefit the animals with CHDs.

REFERENCES

1. Cardiology Service: Congenital heart disease in dogs. http://www.michvet.com/Client%20Education%20Handouts/Cardio/Congenital%20Heart%20Disease.pdf

2. Patterson DF: Epidemiologic and genetic studies of congenital heart disease in the dog. *Circ Res*, 23 (2): 171-202, 1968.

3. Oliveira P, Domenech O, Silva J, Vannini S, Bussadori R, Bussadori C: Retrospective review of congenital heart disease in 976 dogs. *J Vet Intern Med*, 25 (3): 477-483, 2011.

4. Tidholm A: Retrospective study of congenital heart defects in 151 dogs. *J Small Anim Pract*, 38 (3): 94-98, 1997.

5. Stafford-Johnson M: Companion animal practice: Decision making in suspected congenital heart disease in dogs and cats. *In Practice*, 28 (9): 538-543, 2006.

6. MacDonald KA: Congenital heart diseases of puppies and kittens. *Vet Clin Small Anim*, 36 (3): 503-531, 2006.

7. Van Israël N: Congenital heart diseases: How to recognize them? http://www.acapulco-vet.be/service.php?module=library&action=get_file&target=96&options=%7Clng:fr

8. Bellumori TP, Famula TR, Bannasch DL, Belanger JM, Oberbauer AM: Prevalence of inherited disorders among mixed-breed and purebred dogs: 27.254 cases (1995-2010). *J Am Vet Med Assoc*, 242 (11): 1549-1555, 2013.

9. Schneider M, Hildebrandt N, Schweigl T, Wehner M: Transthoracic echocardiographic measurement of patent ductus arteriosus in dogs. *J Vet Intern Med*, 21 (2): 251-257, 2007.

10. Van Israel N, French AT, Dukes-McEwan J, Corcoran BM: Review of left-to-right shunting patent ductus arteriosus and short term outcome in 98 dogs. *J Small Anim Pract*, 43 (9): 395-400, 2002.

11. Birchard SJ, Bonagura JD, Fingland RB: Results of ligation of patent ductus arteriosus: 201 cases (1969-1988). *J Am Vet Med Assoc*, 196 (12): 2011-2031, 1990.

12. White P: Treatment of patent ductus arteriosus by the use of an Amplatz canine ductal occluder device. *Can Vet J*, 50 (4): 401-404, 2009.

13. Tanaka R, Hoshi K, Nagashima Y, Fujii Y, Yamane Y: Detachable coils for occlusion of patent ductus arteriosus in 2 dogs. *Vet Surg*, 30 (6): 580-584, 2001.

14. Smith PJ, Martin MW: Transcatheter embolisation of patent ductus arteriosus using an Amplatzer vascular plug in six dogs. *J Small Anim Pract*, 48 (2): 80-86, 2007.

15. O'Sullivan L: Minimally invasive interventional cardiology: Diagnosis and transcatheter correction of patent ductus arteriosus in dogs. *Small Anim Vet Rounds*, 1 (4): 2012. http://savrounds.ca/crus/147-004%20English.pdf

16. Tanaka R, Nagashima Y, Hoshi K, Yamane Y: Supplemental embolization coil implantation for closure of patent ductus arteriosus in a beagle dog. *J Vet Med Sci*, 63 (5): 557-559, 2001.

17. Singh MK, Kittleson MD, Kass PH, Griffiths LG: Occlusion devices and approaches in canine patent ductus arteriosus: Comparison of outcomes. *J Vet Intern Med*, 26 (1): 85-92, 2012.

18. Saunders AB, Miller MW, Gordon SG, Bahr A: Pulmonary embolization of vascular occlusion coils in dogs with patent ductus arteriosus. *J Vet Intern Med*, 18 (5): 663-666, 2004.

19. Nguyenba TP, Tobias AH: The Amplatz canine duct occluder: A novel device for patent ductus arteriosus occlusion. *J Vet Cardiol*, 9 (2): 109-117, 2007.

20. Reist-Marti SB, Dolf G, Leeb T, Kottmann S, Kietzmann S, Butenhoff K, Rieder S: Genetic evidence of subaortic stenosis in the Newfoundland dog. *Vet Rec*, 170 (23): 597, 2012.

21. Kienle RD, Thomas WP, Pion PD: The natural clinical history of canine congenital subaortic stenosis. *J Vet Intern Med*, 8 (6): 423-431, 1994.

22. Stern JA, Meurs KM, Nelson OL, Lahmers SM, Lehmkuhl LB: Familial subvalvular aortic stenosis in golden retrievers: Inheritance and echocardiographic findings. *J Small Anim Pract*, 53 (4): 213-216, 2012.

23. Kleman ME, Estrada AH, Maisenbacher HW 3rd, Prošek R, Pogue B, Shih A, Paolillo JA: How to perform combined cutting balloon and high pressure balloon valvuloplasty for dogs with subaortic stenosis. *J Vet Cardiol*, 14 (2): 351-361, 2012.

24. Minors SL, O'Grady MR, Williams RM, O'Sullivan ML: Clinical and echocardiographic features of primary infundibular stenosis with intact ventricular septum in dogs. *J Vet Intern Med*, 20 (6): 1344-1350, 2006.

25. Sisson DD, MacCoy DM: Treatment of congenital pulmonic stenosis in two dogs by balloon valvuloplasty. *J Vet Intern Med*, 2 (2): 92-99, 1988.

26. Estrada A, Moïse NS, Erb HN, McDonough SP, Renaud-Farrell S: Prospective evaluation of the balloon-to-annulus ratio for valvuloplasty in the treatment of pulmonic stenosis in the dog. *J Vet Intern Med*, 20 (4): 862-872, 2006.

27. Chetboul V, Charles V, Nicolle A, Sampedrano CC, Gouni V, Pouchelon JL, Tissier R: Retrospective study of 156 atrial septal defects in dogs and cats (2001-2005). *J Vet Med A Physiol Pathol Clin Med*, 53 (4): 179-184, 2006.

28. Sanders RA, Hogan DE, Green HW 3rd, Hoyer MH, Puppel DA: Transcatheter closure of an atrial septal defect in a dog. *J Am Vet Med Assoc*, 227 (3): 430-434, 2005.

29. Gordon SG, Miller MW, Roland RM, Saunders AB, Achen SE, Drourr LT, Nelson DA: Transcatheter atrial septal defect closure with the Amplatzer atrial septal occluder in 13 dogs: Short- and mid-term outcome. *J Vet Intern Med*, 23 (5): 995-1002, 2009.

30. Piantedosi D, Cortese L, Meomartino L, Di Loria A, Ciaramella P: Situs inversus totalis associated with subaortic stenosis, restrictive ventricular septal defect, and tricuspid dysplasia in an adult dog. *Can Vet J*, 52 (11): 1237-1242, 2011.

31. No author listed: Ventricular septal defect (VSD) in dogs and cats. http://www.vetnext.com/search.php?s=aandoening&id=73055143150%2076.

32. Fujii Y, Fukuda T, Machida N, Yamane T, Wakao Y: Transcatheter closure of congenital ventricular septal defects in 3 dogs with a detachable coil. *J Vet Intern Med,* 18 (6): 911-914, 2004.

33. Eyster GE, Whipple RD, Anderson LK, Evans AT, O'Handley P: Pulmonary artery banding for ventricular septal defect in dogs and cats. *J Am Vet Med Assoc*, 170 (4): 434-438, 1977.

34. Shimizu M, Tanaka R, Hoshi K, Hirao H, Kobayashi M, Shimamura S, Yamane Y: Surgical correction of ventricular septal defect with aortic regurgitation in a dog. *Aust Vet J*, 84 (4): 117-121, 2006.

35. Hunt GB, Pearson MR, Bellenger CR, Malik R: Ventricular septal defect repair in a small dog using cross-circulation. *Aust Vet J,* 72 (10): 379-382, 1995.

36. Shimamura S, Kutsuna H, Shimizu M, Kobayashi M, Hirao H, Tanaka R, Takashima K, Machida N, Yamane Y: Comparison of right atrium incision and right ventricular outflow incision for surgical repair of membranous ventricular septal defect using cardiopulmonary bypass in dogs. *Vet Surg*, 35 (4): 382-387, 2006.

37. Peddle GD, Boger L, Van Winkle TJ, Oyama MA: Gerbode type defect and third degree atrioventricular block in association with bacterial endocarditis in a dog. *J Vet Cardiol*, 10 (2): 133-139, 2008.

38. Margiocco ML, Bulmer BJ, Sisson DD: Percutaneous occlusion of a muscular ventricular septal defect with an Amplatzer muscular VSD occluder. *J Vet Cardiol*, 10 (1): 61-66, 2008.

39. Pettersen MD, Neish SR: Pediatric complete atrioventricular septal defects. http://emedicine.medscape.com/article/893914-overview.

40. Yamano S, Uechi M, Tanaka K, Hori Y, Ebisawa T, Harada K, Mizukoshi T: Surgical repair of a complete endocardial cushion defect in a dog. *Vet Surg*, 40 (4): 408-412, 2011.

41. Halit V, Oktar GL, Imren VY, Iriz E, Erer D, Kula S, Tunaoglu FS, Gokgoz L, Olgunturk R: Traditional single patch versus the "Australian" technique for repair of complete atrioventricular canal defects. *Surg Today*, 38 (11): 999-1003, 2008.

42. Myers PO, Cikirikcioglu M, Aggoun Y, Murith N, Kalangos A: No-patch technique for complete atrioventricular canal repair. *Ann Thorac Surg*, 90 (1): 317-319, 2010.

43. Backer CL, Mavroudis C, Alboliras ET, Zales VR: Repair of complete atrioventricular canal defects: Results with the two-patch technique. *Ann Thorac Surg*, 60 (3): 530-537, 1995.

44. Pasławska U, Noszczyk-Nowak A, Cepiel A, Staszczyk M, Janiszewski A: A retrospective study of tetralogy of Fallot in dogs. *Turk J Vet Anim Sci*, 37: 57-60, 2013.

45. Park IC, Lee HS, Kim JT, Lee JS, Lee SG, Hyun C: Pentalogy of Fallot in a Korean Sapsaree dog. *J Vet Med Sci*, 69 (1): 73-76, 2007.

46. Lew LJ, Fowler JD, McKay R, Egger CM, Rosin MW: Open-heart correction of tetralogy of Fallot in an acyanotic dog. *J Am Vet Med Assoc*, 213 (5): 652-657, 1998.

47. Orton EC, Mama K, Hellyer P, Hackett TB: Open surgical repair of tetralogy of Fallot in dogs. *J Am Vet Med Assoc*, 219 (8): 1089-1093,1073, 2001.

48. Yuan SM, Shinfeld A, Raanani E: The Blalock-Taussig shunt. *J Card Surg,* 24 (2): 101-108, 2009.

49. Oguchi Y, Matsumoto H, Masuda Y, Takashima H, Takashima K, Yamane Y: Balloon dilation of right ventricular outflow tract in a dog with tetralogy of Fallot. *J Vet Med Sci*, 61 (9): 1067-1069, 1999.

50. Tobias AH, Thomas WP, Kittleson MD, Komtebedde J: Cor triatriatum dexter in two dogs. *J Am Vet Med Assoc*, 202 (2): 285-290, 1993.

51. Johnson MS, Martin M, De Giovanni JV, Boswood A, Swift S: Management of cor triatriatum dexter by balloon dilatation in three dogs. *J Small Anim Pract*, 45 (1): 16-20, 2004.

52. Chan IP, Lin CC, LinCC, Lin SL: Case report: Cor triatriatum dexter, tricuspid dysplasia and bicuspid pulmonary valve in a miniature pinscher puppy. *Taiwan Vet J*, 39 (4): 267-273, 2013.

53. Nieuwland J, Beijerink N, Szatmári V: Resolution of ascites after successful balloon dilatation of cortriatriatum dexter in a dog. *Tijdschr Diergeneeskd*, 135 (8): 330-333, 2010.

54. Mitten RW, Edwards GA, Rishniw M: Diagnosis and management of cor triatriatum dexter in a Pyrenean mountain dog and an Akita Inu. *Aust Vet J*, 79 (3): 177-180, 2001.

55. Tanaka R, Hoshi K, Shimizu M, Hirao H, Akiyama M, Kobayashi M, Machida N, Maruo K, Yamane Y: Surgical correction of cor triatriatum dexter in a dog under extracorporeal circulation. *J Small Anim Pract*, 44 (8): 370-373, 2003.

56. Leblanc N, Defrancesco TC, Adams AK, Atkins CE, Tou SP, Fudge JC, Keene BW: Cutting balloon catheterization for interventional treatment of cor triatriatum dexter: 2 cases. *J Vet Cardiol*, 14 (4): 525-530, 2012.

57. Adin DB, Thomas WP: Balloon dilation of cor triatriatum dexter in a dog. *J Vet Intern Med*, 13 (6): 617-619, 1999.

58. Takemura N, Machida N, Nakagawa K, Amasaki H, Washizu M, Hirose H: Ebstein's anomaly in a beagle dog. *J Vet Med Sci*, 65 (4): 531-533, 2003.

59. Sousa MG, Gerardi DG, Alves RO, Camacho AA: Tricuspid valve dysplasia and Ebstein's anomaly in dogs: Case report. *Arq Bras Med Vet Zootec*, 58 (5): 762-767, 2006.

60. Choi R, Lee SK, Moon HS, Park IC, Hyun C: Ebstein's anomaly with an atrial septal defect in a jindo dog. *Can Vet J*, 50 (4): 405-410, 2009.

61. Pikula J, Pikulova J, Bandouchova H, Kohout P, Najman K, Tichy F, Treml F: Subaortic stenosis and mitral dysplasia in three Black Russian Terrier puppies. *Vet Med - Czech*, 50 (7): 321-326, 2005.

62. Strickland KN: Chapter 12. Congenital Heart Disease. **In,** Tilley LP, Smith FWK Jr, Oyama M, Sleeper MM (Eds): Manual of Canine and Feline Cardiology. 4th ed., Saunders, St. Louis, Missouri, USA, 2008.

63. Otoni C, Abbott JA: Mitral valve dysplasia characterized by isolated cleft of the anterior leaflet resulting in fixed left ventricular outflow tract

obstruction. *J Vet Cardiol*, 14 (1): 301-305, 2012.

64. White RN, Stepien RL, Hammond RA, Holden DJ, Torrington AM, Milner HR, Cobb MA, Hellens SH: Mitral valve replacement for the treatment of congenital mitral dysplasia in a bull terrier. *J Small Anim Pract*, 36 (9): 407-410, 1995.

65. Behr L, Chetboul V, Sampedrano CC, Vassiliki G, Pouchelon JL, Laborde F, Borenstein N: Beating heart mitral valve replacement with a bovine pericardial bioprosthesis for treatment of mitral valve dysplasia in a Bull Terrier. *Vet Surg*, 36 (3): 190-198, 2007.

66. Kittleson MD: Case 23: Tricuspid Valve Dysplasia Section from "Small Animal Cardiovascular Medicine" On-Line. Case Studies In Small Animal Cardiovascular Medicine. http://www.vetmed.ucdavis.edu/vmth/small_animal/cardio_kittleson/cases/case23/text.htm

67. Hoffmann G, Amberger CN, Seiler G, Lombard CW: Tricuspid valve dysplasia in fifteen dogs. *Schweiz Arch Tierheilkd*, 142 (5): 268-277, 2000.

68. Ohad DG, Avrahami A, Waner T, David L: The occurrence and suspected mode of inheritance of congenital subaortic stenosis and tricuspid valve dysplasia in Dogue de Bordeaux dogs. *Vet J*, 197 (2): 351-357, 2013.

69. Arai S, Griffiths LG, Mama K, Hackett TB, Monnet E, Boon JA, Carter L, Orton EC: Bioprosthesis valve replacement in dogs with congenital tricuspid valve dysplasia: Technique and outcome. *J Vet Cardiol*, 13 (2): 91-99, 2011.

70. Stern JA, Tou SP, Barker PC, Hill KD, Lodge AJ, Mathews KG, Keene BW: Hybrid cutting balloon dilatation for treatment of cor triatriatum sinister in a cat. *J Vet Cardiol*, 15 (3): 205-210, 2013.

71. Bailliard F, Anderson RH: Tetralogy of Fallot. *Orphanet J Rare Dis*, 13, 2, 2009. DOI: 10.1186/1750-1172-4-2

YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup, derleme ve çeviri türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.
2- Dergide yayımlanması istenen yazılar Times New Roman yazı tipi ve 12 punto ile A4 formatında, 1.5 satır aralıklı ve sayfa kenar boşlukları 2.5 cm olacak şekilde hazırlanmalı ve şekil ve tablo gibi görsel öğelerin metin içindeki yerlerine Türkçe ve yabancı dilde adları ve gerekli açıklamaları mutlaka yazılmalıdır.
Dergiye gönderilecek makale ve ekleri (şekil vs) *http://vetdergi.kafkas.edu.tr* adresindeki online makale gönderme sistemi kullanılarak yapılmalıdır.
Başvuru sırasında yazarlar yazıda yer alacak şekilleri online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış *Telif Hakkı Devir Sözleşmesi* editörlüğe gönderilmelidir.
3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.
4- ***Makale Türleri***
Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır. Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 12 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.
Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.
Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.
Gözlem (Olgu Sunumu), uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri Giriş, Olgunun Tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.
Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 2 sayfayı geçmemelidir.
Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri Giriş, Metin, Sonuç ve Kaynaklar bölümlerinden oluşmalı ve 12 sayfayı geçmemelidir.
Çeviri, makalenin orijinal formatı dikkate alınarak hazırlanmalıdır.
Yazarla ilgili kişisel ve kuruma ait bilgiler ana metin dosyasına değil, on-line başvuru sırasında sistemdeki ilgili yerlere unvan belirtilmeksizin eklenmelidir.
5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.
6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır. Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.
Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.
Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.
Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.
Örnek: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.
DOI numarası bulunan kaynaklarda bu bilgi ilgili kaynak künyesinin sonuna eklenmelidir.
Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi, kaynak bilgilerinin sonuna eklenmelidir.
Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.
Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.
7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.
8- Editörlük, dergiye gönderilen yazılar üzerinde gerekli görülen kısaltma ve düzeltmeleri yapabileceği gibi önerilerini yazarlara iletebilir. Yazarlar, düzeltilmek üzere yollanan yazıları online sistemde belirtilen sürede gerekli düzeltmeleri yaparak editörlüğe iade etmelidirler. Editörlükçe ön incelemesi yapılan ve değerlendirmeye alınması uygun görülen makaleler ilgili bilim dalından bir yayın danışmanı ve iki raportörün olumlu görüşü alındığı takdirde yayımlanır.
9- Yayınlanan yazılardan dolayı doğabilecek her türlü sorumluluk yazarlara aittir.
10- Yazarlara telif ücreti ödenmez.
11- Resim ve baskı masrafları için yazarlardan ücret alınır. Ücret bilgileri *http://vetdergi.kafkas.edu.tr/* adresinden öğrenilebilir.
12- Yazarlara 50 adet ayrı baskı ücretsiz olarak yollanır.

ABONE KOŞULLARI

Yıllık: 60 TL
Banka Hesap No: T.C. Ziraat Bankası Kars Şubesi, 26146825-5001 Nolu Hesap, Kars - TÜRKİYE
IBAN: TR830001000147261468255001

INSTRUCTIONS FOR AUTHORS

1- The Journal of the Faculty of Veterinary Medicine, University of Kafkas (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly, covers original papers, short communication and preliminary scientific reports, case reports, observation, letter to the editor, review and translation on all aspects of veterinary medicine and animal science (clinical and paraclinical sciences, biological and basic sciences, zoonoses and public health, animal feeding and nutritional diseases, animal breeding and genetics, hygiene and technology of food from animal sources, exotic animal science). Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table must clearly be written in both Turkish and foreign language and their appropriate position should be indicated in the text.

The manuscript and its supplementary (figure etc.) should be submitted by using online manuscript submission system at the address of *http://vetdergi.kafkas.edu.tr/*

During the submission, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the copyright transfer agreement form signed by all the authors should be send to the editorial office.

3- Authors should indicate the name of institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, editorial board may also request the official document of the ethical commission report.

4- Original (full-length) Manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments. Manuscripts written in Turkish consist of the title in Turkish, summary and keywords in Turkish, Introduction, Material and Methods, Results, Discussion, and References and it should not exceed 12 pages including text, tables and illustrations. Manuscripts written in a foreign language should follow the title in foreign language, summary and keywords in foreign language, title in Turkish, summary and keywords in Turkish and the remaining sections described above for the manuscripts written in Turkish.

Summaries written in Turkish or foreign language should contain 200±20 words.

Short Communication Manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but each of the
summaries should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages. Additionally, they should not contain more than 4 figures or tables.

Preliminary Scientific Reports are short description (maximum 4 pages) of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles.

Case Reports describe rare significant findings encountered in the application, clinic and laboratory of related fields. The title and summary of these articles should be written in the format of full-length original articles and the remaining sections should follow Introduction, Case History, Discussion and References without exceeding the total of 4 pages.

Letters to the Editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases without exceeding 2 pages.

Reviews are original manuscripts gather the literature on current and significant subject along with the commentary and findings of the author on the particular subject. The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should follow Introduction, Text, Conclusion, and References without exceeding 12 page.

Translations should be prepared based on the format of original document being translated.

The information about author/s and institution/s should be added during the online submissoin and the main documant should be free of these information.

5- The necessary descriptive information (thesis, projects, financial supports etc) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of title.

6- References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must be complied with the other references.

Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

7- The Latin expression such as species names of bacterium, virus, parasite and fungus and anatomical terms must be written in italic character keeping their original forms.

8- The editorial board has the right to perform necessary modifications and reduction on the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by editorial board, the article can only be published after the approval of the publication advisor and two referees specialized in the particular field.

9- All responsibilities from published articles merely belong to the authors.

10- There is no copyright fee for the authors.

11- A fee is charged from the authors to cover printing cost and other expenses. This payment information can be found at *http://vetdergi.kafkas.edu.tr/*

12- Reprints (in multiples of 50) of the article are sent to the authors for free.

SUBSCRIPTION

Yearly: $40

Account: T.C. Ziraat Bank, Kars Branch, Account No: 26146825-500

IBAN: TR830001000147261468255004

YAZAR İNDEKSİ için tıklayınız