ISSN 1300 - 6045
e-ISSN 1309 - 2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University

Published Bi-monthly

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

Volume: 25 | Issue: 3 MAY - JUNE | Year: 2019

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

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

JOURNAL OF THE FACULTY OF VETERINARY MEDICINE, KAFKAS UNIVERSITY

(MAY - JUNE)

Volume: 25 Number: 3 Year: 2019

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:

- **Academic Search Premier**
- **CAB Abstracts**
- **DOAJ**
- **EBSCO**
- **Elsevier - Scopus**
- **EMBASE**
- **Thomson Reuters - Zoological Record**
- **TÜBİTAK - ULAKBİM Yaşam Bilimleri Veri Tabanı**
- **Türkiye Atıf Dizini**
- **Veterinary Science Database**

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

ELEKTRONİK BASKI (ELECTRONIC EDITION)
http://vetdergikafkas.org

ONLINE MAKALE GÖNDERME (ONLINE SUBMISSION)
http://submit.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
BOSNAHERSEK CAD. ALTUNALEM YAPI KOOP. ZEMİN KAT - ERZURUM
Tel: +90 442 2334667 E-mail: eserofset25@hotmail.com

Bu Sayının Hakem Listesi *(alfabetik sıra)*
The Referees List of This Issue *(in alphabetical order)*

AKAL Eser	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
AKBAŞ Aykut Asım	Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
AKYOL Çetin Volkan	Uludağ Üniversitesi Veteriner Fakültesi
ALBAY Metin Koray	Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
ALTUNBAŞ Korhan	Afyon Kocatepe Üniversitesi Veteriner Fakültesi
ARI Umut Çağın	Kafkas Üniversitesi Veteriner Fakültesi
ARSLAN Mükremin Özkan	Kafkas Üniversitesi Tıp Fakültesi
BAKİ ACAR Duygu	Afyon Kocatepe Üniversitesi Veteriner Fakültesi
BALYEN Lokman	Kafkas Üniversitesi Tıp Fakültesi
BEŞOLUK Kamil	Selçuk Üniversitesi Veteriner Fakültesi
ÇAKIR İbrahim	Bolu Abant İzzet Baysal Üniversitesi Mühendislik Mimarlık Fakültesi
ÇELEBİ Özgür	Kafkas Üniversitesi Veteriner Fakültesi
ÇIRAK Veli Yılgör	Uludağ Üniversitesi Veteriner Fakültesi
ÇİÇEK Hasan	Afyon Kocatepe Üniversitesi Veteriner Fakültesi
ÇİFTCİ Alper	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
DOKUZEYLÜL Banu	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
ELİTOK Bülent	Afyon Kocatepe Üniversitesi Veteriner Fakültesi
ERDEN Hasan	Adnan Menderes Üniversitesi Veteriner Fakültesi
EREN Hasan	Adnan Menderes Üniversitesi Veteriner Fakültesi
ERDOĞAN Güneş	Adnan Menderes Üniversitesi Veteriner Fakültesi
FİLYA İsmail	Uludağ Üniversitesi Ziraat Fakültesi
GEZER İNCE Nazan	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
GÖNÜLALAN Zafer	Erciyes Üniversitesi Veteriner Fakültesi
GÜL SANCAK İrem	Ankara Üniversitesi Veteriner Fakültesi
GÜLMEZ SAĞLAM Aliye	Kafkas Üniversitesi Veteriner Fakültesi
GÜLŞEN Nurettin	Selçuk Üniversitesi Veteriner Fakültesi
GÜRBÜZ Yavuz	Kahramanmaraş Sütçü İmam Üniversitesi Ziraat Fakültesi
GÜRSOY Oğuz	Mehmet Akif Ersoy Üniversitesi Mühendislik Mimarlık Fakültesi
HAKKI Sema S.	Selçuk Üniversitesi Diş Hekimliği Fakültesi
İKİZ Serkan	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
İNAL Fatma	Selçuk Üniversitesi Veteriner Fakültesi
KAR Sırrı	Namık Kemal Üniversitesi Fen Edebiyat Fakültesi
KARA Erkut	Adnan Menderes Üniversitesi Veteriner Fakültesi
KARABAĞ Kemal	Akdeniz Üniversitesi Ziraat Fakültesi
KAYA Duygu	Kafkas Üniversitesi Veteriner Fakültesi
KAYA Mükerrem	Atatürk Üniversitesi Ziraat Fakültesi
KAYAR Abdullah	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
KILIÇ Servet	Namık Kemal Üniversitesi Veteriner Fakültesi
KIRKPINAR Figen	Ege Üniversitesi Ziraat Fakültesi
KULAKSIZ Recai	Balıkesir Üniversitesi Veteriner Fakültesi
KUM Şadiye	Adnan Menderes Üniversitesi Veteriner Fakültesi
KÜÇÜKÇETİN Ahmet	Akdeniz Üniversitesi Mühendislik Fakültesi
KÜPLÜLÜ Şükrü	Ankara Üniversitesi Veteriner Fakültesi
LAÇİN Ekrem	Atatürk Üniversitesi Veteriner Fakültesi
MERAL Yücel	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
MERHAN Oğuz	Kafkas Üniversitesi Veteriner Fakültesi
MUTLU Zihni	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
OĞRAK Yusuf Ziya	Cumhuriyet Üniversitesi Veteriner Fakültesi
OKUMUŞ Zafer	Atatürk Üniversitesi Veteriner Fakültesi
ÖGE Semih	Ankara Üniversitesi Veteriner Fakültesi

Bu Sayının Hakem Listesi *(alfabetik sıra)*
The Referees List of This Issue *(in alphabetical order)*

ÖNOL Ahmet Gökhan	Adnan Menderes Üniversitesi Veteriner Fakültesi
ÖZDEMİR Derviş	Atatürk Üniversitesi Veteriner Fakültesi
ÖZDEMİR Vural	Afyon Kocatepe Üniversitesi Veteriner Fakültesi
ÖZER Kütşat	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
ÖZFİLİZ Nesrin	Uludağ Üniversitesi Veteriner Fakültesi
ÖZTÜRKLER Yavuz	Kafkas Üniversitesi Veteriner Fakültesi
PABUCCUOĞLU Serhat	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
PEKMEZCİ Didem	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
SANDIKÇI Mustafa	Adnan Menderes Üniversitesi Veteriner Fakültesi
SARI Barış	Kafkas Üniversitesi Veteriner Fakültesi
SAVAŞ Sarıözkan	Erciyes Üniversitesi Veteriner Fakültesi
SİPAHİ Cevat	Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
TAŞDEMİR Umut	Aksaray Üniversitesi Veteriner Fakültesi
TEKELİ Ahmet	Osmangazi Üniversitesi Ziraat Fakültesi
TEKİN Mehmet Emin	Selçuk Üniversitesi Veteriner Fakültesi
TİMURKAAN Sema	Fırat Üniversitesi Veteriner Fakültesi
UMUR Şinasi	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
UZLU Erdoğan	Kafkas Üniversitesi Veteriner Fakültesi
ÜSTÜN Yakup	Erciyes Üniversitesi Diş Hekimliği Fakültesi
YAMAN Mehmet	Mustafa Kemal Üniversitesi Veteriner Fakültesi
YILDIZ Kader	Kırıkkale Üniversitesi Veteriner Fakültesi
YILMAZ Alper	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi

İÇİNDEKİLER (Contents)

Kafkas Univ Vet Fak Derg
25 (3): 283-290, 2019
DOI: 10.9775/kvfd.2018.20471

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Estimation of the Economic Losses Related to Calf Mortalities Kars Province, in Turkey

Pınar AYVAZOĞLU DEMİR [1,a] Erol AYDIN [1,b] Cemalettin AYVAZOĞLU [2,c]

[1] Department of Livestock Economics, Faculty of Veterinary Medicine, Kafkas University, TR-36100 Kars - TURKEY
[2] Göle Vocational High School, Ardahan University, TR-75700 Ardahan - TURKEY
[a] ORCID: 0000-0002-7010-0475; [b] ORCID: 0000-0001-8427-5658; [c] ORCID: 0000-0003-2064-0657

Article ID: KVFD-2018-20471 Received: 04.07.2018 Accepted: 22.02.2019 Published Online: 24.02.2019

How to Cite This Article

Ayvazoğlu Demir P, Aydın E, Ayvazoğlu C: Estimation of the economic losses related to calf mortalities Kars province, in Turkey. *Kafkas Univ Vet Fak Derg,* 25 (3): 283-290, 2019. DOI: 10.9775/kvfd.2018.20471

Abstract

In this study, calf in Turkey live cattle stock in an important position in terms of the Kars dairy cattle-feeding operation, maintenance practices and differences in the level of knowledge and is intended to determine the economic losses due to calf mortalities. The material of the study was constituted by the data obtained from the interviews conducted with 108 dairy cattle business owners in the central villages of Kars. In the interviews, data about 0-180 days old patients and deceased calves were collected from livestock enterprise owners in 2016-2017. In this study, economic losses due to calf mortality were determined by taking into account the calculation methods in the literatüre. In the study, it was determined that 281 (24.65%) of 1140 calves had various diseases in 2017 and 63 (5.52%) of them died. It was calculated that an average of 156.32 TRY ($43.95) was spent per animal and the economic loss due to calves that died was estimated as 4.597 TRY ($1.293). As a result, it has been shown that training studies aiming to increase producer knowledge levels in minimizing calf diseases and deaths are important.

Keywords: *Calf mortality, Economic loss, Treatment and medicine expenses*

Kars İlinde Buzağı Ölümlerine İlişkin Meydana Gelen Ekonomik Kayıpların Tahmini

Öz

Bu araştırmada Türkiye'de canlı sığır stoku açısından önemli bir konumda olan Kars ili süt sığırcılık işletmelerindeki buzağı bakım-besleme uygulamaları ve bilgi düzeyindeki farklılıklar ile buzağı kaybına bağlı ekonomik kayıpların tespit edilmesi amaçlanmıştır. Araştırmanın materyalini Kars merkez köylerinde bulunan toplam 108 adet süt sığırcılık işletme sahiplerinden elde edilen veriler oluşturmuştur. Yapılan görüşmelerde 2016-2017 yıllarında işletme sahiplerinden 0-180 günlük yaştaki hasta ve ölen buzağılara ilişkin veriler toplanmıştır. Çalışmada buzağı ölümlerine bağlı ekonomik kayıplar literatürde yer alan hesaplama yöntemleri dikkate alınarak tahmin edilmiştir. Yapılan çalışmada 2017 yılında 1140 buzağıdan 281 (%24.65) tanesinin hastalandığı, hastalananlardan 63 (%5.52) tanesinin ise öldüğü belirlenmiştir. Yapılan çalışmada 2017 yılında cari fiyatlar üzerinden işletmelerin 281 hasta buzağı için tedavi, ilaç ve bakım masrafı olarak toplam 11.346 TL ($3.190); hayvan başına ortalama 156.32 TL ($43.95) harcama yapıldığı ve ölen bir buzağının ekonomik kaybının tahmini olarak ortalama 4.597 TL ($1.293) olduğu hesaplanmıştır. Sonuç olarak buzağı hastalıkları ve ölümlerinin en aza indirilmesinde üretici bilgi düzeylerinin artırılmasına yönelik eğitim çalışmaları önem arz etmektedir.

Anahtar sözcükler: *Buzağı ölümleri, Ekonomik kayıp, Tedavi ve ilaç masrafları*

INTRODUCTION

Breeding calves for meat and dairy production, which also ensures the continuation of the herd, has economic values for enterprises. The survival of each newborn calf, the income from the calf sale, the increase in the milk yield of cows and the growth of the herd; these three reasons are important for dairy farms [1]. In dairy farming, milk accounts for 60% of the income, while the increase in calves and inventory value makes up 40% of the income. In other words, new-born calves are approximately 40% effective in the incomes of enterprises [2].

İletişim (Correspondence)
+90 474 2426807 Mobile: +90 543 6542232
pinardemir80@hotmail.com

The husbandry and feeding of calves is very important in the first week following their birth. However due to differences in practice during the growth period of calves, the likelihood of disease prevalence in livestock enterprises, calf mortality and cost of growth can be change. In European countries, neonatal calf mortalities have been reported to be 10-15% in livestock enterprises while this rate can reach up to 50% in Turkey [3].

One of the most important problem for dairy farming in Turkey, as in most countries in the world are calf disease. Because economic losses arise resulting from veterinary-treatment costs, husbandry costs and death of a calf due to diseases the calves can be catch. Growth retardation also affects the profitability of the livestock enterprises negatively, leading to loss of meat, milk and/or reproduction [2,4-6]. Due to a lack of records in Turkey, the rate of calf mortality is not exactly known. In this study, economic losses resulting from calf mortalities In this study, the economic loss of calf deaths, one of the most important problems of the livestock sector, which is important for the contribution of the produced animal food products to the economy has been investigated. In this study, the economic losses due to loss calf were identified in the dairy cattle farms are located in Kars province in Turkey.

MATERIAL and METHODS

The material of this study was the data obtained during the interviews conducted with a total of 108 cattle breeding enterprise owners based in central villages of the province of Kars in Turkey. In this study, data on the owners of calves aged between 0 and 180 days were collected to determine the disease and mortality rates in calves in the study region and the economic losses associated with these rates.

In the interviews, a questionnaire was applied to the owners of the calves born in the years 2016 and 2017 and the information about diseased and dead calves were obtained. The questionnaire was consists of two main topics: the owner of the livestock enterprises and the information regarding the calves. The data obtained from the interviews were calculated by using percentage and frequency values, average values and Anova test via the SPSS 16 statistical package program [7].

The disease prevalence ratio (PR) for the study was found using the following formula:

PR (%) = (Total calf number with a disease symptom/Total calf number (calf/year) x 100

The mortality rate (MR) was calculated as follows [8]:

MR (%) = (Number of calf deaths (calf/year)/Total calf number (calf/year) x 100

The economic losses in the participating enterprises were calculated considering only the diseased calves, regardless of the cause of the disease. In the economic analysis, the dead calves were divided into three groups according to their ages (1st group: younger than 30 days, 2nd group: 30-89 days, 3rd group: 90-180 days). The method used to estimate the calf mortalities is given in *Table 1* [9-11]. However, for this study, the calculation methods used in the literature to calculate the economic loss due to calf mortalities were modified according to the research conditions. In the calculation of economic losses related to calf deaths, the loss of the mother's milk yield and the price of heifers and the developmental delays that occurred later were taken into consideration.

In the economic analysis of calf deaths, the financial values of calves, deaths, alternative costs and treatment costs due to diseases were taken into consideration. In interviews, it was determined that the owners of the calves applied traditional animal breeding methods in calves. In this context, it was found that calf mortalities reduced cows' milk yield by 20-30%. For all that, in calculating the economic losses due to calf mortalities within 30 days, milk yield loss was also taken into account. If the calf is alive, it is determined that milk, meat and reproductive performance are lower than diseased calves. For this reason, in calculating the opportunity cost, the value of the replacement heifer was taken into account in relation to the developmental retardation that occurred in the diseased calf. The value of the replacement heifer 65% (60-70%) of the healthy animal value was considered. In this study, 18-month-old animals were considered as heifer in calculating the opportunity cost. Therefore, the average cost of feeding the calves was calculated as a heifer (12, 15, 17 per month). In the analysis, the opportunity cost was calculated by decreasing the estimated maintenance- feed cost from the price of a replacement heifer ($/head).

Table 1. *Economic loss calculation method*

Group	Calculation Method
<30 days old calf	(Dead calf's value) + (250 days * loss of dairy milk * dairy milk price) + (Veterinary and treatment costs) + Opportunity cost (Price of a replacement heifer - estimated 17 months maintenance feed costs)
30-89 days old calf	(Dead calf's value) + (Veterinary and treatment costs) + Opportunity cost (Price of a replacement heifer - estimated 15 months maintenance feed cost)
90-180 days old calf	(Dead calf's value) + (Veterinary and treatment costs) + Opportunity cost (Price of a replacement heifer - estimated 12 months maintenance feed costs)

The technical and financial parameters used in the financial analysis and the values obtained from the relevant producer opinions are given in *Table 2*.

RESULTS

In the interviews conducted, it was found that 108 producers who participated in the survey study had a mean age of 43.24 (min: 18, max: 73), a mean experience of 21.89 years (min: 1, max: 50) and a monthly average income of 1.050 Turkish Lira (TRY)/$287. General information regarding the participants is given in *Table 3*.

The months when births are frequent in the study region are shown in *Fig. 1a*. It can be seen form *Fig. 1a* that the enterprises experience birth in every period of the year, however, births were concentrated between January and May.

Questions regarding calf care asked during interviews are given in *Table 4*. As shown in *Table 4*, it is seen that 89% of the enterprises had a separate compartment for the calves in the barns. In addition, it was determined that 68.5% of the calves drank colostrum within the first 6 h after birth.

The obtained data on the number of animals in the enterprises are given in *Table 5*. In the interviews, it was determined that the total of 313 (29.50%) in 2016 and 281 (24.65%) in 2017, diseased calves in 108 enterprises.

Table 6 shows the general condition of the animal shelters owned by the participants and the frequency of their cleaning owned by the participants. An average of 2.60 calves in 2017 was found to be diseased. In the analysis, no statistically significant relationship was found between educational status and number of diseases calves per

Table 2. *Technical and financial parameters used in estimating calf mortality related losses*

Parameters	Value	Parameters	Value
30-day average calf price	3000 TRY ($ 845)	Cost for 30-day diseased calf (treatment + medication + care)	85 TRY ($24)
30-89 day average calf price	3500 TRY ($ 984)	Cost for 30-89 day diseased calf (treatment + medication + care)	145 TRY ($41)
90-180 day average calf price	4000 TRY ($ 1125)	Costs for 90-180 days of diseased calf (treatment + medication + care)	240 TRY ($68)
Annual milk production (lt/cow)	10	Milk loss from cow due to loss of calf (%)	25% (20-30%)
Price of a replacement heifer	3900 ($ 1097)	1 day calf care-feeding cost	7 TRY ($2)
*1 $ = 3.556 TRY			

Table 3. *General information about the participants*

Education Status	Frequency	%	Age Average	Experience Year	Income (TRY)
Illiterate	2	1.9	59.50	29.00	650.00
Primary school	38	35.2	46.89	25.51	885.71
Middle school	32	29.6	46.12	24.34	987.50
High school	29	26.9	36.93	16.03	953.44
University	7	6.5	31.71	13.85	1226.31

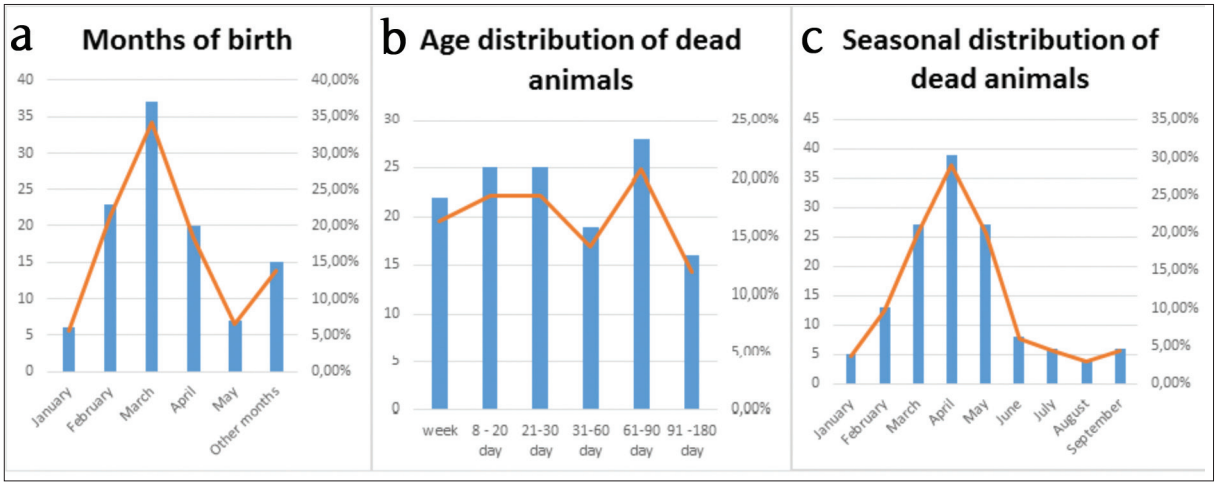


Fig 1. a- Months of birth; **b-** Age distribution of dead animals; **c-** Seasonal distribution of dead animals

Table 4. *Calf care information*

Parameters	Yes		No	
	Frequency	%	Frequency	%
Is there a separate compartment in the barn for the calves?	96	88.9	12	11.1
Are diseased calves separated from other calves?	62	57.4	46	42.6
Are umbilical cords cleaned with disinfectant after birth?	60	55.6	48	44.4
Do you feed calves with colostrum within the first 6 hours of after birth?	74	68.5	34	31.5
Parameters	Min.	Max.	Average	Std. Dev.
Average daily amount of milk the calves drink (L/day)	1.00	5.00	2.20	1.00
Mean duration of weaning of calves (Months)	1.00	8.00	4.07	1.05

Table 5. *Data on animal numbers*

Enterprises Data	Year	Min.	Max.	Total	Average	Std. Dev
Total cattle (head)	2016	3	150	2.584	24.14	19.28
	2017	2	140	2.637	24.65	18.65
Number of cows (head)	2016	2	35	1.380	12.89	6.88
	2017	2	35	1.458	13.50	7.25
Number of calves (head)	2016	1	22	1.061	9.92	5.08
	2017	1	25	1.140	10.65	5.69
Number of diseased calves (head)	2016	0	22	313	29.50	3.25
	2017	0	15	281	24.65	2.97
Cost for diseased calf (TRY/head) (treatment + medicine + care)	2016	20	300	7.279	103.98	64.75
	2017	25	1.000	11.346	156.32	162.99
Number of calf mortality (head)	2016	1	7	72	6.78	1.05
	2017	1	3	63	5.52	0.70

Table 6. *General condition of barns and cleaning frequency*

Condition of the Barn	Frequency	%	Diseases Calf Rate	Std. Error	Cleaning Frequency	Frequency	%	Diseases Calf Rate	Std. Error
Good	23	21.3	1.65	0.375	1 per day	34	31.5	2.73	0.606
Middle	67	62.0	3.05	0.401	2 per day	55	50.9	2.60	0.335
Bad	18	16.7	2.11	0.598	3 per day	19	17.6	2.52	0.646
Total/Mean	108	100.0	2.60	0.283	Total/Mean	108	100.0	2.60	0.283
F/P Value	F=2.301 p=0.105				F=0.30 p=0.971				

enterprise (P>0.05). In addition, there is no statistically significant relationship between the general structure of the barn and the frequency of cleaning and the number of diseases calves per enterprise (P>0.05).

The diseases seen in the calves in 2016 and 2017 are given in *Table 7*. *Table 7* shows that enteritis, respiratory diseases and foot-mouth disease are more common in calves and umbilical cord infections, tympani, meningitis and other causes were frequently seen.

Table 8 provides information on a total of 135 calves that died in 2016 and 2017. In the table, the first cause of death is seen as enteritis with a rate of 45.2%, followed by respiratory diseases with a rate of 22.2% and foot-and-mouth disease with a rate of 19.3%.

It was determined that 53.3% of the dead calves were female and 46.7% were male. No statistically significant difference was found between gender and calf death (P>0.05). In the *Table 8*, when grouped according to breeds, it is apparent that the deaths occurred in earlier days (47 days) for the local breed and in later days (60 days) for the simmental breed. However, no statistically

Table 7. *Diseases seen in calves in 2016 and 2017*

Disease	2016		2017		Average
	Frequency	%	Frequency	%	
Enteritis (Diarrhoea)	23	25.00	24	26.67	25.82
Respiratory Diseases	15	16.30	15	16.67	16.48
Foot-and-Mouth Disease (FMD)	12	13.04	13	14.44	13.74
Umbilical Lesions	4	4.35	4	4.44	4.40
Enteritis - Respiratory Diseases	14	15.22	14	15.56	15.38
Enteritis - Umbilical Lesions	6	6.52	11	12.22	9.34
Other	18	19.57	12	13.33	16.48
Total	92	100.00	90	100.00	100.00

Table 8. *Information on the dead calves*

Parameters		DAK/Local	Hybrid	Brown Swiss	Simmental	Total
Number of calves (head)		31 (23.0%)	46 (34.1%)	41 (30.4%)	17 (12.6%)	135 (100%)
Mean Age of Death (X±SS) (Day)		47.1±44.1	52.8±52.5	57.2±38.1	59.8±56.0	53.78±46.80
Gender (head)	Female	20 (64.5%)	27 (58.7%)	16 (39.0%)	9 (52.9%)	72 (100%)
	Male	11 (35.5%)	19 (41.3%)	25 (61.0%)	8 (47.1%)	63 (100%)
Cause of death (Head)	Enteritis	13 (21.3%)	20 (32.8%)	19 (31.1%)	9 (14.8%)	61 (100%)
	Respiratory diseases	9 (30.0%)	10 (33.3%)	9 (30.0%)	2 (6.7%)	30 (100%)
	FMD	5 (19.2%)	9 (34.6%)	9 (34.6%)	3 (11.5%)	26 (100%)
	Umbilical	0 (0.0%)	1 (25.0%)	1 (25.0%)	2 (50.0%)	4 (100%)
	Tympany	3 (37.5%)	2 (25.0%)	2 (25.0%)	1 (12.5%)	8 (100%)
	Other	0 (0.0%)	1 (16.7%)	2 (33.3%)	3 (50.0%)	6 (100%)

Table 9. *Age of death and causes of death*

Day	Enteritis	Respiratory	FMD	Umbilical	Tympany	Other	Total
1 week	16 (72.7%)	5 (22.7%)	0.0	0.0	0.0	1 (4.6%)	22 (100%)
8 -20 day	14 (56.0%)	6 (24.0%)	3 (12.0%)	0.0	0.0	2 (8.0%)	25 (100%)
21-30 day	14 (56.0%)	4 (16.0%)	2 (8.0%)	3 (12.0%)	2 (8.0%)	0.0	25 (100%)
31-60 day	5 (26.3%)	7 (36.8%)	5 (26.3%)	1 (5.3%)	1 (5.3%)	0.0	19 (100%)
61-90 day	7 (25.0%)	6 (21.4%)	11 (39.3%)	0.0	3 (10.7%)	1 (3.6%)	28 (100%)
91-180 day	5 (31.2%)	2 (12.5%)	5 (31.2%)	0.0	2 (12.5%)	2 (12.5%)	16 (100%)
Total	61 (45.2%)	30 (22.2%)	26 (19.3%)	4 (3.0%)	8 (5.9%)	6 (4.4%)	135 (100%)

Table 10. *Estimated economic loss due to calf mortalities (TRY)*

Groups	Per Animal	Total Loss
<30 day	3.978	194.922
30-89 day	4.395	184.590
90-180 day	5.420	238.480
Mean/Total	4.598	617.992

significant difference was found between race and age of death (P>0.05).

The ages (days) of calves when they died are given in *Fig. 1b*. From the table it can be seen that 53.3% of calves died in less than 30 days, while 20.7% of deaths occurred within 61-90 days, followed by 18.5% within 8-20 days and 21-30 days after birth.

In *Table 9*, the findings regarding age and causes of death

are given. As can be seen from the table, enteritis with a rate of 72.7% and respiratory diseases with a rate of 22.7% were the leading causes of deaths within the first week after birth. More deaths were seen related omphalitis and tympani on the 21st and 30th days following birth and foot-and-mouth disease on the 31st and 90th days.

The breakdown of the deaths of calves seen in the enterprises over the years is shown in *Fig. 1c*. As can be seen from *Fig. 1c*, calf mortalities showed an increase with the start of winter and peaked in spring.

Estimated economic losses from animal and per calf deaths per farm are shown in *Table 10*. As can be seen from the table, economic loss due to calf mortalities is estimated to be 4.597 TRY ($1.293) per animal, with a total loss of 617.992 TRY ($173.788) considering the total of 135 calf mortalities.

DISCUSSION

In the interviews, 62% rate of the participants stated that the current status of their stables is moderate. This result is close to the findings (58%) of the study conducted by Tilki et al.[12]. On the other hand, Demir et al.[9] and Demir et al.[13] reported that the majority of the barns in the region were technically inadequate, traditional and of the same type and that a large part of the barns was inadequate for ventilation and lighting. In this study, it was determined that 89% of the enterprises had calves present in the same barn but in separate sections and the barns were technically inadequate. In the interviews, it was stated that the winter was harsh and the animals were kept together due to heating problems. Bozukluhan and Gökce [14] stated that the septicaemia neonatorum was found in calves because of the contamination of the barns in the region and the presence of many cattle from all age groups in the same barn.

Feeding a calf with sufficient amount of colostrum is very important for health and vitality. However, it was determined that only 68.5% of the enterprises in the region gave calves colostrum within the first 6 h after birth. The low rate is due to the fact that many farmers in the locality think that colostram causes enterit in calves. However, postpartum period is the most common case of the calf with pathogenic microorganisms, and immune substances from the mother can only be transferred via colostrum [14]. In addition, the colostrum protects the offspring from diseases which they may encounter within the first 3-4 months of their lives [15]. From this point of view, it can be said that producers do not have enough knowledge about the importance of colostrum.

In this study, data were collected on 0-180 day old diseased and dead calves in the region. As a matter of fact, studies on diseased animals such as cattle and sheep reported that the highest amount of disease in the entire age group was observed in the 0-6 month period [14,16]. For the cattle brought to the clinic of Kafkas University Veterinary Faculty, the rate of calves was determined as 56.30% [17]. This situation was attributed to an inadequate immune system against bacterial, viral and parasitic infections in calves [18].

In this study, it was determined that average 13 cattle and 10 calves per enterprise. Approximately 30% of 1061 calves in 2016 year and 25% of 1140 calves in 2017 year were found to be diseased. This rate was found to be close to the morbidity rates between 20-30% in other studies [19,20]. But this rate found to be lower than the other study conducted by Erdoğan et al.[21] (51.1%, 36.3%) in 2001 and 2002 in the province of Kars. This situation has been interpreted as increasing the knowledge of livestock enterprises about maintenance and feeding.

In some studies, conducted in developed countries, it is considered that average of 2-12% of calf mortality is acceptable in an enterprise [2,19,20]. In the studies conducted, the general mortality rate in calves was determined as Sivula et al.[19] 11.8%, and Wells et al.[20] 6.3%. In Turkey, the neonatal calf mortalities are reported to be 15-20% in public enterprises. However, in the present study, the calf mortality ratio was calculated as 5.52%. It was found that a significant portion of these mortalities is due to inadequate and incorrect nutrition methods, but with a good husbandry-nutrition method, the mortality rate can be reduced by 3-5% [3,15,22]. Similar to the findings of this study, conducted by Tokgöz et al.[23] found that the mortality rate in calves was 5.45%.

In this study, it was determined that enteritis cases were seen in at least 26% of the enterprises. Similar to the findings of this study, Erdogan et al.[21] found that the ratio of enteritis in neonatal calves was 28.8% and that of Citil et al.[24] was 30.4%.

Among the causes of deaths, enteritis ranks first with a rate of 45.2%. It was determined that 72.7% of the deaths during the first week of birth were due to enteritis. In parallel with this finding, Citil et al.[24] reported that enteritis in new-born calves was more common in the first week after birth in their study of the region. These diseases cause very important economic losses as new-born calves with enteritis and an ongoing disease lead to high mortality and growth retardation [5].

In this study it was found that respiratory system diseases were the other most common disease in calves. Demir and Bozukluhan [9] reported that the frequency of diseases in their study conducted in Kars was 24.4% in calves and cows. Other studies also showed that respiratory system diseases are among the top 3 in terms of the frequency of occurrence in the region [17,25]. In this respect, respiratory diseases are of great importance in terms of aquaculture and they lead to significant economic losses [26]. As a matter of fact, the study by Demir and Bozukluhan [9] reported that the average economic loss due to respiratory tract diseases in the region was $202 per calf.

In this study, mortalities due to respiratory system diseases in calves were observed from the first week following birth, but the highest mortality was found between 31-60 days (37%). As a matter of fact, in the study by Erdogan et al.[25], it was reported that pneumonia cases appeared after the 4th week of birth. Although it is not seen in this study results, it has been reported that calf deaths or high-cost traumatic problems occur due to extraction force in dairy farms [27].

In interviews with producers, it was found that calves also frequently had umblical infections in dairy farms. In the study conducted by Celik [3], reported that, in parallel with this study, umbilical lesions were seen in 5% to 15% of newborn calves. In this study, it is thought that this situation is related to the fact that the rate of navel cord cleaning performed immediately after birth is as low as 55.6%. According to these findings, birth hygiene and nutrition are not fulfilled adequately by producers [28].

In the study conducted, mortalities due to navel infection in calves were found to be more frequent between the 21st and 60th days following birth. Belge et al.[28] reported that 76.8% of the total 112 calf lesions detected in the navel lesion occurred between 0 and 3 months.

In the interviews, it was determined that the enterprises spent an average of 103 TRY ($29) per animal only as treatment, medicine and maintenance costs in 2016, which increased to 184 TRY ($52) in 2017. If the calf smaller than 30 days dies, the cost estimate for this case is calculated as 3.978 TRY ($1.119). This figure was found to be 4.395 TRY ($1.235) in the 30-89-day calf and 5.420 TRY ($1.524) in the 90-180-day calf.

In conclusion, calf diseases, which are one of the most important problems in animal production, cause inefficiency, development retardation, treatment costs and even deaths which, in return, cause significant economic losses in Turkey and around the world. The most important issue in livestock enterprises, as it is in every business, is to be able to produce high quality and healthy production at the maximum amount with a minimum cost. For the profitability and continuity of a dairy farming enterprises, calves need to be grown in a healthy way and at a low cost. In this way, calf mortalities can be reduced and dairy cattle farms can become more profitable.

REFERENCES

1. Akman N, Şen AÖ: Buzağı büyütme ve barındırma. Amasya ili Damızlık Sığır Yetiştiriciliği Birliği Yayınları. 2016. http://www.amasyadsyb.org/docs/Amasya_DSYB Yayin_007.pdf; *Accessed:* 05.07.2017.

2. Erez İ, Göncü S: Siyah alaca buzağılarda erken sütten kesmenin performans üzerine etkileri. *Ç Ü Fen Müh Bil Derg,* 28 (3): 68-78, 2012.

3. Çelik HE: Konya›nın Akşehir, Ilgın ve Kadınhanı ilçelerindeki perinatal buzağı kayıplarının prevalansının belirlenmesi. *Doktora Tezi*, Selçuk Üniv. Sağlık Bil. Enst., 2013,

4. Orro T, Nieminen M, Tamminen T, Sukura A, Sankari S, Soveri T: Temporal changes in concentrations of serum amyloid-A and their associations with gain in neonatal reindeer calves. *Comp Immunol Microbiol Infect Dis,* 29, 79-88, 2006. DOI: 10.1016/j.cimid.2006.01.002

5. Ranjan R, Naresh R, Patra RC, Swarup D: Erythrocyte lipid peroxides and blood zinc ve copper concentrations in acute undifferentiated diarrhoea in calves. *Vet Res Commun,* 30, 249-254, 2006. DOI: 10.1007/s11259-006-3185-8

6. Seyedsharifi R, Ghadimi M, Hedayat Evrigh N, Seifdavati J, Boustan A, Abdi Benamar H: Economic evaluation in traditional and industrial livestock with different levels of milk production in Ardebil province with emphasis on risk criteria. *Kafkas Univ Vet Fak Derg,* 24 (5): 681-689, 2018. DOI: 10.9775/kvfd.2018.19720

7. İkiz F, Püskülcü H, Eren Ş: İstatistiğe Giriş. 8. Baskı. Barış Yayınları, İzmir, 2013.

8. Erganiş O: Veteriner Epidemiyoloji/Temel Bilgiler. Konya: Mimoza Yayınları, 1993.

9. Demir P, Bozukluhan K: Economic losses resulting from respiratory diseases in cattle. *J Anim Vet Adv*, 11 (4): 438-442, 2012. DOI: 10.3923/javaa.2012.438.442

10. Sariozkan S, Yalçın C: Estimating the production losses due to cystic echinococcosis in ruminants in Turkey. *Vet Parasitol,* 163 (4): 330-334, 2009. DOI: 10.1016/j.vetpar.2009.04.032

11. Sariozkan S, Yalçın C: Estimating the total cost of bovine fasciolosis in Turkey. *Ann Trop Med Parasitol,* 105 (6): 439-444, 2011. DOI: 10.1179/1364859411Y.0000000031

12. Tilki M, Sarı M, Aydın E, Işık S, Aksoy AR: Kars ili sığır işletmelerinde barınakların mevcut durumu ve yetiştirici talepleri: I. Mevcut durum. *Kafkas Univ Vet Fak Derg*, 19, 109-116, 2013. DOI: 10.9775/kvfd.2012.7282

13. Demir P, Işık Adıgüzel S, Sarı M, Ayvazoğlu C: Kars ili süt sığırcılık işletmelerinin genel yapısı ve ekonomik boyutu. *F Ü Sağ Bil Vet Derg,* 28 (1): 9-13, 2014.

14. Bozukluhan K, Gokce Hİ: 2000-2007 yılları arasında Kafkas Üniversitesi Veteriner Fakültesi kliniklerine getirilen hayvanların iç hastalıkları yönünden istatistiksel değerlendirilmesi. *Vet Hekim Der Derg,* 80, 45-52, 2009.

15. Erdem H, Atasever S: Yeni doğan buzağılarda kolostrumun önemi. *OMÜ Ziraat Fak Derg,* 20 (2): 79-84, 2005.

16. Kennerman E, Yılmaz Z, Şentürk S: Uludağ Üniversitesi Veteriner Fakültesi *iç hastalıkları kliniğine* getirilen sığır ve koyunların değerlendirilmesi (1990-2000). *Uludag Univ J Fac Vet Med,* 22, 19-25, 2003.

17. Karademir B: KAÜ Veteriner Fakültesi iç hastalıkları kliniklerine 1999 yılında kabul edilen hayvanların genel durumu. *İstanbul Univ Vet Fak Derg*, 27 (2): 377- 383, 2001.

18. Aydın F, Umur Ş, Gökçe G, Genç O, Güler MA: Kars yöresinde ishalli buzağılardan bakteriyel ve paraziter ekenlerin izolasyonu ve identifikasyonu. *Kafkas Univ Vet Fak Derg,* 7, 7-14, 2001.

19. Sivula NJ, Ames TR, Marsh WE, Werdin RE: Descriptive epidemiology of morbidity and mortality in Minnesota dairy heifer calves. *Prev Vet Med,* 27, 155-171, 1996. DOI: 10.1016/0167-5877(95)01000-9

20. Wells SJ, Gerber LP, Hill GW: Health status of preweaned dairy heifers in the United States. *Prev Vet Med*, 29 (3): 185-199, 1997. DOI: 10.1016/S0167-5877(96)01078-1

21. Erdoğan HM, Ünver A, Güneş V, Çitil M: Kars yöresindeki neonatal buzağılarda rotavirus ve coronavirus yaygınlığı. *Kafkas Univ Vet Fak Derg,* 9 (1): 65-68, 2003.

22. Özen N: Süt Sığırlarının Beslenmesi. Akdeniz Üniversitesi Ziraat Fakültesi Yayınları Yardımcı Ders Notu, No: 3, Antalya, 1999.

23. Tokgöz BS, Özdemir R, Turut N, Mirioğlu M, İnce H, Mahanoğlu B, Yoldaş A, Tuzcu N: Adana Bölgesinde görülen neonatal buzağı enfeksiyonlarının morbidite ve mortaliteleri ve risk faktörlerinin belirlenmesi. *AVKAE Derg*, 3 (1): 7-14, 2013.

24. Çitil M, Güneş V, Karademir B: 1996-2001 Yılları arası KAÜ Veteriner Fakültesi iç hastalıkları kliniğine getirilen ishalli buzağılar üzerine retrospektif bir çalışma. *Kafkas Univ Vet Fak Derg,* 9 (1): 39-42, 2003.

25. Erdoğan HM, Ünver A, Çitil M, Güneş V, Arslan MÖ, Tuzcu M, Gökçe Hİ: Dairy farming in Kars district, Turkey: III. Neonatal calf health. *Turk J Vet Anim Sci,* 33, 185-192, 2009. DOI: 10.3906/vet-0708-2

26. Karayel Hacıoğlu İ, Coşkun N, Duran Yelken S, Sevinc S, Alkan F: Phylogenetic analysis of bovine respiratory syncytial virus from calves with respiratory disorders. *Kafkas Univ Vet Fak Derg,* 25 (2): 251-256, 2019. DOI: 10.9775/kvfd.2018.20819

27. Aksoy Ö, Özaydın İ, Kılıç E, Öztürk S, Güngör E, Kurt B, Oral H: Evaluation of fractures in calves due to forced extraction during dystocia: 27 cases (2003-2008). *Kafkas Univ Vet Fak Derg,* 15 (3): 339-344, 2009. DOI: 10.9775/kvfd.2008.100-A

28. Belge A, Bakır B, Atasoy N, Alkan I: Buzağılarda göbek lezyonları. *Y Y Ü Vet Fak Derg,* 7 (1-2): 14-17, 1996.

Kafkas Univ Vet Fak Derg
25 (3): 291-297, 2019
DOI: 10.9775/kvfd.2018.20843

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Long Term Incubation Resilience of Post-Thaw Ram Semen Diluted with Lecithin-Based Extender Supplemented with Bovine Serum Albumin

Selim ALÇAY [1,a] Mehmed Berk TOKER [1,b] Elif GÖKÇE [2,c]
Nail Tekin ÖNDER [1,d] Burcu ÜSTÜNER [1,e] Zekariya NUR [1,f]

[1] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Uludag University, TR-16059 Bursa - TURKEY
[2] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Namık Kemal University, TR-59030 Tekirdag - TURKEY
[a] ORCID: 0000-0002-2472-8157; [b] ORCID: 0000-0003-4033-9749; [c] ORCID: 0000-0002-7678-3289; [d] ORCID: 0000-0001-5141-0008
[e] ORCID: 0000-0001-5999-4685; [f] ORCID: 0000-0002-1438-221X

Article Code: KVFD-2018-20843 Received: 26.08.2018 Accepted: 06.12.2018 Published Online: 06.12.2018

How to Cite This Article

Alçay S, Toker MB, Gökçe E, Önder NT, Üstüner B, Nur Z: Long term incubation resilience of post-thaw ram semen diluted with lecithin-based extender supplemented with bovine serum albumin. *Kafkas Univ Vet Fak Derg,* 25 (3): 291-297, 2019. DOI: 10.9775/kvfd.2018.20843

Abstract

The objective of the study was to determine the optimum concentration of BSA in lecithin-based extender for post-thawing quality and incubation resilience (0 h, 6 h and 10 h) of ram sperm. Ejaculates were collected from five rams via electro ejaculation. Ejaculates were mixed to obtain pooled semen. Then, pooled semen was diluted with soybean lecithin-based extender without BSA (control) or supplemented with different concentrations of BSA (2.5 mg/mL, 5 mg/mL, 7.5 mg/mL and 10 mg/mL), at a final concentration of 150x106 spermatozoon/mL. Sperm motility, plasma membrane functional integrity (HOST), mitochondrial activity (rhodamine123), capacitation status (CTC), and DNA integrity (TUNEL) were evaluated. At the 10 h incubation, motility, plasma membrane functional integrity and mitochondrial function were better preserved in the BSA5 group compared to the control group. It was determined that high doses of BSA (5 mg/mL, 7.5 mg/mL and 10 mg/mL) affected acrosome reaction. The highest acrosome reaction rates were obtained in BSA10 groups in 6 h and 10 h incubation ($P<0.05$). TUNEL assay demonstrated that there were no differences among groups for DNA fragmentation at post-thaw and during incubation periods. The study shows that BSA supplemented extenders may have beneficial effect on ram semen parameters at 0 h, 6 h and 10 h of incubation. The results of the present study demonstrated a remarkable advantage of using 5 mg/mL of BSA in 1% lecithin-based extender.

Keywords: *Bovine serum albumin, Cryopreservation, Incubation resilience, Ram spermatozoa*

Sığır Serum Albumini İlave Edilmiş Lesitin Bazlı Sulandırıcı İle Sulandırılan Koç Spermasının Dondurma-Çözdürme Sonrası Uzun Süreli İnkübasyon Direnci

Öz

Bu çalışmanın amacı koç spermasının dondurma çözdürme sonrası ve inkübasyon direnci göz önüne alınarak (0, 6 ve 10. saatler) lesitin bazlı sulandırıcı için uygun Sığır Serum Albumini BSA konsantrasyonunun belirlenmesidir. Bu amaçla 5 koçtan sperma örnekleri alınmıştır. Alınan spermalar bir araya getirildikten sonra final konsantrasyonu 150x106 spermatozoon/mL olacak şekilde farklı dozlarda BSA içeren (2.5 mg/mL, 5 mg/mL, 7.5 mg/mL and 10 mg/mL) ve içermeyen (kontrol) soya fasülyesi lesitini bazlı sulandırıcılarla sulandırılmıştır. Spermanın değerlendirilmesi amacıyla motilite, plazma membran fonksiyonel bütünlüğü (HOST), mitokondriyal aktivite (Rhodamine123), kapasitasyon statüsü (CTC) ve DNA bütünlüğü değerlerine bakılmıştır. İnkübasyonun sonunda (10. saat) BSA5 grubunun motiliteyi, plazma membran fonksiyonel bütünlüğü ve mitokondriyal fonksiyonu diğer gruplara göre daha fazla koruduğu görülmüştür. BSA'nın yükselen dozları (5 mg/mL, 7.5 mg/mL and 10 mg/mL) akrozom reaksiyonunu etkilemektedir. İnkubasyonun 6 ve 10. saatlerinde en yüksek akrozom reaksiyonu oranları BSA10 grubunda gözlemlenmiştir ($P<0.05$). TUNEL sonuçları göz önüne alındığında; çözdürme ve inkübasyon sonrası gruplar arası DNA bütünlüğü bakımından bir fark olmadığı görülmektedir. Çalışma sonucunda; sulandırıcıya BSA ilave edilmesinin inkubasyonun 0, 6 ve 10. saatlerinde koç sperm parametreleri üzerinde yararlı etkisi olduğu görülmektedir. Çalışma sonucu elde edilen veriler göz önüne alındığında %1 lesitin bazlı sulandırıcıya 5 mg/mL BSA ilavesinin önemli bir avantaj oluşturduğu görülmektedir.

Anahtar sözcükler: *Sığır serum albumini, Kriyoprezervasyon, İnkübasyon drenci, Koç sperması*

İletişim (Correspondence)
+90 224 2941356
salcay@uludag.edu.tr

INTRODUCTION

Semen cryopreservation is the pillar of the reproductive biotechnology [1]. This reversible process brings semen metabolism to a standstill, in this way it is possible to storage genetic material throughout long time [2]. Even though cryopreservation is a reversible operation, there are some detrimental effects (cold shock, ice crystallization, lipid peroxidation etc.) of this process [2,3]. These adverse effects may cause irreversible decrease on motility, viability and fertilization ability of spermatozoa [4,5]. Therefore, the success of semen cryopreservation depends on minimizing the adverse effects and maintaining the post-thaw semen quality [1,5]. There are a lot of research, aim of which was to increase success of ram semen cryopreservation with different procedures and additives [6-10].

Lecithin is a low-density lipoprotein fraction that is mainly found in plants and plays an important role in the regulation of an animal cells' bio-membrane [11]. In addition, it is used for semen cryopreservation either as an ingredient of egg yolk or as an extracted substance [11,12]. Many researchers have obtained acceptable post-thaw semen parameters with using lecithin-based extender [11-16] but upgrading these results is possible with optimized lecithin base extenders.

Bovine Serum Albumine (BSA) has multifunctional effect on sperm with its macromolecular structure and antioxidant capacity. Therefore, BSA increases the post-thaw motility [17,18], protects the membrane against cold shock [17] maintains DNA integrity during freezing-thawing and incubation periods [18]. In addition, it induces the capacitation [19,20] and acrosome reaction [21]. In brief; BSA not only protect the spermatozoa against freezing-thawing process but also stimulates the capacitation and acrosome reaction. Besides, BSA increase the possibility of sperm-zona pellucida interactions and fertility results. As a consequence of these, BSA supplemented extenders are used for cryopreservation or liquid storage of bull [18], ram [22], goat [23,24], stallion [25], buffalo [26], rabbit [27] and turkey semen [28].

Many researchers used lecithin or BSA based extenders to improve post-thaw quality and fertilization ability of spermatozoa [16-18,20,21,28]. However, collaborate using of these ingredients for ram semen cryopreservation has not been performed yet. The aim of this study was determining the appropriate dose of BSA in lecithin-based extenders for improving motility, viability, DNA integrity in 0 h, 6 h and 10 h of incubation. Also, we aimed to recognize the capacitation and acrosome reaction of ram spermatozoa at the same time periods.

MATERIAL and METHODS

Scientific Ethical Committee (Uludag University, Bursa, Turkey) have approved all issues concerning the experimental setups and evaluation techniques (2015-07/03).

Chemicals

All chemicals used in the study were obtained from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA).

Experimental Design

This study was designed to evaluate the efficacy of BSA supplementation to the extender in ram sperm cryopreservation. Therefore, we used various concentrations of BSA (0 mg/mL, 2.5 mg/mL, 5 mg/mL, 7.5 mg/mL or 10 mg/mL) in lecithin-based extenders during non-breeding season.

Semen Extender Preparation

Extenders contained 223.7 mM Tris, 55.5 mM fructose, 66.6 mM citric acid, 100.4 mM Trehalose, 4.03 mM EDTA, 1 mM cysteine, 4 g/L penicillin G, 3 g/L dihydrostreptomycin, 1% lecithin in distilled water. Relevant concentrations of BSA according to experimental design added to each group of extenders, but the control.

Semen Collection and Dilution

Semen collection from Kivircik Rams, which were maintained with same conditions at Uludag University, Faculty of Veterinary Medicine in Bursa, Turkey, was performed by electrically stimulated ejaculation (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand) five times in every other day. After the collection, the ejaculates were transferred to a water bath (37°C). Rapid wave motion and motility evaluated by phase-contrast microscope (Olympus BX51, Olympus Optical Co., Tokyo, Japan) with a warm slide (37°C). Spermatozoa concentration on native semen was evaluated with hemocytometric method. For this purpose, semen was diluted with alcohol (1:200 semen/semen-alcohol dilution) then the solution was dropped to Thoma's Couting Slide and the concentration of spermatozoa were counted. Ejaculates have rapid wave (>+3 on 0-5 scale), >75% motility and >$1.0x10^9$ spermatozoon/mL were chosen for cryopreservation. In order to eliminate individual differences, ejaculates were pooled.

Briefly, pooled ejaculate was split into five equal aliquots and diluted (37°C) to final concentration of approximately $150x10^6$ (spermatozoon/mL) with BSA supplemented extenders and control extender. Within 1 h, diluted semen was gradually cooled to 4°C and then equilibrated for 2 h at 4°C.

Semen Freezing and Thawing

After the equilibration, semen was loaded into 0.25 mL French straws. Semen was frozen at 3°C/min from +5°C to -8°C and at 15°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). The straws were then plunged into liquid nitrogen at -196°C where

they were stored. Three straws from each group were thawed at 37°C for 30 s in a water bath and incubated in humidified air chamber with 5% CO_2 for 10 h at 39°C to evaluate post-thaw semen characteristics.

Semen Evaluation

We evaluated sperm motility, plasma membrane integrity (hypoosmotic swelling test [HOST]), mitochondrial activity, (R123; Invitrogen TM, Eugene, OR, USA), capacitation status (Chlortetra cycline [CTC] staining) and DNA integrity (using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling [TUNEL]) at three-time points (post-thaw at 0 h, 6 h and 10 h). All processes and measurements were conducted by the same person during the study. Sperm motility was assessed subjectively using a phase-contrast microscope (400×) with a warm slide (37°C).

Assessment of plasma membrane functional integrity was performed with HOST test described by Leboef et al.[29] with little modification [30]. Mitochondrial activity was evaluated using a combination of fluorescent stains, Rhodamine 123 (R123) and PI methodology of Fraser et al. was used to performe [31]. Capacitation status evaluation was performed as described by Pérez et al.[32]. For the TUNEL technique, we used the In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with slight modifications [33].

Statistical Analysis

All data obtained from study were analyzed using SPSS (SPSS 20.0 for Windows; SPSS, Chicago, IL, USA). Data were represented as mean ± standard deviation. Shapiro Wilk test was used as normality test. Means of obtained semen parameters were analyzed using Kruskal Wallis test. Statistical differences between the various treatment group means were determined by Mann Whitney U test. Differences with values of $P<0.05$ were considered to be statistically significant.

RESULTS

The standard semen parameters of the BSA were evaluated in 5 independent experiments by the same person. Percentages of sperm motility, plasma membrane functional integrity, mitochondrial activity and DNA fragmentation were indicated in *Table 1*. Capacitation status are showed in *Table 2*.

Sperm motility was progressively reduced after incubation *(Fig. 1)* ($P<0.001$). Post-thaw sperm motility rates in BSA7.5 group was higher than the control and BSA2.5 groups at 0 h ($P<0.05$). After 6 h incubation, BSA2.5 group had higher motility rate than the control group ($P<0.05$). At the end of the 10 h incubation, mean motility rates better preserved in the BSA2.5, BSA7.5 and BSA10 groups than the control groups ($P<0.05$). The highest percentage of motility was observed in BSA5 group at post-thaw and incubation periods ($P<0.05$).

Plasma membrane functional integrity deteriorated after post-thaw and incubation ($P<0.001$). Membrane functional integrity was better preserved in the BSA7.5 and BSA10 groups than the control group at 0 h ($P<0.05$). The BSA2.5 group had higher membrane integrity percentage than the control group at 6 h ($P<0.05$). After 10 h incubation, the similar result was obtained in the BSA2.5, BSA7.5 and BSA10 groups ($P>0.05$). Membrane integrity was protected in BSA5 group compared to the other groups ($P<0.05$).

Mitochondrial function decreased with the freeze-thaw process ($P<0.001$). While, post-thaw mitochondrial function

Table 1. *The mean of studied sperm resilience parameters on different extender groups*

Incubation Period (h)	Group	Motility (%)	HOST (%)	Mitochondrial Function (%)	DNA Fragmentation (%)
0	Control	53.00±2.54^{a}	63.40±3.25^{a}	58.0±1.46^{a}	4.75±0.96
	BSA 2.5	54.00±2.07^{a}	65.40±2.13^{ab}	59.4±2.13^{a}	5.00±0.82
	BSA 5	61.00±2.07^{c}	71.20±3.88^{c}	68.0±2.36^{c}	4.60±0.55
	BSA 7.5	57.00±2.54^{b}	67.00±1.46^{b}	63.6±4.22^{b}	5.00±1.15
	BSA 10	56.00±2.07^{ab}	67.40±2.75^{b}	63.6±3.50^{b}	5.75±1.26
6	Control	22.00±4.14^{a}	32.20±3.17^{a}	26.8±1.78^{a}	9.75±2.50
	BSA 2.5	26.00±3.87^{b}	36.40±1.92^{b}	30.20±2.57^{b}	9.60±1.14
	BSA 5	35.00±3.27^{c}	43.40±4.56^{c}	38.60±2.50^{c}	8.40±1.14
	BSA 7.5	25.00±3.27^{ab}	34.60±3.85^{ab}	29.40±4.37^{ab}	9.00±0.82
	BSA 10	24.00±3.87^{ab}	35.20±4.16^{ab}	30.80±4.00^{b}	10.20±0.84
10	Control	2.00±2.54^{a}	10.40±3.50^{a}	7.20±2.01^{a}	19.67±1.15
	BSA 2.5	6.00±2.07^{b}	13.40±2.41^{b}	11.40±2.32^{b}	18.25±2.22
	BSA 5	12.00±2.54^{c}	21.00±2.17^{c}	20.40±3.62^{c}	18.00±1.41
	BSA 7.5	8.00±2.54^{b}	12.20±2.57^{ab}	14.20±2.88^{b}	19.50±3.32
	BSA 10	7.00±2.54^{b}	13.40±1.80^{b}	13.60±3.96^{b}	19.50±4.20

$^{a, b, c}$ Values with different superscripts in the same column for each of incubation time are significantly different ($P<0.05$)
Data is presented in Mean± S.D.

Table 2. *Effect of BSA on capacitation status of frozen-thawed sperm*

Incubation Period (h)	Group	Uncapacitated (%)	Capacitated (%)	Acrosome Reacted (%)
0	Control	24.40±1.82^{a}	58.20±0.84^{a}	17.80±2.68
	BSA 2.5	22.20±1.30^{a}	58.80±1.48^{a}	19.00±1.41
	BSA 5	22.60±3.05^{a}	59.40±2.30^{a}	18.00±4.47
	BSA 7.5	17.80±1.30^{b}	64.00±2.34^{b}	18.20±1.79
	BSA 10	15.20±2.28^{b}	67.40±2.41^{b}	17.40±1.14
6	Control	14.40±2.61^{a}	66.40±1.52^{a}	19.20±1.92^{a}
	BSA 2.5	15.20±1.10^{ab}	65.40±0.89^{ab}	19.40±1.34^{a}
	BSA 5	17.80±1.30^{b}	61.40±2.07^{b}	20.60±1.52^{a}
	BSA 7.5	11.00±1.41^{c}	63.00±3.08^{ab}	26.00±3.81^{b}
	BSA 10	9.60±0.55^{c}	63.40±2.41^{ab}	27.00±2.12^{b}
10	Control	6.80±0.84^{a}	58.80±1.79	34.40±1.52^{a}
	BSA 2.5	6.60±1.52^{a}	58.60±2.07	34.80±1.10^{a}
	BSA 5	6.00±0.71^{ab}	57.60±1.34	36.40±1.14^{ab}
	BSA 7.5	4.40±1.14^{bc}	56.20±2.59	39.40±1.82^{bc}
	BSA 10	3.60±0.89^{c}	54.60±4.39	41.80±4.09^{c}

^{a,b,c} *Values with different superscripts in the same column for each of incubation time are significantly different (P<0.05)*
Data is presented in Mean± S.D.

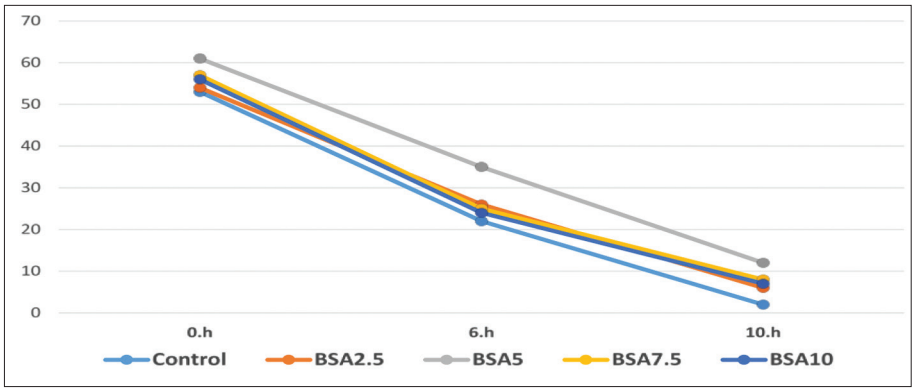


Fig 1. The motility results of the experiment groups during incubation

values at 0 h in BSA7.5 and BSA10 groups were higher than the BSA2.5 and control groups. After 6 h incubation, BSA2.5 and BSA10 group had higher mitochondrial function rate than the control group (P<0.05). At the end of the 10 h incubation, mitochondrial function was successfully preserved in BSA2.5, BSA7.5 and BSA10 groups compared to the control group (P<0.05). Similar to motility and plasma membrane integrity, the best mitochondrial function was obtained for the BSA5 group compared to the other groups (P<0.05).

The post-thaw and after incubation DNA damaged spermatozoa percentages were not statistically significant in all groups (P>0.05).

Post-thaw uncapacitated and capacitated status in control, BSA2.5 and BSA5 groups were higher than the other groups (P<0.05) In addition, acrosome reacted (AR) values were not significant among groups. After 6 h incubation, uncapacitated rates in BSA7.5 and BSA10 groups were lower than the compared to the other groups (P<0.05). Capacitation rates in control group were higher than the BSA5 group (P<0.05). AR rates in BSA7.5 and BSA10 groups were higher than the other groups (P<0.05). At the end of the 10 h incubation, uncapacitated rates in control and BSA2.5 groups were higher than the BSA7.5 and BSA10 groups (P<0.05). Capacitated rates were not significant between groups. AR rates in BSA10 groups were higher than the control, BSA2.5 and BSA5 groups (P<0.05).

DISCUSSION

Cryopreservation process have a detrimental effect on spermatozoa because of temperature change, cold shock, ice crystallization and lipid peroxidation. These restrictive effects provoke to decrease of motility, viability, mitochondrial membrane function, DNA integrity and fertilizing ability of spermatozoa [11,12,16]. Various supplements were tested to minimize the adverse effect of cryopreservation [5-12,14,16,28].

Bovine serum albumin is one of the attempted supplements, because of a good amino acid profile and protective functions [17,21]. In the present study, we compared the effect of exogenous addition of BSA in lecithin-based extender on ram semen quality at post-thaw and during the incubation periods.

Motility is one of the essential semen quality parameters as an indicator of attaining to oocyte [33]. The post-thaw motility values of ram semen cryopreserved with lecithin-based extender ranged between 31.0% - 48.1% [5,11,14,34]. Our post-thaw motility results clearly higher than these studies. Good interaction between lecithin-BSA could be the reason of these results. BSA stimulates sperm motility [17,34] but there is no enlightening explanation about mechanism of its stimulative effect [17]. In our study; although BSA supplementation prompted to clear increase on motility, BSA2.5 group had not sufficient effect to make statistical difference at all incubation times. BSA5 group had better motility values than other groups at post thaw and after incubation ($P<0.05$). When sufficient BSA doses (BSA5, BSA7.5, BSA10) were compared among each other, it was shown that increasing doses of BSA caused gradually decrease on motility.

The sperm-environment interaction is performed with plasma membranes that have an essential role in sperm metabolism [26]. Therefore, plasma membrane integrity is important for capacitation, acrosome reaction and finally oocyte fusion of sperm [35]. However, plasma membrane permeability and integrity get harmed from cold shock and lipid peroxidation (LPO) [33]. Cold shock causes phase transition of membrane lipids and then plasma membrane could lose its selective permeability [36]. The protection against cold shock is possible with increasing the fluidity of membrane [37]. The protective effect of BSA against cold shock is based on this expected impact. BSA attaches to the sperm membrane then changes sperm membrane lipid composition and decreases to phospholipid concentration [19]. The proper protection against cold shock could be evaluated with assessment of plasma membrane functional integrity. HOST is the optimized test for detecting the subtle changes of sperm membrane functional integrity [35]. In the present study, the HOST values in BSA5 group were higher than in the other groups at post-thaw and after incubation ($P<0.05$). The HOST values are in a good agreement with the previous researches [5,11,12,28,33].

The negative effect of cold shock and LPO is not only limited to losing of plasma membrane integrity, but also the organelle membranes suffered from them as well. Membrane integrity loses lead to malfunction or dysfunction of organelles [38]. When reactive oxygen species (the result of cooling, cold shock or cryostress) induce mitochondrial membrane damage for this reason ATP synthesis is interrupted and consequently spermatozoon loss its motility and metabolism function [38]. Therefore, mitochondrial membrane function is important for sperm fertilization ability. In the current study, sperm mitochondrial function was assessed by R123 fluorescent staining. The BSA5 group protected mitochondrial activity properly and this protective effect proceeded reach up to 10 h incubation.

Capacitation and acrosome reaction are essential for the last journey of spermatozoon. It has been related with sperm penetration and fusion to zona pellucida [39]. The trigger of capacitation process is the increment of intracellular calcium concentration so it can be regarded as a key factor that regulating induction of sperm capacitation [40]. The BSA promotes Ca^{+2} to influx into spermatozoon and rises intracellular Ca^{+2} concentration. In this way it stimulates sperm membrane reorganization [41]. In the study, there was no statistical difference between low doses BSA and control for uncapacitated spermatozoa rates at 0 h. It is not an undesirable result because higher rate of capacitated spermatozoa at 0 h may be the indicator of precapacitation. Considering the progressive times of incubation, higher BSA doses decreased the capacitated spermatozoa rates. The decrease of capacitated sperm with higher dose BSA groups was relevant with the increase of acrosome reacted sperm. Briefly, higher doses of BSA was induce acrosome reaction and the highest results of 6 h and 10 h was obtained with BSA10 ($P<0.05$).

The other adverse effect of cryopreservation is the DNA damage [33,42]. On the contrary of the other assessment methods; DNA integrity is not the direct indicator of sperm fertilizing ability [42]. It is important for identification of seriously damaged spermatozoa and embryo development ability [42]. According to our data; there were no difference among DNA integrities of control or BSA groups. In many studies using ram spermatozoa, the post-thaw and after 6 h incubation results of DNA fragmentation rates are in a rate between 3.6%-11.9% [12,35,43] and 5.8%-12.4% [12,43] respectively. In our study, similar DNA fragmentation rates were obtained.

The results of the present study indicated that BSA5 group preserved sperm motility, plasma membrane functional integrity and sustained mitochondrial activity better than both BSA-free and higher doses of BSA supplemented extenders throughout freezing-thawing and incubation period. Considering to all sperm parameters mentioned above; BSA5 group was the optimum for ram semen preservation. Beneficial effect of BSA supplementation looked promising to increase the utility of lecithin-based extender for ram spermatozoa. Future studies should be aimed at confirming the usefulness of the supplementation with BSA5 group regarding field fertility.

Acknowledgements

This work was supported by the Uludag University Scientific

Research Projects Unit, Bursa, Turkey, (BAP) (Project number: KUAP (V)-2015/55

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

1. Salamon S, Maxwell WM: Storage of ram semen. *Anim Reprod Sci,* 62, 77-111, 2000. DOI: 10.1016/S0378-4320(00)00155-X

2. Holt WV, Penfold LM: Fundamental and practical aspects of semen cryopreservation. **In,** Chenoweth P, Lorton S (Eds): Animal Andrology: Theories and Applications, 76-99, CAB International CABI, Wallingford Oxfordshire, 2014. DOI: 10.1079/9781780643168.0076

3. Fiser PS, Fairfull RW: The effects of rapid cooling (cold shock) of ram semen, photoperiod, and egg yolk in diluents on the survival of spermatozoa before and after freezing. *Cryobiology,* 23, 518-524, 1986. DOI: 10.1016/0011-2240(86)90061-1

4. O'Connell M, McClure N, Lewis SEM: The effects of cryopreservation on sperm morphology, motility and mitochondrial function. *Hum Reprod*, 17 (3): 704-709, 2002. DOI: 10.1093/humrep/17.3.704

5. Ustuner B, Alcay S, Toker MB, Nur Z, Gokce E, Sonat FA, Gul Z, Duman M, Ceniz C, Uslu A, Sagirkaya H, Soylu MK: Effect of rainbow trout *(Oncorhynchus mykiss)* seminal plasma on the post-thaw quality of ram semen cryopreserved in a soybean lecithin-based or egg yolk-based extender. *Anim Reprod Sci*, 164, 97-104, 2016. DOI: 10.1016/J.ANIREPROSCI.2015.11.017

6. Yildiz S, Öztürkler Y, Ari UC, Lehimcioglu NC, Atakisi E, Kulaksiz R: The effects of l-ergothioneine, n-acetylcystein and cystein on freezing of ram semen. *Kafkas Univ Vet Fak Derg*, 21 (1): 81-86, 2015. DOI: 10.9775/kvfd.2014.11792

7. Ari UC, Kulaksiz R, Ozturkler Y, Lehimcioglu NC, Yildiz S: Effect of n-acetylcysteine (NAC) on post-thaw semen quality of Tushin rams. *Kafkas Univ Vet Fak Derg,* 22 (6): 883-887, 2016. DOI: 10.9775/kvfd.2016.15558

8. Ari UC, Kulaksiz R, Ozturkler Y, Yildiz S, Lehimcioglu NC: Effect of L -(+)-ergothioneine (EGT) on freezability of ram semen. *Int J Anim Vet Adv*, 4 (6): 378-383, 2012.

9. Ozturkler Y, Ari UC: Addıtives used ın extenders to ımprove the freezabılıty of ram semen ın recent years: A mini review. *Agric Biol*, 52 (2): 242-250, 2017.

10. Ari UC, Kulaksiz R, Ozturkler Y: Freezability of tushin ram semen extended with goat or cow milk based extenders. *Reprod Domest Anim*, 46 (6): 975-979, 2011. DOI: 10.1111/j.1439-0531.2011.01769.x

11. Ustuner B, Alcay S, Nur Z, Soylu MK: Effect of egg yolk and soybean lecithin on tris-based extender in post-thaw ram semen quality and *in vitro* fertility. *Kafkas Univ Vet Fak Derg*, 20, 393-398, 2014. DOI: 10.9775/kvfd.2013.10248

12. Toker MB, Alcay S, Gokce E, Ustuner B: Cryopreservation of ram semen with antioxidant supplemented soybean lecithin-based extenders and impacts on incubation resilience. *Cryobiology,* 72, 205-209, 2016. DOI: 10.1016/J.CRYOBIOL.2016.05.001

13. Masoudi R, Sharafi M, Zare Shahneh A, Towhidi A, Kohram H, Zhandi M, Esmaeili V, Shahverdi A: Effect of dietary fish oil supplementation on ram semen freeze ability and fertility using soybean lecithin-and egg yolk-based extenders. *Theriogenology,* 86, 1583-1588, 2016. DOI: 10.1016/j.theriogenology.2016.05.018

14. Mehdipour M, Daghigh Kia H, Nazari M, Najafi A: Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic and oxidative parameters in ram semen. *Cryobiology*, 78, 34-40, 2017. DOI: 10.1016/J.CRYOBIOL.2017.07.005

15. Motlagh MK, Sharafi M, Zhandi M, Mohammadi-Sangcheshmeh A, Shakeri M, Soleimani M, Zeinoaldini S: Antioxidant effect of rosemary *(Rosmarinus officinalis L.)* extract in soybean lecithin-based semen extender following freeze–thawing process of ram sperm. *Cryobiology,* 69, 217-222, 2014. DOI: 10.1016/J.CRYOBIOL.2014.07.007

16. Najafi A, Daghigh-Kia H, Dodaran HV, Mehdipour M, Alvarez-Rodriguez M: Ethylene glycol, but not DMSO, could replace glycerol inclusion in soybean lecithin-based extenders in ram sperm cryopreservation. *Anim Reprod Sci,* 177, 35-41, 2017. DOI: 10.1016/J.ANIREPROSCI.2016.12.004

17. Uysal O, Bucak MN: Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. *Acta Vet Brno*, 76, 383-390, 2007. DOI: 10.2754/avb200776030383

18. Uysal O, Bucak MN, Yavas I, Varisli O: Effect of various antioxidants on the quality of frozen-thawed bull semen. *J Anim Vet Adv*, 6, 1362-1366, 2007.

19. Davis BK, Byrne R, Hungund B: Studies on the mechanism of capacitation. II. Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin during capacitation *in vitro*. *Biochim Biophys Acta*, 558, 257-266, 1979. DOI: 10.1016/0005-2736(79)90260-8

20. Xia J, Ren D: The BSA-induced Ca^{2+} influx during sperm capacitation is CATSPER channel-dependent. *Reprod Biol Endocrinol*, 7:119, 2009. DOI: 10.1186/1477-7827-7-119

21. Hossain MS, Hyeong LJ, Mıah AG, Tsujıı H: Effect of fatty acids bound to bovine serum albumin-V on acrosome reaction and utilization of glucose in boar spermatozoa. *Reprod Med Biol,* 6, 109-115, 2007. DOI: 10.1111/j.1447-0578.2007.00173.x

22. Gokce E, Alcay S, Gul Z: Positive effect of BSA supplemented soybean lecithin based extender on liquid storage of ram semen at 5°C. *Ankara Univ Vet Fak Derg,* 64, 313-320, 2017.

23. Amidi F, Farshad A, Khor AK: Effects of cholesterol-loaded cyclodextrin during freezing step of cryopreservation with TCGY extender containing bovine serum albumin on quality of goat spermatozoa. *Cryobiology*, 61, 94-99, 2010. DOI: 10.1016/j.cryobiol.2010.05.006

24. Anghel A, Zamfirescu S, Dragomir C, Nadolu D, Elena S, Florica B: The effects of antioxidants on the cytological parameters of cryopreserved buck semen. *Rom Biotechnol Lett*, 15 (3): 26-32, 2010.

25. Ball BA, Medina V, Gravance CG, Baumbe J: Effect of antioxidants on preservation of motility,viability and acrosomal integrity of equine spermatozoa during storage at 5°C. *Theriogenology*, 56, 577-589, 2001. DOI: 10.1016/S0093-691X(01)00590-8

26. Akhter S, Rakha BA, Iqbal R, Ansari MS: Effect of bovine serum albumin on motility, plasmalemma, viability and chromatin ıntegrity of buffalo bull spermatozoa. *Pakistan J Zool*, 46, 115-120, 2014.

27. Sariozkan S, Turk G, Canturk F, Yay A, Eken A, Akcay A: The effect of bovine serum albumin and fetal calf serum on sperm quality, DNA fragmentation and lipid peroxidation of the liquid stored rabbit semen. *Cryobiology*, 67, 1-6, 2013. DOI: 10.1016/j.cryobiol.2013.04.002

28. Bakst MR, Cecil HC: Effect of bovine serum albumin on motility and fecundity of turkey spermatozoa before and after storage. *J Reprod Fertil,* 94, 287-293, 1992. DOI: 10.1530/jrf.0.0940287

29. Leboeuf B, Le Vern Y, Furstoss V, Kerboeuf D, Guıllouet P, Magıstrını M: Response of goat sperm to hypoosmotic steps modelled probit analysis. *Anim Reprod Sci,* 91, 265-274, 2006. DOI: 10.1016/j.anireprosci.2005.04.014

30. Alcay S, Gokce E, Toker MB, Onder NT, Ustuner B, Uzabaci E, Gul Z, Cavus S: Freeze-dried egg yolk based extenders containing various antioxidants improve post-thawing quality and incubation resilience of goat spermatozoa. *Cryobiology,* 72, 269-273, 2016. DOI: 10.1016/j.cryobiol.2016.03.007

31. Fraser L, Lecewicz M, Strzezek J: Fluorometric assessments of viability and mitochondrial status of boar spermatozoa following liquid storage. *Pol J Vet Sci*, 5, 85-92, 2002.

32. Pérez LJ, Valcárcel A, de las Heras MA, Moses DF, Baldassarre H: *In vitro* capacitation and induction of acrosomal exocytosis in ram spermatozoa as assessed by the chlortetracycline assay. *Theriogenology*, 45, 1037-1046, 1996. DOI: 10.1016/0093-691X(96)00031-3

33. 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. DOI: 10.1016/J.THERIOGENOLOGY.2009.12.007

34. Matsuoka T, Imai H, Kohno H, Fukui Y: Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa. *J Reprod Develop*, 52, 675-683, 2006. DOI: 10.1262/jrd.18033

35. Maxwell WM, Salamon S: Liquid storage of ram semen: A review. *Reprod Fertil Dev*, 5, 613-638, 1993. DOI: 10.1071/RD9930613

36. El-Kon I: Testing usability of bovine serum albumin (BSA) for preservation of egyptian buffalo semen. *Am Eurasian J Agric Environ Sci*, 11, 495-502, 2011.

37. Fang L, Bai C, Chen Y, Dai J, Xiang Y, Ji X, Huang C, Dong Q: Inhibition of ROS production through mitochondria-targeted antioxidant and mitochondrial uncoupling increases post-thaw sperm viability in yellow catfish. *Cryobiology*, 69, 386-393, 2014. DOI: 10.1016/j.cryobiol.2014.09.005

38. Bedford JM: Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol Reprod*, 28, 108-120, 1983.

39. Baek S, Lee ST, Hwang JY, Park KH, Yun JI: Identification of capacitation inducers customized to sperm retrieved from inbred mouse epididymis. *Biochem Biophys Res Commun*, 488, 273-277, 2017. DOI: 10.1016/J.BBRC.2017.04.152

40. Visconti PE: Understanding the molecular basis of sperm capacitation through kinase design. *Proc Natl Acad Sci U S A*, 106, 667-668, 2009. DOI: 10.1073/pnas.0811895106

41. López-Fernández C, Fernández JL, Gosálbez A, Arroyo F, Vázquez JM, Holt WV, Gosálvez J: Dynamics of sperm DNA fragmentation in domestic animals III. Ram. *Theriogenology*, 70, 898-908, 2008. DOI: 10.1016/j.theriogenology.2008.04.055

42. Alcay S, Ustuner B, Nur Z: Effects of low molecular weight cryoprotectants on the post-thaw ram sperm quality and fertilizing ability. *Small Ruminant Res*, 136, 59-64, 2016. DOI: 10.1016/J.SMALLRUMRES.2016.01.009

43. Alcay S, Toker MB Gokce E, Gul Z, Onder NT, Ustuner B, Nur Z, Sagirkaya H, Soylu MK: Improvement of incubation resilience with various antioxidants in cryopreserved ram semen. *Erciyes Üniv Vet Fak Derg,* 14 (3): 183-190, 2017.

Kafkas Univ Vet Fak Derg
25 (3): 299-303, 2019
DOI: 10.9775/kvfd.2018.20853

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Genotypic Identification of Lactic Acid Bacteria in Pastirma Produced with Different Curing Processes

Kübra ÇİNAR [1,a] Kübra FETTAHOĞLU [2,b] Güzin KABAN [2,c]

[1] Bayburt University, Faculty of Engineering, Department of Food Engineering, TR-69000 Bayburt - TURKEY
[2] Atatürk University, Faculty of Agriculture, Department of Food Engineering, TR-25100 Erzurum - TURKEY
[a] ORCID: 0000-0002-3715-8739; [b] ORCID: 0000-0002-9464-0660; [c] ORCID: 0000-0001-6720-7231

Article ID: KVFD-2018-20853 Received: 28.08.2018 Accepted: 04.12.2018 Published Online: 04.12.2018

How to Cite This Article

Çinar K, Fettahoğlu K, Kaban G: Genotypic identification of lactic acid bacteria in pastirma produced with different curing processes. *Kafkas Univ Vet Fak Derg,* 25 (3): 299-303, 2019. DOI: 10.9775/kvfd.2018.20853

Abstract

The lactic acid bacteria isolated from pastirma, produced under controlled conditions using two different curing temperatures (4°C or 10°C) and two different curing agents (150 mg/kg sodium nitrite or 300 mg/kg potassium nitrate), were subjected to genotypic (16S rRNA sequecing) identification. According to the identification results, 68 of 87 isolates (78.16%) was identified as *Pediococcus pentosaceus.* This species was followed by *P. acidilactici* (14.94%), *Lactobacillus sakei* (4.60%) and *L. plantarum* (2.30%), respectively. *P. pentosaceus* was dominant species in all curing applications (4°C/nitrate or nitrite or 10°C/nitrate or nitrite). Another species determined in all groups was *P. acidilactici.* While *L. plantarum* was only isolated from samples produced with nitrate (4°C or 10°C), *L. sakei* was isolated from samples produced with nitrite (4°C or 10°C). The effect of the curing agent on the biodiversity of lactic acid bacteria in pastirma was more effective than the curing temperature.

Keywords: *Pastirma, Nitrate, Nitrite, Pediococcus, Lactobacillus, 16S rRNA*

Farklı Kürleme İşlemleri İle Üretilen Pastırmada Laktik Asit Bakterilerinin Genotipik İdentifikasyonu

Öz

İki farklı kürleme sıcaklığı (4°C veya 10°C) ve iki farklı kürleme ajanı (150 mg/kg sodium nitrit veya 300 mg/kg potasyum nitrat) kullanılarak kontrollü şartlar altında üretilen pastırmadan izole edilen laktik asit bakterileri, genotipik (16S rRNA) identifikasyona tabi tutulmuştur. İdentifikasyon sonuçlarına göre, 87 izolatın 68'i (%78.16) *Pediococcus pentosaceus* olarak tanımlanmıştır. Bu türü sırasıyla *P. acidilactici* (%14.94), *Lactobacillus sakei* (%4.60) ve *L. plantarum* (%2.30) takip etmiştir. Tüm kürleme uygulamalarında (4°C/nitrat veya nitrit veya 10°C/nitrat veya nitrit), *P. pentosaceus* dominant türdür. Tüm gruplarda belirlenen diğer bir tür ise *P. acidilactici*'dir. *L. plantarum* yalnız nitrat (4°C veya 10°C) ile üretilen örneklerden identifiye edilirken, *L. sakei* yalnızca nitrit (4°C veya 10°C) kullanılarak üretilen örneklerde tanımlanmıştır. Pastırmada laktik asit bakterilerinin biyoçeşitliliği üzerine kürleme ajanının etkisi, kürleme sıcaklığınından daha etkili olmuştur.

Anahtar sözcükler: *Pastırma, Nitrat, Nitrit, Pediococcus, Lactobacillus, 16S rRNA*

INTRODUCTION

Pastirma, a traditional Turkish dry-cured meat product, is made from beef or water buffalo meat. Its production stages consist of curing, drying, pressing and çemen covering. The production of pastirma takes about one month and the heating or smoking stages do not include in the process [1]. Curing process is one of the most important stages in the production of pastirma. Product-type and process conditions are considered in the selection of curing agent in meat products [2]. European Parliament and Council Directive 2014/601/EC (section 08.3.1) allows use of 150 mg nitrite (ingoing amount)/kg and 150 mg nitrate (ingoing amount)/kg in non-heat-treated meat products [3]. In the same fashion, Turkish Food Codex Regulation on Food Additives (2013/28693) also allows the use of 150 mg/kg nitrite in non-heat-treated meat products while it forbids the use of nitrate in pastirma [4]. However, nitrate is the most commonly used curing agent in pastirma [1,2,5-9]. These curing agents are important additivies for cured

İletişim (Correspondence)
+90 442 2312425 Fax: +90 442 2315878
gkaban@atauni.edu.tr

meat products due to formation of color and flavor, antioxidant and antimicrobial properties [9,10]. On the other hand, nitrate must be converted to nitrite for observation of the expected effects from nitrate in the processes with nitrate [11].

In pastirma microbiota, Gr (+), catalase-positive cocci (coagulase-negative *Staphylococcus* and *Kocuria*) and lactic acid bacteria constitute two important microorganism groups [12]. Lactic acid bacteria are important microorganisms in terms of technological properties and food safety. They produce antimicrobial compounds such as bacteriocins and organic acids [13]. The number of lactic acid bacteria in the final product may vary depending on the process conditions of the enterprise where production is made because pastirma is produced by traditional methods. It is possible to face very low numbers during production performed under controlled conditions from raw material with quite a low number of initial microorganisms. On the other hand, it is also possible to encounter high numbers of lactic acid bacteria in pastirma although it is rare. Although the number of lactic acid bacteria varied between 10^4-10^8 cfu/g in some studies [14,15], counts varying between 10^3-10^6 cfu/g [16], 10^2-10^7 cfu/g [17], 10^3-10^7 cfu/g [18] were reported in other studies.

Various species belonging to *Lactobacillus, Enterococcus, Pediococcus, Leuconostoc and Weisella* genera were determined in studies for the isolation and identification of the lactic acid bacteria in pastirma [18-20]. There is a study on the biodiversity of lactic acid bacteria in pastirma under different curing processing, in which lactic acid bacteria were identified phenotypically using API CHL [21]. The aim of this study was to genotypically identify the isolates of lactic acid bacteria from pastirma produced with different curing agents and curing temperatures (4°C or 10°C).

MATERIAL and METHODS

Isolates

Eighty seven lactic acid bacteria isolates, obtained from pastirma produced under controlled conditions using two different curing temperatures (4°C or 10°C) and two different curing agents (150 mg/kg sodium nitrite or 300 mg/kg potassium nitrate) [21] were subjected to genotypic (16S rRNA sequecing) identification.

Genotypic Identification

A High Pure PCR template preparation kit (Roche, Indianapolis, IN) according to the manufacturer's protocol was used to isolation of genomic DNA. 16S rRNA coding region sequence was selected and amplified by PCR (TC-4000 Techne). Faststart High Fidelity PCR System dNTPack kit (Roche) was used in PCR and 27F (5'-AGAGTTTGATCM TGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGA CTT-3') universal primers were also used to amplify the 16S rRNA gene. The amplification program was initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1.5 min; and a final extension step at 72°C for 7 min. Cycle sequencing reaction products were purified with a Sephadex column. 16S rRNA sequence analysis of PCR products was carried out by the Macrogen company (Netherlands). The sequence results obtained were aligned with the NCBI database using the BLAST program *(http://blast.ncbi.nlm.nih.gov)*.

RESULTS

A total of 87 lactic acid bacteria isolates belonging to four different species and two different genera were identified. As a result of identification, 68 isolates were identified as *Pediococcus pentosaceus*, 13 isolates as *Pediococcus acidilactici*, 4 isolates as *Lactobacillus sakei* and 2 isolates as *Lactobacillus plantarum (Table 1)*. When they were evaluated on the basis of species, it was determined that 93.10% of the isolates consisted of two species belonging to *Pediococcus* genera, and 6.90% of the isolates consisted of two species belonging to *Lactobacillus*. According to the results, *P. pentesaceus* (78.16%) was found as dominant species in pastirma, and this species was followed by *P. acidilactici* (14.94%). The isolation ratios of *L. sakei* (4.60%) and *L. plantarum* (2.30%) species were quite low.

Twenty four isolates were identified in pastirma samples in which nitrite as the curing agent and the curing temperature of 4°C were used; 22 isolates were identified in pastirma samples in which nitrite as the curing agent and the curing temperature of 10°C were used. Twenty isolates were identified in pastirma samples in which nitrate as the curing agent and the curing temperature of 4°C were used; a total of 21 isolates were identified in pastirma samples in which nitrate was used at 10°C *(Table 2)*. Twenty isolates in the combination of nitrite and curing temperature of 4°C and 16 isolates in the combination of nitrite and curing temperature of 10°C were identified as *P. pentosaceus.* In the groups in which nitrate was used as the curing agent, 18 isolates at 4°C curing temperature and 14 isolates at 10°C curing temperature were identified as *P. pentosaceus (Table 2)*.

Four different species *(P. acidilactici, P. pentosaceus, L. plantarum, L. sakei)* both at 4°C curing temperature and at 10°C curing temperature were identified independently of the curing agent. The isolates were identified as *P. acidilactici, P. pentosaceus* and *L. sakei* in nitrite-curing process, while *P. acidilactici, P. pentosaceus* and *L. plantarum* were identified in the presence of nitrate. According to these results, *L. plantarum* was only identified in case of using nitrate, and *L. sakei* was only identified in the presence of nitrite.

DISCUSSION

In the studies conducted on commercially available pastirma

Table 1. *Genotypic identification results of lactic acid bacteria isolated from pastirma produced under different curing temperature and curing agents*

Isolate No	Code	Species	Number of Base	Identitiy (%)	Isolate No	Code	Species	Number of Base	Identitiy (%)	Isolate No	Code	Species	Number of Base	Identitiy (%)
1	K4	*P. pentosaceus*	1133	99	30	K52	*P. pentosaceus*	1100	100	59	K98	*P. acidilactici*	1011	100
2	K5	*P. pentosaceus*	1388	100	31	K53	*P. acidilactici*	1014	97	60	K99	*P. acidilactici*	1268	10
3	K6	*P. pentosaceus*	1258	100	32	K54	*P. pentosaceus*	1114	99	61	K100	*P. acidilactici*	1155	100
4	K7	*P. pentosaceus*	1169	99	33	K55	*P. pentosaceus*	1025	100	62	K101	*P. pentosaceus*	1054	98
5	K8	*P. pentosaceus*	1141	99	34	K56	*P. pentosaceus*	1016	100	63	K102	*P. pentosaceus*	1077	100
6	K9	*P. pentosaceus*	1136	100	35	K57	*P. acidilactici*	1035	99	64	K103	*L. sakei*	1074	98
7	K10	*P. pentosaceus*	1123	100	36	K58	*P. acidilactici*	876	99	65	K104	*P. pentosaceus*	1110	99
8	K13	*P. pentosaceus*	1048	100	37	K59	*P. acidilactici*	1120	100	66	K105	*P. pentosaceus*	1153	99
9	K14	*P. pentosaceus*	1267	100	38	K60	*P. acidilactici*	1075	100	67	K106	*P. pentosaceus*	1062	100
10	K15	*P. pentosaceus*	1052	100	39	K61	*P. pentosaceus*	1158	100	68	K107	*P. pentosaceus*	1045	100
11	K21	*P. pentosaceus*	1284	100	40	K62	*P. acidilactici*	1019	100	69	K108	*L. sakei*	1098	99
12	K22	*P. pentosaceus*	1169	100	41	K63	*P. acidilactici*	1113	98	70	K109	*L. sakei*	1127	99
13	K23	*P. pentosaceus*	1297	100	42	K64	*P. acidilactici*	1254	100	71	K110	*P. pentosaceus*	1240	100
14	K24	*P. pentosaceus*	1086	100	43	K66B	*P. pentosaceus*	1266	100	72	K111	*L. sakei*	994	100
15	K31	*P. pentosaceus*	1143	10	44	K66S	*P. pentosaceus*	1144	99	73	K112	*P. pentosaceus*	919	100
16	K32	*P. pentosaceus*	1109	97	45	K67	*P. pentosaceus*	1045	100	74	K113	*P. pentosaceus*	1139	100
17	K33	*P. pentosaceus*	989	99	46	K72	*L. plantarum*	1076	99	75	K114	*P. pentosaceus*	1109	99
18	K34	*P. pentosaceus*	1048	99	47	K73	*L. plantarum*	934	99	76	K115	*P. pentosaceus*	1069	98
19	K35	*P. pentosaceus*	1126	99	48	K74	*P. pentosaceus*	1046	100	77	K116	*P. pentosaceus*	1041	100
20	K36	*P. pentosaceus*	1113	99	49	K75	*P. pentosaceus*	1217	100	78	K117	*P. pentosaceus*	1029	99
21	K37	*P. pentosaceus*	1288	100	50	K76	*P. pentosaceus*	1034	100	79	K118	*P. pentosaceus*	1190	99
22	K38	*P. pentosaceus*	1061	100	51	K79	*P. pentosaceus*	1072	99	80	K119	*P. acidilactici*	1188	99
23	K39	*P. pentosaceus*	1214	100	52	K81	*P. pentosaceus*	1109	100	81	K120	*P. pentosaceus*	1155	99
24	K40	*P. pentosaceus*	1089	100	53	K82	*P. pentosaceus*	1041	100	82	K121	*P. pentosaceus*	1106	99
25	K41	*P. pentosaceus*	1185	100	54	K83	*P. acidilactici*	1132	99	83	K122	*P. pentosaceus*	1143	100
26	K42	*P. pentosaceus*	1087	99	55	K85	*P. pentosaceus*	1204	100	84	K124	*P. pentosaceus*	1177	99
27	K44	*P. pentosaceus*	1022	99	56	K86	*P. pentosaceus*	1123	99	85	K127	*P. pentosaceus*	1023	100
28	K45	*P. pentosaceus*	1004	100	57	K87	*P. pentosaceus*	1235	100	86	K128	*P. pentosaceus*	1259	100
29	K51	*P. pentosaceus*	1145	100	58	K97	*P .pentosaceus*	1041	100	87	K129	*P. pentosaceus*	1090	100

Table 2. *Diversity and prevalence of lactic acid bacteria isolated and genetically identificated from pastirma produced under different curing temperature and curing agents*

Isolates	Curing Temperature								Total Number of Isolates (%)
	4°C				10°C				
	Nitrate		Nitrite		Nitrate		Nitrite		
	Isolates	%	Isolates	%	Isolates	%	Isolates	%	
P. pentosaceus	18	90	20	83.33	14	66.67	16	72.72	68 (78.16)
P. acidilactici	1	5.0	3	12.50	6	28.57	3	13.64	13 (14.94)
L. sakei	-	-	1	4.17	-	-	3	13.64	4 (4.60)
L. plantarum	1	5.0	-	-	1	4.76	-	-	2 (2.30)
Total	**20**	**100**	**24**	**100**	**21**	**100**	**22**	**100**	**87 (100)**

samples, quite different numbers of lactic acid bacteria were determined [14-16,18]. On the other hand, in a study investigating the effect of different levels of sodium and potassium nitrate on the quality properties of pastirma, it was found that the use and levels of sodium or potassium nitrate did not cause any differences in the microbiological properties of the product [22]. *Pediococcus* spp. which are dominantly available in experimental pastirma were also found in samples taken from the market. Sınmaz et al.[18] identified 5.7% of the lactic acid bacteria isolated from pastirma as *P. pentosaceus* and 4.7% of them as *P. acidilactici*. Dinçer and Kıvanç [20] isolated *P. acidilactici* as well as *L. plantarum*, *L. sakei* and *Enterococcus faecium* from pastirma. However, *Pediococcus* species were not found in pastirma samples in the study carried out by Özdemir and Siriken [19]. In another study on lactic acid bacteria isolated from pastirma produced with different curing condition, 72.41% of the isolates were phenotypically identified as *P. pentosaceus* [21]. Similar result (78.16%) was also observed in the present study where genotypic identification was used.

Pediococci are homofermentative microorganisms and they are important microorganisms for food microbiology in terms of being tolerant to salt and also developing in a wide temperature range [23,24]. It is stated that the optimum growth temperature of pediococci varies between 25 and 40°C [25]. In addition, it is also indicated that some species show a wide tolerance against pH and salt as well as temperature [26]. In this present study, it is thought that pediococci show tolerance to salt at the curing and drying stages, and could survive at later stages.

In this present study, *L. plantarum* and *L. sakei* were also identified. These species belonging to *Lactobacillus* genera are facultative heterofermentative and produce a high proportion of lactic acid by following Embden-Meyerhoff-Parnas (EMP) called glycolysis [27]. Özdemir and Siriken [19] identified 5 isolates from 40 isolates which were isolated from pastirma by them as *L. plantarum* and 8 isolates as *L. curvatus*. Dinçer and Kıvanç [20] identified *L. plantarum* as the dominant species as a result of the biochemical tests on 92 isolates obtained from pastirma samples by using API50 CH. Çinar [21] reported that *L. sakei* was not determined in pastirma sample, while *L. plantarum* (2.30%), *L. curvatus* (2.30%), *L. brevis* (2.30%) and *L. collinoides* (1.15%) were isolated from pastirma samples. In the present study, *Lactobacillus* isolates were identified as *L. sakei* and *L. plantarum*. Sınmaz et al.[18] identified *L. plantarum* only in one sample and *L. paraplantarum* in another sample as a result of 16S rRNA sequence analysis on 14 pastirma samples. In the same study, *L. curvatus* was identified in two samples and it was stated that *L. sakei* was the dominant species.

As a conclusion, *P. pentosaceus* was the dominant species in pastirma under different curing conditions. In addition, the effect of the curing agent on the biodiversity of lactic acid bacteria in pastirma was more effective than the curing temperature. The species of *P. acidilactici, P. pentosaceus, L. sakei* and *L. plantarum* were detected both at 4°C and at 10°C. However, *L. plantarum* was only identified from pastirma cured with nitrate, and *L. sakei* was only identified from the samples cured with nitrite.

REFERENCES

1. Akköse A, Kaban G, Karaoğlu MM, Kaya M: Characteristics of pastırma types produced from water buffalo meat. *Kafkas Univ Vet Fak Derg*, 24 (2): 179-185, 2018. DOI: 10.9775/kvfd.2017.18551

2. Gökalp HY, Kaya M, Zorba Ö: Et Ürünleri İşleme Mühendisliği. Atatürk Üniversitesi Yayın No: 320, 3. Baskı, Erzurum, Türkiye, 2002.

3. European Commission Regulation (EU) no 601/2014 of 4 June 2014 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council as regards the food categories of meat and the use of certain food additives in meat preparations. *Official Journal of the European Union,* 11-166, 2014.

4. Turkish Food Codex Regulation on Food Additives: Republic of Turkey Ministry of Agriculture and Forestry, Official Gazette Issue and Date: 28693 and 30 June 2013, Ankara, Turkey, 2013.

5. Anıl N: Türk pastırması: Modern yapım tekniğinin geliştirilmesi ve vakumla paketlenerek saklanması. *Selçuk Üniv Vet Fak Derg*, 4 (1): 363-375, 1988.

6. Tekinşen OC, Doğruer Y: Her Yönüyle Pastırma. Selçuk Üniversitesi Basımevi, 25-28, Konya, 2000.

7. Doğruer Y, Güner A, Gürbüz Ü, Uçar G: Sodyum ve potasyum nitratın üretim periyodu süresince pastırmanın kalitesine etkisi. *Turk J Vet Anim*

Sci, 27, 805-811, 2003.

8. Kaban G: Changes in the composition of volatile compounds and in microbiological and physicochemical parameters during pastırma processing. *Meat Sci*, 82, 17-23, 2009. DOI: 10.1016/j.meatsci.2008.11.017

9. Akköse A, Ünal N, Yalınkılıç B, Kaban G, Kaya M: Volatile compounds and some physico chemical properties of pastırma produced with different nitrate levels. *Asian-Australas J Anim Sci,* 30 (8): 1168-1174, 2017. DOI: 10.5713/ajas.16.0512

10. Büyükünal SK, Şakar FŞ, Turhan İ, Erginbaş Ç, Sandıkçı Altunatmaz S, Yılmaz Aksu F, Yılmaz Eker F, Kahraman T: Presence of *Salmonella* spp., *Listeria monocytogenes, Escherichia coli* O157 and nitrate-nitrite residue levels in Turkish traditional fermented meat products (sucuk and pastırma). *Kafkas Univ Vet Fak Derg*, 22 (2): 233-236, 2016. DOI: 10.9775/kvfd.2015.14238

11. Honikel KO: Principles of curing. **In,** Tolda F (Ed): Handbook of Fermented Meat and Poultry. 17-30, Blackwell Publishing, UK, 2008.

12. Kaban G: Sucuk and pastırma: Microbiological changes and formation of volatile compounds. *Meat Sci*, 95, 912-918, 2013. DOI: 10.1016/j.meatsci.2013.03.021

13. Sezer Ç, Güven A: Investigation of bacteriocin production capability of lactic acid bacteria isolated from foods. *Kafkas Univ Vet Fak Derg,* 15 (1): 45-50, 2009. DOI:10.9775/kvfd.2008.56-A

14. El-Khateib T, Schmidt U, Leistner L: Mikrobiologisch estabilität von Türkischer pastırma. *Fleischwirtsch,* 67 (1): 101-105, 1987.

15. Özdemir H, Sireli UT, Sarımehmetoğlu B, Inat G: Ankara'da tüketime sunulan pastırmalarda mikrobiyal floranın incelenmesi. *Turk J Vet Anim Sci*, 23 (Suppl. 1): 57-62,1999.

16. Kaban G, Kaya M: Pastırmadan katalaz pozitif kokların izolasyonu ve identifikasyonu. *Türkiye 9. Gıda Kongresi,* 24-26 Mayıs, Bolu, 2006.

17. Aksu Mİ, Kaya M: Erzurum piyasasında tüketime sunulan pastırmaların bazı fiziksel, kimyasal ve mikrobiyolojik özellikleri. *Turk J Vet Anim Sci*, 25, 319-326, 2001.

18. Oz E, Kaban G, Barış Ö, Kaya M: Isolation and identification of lactic acid bacteria from pastırma. *Food Cont*, 77, 158-162, 2017. DOI: 10.1016/j.foodcont.2017.02.017

19. Özdemir H, Siriken B: Pastırmalardan izole edilen laktobasillerin bazı biyokimyasal ve fizyolojik özellikleri. *10. KÜKEM Kongresi,* 20 (3): 74-75, 1997.

20. Dinçer E, Kıvanç M: Characterization of lactic acid bacteria from Turkish pastirma. *Annal Microbiol,* 62 (3): 1155-1163, 2012. DOI: 10.1007/s13213-011-0355-x

21. Çinar K: Farklı kürleme sıcaklıkları ve farklı kürleme ajanları kullanılarak üretilen pastırmaların laktik asit bakteri florası ve diğer bazı özellikleri. *Yüksek Lisans Tezi*, Atatürk Üniversitesi, Fen Bilimleri Enstitüsü, 2014.

22. Doğruer Y, Yalçın S, Gürbüz U, Güner A: Sodyum ve potasyum nitratın depolama süresince pastırmanın kalitesine etkisi. *Vet Bilim Derg*, 17 (4): 37-42, 2001.

23. Turantaş F: Fermentasyonda rol oynayan mikroorganizmalar. **In,** Ünlütürk A, Turantaş F (Eds): Gıda Mikrobiyolojisi. 425-445, Mengi Tan Basımevi, Çınarlı-İzmir,1999.

24. Ayhan K: Gıdalarda bulunan mikroorganizmalar. Gıda Mikrobiyolojisi ve Uygulamaları. Bölüm 2, 48, Sim Matbaacılık Ltd. Şti., Ankara, 2000.

25. Raccach M: *Pediococcus*. **In,** Batt CA, Tortorello ML (Eds): Encyclopedia of Food Microbiology. 1-6, Elsevier Ltd., USA, 2014.

26. Stiles ME, Holzapfel WH: Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol,* 36, 1-29,1997. DOI: 10.1016/S0168-1605(96)01233-0

27. Kaban G, Kaya M, Lücke FK: Meat starter cultures. **In,** Encyclopedia of Biotechnology in Agriculture and Food. 1-4, Taylor and Francis, New York, 2012.

Kafkas Univ Vet Fak Derg
25 (3): 305-309, 2019
DOI: 10.9775/kvfd.2018.20864

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Associations Between Forkhead Box L2 Expression and Ovary Development in Laying Hens

Shouping ZHANG [1,2,a] Xiaojing XIA [1,b] Lirong WANG [1,c] Renfeng LI [1,d] Meng YANG [1] Sanhu WANG [1,e]

[1] College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, CHINA
[2] Postdoctoral Research Base, Henan Institute of Science and Technology, Xinxiang 453003, CHINA
[a] ORCID: 0000-0003-1774-1924; [b] ORCID: 0000-0001-7717-8411; [c] ORCID: 0000-0003-3833-4703; [d] ORCID: 0000-0003-4101-5922
[e] ORCID: 0000-0002-1300-3185

Article ID: KVFD-2018-20864 Received: 29.08.2018 Accepted: 15.02.2019 Published Online: 16.02.2019

How to Cite This Article

Zhang S, Xia X, Wang L, Li R, Yang M, Wangs S: Associations between forkhead box L2 expression and ovary development in laying hens. *Kafkas Univ Vet Fak Derg,* 25 (3): 305-309, 2019. DOI: 10.9775/kvfd.2018.20864

Abstract

Make sure healthy ovary or follicle is critical for extending egg laying performance in poultry. Transcription factor forkhead box L2 (FOXL2) gene have key role in regulate development of ovary. In the present research, different aged Hy-line Brown hens were maintained to explore relationships between ovarian developing and FOXL2 expression. Through histological observation, different quantities of follicles from various phases of age were observed. It was displayed that FOXL2 expression and number mature follicle were increased as the days of age increased and then decreased. In comparison, the expression of FOXL2 in hypothalamus and eyelid were remained in a relative stable level. Taken together, these data in our research establish a framework for understanding the potential functions of FOXL2 in regulate chicken ovarian developing and may provide a new perspective on the theory and practice to increase egg production or others.

Keywords: *FOXL2, Follicle, Ovary, Laying, Poultry*

Yumurtacı Tavuklarda Ovaryum Gelişimi İle Forkhead Box L2 Ekspresyonu Arasındaki İlişki

Öz

Kanatlılarda yumurtlama performansını artırmada sağlıklı ovaryum veya foliküller kritik öneme sahiptir. Transkripsiyon faktörü forkhead box L2 (FOXL2) geni, ovaryum gelişimini düzenlemede anahtar rol oynamaktadır. Bu araştırmada, ovaryum gelişimi ile FOXL2 ekspresyonu arasındaki ilişkiyi araştırmak için farklı yaşlarda Hy-line Brown tavuklar kullanılmıştır. Histolojik olarak, değişik yaş evrelerindeki folikül miktarları incelendi. FOXL2 ekspresyonu ve olgunlaşmış folikül sayısının, yaşla birlikte arttığı ve sonrasında ise azaldığı gözlemlenmiştir. Hipotalamus ve gözkapağında FOXL2 ekspresyonu karşılaştırıldığında göreceli olarak sabit kaldıkları belirlenmiştir. Sonuçlar birlikte değerlendirildiğinde, çalışmada elde edilen veriler tavuklarda ovaryum gelişimini düzenlemede FOXL2'nin muhtemel fonksiyonunu anlamada bir çerçeve oluşturabilir, teorik ve uygulamada yumurta üretimini artırmada yeni bir bakış açısı geliştirmeye yardımcı olabilir.

Anahtar sözcükler: *FOXL2, Folikül, Ovaryum, Yumurtacı, Kanatlı*

INTRODUCTION

As egg laying poultry, the most important economic trait is egg production, which is influenced by a variety of factors, including genetics, nutrition, and environment conditions (such as light time and intensity). All of these factors influence egg production could be attributed to affecting ovarian development. The ovarian development of chickens starts from the early stage of embryos and continues until the end of the whole reproductive cycle. And the growth, maturation, and differentiation of follicles in ovary were regulated under the synergistic of local regulatory factors (growth factors and cytokines) and/or exogenous hormones (follicle stimulating hormone and luteinizing hormone) [1,2].

FOXL2 is the first discovered factors that participate in ovarian development in a variety of animals such as mammals, birds, reptiles and fish. And it's a highly conserved gene that continuously expressed in mammalian ovaries

İletişim (Correspondence)
+86 0373 3040718
vet_sanhu@sina.com

from sexual differentiation to adulthood especially in granulosa cells. In addition to causing Blepharophimosis-ptosis-epicanthus-inversus syndrome BPES, mutations in FOXL2 cause many ovarian diseases. Over 95% granulosa cell tumors (GCTs) are associated with abnormal expression of FOXL2 [3,4]. In many animals, FOXL2 directly activates the transcription of CYP19A1 to regulate estrogen synthesis [5,6]. FOXL2 is a molecular marker of the early mammalian ovary, which begins to express in the mouse embryo of 12.5 d old and has female specificity. Further studies identified FOXL2 mice cannot form primordial follicles that became sterile [7,8]. These data demonstrated that FOXL2 plays an important role in the process of sex differentiation and and ovarian granulosa cell differentiation.

Although the function of FOXL2 has been well studied in mammals, it remains unclear in chicken. Studies in chicken embryos have found that FOXL2 and CYP19A1 are both female-specific and the expression patterns are highly correlated. The expression of FOXL2 was decreased in chicken embryos supplemented with CYP19A1 inhibitors, but did not completely disappear or have obvious sexual reversal, while over expression of CYP19A1, the expression of FOXL2 was increased [9,10]. Therefore, it can be speculated that during embryonic period, FOXL2 has a certain interaction with aromatase and participates in the sex determination of chicken embryo gonads. On the other side, a non-synonymous replacement of FOXL2 SNP A238G causes isoleucine 77-proline mutations associated with egg production and egg weight in Chinese Big Bone Chicken, and FOXL2 can enhance the regulate role of GDF9 in pre-follicular cells proliferation [11,12]. However, there is no systematic study on the role of FOXL2 in the development of chicken embryo gonads or in the development of adult ovarian follicles.

The laying performance of hens is a compelling problem in poultry production, and egg production performance is closely related to the development of hen ovary. Ovarian development is a dynamic process that continues the entire process of female reproductive life. FOXL2 has been considered as a key factor in controlling normal reproductive physiology in mammals. Therefore, we identified FOXL2 as a candidate gene for controlling ovarian development in poultry, and systematically studied its role in the development of poultry ovary. In this research, we will compare the expression of FOXL2 and follicle developing at different age in egg-laying hens that further provide a suggestion for extending egg production stage.

MATERIAL and METHODS

Ethics Statement

Experimentation with animals was approved by the Experimental animal management methods of Xinxiang Medical University (Approval number 201206078) and followed the Regulations of Experimental Animals of Henan Authority.

Animals and Sample Collection

Several different-aged Hy-Line Brown hens were purchased from Siqing chicken farm (Xinxiang, China) and maintained on open floor space under free food and water intake. Chickens were divided into 8 groups according to their age and 3 chickens in each group (30 d, 60 d, 90 d, 120 d, 160 d, 220 d, 330 d, 480 d). At times, the hens was selected and euthanized by decapitation. Ovary was removed and weighted at different age to investigate the follicle development of hens. At the same time, different stage follicles in ovary were counted. While other organ such as hypothalamus and eyelid were excised at the indicated ages. Scissors cut appropriate size of organ (50-100 mg) and stored in liquid nitrogen until used for RNA extraction.

Histopathological Analysis

A part of ovary (1 cm^3) were removed and fixed with 4% neutral formalin at room temperature for 48 h. Serial tissue sections were cut to 5-μm thickness after embedding in paraffin. Each slide was stained with hematoxylin and eosin (H&E) and then examined by light microscopy (Olympus BX41, Olympus Optical Co., Tokyo, Japan).

Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was prepared from 10 mg of collected organ homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNaseI-treated RNA (0.2 μg) was reverse-transcribed into cDNA via an EasyScript First-Strand cDNA Synthesis Super Mix (TransGen Biotech, Beijing, China). The following primers were used in the qPCR: Foxl2 forward primer, 5′-CTACT CCTACGTGGCCCTGA-3′, and reverse primer, 5′-TGATGAAG CACTCGTTGAGG-3′; β-actin forward primer, 5′-AGTACCCC ATTGAACACGGT-3′, and reverse primer, 5′-ATACATGGCT GGGGTGTTGA-3′. The reaction was run on a 7500 thermal cycler (Applied Biosystems) with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 40 s. The expression of FOXL2 was determined using the relative quantification method and normalized to control using the $2^{-\Delta\Delta CT}$ method with β-actin as an internal standard.

Statistical Analysis

Data are expressed as means ± standard error (SE). Differences in variability among different groups were determined by one-way tests of variance using the GraphPad Prism (version 6.0; GraphPad Software, San Diego, CA, USA); statistical significance was set at $P<0.05$.

RESULTS

It was demonstrated that the weight of ovary was obviously

increased as the days of age increasing and reached a peak value until 220 d and then began a slow decline during aging (30, 60, 90, 120, 160, 220, 330, 480) *(Fig. 1)*. Before age of 160 d, we could hardly see any mature egg in ovary.

An obvious different morphology of follicle was observed in different age's hens by using HE staining. To further explore the relationships between laying and ovary weight, the histopathological features of the different aged ovary are shown in *Fig. 2*. Histologically, the normal ovarian cavities were infiltrated with small homogeneous follicles in low-aged chicken. While the higher aged hens contained with a comparatively high number of primordial and early follicles. To further deeply exploit follicle developing kinetics, the number of primary follicles and secondary follicles were detected according to the follicle diameter. The table exhibit secondary follicles number was obviously increased as the days of age increasing and reached a peak value until 160 d and then began a slow decline between different ages *(Table 1)*. Above these data, it was exhibited that the development of ovary has a relationship with hens' age.

Many researchers reported that FOXL2 is one of the most important sex determination genes. To examine the role of FOXL2 on development of follicle or egg production, analyses of its expression in ovary were performed by real-time PCR on different age's hens. As shown in *Fig.2a*, the expression of FOXL2 was up-regulated and reached a peak value at 120 d, thereafter with a continuous down-regulated expression. To further confirm its regulated role, the expression of FOXL2 in eyelid and hypothalamus were also detected *(Fig. 3)*. Different from in ovary, FOXL2 expression was maintained at a constant level. In hypothalamus, FOXL2 high expression kinetics started earlier than 60 d, and sustained a peak value at 60-120 d, and there after returning to near basal levels at high age. Based

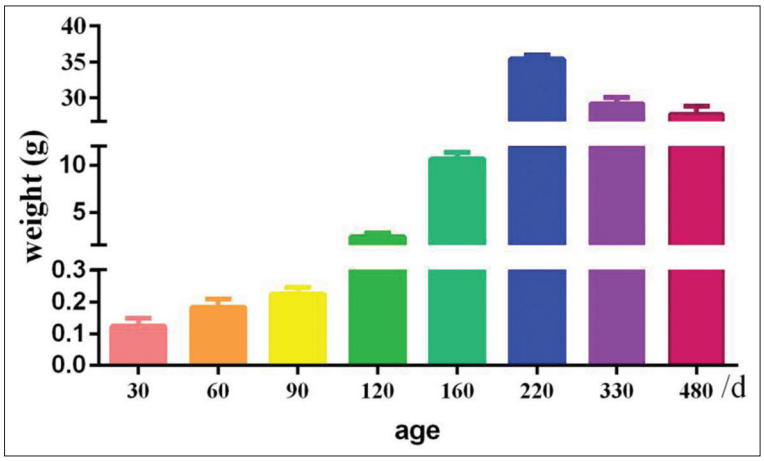


Fig 1. Weight of ovary. The weight of ovary were measured at indicated age. (N= 3)

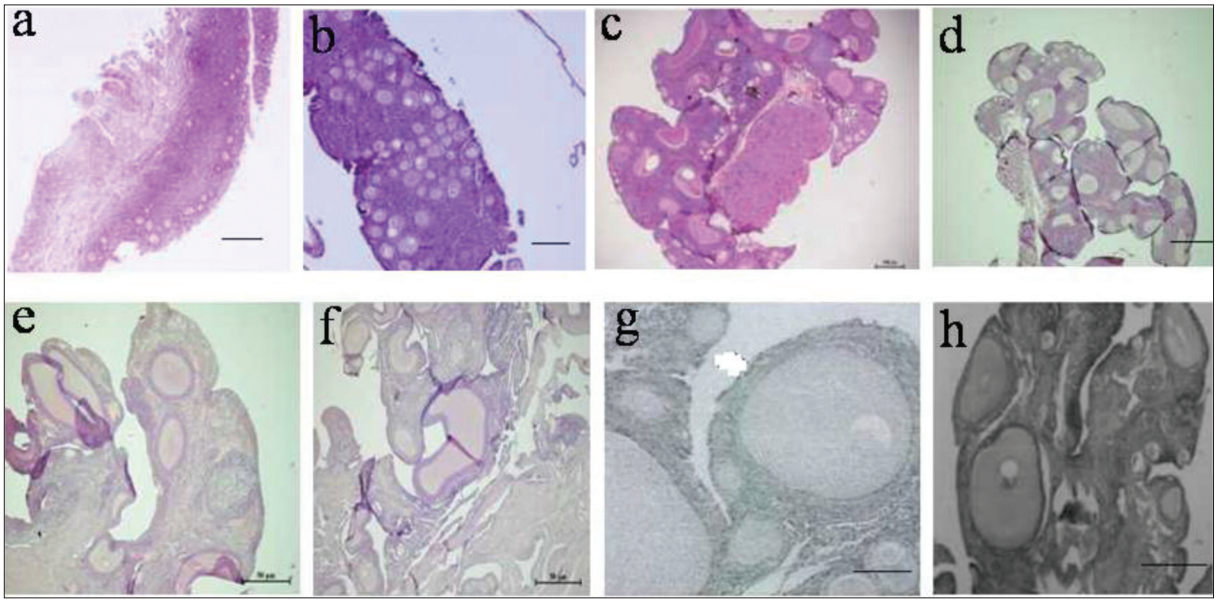


Fig 2. Ovary histopathology at day indicated age. Ovary were subjected to H&E staining. a:30d; b:60d; c:90d; d:120d; e:160d; f:220d; g:330d; h:480d

Table 1. *Diameter of follicle in ovary*

Age	Primary Follicle	Secondary Follicle	Shrinkage of Secondary Follicle
30	88165.27±4252.37	648.55±866.71	1.57±0.34
60	78231.87±6106.40	5595.47±886.64	2.07±0.64
90	62820.34±11434.69	7553.120±2302.44	2.53±0.65
120	63018.30±10068.41	8684.20±1875.39	2.48±0.53
160	61380.10±7743.69	9868.10±1206.37	2.60±0.50
220	61380.10±7743.69	8168.70±1306.33	3.30±0.49
330	56230.20±8206.77	7168.80±1236.19	3.60±0.59
480	40724.10±3240.64	5164.65±1024.26	5.12±0.84

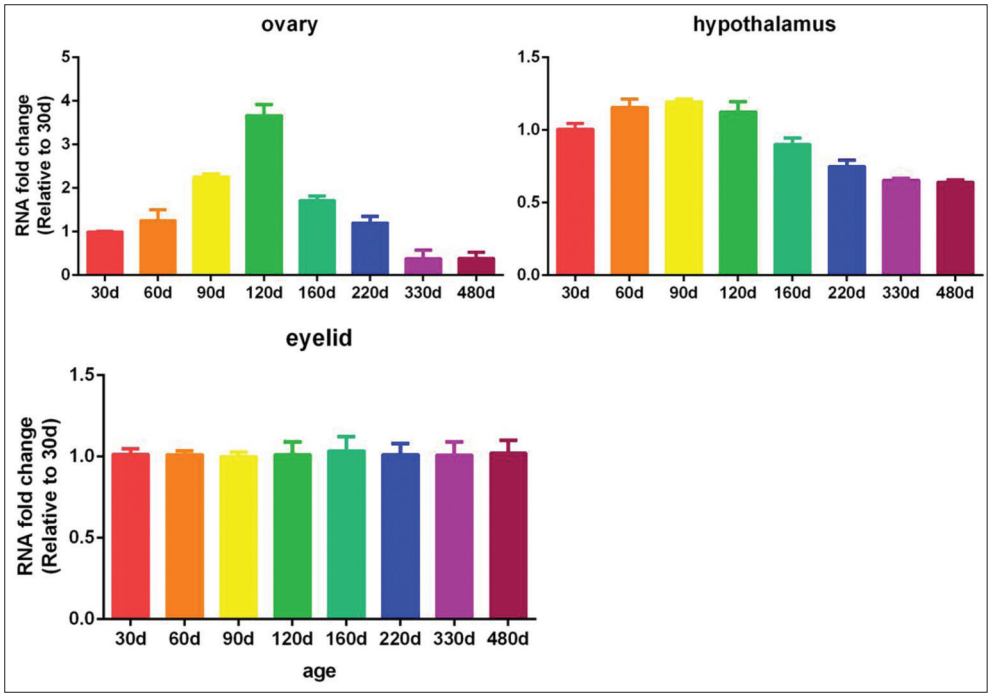


Fig 3. The expression of FOXL2 gene in different organ. The mRNA levels of FOXL2 in the ovary, eyelid, hypothalamus at indicated age were determined by real-time PCR (N=3)

on these data, it's suggested that a relationship between FOXL2 and ovarian developing.

DISCUSSION

Ovarian reserve is a critical factor affecting the function of ovary in human being. At present, the ovarian reserve function is usually evaluated based on the age, the number of follicles. Age was the only independent factor that affected the ovarian reserve, the number of follicle and high quality embryo could be reduced with advancing age [13]. Therefore, the physiological age of women is sometimes not exactly compatible with ovarian reserve function. FOXL2 is an important regulator in early stage of human ovarian differentiation and involved in the proliferation and differentiation of granulosa cells. Studies have shown that the expression level of FOXL2 in ovarian granulosa cells is negatively correlated with serum basal FSH, indicating that the expression level of FOXL2 in luteinized granulosa cells decreases with the increase of FSH, suggesting that the decrease of FOXL2 mRNA expression may reflect the decrease of ovarian reserve function [14]. Fuhrer et al.[15] reported that FOXL2 may have an anti-follicular apoptosis effect, and the reduced expression of FOXL2 may promote apoptosis of follicles and decrease the number of follicles in the ovary, leading to a decrease in ovarian reserve function. Therefore, FOXL2 can be used as a direct indicator of ovarian reserve function.

Similarly, there are currently no specific markers for independent evaluation of ovarian reserve function and ovarian response in laying hens. The follicular development process of mature ovary in poultry is different from that of mammals and has priority characteristics. The expression level of FOXL2 is up-regulated during this process, indicating that it has a certain effect on the differentiation of granulosa cells during chicken follicle selection [16]. The results were consistent with the results of Govoroun et al.[17] and Qin et al.[12]. Thus, similar to the role in mammals, FOXL2 may also affect follicular development

by participating in the regulation of the function of ovarian granulosa cells in sexually mature chicken ovaries. In our study, we aimed to explore the relationship between FOXL2 and ovarian developing in Hy-Line chicken. From our data, with growth of age, the expression of FOXL2 in ovarian was increased, and then slowly decreased. By comparison, the expression of FOXL2 in hypothalamus and eyelid was not change very much over time. Our data was identical with other research that expression of FOXL2 has critical roles in the regulation of hen ovarian development and may be used as a indicator.

In summary, this experiment demonstrated that FOXL2 plays an important role in the development of chicken ovary, but its specific regulatory mechanism needs further research. In depth study of the gene regulatory network of chicken ovary development and candidate key genes can provide ideas for understanding the regulation mechanism of chicken follicle development, and provide a theoretical basis for genetic improvement of chicken laying performance.

Acknowledgements

This work was supported by the Research Program of Henan province Basic and Advanced Technology (No. 132300410014) and 60th General Financial Grant from the China Postdoctoral Science Foundation (No. 2016M602243).

REFERENCES

1. Hsueh AJW, Kawamura K, Cheng Y, Fauser BCJM: Intraovarian control of early folliculogenesis. *Endocr Rev,* 36 (1): 1-24, 2015. DOI: 10.1210/er.2014-1020

2. Liu C, Peng J, Matzuk MM, Yao HH: Lineage specification of ovarian theca cells requires multicellular interactions via oocyte and granulosa cells. *Nat Commun,* 6:6934, 2015. DOI: 10.1038/ncomms7934

3. Geiersbach KB, Jarboe EA, Jahromi MS, Baker CL, Paxton CN, Tripp SR, Schiffman JD: FOXL2 mutation and large-scale genomic imbalances in adult granulosa cell tumors of the ovary. *Cancer Genet,* 204 (11): 596-602, 2011. DOI: 10.1016/j.cancergen.2011.10.002

4. Shah SP, Kobel M, Senz J, Morin RD, Clarke BA, Wiegand KC, Leung G, Zayed A, Mehl E, Kalloger SE, Sun M, Giuliany R, Yorida E, Jones S, Varhol R, Swenerton KD, Miller D, Clement PB, Crane C, Madore J, Provencher D, Leung P, DeFazio A, Khattra J, Turashvili G, Zhao Y, Zeng T, Glover JN, Vanderhyden B, Zhao C, Parkinson CA, Jimenez-Linan M, Bowtell DD, Mes-Masson AM, Brenton JD, Aparicio SA, Boyd N, Hirst M, Gilks CB, Marra M, Huntsman DG: Mutation of FOXL2 in granulosa-cell tumors of the ovary. *N Engl J Med,* 360 (26): 2719-2729, 2009. DOI: 10.1056/NEJMoa0902542

5. Park M, Shin E, Won M, Kim JH, Go H, Kim HL, Ko JJ, Lee K, Bae J: FOXL2 interacts with steroidogenic factor-1 (SF-1) and represses SF-1-induced CYP17 transcription in granulosa cells. *Mol Endocrinol,* 24 (5): 1024-1036, 2010. DOI: 10.1210/me.2009-0375

6. Zhang X, Li M, Ma H, Liu X, Shi H, Li M, Wang D: Mutation of foxl2 or cyp19a1a results in female to male sex reversal in XX Nile Tilapia. *Endocrinology,* 158 (8): 2634-2647, 2017. DOI: 10.1210/en.2017-00127

7. Ottolenghi C, Omari S, Garcia-Ortiz JE, Uda M, Crisponi L, Forabosco A, Pilia G, Schlessinger D: Foxl2 is required for commitment to ovary differentiation. *Hum Mol Genet,* 14 (14): 2053-2062, 2005. DOI: 10.1093/hmg/ddi210

8. Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, Treier M: The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development,* 131 (4): 933-942, 2004. DOI: 10.1242/dev.00969

9. Ayers KL, Lambeth LS, Davidson NM, Sinclair AH, Oshlack A, Smith CA: Identification of candidate gonadal sex differentiation genes in the chicken embryo using RNA-seq. *BMC Genomics,* 16:704, 2015. DOI: 10.1186/s12864-015-1886-5

10. Lambeth LS, Cummins DM, Doran TJ, Sinclair AH, Smith CA: Overexpression of aromatase alone is sufficient for ovarian development in genetically male chicken embryos. *PLoS One,* 8 (6): e68362, 2013. DOI: 10.1371/journal.pone.0068362

11. Qin N, Fan XC, Xu XX, Tyasi TL, Li SJ, Zhang YY, Wei ML, Xu RF: Cooperative effects of FOXL2 with the members of TGF-beta superfamily on FSH receptor mRNA expression and granulosa cell proliferation from hen prehierarchical follicles. *PLoS One,* 10 (10): e0141062, 2015. DOI: 10.1371/journal.pone.0141062

12. Qin N, Liu Q, Zhang YY, Fan XC, Xu XX, Lv ZC, Wei ML, Jing Y, Mu F, Xu RF: Association of novel polymorphisms of forkhead box L2 and growth differentiation factor-9 genes with egg production traits in local Chinese Dagu hens. *Poult Sci,* 94 (1): 88-95, 2015. DOI: 10.3382/ps/peu023

13. Steiner AZ, Pritchard D, Stanczyk FZ, Kesner JS, Meadows JW, Herring AH, Baird DD: Association between biomarkers of ovarian reserve and infertility among older women of reproductive age. *JAMA,* 318 (14): 1367-1376, 2017. DOI: 10.1001/jama.2017.14588

14. Yong PYK, Baird DT, Thong KJ, McNeilly AS, Anderson RA: Prospective analysis of the relationships between the ovarian follicle cohort and basal FSH concentration, the inhibin response to exogenous FSH and ovarian follicle number at different stages of the normal menstrual cycle and after pituitary down-regulation. *Hum Reprod,* 18 (1): 35-44, 2003.

15. Fuhrer D: Lessons from studies of complex genetic disorders: identification of FOXL2 - A novel transcription factor on the wing to fertility. *Eur J Endocrinol,* 146 (1): 15-18, 2002.

16. Wang J, Zhao C, Li J, Feng Y, Gong Y: Transcriptome analysis of the potential roles of FOXL2 in chicken pre-hierarchical and pre-ovulatory granulosa cells. *Comp Biochem Physiol Part D Genomics Proteomics,* 21, 56-66, 2017. DOI: 10.1016/j.cbd.2016.12.003

17. Govoroun MS, Pannetier M, Pailhoux E, Cocquet J, Brillard JP, Couty I, Batellier F, Cotinot C: Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. *Dev Dyn,* 231 (4): 859-870, 2004. DOI: 10.1002/dvdy.20189

Kafkas Univ Vet Fak Derg
25 (3): 311-320, 2019
DOI: 10.9775/kvfd.2018.20889

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Efficacy of Probiotics on Health Status and Growth Performance of *Eimeria tenella* Infected Broiler Chickens

S. Zerrin ERDOĞMUŞ [1] Nurhayat GÜLMEZ [2] Ayfer FINDIK [3] Hüseyin ŞAH [2] Murat GÜLMEZ [4]

[1] Department of Parasitology, Veterinary Faculty, Near East University, Nicosia, CYPRUS
[2] Department of Histology and Embriology, Veterinary Faculty, Near East University, Nicosia, CYPRUS
[3] Department of Virology, Veterinary Faculty, Near East University, Nicosia, CYPRUS
[4] Asilçağ Trading Ltd. 7th St. No 14. Industrial Zone, Nicosia, CYPRUS

Article Code: KVFD-2018-20889 Received: 02.09.2018 Accepted: 09.12.2018 Published Online: 09.12.2018

How to Cite This Article

Erdoğmuş SZ, Gülmez N, Fındık A, Şah H, Gülmez M: Efficacy of probiotics on health status and growth performance of *Eimeria tenella* infected broiler chickens. *Kafkas Univ Vet Fak Derg,* 25 (3): 311-320, 2019. DOI: 10.9775/kvfd.2018.20889

Abstract

A probiotic containing *Pediococcus acidilactici* and *Bacillus subtilis* (Smart ProLive) at a $1x10^7$ CFU/mL dose in drinking water were given continuously from the d 14 to the end of the treatment (d 35) in broiler chickens. Experimental infection was produced by oral gavage of sporulated *E. tenella* oocysts at 14^{th} d of age. Feed consumption (FC), live body weight (LBW) and feed consumption rate (FCR) were measured at weekly basis. Villus height and crypt depth in cecum and ileum, and antibody titers in the blood were performed at 28-day-old. Probiotics appeared to be superior to salinomycin on the villus height and crypt depth of cecum and ileum ($P<0.05$). A numerical, but not significant ($P>0.05$) improvement on the LBW was determined at the groups of probiotic and salinomycin+probiotic than that of control and salinomycin groups. Nevertheless, FC and FCR results of the probiotic and salinomycin+probiotic groups were good than that of control and salinomycin groups. Probiotics were effective on the villus heights and crypt depths than that of salinomycin alone. Salinomycin appeared to be good only than control group in all the parameters. Not a significant difference from antibody titers was existed among the groups. Based on these results it can be concluded that a good source of probiotics can be used as natural antimicrobial growth promoters in replacement with forbidden anticoccidials in broiler rearing.

Keywords: *Broiler, Coccidiosis, Eimeria tenella, Probiotics, Salinomycin, Histology, Antibody*

Probiyotiklerin *Eimeria tenella* İle Enfekte Broiler Piliçlerin Sağlık Durumu ve Verim Performansı Üzerine Etkileri

Öz

Pediococcus acidilactici ve *Bacillus subtilis* içeren bir probiyotik (Smart ProLive) 14. günden 35. güne kadar içme suyu ile birlikte sürekli olarak $1x10^7$ KOB/mL dozunda broiler civcivlere verildi. Deneysel enfeksiyon 14 günlük civcivlere sporlu *E. tenella* ookistleri ağız yoluyla verilerek yapıldı. Yem tüketimi, canlı ağırlıklar ve yemden yararlanma oranları haftalık olarak takip edildi. Sekum ve ileumda villus yüksekliği ve kript derinliği ile antikor titre analizi 28 günlük civcivlerde yapıldı. Probiotiklerin sekum ve ileumda villus yüksekliği ve kript derinliği üzerindeki olumlu etkisi salinomisinden daha üstün bulundu ($P<0.05$). İstatistiki önem ortaya çıkmamakla birlikte ($P>0.05$) canlı ağırlık artışı probiyotik ve probiyotik+salinomisin grubunda control ve salinomisin grubuna göre daha yüksek bulundu. Ancak, yem tüketimi ve yemden yararlanma konusunda probiyotik ve probiotik+salinomisin grupları kontrol ve salinomisin gruplarındakinden üstündü. Salinomisin tüm parametrelerde sadece control grubundan üstün olabildi. Antikor titreleri bakımından gruplar arasında fark gözlenmedi. Elde edilen bulgular ışığında iyi bir probiyotiğin kullanımı riskli ve direnç oluşumuna neden olabilecek antikoksidiyaller yerine doğal antimikrobiyal büyütme faktörü olarak kullanılabileceği öne sürülebilir.

Anahtar sözcükler: *Broiler, Koksidiyozis, Eimeria tenella, Probiyotik, Salinomisin, Histoloji, Antikor*

INTRODUCTION

The poultry industry is one of the most important food of animal origin suppliers in the world. The global poultry production has been stated to be 111.000 thousand metric tons in 2015, and world poultry production is projected to increase by 24% over the next decades, reaching 131.255 thousand metric tons in 2025. Poultry meat production will be dominating more than half of the growth of all the additional meat produced by 2025 [1]. The poultry meat

İletişim (Correspondence)
+90 392 2236464; Fax: +90 392 2236461
mgulmez@hotmail.com

market is growing fast, with a significant increase in production with time [2-4].

Coccidiosis is a major parasitic disease of poultry with great economic impact, which mainly affects the intestinal tract of birds. The clinical and economic importance of coccidiosis is likely to remain unchanged during the coming decades as long as commercial poultry is reared in large numbers at high densities, which seems necessary to make the poultry industry profitable [5]. Anticoccidial drugs play a major role in combating this disease caused by protozoan parasites of the genus *Eimeria* both therapeutically and prophylactically [6,7]. Nevertheless, extensive use of anticoccidials has led to the development of anticoccidial drug resistance [8]. Even with the shuttle and rotation programs there is no method to fully prevent drug resistance [2,9].

Fortification of feeds of food animals with sub-therapeutic doses of antibiotics to protect against infections and to promote yield performances has recently been an unwanted situation in the point of the view of the public health [10]. Sub-therapeutic uses of drugs are perceived to lead to microbial resistance, as well as consumer concerns regarding residues in food products. The relatively recent ban of sub-therapeutic doses of certain antibiotics as feed additives in the European Union led to a general decline in animal health [11].

Drug resistance and consumer concerns regarding drug usage has been a motivating factor to the practice of live vaccines to control coccidiosis. Vaccines have been stated to provide an alternative for disease protection, capable of limited efficacy as they induce specific protective immunity by exposing the chicken's immune system to *Eimeria* antigens [9,12-14]. However, some drawbacks to live vaccines have been stated to occur. Subunit vaccines may circumvent most shortcomings of live vaccines; however, at present these products has stated to be underperform due to the lack of immunogenicity [5]. Immunity to avian coccidiosis has been stated to be strongly species-specific, therefore the bird will only develop immunity to the species of *Eimeria* present in the vaccine [12,13]. Also, vaccine application to the post-hatch chickens has not found to be so easy to apply [15]. Some secondary infections such as necrotic enteritis may occur after vaccine application [9,14].

The ban on the use of antibiotic growth promoters results in higher feed costs [14]. It has been concluded that future coccidiosis control is unlikely to be achieved solely by using anticoccidial products as feed additives and/or through feed composition and management [5]. Use of anticoccidial drugs and vaccines are generally considered to be successful. Due to the issues related to the, as well as the impending ban on animal feed additives, researchers has recently focused on 'natural' alternatives of drugs to controlling and managing coccidiosis [5].

Alternative controls include nutritionals and probiotics (immunomodulators) or natural feed additives [16-19]. Some of the bacteria used as probiotics are *Lactobacillus, Pediococcus, Bacillus*, *Saccharomyces cerevisiae* and *Enterococcus faecium*. Direct fed microbials (DFM) are include *Aspergillus oryzae* and *Bacillus subtilis* and also found to be useful [20]. In *E. acervulina* infected broilers, lower intestinal development of coccidiosis and lower oocyst production have been explained by enhanced local cell-mediated immunity when a *Lactobacillus*-based probiotic supplemented diet has been used [21]. In a study performed with a *Pediococcus*-based commercial probiotic given to birds infected with an *E. acervulina* or *E. tenella* infection, increased resistance of birds against coccidiosis and a partial protection against growth retardation has been demonstrated [22]. In another study, a *Pediococcus*- and *Saccharomyces*-based probiotic has given to birds infected with 5000 oocysts of either *E. acervulina* or *E. tenella* and less oocyst shedding and a better antibody response has been found in probiotic fed birds compared to non-probiotic controls [23]. Probiotic supplementation is one option currently being explored as a means of reducing the amount and severity of enteric diseases in poultry and subsequent contamination of poultry products for human consumption [24,25].

Numerous efforts to date have been implemented in the control of avian coccidiosis caused by the *Eimeria* parasite. Since the appearance of anticoccidial chemical compounds, the search for new alternatives continues. Today, no product is available to cope with the disease; however, the number of products commercially available is constantly increasing [2]. The objective of this study was to comparatively evaluate the effect of a commercial probiotic product (in the manufacturers' demonstration on the bag, it contains $\geq 1\times10^{11}$ CFU/mL probiotics, *Pediococcus acidilactici* and *Bacillus subtilis)*, alone or in combination with the anticoccidial medicine salinomycin on broiler performance and intestinal health to *E. tenella* infection as evaluated by growth parameters, histological alterations within the intestine, and response to routine vaccines.

MATERIAL and METHODS

Preparation of Sporulated E. tenella Oocyst Suspension (inoculum)

For preparation of artificial infection material of coccidiosis, bloody fecal materials from 15 d old free-range broiler chickens in a local flock were collected and mixed in plastic bags. Then the bags brought to the lab and examined for the presence of presumptive *E. tenella* oocysts in reference to Conway and McKenzie [26]. The positive stool samples were used as the primary oocyst source in our preliminary study. The samples were filtered, centrifuged and sporulated in potassium dichromate at room temperature for seven days. The oocysts were recovered by centrifugation in saturated NaCl solution by washing with distilled water. Then, the material was concentrated by centrifugation and stored

in potassium dichromate solution, quantified in *Neubauer chamber* and stored at 4°C [27,28]. In our preliminary study, sporulated oocyst suspension was passaged in 9 broiler chickens with the age of 7 days for checking pathogenity and cecal localization. Each chicken was placed in one separate plastic pen with plastic mesh bedding. After 10 days of oocyst inoculation, all the birds were euthanized and bloody content and deformations were clearly seen from all the 9 ceca. Not a visual sign of coccidiosis was seen in other parts of the intestines. All the cecal content of the 9 euthanized chickens were collected and sporulated oocysts suspension was prepared as mentioned above and kept in a refrigerator at 4°C until use in the study.

Preparation of Probiotic Drinking Water

A commercial probiotic (Smart ProLive) in the form of 50 g water soluble powder in aluminum bags was purchased from a local Veterinary clinic. It is added to the sterile saline solution (0.9% NaCl, w/v) at recommended dose of 50 mg/L of water, and gently mixed in a sterile Erlenmeyer flask. Total aerobic bacteria were counted from this water. The 10-fold increment serial dilution technique was conducted according to Miller and Wolin [29]. One milliliter of the homogenized suspension was then transferred into 9 mL of 0.9% saline solution (NaCl) and serially diluted from 10^{-1} to 10^{-8} by using the same saline solution tubes. From the last three diluted samples, 0.1 mL each was plated on the Trypticase soy agar (TSA, Merck, Germany) plates and the plates incubated at 37°C for 48 h. All the colonies grown on the plates were counted and results were expressed as $\log_{10}$ colony forming units (CFU) per gram probiotic product. A total of 1.1 x10^{11} CFU/g live bacteria were detected in the probiotic source. After the count of CFU/g of probiotic product, the drinking water of chickens was fortified by addition of 1 g powdered probiotic to 10 L of drinking water to make a probiotic water including 1.1 x10^{7} CFU/mL live probiotic bacteria in it. The probiotic drinking water of the chickens were refreshed 3 d intervals during the experiments. The bags of 50 g probiotic source used in the study kept at room temperature during use as recommended.

Experimental Design and Treatments

The study has been permitted by NEU Ethical Board at Meeting No: 2016/2 held at 12th May 2016. The study performed was a 35-day grow-out with 90 Ross 308 mix sexed broilers housed on 4 plastic mesh cages with 3 replicate pens in each. The birds at 10th day of breeding were purchased from a local farm and transferred to the cages after a 30 min journey. A total of 10 birds (5 male and 5 female) were located in each of 9 pens. The 1st cage was received as the two separate groups such that, the upper pen of the 1st cage was received as control that no medication, probiotics and infection were applied (Control group). The chickens of middle and bottom pen of the 1st cage were fed with salinomycin added feed (0.5 g/kg of feed) during the course (Group S). All the chickens in the three pens of 2nd cage were fed with probiotics via drinking water (Group P). All the three flats of 3rd cage were fed with probiotics and salinomycin (Group SP).

Each of ten birds in each pen was marked by using 10 different colors. The male and female birds were recorded. Weight gain of each separate bird and also feed consumption of each separate pen was recorded weekly. At 14 d of age, the birds were infected with sporulated *E. tenella* oocysts by administering them directly into the crop via an oral gavage of the oocysts suspension by a rubber tube adjusted to a plastic syringe [30]. So, except for Control group, four chickens from each pen that marked with the same colors (2 male and 2 female) were artificially infected with 9×10^{4} *Eimeria* oocysts. The oocysts doses were prepared by the section of parasitology. Mortality was recorded during the experiments. Routine vaccination program was applied for immunization. Air conditioner was used to standardize room temperature to meet Ross 308 handbook [31].

All birds had access ad libitum to their particular diets during all the growth period. Both salinomycin via feed and probiotic via drinking water were given to the chickens from 10th day to 35th day of the experiment. The basal diet was a typical mash corn-soybean meal diet that was bought a local commercial broiler feed producer. The formula of the feed is demonstrated in the *Table 1*.

Performance Parameters

All the chickens were individually weighed at 10 (the d of allocation into the pens), 14, 21, 28 and 35th day. The diets were removed and weighed prior to the weighting of the birds. Weighting was completed in one h in each time. Feed consumption (FC) and live body weights (LBW) were recorded weekly. So, Feed Conversion Ratio (FCR) of each separate pen was calculated by dividing weekly FC to LBW.

Oocyst Shedding

The feces of the chickens were checked for oocyst shedding at daily intervals. Feces samples were taken simultaneously from feces trays of each 9 separate pen once a d after 3 day of oocyst inoculation. The feces samples were examined by using the Fulleborn's saturated salt solution method [32].

Histological Examinations

On d 28, a total of the 36 birds, 4 (one infected male, one infected female, one non-infected male and one non-infected female) from each of 9 pens were sacrificed (Ethical Commission report No. 1324/13.06.2017). Then, the ceca incised. After emptying the content the ceca were washed under mild flowing tap water. For micro morphometric examinations, the entire segments of the ceca were fixed in 10% formalin, embedded in paraffin wax, and sectioned to

Table 1. *Ingredients and calculated nutrients, energy of diet*

Ingredients	g/kg (as feed basis)
Maize	580
Soybean meal	310
Soybean oil	42
Monocalcium phosphate	16
Limestone	17
DL-methionine	2
Lysine HCl	0.7
Chromic oxide marker	25
Vitamin-mineral premix*	3
Salt	4
Calculated nutrients and energy	
Protein	201
ME (Mj/kg)	13.4
Ca	10
P	7
Na-phytate	4.5
Ca-tP	1.4

** Supplied following per kg of diet: retinol, 5.400 IU; cholecalciferol, 2.600 IU; α-tochopherol, 11 IU; menadoine sodium bisulphate; 4,4 mg; riboflavin, 5.49 mg; D-pantothenic acid, 11 mg; niacin, 44 mg; choline chloride, 770 mg; cyanocobalamin, 13 µg; biotin, 55 µg; thiamine mononitrate, 2.2 mg; folic acid, 1 mg; pyridoxine hydrochloride, 3.3 mg; I, 1 mg; Mn, 66 mg; Cu, 4.4 mg; Fe, 44 mg; Zn, 44 mg; Se, 0.3 mg*

give 4-µm-thick serial paraffin sections. Then, sections were stained with hematoxylin-eosin to measure the height of intestinal villi and the depth of intestinal crypts under a light microscopy [30]. Histological sections were examined with a Leica DM500 light- microscope coupled with a Leica Microsystem Framework integrated digital imaging analysis system (Leica ICCSO HD, Heerbrugg, Switzerland). The villous height was estimated by measuring the vertical distance from the villous tip to the villous-crypt junction level for 30 villi per section. The crypt depth (the vertical distance from the villous-crypt junction to the lower limit of the crypt) was estimated for 30 corresponding crypts per section [30].

Immunological Examinations

Broiler chickens were vaccinated with live attenuated vaccines against Newcastle Disease Virus (NDV) Avinew© VG/GA strain (Merial-Lyon-France) and Infectious Bronshitis Virus (IBV) Nobilis© IB 4/91 strain (Intervet International BV.-Boxmeer/Netherland). The vaccination was performed at day 1, day 10 with Nobilis and day 1, day 10 and day 18 with Avinew respectively according to the manufacturer's instructions. On day 28, blood samples from the 36 birds that sacrificed for histological analyses were collected and used for analysis of immune response against NDV vaccine and IBV vaccine. The antibody titers were determined by using commercial ELISA test kits against NDV (Biotech, TW4 5PY Hounslow, UK) and IBV (SL5 8BP Ascot, UK).

Statistical Analysis

The results of the study were subjected to one-way analysis of variance (ANOVA) using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences in experimental treatments were tested using Turkey's honestly significant difference following ANOVA with significance reported at $P<0.05$.

RESULTS

The infection dose determined in our preliminary study, a total of 5000-6000 sporulated presumptive *E. tenella* oocysts were given per chicken to cause the signs of an apparent infection. Oocyst suspensions were given to the each separate 14 day-old chickens via intra-crop tube inoculation to the experiment groups except for the Control Group. In these studies, it was seen that the ceca were highly infected 6th day after inoculation *(Fig. 1)*. The first oocyst shedding in the feces was seen after 6 d of the oocyst inoculation made at 14th day. Except for the Control group, all the groups shed oocysts in the faces from 20th day to 35th day. No oocyst contamination from other groups to the Control group was detected (data has not been shown). Both at the preliminary study and at the experimental study, visual signs of coccidiosis have been determined in the parts of intestines except for the ceca. During the 1st and 2nd weeks of the experiment, feed consumption of Control group was higher than that of other groups. In the other 3 groups, not a significant difference was appeared during this time period. Nevertheless, at the 3th week of the experiment, FC of P and SP groups were significantly lower than that of Control and S groups *(Fig. 2a)*.

At the start d of experiment (d 14), the average LBWs of the chickens were ranged from 396 to 417 and there were no statistically significance between the groups ($P>0.05$). After that week of infection, LBW of the groups differed slightly from each other during experiment. Since LBW of group P and SP were higher than that of Control and S groups, there were no statistical significance during all the time periods of the experiment ($P>0.05$). The Mean ±SD values of LBW of the groups were such that Control (1207±121 g), S (1309±87 g), P (1393±63 g) and SP (1372±126 g) at the d 35 *(Fig. 2b)*.

The FCR of Control group appeared to be higher than that of the other groups during the experiment period. Since the FCR of all groups were high at the 1st week of the experiment, it decreased gradually after this time period. The best result was seen in the P group, and 1.56±0.30 FCR has recorded in the last week of breeding. The second FCR was recorded in the SP group (1.79±0.36) at the same week. The FCR result of salinomycin applied group (Group S) was 2.08±0.49. The results of S group was not better than that of group P or SP. Since the positive effect of probiotics

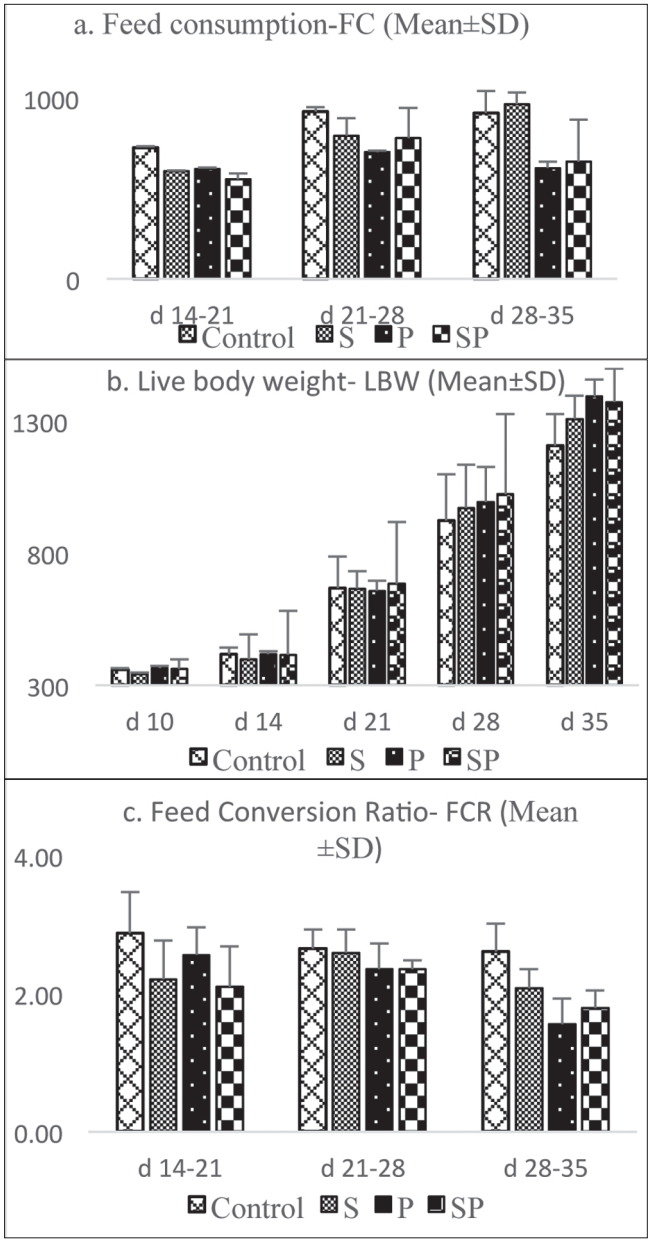


Fig 1. Feed consumption (a), live weight gain (b) and feed conversion ratio (c) results from the study of anticoccidial efficacy of some probiotics in comparison with the anticoccidial medicine salinomycin. Control: control group chickens that neither oocysts nor salinomycin and probiotics were given, S: salinomycin by adding to the feed at a 2.5 g/kg dose, P: probiotics added to drinking water at a dose $1.1x10^7$ CFU/mL, SP; both S and P at the same doses of the groups of S and P were given

alone (the group P) on the FCR was better than that of S and SP groups at the week of oocyst gavage, the positive effect of this group on the FCR was good than that of S and SP groups at last 2 weeks of the experiment. The Control group (FCR 2.61±0.72) and the group S (FCR 2.08±0.49) represented higher results of FCR than that of groups P and SP at the last week of the experiment *(Fig. 2c)*.

In our preliminary studies, ceca of the artificially infected chickens by using *E. tenella* oocysts demonstrated highly infected and were full of blood after 6 days post-inoculation

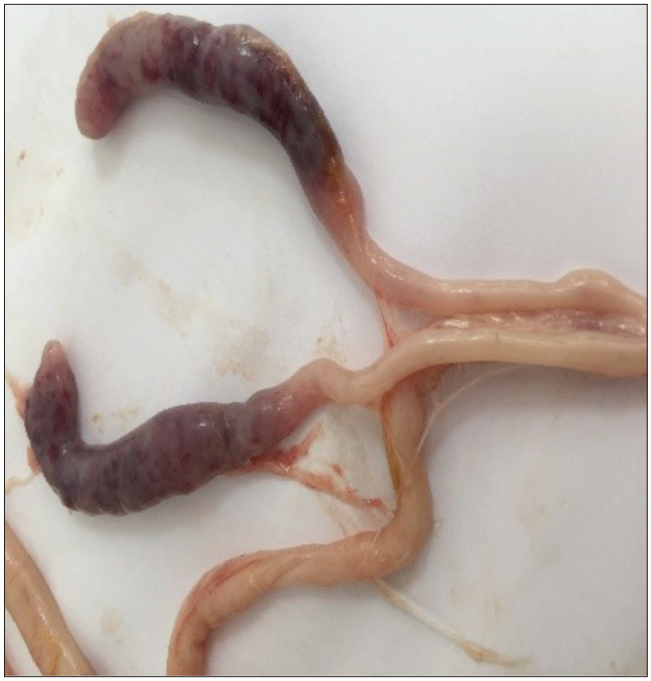


Fig 2. The cecum of a 20 d old broiler chicken infected with *E. tenella* oocysts at the 14th d of its life

(Fig. 1). At the 6th day of post-inoculation of chickens with *E. tenella*, gametocytes and numerous intracellular schizonts containing merozoites were observed between crypt epithelial cells of the cecum. Severe bleeding and erosions from luminal epithelial tissues were seen *(Fig. 3a)*.

The chickens were sacrificed at the day of 28 of the breeding and histological examinations were made. Severe inflammation, infiltrating neutrophils, eosinophil and mononuclear leukocytes were observed in the lamina propria of the ceca of the infected animals *(Fig. 3a)*. In these animals, the villi were partially lost their surface epithelial cells and became atrophic. Some of intestinal glands have also became atrophic and turned to vacuoles *(Fig. 3b)*.

In all the infected groups, the epithelial cells of the villi were mostly prismatic and some flattened. Nevertheless, in the non-infected animals in same group, fully prismatic epithelial layers were seen *(Fig. 3c)*. These results have demonstrated that epithelial tissue disposition and damage have seen clearly in the infected animals. Histological results have demonstrated no shedding of the infection in each pen from infected to non-infected chickens.

Neither villus heights nor crypt depths were different between the cecum of control and S group chickens ($P>0.05$). Also, neither villus heights nor cd were different between the cecum of P and PS group chickens ($P>0.05$). No statistically significant difference have existed between the groups in the point of view of crypt depths of ceca of infected chickens ($P>0.05$). Both P and SP have demonstrated a positive effect on the villus heights of ceca of infected and non-infected chickens ($P<0.05$). P

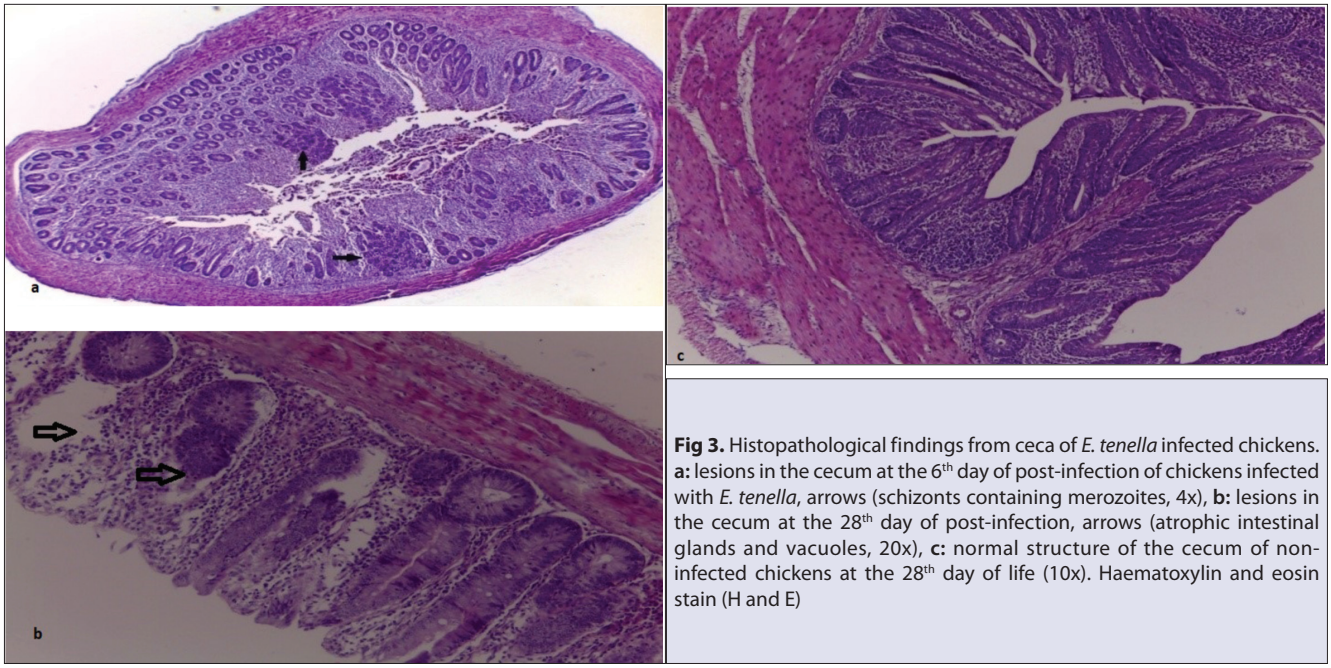


Fig 3. Histopathological findings from ceca of *E. tenella* infected chickens. **a:** lesions in the cecum at the 6th day of post-infection of chickens infected with *E. tenella*, arrows (schizonts containing merozoites, 4x), **b:** lesions in the cecum at the 28th day of post-infection, arrows (atrophic intestinal glands and vacuoles, 20x), **c:** normal structure of the cecum of non-infected chickens at the 28th day of life (10x). Haematoxylin and eosin stain (H and E)

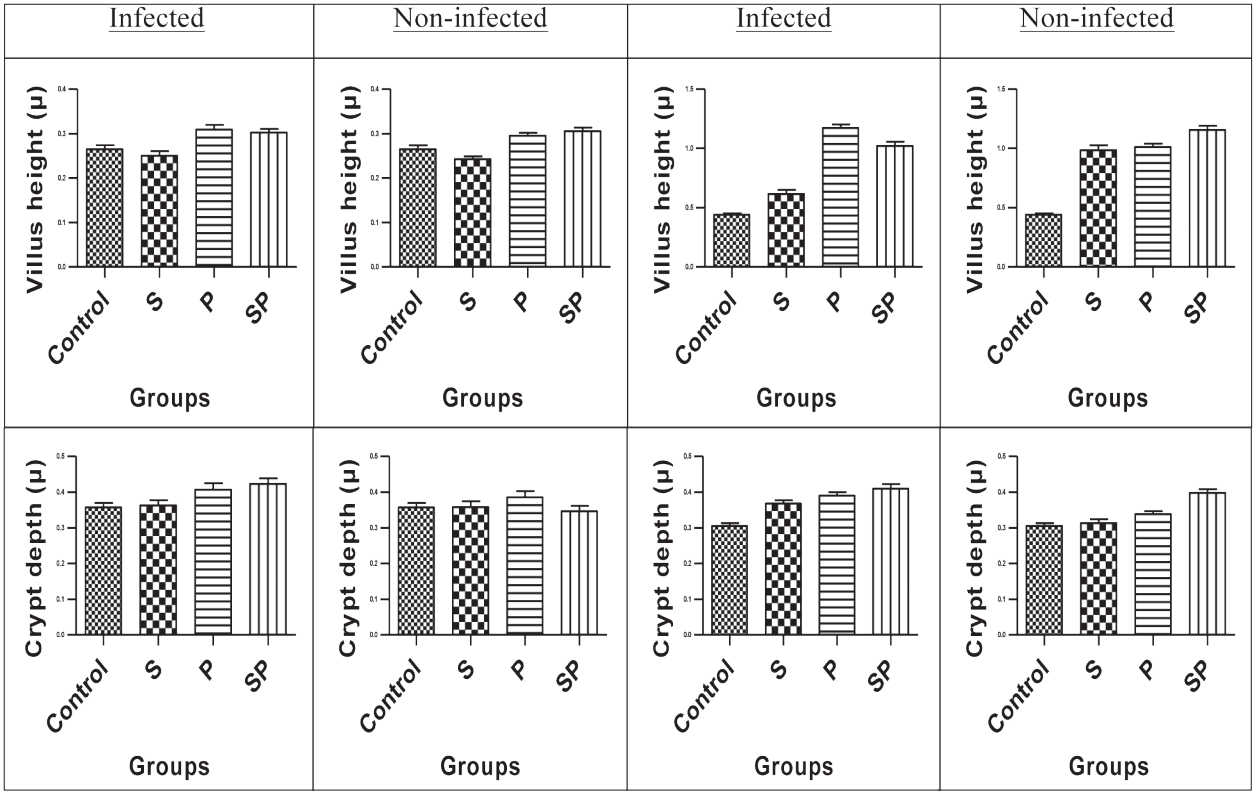


Fig 4. Villus heights and crypt depths (Mean±SEM) of ceca and ileum of infected and non-infected (infected by using *E. tenella* oocysts at the 14th day of life) chickens of 28th day age. Control: control group chickens that neither oocysts nor salinomycin and probiotics were given, S: salinomycin by adding to the feed at a 2.5 g/kg dose, P: probiotics added to drinking water at a dose $1.1x10^7$ CFU/mL, SP: both S and P at the same doses of the groups of S and P were given

appeared to be more effective on the villus heights of ceca of chickens *(Table 2)*.

All the three of S, P and SP appeared to be effective on the villus heights of ileum of infected and non-infected chickens *(Fig. 4)*. Except for a result of no statistical difference between S and P on the villus height of ileum of infected chickens, SP and P appeared to be more effective than S and control samples on the villus heights of ileum of infected and non-infected chickens *(Table 2)*.

Table 2. *Statisitical analysis summary of the villus heights and crypt depths of ceca and ileum of infected and non-infected chickens*

Compared Groups	Cecum				Ileum			
	Villus Heights		Crypt Depths		Villus Heights		Crypt depths	
	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected
Control vs S	No	No	No	No	Yes***	Yes***	No	Yes***
Control vs P	Yes*	Yes**	No	No	Yes***	Yes***	Yes*	Yes***
Control vs SP	Yes**	Yes**	No	Yes**	Yes***	Yes***	Yes***	Yes***
S vs P	Yes***	Yes***	No	No	No	Yes***	No	No
S vs SP	Yes***	Yes**	No	Yes**	Yes***	Yes***	Yes***	Yes***
P vs SP	No	No	No	No	Yes***	Yes***	Yes*	No

Tukey's Multiple Comparison Test; Significant, P<0.05

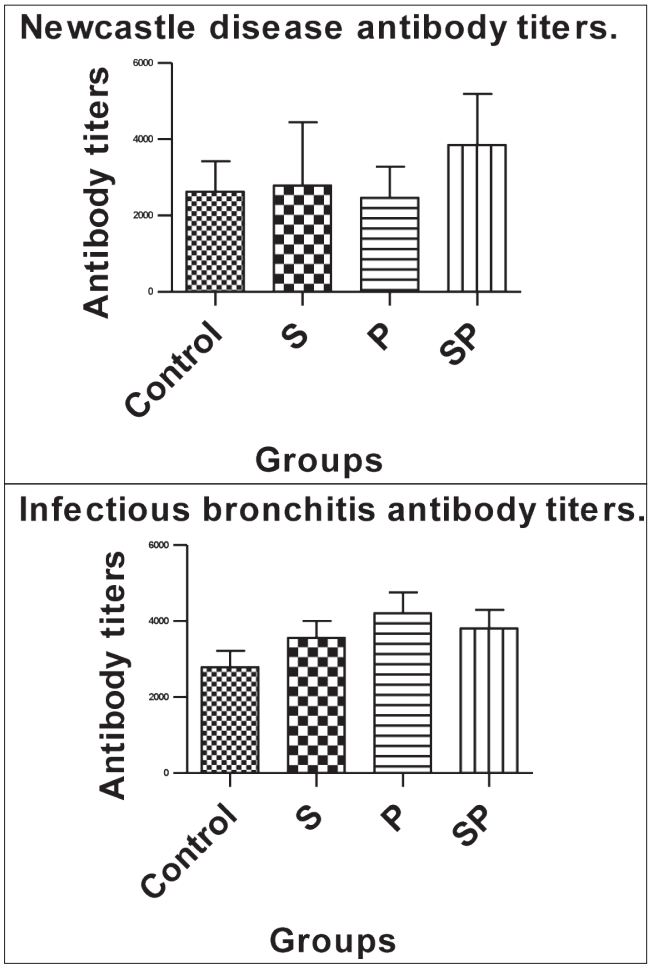


Fig 5. Antibody titers (Mean±SEM) of chickens of 28th day age. Control: control group chickens that neither oocysts nor salinomycin and probiotics were given, S: salinomycin by adding to the feed at a 2.5 g/kg dose, P: probiotics added to drinking water at a dose $1.1x10^7$ CFU/mL, SP: both S and P at the same doses of the groups of S and P were given

Combined effect of S and P appeared to be more effective than that of S or P alone *(Table 2)*. The S or P alone has not demonstrated a good action on the crypt depths of ileum of infected chickens. All the three of S, P and SP appeared to be effective on the crypt depths of ileum of non-infected chickens *(Table 2)*. No statistical difference existed between S and P groups, also between P and SP groups in the crypt depths of ileum of non-infected chickens (P>0.05). The *Fig. 4* is represented to check out the numerical results as figures.

No statistically significant difference has existed among the groups in the point of view of Newcastle or infectious bronchitis disease antibodies *(Fig. 5)*.

DISCUSSION

Poultry meat industry is one of the leading meat producers almost all over the world. Both economical and feasible impacts of the industry are forcing it to grow fast. One cause for this is also fast growing World's human population and accordingly growing the demand of protein of animal origin [4]. The future challenges of the poultry-meat industry regarding sustainability, social acceptance of intensive animal production, and the introduction and dissemination risk of highly infective poultry diseases. Breeding of meat poultry has many problems to solve. One leading problem is disease and accordingly economical loses [33]. Although it is differed from country to country, the most occasionally prevailed diseases are respiratory and digestive system diseases. These diseases include necrotic enteritis, coli-septicemia, infectious bronchitis, chronic respiratory disease, infectious bursal disease (IBD) and Newcastle disease [34]. One of the most important digestive system diseases of broiler chickens is coccidiosis. *E. tenella* is one of the most prevailed causative agents of coccidiosis of the broiler chickens [35]. This protozoon is located basically to the ceca of the chickens. The disease causes to death or sub-latent chronic disease. Even though chicken is not died, the chronic form and sub-clinic form of the disease may cause poor LBW, high FCR and secondary diseases [20,36]. In this study, the visible signs of coccidiosis infection in the ceca of chickens at 6th day of post-infection has clearly demonstrated *(Fig. 2)*.

After the ban of the most of anticoccidial drugs and antibiotic growth promoters, the industry came to face with breeding performance problems and also disease control problems. From the date of 2006, when such restrictions on the use of anticoccidials and AGP's took place, an

emergence for research on new friendly anticoccidials and replacer for AGP have occurred. After the rest of 12 year of this new period many researches have conducted on the subject. Some plant based extracts, live beneficial microorganisms (probiotics) and vaccine applications have been recommended by researchers [2,37,38].

Not a significant difference from antibody titers was existed among the groups *(Fig. 5)*.

AGPs are used as growth enhancers and health promoter of digestive system of meat chickens [8,38,39]. Probiotics also have been recommended as natural grow promoting agents in replacing with AGPs [37,40]. Until now, there is no officially recommended probiotic formula or application method [30,41]. Thus, research results and recommendations have been different from one researcher to another. In this study we have used a combination of two live probiotic strains *(B. subtilis* and *P. acidilactici)* by adding drinking water of broiler chickens at a dose of $1.1x10^7$ CFU/mL from 14 d to 35 d. The results have demonstrated that probiotic use may help problems caused by *E. tenella* infection in broiler chickens. All the results determined in that study have demonstrated superiority of probiotics over salinomycin use. FC, LBW, FCR, villus height and crypt depth values appeared to be good in P and SP groups when compared with the Control and S groups *(Fig. 2, Fig. 4)*.

Health promotion and growth enhancing effect of probiotics have been well documented [36]. Nevertheless, there are some researchers that have not confirmed positive probiotic effect on the broiler chicken growth performance or health status [42-44]. Bino Sunder *et al*.[45] have reviewed that anticoccidial resistance is a big problem for broiler chicken breeders all over the world and probiotic use is one of the promising solutions. The researchers have summarized that probiotics modify receptors on enterocytes and this impairs or destroys sporozites and/or merozoites from pathogenity on enterocytes. Chen *et al*.[38] have also demonstrated that probiotics have been effective on the growth rate and the inflammation of broiler chickens caused by *E. tenella* infection. Health promotion *(Table 2, Fig. 3, Fig. 4)* and growth enhancing effect *(Fig. 2)* of probiotics used in this study were determined clearly and the results have confirmed many other researchers' results [2,36,37,41,46]. At the 3rd week of the experiment, FC and FCR of P and SP groups were significantly lower than that of Control and S groups *(Fig. 2a,c)*. A good source of probiotics applied continuously during all the breeding time period may be a good alternative to AGPs and anticoccidial health promoters in broilers.

Broiler chickens are fast growing animals and bred intensively. The conditions leads the stress and thus immunity of the body and especially the digestive system is of importance. Also, consistency of mucosal layer of intestines, villus heights and also the crypt depths in that absorptive layer are so important both on health and growth performances. Heak *et al*.[20] have evaluated the results of 49 different studies made on the effect of probiotics on the epithelial tissue of the small intestines of chickens and only 32 of them have favored the DFM over control on villus heights. Nevertheless, the researchers has not been determined the positive effect of DFM on the crypt depth when checked the 96 studies made before. Our results are in agreement with that 32 studies, and we also demonstrated the positive effect of probiotics on villus heights of cecum and ileum *(Table 2, Fig. 4)*. Taheri *et al*.[42] have also determined positive effect of probiotics on villus height. Ştef *et al*.[47] have also demonstrated the positive effect of probiotics on the growth performances, gut health and disease prevention. Heak *et al*.[20] have demonstrated that there have not been a significant positive effects of probiotics on the crypt depths bot in cecum and ileum (n=96 comparisons in research studies). We determined in this study that probiotics were more effective on the villus heights and crypt depths both in the ceca and ileum of chickens both infected and not. Differences among the results of the studies may be due to difference from analysis days, or any other factor such as breeding strategy, difference between probiotic strains, etc.

Almost all the researchers have chosen about 7th d after oocyst gavage in their hispathological studies. These researchers have occasionally chosen that day for scoring the gross lesions of intestines visually [38]. In this study, we chosen the day of histological examination day as the 28th day of broiler life. At the 14 d post-infection, examination of ceca might be more valuable since gross lesions would be recovered and health of the ceca and ileum after recover from infection would be more efficiently determined by histological examinations. Neither infected nor control chicken have demonstrated visible lesion from their intestines (data has not shown). In our preliminary studies, we demonstrated the difficulty of analysis of the ceca at the week of infection due to gross lesions and bleedings. So, we think that not the week of infection but 1 week after oocyst shedding would be chosen for histological examinations of intestines to check out the effect of probiotics on the health status of intestines of broiler chickens.

In this study, we determined the positive effect of daily water based feeding with a mix strain probiotic source at a $1.1x10^7$ CFU/mL in the drinking water during whole feeding period can enhance the natural resistance to the *E. tenella* infection. Giannenas *et al*.[41] have also demonstrated such results in their study. The researchers recommended a multi-strain probiotic use for a natural protection against coccidiosis in broiler chickens. Ritzi *et al*.[18] have also suggested in their study that probiotic supplementation via drinking water can be alternative to AGPs and can enhance performance and help alleviate the negative effects of a mixed *Eimeria* infection. Ariyadi and Harimurti [48] have also suggested that probiotics may

stimulate proliferation of intestinal epithelium regulate mucosal barrier formed by mucin in the intestine of broiler chickens. Giannenas *et al*.[30] have also suggested that a mixture of probiotic substances has given considerable improvement in both growth performance and intestinal health in comparison with infected control birds an fairly similar improvement to an approved anticoccidial during a mixed *Eimeria* infection. Contrary to these findings, Lu *et al*.[49] have demonstrated the superiority of salinomycin to a commercial probiotic and some other natural DFM alternatives.

In conclusion, the results of the present study suggest that in the absence of in-feed anticoccidial drugs, treatment with probiotics could alleviate impact of coccidiosis infection on broiler chickens. Beneficial effects of probiotics on the intestinal health could minimize the side effects of coccidiosis. Economical loses due to the infection and also public health concerns due to use of DFMs could be minimized. Future researches on the use of probiotic sources as alternative to AGPs and anticoccidial drugs can support growing regimes that include no AGPs and such medicines.

Acknowledgements

The authors appreciate the research support provided by a grant from the Near East University, Nicosia/ Turkish Republic of North Cyprus (Grant no. SAG-2016-04030).

REFERENCES

1. Global Industry Statistics: http://www.poultryhub.org/production/industry-structure-and-organisations/global-industry-statistics/; *Accessed:* 08.05.2018.

2. Quiroz-Castañeda RE, Dantán-González E: Control of avian coccidiosis: Future and present natural alternatives**.** *Biomed Res Int*, 2015:430610, 2015. DOI: 10.1155/2015/430610

3. Krishnasamy V, Otte J, Silbergeld E: Antimicrobial use in Chinese swine and broiler poultry production. *Antimicrob Resist Infect Control,* 4:17, 2015. DOI: 10.1186/s13756-015-0050-y

4. Livestock and Poultry: World Markets and Trade China's Meat and Poultry Import Forecast 2018: Decline and Constrained Growth. https://apps.fas.usda.gov/psdonline/circulars/livestock_poultry.pdf/ *Accessed:* 20.06.2018.

5. Resistance to anticoccidial drugs: Alternative strategies to control coccidiosis in broilers. https://dspace.library.uu.nl/bitstream/handle/1874/40109/peek.pdf/; *Accessed:* 12 June, 2018.

6. Hayakawa T, Masuda T, Tsukahara T, Nakayama K, Maruyama K: Morphometric and histopathological evaluation of a probiotic and its synergism with vaccination against coccidiosis in broilers. *Anim Sci Lett,* 1 (1): 33-49, 2014.

7. Tewari AK, Maharana BR: Control of poultry coccidiosis: Changing trends. *J Parasit Dis*, 35 (1): 10-17, 2017. DOI: 10.1007/s12639-011-0034-7

8. Usman JG, Gadzama UN, Kwaghe AV, Madziga HA: Anticoccidial resistance in poultry: A review. *New York Sci J*, 4 (8): 102-109, 2011.

9. Stringfellow K, Caldwell D, Lee J, Mohnl M, Beltran R, Schatzmayr G, Fitz-Coy S, Broussard C, Farnell M: Evaluations of probiotic administration on the immune response of coccidiosis-vaccinated broilers. *Poult Sci*, 90 (8): 1652-1658, 2011. DOI: 10.3382/ps.2010-01026

10. Food-safety concerns in the poultry sector of developing countries. http://www.fao.org/ag/againfo/home/events/bangkok2007/docs/part2/2_8.pdf/; *Accessed*: 28.06.2018.

11. Castanon JIR: History of the use of antibiotic as growth promoters in European poultry feeds. *Poult Sci*, 86 (11): 2466-2471, 2007. DOI: 10.3382/ps.2007-00249

12. Dalloul RA, Lillehoj HS: Poultry coccidiosis: Recent advancements in control measures and vaccine development. *Expert Rev Vaccines,* 5 (1): 143-163, 2006. DOI: 10.1586/14760584.5.1.143

13. Williams RB: Anticoccidial vaccines for broiler chickens: Pathways to success. *Avian Pathol,* 31 (4): 317-353, 2002. DOI: 10.1080/03079450220148988

14. Li GQ, Kanu S, Xiao SM, Xiang FY: Responses of chickens vaccinated with a live attenuated multi-valent ionophore-tolerant *Eimeria* vaccine. *Vet Parasitol*, 129 (3-4): 179-186, 2005. DOI: 10.1016/j.vetpar.2004.09.034

15. Dalloul RA, Lillehoj HS: Recent advances in immunomodulation and vaccination strategies against coccidiosis. *Avian Dis*, 49 (1): 1-8, 2005. DOI: 10.1637/7306-11150R

16. Khaliq K, Akhtar M, Awaıs MM, Anwar MI: Evaluation of immunotherapeutic effects of *Aloe vera* polysaccharides against coccidiosis in chicken. *Kafkas Univ Vet Fak Derg*, 23 (6): 895-901, 2017. DOI: 10.9775/kvfd.2017.17957

17. Jamil M, Hussain N, Gul J, Harman Y, Ahmed A, Nawz S, Saddam M: Role of probiotics in control of avian coccidiosis. *Br J Poult Sci*, 6 (2): 26-28, 2017.

18. Ritzi MM, Abdelrahman W, Mohnl M, Dalloul RA: Effects of probiotics and application methods on performance and response of broiler chickens to an *Eimeria* challenge. *Poult Sci,* 93 (11): 2772-2778, 2014. DOI: 10.3382/ps.2014-04207

19. Ullah MI, Akhtar M, Awaıs MM, Anwar MI, Khaliq K: Immunological and anti-*eimeria* effects of hot water and methanolic extracts of *Pleurotus sajor-caju* in broiler. *Kafkas Univ Vet Fak Derg*, 24 (6): 893-898, 2018. DOI: 10.9775/kvfd.2018.20232

20. Heak C, Sukon P, Kongpechr S, Tengjaroenkul B, Chuachan K: Effect of direct-fed microbials on intestinal villus height in broiler chickens: A systematic review and meta-analysis of controlled trials. *Int J Poult Sci,* 16 (10): 403-414, 2017. DOI: 10.3923/ijps.2017.403.414

21. Dalloul RA, Lillehoj HS, Shellem TA, Doerr JA: Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. *Poult Sci*, 82 (1): 62-66, 2003. DOI: 10.1093/ps/82.1.62

22. Lee S, Lillehoj HS, Park DW, Hong YH, Lin JJ: Effects of *Pediococcus*- and *Saccharomyces*-based probiotic (MitoMax®) on coccidiosis in broiler chickens. *Comp Immunol Microbiol Infect Dis,* 30 (4): 261-268, 2007a. DOI: 10.1016/j.cimid.2007.02.002

23. Lee SH, Lillehoj HS, Dalloul RA, Park DW, Hong YH, Lin JJ: Influence of *Pediococcus*-based probiotic on coccidiosis in broiler chickens. *Poult Sci*, 86 (1): 63-66, 2007b. DOI: 10.1093/ps/86.1.63

24. Patterson JA, Burkholder KM: Application of prebiotics and probiotics in poultry production. *Poult Sci*, 82 (4): 627-631, 2003. DOI: 10.1093/ps/82.4.627

25. Eckert NH, Lee JT, Hyatt D, Stevens SM, Anderson S, Anderson PN, Beltran R, Schatzmayr G, Monhl M, Caldwell DJ: Influence of probiotic administration by feed or water on growth parameters of broilers reared on medicated and nonmedicated diets. *J Appl Poult Res*, 19 (1): 59-67, 2010. DOI: 10.3382/japr.2009-00084

26. Conway DP, McKenzie ME: Poultry Coccidiosis, Diagnostic and Testing Procedures. 3rd edn., 37-40, Ames, Iowa, Blackwell publishing. IA, USA, 2007.

27. Carvalho FS, Wenceslau AA, Teixeira M, Matos Carneiro JA, Melo ADB, Albuquerque GR: Diagnosis of *Eimeria* species using traditional and molecular methods in field studies. *Vet Parasitol*, 176 (2-3), 2011. DOI: 10.1016/j.vetpar.2010.11.015

28. Holdsworth PA, Conway DP, McKenzie MA, Dayton AD, Chapman HD, Mathis GF, Skinner JT, Mundt HC, Williams RB: World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the efficacy of anticoccidial drugs in chickens and turkeys. *Vet Parasitol*, 121 (3-4): 189-212. DOI: 10.1016/j.vetpar.2004.03.006

29. Miller TL, Wolin MJ: A serum bottle modification of the hungate technique for cultivating obligate anaerobes. *Appl Microbiol*, 27 (5): 985-987, 1974.

30. Giannenas I, Tsalie E, Triantafillou E, Hessenberger S, Teichmann K, Mohnl M, Tontis D: Assessment of probiotics supplementation via feed or water on the growth performance, intestinal morphology and microflora of chickens after experimental infection with *Eimeria acervulina, Eimeria maxima* and *Eimeria tenella*. *Avian Pathol,* 43 (3): 209-216, 2014. DOI: 10.1080/03079457.2014.899430

31. The ROSS Management Handbook 2014. http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-Broiler-Handbook-2014i-EN.pdf/; *Accessed:* 25.07.2017.

32. Georgis' Bowman D: Parasitology for Veterinarians. 8th ed., U.S.A. Saunders. Broiler chicks Gallus domesticus in Riyadh city. Saudi Arabia *J King Saud*, 9 (1): 12-19, 2003.

33. Hovhannisyan SV, Grigoryan KA: The main problems and features of the global and local meat production. *Ann Agrar Sci,* 14 (4): 315-318, 2016. DOI: 10.1016/j.aasci.2016.09.008

34. Biggs PM: The world of poultry disease. *Avian Pathol,* 11 (2): 281-300, 1982. DOI: 10.1080/03079458208436101

35. Györke A, Kalmár Z, Pop LM, Şuteu OL: The economic impact of infection with *Eimeria* spp. in broiler farms from Romania. *R Bras Zootec*, 45 (5): 273-280, 2016. DOI: 10.1590/S1806-92902016000500010

36. Ohimain EI, Ofongo RTS: The effect of probiotic and prebiotic feed supplementation on chicken health and gut microflora: A Review. *Int J Anim Vet Adv,* 4 (2): 135-143, 2012.

37. Firouzi S, Mosleh N, Far SST,Taebipur MJ, Kish GF: Efficacy of anticoccidial vaccination of chickens via different routes: A comparative study. *Bulg J Vet Med*, 17 (4): 293-301, 2014.

38. Chen CY, Chuang LT, Chiang YC, Lin CL, Lien YY, Yang HY: Use of a probiotic to ameliorate the growth rate and the inflamation of broiler chickens caused by *Eimeria tenella* infection. *J Anim Res Nutr,* 1 (2-10): 1-7, 2016. DOI: 10.21767/2572-5459.100010

39. Sarı B, Çakmak A: Etlik piliçlerde coccidiosis'den korunmada anticoccidial katkılı yem uygulamalarının etkisi. *Kocatepe Vet J*, 1, 1-10, 2008.

40. Konosonoka IH, Osmane B,Cerina S, Krastina V,Vitina II, Valdovska A: Feeding technology impact on broiler productivity and intestinal tract microflora. *Eng Rural Dev,* 20, 126-132, 2015.

41. Giannenas I, Papadopoulos E, Tsalie E, Triantafillou E, Henikl S, Teichmann K, Tontis D: Assessment of dietary supplementation with probiotics on performance, intestinal morphology and microflora of chickens infected with *Eimeria tenella*. *Vet Parasitol*, 188 (1-2): 31-40, 2012. DOI: 10.1016/j.vetpar.2012.02.017

42. Taheri V, Seidavi A, Asadpour L, Phillips CJC: A comparison of the effects of antibiotics, probiotics, synbiotics and prebiotics on the performance and carcass characteristics of broilers. *Vet Res Commun*, 42 (3): 195-207, 2018. DOI: 10.1007/s11259-018-9724-2

43. Johnson TJ, Youmans BP, Noll S, Cardona C, Evans NP, Karnezos TP, Ngunjiri JM, Abundo MC, Lee CW: A consistent and predictable commercial broiler chicken bacterial microbiota in antibiotic-free production displays strong correlations with performance. *Appl Environ Microbiol*, 84:e00362-18, 2018. DOI: 10.1128/AEM.00362-18

44. Abu- Akkada SS, Awad AM: Protective effects of probiotics and prebiotics on *Eimeria tenella*-infected broiler chickens. *Pak Vet J*, 35 (4): 446-450, 2015.

45. Bino Sundar ST, Harikrishnan TJ, Latha BR, Sarath Chandra G, Senthil Kumar TMA: Anticoccidial drug resistance in chcken coccidiosis and promising solutions: A review. *J Entomol Zool Stud,* 5 (4): 1526-1529, 2017.

46. Getachew T: A review on effects of probiotic supplementation in poultry performance and cholesterol levels of egg and meat. *J World Poult Res,* 6 (1): 31-36, 2016.

47. Ştef L, Dumitrescu G, Simiz E, Cean A, Julean C, Ştef DS, Pet E, Peţ I, Gherasim V, Corcionivoschi N: The effect of probiotics on broiler growth and intestinal morphology when used to prevent *Campylobacter jejuni* colonization. *Anim Sci Biotech*, 48 (1): 43-50, 2015.

48. Ariyadi B, Harimurti S: Effect of indigenous probiotic lactic acid bacteria on the small intestinal histology structure and expression of mucins in the ileum of broiler chickens. *Int J Poult Sci,* 14 (5): 276-278, 2015. DOI: 10.3923/ijps.2015.276.278

49. Lu H, Adedokun SA, Adeola L, Ajuwon KM: Anti-inflammatory effects of non-antibiotic alternatives in coccidia challenged broiler chickens. *J Poult Sci*, 51, 14-21, 2014. DOI: 10.2141/jpsa.0120176

Kafkas Univ Vet Fak Derg
25 (3): 321-328, 2019
DOI: 10.9775/kvfd.2018.20930

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Hawthorn *(Crataegus oxyacantha)* Flavonoid Extract as an Effective Medicinal Plant Derivative to Prevent Pulmonary Hypertension and Heart Failure in Broiler Chickens

Behnam AHMADIPOUR [1,†] Majid KALANTAR [2,†] Seyed Mahdi HOSSEINI [3] Zia ur REHMAN [3,5]
Farmanullah FARMANULLAH [3] Mohammad Hassan KALANTAR [4] LiGuo YANG [3]

[†] *Authors have equal contribution in the article*
[1] Department of Animal Science, Shahrekord University, Shahrekord 88186-34141, IRAN
[2] Animal Science Department, Qom Agricultural and Natural Source Research and Education Center (AREEO) Qom Iran, IRAN
[3] Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction, Education Ministry of China, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, CHINA
[4] Student Research Committee, Arak University of Medical Science, Arak, IRAN
[5] Department of Animal Health, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture Peshawar, PAKISTAN

Article ID: KVFD-2018-20930 Received: 09.09.2018 Accepted: 15.02.2019 Published Online: 25.02.2019

How to Cite This Article

Ahmadipour B, Kalantar M, Hosseini SM, Rehman ZU, Farmanullah F, Kalantar MH, Yang L: Hawthorn *(Crataegus oxyacantha)* flavonoid extract as an effective medicinal plant derivative to prevent pulmonary hypertension and heart failure in broiler chickens. *Kafkas Univ Vet Fak Derg,* 25 (3): 321-328, 2019. DOI: 10.9775/kvfd.2018.20930

Abstract

The aim of this study was to investigate the effect of crateagus flavonoid extract in preventing pulmonary hypertension syndrome (PHS) in broiler chickens reared at high altitude, encountered ascites was evaluated. A 225 day-old broiler chickens (Ross-308) were randomly assigned to three treatments including different drinking levels of crateagus flavonoid extract (0, 0.1, and 0.2 mL per liter of drinking water) in a 42-day trial. Body weight gain were increased and feed conversion ratio were decreased significantly (P<0.05) when crateagus flavonoid extract was consumed by broiler chickens at levels of 0.1 and 0.2 mL per liter of drinking water in the both starting and growing stages, and throughout the trial. Over-expression of inducible nitric-oxide synthase in the heart was observed in chickens consumed different levels of crateagus flavonoid extract. Birds received crateagus flavonoid extract at levels of 0.1 and 0.2 mL had significantly (P<0.05) higher circulatory concentrations of nitric oxide but significantly (P<0.05) lower serum malondialdehyde concentration, hematocrit and heterophil to lymphocyte ratio compared to control group. Consuming crateagus flavonoid extract at levels of 0.1 and 0.2 mL reduced incidence of right ventricular hypertrophy and led to a significant decline in mortality from PHS. It was concluded that crateagus flavonoid extract is an effective medicinal plant derivative to prevent PHS and ascites in broiler chickens by lowering pulmonary blood pressure and increasing serum antioxidant capacities.

Keywords: *Chicken, Crateagus flavonoid extract, Cardiac disorder, Gene expression, Ascites*

Etlik Piliçlerde Pulmoner Hipertansiyon ve Kalp Yetmezliğini Engellemede Etkili Bir Tıbbi Bitki Türü Olarak Alıç *(Crataegus oxyacantha)* Flavanoid Ekstraktı

Öz

Bu çalışmanın amacı, yüksek irtifada yetiştirilen broiler tavuklarında pulmoner hipertansiyon sendromunun (PHS) önlenmesinde alıç flavonoid ekstraktının etkisini araştırmaktır. 42 gün süreli çalışmada, 225 adet 1 günlük broiler tavuk (Ross-308) farklı içme suyu seviyelerindeki alıç flavanoid ekstraktlarına göre (0, 0.1 ve 0.2 mL/L içme suyu) rastgele üç uygulama grubuna ayrıldı. Başlangıç ve büyüme evrelerinde tüm çalışma süresince 0.1 ve 0.2 mL/L içme suyu oranında alıç flavanoid ekstraktı tüketen broiler tavuklarda anlamlı derecelerde vücut ağırlık kazanımı artarken yem konversiyon oranı azaldı (P<0.05). Farklı seviyelerde alıç flavanoid ekstraktı tüketen tavukların kalplerinde indüklenebilir nitrik oksit sentazın fazla ekspresyonu gözlemlendi. Kontrol grubu ile karşılaştırıldığında 0.1 ve 0.2 mL oranında alıç flavanoid ekstraktı tüketen tavuklarda anlamlı derecelerde nitrik oksitin daha yüksek dolaşım konsantrasyonuna sahip olduğu (P<0.05) ve serum malondialdehit konsantrasyonu, hematokrit ve heterofil/lenfosit oranlarının daha düşük olduğu (P<0.05) belirlendi. 0.1 ve 0.2 mL oranında alıç flavanoid ekstraktı tüketilmesi sağ ventriküler hipertrofi insidansını azalttı ve PHS'ye bağlı mortaliteyi anlamlı derecede azalmaya neden oldu. Alıç flavanoid ekstraktının, pulmoner kan basıncını düşürmek ve serum antioksidan kapasitesini artırmak suretiyle broiler tavuklarda PHS ve aşitesi önlemede etkili bir tıbbi bitki türü olduğu sonucuna varıldı.

Anahtar sözcükler: *Tavuk, Alıç flavanoid ekstraktı, Kardiyak bozukluk, Gen ekspresyonu, Ascites*

İletişim (Correspondence)
+86 27 87281813; Fax: +86 27 87281813
ylg@mail.hzau.edu.cn

INTRODUCTION

Rapid growth in modern broiler chickens has disposed these birds to pulmonary hypertension syndrome (PHS) due to the imbalance between oxygen-demanding muscles and oxygen-supplying organs such as heart and lungs [1]. Intensive genetic selection through the past decades in broiler chickens for rapid growth has reduced the heart and lungs ratio against body muscles mass, whereas increased sensitivity of broiler chickens to PHS will be increase if they are raised at high altitudes with limited atmospheric oxygen supply [2,3]. Succeed vasoconstriction of arterioles will be respond to hypoxia and broiler chickens develops pulmonary hypertension with subsequent right ventricular failure (RVF) that finally leads to ascites and pulmonary vascular remodeling which results from pulmonary hypertension [4,5]. Research has demonstrated the impact of different factors on the development of PHS [6-8]. It is of particularly important to know the effects of herbal medicine in prevention and control of PHS in broiler chickens due to negative effects of PHS on the world broiler chickens industry.

Crataegus oxyacantha (common hawthorn) is an endemic member of the *Rosaceae* family that grows in Europe, Africa, and Asia, where is commonly found as a shrub or small tree 5-10 m tall [9]. Scientific evidence has demonstrated that hawthorn fruit, leaves, and flowers possesses potent antioxidant and free radical scavenging activities, due to the presence of different bioactive compounds, such as epicatechin, hyperoside, and chlorogenic acid [9]. These compounds are reported to have many pharmacological effects, including neuroprotective, hepatoprotective, cardioprotective, and nephroprotective [9,10]. Furthermore, hawthorn fruit possesses tonic effects on the heart and could reduce cardiovascular occurrence [11].

In broiler chickens potential of free radicals in creation of PHS has been addressed [12]. Antioxidants play a vital role in protecting cells against reactive oxygen species (ROS) by reducing chemical radicals and disrupting the process of lipid peroxidation [13]. Low quantities of antioxidants in the body of birds with PHS could therefore lead to an inability to control lipid peroxidation [12]. Cawthon et al.[14] observed lower levels of primary antioxidants, and α-tocopherol, and glutathione (GSH) in the mitochondria in the liver of birds with PHS. Dietary supplementation of vitamin E [15], or as an implant [12], and vitamin C in the diet [15,16] have been used to improve body antioxidant status and to prevent ascites.

Ahmadipour et al.[1] showed that body weight gain and feed to gain responses improved when *Kelussia odoratissima Mozzaf* (KOM) was included in broiler diets at 0.05 and 0.75% in the growing stage and throughout the trial. Over-expression of inducible nitric oxide (iNOS) synthase in the heart, higher circulatory concentrations of NO, but lower serum MDA concentration, hematocrit and heterophil to lymphocyte ratio were observed in chickens fed KOM compared to the birds fed the control diet. Feeding KOM prevented from right ventricular hypertrophy and led to a significant decline in mortality from PHS ($P<0.05$).

Based on the report of Tekeli [17] the use of 10 and 20 g/kg of rosehip in the rations under cold stress conditions in broiler chickens significantly reduced T3 hormone, Na, cholesterol, RBC, HCT and HGB compared to the control group ($P<0.05$).

There is no information about the antioxidant effect of crateagus flavonoid extract on the antioxidant status, PHS and ascites incidence in broiler chickens. According to the facts that some compounds in *crataegus oxyacantha* have strong antioxidant potential and some of its compounds have lowering blood pressure effects, the objectives of the present study were to examine the effects of different drinking levels of crateagus flavonoid extract in preventing pulmonary PHS of broiler chickens. To the best of our knowledge, there has been no report on the effect of *crataegus oxyacantha* on pulmonary hypertension in birds.

MATERIAL and METHODS

Experimental Facility and Hypoxic Condition

The experiment was conducted in the experimental facility of Shahrekord University, Shahrekord, Iran. The study was ethically approved by the Ethical Review Committee of College of Public Health and Medical Sciences of Shahrekord University, Shahrekord, Iran. Management of the chickens in the experimental setting followed the guidelines for animal handling, care and use as prescribed by the Ethical Review Committee at Shahrekord University.

Birds were reared at altitude of 2.100 m above sea level under hypoxic conditions known as hypobaric hypoxia faced with ascites. Hypoxic condition was defined as reduced partial pressure of oxygen that occurs at high altitude as the altitude increases up to 1.800 m [1]. The partial pressure of oxygen falls down 7 mmHg for each 1.000 m altitude approximately. This is equal to a reduction of approximately 2.5% of the air oxygen for each 1.000 m altitude [18]. Therefore, compared to sea level with partial pressure of oxygen equal to 21%, the partial pressure of oxygen in the experimental facility of Shahrekord University was calculated to be 15.75%. At this altitude, hypobaric hypoxia will be associated with a high degree of PHS occurrence and could be leads to ascites [5].

Birds and Management

A total of 225 day-old mixed broiler chickens (Ross 308) from a parent stock of age 42 weeks were randomized across 15 floor pens with 2 square meter area (15 birds per pen). All chicks were allocated to pens so that all pens

had equal average body weights (46.8±1.2 g). Each pen was equipped with a bell drinker and a feed trough. The temperature of the experimental house was set at about 32°C during week 1, then at a rate of 3°C reduced through week 2 to week 4, and finally fixed at 22°C until the end of trail. All chicks had free access to feed and water and provided with 23 h light and 1 h dark throughout the trial.

Treatments

A mash diet based on corn and soybean meal were formulated for the starting (1-3 weeks of age, AME:CP=139) and growing (3-6 weeks of age, AME:CP=160) stages according to NRC (1994) recommendations for all treatments *(Table1)*. Experimental treatments were prepared by adding 0.0, 0.1 and 0.2 mL of crateagus flavonoid extract (HE 00152, Crateagus-Drop 6260) per liter of drinking water (pH=7.05; TDS=2.000 ppm) of broiler chickens. So each liter of drinking water contained 0.25 and 0.50 mg of total flavonoids compounds. In this way, birds in groups of 0.1 and 0.2 received 0.05 to 0.10 mg of total flavonoids compounds daily. Generally, flavonoid extract of *crataegus oxyacantha* containing biologically active flavonoid compounds (polyphenols) like anthocyanidins and proanthocyanidins (also known as bioflavones or procyanidins). Each mL of oral crataegus-Drop 6260 contained 2.5 mg of total flavonoids compounds in form of hyperoside (21.4% polyphenols and 19.7% procyanidins), produced by Iran-Darouk Pharmacy Co, under production code of 3067-88-02. Determination of total phenolic compounds in crataegus-Drop 6260 was done through colorimetric method according to the standard extraction procedure by mentioned company [19].

Measurements

Mass body weight of birds in each pen was obtained at 21 and 42 days of age. Body weight gain and feed intake were calculated for 1-21 day, 21-42 day, and 1-42 day periods. Feed conversion ratio (FCR) data corrected for mortality weights, was also calculated for all of periods. At 42 days of age, 10 birds per treatment were selected for blood collection and processing. The selected birds had body weights within approximately 5% of the average pen body weight. Blood samples (3 mL) were collected from the brachial vein and centrifuged at 2500 g for 10 min to obtain sera. Serum samples were used for the determination of NO and MDA. Serum NO was measured according to the method described by Chapman and Wideman [20]. Serum MDA concentration as biomarker of oxidative stress was assayed by the method of Nair and Turner [21].

For measuring hematocrit, samples of blood were collected in micro-hematocrit tubes. An aliquot of blood was also obtained on glass slides to prepare the blood smear for the determination of differential leukocyte count. Thereafter the May-Grunwald and Giemsa staining, 100 leukocytes, including granular (heterophils) and non-granular (lymphocytes) were enumerated and the heterophil to lymphocyte ratio (H:L) was calculated. All chemical reagents were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Co., St. Louis, MO, USA). After the blood collection, the birds were killed by decapitation. Data obtained at processing time were included live body weight, hot carcass weight, breast weight, and thigh weight. The hearts were also removed and the ventricles were dissected and weighed to calculate the right-to-total ventricular weight ratio (RV: TV ratio). The RV: TV is indicative of pulmonary hypertension [7]. In addition, total mortality and mortality from PHS was

Table1. *Composition of the basal diet for broiler chickens during starter and grower stages*

Item (% Unless Noted)	**Starter (1-21 Days)**	**Grower (22-42 Days)**
Corn	47.4	55.8
Soybean meal (44% CP)	37.3	33.5
Fish meal (60% CP)	3.6	1.1
Wheat bran	0.5	1.3
Soy oil	7.5	4.7
Dicalcium phosphate	1.3	1.2
Oyster shell	1.45	1.5
Salt	0.35	0.3
DL-Methionine	0.1	0.1
L-Lysine	-	-
Mineral supplement[a]	0.25	0.25
Vitamin supplement[b]	0.25	0.25
Calculated composition		
AME (kcal/kg)	3200	3200
CP	23	20
AME:CP	139	160
Met	0.52	0.41
Met+Cys	0.86	0.74
Lys	1.3	1.06
Thr	1	0.91
Arg	1.46	1.29
Ca	1	0.91
Available P	0.45	0.35
Na	0.18	0.15
Cl	0.27	0.29
K	0.91	0.92
Na + K – Cl (mEq/kg)	237	238

[a] Provided the following per kg of diet: vit. A (trans retinyl acetate), 3600 IU; vit. D_3 (cholecalciferol), 800 IU; vit. E (dl-α-tocopheryl acetate), 7.2 mg; vit. K_3, 1.6 mg; thiamine, 0.72 mg; riboflavin, 3.3 mg; niacin, 0.4 mg; pyridoxin, 1.2 mg; cobalamine, 0.6 mg; folicacid, 0.5 mg; choline chloride, 200 mg.
[b] Provided the following per kg of diet: Mn (from $MnSO_4.H_2O$), 40 mg; Zn (from ZnO), 40 mg; Fe (from $FeSO_4.7H_2O$), 20 mg; Cu (from $CuSO_4.5H_2O$), 4 mg; I [from $Ca(IO_3)_2.H_2O$], 0.64 mg; Se (from sodium selenite),0.08 mg

checked daily throughout the trial and whenever the RV: TV was greater than 0.25 are considered as pulmonary hypertension [22].

PCR Analysis

At the end of trail (42 days of age), 10 chickens from the control group and the groups received different levels of crateagus flavonoid extract were randomly selected, weighed and killed by decapitation. The hearts were harvested and the right ventricles were dissected and immediately frozen in liquid nitrogen and stored at -70°C for subsequent RNA analysis. Specific primers of SOD1, iNOS and β-actin were designed with Primer-Blast (NCBI). Details of the primers are listed in *Table 2*.

Polymerase chain reactions (PCRs) were carried out in a realtime PCR cycler (Rotor Gene Q6000, Qiagen, USA) in three replicates for each sample of ventricles. The quantitative polymerase chain reaction (qPCR) methodology was followed as explained with slight modification [23]. One microliter cDNA (complementary DNA) was added to the 10 µL of SYBR® Premix Ex Taq II Mix and 1 µL of each specific primer in a total volume of 20 µL. The thermal profile was 95°C for 30 s, 40 cycles of 94°C for 40 s, 64°C for 35 s and 72°C for 30 s. At the end of each phase, the measurement of fluorescence was done and used for quantitative objectives. Gene expression data were normalized to β-actin. Data were analyzed using LinReg PCR software version 2012.0 (Amsterdam, Netherland), to give the threshold cycle number and reaction efficiency [24]. Relative transcript levels and fold changes in transcript abundance were calculated using efficiency adjusted Paffl methodology [25].

Statistical Analysis

Results were analyzed by GLM procedure using SAS (2007) software in a completely randomized design. Data were subjected to a nested design when there was sampling effect within pens. The statistical model used for growth performance data was $Y_{ij} = \mu + T_i + e_{ij}$. For other traits, the model was $Y_{ijk} = \mu + T_i + e_{ij} + \varepsilon_{ijk}$. In these models, Y_{ij} and Y_{ijk} are observations; µ is the general mean; Ti is the effect of treatment i; e_{ij} is random error; and ε_{ijk} is subsampling error. Means were separated by Duncan's multiple range test.

RESULTS

Effects of different drinking levels of crateagus flavonoid extract on broiler chickens growth performance and the rate of mortality are shown in *Table 3*. Body weight gain and FCR improved when drinking crateagus flavonoid extract was used by broiler chickens at levels of 0.1 and 0.2 mL throughout the trial (P<0.05). However, no significant effect was observed among treatments in terms of feed intake in 1-21 days of age. Significant decline in mortality percentage of birds was observed through different stages of trail in the groups received 0.1 and 0.2 mL of crataegus flavonoid extract compared to the control group (P<0.05).

Table 4 indicates blood and serum variables of broiler chickens received different levels of drinking crateagus flavonoid extract. Broiler chickens received drinking crateagus flavonoid extract at levels of 0.1 and 0.2 mL had higher concentrations of NO, but lower concentrations of MDA than that of control group (P<0.05). Both levels of drinking crateagus flavonoid extract caused a reduction in heterophil to lymphocyte ratio and hematocrit when compared to the control (P<0.05).

The expression of SOD1, iNOS, and ET-1 genes in the heart of broiler chickens affected by different levels of drinking crateagus flavonoid extract *(Table 5)*. Superoxide dismutase-1 was highly over-expressed in broiler chickens consumed drinking crateagus flavonoid extract at both levels of 0.1 and 0.2 mL. Inducible nitricoxide synthase was also highly over-expressed in the right ventricle of birds consumed drinking crateagus flavonoid extract at levels of 0.1 and 0.2 mL. On the other hand, crateagus flavonoid extract significantly suppressed the expression of ET-1.

Table 6 depicts the carcass characteristics of broiler chickens consumed different levels of drinking crateagus flavonoid extract at 42 days of age. Carcass yield was higher in broiler chickens consumed levels of 0.1 and 0.2 drinking crateagus flavonoid extract compared to control group, but breast and thigh yields were not affected by different levels of drinking crateagus flavonoid extract. However, inclusion of crateagus flavonoid extract in drinking water of broiler chickens reduced the proportions of liver, heart and abdominal fat when compared to the

Table 2. *Details of the primers used for quantitative real time PCR analysis of chicken mRNAs*

Target	Primers	PCR Product (bp)	Accession No
β-Actin	5′-AGCGAACGCCCCCAAAGTTCT-3′ 5′-AGCTGGGCTGTTGCCTTCACA-3′	13	NM_205518.1
SOD1	5′-CACTGCATCATTGGCCGTACCA-3′ 5′-GCTTGCACACGGAAGAGCAAGT-3′	223	NM_205064.1
iNOS	5′-AGGCCAAACATCCTGGAGGTC-3′ 5′-TCATAGAGACGCTGCTGCCAG-3′	371	U46504
ET-1	5′-GGACGAGGAGTGCGTGTATT-3′ 5′-GCT CCAGCAAGCATCTCTG-3′	141	XM418943

SOD1: superoxide dismutase 1; iNOS: inducible nitric oxide synthase; ET-1: endothelin 1; bp: base pair

Table 3. *Effects of drinking crataegus flavonoid extract on broiler's growth performance and mortality percentage*

Parameter	Age	Drinking Levels of *Crataegus* Flavonoid Extract			SEM
		Control (0 mL)	0.1 (mL)	0.2 (mL)	
Weight gain (g/bird)	1-21 days of age	664.13^{b}	713.00^{a}	703.49^{a}	16.84
	22-42 days of age	1334.09^{b}	1473.64^{a}	1534.79^{a}	41.64
	1-42 days of age	1998.22^{b}	2186.64^{a}	2238.18^{a}	40.47
Feed intake (g/bird)	1-21 days of age	1051.75	1031.25	1012.93	26.42
	22-42 days of age	2860.96^{b}	2945.33^{a}	2898.24^{ab}	33.61
	1-42 days of age	3887.71^{b}	4001.58^{a}	3961.17^{ab}	41.48
Feed conversion ratio	1-21 days of age	1.58^{a}	1.45^{b}	1.44^{b}	0.02
	22-42 days of age	2.14^{a}	2.00^{b}	1.89^{c}	0.02
	1-42 days of age	1.95^{a}	1.83^{b}	1.77^{c}	0.03
Mortality percentage (%)	1-21 days of age	8.25^{a}	6.09^{b}	5.98^{b}	0.38
	22-42 days of age	23.27^{a}	16.43^{b}	16.17^{b}	0.66
	1-42 days of age	31.52^{a}	22.52^{b}	22.15^{c}	0.91

Table 4. *Effect of drinking crataegus flavonoid extract on serum and blood variables in broiler chickens measured at 42 days of age*

Parameter	Drinking Levels of *Crataegus* Flavonoid Extract			SEM
	Control (0 mL)	0.1 (mL)	0.2 (mL)	
Plasma nitric oxide (µmol/L)	5.32^{c}	6.71^{b}	8.06^{a}	0.36
Malondialdehyde (µmol/L)	2.09^{a}	1.06^{b}	0.84^{c}	0.11
Heterophil to lymphocyte (%)	1.03^{a}	0.70^{b}	0.61^{b}	0.16
Hematocrit (%)	39.75^{a}	36.13^{b}	32.50^{c}	1.65

Superscripts in the same row with different letters are statistically different (P<0.05)
Each mean represents values from 10 replicates

Table 5. *Effect of drinking crataegus flavonoid extract on expression of SOD1, iNOS, and ET-1 genes in the right ventricle of broiler chickens measured at 42 days of age*

Item	Control (T1)	0.1 (T2)	0.2 (T3)	T2/T1 Ratio	T3/T1 Ratio	SEM
SOD1	0.0001^{c}	0.006^{b}	0.021^{a}	60	210	0.008
iNOS	0.001^{c}	0.024^{b}	0.603^{a}	24	603	0.016
ET-1	0.047^{a}	0.001^{b}	0.001^{b}	0.021	0.021	0.024

Superscripts in the same column with different letters are statistically different (P<0.05). SOD1: superoxide dismutase1; iNOS: inducible nitricoxide; ET-1:endothelin1; CAT: Catalase. Number of observation=20

Table 6. *Effect of drinking crataegus flavonoid extract on carcass characteristics of broiler chickens at 42 days of age (as % of carcass weight)*

Item (%)	Drinking Levels of *Crataegus* Flavonoid Extract			SEM
	Control (0 mL)	0.1 (mL)	0.2 (mL)	
Carcass yield	67.68^{b}	70.78^{a}	70.66^{a}	1.39
Breast yield	35.27	35.05	36.35	0.87
Thigh yield	30.21	30.54	30.22	0.45
Abdominal fat	1.39^{a}	1.14^{b}	1.05^{b}	0.10
Liver	2.88^{a}	2.52^{b}	2.26^{c}	0.07
Heart	0.83^{a}	0.71^{ab}	0.63^{b}	0.06
RV:TV (ratio)	0.32^{a}	0.25^{b}	0.22^{b}	0.02

Superscripts in the same row with different letters are statistically different (P<0.05)
Each mean represents values from 10 replicates. RV:TV right ventricle to total ventricle weight ratio

Table 7. *Effect of drinking crataegus flavonoid extract on total mortality and mortality from PHS in broiler chickens reared up to 42 days of age*

Item (%)	Drinking Levels of *Crataegus* Flavonoid Extract			SEM
	Control (0 mL)	0.1 (mL)	0.2 (mL)	
Total mortality	31.52[a]	25.52[b]	22.15[b]	2.91
PHS mortality	28.72[a]	22.32[b]	18.41[b]	2.88

Superscripts in the same row with different letters are statistically different (P<0.05)

control. In addition, using of drinking crateagus flavonoid extract decreased the RV:TV ratio (P<0.05).

In *Table 7* compared the total mortality with mortality from PHS in broiler chickens consumed different levels of drinking crateagus flavonoid extract up to 42 days of age. Consuming different levels of drinking crateagus flavonoid extract at both levels of 0.1 and 0.2 mL caused a reduction in PHS mortality (P<0.05).

DISCUSSION

In this study increase of body weight gain and decrease in FCR was observed in broiler chickens after consuming different levels of drinking crateagus flavonoid extract which can be attributed to positive effects of this compound. As a rule, the RV:TV ratio is an index of pulmonary hypertension in chickens so that the RV:TV values greater than 0.25 regards as pulmonary hypertension [1,6]. In the control group the mean value of RV: TV was greater than 0.25, implicated to further number of birds in this group which suffered from pulmonary hypertension. Increased growth performance of birds in the groups received drinking crateagus flavonoid extract can also be attributed to the polyphenols (flavonoids) and oligomeric proanthocyanidins (OPCs) compounds in *crataegus oxyacantha*. The plant polyphenols including flavonoids and non-flavonoids exhibit a broad spectrum of beneficial biological properties such as growth-promoting, anti-oxidative, sedative, antibacterial and anti- viral actions [26]. Increased serum concentration of NO as a result of consuming different levels of crateagus flavonoid extract to broiler chickens is due to the over- expression of iNOS gene in broiler's heart. It has been demonstrated that iNOS gene is normally expressed in the heart of broiler chickens and contributed in normal NO production in myocardio-cytes. NO is an important regulator of cardiac function by involvement in the control of myocardial energetics, myocardial regeneration, hyper- trophic remodeling and improvement of ventricular diastolic distensibility [1,27]. It has been suggested that impaired NO synthesis and local reduction of iNOS gene expression in the heart ventricles are involved in the pathophysiology of cardiac failure in broiler chickens with pulmonary hypertension [28]. On the other hand, consuming different levels of crateagus flavonoid extract especially at level of 0.2 mL per liter in drinking water of broiler chickens caused significant reductions in circulatory level of MDA. MDA is an indicator of lipid oxidation in the body and it is an index of oxidative stress. It is clear that chickens are very susceptible to oxidative stress because of their higher metabolic rate [29]. Increased metabolic rate resulted in higher production of ROS [1,12]. Moreover, birds have a body temperature about 3°C higher than in mammals, which expands the production of ROS [30,31].

As well as, chicken's blood glucose concentration as a potent oxidative factor is at least twice as high as that of mammals [32], so antioxidant potency is crucial to broiler chickens against oxidative agents [33]. Some of compounds in crateagus flavonoid extract such as flavonoids, particularly OPCs contribute to the productive roles against oxidative stress and lipid peroxidation (reduced MDA concentration) along with increase the activities of the antioxidant enzymes glutathione peroxidase, super-oxide dismutase and catalase, which counteract the oxidative stress [9,34]. Additionally, some organic acids such as chlorogenic acid and ferulic acid has ferric reducing ability and by involvement in Fe^{2+}/H_2O_2 systems exerts antioxidant activity [34-36]. Following mechanisms through using flavonoids can also prevent oxidative stress including: direct scavenging of ROS, activation of anti-oxidant enzymes, metal chelating activity, reduction of α-tocopheryl radicals, inhibition of oxidases, and increase in uric acid level [3,26]. Flavonoids have great amount of vitamin P (citrin bioflavonoid) which can synergistically act with vitamin C which counteract the oxidative products [37,38]. Significant reductions observed in the H:L ratio and hematocrit in birds consumed different levels of crateagus flavonoid extract are in accordance with decreased oxidative stress. The H:L ratio is an index to describe stress in the chicken [39]. Therefore, consuming crateagus flavonoid extract suppresses ROS production and deduces the oxidative stress of birds, which led to increase the growth performance and reducing the MDA level and H:L ratio.

Also in this study similar to previous experiment [1], abdominal fat deposition and liver percentage was significantly reduced in chickens consumed different levels of crateagus flavonoid extract. crateagus flavonoid extract has lipolytic effect [40,41]. Lipolytic effect of crateagus flavonoid extract is attributed to flavonoids and OPCs as well as phenolic compounds [40,42]. Lipid-lowering effects of flavonoids have been well documented [43]. Reduced liver percentage compare to live body weight in chickens

consumed different levels of crateagus flavonoid extract is in line with decreased lipogenesis as appeared in reduced abdominal fat. Liver in the chicken is the primary site of lipogenesis [44] and declined liver weight reflects lower lipogenesis due to the consuming of crateagus flavonoid extract in broiler chickens diets. The proportion of heart percentage to live body weight and RV:TV ratio have been reduced by consuming of crateagus flavonoid extract to birds. These observations confirm ability of *crataegus oxyacantha* flavonoid extract to prevent heart hypertrophy and particularly right ventricular hypertrophy. It is evident that birds of the control group (RV:TV more than 0.25) are in pre-ascitic condition and this situation has been improved when birds consumed crateagus flavonoid extract at levels of 0.1 and 0.2 mL per liter of drinking water. Although other research findings in this regard point to this fact that the RV:TV more than 0.27 can be considered as ascetic condition [43]. In this regard, a significant decline in total mortality and mortality from PHS was observed in the groups received consumed different levels of crateagus flavonoid extract when compared to the control group.

Crataegus oxyacantha flavonoid extract significantly promoted over expression of SOD in the heart of chickens. Research has shown that over expression of SOD reduces hypertension, increases availability of NO and endothelium-dependent relaxation in different models of hypertension [1,45]. This finding explains significant reduction in the incidence of PHS in birds consumed crateagus flavonoid extract at levels of 0.1 and 0.2 mL per liter of drinking water. According to previous reports, the vascular remodeling in lung vessel beds contributes to mortality of broiler chickens with PHS [5,7]. Flavonoid content of crateagus extract prevents the proliferation of vascular smooth muscle cells and inhibits thickening of the intima and narrowing of the vessels, as well as exerts considerable collagen stabilizing effect [46,47]. Thus, flavonoids and OPCs contribute to be the effective factor in preventing cardiovascular diseases [11,47]. Flavonoids and OPCs has also vaso-relaxant potential and considering the fact that flavonoids and OPCs is the main constitute of *crataegus oxyacantha*, this medicinal plant could effectively prevent PHS in broiler chickens. Furthermore, flavonoids as the main constitute of *crataegus oxyacantha* have endothelium-independent vaso-dilating effects and by possessing lowering blood pressure potential [33,48] further improved cardio-pulmonary function and helped to prevent PHS [11,46]. It is worth noting that the vasodilatory effect of flavonoid compounds may be intensified by over production of NO synthesis [1,48]. Significant decrease in the expression of ET-1 by consuming of crateagus flavonoid extract further suggests the potential of this plant extract in preventing pulmonary hypertension.

In conclusion, consuming different levels of crateagus flavonoid extract could significantly prevent PHS and cardiac disorders in broiler chickens reared at high altitudes encountered to ascites. Beneficial effects of crateagus flavonoid extract are attributed to antioxidant actions that mediated through flavonoids and OPCs bioactive compounds. Therefore, crateagus flavonoid extract is an effective medicinal plant derivative to prevent pulmonary hypertension in broiler chickens under the terms of ascites and reared at high altitude.

Acknowledgments

This study was financially supported by the Shahrekord University, Shahrekord, Iran. Authors wish to thanks for technical helps and lab supports of animal science department of agricultural research center of Qom (QARC), Qom, Iran.

Authors Conflict

All the authors have not conflict.

REFERENCES

1. Ahmadipour B, Hassanpour H, Asadi E, Khajali F, Rafiei F, Khajali F: Kelussia odoratissima Mozzaf - A promising medicinal herb to prevent pulmonary hypertension in broiler chickens reared at high altitude. *J Ethnopharmacol,* 159, 49-54, 2015. DOI: 10.1016/j.jep.2014.10.043

2. Khajali F, Fahimi S: Influence of dietary fat source and supplementary alpha-tocopheryl acetate on pulmonary hypertension and lipid peroxidation in broilers. *J Anim Physiol Anim Nutr,* 94 (6): 767-772, 2010. DOI: 10.1111/j.1439-0396.2009.00959.x

3. Behrooj N, Khajali F, Hassanpour H: Feeding reduced-protein diets to broilers subjected to hypobaric hypoxia is associated with the development of pulmonary hypertension syndrome. *Br Poult Sci,* 53 (5): 658-664, 2012. DOI: 10.1080/00071668.2012.727082

4. Khajali F, Saedi M: The effect of low chloride and high bicarbonate diets on growth, blood parameters, and pulmonary hypertensive response in broiler chickens reared at high altitude. *Archiv für Geflügelkunde,* 75, 235-238, 2011.

5. Wideman RF, Rhoads DD, Erf GF, Anthony NB: Pulmonary arterial hypertension (ascites syndrome) in broilers: A review. *Poult Sci,* 92 (1): 64-83, 2013. DOI: 10.3382/ps.2012-02745

6. Izadinia M, Nobakht M, Khajali F, Faraji M, Zamani F, Qujeq D, Karimi I: Pulmonary hypertension and ascites as affected by dietary protein source in broiler chickens reared in cool temperature at high altitudes. *Anim Feed Sci Technol,* 155 (2-4): 194-200, 2010. DOI: 10.1016/j.anifeedsci.2009.12.009

7. Khajali F, Liyanage R, Wideman RF: Methylglyoxal and pulmonary hypertension in broiler chickens. *Poult Sci,* 90 (6): 1287-1294, 2011. DOI: 10.3382/ps.2010-01120

8. Saki A, Haghighat M, Khajali F: Supplemental arginine administered in ovo or in the feed reduces the susceptibility of broilers to pulmonary hypertension syndrome. *Br Poult Sci,* 54 (5): 575-580, 2013. 10.1080/00071668.2013.811716

9. Chang Q, Zuo Z, Harrison F, Chow MSS: Hawthorn. *J Clin Pharmacol,* 42 (6): 605-612, 2002. DOI: 10.1177/00970002042006003

10. Kirakosyan A, Seymour E, Kaufman PB, Warber S, Bolling S, Chang SC: Antioxidant capacity of polyphenolic extracts from leaves of *Crataegus laevigata* and *Crataegus monogyna* (Hawthorn) subjected to drought and cold stress. *J Agric Food Chem,* 51 (14): 3973-3976, 2003. DOI: 10.1021/jf030096r

11. Salehi S, Long SR, Proteau PJ, Filtz TM: Hawthorn *(Crataegus monogyna* Jacq.) extract exhibits atropine-sensitive activity in a cultured cardiomyocyte assay. *J Nat Med,* 63 (1): 1-8, 2009. DOI: 10.1007/s11418-

008-0278-4

12. Bottje WG, Wideman RF: Potential role of free radicals in the pathogenesis of pulmonary hypertension syndrome. *Poult Avian Biol Rev,* 6, 211-231, 1995.

13. Yu BP: Cellular defenses against damage from reactive oxygen species. *Physiol Rev,* 74 (1): 139-162, 1994. DOI: 10.1152/physrev.1994.74.1.139

14. Cawthon D, Beers K, Bottje WG: Electron transport chain defect and inefficient respiration may underlie pulmonary hypertension syndrome (ascites)-associated mitochondrial dysfunction in broilers. *Poult Sci,* 80 (4): 474-484, 2001. DOI: 10.1093/ps/80.4.474

15. Nain S, Wojnarowicz C, Laarveld B, Olkowski AA: Effects of dietary vitamin E and C supplementation on heart failure in fast growing commercial broiler chickens. *Br Poult Sci,* 49 (6): 697-704, 2008. DOI: 10.1080/00071660802415658

16. Ladmakhi MH, Buys N, Dewil E, Rahimi G, Decuypere E: The prophylactic effect of vitamin C supplementation on broiler ascites incidence and plasma thyroid hormone concentration. *Avian Pathol,* 26 (1): 33-44, 1997. DOI: 10.1080/03079459708419191

17. Tekeli A: Effect of rosehip fruit *(Rosa canina* l.) supplementation to rations of broilers grown under cold stress conditions on some performance, blood, morphological, carcass and meat quality characteristics. *Europ Poult Sci,* 78, 1612-9199, 2014. DOI: 10.1399/eps.2014.19

18. Julian RJ: The response of the heart and pulmonary arteries to hypoxia, pressure, and volume. A short review. *Poult Sci,* 86 (5): 1006-1011, 2007. DOI: 10.1093/PS/86.5.1006

19. Seevers PM, Daly JM: Studies on wheat stem rust resistance controlled at the Sr6 locus. II. Peroxidase activities. *Phytopathology* 60 (11): 1642-1647, 1970. DOI: 10.1094/Phyto-60-1642

20. Chapman ME, Wideman Jr RF: Evaluation of total plasma nitric oxide concentrations in broilers infused intravenously with sodium nitrite, lipopolysaccharide, aminoguanidine, and sodium nitroprusside. *Poult Sci,* 85 (2): 312-320, 2006. DOI: 10.1093/ps/85.2.312

21. Nair V, Turner GA: The thiobarbituric acid test for lipid peroxidation: Structure of the adduct with malondialdehyde. *Lipids,* 19 (10): 804-805, 1984. DOI: 10.1007/bf02534475

22. Saedi M, Khajali F: Blood gas values and pulmonary hypertension as affected by dietary sodium source in broiler chickens reared at cool temperature in a high-altitude area. *Acta Vet Hung,* 58 (3): 379-388, 2010. DOI: 10.1556/AVet.58.2010.3.10

23. Rehman ZU, Worku T, Davis JS, Talpur HS, Bhattarai D, Kadariya I, Hua G, Cao J, Dad R, Farmanullah, Hussain T, Yang L: Role and mechanism of AMH in the regulation of Sertoli cells in mice. *J Steroid Biochem Mol Biol*, 174, 133-140, 2017. DOI: 10.1016/j.jsbmb.2017.08.011

24. Ruijter JM1, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, Moorman AF: Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res,* 37 (6): e45, 2009. DOI: 10.1093/nar/gkp045

25. Dorak M: Real Time PCR. Taylor & Francis, Oxford, UK, 2006.

26. Surai PF: Polyphenol compounds in the chicken/animal diet: From the past to the future. *J Anim Physiol Anim Nutr,* 98 (1): 19-31, 2014. DOI: 10.1111/jpn.12070

27. Belge C, Massion PB, Pelat M, Balligand JL: Nitric oxide and the heart: Update on new paradigms. *Ann N Y Acad Sci,* 1047 (1): 173-182, 2005. DOI: 10.1196/annals.1341.016

28. Hassanpour H, Yazdani A, Khabir Soreshjani K, Asgharzadeh S: Evaluation of endothelial and inducible nitric oxide synthase genes expression in the heart of broiler chickens with experimental pulmonary hypertension. *Br Poult Sci,* 50 (6): 725-732, 2009. DOI: 10.1080/00071660903141005

29. Munshi-South J, Wilkinson GS: Bats and birds: Exceptional longevity despite high metabolic rates. *Ageing Res Rev,* 9 (1): 12-19, 2010. DOI: 10.1016/j.arr.2009.07.006

30. Sohal RS, Weindruch R: Oxidative stress, caloric restriction, and aging. *Science,* 273 (5271): 59-63, 1996. DOI: 10.1126/science.273.5271.59

31. Nagy KA: Field metabolic rate and body size. *J Exp Biol,* 208: 1621-1625, 2005. DOI: 10.1242/jeb.01553

32. Braun EJ, Sweazea KL: Glucose regulation in birds. *Comp Biochem Physiol B Biochem Mol Biol,* 151 (1): 1-9, 2008. DOI: 10.1016/j.cbpb.2008.05.007

33. Jorge VG, Angel JR, Adrian TS, Francisco AC, Anuar SG, Samuel ES, Angel SO, Emmanuel HN: Vasorelaxant activity of extracts obtained from Apium graveolens: possible source for vasorelaxant molecules isolation with potential antihypertensive effect. *Asian Pac J Trop Biomed,* 3 (10): 776-779, 2013. DOI: 10.1016/s2221-1691(13)60154-9

34. Barros L, Carvalho AM, Ferreira ICFR: Comparing the composition and bioactivity of *Crataegus Monogyna* flowers and fruits used in folk medicine. *Phytochem Anal,* 22 (2): 181-188, 2011. DOI: 10.1002/pca.1267

35. Liu J: Pharmacology of oleanolic acid and ursolic acid. *J Ethnopharmacol,* 49 (2): 57-68, 1995. DOI: 10.1016/0378-8741(95)90032-2

36. Sajjadi SE, Shokoohinia Y, Moayedi NS: Isolation and identification of ferulic acid from aerial parts of *Kelussia odoratissima* Mozaff. *Jundishapur J Nat Pharm Prod,* 7 (4): 159-162, 2012.

37. Mills S, Bone K: Principles and practice of phytotherapy. Modern Herbal Medicine. Churchill Livingstone, 2000.

38. Özcan M, Hacıseferoğulları H, Marakoğlu T, Arslan D: Hawthorn (Crataegus spp.) fruit: Some physical and chemical properties. *J Food Eng,* 69 (4): 409-413, 2005. DOI: 10.1016/j.jfoodeng.2004.08.032

39. Zulkifli I, Che Norma MT, Chong CH, Loh TC: Heterophil to lymphocyte ratio and tonic immobility reactions to preslaughter handling in broiler chickens treated with ascorbic acid. *Poult Sci,* 79 (3): 402-406, 2000. DOI: 10.1093/ps/79.3.402

40. Rajendran S, Deepalakshmi PD, Parasakthy K, Devaraj H, Devaraj SN: Effect of tincture of Crataegus on the LDL-receptor activity of hepatic plasma membrane of rats fed an atherogenic diet. *Atherosclerosis,* 123 (1-2): 235-241, 1996.

41. Zhang Z, Ho WKK, Huang Y, James AE, Lam LW, Chen ZY: Hawthorn fruit is hypolipidemic in rabbits fed a high cholesterol diet. *J Nutr,* 132 (1): 5-10, 2002. DOI: 10.1093/JN/132.1.5

42. Long SR, Carey RA, Crofoot KM, Proteau PJ, Filtz TM: Effect of hawthorn *(Crataegus oxycantha)* crude extract and chromatographic fractions on multiple activities in a cultured cardiomyocyte assay. *Phytomedicine,* 13 (9-10): 643-650, 2006. DOI: 10.1016/J.PHYMED.2006.01.005

43. Chen JJ, Li XR: Hypolipidemic effect of flavonoids from mulberry leaves in triton WR-1339 induced hyperlipidemic mice. *Asia Pac J Clin Nutr,* 16 (Suppl. 1): 290-294, 2007.

44. Kouba M, Catheline D, Leclercq B: Lipogenesis in turkeys and chickens: A study of body composition and liver lipogenic enzyme activities. *Br Poult Sci,* 33 (5): 1003-1014, 1992. DOI: 10.1080/00071669208417543

45. Chu Y, Iida S, Lund DD, Weiss RM, DiBona GF, Watanabe Y, Faraci FM, Heistad DD: Gene transfer of extracellular superoxide dismutase reduces arterial pressure in spontaneously hypertensive rats. *Circ Res,* 92 (4): 461-468, 2003. DOI: 10.1161/01.RES.0000057755.02845.F9

46. Brixius K, Willms S, Napp A, Tossios P, Ladage D, Bloch W, Mehlhorn U, Schwinger RHG: Crataegus special extract WS® 1442 induces an endothelium-dependent, NO-mediated vasorelaxation via eNOS-phosphorylation at serine 1177. *Cardiovasc Drugs Ther,* 20 (3): 177-184, 2006. DOI: 10.1007/s10557-006-8723-7

47. Lu Q, Qiu TQ, Yang H: Ligustilide inhibits vascular smooth muscle cells proliferation. *Eur J Pharmacol,* 542 (1-3): 136-140, 2006. DOI: 10.1016/j.ejphar.2006.04.023

48. Mladěnka P, Zatloukalová L, Filipský T, Hrdina R: Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. *Free Radic Biol Med,* 49 (6): 963-975, 2010. DOI: 10.1016/j.freeradbiomed.2010.06.010

Kafkas Univ Vet Fak Derg
25 (3): 329-333, 2019
DOI: 10.9775/kvfd.2018.20937

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

The Biometric Ratios on the Tarsus of the Chinchilla *(Chinchilla lanigera)* Based on 3D Reconstructed Images

Sema ÖZKADİF [1,a] Emrullah EKEN [2,b] Ayşe HALIGÜR [1,c]

[1] Cukurova University, Faculty of Ceyhan Veterinary Medicine, Department of Anatomy, TR-01930 Adana - TURKEY
[2] Selcuk University, Faculty of Veterinary Medicine, Department of Anatomy, TR-42003 Konya - TURKEY
[a] ORCID: 0000-0002-5398-9874; [b] ORCID: 0000-0001-7426-5325; [c] ORCID: 0000-0002-3668-4286

Article ID: KVFD-2018-20937 Received: 11.09.2018 Accepted: 04.02.2019 Published Online: 07.02.2019

How to Cite This Article

Özkadif S, Eken E, Halıgür A: The biometric ratios on the tarsus of the Chinchilla *(Chinchilla lanigera)* based on 3D reconstructed images. *Kafkas Univ Vet Fak Derg,* 25 (3): 329-333, 2019. DOI: 10.9775/kvfd.2018.20937

Abstract

This study was undertaken to perform a three-dimensional (3D) reconstruction of the tarsal bones of chinchillas using multidetector computed tomography (MDCT) images and reveal biometric ratio of the bones and compare between sexes. For this purpose, a total of 12 adult chinchillas *(Chinchilla lanigera)* of both sexes (six males and six females) were used. After anesthetizing the animals, MDCT images were obtained in DICOM format, and 3D reconstruction was performed on a computer using the Mimics 13.1 program. The volumes and surface areas of each of the bones that constitute the tarsus of the chinchilla were automatically measured by the program based on the 3D model. After all values of each tarsal bone were expressed as ratios with in tarsus, they were analyzed statistically to reveal differences between sexes. The results showed that there were statistical differences ($P<0.05$) in calcaneus, talus, central tarsal bone and tarsal bone IV in term of volume ratio and in central tarsal bone, tarsal bone I and tarsal bone IV in term of surface area ratio between sexes. It is considered that 3D tarsus models are useful in revealing anatomic structures and also in assisting clinical diagnosis and treatment.

Keywords: *Tarsus, Chinchilla, 3D imaging, Anatomy*

Chinchilla *(Chinchilla lanigera)* Tarsus'unda Üç Boyutlu Rekontrüksiyon Görüntülerine Dayalı Biyometrik Oranlar

Öz

Bu çalışma şinşillanın tarsal kemiklerinin multidedektör bilgisayarlı tomografi (MDBT) görüntülerini kullanarak üç boyutlu (3B) rekonstrüksiyonunu yapmak ve kemiklerin biyometrik oranlarını ortaya koymak ve cinsiyetler arasında karşılaştırmak amacıyla gerçekleştirildi. Bunun için her iki cinsiyetten (6 erkek, 6 dişi) toplam 12 adet yetişkin şinşilla *(Chinchilla lanigera)* kullanıldı. Anestezi altında hayvanların MDBT görüntüleri alındıktan sonra DICOM formatında depolandı ve Mimics 13.1 programının olduğu bir bilgisayarda 3B rekonstrüksiyonları gerçekleştirildi. 3B modeli ortaya konulan şinşilla tarsus'unu oluşturan tarsal kemiklerin her birinin hacimleri ve yüzey alanları otomatik olarak program tarafından ölçüldü. Her bir tarsal kemik değerinin tarsus'daki oranları belirtildikten sonra, cinsiyetler arasındaki farklılıkları ortaya çıkarmak için istatistiki analiz yapıldı. Sonuçlar hacim oranına göre calcaneus, talus, os tarsi centrale ve os tarsale IV'de ve yüzey alanı oranına göre os tarsi centrale, os tarsale I ve os tarsale IV'de cinsiyetler arasında istatistiki farkın ($P<0.05$) olduğunu gösterdi. 3B tarsus modellerinin anatomik yapıları ortaya çıkarmada ve ayrıca klinik tanı ve tedaviye yardımcı olmada yararlı olduğu düşünülmektedir.

Anahtar sözcükler: *Tarsus, Şinşilla, 3D görüntüleme, Anatomi*

INTRODUCTION

The skeletal dimensions are important when there are no key points that allow the body to be recognized. Sex discrimination is important in the recognition of the body [1]. In forensic medicine anatomically, short bones have some advantages than other bones [2]. Measurements of hand and tarsal bones have been shown to be sexually dimorphic by previous researchers [3,4].

The tarsal bones are morphologically less recognizable than long bones by non-specialists and can be easily misidentified due to their similarities in animals of similar sizes [5]. Three-dimensional (3D) models of the tarsal bones

İletişim (Correspondence)
+90 322 6133507
semaerten80@gmail.com

assist in determining the shape and size of these bones, as well as the joint geometries, by observing the relationship between the different bones. These models also facilitate the diagnosis and treatment of foot deformities [6].

Measurements obtained from 3D model of bones uses in sexual dimorphism [7]. Computer-based volume calculations from 3D models and volumetric ratios are significant in determining the gender [8].

A review of the literature reveals studies on the tarsal bones on the leopard *(Panthera pardus)* [9], the Indian blackbuck *(Antilope cervicapra)* [10], rabbit [11], the grasscutter *(Thryonomys swinderianus)* [12] and the Indian spotted deer *(Axis axis)* [13]; computerized tomography imaging in dogs [14] and African hedgehogs *(Atelerix albiventris)* [15]; and the 3D reconstruction of the tarsal joint in mice [16], laboratory mice, white-footed mice, rats [17], and red-footed tortoises *(Chelonoidis carbonaria)* [18]. Furthermore, research has been undertaken for the 3D reconstruction of human foot bones, and the 3D reconstructed images of the tarsal bones have been utilized in clinical trials, as well as anatomical studies [17,19].

The anatomy of a lot of domestic rodents such as guinea pigs, rats, mice, and hamsters, has been well described. Chinchillas are being popularity as pets [20]. Çevik-Demirkan et al.[21] investigated the anatomy of the hindlimb of the chinchilla. In another study, the radiological images of the chinchilla skeleton were analyzed and provided osteological contribution [22]. Also 3D reconstruction of femur and vertebral column performed and morphometric measurements revealed [23,24]. However, to the best of our knowledge, no study has been conducted to perform 3D reconstruction of the chinchilla's tarsal bones, identify their volume and surface area ratios and determine whether there are any differences between the sexes. This current study was carried out to fill this field in the literature.

MATERIAL and METHODS

This study was accepted by the ethics committee of the Veterinary Faculty of Selcuk University on April 27, 2018 (Decision number: 2018/39). In the study, a total of 12 adult chinchillas *(Chinchilla lanigera)* of both sexes weighing from 500 to 600 g. were used. The 3D models of the tarsal bones were obtained with the Multimodal Immersive Motion rehabilitation with Interactive Cognitive Systems (Mimics) 13.1 software. In order to obtain 3D reconstruction via this program, the MDCT images of the tarsal bones were obtained at high resolution. The animals from which the images were to be taken were anesthetized with a mixture of 60 mg/kg ketamin (Ketalar, Pfizer®) and 6 mg/kg xylazine (Rompun, Bayer®) intravenously. Under anesthesia, the MDCT images were taken of the animals in a prone position. The parameters of the MDCT instrument (Somatom Sensation 64; Siemens Medical Solutions, Germany) were adjusted as; physical detector collimation, 32 x 0.6 mm; final section collimation, 64 x 0.6 mm; section thickness, 0.50 mm; gantry rotation time; 330 msec; kVp; 120; mA, 300; resolution, 512 x 512 pixel; and resolution range, 0.92 x 0.92. The dosage parameters and scans were performed by utilizing standard protocols and taking the literature [25,26] into consideration. Radiometric resolution (MONOCHROME2; 16 bits) was obtained at the lowest radiation level and with optimum image quality. The images were stored in DICOM format and transferred to a personal computer installed with Mimics 13.1.

Two of the experts in the field of anatomy obtained similar results by performing reconstructions of tarsal bones at different times. In the automatic segmentation process, the limits of tarsal bones were determined and were assigned different colors *(Fig. 1)*. The limits of the images were determined, and the reconstruction of the tarsal bones was carried out using the 3D transformer component of Mimics 13.1. The volume and surface area of all tarsal bones in the chinchilla both right and left side were measured automatically using the 3D program. After all values of each tarsal bone were expressed as ratios with in tarsus, they were analyzed statistically to reveal differences between sexes. The materiality control of the differences between the average values was undertaken using the SPSS 16.00 software program and an independent t- test.

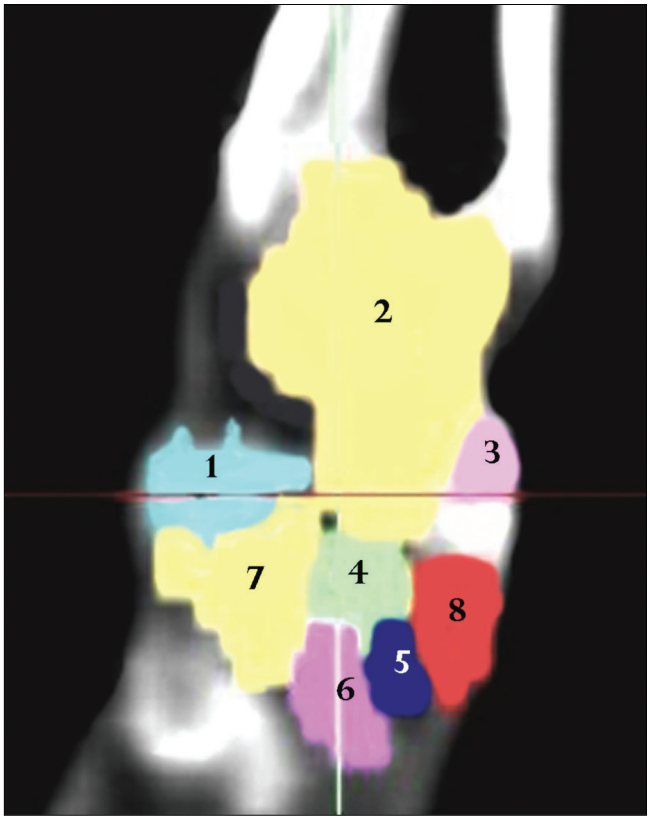


Fig 1. Limitation of tarsal bones on coronal section with different colors
1: Calcaneus, 2: Talus, 3: Medial tibial tarsal bone, 4: Central tarsal bone, 5: Tarsal bone II, 6: Tarsal bone III, 7: Tarsal bone IV, 8: Tarsal bone I

RESULTS

The volume and surface area of the chinchilla tarsal bones were obtained from 3D reconstruction formed using the Mimics 13.1 program to process the MDCT images *(Fig. 2, Fig. 3)*. The statistical results the ratio of the mean values were found significant at the level of P<0.05 *(Table 1, Table 2)*.

The 3D reconstructed images of the tarsal bones of the chinchilla revealed eight bones. The proximal row of the tarsus consisted of the calcaneus articulating with the fibula, the talus articulating with the tibia, and the medial tibial tarsal bone in the medial of the talus. In the distal row were the tarsal bone I to IV. In both proximal and distal rows, the central tarsal bone was observed *(Fig. 1, Fig. 2, Fig. 3)*. It was determined that the central tarsal bone did not articulate with the calcaneus and medial tibial tarsal bone.

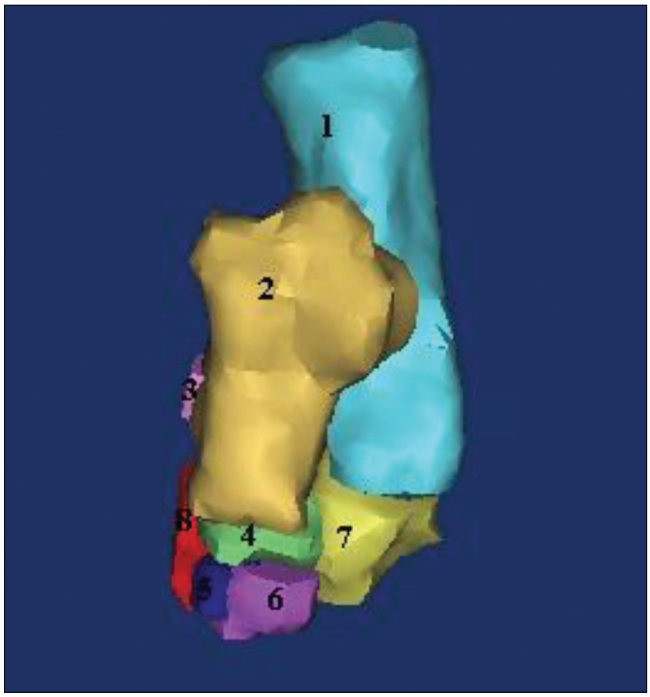


Fig 2. Dorsal view of 3D model of tarsal bones
1: Calcaneus, 2: Talus 3: Medial tibial tarsal bone, 4: Central tarsal bone, 5: Tarsal bone II, 6: Tarsal bone III, 7: Tarsal bone IV, 8: Tarsal bone I

Both right and left side of the tarsal bones a statistically significant difference was found for calcaneus, talus, central tarsal bone and tarsal bone IV in term of volume ratio between sexes. Also for central tarsal bone, tarsal bone I and tarsal bone IV was seen statistical difference between sexes in term of surface area ratio *(Table 1, Table 2)*.

For both male and female chinchillas, the order of the tarsal bones from the greatest to the smallest volume was as follows: the calcaneus, talus, tarsal bone IV, central tarsal bone, tarsal bone III, tarsal bone I, tarsal bone II, and medial tibial tarsal bone. The order of the tarsal bones according to their surface area from the largest to the smallest was; the calcaneus, talus, tarsal bone IV, tarsal bone III, central tarsal bone, tarsal bone I, tarsal bone II, and medial tibial tarsal bone for female chinchillas, and the calcaneus, talus, tarsal bone IV, central tarsal bone, tarsal bone III, tarsal bone I, tarsal bone II, and medial tibial tarsal bone in male chinchillas *(Table 1, Table 2)*.

DISCUSSION

In this study, 3D model obtained from the MDCT images of the tarsal bones in the chinchilla. Three-dimensional reconstructions for bone are clearer and more useful and it is used in tarsal bones [14].

Female chinchillas are larger than male chinchillas. They born larger and grow for a longer time [27]. Depending on gender, there will be biometric differences between male and female. The most important thing is the difference in the ratio of the measured values.

In this study, the volume and surface area ratio of the tarsal bones differed between the male and female chinchillas. This is consistent with the results of previous study indicating that sexuel dimorphism in chinchilla [27]. Also sexuel dimorphism were showed in tarsal bones in human [4].

The limitation of this study is the number of the animal. In

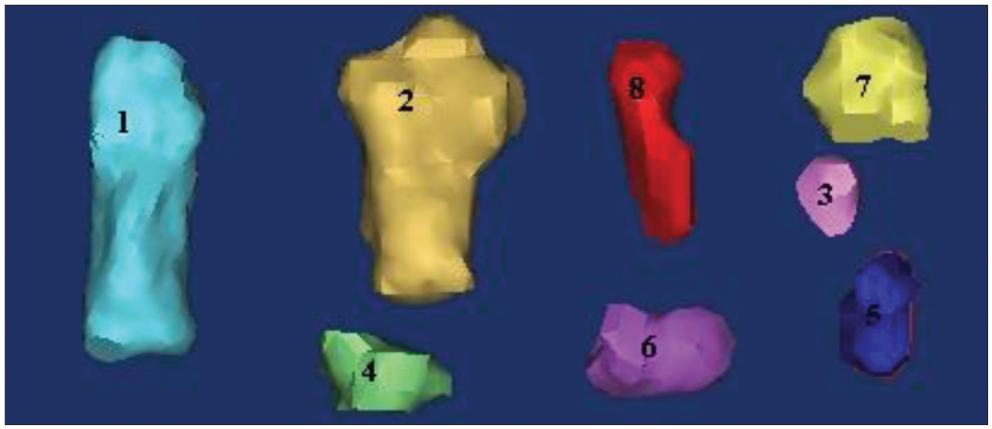


Fig 3. 3D model of tarsal bones
1: Calcaneus, 2: Talus, 3: Medial tibial tarsal bone, 4: Central tarsal bone, 5: Tarsal bone II, 6: Tarsal bone III, 7: Tarsal bone IV, 8: Tarsal bone I

Table 1. *Statistical analysis performed by taking percentage rates of volume means obtained from 3D images of tarsal bones (Mean ± SD)*

Measurements	Right			Left		
	Female (n=6)	Male (n=6)	P	Female (n=6)	Male (n=6)	P
Calcaneus	42.22±0.15	44.16±0.24	0.000	42.35±0.17	44.08±0.18	0.000
Talus	31.26±0.21	30.43±0.45	0.002	31.31±0.17	30.47±0.41	0.001
Central tarsal bone	5.79±0.07	5.31±0.22	0.001	5.70±0.06	5.43±0.21	0.016
Medial tibial tarsal bone	1.38±0.05	1.32±0.06	0.080	1.37±0.05	1.32±0.10	0.313
Tarsal bone I	3.41±0.07	3.42±0.33	0.937	3.43±0.09	3.40±0.31	0.789
Tarsal bone II	2.93±0.08	2.82±0.14	0.135	2.91±0.08	2.74±0.20	0.087
Tarsal bone III	5.46±0.07	5.26±0.28	0.124	5.44±0.06	5.29±0.21	0.125
Tarsal bone IV	7.50±0.13	7.25±0.15	0.012	7.45±0.09	7.23±0.18	0.028

Table 2. *Statistical analysis performed by taking percentage rates of surface area means obtained from 3D images of tarsal bones (mean ± SD)*

Measurements	Right			Left		
	Female (n=6)	Male (n=6)	P	Female (n=6)	Male (n=6)	P
Calcaneus	35.56±0.13	35.42±0.30	0.340	35.61±0.15	35.41±0.34	0.211
Talus	25.47±0.11	25.50±0.21	0.771	25.42±0.10	25.48±0.21	0.612
Central tarsal bone	7.60±0.12	7.79±0.08	0.009	7.58±0.13	7.89±0.28	0.034
Medial tibial tarsal bone	2.49±0.08	2.57±0.12	0.264	2.50±0.08	2.54±0.08	0.356
Tarsal bone I	6.46±0.12	6.77±0.18	0.007	6.45±0.13	6.77±0.20	0.011
Tarsal bone II	5.33±0.06	5.33±0.14	0.899	5.35±0.09	5.35±0.16	0.966
Tarsal bone III	7.72±0.11	7.68±0.15	0.681	7.71±0.12	7.63±0.15	0.356
Tarsal bone IV	9.34±0.11	8.90±0.13	0.000	9.34±0.09	8.89±0.12	0.000

this study we used 12 chinchillas. If we have more animals we would be able to get strengthen statistical result. Three-dimensional reconstruction method helps user to better understand the anatomical structures that are difficult to understand with other methods by allowing the user to transform 3D image into what they need [28]. Three-dimensional reconsructive models uses in anatomical studies [23,24,29-31] and clinical studies [32-34]. The validity and reliability of 3D models were proven on comparison of biometric measurement values [35]. Three- dimensional reconstruction with small bones the section thickness of the MDCT images should be very little.

In conclusion, this was the first study to perform biometric ratios on the tarsus of the chinchilla based on 3D re-constructed images. The 3D volume and surface area ratios of tarsal bones in chinchilla revealed and sexuel dimorphism showed on chinchilla tarsus. Three-dimensional tarsus models can be useful for the investigation of the anatomy and morphology of the tarsal bones with a rather small and complex structure, help clinicians in the diagnosis and treatment processes, assist surgeons in planning operations and in forensic medicine. In further studies the the tarsal joint should be study with its ligaments.

REFERENCES

1. Siddiqi N, Norrish M: Sexual dimorphism from femoral bone dimensions parameters among African Tribes and South Africans of European descent. *Int J Forensic Sci Sexual*, 2 (3): 1-11, 2018.

2. Navsa N, Steyn M, Iscan MY: Sex determination from the metacarpals in a modern South African male and female sample UPS Space University, Pretoria, 2008. www.up.ac.az/dspace/handle.net; *Accessed:* 23 September 2018.

3. Eshak GA, Ahmed HM, Abdel Gawad EA: Gender determination from hand bones length and volume using multidetector computed tomography: A study in Egyptian people. *J Forensic Leg Med*, 18, 246-252, 2011. DOI: 10.1016/j.jflm.2011.04.005

4. Harris SM, Case DT: Sexual dimorphism in the tarsal bones: Implications for sex determination. *J Forensic Sci*, 57, 295-305, 2012. DOI: 10.1111/j.1556-4029.2011.02004.x

5. Smart TS: Carpals and tarsals of mule deer, black bear and human: an osteology guide for the archaeologist. *MSc Thesis,* Western Washington University, 2009.

6. Jain ML, Dhande SG, Vyas NS: Computer aided diagnosis of human foot's bones. *IJBES*, 1, 17-26, 2014.

7. Brzobohata H, Krajicek V, Horak Z, Veleminska J: Sexual dimorphism of the human tibia through time: Insights into shape variation using a surface-based approach. *PLoS One,* 11 (11): e0166461, 2016. DOI:10.1371/journal.pone.0166461

8. Shearer BM, Sholts SB, Garvin HM, Wärmländer SKTS: Sexual dimorphism in human browridge volume measured from 3D models of dry crania: A new digital morphometrics approach. *Forensic Sci Int,* 222 (1-3): 400.e1-400.e5, 2012. DOI: 10.1016/j.forsciint.2012.06.013

9. Podhade DN, Shrivastav AB, Vaish R, Tiwari Y: Morphology and morphometry of tarsals of the leopard *(Panthera pardus). Res J Anim Vet Fishery Sci*, 2, 20-21, 2014.

10. Choudhary OMP, Ishwer S, Bharti SK: Gross and biometrical studies on the tarsal bones of Indian blackbuck *(Antilope cervicapra). IJBAAR*, 13,

453-456, 2015.

11. Ajayi IE, Shawulu JC, Zachariya TS, Ahmed S, Adah BMJ: Osteomorphometry of the bones of the thigh, crus and foot in the New Zealand white rabbit *(Oryctolagus cuniculus)*. *Ital J Anat Embryol*, 117, 125-134, 2012.

12. Onwuama KT, Ojo SA, Hambolu JO, Dzenda T, Zakari FO, Salami SO: Macro-anatomical and morphometric studies of the hindlimb of grasscutter *(Thryonomys swinderianus*, Temminck-1827). *Anat Histol Embryol*, 47, 21-27, 2018. DOI: 10.1111/ahe.12319

13. Yadav S, Joshi S, Mathur R, Choudhary OP: Morphometry of tarsal and metatarsal of Indian Spotted Deer *(Axis axis)*. *Indian Vet J*, 92, 43-46, 2015.

14. Gielen IM, De Rycke LM, Van Bree HJ, Simoens PJ: Computed tomography of the tarsal joint in clinically normal dogs. *Am J Vet Res*, 621 (2): 1911-1915, 2001. DOI: 10.2460/ajvr.2001.62.1911

15. Girgiri IA, Yahaya A, Gambo BG, Majama YB, Sule A: Osteomorphology of the appendicular skeleton of four-toed african hedgehogs *(Atelerix albiventris)* Part (2): Pelvic limb. *Glob Vet*, 16, 413-418, 2016.

16. Kai Y, Matsumoto K, Kameoka S, Arai S, Matsumoto N, Komiyama K, Shimba S, Honda K: Observation of the tarsus joint in the Mop-3/Bmal-1 gene knock-out mouse using "*In vivo*" Micro-CT: Influence of diet and sex on calcification of the tendon of the tarsus joint. *J Hard Tissue Biol*, 21, 133-140, 2012. DOI: 10.2485/jhtb.21.133

17. Richbourg HA, Martin MJ, Schachner ER, Mcnulty MA: Anatomical variation of the tarsus in common inbred mouse strains. *Anat Rec*, 300, 450-459, 2017. DOI 10.1002/ar.23493

18. Bortolini Z, Lehmkuhl RC, Ozeki LM, Tranquilim MV, Sesoko NF, Teixeira CR, Vulcano LC: Association of 3D reconstruction and conventional radiography for the description of the appendicular skeleton of chelonoidis carbonaria (Spix, 1824). *Anat Histol Embryol*, 41, 445-452, 2012. DOI: 10.1111/j.1439-0264.2012.01155.x

19. Getman LM, Ross MW, Smith MA: Surgical repair of fractures of the lateral and medial tibial malleoli in a yearling Arabian filly. *Equine Vet Educ*, 24, 496-502, 2012. DOI: 10.1111/j.2042-3292.2011.00328.x

20. Brenner SZG, Hawkins MG, Tell LA, Hornof WJ, Plopper CG, Verstraete FJM: Clinical anatomy, radiography, and computed tomography of the chinchilla skull. *Comp Cont Educ Pract,* 27, 933-942, 2005.

21. Çevik-Demirkan A, Özdemir V, Demirkan I: Anatomy of the hind limb skeleton of the chinchilla *(Chinchilla lanigera)*. *Acta Vet Brno*, 76, 501-507, 2007. DOI: 10.2754/avb200776040501

22. Gasse CAS: Contribution radiologique et ostéologique à la connaissance du chinchilla (*Chinchilla lanigera*). These pour obtenir le grade de Docteur Veterinaire. Ministere de L'agriculture et de la Peche Ecole Nationale Veterinaire de Toulouse. France. 94-97. 2008.

23. Ozkadif S, Varlik A, Kalayci I, Eken E: Morphometric evaluation of chinchillas *(Chinchilla lanigera)* femur with different modelling techniques. *Kafkas Univ Vet Fak Derg*, 22, 945-951, 2016. DOI: 10.9775/kvfd.2016.15683

24. Ozkadif S, Eken E, Dayan MO, Besoluk K: Determination of sex-related differences based on 3D reconstruction of the chinchilla *(Chinchilla lanigera)* vertebral column from MDCT scans. *Vet Med-Czech,* 62, 204-210, 2017. DOI: 10.17221/19/2015-VETMED

25. Prokop M: General principles of MDCT. *Eur J Radiol*, 45, S4-S10, 2003. DOI: 10.1016/S0720-048X(02)00358-3

26. Kalra MK, Maher MM, Toth TL, Hamberg LM, Blake MA, Shepard J, Saini S: Strategies for CT radiation dose optimization. *Radiology*, 230, 619-628, 2004. DOI: 10.1148/radiol.2303021726

27. Lammers AR, Dziech HA, German RZ: Ontogeny of sexual dimorphism in *Chinchilla lanigera* (Rodentia: Chinchillidae). *J Mammal,* 82, 79-189, 2001. DOI: 10.1644/1545-1542(2001)082<0179:OOSDIC>2.0.CO;2

28. Yamada K, Taniura T, Tanabe S, Yamaguchi M, Azemoto S, Wisner ER: The use of multi- detector row computed tomography (MDCT) as an alternative to specimen preparation for anatomical insrtuction. *J Vet Med Educ*, 34:143-150, 2007.

29. Jaeger M, Briand D, Borianne P, Bonnel F: Knee anatomy 3D reconstruction and visualization from CT scans. *Surg Radiol Anat,* 15 (3): 231-231, 1993.

30. Gezer İnce N, Demircioğlu İ, Yılmaz B, Ağyar A, Dusak A: Martılarda *(Laridae* spp.) cranium'un üç boyutlu modellemesi. *Harran Üniv Vet Fak Derg*, 7, 98-101, 2018.

31. Özkadif S, Eken E, Kalaycı I: A three-dimensional reconstructive study of pelvic cavity in the New Zealand rabbit *(Oryctolagus cuniculus)*. *Sci World J*, 2014:489854, 2014. DOI: 10.1155/2014/489854

32. Watanabe Y, Ikegami R, Takasu K, Mori K: Three-dimensional computed tomographic images of pelvic muscle in anorectal malformations. *J Pediatr Surg,* 40, 1931-1934, 2005. DOI: 10.1016/j.jpedsurg.2005.08.010

33. Jun BC, Song SW, Cho JE, Park CS, Lee DH, Chang KH, Yeo SW: Three-dimensional reconstruction based on images from spiral high-resolution computed tomography of the temporal bone: Anatomy and clinical application. *J Laryngol Otol*, 119, 693-698, 2005.

34. Miyamoto R, Tadano S, Sano N, Inagawa S, Adachi S, Yamamoto M: The impact of three-dimensional reconstruction on laparoscopic-assisted surgery for right-sided colon cancer. *Wideochir Inne Tech Maloinwazyjne*, 12, 251-256, 2017. DOI: 10.5114/wiitm.2017.67996

35. Kim M, Huh KH, Yi WJ, Heo MS, Lee SS, Choi SC: Evaluation of accuracy of 3D reconstruction images using multi-detector CT and cone-beam CT. *Imaging Sci Dent*, 42, 25-33, 2012. DOI: 10.5624/isd.2012.42.1.25

Kafkas Univ Vet Fak Derg
25 (3): 335-339, 2019
DOI: 10.9775/kvfd.2018.20950

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

The Effect of Hot-Iron Disbudding on Thiol-Disulphide Homeostasis in Calves

Hasan ERDOĞAN [1,a] İlker ÇAMKERTEN [2,b] Güzin ÇAMKERTEN [3,c] Kerem URAL [1,d]
Songül ERDOĞAN [1,e] İsmail GÜNAL [4,f] Özcan EREL [5,g]

[1] Adnan Menderes University, Veterinary Faculty, Internal Medicine, TR-09016 Aydın - TURKEY
[2] Aksaray University, Veterinary Faculty, Internal Medicine, TR-68100 Aksaray - TURKEY
[3] Aksaray University, Veterinary Faculty, Biochemistry, TR-68100 Aksaray - TURKEY
[4] Harran University, Veterinary Faculty, Internal Medicine, TR-63000 Şanlıurfa - TURKEY
[5] Yıldırım Beyazıt University, Faculty of Medicine, Biochemistry, TR-06800 Ankara - TURKEY
[a] ORCID: 0000-0001-5141-5108; [b] ORCID: 0000-0002-6952-7703; [c] ORCID: 0000-0003-2732-9490; [d] ORCID: 0000-0003-1867-7143
[e] ORCID: 0000-0002-7833-5519; [f] ORCID: 0000-0003-3679-4132; [g] ORCID: 0000-0002-2996-3236

Article Code: KVFD-2018-20950 Received: 12.09.2018 Accepted: 25.12.2018 Published Online: 26.12.2018

How to Cite This Article

Erdoğan H, Çamkerten İ, Çamkerten G, Ural K, Erdoğan S, Günal İ, Erel Ö: The effect of hot-iron disbudding on thiol-disulphide homeostasis in calves. *Kafkas Univ Vet Fak Derg,* 25 (3): 335-339, 2019. DOI: 10.9775/kvfd.2018.20950

Abstract

We aimed to examine the effect of hot-iron disbudding on serum thiol-disulphide homeostasis levels as a marker of oxidant stress in relationship with trauma in calves under sedation, local anaesthesia, and the non-steroidal anti-inflammatory drug ketoprofen. A total of 30 Holstein calves were enrolled in the study and allocated into three groups: disbudded following sedation with xylazine (n=10) (group I); disbudded following sedation (xylazine) and local anaesthesia with lidocaine (n=10) (group II); and disbudded after sedation (xylazine), local anaesthesia (lidocaine), and ketoprofen (n=10) (group III). Blood samples were withdrawn before (0. min) and 30, 60, 90, and 120 min after dehorning. Serum native thiols, total thiols, and disulphide levels were detected with a novel assay. Native thiol and total thiol levels were reduced in all groups without any significance during the study period. At the 90th min of the study, native thiol levels in group II were significantly lower than in groups I and III. There were no significant alterations in total thiol levels in both groups. Disulphide levels showed no significant changes in group, time, and group by time interactions, but at the 60th min, groups I and III had the lowest levels. Disulphite/native thiol, disulphite/total thiol, and native thiol/total thiol levels had significant group alterations in the 60th min. The reduction of native thiol and total thiol levels in all groups without significance might be related to the antioxidant activity of plasma; however, it is thought that the pain management procedures should be related to the sensitive oxidative balance by thiols.

Keywords: *Calf, Disbudding, Sedation, Thiol-disulphide*

Buzağılarda Sıcak Koter İle Boynuzsuzlaştırmanın Tiyol-Disülfit Homeostazı Üzerine Etkisi

Öz

Bu çalışmada sedasyon, lokal anestezi ve non-steroidal bir ilaç olan ketoprofen uygulanmış sıcak koterizasyon işlemi ile boynuzsuzlaştırılan buzağılarda oksidatif stresin değerlendirilmesinde tiyol-disülfüt homeostazına olan etkilerinin belirlenmesi amaçlandı. Çalışmaya toplam 30 adet Holstein buzağı dahil edilerek üç gruba ayrıldı: sedasyon (xylazine) işlemini takiben boynuzsuzlaştırılan (n=10) (grup I); sedasyon (xylazine) ve lidokain ile lokal anestezi işlemi sonrasında boynuzsuzlaştırılan (n=10) (grup II); ve sedasyon (xylazine), local anestezi (lidocaine) ve ketoprofen uygulaması sonrasında boynuzsuzlaştırılan (n=10) (group III). Kan örnekleri, boynuzsuzlaştırmadan önce (0. dak) ve 30, 60, 90 ve 120 dakika sonra olacak şekilde toplandı. Serum natif tiyol, total tiyol ve disülfür seviyeleri yeni bir test ile belirlendi. Çalışma süresince natif tiyol ve total tiyol seviyelerinde istatistiksel anlamlı olmayan azalmalar belirlendi. Araştırmanın 90. dakikasında grup II'de bulunan hayvanların natif tiyol seviyelerinin grup I ve III'e göre istatistiksel anlamlı düşüktü. Total tiyol seviyelerinde ise gruplar arasında istatistiksel anlamlı farklılıkların olmadığı belirlendi. Disülfit seviyelerinde grup, zaman ve grup zaman arasında farklılıklar belirlenmezken, araştırmanın 60. dakikasında grup I ve III'te en düşük seviyelere geldiği belirlendi. Disülfit/natif tiyol, disülfit/total tiyol ve natif tiyol/total tiyol seviyelerininde 60. dakikada gruplar arasında istatistiksel anlamlı değişimler gösterdiği belirlendi. Natif tiyol ve total tiyol seviyelerindeki bu azalmaların plazmadaki antioksidan kapasite ile ilişkili olabileceği, ancak uygulanan ağrı yönetimi prosedürlerinin tiyol ile oluşturulan hassas oksidatif dengeyi etkilediği düşünüldü.

Anahtar sözcükler: *Buzağı, Boynuzsuzlaştırma, Sedasyon, Tiyol-disülfit*

İletişim (Correspondence)
+90 256 2470700/372 Fax: +90 256 2470720
hasan.erdogan@adu.edu.tr

INTRODUCTION

Dehorning is one of the frequently applied practices in livestock and is depended on to keep animals safe from injuries. Dehorning is a stressful and painful process that results in many homeostatic changes in animals [1]. The disbudding procedure is another term for dehorning in calves up to 3 months of age. The disbudding procedure can be applied to calves in different ways, such as hot-iron disbudding, chemical disbudding with caustic pastes, and surgical disbudding using scoop dehorners. In calves up to 8 weeks of age, hot-iron disbudding can be used [2]. However, this provokes third-degree burns in the area where it is performed [3]. Along with hot-iron disbudding, inflammatory changes, severe pain, behavioural/physiological alterations, and acute stress responses exist in calves [2,4-6]. Numerous reports have described behavioural and physiological reactions to disbudding in calves [1,7-9]. Many inflammatoric conditions cause an increase in oxidative stress mediators that are provoked by pro-inflammatory cytokines in castrated and dehorned calves [10,11]. Previous studies and the American Veterinary Medical Association indicate the essentiality of pain management by pharmacological agents. These studies point out several methodologies, such as local anaesthesia [4,12,13], non-steroidal anti-inflammatory drugs (NSAIDs) [13-15], and sedatives [16,17].

Oxidative balance is described as the equilibrium among free radical eradication and production. Cell damage initiated by free radicals is limited to oxidative balance, and the imbalanced free radical production derives oxidative stress. Thiol is a novel and substantial antioxidant used to eliminate reactive oxygen via non-enzymatic and enzymatic pathways [18,19]. The plasma thiol pool includes both low molecular weight thiols (e.g. glutathione, cysteine, and homocysteine) and protein thiols. Thiols have an antioxidant role in oxidation reactions by composing disulphide bonds. Dynamic thiol/disulphide homeostasis is crucial for detoxification, apoptosis, and the processes of controlling enzymatic reactions and signalling pathways. Many inflammatoric conditions are initiated by anomalous thiol/disulphide levels [20-25]. Korkmaz et al.[9] described the alterations of oxidative stress parameters in calves and mature cows undergoing hot iron dehorning, and defined the amputation process [9,26]. To our knowledge, dynamic thiol/disulphide homeostasis has not been studied previously in calves. Therefore, in the present study, we aimed to evaluate the alterations of thiol/disulphide homeostasis in calves undergoing disbudding with different analgesia and anaesthesia procedures.

MATERIAL and METHODS

Experimental Design, Calves, and Treatments

The study included 30 Holstein calves from both sexes (17 male and 13 female) at 10 weeks of age (BW= 85.6±8.9 kg). All calves were assigned to individual pens 7 days prior to the study and weaned at 7 weeks of age. Calves were fed with ad libitum access to water and a calf starter during the entire period. Study procedures was approved by Local Animal Ethic Committee of Adnan Menderes University with a number of 2017-058.

Calves were randomly divided into three groups. Study groups were designed as the treatment procedure: group I (n=10) xylazine group (with disbudding under sedation with an intramuscular injection of xylazine [Xylazinbio®, Interhas, Czech Republic (dose of 0.25 mg/kg)]); group II (n=10) to those of calves administered xylazine and local anaesthesia [with disbudding under xylazine sedation and subcutaneous infiltration of 20 mg of lidocain (Adokain®, Sanovel, Turkey) for horn buds prior to disbudding]; and group III (n=10) received xylazine, local anaesthesia, and subcutaneous injection of meloxicam (Maxicam®, Sanovel, Turkey) with a dose of 0.5 mg/kg before dehorning. All treatment and disbudding procedures were performed by the same researcher. Furthermore, the local anaesthesia procedure was carried out with a ring block and corneal nerve block between the horn bud and lateral canthus of the eye. Calves were deprived from calf starter 12 h prior to the study (in an attempt to decrease the risk of bloat whereas personnel controlled the calves during reanimation.

Sample Collection and Analysis

Peripheral blood specimens were withdrawn from *Vena jugularis* starting prior to the dehorning and after drug application at 0, 30, 60, 90, and 120 min in lithium heparinised tubes. Immediately after the blood samples were taken, plasma samples were removed using a portable centrifuge in the eppendorf tubes. Plasma samples were stored at -80°C, then moved to the laboratory and analysed.

Total thiol (-S-S- + -SH) includes native and reduced thiol. A novel automatic and spectrophotometric technique established by Erel and Neselioglu [27] was used to determine the thiol/disulphide concentrations. The principle of this method is based on the degradation of dynamic disulphide bonds (–S–S–) to functional thiol groups (–SH) with a sodium borohydride ($NaBH_4$) solution. The remaining $NaBH_4$ residue was totally removed by formaldehyde. Thus, this inhibited extra reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) along with any disulphide bonds resulting from the reaction with DTNB. The following reaction with the DTNB-modified Ellman reagent was used to detect the amount of total thiol. The disulphide levels were counted automatically as half of the quantity of total thiol and native thiol. Disulphide/total thiol percent ratios, disulphide/native thiol percent ratios, and disulphide/total thiol percent ratios were calculated from the measured disulphide, total thiol, and native thiol parameters.

Statistical Analyses

All repeated measurements were tabulated as means and standard errors according to descriptive statics. Normality tests were confirmed using the Shapiro-Wilk test. Obtained data were evaluated using both parametric repeated measures of ANOVA and non-parametric and Kruskall Wallis tests for group, time, and group-time interactions. The SPSS 22.0 packet program was used for all tests and $P<0.05$ was considered significant.

RESULTS

There was no statistical difference in native thiol levels in any of the calves in the study groups. Native thiol levels were determined to be statistically lower in group I and III compared to group II at the 90th min following disbudding. The native thiol levels decreased in a statistically insignificant manner regarding all the treatment groups depending on time. Total thiol levels were found to be lowest at the 60th min of the study, while no significant differences were observed in group, time or group-time interaction in any treatment group. The total thiol levels increased from the 60th min of administration to the 90th and 120th min, but were lower than values measured at min 0. In the disulphide levels, the differences in terms of group, time, and group-time interaction in the application groups were not statistically significant. The disulphide concentrations decreased to the lowest level in groups I and III at the 60th min of application but increased in group II compared to min 0. There was no statistical difference in terms of time and group-time interactions in any of the disulphite/native thiol, disulphite/total thiol, and native thiol/total thiol levels. There were differences at the 60th min of application between all groups *(Table 1)*.

DISCUSSION

In livestock, the pain management of dehorning or disbudding procedures is an important animal welfare issue [28]. In addition to local anaesthesia, NSAID analgesia appears to be generally beneficial, but the lack of specific recommendations for analgesic protocols may reflect the diversity studied in the literature. It is the current recommendation of clinicians and veterinarians that local anaesthetic and NSAIDs in North America can be obtained in full compliance [29,30]. Different methodologies might be used for dehorning (e.g. surgical amputation, chemical methods, or cautery), but disbudding with a cautery is still the most preferred method by livestock producers in the United States, Canada, and North America [29-31]. For managing pain and cortisol spikes in calves after the cautery disbudding process, local anaesthetic agents, non-steroidal anti-inflammatory drugs, and sedatives are used together or solely [30]. Stock et al.[1] reported that the suppression of increases in cortisol levels reduced the pain-related inflammatory response. In our study, the calves undergoing the disbudding process were divided into groups based on commonly used pain management methods.

In a study that evaluated serum oxidant and antioxidant status, the concentrations of nitric oxide (NO) and malondialdehyde (MDA) levels did not reveal any difference

Table 1. *Time-dependent disulfide concentrations pursuant to treatment groups*

Parameter	Group	0. min	30. min	60. min	90. min	120. min	Interactions	P value
Native thiol (μmol/L)	Group I	273.4±13.2	271.5±11.7	249.1±19.6	254.1±16.8	247.6±9.3	Group	0.028
	Group II	240.0±11.6	230.0±11.9	221.4±16.9	224.7±9.2	232.7±14.4	Time	0.211
	Group III	269.6±8.1	254.4±5.4	260.2±2.7	263.3±2.9	251.7±13.2	Group & time	0.779
Total thiol (μmol/L)	Group I	317.2±12.7	318.3±14.8	284.1±24.0	299.4±19.2	290.3±12.2	Group	0.134
	Group II	285.4±14.8	274.6±17.2	269.7±19.1	268.8±8.1	281.6±11.7	Time	0.168
	Group III	313.5±7.2	289.2±9.6	287.8±4.2	292.5±2.8	294.1±16.5	Group & time	0.823
Disulphide (μmol/L)	Group I	21.9±3.7	23.4±3.4	17.5±4.2	22.5±3.3	21.3±2.8	Group	0.106
	Group II	22.7±2.3	22.3±3.0	24.2±2.8	22.0±2.8	24.4±2.1	Time	0.271
	Group III	22.0±1.6	17.4±2.7	13.8±2.1	14.6±1.5	21.2±2.4	Group & time	0.451
Disulphide/native thiol (%)	Group I	7.4±1.4	8.6±1.3	6.9±1.6	9.1±1.3	8.6±1.1	Group	0.014
	Group II	9.4±0.8	9.5±1.0	11.2±1.4	10.1±1.6	11.0±1.5	Time	0.435
	Group III	8.2±0.8	6.8±1.0	5.3±0.8	5.6±0.6	8.4±0.7	Group & time	0.284
Disulphide/total thiol (%)	Group I	6.7±1.1	7.3±0.9	5.8±1.2	7.5±0.9	7.3±0.8	Group	0.017
	Group II	7.9±0.6	7.9±0.7	9.0±1.0	8.2±1.9	8.9±1.0	Time	0.342
	Group III	7.0±0.6	5.9±0.8	4.8±0.7	5.0±0.5	7.1±0.5	Group & time	0.312
Native thiol/total thiol (%)	Group I	86.3±2.3	85.5±1.9	88.4±2.5	84.8±1.8	85.5±1.6	Group	0.019
	Group II	84.2±1.1	84.2±1.5	82.0±1.9	83.6±2.2	82.3±2.0	Time	0.342
	Group III	85.9±1.1	88.2±1.6	90.5±1.3	90.1±1.0	85.7±1.0	Group & time	0.285

between control and Dex (dexketoprofen trometamol) groups. In addition, glutathione (GSH) significantly increased at 15 min after disbudding in the Dex group, and total antioxidant activity did not show any difference between groups. There were no significant alterations in the examined parameters in both groups during the study period [9]. Among the adverse consequences of stress, oxidative stress, which is characterised by the accumulation of radical oxygen species (ROS), can affect life [32].

The thiol-disulphide homeostasis situation has important responsibilities in antioxidant protection, apoptosis, signal transduction, detoxification, regulation of enzymatic activity, and cellular signalling mechanisms [20,21]. Furthermore, thiol-disulphide homeostasis is increasingly being evaluated in various medical conditions in humans, such as diabetes [22], rheumatoid arthritis [23], cancer [25], multiple sclerosis [33], hepatic disorders [24], and surgery [34]. The plasma thiol pool is constituted by cysteine, GSH, homocysteine, and albumin. In expanded oxidative stress conditions, thiol concentrations are decreased to compensate for the reactive oxygen radicals, wherein the sulfhydryl groups of the thiols play an important role [35]. Under oxidative stress conditions, thiol molecules engage disulphide bonds, which are reduced back to thiols to tolerate thiol/disulphide homeostasis [36]. In our study, thiol-disulphide homeostasis was evaluated in calves undergoing the dehorning process with different pain management regimens. Native thiol and total thiol concentrations were decreased in all groups of calves during the study period. Native thiol concentrations were found to be significantly different at the 90th min of the study period between group II and both groups I and III. In contrast, total thiol levels showed no significant alterations in group, time, or group-time interactions, but the lowest levels were examined at the 60th min of the study. This might be explained by thiols' negative reduction properties as electron acceptors. Thiol groups interact with oxidants and are neutralised to a less toxic product called disulphide. Total thiol and native thiol concentrations have been shown to be reduced by oxidation [27]. In our study, another interesting finding was detected in disulphide levels. There were no significant alterations in disulphide concentrations in both groups. In the 60th min of the study, the disulphide concentrations reached minimum levels in groups I and III. However, in group II, the disulphide concentrations were greater than at the beginning of the study. Reductions of thiol concentrations without a rise in disulphide levels might be the outcome of inadequate intake or increased devastation because of its use in other syntheses instead of conversion to disulphide [37]. The calculated parameters of disulphite/native thiol, disulphite/total thiol, and native thiol/total thiol levels were significant in group interactions at the 60th min of the study. In this study, decreases in native thiol and total thiol concentrations without increases in disulphide levels might be related to nutritional factors instead of oxidative stress. Furthermore, oxidative stress might be suppressed by pain management strategies. This study is thought to be limited by the fact that pain management cannot be measured by cortisol levels.

In conclusion, to the best of our knowledge, this is the first study to examine thiol disulphide homeostasis in calves undergoing dehorning. Examining thiol/disulphide homeostasis during dehorning with different pain management procedures might be beneficial as an early evaluation test to recognise the best strategies in calves. Further studies are warranted to understand the association between oxidative stress and dehorning.

Declaration of Conflicting Interest

All authors have declared to be any financial and personal contest effected this study by other people or organizations.

REFERENCES

1. Stock ML, Baldridge SL, Griffin D, Coetzee JF: Bovine dehorning: Assessing pain and providing analgesic management. *Vet Clin North Am Food Anim Pract*, 29 (1): 103-133, 2013. DOI: 10.1016/j.cvfa.2012.11.001

2. Stafford KJ, Mellor DJ: Dehorning and disbudding distress and its alleviation in calves. *Vet J,* 169 (3): 337-349, 2005. DOI: 10.1016/j.tvjl.2004.02.005

3. Taschke AC, Fölsch DW: Ethological, physiological and histological aspects of pain and stress in cattle when being dehorned. *Tierarztl Prax,* 25 (1): 19-27, 1997.

4. McMeekan CM, Stafford KJ, Mellor DJ, Bruce RA, Ward RN, Gregory NG: Effects of regional analgesia and/or a non-steroidal anti-inflammatory analgesic on the acute cortisol response to dehorning in calves. *Res Vet Sci,* 64 (2): 147-150, 1998. DOI: 10.1016/S0034-5288(98)90010-8

5. Weary DM, Niel L, Flower FC, Fraser D: Identifying and preventing pain in animals. *Appl Anim Behav Sci,* 100 (1-2): 64-76, 2006. DOI: 10.1016/j.applanim.2006.04.013

6. Doherty TJ, Kattesh HG, Adcock RJ, Welborn MG, Saxton AM, Morrow JL, Dailey JW: Effects of a concentrated lidocaine solution on the acute phase stress response to dehorning in dairy calves. *J Dairy Sci,* 90 (9): 4232-4239, 2007. DOI: 10.3168/jds.2007-0080

7. Molony V, Kent JE, Robertson IS: Assessment of acute and chronic pain after different methods of castration of calves. *Appl Anim Behav Sci,* 46 (1-2): 33-48, 1995. DOI: 10.1016/0168-1591(95)00635-4

8. Caray D, Des Roches AD, Frouja S, Andanson S, Veissier I: Hot-iron disbudding: stress responses and behavior of 1-and 4-week-old calves receiving anti-inflammatory analgesia without or with sedation using xylazine. *Livest Sci,* 179, 22-28, 2015. DOI: 10.1016/j.livsci.2015.05.013

9. Korkmaz M, Sarıtaş ZK, Bülbül A, Demirkan I: Effect of pre-emptive dexketoprofen trometamol on acute cortisol, inflammatory response and oxidative stress to hot-iron disbudding in calves. *Kafkas Univ Vet Fak Derg,* 21 (4): 563-568, 2015. DOI: 10.9775/kvfd.2015.12963

10. Earley B, Crowe MA: Effects of ketoprofen alone or in combination with local anesthesia during the castration of bull calves on plasma cortisol, immunological, and inflammatory responses. *J Anim Sci,* 80 (4): 1044-1052, 2002. DOI: 10.2527/2002.8041044x

11. Ting STL, Earley B, Hughes JML, Crowe MA: Effect of ketoprofen, lidocaine local anesthesia, and combined xylazine and lidocaine caudal epidural anesthesia during castration of beef cattle on stress responses, immunity, growth, and behavior. *J Anim Sci,* 81, 1281-1293, 2003. DOI: 10.2527/2003.8151281x

12. McMeekan C, Stafford KJ, Mellor DJ, Bruce RA, Ward RN, Gregory N: Effects of a local anaesthetic and a non-steroidal anti-inflammatlory

analgesic on the behavioural responses of calves to dehorning. *N Z Vet J,* 47 (3): 92-96, 1999. DOI: 10.1080/00480169.1999.36120

13. Duffield TF, Heinrich A, Millman ST, DeHaan A, James S, Lissemore K: Reduction in pain response by combined use of local lidocaine anesthesia and systemic ketoprofen in dairy calves dehorned by heat cauterization. *Can Vet J,* 51 (3): 283-288, 2010.

14. Heinrich A, Duffield TF, Lissemore KD, Millman ST: The effect of meloxicam on behavior and pain sensitivity of dairy calves following cautery dehorning with a local anesthetic. *J Dairy Sci,* 93 (6): 2450-2457, 2010. DOI: 10.3168/jds.2009-2813

15. Stilwell G, Lima MS, Carvalho RC, Broom D: Effects of hot-iron disbudding using regional anesthesia with and without carprofen, on cortisol and behavior of calves. *Res Vet Sci,* 92, 338-341, 2012. DOI: 10.1016/j.rvsc.2011.02.005

16. Mintline EM, Stewart M, Rogers AR, Cox NR, Verkerk GA, Stookey JM, Tucker CB: Play behavior as an indicator of animal welfare: Disbudding in dairy calves. *Appl Anim Behav Sci,* 144 (1-2): 22-30, 2013. DOI: 10.1016/j.applanim.2012.12.008

17. Stafford KJ, Mellor DJ: Addressing the pain associated with disbudding and dehorning in cattle. *Appl Anim Behav Sci,* 135, 226-231, 2011. DOI: 10.1016/j.applanim.2011.10.018

18. Cadenas E: Biochemistry of oxygen toxicity. *Annu Rev Biochem,* 58, 79-110, 1989. DOI: 10.1146/annurev.bi.58.070189.000455

19. Young IS, Woodside JV: Antioxidants in health and disease. *J Clin Pathol,* 54 (3): 176-186, 2001. DOI: 10.1136/jcp.54.3.176

20. Biswas S, Chida AS, Rahman I: Redox modifications of protein–thiols: Emerging roles in cell signaling. *Biochem Pharmacol,* 71 (5): 551-564, 2006. DOI: 10.1016/j.bcp.2005.10.044

21. Circu ML, Aw TY: Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med,* 48 (6): 749-762, 2010. DOI: 10.1016/j.freeradbiomed.2009.12.022

22. Matteucci E, Giampietro O: Thiol signalling network with an eye to diabetes. *Molecules,* 15 (12): 8890-8903, 2010. DOI: 10.3390/molecules15128890

23. Tetik S, Ahmad S, Alturfan AA, Fresko I, Disbudak M, Sahin Y, Aksoy H, Yardimci KT: Determination of oxidant stress in plasma of rheumatoid arthritis and primary osteoarthritis patients. *Indian J Biochem Biophys,* 47, 353-358, 2010.

24. Kuo LM, Kuo CY, Lin CY, Hung MF, Shen JJ, Hwang TL: Intracellular glutathione depletion by oridonin leads to apoptosis in hepatic stellate cells. *Molecules,* 19 (3): 3327-3344, 2014. DOI: 10.3390/molecules19033327

25. Prabhu A, Sarcar B, Kahali S, Yuan Z, Johnson JJ, Adam KP, Chinnaiyan P: Cysteine catabolism: A novel metabolic pathway contributing to glioblastoma growth. *Cancer Res,* 74 (3): 787-796, 2013. DOI: 10.1158/0008-5472.CAN-13-1423

26. Fidan AF, Pamuk K, Ozdemir A, Saritas ZK, Tarakci U: Effects of dehorning by amputation on oxidantantioxidant status in mature cattle. *Rev Med Vet,* 161 (11): 502-508, 2010.

27. Erel O, Neselioglu S: A novel and automated assay for thiol/disulphide homeostasis. *Clin Biochem*, 47 (18): 326-332, 2014. DOI: 10.1016/j.clinbiochem.2014.09.026

28. Ventura BA, von Keyserlingk MAG, Weary DM: Animal welfare concerns and values of stakeholders within the dairy industry. *J Agric Environ Ethics,* 28, 109-126, 2015. DOI: 10.1007/s10806-014-9523-x

29. Adams AE, Lombard JE, Shivley CS, Urie NJ, Roman-Muniz IN, Fossler CP, Kopral CA: Management practices that may impact dairy heifer welfare on US dairy operations. *J Dairy Sci,* 98 (2): 105, 2015.

30. Winder CB, LeBlanc SJ, Haley DB, Lissemore KD, Godkin MA, Duffield TF: Practices for the disbudding and dehorning of dairy calves by veterinarians and dairy producers in Ontario, Canada. *J Dairy Sci,* 99 (12): 10161-10173, 2016. DOI: 10.3168/jds.2016-11270

31. Vasseur E, Borderas F, Cue RI, Lefebvre D, Pellerin D, Rushen J, Wade KM, De Passillé AM: A survey of dairy calf management practices in Canada that affect animal welfare. *J Dairy Sci,* 93, 1307-1315, 2010. DOI: 10.3168/jds.2009-2429

32. Chirase NK, Greene LW, Purdy CW, Loan RW, Auvermann BW, Parker DB, Walborg EF, Stevenson DE, Xu Y, Klaunig JE: Effect of transport stress on respiratory disease, serum antioxidant status, and serum concentrations of lipid peroxidation biomarkers in beef cattle. *Am J Vet Res,* 65 (6): 860-864, 2004. DOI: 10.2460/ajvr.2004.65.860

33. Calabrese M, Magliozzi R, Ciccarelli O, Geurts JJG, Reynolds R, Martin R: Exploring the origins of grey matter damage in multiple sclerosis. *Nat Rev Neurosci,* 16 (3): 147-158, 2015. DOI: 10.1038/nrn3900

34. Polat M, Ozcan O, Sahan L, Üstündag Budak Y, Alisik M, Yilmaz N, Erel Ö: Changes in thiol-disulfide homeostasis of the body to surgical trauma in laparoscopic cholecystectomy patients. *J Laparoendosc Adv Surg Tech A,* 26 (12): 992-996, 2016. DOI: 10.1089/lap.2016.0381

35. Erkus ME, Altiparmak IH, Demirbag R, Gunebakmaz O, Kaya Z, Taskin A, Neselioglu S, Erel O: The ınvestigation of the dynamic thiol-disulfide homeostasis in acute coronary syndromes. *Am J Cardiol,* 115 (Suppl 1), S117-S118, 2015. DOI: 10.1016/j.amjcard.2015.01.401

36. Jones DP, Liang Y: Measuring the poise of thiol/disulfide couples *in vivo. Free Radic Biol Med,* 47 (10): 1329-1338, 2009. DOI: 10.1016/j.freeradbiomed.2009.08.021

37. Durrieu G, LLau ME, Rascol O, Senard JM, Rascol A, Montastruc JL: Parkinson's disease and weight loss: A study with anthropometric and nutritional assessment. *Clin Auton Res,* 2, 153-157, 1992. DOI: 10.1007/BF01818955

Kafkas Univ Vet Fak Derg
25 (3): 341-348, 2019
DOI: 10.9775/kvfd.2018.20952

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Isolation and Molecular Characterization of Thermophilic *Campylobacter* spp. in Dogs and Cats [1]

Özkan ASLANTAŞ [1,a]

[1] *The study was partly supported by Hatay Mustafa Kemal University Scientific Research Fund (Project Number: 12860)*
[1] Department of Microbiology, Faculty of Veterinary Medicine, Hatay Mustafa Kemal University, TR-31060 Hatay - TURKEY
[a] ORCID: 0000-0003-0407-8633

Article Code: KVFD-2018-20952 Received: 13.09.2018 Accepted: 18.12.2018 Published Online: 18.12.2018

How to Cite This Article

Aslantaş Ö: Isolation and molecular characterization of thermophilic *Campylobacter* spp. in dogs and cats. *Kafkas Univ Vet Fak Derg,* 25 (3): 341-348, 2019. DOI: 10.9775/kvfd.2018.20952

Abstract

This study aimed to evaluate the occurence, virulence properties, genetic diversity, antimicrobial susceptibilities and genetic determinants of resistance of thermophilic *Campylobacters* from dogs and cats under different housing conditions. Rectal swabs were taken from 136 dogs (household dogs, n=56; shelter-housed dogs, n=80), and 14 shelter-housed cats. Antimicrobial susceptibilities of the isolates were performed by disc diffusion method. Tetracycline *(tet*O), ampicillin *(bla$_{OXA}$-61),* aminoglycoside (*aph-3-1)* resistance and multidrug efflux pump *(cme*B) genes were investigated by multiplex polymerase chain reaction (mPCR). The genetic diversity among the isolates was determined by sequence analysis of short variable regions (SVRs) of *fla*A gene. The presence of virulence and toxin genes was also investigated by PCR. *Campylobacter* spp. were isolated from 33.8% of dogs and 28.6% of cats. *C. jejuni* was the most common species in both dogs (52.2%) and cats (100%), followed by *C. coli,* which was isolated from 41.3% of dogs. High rates of resistance against nalidixic acid (78.7%), ciprofloxacin (74.5%), ampicillin (68.1%), tetracycline (53.2%) were observed. The frequency of *fla*A, *vir*B11, *cdt*A, *cdt*B, *cdt*C, *rac*R, *cad*F, *cia*B, *dnaJ* and *pld*A genes was 100%, 2.1%, 83%, 72.3%, 72.3%, 57.4%, 93.6%, 12.8%, 53.2% and 44.7%, respectively. Based on *fla*A-SVR typing, 17 different alleles were determined among the isolates. The results of this study suggested that pet animals were colonized with antimicrobial resistant thermophilic *Campylobacter* spp. having high pathogenic potential and genetic diversity.

Keywords: *Thermophilic campylobacter, Antimicrobial resistance, Virulence, flaA-SVR typing*

Köpek ve Kedilerden Thermofilik *Campylobacter* İzolasyonu ve Moleküler Karakterizasyonu

Öz

Bu çalışmada, farklı koşullarda barındırılan köpekler ve kedilerde termofilik *Campylobacter* türlerinin varlığı, virülans özellikleri, genetik çeşitliliği, antimikrobiyal duyarlılık ve direnç genlerinin belirlenmesi amaçlandı. Rektal svab örnekleri 136 köpekten (sahipli, n=56; barınak, n=80) ve 14 barınak kedisinden alındı. İzolatların antimikrobiyallere olan duyarlılıkları disk difüzyon metodu ile belirlendi. Tetrasiklin *(tet*O), ampisilin *(bla$_{OXA}$-61)*, aminoglikozid *(aph-3-1)* direnç ve multidrug efluks pompası *(cme*B) genleri multipleks polimeraz zincir reaksiyonu (mPZR) ile araştırıldı. İzolatlar arasındaki genetik farklılık, *fla*A geninin kısa değişken bölgelerinin (SVR'ler) dizi analizi ile belirlendi. İzolatlar arasında virülans ve toksin genlerinin varlığı ise PZR ile araştırıldı. *Campylobacter* spp., köpeklerin %33.8'inden ve kedilerin %28.6'sından izole edildi. *C. jejuni* hem köpeklerde (%52.2) hem de kedilerde (%100) en sık izole edilen tür iken; *C. coli* sadece köpeklerin %41.3'ünden izole edildi. Nalidiksik asit (%78.7), siprofloksasin (%74.5), ampisilin (%68.1) ve tetrasikline (%53.2) karşı yüksek oranlarda direnç gözlendi. *fla*A, *vir*B11, *cdt*A, *cdt*B, *cdt*C, *rac*R, *cad*F, *cia*B, *dnaJ* ve *pld*A virulens genleri sırasıyla %100, %2.1, %83, %72.3, %72.3, %57.4, %93.6, %12.8, %53.2 ve %44.7 oranlarında saptandı. *fla*A-SVR tiplendirme metodu ile *C. jejuni* ve *C. coli* izolatları arasında 17 farklı allel belirlendi. Bu çalışmanın sonuçları, pet hayvanlarının antimikrobiyal dirençli, yüksek patojenik potansiyele ve genetik çeşitliliğe sahip termofilik *Campylobacter* spp. ile kolonize olduğunu göstermektedir.

Anahtar sözcükler: *Termofilik campylobacter, Antimikrobiyal direnç, Virulens, flaA-SVR tiplendirme*

INTRODUCTION

Campylobacter spp. are among the most frequently reported causes of foodborne gastroenteritis in the world. The vast majority of human infections were attributed to consumption of contaminated poultry meat [1]. However, repeated contact with dogs and cats has also been identified as an important source of *Campylobacter* infection to their

İletişim (Correspondence)
+90 3262458545/1523 Fax: +90 326 2455704
ozkanaslantas@yahoo.com

owners [2-4], and human infections originating from pet animals have been reported [5]. Dogs and cats are mostly subclinical hosts of *Campylobacter* spp., infected mainly by *C. helveticus*, *C. upsaliensis*, *C. jejuni* and *C. coli* [6,7]. However, gastroenteritis cases related with these agents have also been reported in dogs and cats [4]. The most of *Campylobacter* infections are self-limited and do not require antimicrobial treatment, however, in severe cases, fluoroquinolones (FQ) and macrolides are drug of choise used for the treatment of clinical campylobacteriosis. However, increasing prevalence of antibiotic-resistant *Campylobacter* from various sources such as humans, animals and food, especially FQ, became as serious threat to public health [8-10].

Campylobacter produce a number of virulence factors playing important role in their pathogenesis. The factors involved in pathogenesis of *Campylobacter* include flagella mediated motility, chemotaxis, adhesion to intestinal mucosa, invasion, translocation and production of toxin and secreted proteins [11].

Many molecular methods have been developed to investigate the diversity within *C. jejuni* and *C. coli* isolates. Each molecular method has advantages and disadvantages to determine the genetic relatedness of the *Campylobacter* isolates [12]. Of these methods, sequence analysis of short variable regions (SVRs) of *fla*A gene is widely used method for genotyping of *Campylobacter* isolates [13,14]. This method was reported as one of the effective and reliable methods for typing of *Campylobacter* spp. and has discriminatory power comparable to Pulsed Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST) [15]. In addition, the depositions of the *fla*A-SVR nucleotide alleles in a central web site *(http://pubmlst.org/)* make access to the *fla*A-SVR allele types of *Campylobacter* spp. possible.

The studies on the occurence of *Campylobacter* spp. in pets in Turkey are scarce, and have mainly focused on poultry [9,16,17]. Investigation of the prevalence and other characteristics of *Campylobacter* in cats and dogs is an important step to assess their role as a potential source of human infections. Therefore, the present study aimed (i) to determine the prevalence and antimicrobial susceptibilities of thermophilic *Campylobacter* spp. in stray and household pets and its resistance mechanisms, (ii) to investigate genetic diversity of *C. coli* and *C. jejuni* isolates using *fla*A-SVR sequence-based typing and (iii) to determine the presence and frequency of these virulence genes.

MATERIAL and METHODS

Ethical Statement

The study was approved by Mustafa Kemal University Animal Ethic Committee (2016-2/3).

Study Area and Sample Collection

From March 2016 to June 2016, individual rectal swab specimens were taken from owned household pets (dogs= 56), unowned pets (dogs, n=80; cats, n=14). Unowned pets were housed at Hatay Metropolitan Municipality Kennel. Age and sex distribution of dogs and cats were recorded during the sampling. Age proportion of male/ female dogs and cats were 63/73 and 9/5, respectively. Immediately after sampling, the swabs were placed in Amies Transport Medium with charcoal (LP Italiana, 11898, Italy) and transported to the laboratory and processed immediately upon arrival.

Isolation of *Campylobacter* spp.

The rectal swabs were directly streaked on modified charcoal cefoperazone deoxycholate agar (mCCDA), containing CCDA selective supplement for primary isolation. The plates were incubated at 41.5ºC for 36-48 h under microaerophilic conditions. One presumptive colony from each mCCDA plate was subcultured onto blood agar supplemented with 5% defibrinated sheep blood. The isolates, microscopically curved Gram negative rods with characteristic seagull-winged morphology, catalase and oxidase positive were accepted as *Campylobacter* spp. and stored within cryo-beads in deep freeze (-80ºC) untill use.

DNA Extraction and PCR Analysis for Identification of Genus/Species Level

Chromosomal DNA was obtained by boiling method as previously described Wang et al.[18]. Briefly, one colony was suspended in 200 µL RNase and DNase free water and heated at 100ºC for 10 min and centrifuged at 10.000 g for 10 min. Supernatant was transferred to another steril eppendorf tube and used as template DNA.

For genus confirmation and species determination, a multiplex polymerase chain reaction (mPCR) assay targeting *Campylobacter* genus, *C. jejuni* and *C. coli* was performed using primers and reaction conditions described by Wang et al.[18].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibilities of the isolates were determined by disc diffusion method according to Clinical Laboratory Standards Institute (CLSI, 2008) guidelines [19]. Following antimicrobial discs were used: nalidixic acid (NA, 30 µg), ciprofloxacin (CIP, 5 µg), ampicillin (AM, 10 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg), gentamicin (CN, 10 µg), and erythromycin (E, 15 µg). *C jejuni* (NCTC 12500) and *C. coli* (NCTC 12525) were used as control strains for antimicrobial susceptibility testing.

Detection of Antimicrobial Resistance Genes

All *Campylobacter* spp. were tested for the presence of *tet*O (tetracycline), *aph-3-1* (aminoglycoside), bla_{OXA-61} (ampicillin)

and *cme*B (multi-drug efflux pump) genes by mPCR as previously reported by Obeng et al.[20].

Genotyping by flaA-SVR

PCR amplification of a fragments of 641 bp of the *fla*A gene comprising the SVRs were performed following the procedures described by Lévesque et al.[21]. The *fla*A types were determined by comparing the nucleotide sequences with those in the PubMLST *Campylobacter* database *(http://pubmlst.org/campylobacter/)*.

Detection of Virulence Genes

Presence of putative virulence genes responsible for adhesion, colonization, invasion and toxin production were investigated by PCR as previously described by Bang et al.[22], Konkel et al.[23], Bacon et al.[24], Datta et al.[25], and Nachamkin et al.[26].

Statistical Analysis

Differences in frequencies of isolation rates according to age groups and genders were evaluated using Pearson's chi-square test. SPSS 14.01 was used for statistical analysis. Any P value equal to/or less than <0.05 was accepted as statistically significant.

RESULTS

Overall, 33% (50/150) of the samples tested were positive for *Campylobacter* spp. including 33.8% (46/136) of dog samples and 28.6% (4/14) of cat samples. Of the *Campylobacter* isolates, 56% (28/50) were identified as *C. jejuni*, and 38% (19/50) were determined to be *C. coli* by PCR. The remaining three isolates (6%) were different than *C. jejuni* and *C. coli* and were not characterized further to species level.

The results of antimicrobial susceptibilities of *Campylobacter* isolates are given in *Table 1*. *C. jejuni* isolates from dogs showed high resistance rate to nalidixic acid (79.2%), ciprofloxacin (75%), tetracycline (66.7%) and ampicillin (62.5%), while low resistance were observed to erythromycin (12.5%), gentamicin (12.5%) and chloramphenicol (4.2%). Considering *C. coli* isolates from dogs, similarly high resistance rates to nalidixic acid (78.9%), ciprofloxacin (73.7%), ampicillin (68.4%) and tetracycline (31.6%), but low resistance rates to erythromycin (21.1%), gentamicin (15.8%) and chloramphenicol (5.3%) were recorded. *C. jejuni* isolates from cats were resistant to ampicillin (100%), nalidixic acid (75%), ciprofloxacin (75%), tetracycline (75%), gentamicin (25%), and erythromycin (25%), except chloramphenicol.

Multi drug resistance (MDR) was frequently observed in *C. jejuni* and *C. coli* isolates. The most common multidrug pattern detected among *C. jejuni* isolates was nalidixic acid, ciprofloxacin, ampicillin and tetracycline, which was observed in 35.7% of the isolates, whereas the most common MDR pattern among *C. coli* isolates was nalidixic acid, ciprofloxacin and ampicillin, which was observed in 26.3% of *C. coli* isolates *(Table 2)*.

Of the 19 tetracycline resistant *C. jejuni* isolates, 16 carried *tet*O, and two *C. jejuni* isolates, despite carrying *tet*O gene, were susceptible to tetracycline. *aph-3-1* gene was detected in one phenotypically resistant *C. coli* isolates. Among the ampicillin resistant 19 *C. jejuni* isolates, 14 were found to carry bla_{OXA-61}. While bla_{OXA-61} was found in 8 ampicillin resistant *C. coli* isolates, one isolate that harbored bla_{OXA-61} was susceptible to ampicillin. Four of the ampicillin resistant isolates did not carry bla_{OXA-61}. *cme*B gene was only detected in *C. coli* (89.5%, 17/19) isolates *(Table 3) (Fig. 1)*.

The results of *fla*A-SVR sequence typing of the 28 *C. jejuni* and 19 *C. coli* isolates are given in *Table 4*. Among *C. jejuni* isolates, nine alleles were detected. In *C. coli* isolates, eight alleles were identified. Two *fla*A alleles (alleles 23 and 120) were identical in both *C. jejuni* and *C. coli* isolates.

The frequency of virulence genes detected in the isolates is given in *Table 5*. Among 28 *C. jejuni* isolates, 12 virulence associated gene profile was detected. Whereas 10 virulence associated gene profiles were detected among 19 *C. coli* isolates. The frequency of *fla*A, *vir*B11, *cdt*A, *cdt*B, *cdt*C,

Table 1. *Antimicrobial resistance of C. jejuni and C. coli isolates from dogs and cats*

Antimicrobial	Shelter			Household	
	Cats	Dogs		Dogs	
	C. jejuni (n=4)	*C. jejuni* (n=18)	*C. coli* (n=15)	*C. jejuni* (n=6)	*C. coli* (n=4)
Nalidixic Acid	3 (75)	14 (77.8)	13 (6.7)	5 (83.3)	2 (50)
Ciprofloxcin	3 (75)	13 (72.2)	11 (73.3)	5 (83.3)	3 (75)
Ampicillin	4 (100)	13 (72.2)	9 (60)	2 (33.3)	4 (100)
Tetracycline	3 (75)	11 (61.1)	5 (33.3)	5 (83.3)	1 (25)
Chlorampenicol	0 (0)	1 (5.6)	1 (6.7)	0 (0)	0 (0)
Gentamicin	1 (25)	2 (11.1)	2 (13.3)	1 (16.7)	1 (25)
Erythromycin	1 (25)	3 (16.7)	2 (13.3)	0 (0)	2 (50)

Table 2. *Multidrug resistance patterns determined among C. jejuni and C. coli isolates from dogs and cats*

Resistance Profile	Shelter			Household	
	Dogs		Cats	Dogs	
	C. jejuni (n=18)	*C. coli* (n=15)	*C. jejuni* (n=4)	*C. jejuni* (n=6)	*C. coli* (n=4)
NA, CIP, AM, TE, CN, E	1	1	1	-	-
NA, CIP, AM, CN, E	-	-	-	-	1
AM, TE, CN, C, E	1	1	-	-	-
NA, CIP, TE, CN	-	-	-	1	-
NA, CIP, AM, TE	6	-	2	2	1
NA, CIP, TE	1	2	-	2	-
NA, CIP, AM	3	5	-	-	1
NA, AM, TE	1	1	-	-	-
NA, CIP	1	4	1	-	-
AM, E	1	-	-	-	1
NA	1	1	-	-	-
TE	1	-	-	-	-
Susceptible	1	-	-	1	-

Table 3. *Distribution of resistance genes among the isolates*

Source	Species	Resistance Phenotype and the Occurence of Related Gene	No of The Isolates			
			tetO	*bla*$_{OXA-61}$	*aph-3-1*	*cmeB*
Household	*C. jejuni* (n=6)	Resistant with genes	5	3	-	-
		Resistant without genes	1	1	-	-
		Susceptible with genes	-	1	-	-
	C. coli (n=4)	Resistant with genes	1	2	1	4
		Resistant without genes	-	2	-	-
		Susceptible with genes	-	-	-	-
Shelter	*C. jejuni* (n=22)	Resistant with genes	11	11	-	-
		Resistant without genes	-	2	-	-
		Susceptible with genes	2	1	-	-
	C. coli (n=15)	Resistant with genes	3	6	-	13
		Resistant without genes	-	2	-	-
		Susceptible with genes	2	1	-	-

*rac*R, *cad*F, *cia*B, *dna*J and *pld*A was 100%, 2.1%, 83%, 72.3%, 72.3%, 57.4%, 93.6%, 12.8%, 53.2% and 44.7%, respectively.

DISCUSSION

The findings from this study revealed that 19.6% of household dogs, 43.8% of stray dogs and 28.6% of stray cats were colonized with *Campylobacter* spp. Such a high level colonization rates were not reported in earlier studies conducted in different countries. In Italy, Giacomelli et al.[27] reported a prevalence of *Campylobacter* spp. of 11% in household dogs, 26% in shelter dogs and shelter cats. Another study carried out in Italy, Gargiulo et al.[28] isolated *C. jejuni* with a prevalence rate of 16.8% in stray cats. In Taiwan, Tsai et al.[29] found that 2.7% of household dogs and 23.8% of stray dogs were positive for *Campylobacter* spp. In New Zealand, Bojanić et al.[30] reported prevalence of *Campylobacter* spp. in household dogs and cats as 36% and 16%, respectively. In Korea, Cho et al.[31] reported prevalence of thermophilic *Campylobacter* spp. in stray, breeding and household dogs as 25.2%, 12% and 8.8%, respectively. In Malaysia, Goni et al.[6] reported frequency of *Campylobacter* in stray dogs and cats as 16.3% and 32.6% respectively, while in household dogs and cats as 12.5% each. These findings clearly indicate that dogs and cats were imporant reservoirs of *Campylobacter* spp. in Turkey.

The species distribution of *Campylobacter* from dogs and cats differs considerably according to populations studied,

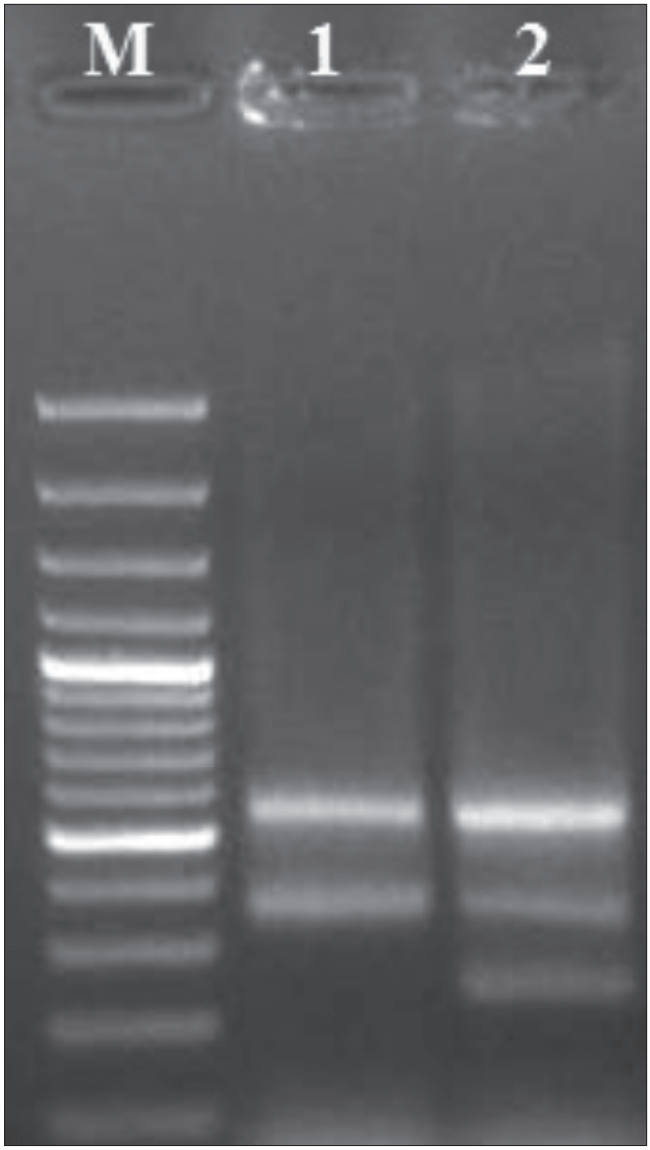


Fig. 1. Agarose gel electrophoresis showing antibiotic resistance genes. Lane M: 100 bp plus molecular marker, Lane 1: *tet*O (559 bp)-bla_{OXA-61}; Lane 2: *tet*O (559 bp)-bla_{OXA-61}-*cme*B (241 bp)

Table 4. *flaA alleles detected in C. jejuni and C. coli isolates*

Source	Animal	Species	*flaA*-SVR Allel	Number of the Isolates
Shelter	Dog	*C. jejuni*	120	4
			85	4
			82	4
			43	3
			80	1
			23	11
			41	1
	Cat	*C. jejuni*	41	1
			44	1
			80	1
			82	1
		C. coli	90	5
			51	3
			62	2
			84	1
			120	1
			23	1
			61	1
			118	1
Household	Dog	*C. jejuni*	82	2
			36	1
			23	1
			41	1
			43	1
		C. coli	118	2
			51	1
			90	1

isolation protocols, region, fastidious character of the agent and years [6,32]. In different studies, *C. upsaliensis* [6,30,33] and *C. jejuni* [27,29] have been reported to be most prevalent species in dogs and cats. In this study, *C. jejuni* was found as the most prevalent species among *Campylobacter*-positive dogs and cats. This is an important finding from public health point of view, since *C. jejuni* is the species most frequently associated with human gastroenteritis [34].

Housing conditions were defined as a risk factor for *Campylobacter* infection in dogs and cats. Unsanitary conditions observed in shelters may increase the spread of infection among sheltered dogs [27]. Acke et al.[35] reported that close contact between shelter-housed animals facilitates infection transmission. Humprey [36] suggested that animals under stresfull conditions could produce noradrenaline, leading to increased susceptibility to infectious agents. Abovementioned factors could explain the higher prevalence of *Campylobacter* infection in shelter-housed dogs compared to household dogs.

The ages of pets animals have been reported to be a risk factor and association between age and *Campylobacter* carriage [6,37]. Similarly, in this study, significantly higher carriage rate found in younger dogs compared with older dogs (P<0.0001). However, a conradictory finding reported by Rahimi et al.[3], who found no significant influence of the age of dogs and cats on *Campylobacter* infection.

Rising trend of antimicrobial resistance have been observed in *Campylobacter* isolates [38,39]. FQs (danofloxacin and enrofloxacin) are frequently used drugs in veterinary field for the treatment and control of infectious diseases of pets and food-producing animals in Turkey. In this study, high resistance rate was determined against ciprofloxacin (73.7% in *C. coli* and 76.9% in *C. jejuni)*. Higher resistance rate to ciprofloxacin is highly important, because the FQs are drug of choise for the treatment of *Campylobacter*

Table 5. *The frequency of virulence genes detected among the isolates*

Virulence Gene Patterns	No of The Isolates	Species	
		C. jejuni	*C. coli*
*fla*A, *dna*J, *cad*F, *pld*A, *rac*R, *cdt*A, *cdt*B, *cdt*C, *vir*B11	1	1	-
*fla*A, *dna*J, *cad*F, *pld*A, *rac*R, *cdt*A, *cdt*B, *cdt*C, *cia*B	3	2	1
*fla*A, *dna*J, *cad*F, *pld*A, *rac*R, *cdt*A, *cdt*B, *cdt*C	13	10	3
*fla*A, dnaJ, *cad*F, *pld*A, *rac*R, *cdt*A, *cdt*C, *cia*B	1	1	-
*fla*A, *dna*J, *cadF*, *racR*, *cdt*A, *cdt*B, *cdt*C, *cia*B	2	1	1
*fla*A, *dna*J, *cad*F, *rac*R, *cdt*A, *cdt*B, *cdt*C	5	4	1
*fla*A, *cad*F, *pld*A, *rac*R, *cdt*A, *cdt*B, *cdt*C	2	1	1
*fla*A, *cad*F, *rac*R, *cdt*A, *cdt*B, *cdt*C	1	1	-
*fla*A, *dna*J, *cad*F, *cdt*A, *cdt*B, *cdt*C	1	-	1
*fla*A, *cad*F, *cdt*A, *cdt*B, *cdt*C	6	1	5
*fla*A, *cad*F, *cdt*A, *cdt*B, *cdt*C	3	-	3
*fla*A, *cad*F, *cdt*A	4	4	-
*fla*A, *cad*F, *cdt*C	2	-	2
*fla*A, *cad*F, *pld*A	1	1	-
*fla*A, *cdt*C	2	1	1

infections. These findings are almost similar to previous studies in broilers [9], chicken meat [8] and humans [10] in Turkey.

Resistance to ampicillin in *Campylobacters* are mainly due to synthesis of beta-lactamases, low affinty binding of the beta-lactams to the target [penicillin binding proteins (PBP)] or reduced permeability of outer membran porins [40]. In this study, high level ampicillin resistance observed in *Campylobacters* might be due to the wide-spread use of beta-lactams or combination of beta-lactams with other antimicrobials for the treatment of infections in pet animals. Besides, resistance to ampicillin in 68.8% (22/32) of the isolates were found to be associated with enzymatic inactivation by bla_{OXA-61}. Therefore, it should be noted that *Campylobacter* resistance to ampicillin is not only associated with enzymatic inactivation by bla_{OXA-61}, but also other resistance mechanisms mentioned above.

In this study, 20 out of 25 (80%) tetracycline-resistant isolates were found to possess *tet*O. The frequent detection of *tet*O in tetracycline-resistant isolates is also reported previous studies [9,20]. However, *tet*O was also detected in four tetracycline-susceptible *Campylobacter* (two *C. jejuni* and two *C. coli*), and the gene was not detected in one tetracycline-resistant *C. coli* isolate. This finding is not surprising because similar findings have already been reported by some investigators [9,20]. Guévremont et al.[41] reported that *tet*O might be present in tetracycline resistant isolates but might be detected by primers used. Another study conducted by Abdi-Hachesoo et al.[42], presence of tetA gene was reported in some tetracycline resistant *Campylobacter* isolates.

In this study, low levels of resistane were observed for chloramphenicol (4.3%), gentamicin (14.9%), and erythromycin (14.9%) in *Campylobacter* spp. isolates. These findings are also similar to previous studies carried out in Iran [3,38].

Several virulence factors have been documented for *Campylobacter* spp. contributing its pathogenicity. Of these virulence factors, *fla*A gene is necessary for colonization [43], which was detected in all *Campylobacter* isolates in present study. Similarly, Cho et al.[31] reported this gene in 100% of *C. jejuni* and *C. coli* isolates. Other virulence genes responsible for adherence and colonization *(cad*F, *rac*R and *dnaJ*) and invasion *(pld*A, *cia*B and *vir*B11) were found at varying rates. Frequency of *cad*F, *rac*R, *dnaJ*, *pld*A and *cia*B genes in *C. jejuni* and *C. coli* isolates were 91.9%-94.7%, 75%-36.8%, 64.3%-31.6%, 57.126.3%, 14.3%-10.5%, respectively. In contrast, Cho et al.[31] reported higher prevalence rate for *rac*R, *dnaJ*, *cad*F, *pld*A and *cia*B genes in *C. jejuni* and *C. coli* isolates as 73.2%-0%, 100%-100%, 100%-100%, 78%-0%, and 73.2%-0%, respectively. The *vir*B11 gene was only detected in one (2.1%) *C. jejuni* isolate from shelter-housed of dog.

CDT is a bacterial protein toxin consisting of three subunits encoded by the *cdt*A, *cdt*B and *cdt*C genes that products of all three gene are required for functionally active toxin. The toxin exerts its effect by inhibiting transition of the cell from G-2 phase-mitosis [44]. Cho et al.[31] detected *cdt* genes in 100% of the isolates. However, the authors found that only some of these isolates show CDT production in the HEp-2 cell cytotoxin assay. Similar observation was also reported by Açık et al.[45]. Since cytotoxity assays are influenced by *in vitro* factors such as repeated subcultures

of isolates, cell types, therefore, it has been suggested that more sensitive methods should be applied to cytotoxity assays for accurate determination cytotoxic activity of isolates [31].

*fla*A gene-based typing methods have been used for genotyping of *Campylobacter* for a long time. Of these methods, *fla*A-SVR typing has been reported as reliable method, giving reliable and reproducible results comparable to PFGE analysis [46]. In this study, discriminatory power (DI) of *fla*A-SVR analysis for *C. jejuni* isolates were 0.845 and 0.8538 for *C. coli* isolates.

In conclusion, to the author's knowledge, the study is the first to investigate the occurence, antimicrobial susceptibility, virulence properties and *fla*A-SVR typing of *C. jejuni* and *C. coli* in dogs and cats under different housing conditions in Turkey. Regardless of their origin, dogs and cats was found a significant source of *Campylobacter* infection in humans. The high antimicrobial resistance to some antimicrobials, particularly FQ, is another striking finding, making treatment options of *Campylobacter* infections very limited. Therefore, continuous surveillance is needed to determine the emergence and dissemination of resistant *Campylobacter* in different origin of animals. Occurence of high rate of virulence genes observed in this study indicate potential pathogenicity of the isolates. Given cohabitation of dogs and cats with humans, good hygiene paratices should be promoted, contact with stray pet animals should be reduced to minimise the risk of transmission.

REFERENCES

1. Sahin O, Kassem II, Shen Z, Lin J, Rajashekara G, Zhang Q: *Campylobacter* in poultry: Ecology and potential interventions. *Avian Dis*, 59, 185-200, 2015. DOI: 10.1637/11072-032315-Review

2. Mughini Gras L, Smid JH, Wagenaar JA, Koene MGJ, Havelaar AH, Friesema IHM, French NP, Flemming C, Galson JD, Graziani C, Busani L, van Pelt W: Increased risk for *Campylobacter jejuni* and *C. coli* infection of pet origin in dog owners and evidence for genetic association between strains causing infection in humans and their pets. *Epidemiol Infect*, 141, 2526-2535, 2013. DOI: 10.1017/S0950268813000356

3. Rahimi E, Chakeri A, Esmizadeh K: Prevalence of *Campylobacter* species in fecal samples from cats and dogs in Iran. *Slov Vet Res*, 49, 117-122, 2012.

4. Acke E: Campylobacteriosis in dogs and cats: A review. *N Z Vet J*, 66, 221-228, 2018. DOI: 10.1080/00480169.2018.1475268

5. Kittl S, Heckel G, Korczak BM, Kuhnert P: Source attribution of human *Campylobacter* isolates by MLST and *fla*-typing and association of genotypes with quinolone resistance. *PLoS One,* 8(11):e81796, 2013. DOI: 10.1371/journal.pone.0081796

6. Goni MD, Abdul-Aziz S, Dhaliwal GK, Zunita Z, Bitrus AA, Jalo IM, Aung WW, Mohamed MA, Aliyu AB: Occurence of *Campylobacter* in dogs and cats in Selangor Malaysia and the associated risk factors. *Malays J Microbiol,* 13, 164-171, 2017.

7. Parsons BN, Williams NJ, Pinchbeck GL, Christley RM, Hart CA, Gaskell RM, Dawson S: Prevalence and shedding patterns of *Campylobacter* spp. in longitudinal studies of kennelled dogs. *Vet J,* 190, 249-254, 2011**.** DOI: 10.1016/j.tvjl.2010.10.006

8. Issa G, Başaran Kahraman B, Adıgüzel MC, Yılmaz Eker F, Akkaya E, Bayrakal GM, Koluman A, Kahraman T: Prevalence and antimicrobial resistance of thermophilic *Campylobacter* isolates from raw chicken meats. *Kafkas Univ Vet Fak Derg,* 24, 701-707, 2018. DOI: 10.9775/kvfd.2018.19741

9. Aslantaş Ö: Genotypic, antimicrobial resistance and virulence profiles of thermophilic *Campylobacter* isolates in broilers. *Kafkas Univ Vet Fak Derg*, 23, 547-554, 2017. DOI: 10.9775/kvfd.2016.17261

10. Abay S, Kayman T, Otlu B, Hizlisoy H, Aydin F, Ertas N: Genetic diversity and antibiotic resistance profiles of *Campylobacter jejuni* isolates from poultry and humans in Turkey. *Int J Food Microbiol*, 178, 29-38, 2014. DOI: 10.1016/j.ijfoodmicro.2014.03.003

11. Bolton DJ: *Campylobacter* virulence and survival factors. *Food Microbiol*, 48, 99-108, 2015. DOI: 10.1016/j.fm.2014.11.017

12. Wassenaar TM, Newell DG: Genotyping of *Campylobacter* spp. *Appl Environ Microbiol*, 66, 1-9, 2000. DOI: 10.1128/AEM.66.1.1-9.2000

13. Giacomelli M, Andrighetto C, Rossi F, Lombardi A, Rizzotti L, Martini M, Piccirillo A: Molecular characterization and genotypic antimicrobial resistance analysis of *Campylobacter jejuni* and *Campylobacter coli* isolated from broiler flocks in northern Italy. *Avian Pathol*, 41, 579-588, 2012. DOI: 10.1080/03079457.2012.734915

14. Wassenaar TM, Fernández-Astorga A, Alonso R, Marteinsson VT, Magnússon SH, Kristoffersen AB, Hofshagen M: Comparison of *Campylobacter fla*-SVR genotypes isolated from humans and poultry in three European regions. *Lett Appl Microbiol*, 49, 388-395, 2009. DOI: 10.1111/j.1472-765X.2009.02678.x

15. Pittenger LG, Englen MD, Parker CT, Frye JG, Quiñones B, Horn ST, Son I, Fedorka-Cray PJ, Harrison MA: Genotyping *Campylobacter jejuni* by comparative genome indexing: An evaluation with pulsed-field gel electrophoresis and *fla*A SVR sequencing. *Foodborne Pathog Dis*, 6, 337-349, 2009. DOI: 10.1089/fpd.2008.0185

16. Cokal Y, Caner V, Sen A, Cetin C, Karagenc N: *Campylobacter* spp. and their antimicrobial resistance patterns in poultry: An epidemiological survey study in Turkey. *Zoonoses Public Health*, 56, 105-110, 2009. DOI: 10.1111/j.1863-2378.2008.01155.x

17. Yıldırım M, İstanbulluoğlu E, Ayvalı B: Prevalence and antibiotic susceptibility of thermophilic *Campylobacter* species in broiler chickens. *Turk J Vet Anim Sci***,** 29, 655-660, 2005.

18. Wang G, Clark CG, Taylor TM, Pucknell C, Barton C, Price L, Woodward DL, Rodgers FG: Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. *J Clin Microbiol,* 40, 4744-4747, 2002. DOI: 10.1128/JCM.40.12.4744-4747.2002

19. Clinical and Laboratory Standards Institute: Performance standards for sntimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: Informational Supplement. CLSI Document M31-A3. Clinical and Laboratory Standards Institute, Wayne, PA, 2008.

20. Obeng AS, Rickard H, Sexton M, Pang Y, Peng H, Barton M: Antimicrobial susceptibilities and resistance genes in *Campylobacter* strains isolated from poultry and pigs in Australia. *J Appl Microbiol*, 113, 294-307, 2012. DOI: 10.1111/j.1365-2672.2012.05354.x

21. Lévesque S, Frost E, Arbeit RD, Michaud S: Multilocus sequence typing of *Campylobacter jejuni* isolates from humans, chickens, raw milk, and environmental water in Quebec, Canada. *J Clin Microbiol*, 46, 3404-3411. DOI: 10.1128/JCM.00042-08

22. Bang DD, Nielsen EM, Scheutz F, Pedersen K, Handberg K, Madsen M: PCR detection of seven virulence and toxin genes of *Campylobacter jejuni* and *Campylobacter coli* isolates from Danish pigs and cattle and cytolethal distending toxin production of the isolates. *J Appl Microbiol*, 94, 1003-1014, 2003. DOI: 10.1046/j.1365-2672.2003.01926.x

23. Konkel ME, Garvis SG, Tipton SL, Anderson DE Jr, Cieplak W Jr : Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Mol Microbiol*, 24, 953-963, 1997. DOI: 10.1046/j.1365-2958.1997.4031771.x

24. Bacon DJ, Alm RA, Burr DH, Hu L, Kopecko DJ, Ewing CP, Trust TJ, Guerry P: Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. *Infect Immun*, 68, 4384-4390, 2000. DOI: 10.1128/IAI.68.8.4384-

4390.2000

25. Datta S, Niwa H, Itoh K: Prevalence of 11 pathogenic genes of *Campylobacter jejuni* by PCR in strains isolated from humans, poultry meat and broiler and bovine faeces. *J Med Microbiol*, 52, 345-348, 2003. DOI: 10.1099/jmm.0.05056-0

26. Nachamkin I, Bohachick K, Patton CM: Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. *J Clin Microbiol*, 31, 1531-1536, 1993.

27. Giacomelli M, Follador N, Coppola LM, Martini M, Piccirillo A: Survey of *Campylobacter* spp. in owned and unowned dogs and cats in Northern Italy. *Vet J,* 204, 333-337, 2015. DOI: 10.1016/j.tvjl.2015.03.017

28. Gargiulo A, Rinaldi L, D'Angelo L, Dipineto L, Borrelli L, Fioretti A, Menna LF: Survey of *Campylobacter jejuni* in stray cats in southern Italy. *Lett Appl Microbiol*,46 (2): 267-270, 2008. DOI: 10.1111/j.1472-765X.2007.02295.x

29. Tsai HJ, Huang HC, Lin CM, Lien YY, Chou CH: Salmonellae and *campylobacters* in household and stray dogs in northern Taiwan. *Vet Res Commun*, 31, 931-939, 2007. DOI: 10.1007/s11259-007-0009-4

30. Bojanić K, Midwinter AC, Marshall JC, Rogers LE, Biggs PJ, Acke E: Isolation of *Campylobacter* spp. from client-owned dogs and cats, and retail raw meat pet food in the Manawatu, New Zealand. *Zoonoses Public Health*, 64, 438-449, 2017. DOI: 10.1111/zph.12323

31. Cho HH, Kim SH, Min W, Ku BK, Kim JH, Kim YH: Characterization of antimicrobial resistance and application of RFLP for epidemiological monitoring of thermophilic *Campylobacter* spp. isolated from dogs and humans in Korea. *Korean J Vet Res*, 54, 91-99, 2014. DOI: 10.14405/kjvr.2014.54.2.91

32. Byrne CM, Clyne M, Bourke B: *Campylobacter jejuni* adhere to and invade chicken intestinal epithelial cells *in vitro. Microbiology,* 153, 561-569, 2007. DOI: 10.1099/mic.0.2006/000711-0

33. Engvall EO, Brändstrom B, Andersson L, Båverud V, Trowald-Wigh G, Englund L: Isolation and identification of thermophilic *Campylobacter* species in faecal samples from Swedish dogs. *Scand J Infect Dis,* 35 (10): 713-718, 2003. DOI: 10.1080/00365540310014558

34. European Food Safety Authority (EFSA): The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. *EFSA J*, 13 (1):3991, 2015. DOI: 10.2903/j.efsa.2015.3991

35. Acke E, Whyte P, Jones BR, McGill K, Collins JD, Fanning S: Prevalence of thermophilic *Campylobacter* species in cats and dogs in two animal shelters in Ireland. *Vet Rec*, 158, 51-54, 2006. DOI: 10.1136/vr.158.2.51

36. Humphrey T: Are happy chickens safer chickens? Poultry welfare and disease susceptibility. *Br Poult Sci,* 47, 379-391, 2006. DOI: 10.1080/00071660600829084

37. Hald B, Pedersen K, Wainø M, Jørgensen JC, Madsen M: Longitudinal study of the excretion patterns of thermophilic *Campylobacter* spp. in young pet dogs in Denmark. *J Clin Microbiol*, 42, 2003-2012, 2004. DOI: 10.1128/JCM.42.5.2003-2012.2004

38. Torkan S, Vazirian B, Khamesipour F, Dida GO: Prevalence of thermotolerant *Campylobacter* species in dogs and cats in Iran. *Vet Med Sci*, 4, 296-303, 2018. DOI: 10.1002/vms3.117

39. Kumar R, Verma AK, Kumar A, Srivastava M, Lal HP: Prevalence and antibiogram of *campylobacter* infections in dogs of Mathura, India. *Asian J Anim Vet Adv*, 7, 434-440, 2012. DOI: 10.3923/ajava.2012.434.440

40. Engberg J, Keelan M, Gerner-Smidt P, Taylor DE: Antimicrobial resistance in *Campylobacter*. **In,** Aarestrup FM (Ed): Antimicrobial Resistance in Bacteria of Animal Origin. 274-277, ASM Press, Washington DC, 2006.

41. Guévremont E, Nadeau E, Sirois M, Quessy S: Antimicrobial susceptibilities of thermophilic *Campylobacter* from humans, swine, and chicken broilers. *Can J Vet Res*, 70, 81–86, 2006.

42. Abdi-Hachesoo B, Khoshbakht R, Sharifiyazdi H, Tabatabaei M, Hosseinzadeh S, Asasi K: Tetracycline resistance genes in *Campylobacter jejuni* and *C. coli* isolated from poultry carcasses. *Jundishapur J Microbiol*, 7:e12129, 2014. DOI: 10.5812/jjm.12129

43. Wassenaar TM, van der Zeijst BAM, Ayling R, Newell DG: Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. *J Gen Microbiol,* 139, 1171-1175, 1993. DOI: 10.1099/00221287-139-6-1171

44. Gomes CN, Souza RA, Passaglia J, Duque SS, Medeiros MIC, Falcão JP: Genotyping of *Campylobacter coli* strains isolated in Brazil suggests possible contamination amongst environmental, human, animal and food sources. *J Med Microbiol*, 65, 80-90, 2016. DOI: 10.1099/jmm.0.000201

45. Heywood W, Henderson B, Nair SP: Cytolethal distending toxin: Creating a gap in the cell cycle. *J Med Microbiol*, 54, 207-216, 2005. DOI: 10.1099/jmm.0.45694-0

46. Acik MN, Karahan M, Ongor H, Cetinkaya B: Investigation of virulence and cytolethal distending toxin genes in *Campylobacter* spp. isolated from sheep in Turkey. *Foodborne Pathog Dis*. 10, 589-594, 2013. DOI: 10.1089/fpd.2012.1447

Kafkas Univ Vet Fak Derg
25 (3): 349-356, 2019
DOI: 10.9775/kvfd.2018.20969

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Polymorphisms of *MBL* Gene Introns and Their Association with *MBL* Serum Levels in Hu Sheep

Mengting ZHAI [1†] Jian MOU [1†] Mengting ZHU [1] Yanping LIANG [1]
Mingyuan WANG [1] Zongsheng ZHAO [1] Hongmei ZHANG [2]

[†] *These authors contributed equally to this work*
[1] College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang, 832003, PR CHINA
[2] Department of Clinical Laboratory, First Affiliated Hospital, Shihezi University, Xinjiang, 832003, PR CHINA

Article ID: KVFD-2018-20969 Received: 16.09.2018 Accepted: 01.02.2019 Published Online: 01.02.2019

How to Cite This Article

Zhai M, Mou J, Zhu M, Liang Y, Wang M, Zhao Z, Zhang H: Polymorphisms of *MBL* gene introns and their association with *MBL* serum levels in Hu Sheep. *Kafkas Univ Vet Fak Derg,* 25 (3): 349-356, 2019. DOI: 10.9775/kvfd.2018.20969

Abstract

PCR single-strand conformation polymorphism (SSCP) and DNA sequencing techniques were used to analyze the genetic polymorphism of mannose-binding lectin *(MBL)* gene 3 introns in Hu sheep. The results showed that 3 introns of *MBL* gene had polymorphism, 3 genotypes were identified in intron1, which were controlled by 2 alleles, respectively; 3 genotypes were identified in intron2, which were controlled by 2 alleles, respectively; 3 genotypes were identified in intron3, which were controlled by 2 alleles, respectively; 6 new single nucleotide polymorphisms(SNPs) mutation sites were found, g.288T>A in intron1, g. 1091 T>C, g.1096A>C, g.1770G>C in intron 2, g.2297C>T, g.2331G>A in intron3. Use ELISA to detect MBL serum level in Hu sheep, then use One-way ANOVA analyze the relationship of different genotypes and *MBL* serum level. And the result showed that the *MBL* serum level of AA genotype was higher than BB genotype ($P<0.05$) in intron1, CC genotype was higher than DD, GG genotype was higher than HH ($P<0.05$) in intron2. So, AA, CC and GG genotypes may be related to disease resistance; BB, DD and HH genotypes be related to disease susceptibility. The point mutation in intron3 couldn't lead to the change of MBL serum level.

Keywords: *Sheep, MBL, Mycoplasma pneumonia, PCR-SSCP, ELISA, Introns*

Hu Koyunlarında MBL Gen İntronlarının Polimorfizmi ve MBL Serum Seviyeleri ile İlişkisi

Öz

PCR single-strand conformation polymorphism (SSCP) ve DNA sekanslama teknikleri, Hu koyunlarında mannoz bağlayan lektin *(MBL)* gen 3 intronunda genetik polimorfizmi analiz etmede kullanılmıştır. Sonuçlar *MBL* geninin 3 intronunda polimorfizmin olduğunu gösterdi, ve intron 1'de sırayla 2 allel tarafından kontrol edilen 3 genotip, intron 2'de sırayla 2 allel tarafından kontrol edilen 3 genotip, intron 3'de de sırayla 2 allel tarafından kontrol edilen 3 genotip tespit edildi. 6 yeni tek nükleotid polimorfizm (SNPs) mutasyon alanı (g.288T>A intron 1'de, g. 1091 T>C, g.1096A>C, g.1770G>C intron 2'de, g.2297C>T, g.2331G>A intron 3'de) bulundu. Hu koyunlarında *MBL* serum seviyelerini belirlemek amacıyla ELİSA uygulandı, sonrasında Tek yönlü ANOVA kullanılarak farklı genotipler ile *MBL* serum seviyeleri arasındaki ilişki analiz edildi. Sonuçlar intron 1'de AA genotipinin *MBL* serum seviyesinin BB genotipinden daha fazla olduğunu ($P<0.05$), intron 2'de CC genotipinin DD genotipinden, GG genotipinin HH genotipinden daha fazla olduğunu ($P<0.05$) gösterdi. Bu nedenle AA, CC ve GG genotipleri hastalık dirençliliği ile ilişkili olabilirken BB, DD ve HH genotipleri hastalık duyarlılığı ile ilişkili olabilir. İntron 3'de nokta mutasyon MBL serum seviyesinde değişime neden olamaz.

Anahtar sözcükler: *Koyun, MBL, Mycoplasma pneumonia, PCR-SSCP, ELISA, İntronlar*

INTRODUCTION

Mannose-binding lectin *(MBL)* is a serum protein mainly produced by the liver and belongs to the C-type calcium ion-dependent lectin, which plays an important role in innate immunity. *MBL* function involves the formation of a complex-activated complement system, which binding to a serine protease associated with MBL in the lectin pathway [1]. It plays an important role in adaptive immune responses, and inflammatory responses by affecting

İletişim (Correspondence)
+86 1356 5735767; Fax: +86 0993 2058722
zhaozongsh@shzu.edu.cn (Z. Zhao); zhanghmay@126.com (H. Zhang)

cytokine release [2]. Sheep *MBL* gene has 4 exons and 3 introns with a full length 4462 base pair (bp) [3]. The study found that there are 5 exons in the *MBL* gene, and 6 single nucleotide polymorphisms (SNPs) associated with *MBL* expression levels [4]. The above polymorphism and their haplotype have the greatest influence on serum *MBL2* content, at the same time, it's essential for its anti-infective effect to maintain a certain level of *MBL* serum concentration, so *MBL* polymorphism has become a hot topic in current research. The study has been shown that the level of *MBL* in the blood is mainly determined by the structure of the *MBL* gene, which is affected by the variation of the gene structure region and the regulation of the activity of the gene promoter region [5]. For example, three SNPs in the exon I of the human *MBL* gene, known as the D-allele (Arg52Cys), B-allele (Gly54Asp) and C-allele (Gly57Glu), interfere with the formation of high *MBL* oligomers, It is speculated that it can affect the level of serum *MBL* by initiating different levels of gene expression [6-8].

Promoter is a cis-actin element of eukaryotic gene expression regulation, contains important information of gene expression regulation network, determines the degree of gene expression and its specificity [9,10]. On the other hand, the mutation of the intron area may influence the transcription, which results in incomplete translation in the functional areas. Therefore, this makes *MBL* protein to change in structure, and hinders the realization of *MBL* biological function. Resulting in a significant decrease in *MBL* serum levels of the body, and ultimately the disease resistance is reduced due to weakening of the body's immunity.

We use PCR-SSCP and DNA sequencing techniques to analyze the genetic polymorphism of 3 introns in the Hu sheep's *MBL* gene, and conduct the statistical analysis on the association between different genotypes in the *MBL* gene and *MBL* serum levels in Hu sheep. In order to lay the foundation for comprehensive study, the study on the correlation between the polymorphisms of the sheep *MBL* gene and diseases.

MATERIAL and METHODS

Collection of Sheep Blood Samples

Whole blood was collected from 105 healthy individuals of the Hu sheep that aged 4 months and weighted 10-12 kg were from different sheep farms in the ninth agricultural unit of the Xinjiang Production and Construction Corps, Tacheng, China. Fresh blood samples were mixed immediately with EDTA buffer. Sera were separated by centrifugation at 3000×g for 10 min, and were then transferred to 1.5 mL Eppendorf tubes and stored at -80°C.

DNA Extraction, Primer Design and PCR Amplification

Genomic DNA was extracted from EDTA anticoagulated blood samples using phenol/chloroform method [11]. Primer sequences of sheep *MBL* introns-1,2,3 and annealing temperatures of the PCR *(Table 1)* were designed with Primer 5.0 from the sequences of the MBL gene of sheep available in GenBank (accession numbers FJ977629). Primers were synthesized at Sangon Biological Engineering Technology Company (SBETC, Shanghai, China) and were used in a 25 µL PCR reaction to amplify a some sections of the intron-1,2,3 region of the *MBL* gene *(Table 1)*. A 25 µL PCR reaction contains 1 µL (50 ng) of genomic DNA extracted from an individual Hu sheep, 2.5 µL 10× PCR buffer, 1 µL (5mM) of each primer, 2.5 µL dNTPs (2.5 mM), 1.5 µL $MgCl_2$ (15 mM), 0.6 µL (1.5 units) Taq DNA polymerase, and 14.9 µL MilliQ H_2O. The PCR reagents were supplied by the SBETC. The conditions for PCR reactions are 94°C for 5min, followed by 30 cycles of 30 s at 94°C, 45 s at annealing temp *(Table 1)*, 30 s at 72°C, and a final extension at 72°C for 10 min. PCR products were electrophoresed on 1.5% agarose-gel using 0.5×TBE buffer; the agarose gel was stained with ethidium bromide.

PCR Single-Strand Conformation Polymorphism Analysis

PCR products were analysed by SSCP, following protocols described [12]. Aliquots of 2 µL PCR products were mixed with 8 µL denaturing solution (98% formamide, 25 mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), were incubated at 98°C for 10 min and were then chilled on ice for 10 min. Denatured PCR products were electrophoresed on 12% PAGE gel (80 mm×73 mm×0.75 mm) in 0.5×TBE buffer at 140V and 12°C for 20 h. The gel was stained with 0.1% silver nitrate solution.

Cloning of PCR Products and DNA Sequencing

PCR products representative of different SSCP patterns in the Hu sheep were cloned using pGEM-T Easy Vector System (Promega) and competent *Escherichia coli* cells following the manufacturer's instruction. 6 to 12 colonies were selected for each SSCP pattern and cultured overnight in Terrific Broth medium that contained 50 mg/mL ampicillin. To isolate plasmids, a 50-mL aliquot of the overnight culture was centrifuged at 13.000 rpm for 2 min; the supernatant was discarded. The pellet was mixed with 30 mL (10×) TE buffer, was boiled for 10 min, and was then centrifuged at 13.000 rpm for 2 min. One µL of the supernatant was used in a PCR with primers MBLF and MBLR (see above for primer sequences). The PCR products from isolated plasmids were electrophoresed on 12% PAGE gels under the same conditions described above for the PCR products from the genomic DNA. The PCR products with *MBL*F and *MBL*R from both isolated plasmids and genomic DNA were sequenced at BGI (Beijing, China; http://www.genomics.cn).

Measurement of MBL Protein Levels in Serum

Serum samples from the Hu sheep were stored at −80°C. *MBL* levels in serum samples were measured using the *MBL*

Table 1. *Primer sequences Information of MBL Gene*

Serial Number	Loci	Sequence	Location	Length/bp	Annealing Temperature/°C
1	Intron1	F: GTGATGGTGCCAAGGGAGAA R: GGGATGCCAGAATCAGAGCC	1145-1329	185	58
2		F: ATCATTTGAAACAGAGGCACG R: TCCCAGGGGAAAGGAGACAC	1289-1494	206	56
3	Intron2	F: GTTTACTTTAGCAAGGTCCAG R: CAGGCATCTCACAAGGGTTT	1696-1917	222	59
4		F: AGCCAAACCCTTGTGAGATG R: ACAATAGCCAGCGTGTAAGT	1894-2111	218	58
5		F: GTCTCACTTACACGCTGGCTAT R: AATACAACGTGGTGGAAGCA	2087-2290	204	59
6		F: TGCTTCCACCACGTTGTATT R: TCCCTGAGTTTGTCCTGTTAA	2271-2478	208	59
7		F: TAACAGGACAAACTCAGGGA R: TGCCAAGCTACTCACTAATT	2458-2650	193	60
8		F:AGTAGCTTGGCATGTGGAGA R:GGGGTAGGGTACCTTTTGAA	2639-2914	276	60
9	Intron3	F: CTGAAGTTTGGTAAAGTGAA R: CTCATTAGTTCTATGCGTTT	3062-3231	170	60
10		F: GCATAGAACTAATGAGTAGCA R: TCACTTGGGTCAGTCGTGTC	3215-3488	270	59
11		F: CGACTGACCCAAGTGAGCAT R: GTCTCAGGGCAAGCAACAGG	3473-3653	181	60
12		F: CACCTCTTTCCCTTTGTTATG R GGTTAAATCTAGCAGCCCTAA	3583-3800	218	57
13		F:TGTTCAGATTAGGGCTGCTAGA R:GCCGCATAAAATATGGTATGTCC	3771-3978	208	59

Oligomer ELISA Kit (ADL, America), which contains a 96-well test plate, standards of known *MBL* concentrations, wash buffers, a *MBL* antigen and a biotinylated monoclonal antibody specific to *MBL*, an enzyme (streptavidin-peroxidase) and a substrate solution. Serum samples from the Hu sheep and standards of known *MBL* concentrations were loaded into the wells on the test plate: 50 µL of each serum sample or standard per well. The *MBL* antigen and the biotinylated monoclonal antibody specific to *MBL* were added to each well and were incubated at 37°C for 60 min. The wells were washed and the enzyme, streptavidin-peroxidase, was added. After incubation at 37°C for 30 min, the wells were washed to remove unbound enzymes; the substrate solution, which reacted with the bound enzyme to induce a colour, was added. The intensity of the colour was proportional to the concentration of *MBL* protein present in the serum samples. It was measured with an ELISA reader at 450 nm and was then converted into *MBL* concentration (µg/L) in serum, using an established human antigenic *MBL* level of 1670 µg/L as a reference.

Statistical Analysis of the Association Between Polymorphisms in MBL Gene and MBL Protein Levels in Serum

Differences in haplotype frequencies were analysed using a chi-square test (χ^2 test). The association between polymorphisms in *MBL* gene and *MBL* protein levels in serum were evaluated using One-way ANOVA test. All statistical analyses were performed with SPSS for Windows (version 17.0).

RESULTS

PCR products for *MBL* gene had 13 specific fragments containing 3 introns. They were subject to a test by 1.5% agarose gel electrophoresis and then found to be consistent with the results of the target fragment without specific bands so could be subject to SSCP analysis.

PCR-SSCP analysis results showed that 1, 4, 6, 8,10 and 13 primers had polymorphisms. Primers 1 is intron 1 had 3 genotypes, respectively defined as AA, BB, AB, which were controlled by A and B alleles. primers 3 is intron 2-1 had 2 genotypes, respectively defined as PP and OP, which were controlled by O and P alleles. primers 4 is intron 2-2 had 3 genotypes, respectively defined as of CC, CD, and DD, which were controlled by C and D alleles. primers 6 is intron 2-4 had 3 genotypes, respectively defined as EE, EF and FF, which were controlled by E and F alleles. primer 8 is intron 2-5 had 3 genotypes, respectively defined as GG, GH and HH, which were controlled by G and H alleles. primers 10 is intron 3-2 had 3 genotypes, respectively defined as II, IJ and JJ, which were controlled by I and J alleles. primers 13 is intron 3-5 had 3 genotypes, respectively defined as KK, KM and MM,

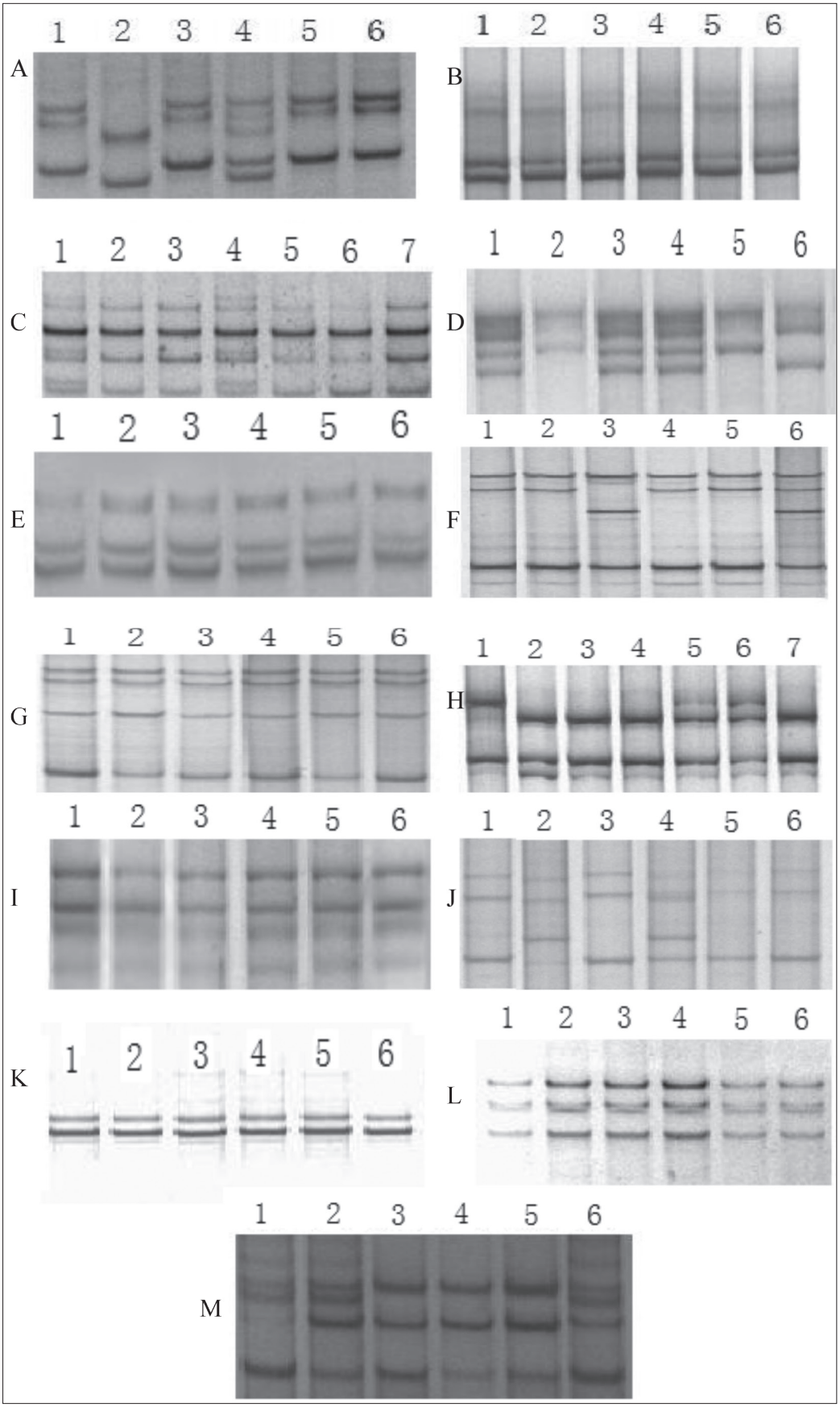


Fig 1. SSCP analysis of PCR products amplified with primers. Each product is represented by one of these letters A-M. These letters correspond to the primer 1-13, respectively

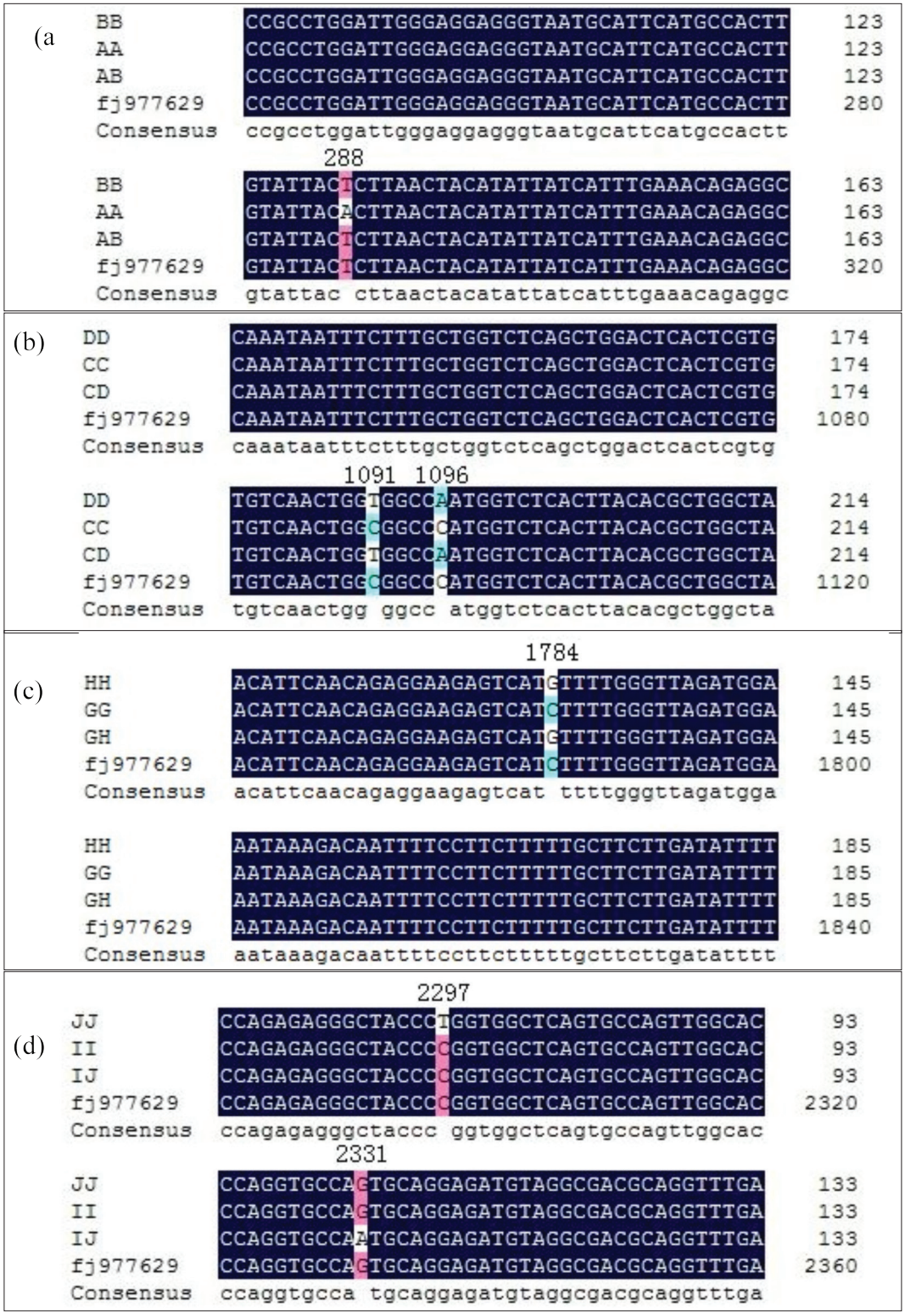


Fig 2. Comparison of sequences of different genotypes of MBL intron primer in Hu sheep (a- primer1; b- primer4; c- primer8; d- primer10)

which were controlled by K and M alleles. Primer 2, 5, 7, 9 were not found to have genetic polymorphisms *(Fig. 1)*.

Let the PCR fragments of different genotypes for all paired primers be cloned and sequenced. The comparison of sequencing results indicated that Hu sheep *MBL* gene intron had 6 single nucleotide mutation points, respectively being intron 1's g.288T> A *(Fig. 2-a)*; intron 2's g. 1091 T> C, g.1096A> C *(Fig. 2-b)*, and g.1784G> C *(Fig. 2-c)*; intron 3's g.2297C> T and g.2331G> A *(Fig. 2-d)*.

Table 2. *Statistics of frequency of genotype and allele at the MBL gene intron in Hu sheep*

Primer Number	Genotype	Genotype Frequency	Allele	Allele Frequency	χ^2
1	AA	0.467 (49)	A	0.619	13.147**
	AB	0.305 (32)	B	0.381	
	BB	0.229 (24)			
4	CC	0.343 (36)	C	0.552	2.445**
	CD	0.419 (44)	D	0.448	
	DD	0.238 (25)			
6	EE	0.238 (25)	E	0.571	13.692**
	EF	0.333 (70)	F	0.429	
	FF	0.095 (10)			
8	GG	0.181 (19)	G	0.519	13.184**
	GH	0.676 (71)	H	0.481	
	HH	0.143 (15)			
10	II	0.553 (58)	I	0.710	5.925**
	IJ	0.314 (33)	J	0.290	
	JJ	0.133 (14)			
13	KK	0.114 (12)	K	0.443	11.551**
	KM	0.657 (69)	M	0.557	
	MM	0.229 (24)			

*$\chi^2_{0.01(100)} = 119.56$; ** $P<0.01$*

According to the phenotype indicated in the electrophoretogram, then statisticed genotype frequencies. Carried out χ^2 test on different genotypes of 6 pairs of primers distributed in Hu sheep, and results showed all pair of primers had a genotype frequency distributed in Hu sheep which was characterized by an extremely significant difference ($P<0.01$), respectively *(Table 2)*.

According to the introns and sheep *MBL* ELISA test kit results, it was indicated that in this study with 105 Hu sheep, *MBL* gene intron had 3 genotypes, i.e. AA, BB and AB genotypes whose number was respectively 49, 24, and 32. According to the different genotype of intron 1, the single factor ANOVA analysis was made for *MBL* serum levels; the results showed that there were extremely significant differences between AA and AB ($P<0.01$), AB and BB ($P<0.01$), AA and BB ($P<0.01$) *(Fig. 3)*.

According to the introns and sheep *MBL* ELISA test kit results, it was indicated that in this study with 105 sheep, *MBL* gene intron 2-2 had 3 genotypes i.e. CC, CD and DD. The number of CC, CD, and DD was respectively 36, 44 and 25. According to the different genotype of intron 1, the single factor ANOVA analysis was made for the *MBL* serum level; the results showed that there were extremely significant differences between CC and CD ($P<0.01$), CD and DD ($P<0.01$), CC and DD ($P<0.01$) *(Fig. 3)*.

Mannose-binding lectin gene intron 2-6 had 3 genotypes i.e. GG, GH and HH whose number was respectively 19, 71 and 15. According to the different genotype of intron 2-6, the single factor ANOVA analysis was made for the *MBL* serum level; the results showed that there were extremely significant differences between GG and GH ($P<0.01$), GH and HH ($P<0.01$), GG and HH ($P<0.01$) *(Fig. 3)*.

Mannose-binding lectin gene intron 3 had 3 genotypes i.e. II, IJ, and JJ whose number was respectively 58, 33, and 14. According to the different genotype of intron 3, the One-way ANOVA analysis was made for the *MBL* serum level; the results didn't show significant differences between II and IJ ($P>0.05$), ($P>0.05$), II and JJ ($P>0.05$) *(Fig. 3)*.

DISCUSSION

Mannose-binding lectin is the most important natural anti-infective immune molecule in humans and animals [13]. It is secreted by the liver and secreted into the blood. It induces and activates the body's immune response before the antigen-antibody reacts specifically. *MBL's* protection features are closely related to *MBL* levels. In other words, a certain concentration of circulating *MBL* is maintained. Level is the basis of its physiological function. The lower the *MBL* level, the higher the susceptibility to pathogenic microorganisms. The expression level of MBL in serum is closely related to the *MBL* gene polymorphism, that is, *MBL* serum level is mainly removed by *MBL* gene. Affected by structural gene mutations and promoter region activity, mutations in the *MBL* gene can result in decreased *MBL* levels in serum, affecting the complement activation system [5,14].

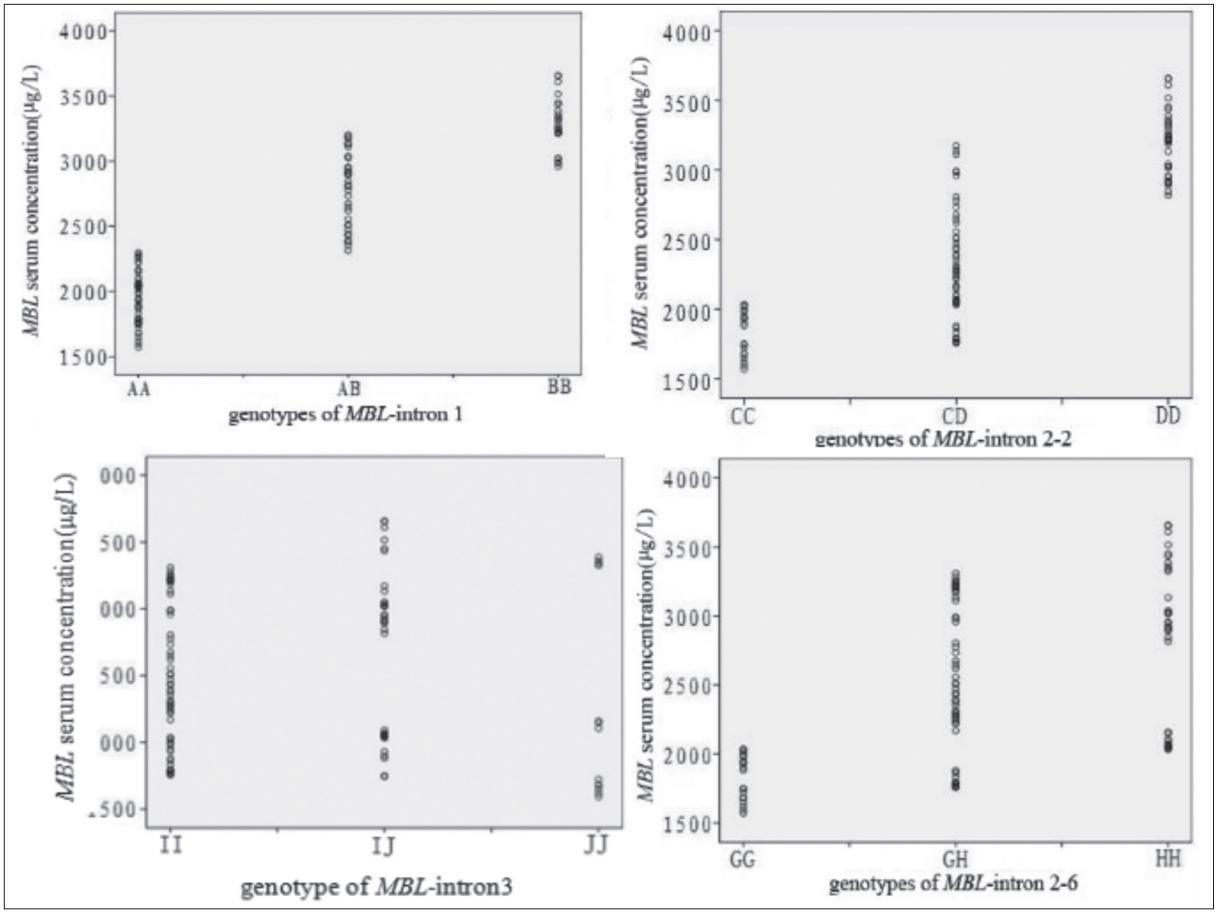


Fig 3. Comparison of MBL serum concentration in different genotypes of MBL

An intron, corresponds to an exon and is a special type of non-coding DNA sequence. The exons are alternately arranged to form an intervening gene [15]. It does not occur in mature mRNA sequences as it is cleaved during transcriptional translation of the precursor RNA. Very few connates have been found in prokaryotic genome sequences, and almost all introns are included in eukaryotic genome sequences, except that inferior eukaryotes have relatively few intron sequences. Therefore, in this experiment, 3 introns of the *MBL* gene were not involved in protein synthesis, but their mutations affected the exon coordination and the encoded protein. There also may be a position effect, it also may affect the correct shearing in the *MBL* gene expression. Thereby affecting the translation of the protein, leading to low *MBL* serum levels, ultimately making the body susceptible to the disease [16]. However, the specific function needs a further research.

This paper carried out the polymorphism analysis for 3 intron areas of the *MBL* gene in Hu sheep, detected and compared all the *MBL* serums which corresponded with genotypes. The results showed that in the Hu sheep *MBL* gene, intron 1's AA-type corresponds to a low level of *MBL* serum concentration, BB-type and AB-type correspond to a high level, so it can be predicted that BB is resistant and AA susceptible. Similarly, intron 2-2's CC-type is susceptible and DD resistant; intron 2-6's GG-type is resistant and HH susceptible; in intron 3, the difference between II and JJ is not significant, so the mutations affect the *MBL* serum level. From the above, it is indicated that in the *MBL* gene, AA of intron 1 and CC and CG of intron 2 are resistant, while BB, DD and HH are susceptible. Intron 1 and 2 genotype differences in *MBL* serum levels are significant, which indicates that *MBL* gene can be used as candidate gene for disease resistance.it found that +328 site of the pig *MBL*1 intron has a C/T mutation and that different genotypes have a significant difference in serum C3c concentration and complement hemolytic activity [17], further confirming that the pig *MBL*1 gene can be used as a function and a positional candidate gene for complement hemolytic activity.

As conclusion, the Hu sheep's *MBL* gene intron has a wealth of genetic polymorphisms, and lays the genetic basis for relevant genetic markers in screening, generation, disease resistance or susceptibility. According to the analysis for the correction of Hu sheep intron polymorphisms with *MBL* serum levels, this experiment screens the resistant and susceptible alleles to lay a solid foundation for the further validation of whether resistant alleles can be used as genetic markers for resistance to mycoplasma pneumoniae in sheep.

REFERENCES

1. Degn SE, Thiel S: Humoral pattern recognition and the complement system. *Scand J Immunol*, 78 (2): 181-193, 2013. DOI: 10.1111/sji.12070

2. Wang M, Zhang Y, Chen Y, Zhang L, Lu X, Chen Z: Mannan-binding lectin regulates dendritic cell maturation and cytokine production induced by lipopolysaccharide. *BMC Immunol*, 12:1, 2011. DOI: 10.1186/1471-2172-12-1

3. Zhao F, Zhao Z, Yan G, Wang D, Ban Q, Yu P, Zhang W, Luo Y: Polymorphisms in mannose-binding lectin *(MBL)* gene and their association with *MBL* protein levels in serum in the Hu sheep. *Vet Immunol Immunopathol*, 140, 297-302, 2011. DOI: 10.1016/j.vetimm.2010.12.009

4. Smithson A, Perello R, Aibar J, Espinosa G, Tassies D, Freire C, Castro P, Suarez B, Lozano F, Nicolas JM: Genotypes coding for low serum levels of mannose-binding lectin are underrepresented among individuals suffering from noninfectious systemic inflammatory response syndrome. *Clin Vaccine Immunol*, 17 (3): 447-453, 2010. DOI: 10.1128/CVI.00375-09

5. Turner MW: The role of mannose binding lectin in health and disease. *Mol Immunol*, 40, 423-429, 2003. DOI: 10.1016/S0161-5890(03)00155-X

6. Madsen HO, Garred P, Kurtzhals JAL, Lamm LU, Ryder LP, Thiel S, Svejgaard A: A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. *Immunogenetics*. 40, 37-44, 1994. DOI: 10.1007/BF00163962

7. Sumiya MTabona P, Arai T, Summerfield JA, Super M, Levinsky RJ, Turner MW: Molecular basis of opsonic defect in immunodeficient children. *Lancet*, 337, 1569-1570, 1991. DOI: 10.1016/0140-6736(91)93263-9

8. Lipscombe RJ, Sumiya M, Summerfield JA, Turner MW: Distinct physicochemical characteristics of human mannose binding protein expressed by individuals of differing genotype. *Immunology*, 85, 660-667, 1995.

9. Wang HM, Zhang LB, Hou MH, Wang CF, Wang LL, Sun T, He HB, Zhong JF: Cloning and activity analysis of bovine natural resistance associated macrophage protein1(Nramp 1)gene promoter. *Scicntia Agric Sinica*, 21, 2011.

10. Ghang X, Luo J, Li JH, et al: Cloning and activity dct-urination of fatty acid synthasc gene promoter of xinong saancn dairy goat. *Scicntia Agric Sinica*, 28, 2010.

11. Sambrook J, Russell DW: Molecular Cloning: A Laboratory Manual. Vol. 3, 3rd ed., Cold Spring Harbor Laboratory Press, *New York*, 49-56, 2001.

12. Lan XY, Pan CY, Chen H, Zhao M, Li JY, Yu J, Zhang CL, Lei CZ, Hua LS, Yang XB: The novel SNPs of the IGFBP3 gene and their associations with litter size and weight traits in goat. *Arc Anim Breed,* 50, 223-224,2007.

13. Gadjeva M, Takahashi K, Thiel S: Mannan-binding lectin-a soluble pattern recognition molecule. *Mol Immunol*, 41 (2-3): 113-121, 2004. DOI: 10.1016/j.molimm.2004.03.015

14. Thiel S, Frederiksen PD, Jensenius JC: Clinical manifestations of mannan-binding lecain deficiency. *Mol Immunol*, 43 (1-2): 86-96, 2006. DOI: 10.1016/j.molimm.2005.06.018

15. Chatterjee A, Rathore A, Yamamoto N, Dhole TN: Mannose-binding lectin (+54) exon-1 gene polymorphism influence human immuno-deficiency virus-1 susceptibility in North Indians. *Tissue Antigens*, 77, 18-22, 2011. DOI: 10.1111/j.1399-0039.2010.01563.x

16. Michelow IC, Lear C, Scully C, Prugar LI, Longley CB, Yantosca LM, Ji X, Karpel M, Brudner M, Takahashi K, Spear GT, Ezekowitz RA, Schmidt EV, Olinger GG: High-dose mannose-binding lectin therapy for Ebola virus infection. *J Infect Dis*, 203, 175-179, 2011. DOI: 10.1093/infdis/jiq025

17. Storgaard P, Nielsen EH, Andersen O, Skriver E, Mortensen H, Hojrup P, Leslie G, Holmskow U, Svehag SE: Isolation and characterization of porcine mannan-binding proteins of different size and ultrastructure. *Scand J Immunol*, 43, 289-296, 1996. DOI: 10.1046/j.1365-3083.1996.d01-39.x

Kafkas Univ Vet Fak Derg
25 (3): 357-363, 2019
DOI: 10.9775/kvfd.2018.20982

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Evaluation of Intramammary Platelet Concentrate Efficacy as a Subclinical Mastitis Treatment in Dairy Cows Based on Somatic Cell Count and Milk Amyloid A Levels [1][2]

Gamze EVKURAN DAL [1,a] Ahmet SABUNCU [1,b] Deniz AKTARAN BALA [2,c]
Sinem Özlem ENGİNLER [1,d] Ali Can ÇETİN [1,e] Baran ÇELİK [3,f] Ömür KOÇAK [4,g]

[1] *This study was funded by Istanbul University with the project number 24040*
[2] *The preliminary result of the study was presented at "Turkish Veterinary Gynaecology Association VII. National & I. International Congress", 12-15 October 2017, Marmaris, Turkey*

[1] Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, TR-34320 Avcilar, İstanbul - TURKEY
[2] Istanbul University-Cerrahpasa, Vocational School of Veterinary Medicine, Food Processing Department, Food Technology Programme, TR-34320 Avcilar, İstanbul - TURKEY
[3] Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Microbiology, TR-34320 Avcilar, İstanbul - TURKEY
[4] Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Animal Breeding and Husbandry, TR-34320 Avcilar, İstanbul - TURKEY
[a] ORCID: 0000-0002-9996-3290; [b] ORCID: 0000-0001-7905-421X; [c] ORCID: 0000-0003-1512-8552; [d] ORCID: 0000-0002-2352-876X
[e] ORCID: 0000-0003-4617-8544; [f] ORCID: 0000-0001-9122-0284; [g] ORCID: 0000-0002-2827-4471

Article ID: KVFD-2018-20982 Received: 18.09.2018 Accepted: 04.01.2019 Published Online: 04.01.2019

How to Cite This Article

Evkuran Dal G, Sabuncu A, Aktaran Bala D, Enginler SÖ, Çetin AC, Çelik B, Koçak Ö: Evaluation of intramammary platelet concentrate efficacy as a subclinical mastitis treatment in dairy cows based on somatic cell count and milk amyloid A levels. *Kafkas Univ Vet Fak Derg,* 25 (3): 357-363, 2019. DOI: 10.9775/kvfd.2018.20982

Abstract

The intramammary administration of platelet concentrate is expected to treat subclinical mastitis and prevent disease recurrence effectively; therefore, it was aimed to evaluate its efficacy in terms of somatic cell count (SCC) and Milk Amyloid A (MAA) measurements. A total of 120 cow mammary lobes with subclinical mastitis were randomly assigned to one of the following three groups: Antibiotic Group (ABG, n=40), Platelet Concentrate Group (PCG, n=40) or Combined Group (CG, n=40). Platelet concentrates were prepared by the double centrifugation method from blood collected from donor cows. All groups received intramammary treatments for 3 days. Analysis of MAA using a commercially available ELISA method and measurement of SCC were performed from milk samples collected on days 0, 7, 14, and 21. Treatment success and absence of recurrence were found to be statistically significant for all three treatment protocols (P<0.001). It is concluded that intramammary platelet concentrate administration can be an effective alternative to intramammary antibiotic use for the treatment of subclinical mastitis.

Keywords: *Subclinical mastitis, Platelet concentrate, Milk amyloid A, Somatic cell count, Dairy cow*

Sütçü İneklerde Subklinik Mastitis Tedavisinde Meme İçi Platelet Konsantresi Etkinliğinin Somatik Hücre Sayımı ve Süt Amiloid A Seviyeleri İle Değerlendirilmesi

Öz

Meme içi platelet konsantresi uygulamasının, subklinik mastitisi tedavi etme ve hastalığın rekürrensini engellemede etkin olabileceği beklenmekte olup; uygulamanın etkinliğinin somatic hücre sayısı (SHS) ve Süt Amiloid A (Milk Amyloid A: MAA) ölçümlerine dayandırılarak değerlendirilmesi amaçlanmıştır. Subklinik mastitisli ineklere ait 120 meme lobu rastgele Antibiyotik Grubu (ABG, n=40), Platelet Konsantresi Grubu (PKG, n=40) ve Kombine Grup (KG, n=40) olmak üzere üç gruba ayrıldı. Platelet konsantreleri donör ineklerden alınan kandan çift santrifüj metoduyla hazırlandı. Gruplara 3 gün boyunca meme içi tedavi uygulandı. Çalışmanın 0, 7, 14 ve 21. günlerinde alınan süt örneklerinde SHS ölçümü ve ELISA yöntemiyle MAA analizi yapıldı. Uygulanan her üç tedavi protokolü için de iyileşme başarısı ve rekürrens görülmemesi durumu istatistiksel açıdan önemli bulundu (P<0.001). Subklinik mastitis tedavisinde meme içi platelet konsantresi uygulamasının meme içi antibiyotik kullanımına alternatif olabileceği sonucuna ulaşıldı.

Anahtar sözcükler: *Subklinik mastitis, Platelet konsantresi, Süt amiloid A, Somatik hücre sayısı, Sütçü inek*

İletişim (Correspondence)
+90 212 4737070/17317
ahmetsabuncu1968@yahoo.com

INTRODUCTION

Milk and dairy products are important sources of food consumption for the vast majority of the world's population [1]. The inflammation of mammary tissue is called mastitis, usually caused by intramammary bacterial infections [2]. Subclinical mastitis is characterized by inflammation with no clinical findings in milk or mammary tissues [3,4].

Mastitis is the most common pathology seen in the dairy industry and is the main cause of antibiotic use and economic loss [1,5,6]. Most cases of mastitis are subclinical with almost 20-50 cases of subclinical mastitis recorded for every clinical mastitis case [7]. Intramammary administration of antibiotics is used to reach the highest drug concentration in cases of clinical mastitis and in almost all subclinical mastitis cases, except for those where the mammary tissue has excessive swelling or fibrosis [8]. Mastitis is difficult to treat due to various direct and indirect issues including treatment costs, unusable milk, labor and time loss, recurrent mastitis, a decrease in milk production and quality, increase in the number of discarded animals, affected animal welfare, etc. [1,3,5]. Among the problems caused by administering antibiotics to animals produced for consumption are antibiotic residue in food and micro-organisms acquiring antibiotic resistance [1,6].

Platelet activation is the first step of healing process that occurs after tissue damage. This process consists the release of several bioactive factors that plays role in the recruitment of cells associated with healing to the damaged tissue. New treatment protocols of platelet concentrate administration, so the highly concentrated bioactive factors, has become popular in recent years. It has several advantages as being safe, easy and has a wide range of application area [9]. Platelets contain growth factors, chemokines, cytokines and active metabolites which are required for rapid wound healing and tissue regeneration. Local application of these growth factors at high concentrations through platelet concentrate increases the repair rate of the tissue by optimizing the area of healing [10]. Alpha-granules of platelets contain various factors as transforming growth factors (TGF), platelete-derived growth factors, epidermal growth factors, insulin-like growth factor-1, chemokins and cytokines which play role in healing process and tissue regeneration [11]. Throughout these factors, TGF-α was reported to have an impact on mammary epithelial proliferation and morphogenesis of the mammary gland [12] which may play important role during healing from mastitis.

Blood products containing intensive amounts of platelets have become widely used in many areas of human medicine. Although there is limited information on their use in veterinary medicine, platelet-rich plasma applications have also become popular in recent years, especially for tendon injuries in equine medicine. The use of platelet concentrate obtained through a double centrifugation method for the treatment of mastitis is of current research interest as an alternative method to stimulate the regeneration of glandular tissue by providing growth factors at supraphysiological concentrations [10].

Regarding the evaluation of mastitis, the use of various parameters such as haptoglobin, milk amyloid A (MAA), lactoferrin, lysozyme, lactate dehydrogenase enzyme, nitric oxide, and heat shock proteins have become widespread in bovine medicine [13]. One of these parameters, MAA, is a specific isoform of serum amyloid A and is secreted only in the presence of inflammation directly from mammary epithelial cells. It is a highly sensitive marker that allows the detection of subclinical mastitis in milk [13,14].

In addition to the use of antibiotics for the treatment of mastitis, which is the most important and common problem in dairy farms, there is a need for new antimicrobial treatment methods. Optimally, such new methods would not create a basis for bacterial resistance and would not leave antibiotic residue in nature and animal-origin food and, therefore, would not threaten human health. Considering the growing interest in organic livestock and organic animal-origin food production in recent years, the development of a novel approach to mastitis treatment could be regarded as a milestone. The absence of information on the use of platelet concentrate in subclinical mastitis screening in the literature confirms the authenticity of our study and the lack of knowledge on this subject.

The intramammary administration of platelet concentrate, which was previously prepared for use and stored in a laboratory environment, is expected to treat subclinical mastitis and prevent disease recurrence effectively; therefore, it was aimed to evaluate its efficacy in terms of somatic cell count (SCC) and MAA measurements.

MATERIAL and METHODS

Study Design

The study was approved by Istanbul University Local Committee on Animal Research Ethics (Permit no. 2016/79).

Milk samples were collected from 3- to 6-year-old cows in the 2nd lactation period [15] with subclinical mastitis, at a private Holstein dairy cattle farm in Istanbul. The mean milk yield of the animals were 24±3.4 L/day. They were kept in 10 m^2 barns per animal and milked by machine twice per day in milking parlors. The machine milking procedure applied in the private farm was consisted of these following steps: preparation of the staff, cleaning of teats with a pre-milking germicide dip solution, drying of teats, foremilk stripping, application of the machine, milking, detaching of the machine and finally post-milking teat germicide dipping [16]. The animals received a complete diet prepared according to their nutritional requirements by the Department of Animal Breeding. The study was

performed during autumn (October-December).

Mastitis screening was performed at a pre-visit (Dpre) to the farm by evaluating clinical examination findings and California Mastitis Test (CMT) results. Milk samples were aseptically collected from 210 mammary lobes with no clinical findings, pre-diagnosed with subclinical mastitis by evaluating CMT scores as +, ++, and +++. Subclinical mastitis diagnoses were confirmed in 187 samples having ≥200.000 cells/mL according to SSC results. Bacteriological analysis was performed on milk samples for isolation of microbial pathogens and antibiotic selection. Of these, 120 mammary lobes with bacterial growth were randomly selected to create study groups consisting of the Antibiotic Group (ABG, n = 40), the Platelet Concentration Group (PCG, n = 40), and the Combination Group (CG, n = 40) which was a combination of both treatment protocols, AB and PC. On the initial day of treatment (D0), two milk samples were taken from each mammary lobe following aseptic conditions. After the mammary lobes were completely emptied, depending on their assigned group, the applications, (intramammary antibiotics, intramammary platelet concentrate, or intramammary antibiotics + platelet concentrate) were performed. Intramammary antibiotic solutions were applied using commercial injectors (amoxicillin + clavulanic acid, Synulox LC, Pfizer). The platelet concentrate in a 5-mL sterile syringe was administered intramammary after the sterile teat catheter was advanced halfway into the teat [10]. The treatment was continued for 12 days at 3-day intervals and teat dipping was applied after each application. Milk sampling was repeated on the 7th, 14th, and 21st (D7, D14, D21) days of the study. SCC and MAA measurements were performed on the milk samples.

Milk Sample Collection

Before taking the milk samples, the mammary quarter was washed, cleaned with 70% alcohol, dried, and the first three milking streams were discarded. Milk samples were collected in sterile tubes under aseptic conditions, preserved cold and delivered to the laboratory within 2 h. Milk samples were collected for SCC measurement and microbiological analysis on Dpre and for SCC and MAA measurements on D0, D7, D14, and D21.

SCC Measurement

Somatic cell count measurements were performed on milk samples for the verification of subclinical mastitis diagnosis on Dpre and to evaluate treatment success on D0, D7, D14, and D21. SCC measurements were performed using a Fossomatic 90 cell counter (Foss Electric, Hillerod, Denmark) after heat treatment at 40°C for 15 min.

Bacteriological Analysis

Milk samples were double inoculated onto 5% sheep blood Columbia Agar, MacConkey agar, and Sabouraud dextrose agar. The inoculants were incubated at 37°C for 24-72 h under aerobic and microaerobic conditions. Morphology and colony characteristics formed at the end of incubation were examined and the samples with three or more types of colonies were evaluated as contaminated. Gram staining was performed on the colonies in the samples suitable for isolation and identification. The isolated microorganisms using pure culture were identified by classical methods [17].

Preparation of Platelet Concentrates

Platelet concentrates were prepared in the Accredited Blood Bank Laboratory of Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine.

To prepare allogeneic platelet concentrate, whole blood from *v. jugularis* of healthy, non-pregnant, non-lactating cows that received no antibiotic treatment in the last two months, was transferred with 16-gauge needles into 450 mL blood transfusion bags containing citrate-phosphate-dextrose-adenine (CPDA-1) [10,18]. Blood samples were taken to the laboratory within two hours under cold conditions. The blood bags were weighed on a precise balance (Precisa XT 6200C, Dietikon, Switzerland) and confirmed to be of equal weight. The blood bags were placed perpendicularly in a centrifuge (Beckman Coulter J6-M1, JS-4.2, California, USA) set at 4200g for 5 min at 22°C with acceleration and deceleration cycles set to 5 min. The blood bags were then carefully removed from the centrifuge, placed in a plasma extractor (Terumo Teruflex ACS201, Tokyo, Japan) from which platelet-rich plasma samples were transferred into 50 mL Falcon tubes. The second centrifugation for preparing the platelet concentrate was performed at 1500 g for 10 min at 4°C. Cell counts of prepared platelet concentrates were performed using a blood counting device (Abacus Junior Vet, Diatron, Budapest, Hungary) and platelet concentrates with the standard of 1×10^9 platelets/mL [10] were stored in 50 mL sterile Falcon tubes.

The prepared platelet concentrates were frozen three times at -80°C (Sanyo MDF-U2086S, Tokyo, Japan), and thawed at 37°C in a plasma heater (DH2 QuickThaw Plasma Thawing System, Helmer, Noblesville, IN, USA) to release platelet-derived factors, then stored at -20°C [10]. Various preparation stages of platelet concentrate are shown at *Fig. 1*.

MAA Analysis

Milk Amyloid A concentrations of D0, D7, D14, and D21 milk samples were determined using a commercial ELISA kit according to the manufacturer's instructions (Milk Amyloid A (MAA) ELISA Kit, Cat. No.: TP-807, Tridelta Development Ltd., Ireland). The sensitivity of the assay is 0.10 µg/mL, the intra-assay and inter-assay coefficient of variations are 6.62% and 9.99%, respectively. Optical densities were read on an automatic plate reader (BioTek EL×808 Absorbance Microplate Reader, USA) at 450 nm and reference 630 nm. MAA concentrations were

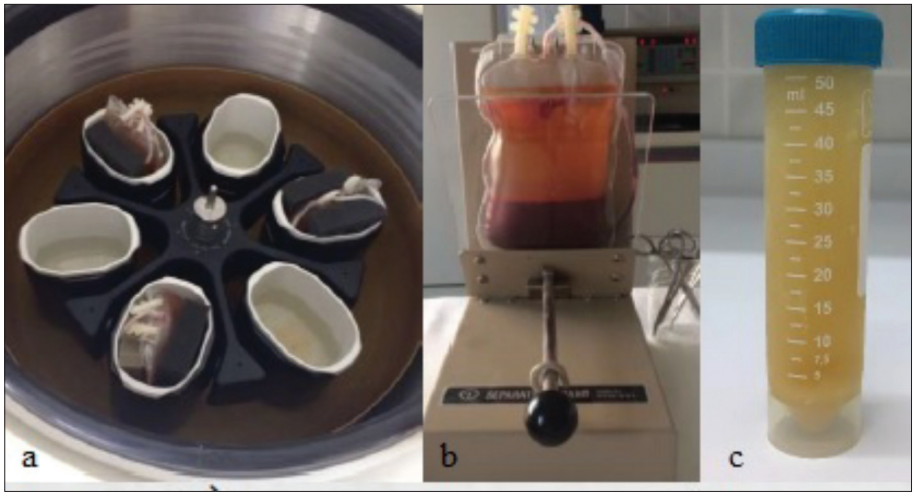


Fig 1. Platelet concentrate preparation. **a-** blood bags placed in the centrifuge; **b-** blood bags placed in the plasma extractor after the first centrifugation; **c-** platelet concentrate obtained after the second centrifugation stored in a Falcon tube

Table 1. *SCC measurement (×10³ cell/mL) per study groups according to sampling day*

Groups	Days				Significance
	D0 Mean±SE	D7 Mean±SE	D14 Mean±SE	D21 Mean±SE	
ABG	748.48±37.27^{Aa}	402.98±22.66^{Ba}	265.23±14.84^{C}	223.38±12.89^{C}	***
PCG	623.83±32.14^{ab}	350.45±19.33^{Bab}	256.05±13.19^{C}	213.68±8.68^{C}	***
CG	667.77±33.27^{ab}	318.75±19.69^{Bb}	228.08±9.23^{C}	206.70±7.45^{C}	***
Significance	*	*	NS	NS	

*SE: Standard Error; NS: Not Significant (P>0.05); * P<0.05; *** P<0.001*
^{a,b} Indicates the significance controls in the same column; ^{A,B,C} Indicates the significance controls in the same row

Table 2. *MAA measurement results of the study groups according to sampling day*

Groups	Days				Significance
	D0 Mean±SE (ng/mL)	D7 Mean±SE (ng/mL)	D14 Mean±SE (ng/mL)	D21 MEan±SE (ng/mL)	
ABG	6198.38±228.49^{Aa}	2851.28±238.70^{B}	1530.00±150.60^{C}	1113.85±109.88^{C}	***
PCG	4762.78±358.85^{Ab}	2935.13±292.56^{B}	1826.08±215.40^{C}	1344.68±160.68^{C}	***
CG	4999.83±309.64^{Ab}	2828.35±262.57^{B}	1497.75±134.88^{C}	1137.38±93.64^{C}	***
Significance	***	NS	NS	NS	

*SE: Standard Error; NS: Not Significant (P>0.05); * P<0.001*
^{a,b} Indicates the significance controls in the same column; ^{A,B,C} Indicates the significance controls in the same row

calculated based on a standard curve using references provided by the manufacturer. Samples were diluted 1:50 as mentioned in the manufacturer's instructions.

Statistical Analyses

Statistical analyses of the collected data were performed to evaluate the success and recurrence status of the intramammary treatments. The results of the MAA measurements were evaluated using a Kruskal-Wallis test, while SSC results were evaluated by one-way ANOVA. The significance control of the groups was tested by the Duncan method and the statistical software program SPSS 13.0 was used for the analyses.

RESULTS

Microbiological analysis of the milk samples revealed *Staphylococcus* sp. (71.5%), *Streptococcus* sp. (14.5%), *Escherichia coli* (3.5%), *Pasteurella* sp. (3.5%), *Corynebacterium* sp. (3.5%), and *Enterococcus* sp. (3.5%). In the study, for mammary lobes assigned to ABG and CG, an intramammary suspension (Synulox LC, Pfizer) containing amoxicillin + clavulanic acid was preferred as an antibiotic. The compatibility between the bacterial species that amoxicilin + clavulonic acid combination has bactericidal effect and the microbiological results obtained from our study was effective at our antibiotic selection.

Treatment groups were formed with 120 mammary lobes selected from the milk samples with SCC over 200×10^3 cells/mL on Dpre. The SCC measurement results of the collected milk samples at the beginning of the treatment and at the follow-up days are detailed in *Table 1*.

The MAA measurement results of the collected milk samples at the beginning of the treatment and on the follow-up days are detailed in *Table 2*.

In the milk samples taken from ABG mammary lobes, SCC, which was 748.48±37.27 × 10^3 cells/mL before treatment, decreased to 402.98±22.66 × 10^3 cells/mL on D7 as a result of intramammary antibiotic administration (P<0.001). Also, MAA measurements for the same days decreased from 6198.38±228.49 ng/mL to 2851.28±238.70 ng/mL (P<0.001). SCC and MAA measurements of milk samples taken on the 14th and 21st days after the beginning of treatment to evaluate the recurrence status of disease were similar to each other and significantly lower than those obtained on D0 and D7 (P<0.001).

For the PCG samples, SCC measurements and MAA concentrations at D0 and D7 significantly decreased from 623.83±32.14 x 10^3 cells/mL to 350.45±19.33 x 10^3 cells/mL (P<0.001), and from 4762.78±358.85 ng/mL to 2935.13±292.56 ng/mL (P<0.001), respectively. SCC and MAA measurements for D14 and D21 were similar to each other but significantly lower than those obtained on D0 and D7 (P<0.001).

Similar results were obtained for CG samples as those for the other treatment protocols. SCC which was determined to be 667.77±33.27 × 10^3 cells/mL on D0 decreased to 318.75±19.69 × 10^3 cells/mL (P<0.001) on D7. The MAA concentration also decreased from 4999.83±309.64 ng/mL to 2828.35±262.57 ng/mL (P<0.001). SCC and MAA measurements on D14 and D21 which evaluated the recurrence status with the combined treatment were similar to each other and lower than those on D0 and D7 (P<0.001).

DISCUSSION

This study was planned considering the need for new alternative approaches in the treatment of sub-clinical mastitis. The efficacy of intramammary platelet concentrate treatment was evaluated through SCC and MAA measurements. According to the current literature, although it is recognized that the application of intra-mammary platelet concentrate provides a very new perspective in the treatment of clinical mastitis [10], no studies were found on animals with subclinical mastitis. On the other hand, the presence of studies examining methods such as the application of various fruit and plant extracts [8,19-21], photodynamic therapy [22], and nitric oxide-releasing solutions [23] for subclinical mastitis treatment indicates the search for alternative methods to antibiotic use worldwide. The lack of new information on mastitis treatment by platelet concentrate applications shows the original value of this study.

The causes of mastitis are divided into two as environmental or contagious microorganisms. *Klebsiella* sp., *E. coli*, and Streptococci are the most frequently isolated environmental factors. *Staphylococcus aureus* and *Str. agalactiae* are the most frequently isolated contagious microorganisms [4,5]. In our study, the most detected species was *Staphylococcus* sp., (71.5%) followed by *Streptococcus* sp. (14.5%). The fact that the majority of the identified microorganisms were contagious factors suggests that the disease may spread due to inadequate sanitation.

Intramammary antibiotics are used as a routine protocol to treat mastitis and to prevent the spread of infectious factors causing this disease [1,5,6,8]. Blood products containing high levels of platelets are thought to exhibit antibiotic action by secreting antimicrobial peptides as well as inducing cell regeneration by stimulating cell proliferation, angiogenesis, and cell migration [10,24]. In an *in vitro* study on the antimicrobial efficacy of human platelet-rich gelatin, it was shown that this application was highly effective on various microorganisms, especially *S. aureus* [25]. In addition, anti-inflammatory and analgesic effects have also been reported [24]. In a study by Lange-Consiglio et al.[10], where the results were evaluated according to SCC, combined treatment in clinical mastitis was reported to be more successful than sole antibiotic or sole platelet concentrate applications, and it was stated that platelet concentrate application was as effective as intramammary antibiotic administration. According to the data obtained from the present study, all three protocols, antibiotic, platelet concentrate, and combined treatment were found to be successful in the treatment of subclinical mastitis. In all three application groups, a decrease of nearly half was detected between pre-treatment (D0) and post-treatment SCC measurements (D7) (P<0.001), and this decrease continued at other measurement days. Although SCC did not decrease below 200 × 10^3 cells/mL, it was determined to be very close to this value. This decrease in SCC since the beginning of the treatment was evaluated as recovery. Examining the recurrence status of the chronic cases in the study mentioned previously, sole platelet concentrate application was more successful than other treatment applications [10]. In our study, the three treatment protocols were also found to be successful in terms of controlling subclinical mastitis recurrence. This new treatment protocol may easily replace antibiotic usage considering the stages of platelet concentrate preparation. It is a simple, uncomplicated and economically affordable method. It can be performed in the office setting of the farm with very limited consumable material supply when the infrastructure is created as well as the laboratory. However, attention must be paid to work sterile. Lysing or damaging platelets should be avoided during process.

Considering that mastitis cases are mostly subclinical [7], it is obvious that the detection of this disease, which can spread to the herd without being noticed, is the most important step. Accordingly, many parameters can be evaluated including CMT; SCC measurements; factor isolation and identification; evaluation of electrical conductivity, density, freezing point and mineral percentage of milk [26,27]. Among these parameters, the one most frequently evaluated is SCC. However, it should be remembered that SCC may vary depending on the species of microbial agent, lactation period and the number of lactations, age and breed of the cow, milk yield, milking frequency, season and the geographical region that the herd is located, as well as non-inflammatory factors [28]. According to the results of a study by Risvanlı and Kalkan [29], there was no strong correlation between age and breed of cows and SCC values in subclinical mastitis. In the present study, factors affecting SCC were not evaluated. However, since animals were kept at the same farm, being the same breed, of a similar age range and lactation period, all milked twice a day, and not affected by any disease other than subclinical mastitis, we decided that SCC results were minimally affected by other factors and accurately reflect subclinical mastitis levels.

In recent years, studies on acute phase protein measurements that give more precise results than SCC for subclinical mastitis diagnosis and follow-up have become widespread. In the present study, MAA, as well as SCC, were preferred for evaluating the success of subclinical mastitis treatment protocols and recurrence status. In a study in which amyloid A was evaluated by a serum ELISA test kit in serum samples and by both serum and milk ELISA test kits in milk samples, it was determined that the most sensitive kit for subclinical mastitis diagnosis was the milk MAA ELISA test kit [30]. In a study on the relationship between subclinical mastitis pathogens and MAA measurements, the lower limit value was 3.9 µg/mL for major pathogens and 1.6 µg/mL for all other pathogens [31]. The MAA data obtained from the present study were consistent with the published literature data and MAA concentrations of AB, PCG, and CG before treatments were 6198.38±228.49 ng/mL, 762.78±358.85 ng/mL, and 999.83±309.64 ng/mL, respectively. Statistically significant reductions were determined in MAA concentrations after treatment in all three study groups ($P<0.001$). MAA concentrations continued to decrease at D14 and D21. D14 and D21 measurements of each group were similar ($P>0.05$) and significantly lower than D0 and D7 ($P<0.001$). All three treatment protocols showed improvement and no recurrence was determined during the 21-day period from the start of treatment.

Examining the relationship between D0 measurement results of all treatment groups, it was seen that the ABG had the highest SCC and MAA results ($P<0.05$ and $P<0.001$, respectively). As a result of this coincidental outcome, which was due to the randomization of the groups, it was decided that the SCC and MAA measurements actually showed a similar pattern in detecting mammary tissue inflammation and also MAA measurements yielded much more sensitive results. Since SCC and MAA measurements are both indicators of healing from mastitis, bacteriological examination was not repeated in this study.

Platelet concentrate heals the mammary tissue by peptide growth factors which lead to tissue protection and/or repair processes through stimulation of glandular tissue regeneration. Additionally, these growth factors increase the infiltration of neutrophils and macrophages to fight against the microorganisms related to mastitis [11,32]. In case of complete healing from mastitis and the lack of recurrence, the factors secreted after inflammation and platelet concentrate administration are expected to be decreased. However, SCC and MAA measurements were the target parameters for treatment evaluation in this study. Nevertheless, repeating the microbiological examinations are considered to be useful for future correspondence.

In mastitis cases, the migration of polymorphonuclear neutrophils (PMNs) from blood to mammary glands is triggered following the invasion of the pathogenic factor into the mammary gland. Activated PMNs, while destroying pathogens, can cause tissue damage as a result of producing reactive oxygen metabolites and granular enzyme release, resulting in the disruption of mammary function. Antibiotics frequently used for the treatment of mastitis can not protect the mammary gland from damage [2]. Therefore, it is understood that damage caused at mammary epithelial cells can not be repaired by using antibiotics, but it is possible to repair damage due to platelet concentrate, which is rich in cytokines, chemokines, and various growth factors. The authors of this study suggest that, although there are no visible pathological changes in subclinical mastitis, changes in the cellular basis that occur during treatment with intramammary platelet concentrate after the invasion of the mammary gland by pathogenic factors can be studied with invasive methods. Reexamination of bacteriological evaluation is considered to be needed to detect the treatment efficiency of platelet concentrate with histopathological examinations for future correspondence.

Results of the present study suggest that intramammary platelet concentrate may be an alternative to antibiotic use in the treatment of subclinical mastitis. It is predicted that the risk of antibiotic residues can be reduced in nature as in the milk offered for human consumption, and the resistance of microorganisms to antibiotics can be partially prevented. In addition, this method can become a treatment protocol for management systems that produce organic milk and dairy products by organic livestock farming, which have become increasingly popular in recent years.

Acknowledgments

This study was funded by Istanbul University with the project number 24040.

Conflict of Interest

There is no conflict of interest in the present study.

REFERENCES

1. De Vliegher S, Fox LK, Piepers S, McDougall S, Barkema HW: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention, and control. *J Dairy Sci*, 95, 1025-1040, 2012. DOI: 10.3168/jds.2010-4074

2. Zhao X, Lacasse P: Mammary tissue damage during bovine mastitis: Causes and control. *J Anim Sci*, 86 (Suppl. 13): 57-65, 2008. DOI: 10.2527/jas.2007-0302

3. Sharma N, Jeong DK: Stem cell research: A novel boulervard towards improved bovine mastitis management. *Int J Biol Sci*, 9 (8): 818-829, 2013. DOI: 10.7150/ijbs.6901

4. Thompson-Crispi K, Atalla H, Miglior F, Mallard BA: Bovine mastitis: Frontiers in immunogenetics. *Front Immunol*, 5:493, 2014 DOI: 10.3389/fimmu.2014.00493

5. Gomes F, Henriques M: Control of bovine mastitis: Old and recent therapeutic approaches. *Curr Microbiol*, 72, 377-382, 2016. DOI: 10.1007/s00284-015-0958-8

6. Lago A, Godden SM, Bey R, Ruegg PL, Leslie K: The selective treatment of clinical mastitis based on on-farm culture results: I. Effects on antibiotic use, milk withholding time, and short-term clinical and bacteriological outcomes. *J Dairy Sci*, 94, 4441-4456, 2011. DOI: 10.3168/jds.2010-4046

7. Abay M, Bekyürek T: Laktasyondaki sütçü ineklerde Staphylococcus aureus'un neden olduğu subklinik mastitislerin tedavisinde sefkuinom ve amoksisilin + kalvulanik asit'in etkinliklerinin karşılaştırılması. *J Health Sci*, 15 (3): 189-193, 2006.

8. Kuru M, Oral H: Mastitis tedavisinde fitoterapi ve homeopatinin kullanımı. *Harran Üniv Vet Fak Derg*, 2 (2): 112-116, 2013.

9. Yung YL, Fu SC, Cheuk YC, Qin L, Ong MTY, Chan KM, Yung PSH: Optimisation of platelet concentrates therapy: Composition, localisation, and duration of action. *Asia Pac J Sports Med Arthrosc Rehabil Technol*, 7, 27-36, 2017. DOI: 10.1016/j.asmart.2016.11.003

10. Lange-Consiglio A, Spelta C, Garlappi R, Luini M, Cremonesi F: Intramammary administration of platelet concentrate as an unconventional therapy in bovine mastitis: First clinical application. *J Dairy Sci*, 97, 6223-6230, 2014. DOI: 10.3168/jds.2014-7999

11. Anitua E, Andia I, Ardanza B, Nurden P, Nurden AT: Autologous platelets as a source of proteins for healing and tissue regeneration. *Thromb Haemost*, 91, 4-15, 2004. DOI: 10.1160/TH03-07-0440

12. Derynck R: The physiology of transforming growth factor-alpha. *Adv Cancer Res*, 58, 27-52, 1992. DOI: 10.1016/S0065-230X(08)60289-4

13. Anwer AM, Asfour HAE, Gamal IM: Apoptosis in somatic cells and immunological bioactive parameters of cow's milk and their relation to subclinical mastitis. *Alexandria J Vet Sci*, 49 (2): 31-41, 2016. DOI: 10.5455/ajvs.209718

14. Kovac G, Tothova C, Nagy O, Seidel H: Milk amyloid A and selected serum proteins in cows suffering from mastitis. *Acta Vet Brno*, 80, 3-9, 2011. DOI: 10.2754/avb201180010003

15. Polat Ü, Çetin M: The changes in some biochemical blood parameters during various lactation stages and dry period in dairy cows. *J Fac Vet Med*, 20, 33-39, 2001.

16. Baştan A: Meme başının dezenfeksiyonu. **In,** İneklerde Meme Sağlığı ve Sorunları. 272-280, Ankara Kardelen Ofset Matbaacılık Tanıtım Hizmetleri San. Tic. Ltd. Şti, Ankara, Türkiye, 2010.

17. Quinn PJ, Markey BK, Leonard FC, Hartigan P, Fanning S, Fitz Patrick ES: Veterinary Microbiology and Microbial Disease. 2nd ed., 1-928, Wiley-Blackwell, John Wiley & Sons, New Jersey, USA, 2002.

18. Bell G: Blood transfusion in cattle. *Livestock*, 11 (3): 39-43, 2006. DOI: 10.1111/j.2044-3870.2006.tb00024.x

19. Khan A, Ahmed T, Rizwan M, Khan N: Comparative therapeutic efficacy of *Phyllanthus emblica* (Amla) fruit extract and procaine penicillin in the treatment of subclinical mastitis in dairy buffaloes. *Microb Pathog*, 115, 8-11, 2018. DOI: 10.1016/j.micpath.2017.12.038

20. Mushtag S, Shah AM, Shah A, Lone SA, Hussain A, Hassan QP, Ali MN: Bovine mastitis: An appraisal of its alternative herbal cure. *Microb Pathog*, 114, 357-361, 2018. DOI: 10.1016/j.micpath.2017.12.024

21. Oral H, Çolak A, Polat B, Cengiz M, Cengiz S, Baştan A, Kaya S: Sütçü ineklerde subklinik mastitis tedavisinde aloe vera kullanımının etkinliği. *Erciyes Üniv Vet Fak Derg*, 11 (3): 157-161, 2014.

22. Moreira LH, Pereira de Souza JC, José de Lima C, Salgado MAC, Fernandes AB, Andreani DIK, Villaverde AB, Zângaro RA: Use of photodynamic therapy in the treatment of bovine subclinical mastitis. *Photodiagnosis Photodyn Ther*, 21, 246-251, 2018. DOI: 10.1016/j.pdpdt.2017.12.009

23. Regev G, Martins J, Sheridan MP, Leemhuis J, Thompson J, Miller C: Feasibility and preliminary safety of nitric oxide releasing solution as a treatment for bovine mastitis. *Res Vet Sci*, 118, 247-253, 2018. DOI: 10.1016/j.rvsc.2018.02.009

24. Amable PR, Carias RBV, Teixeira MVT, Pacheco IC, Amaral RJFC, Granjeiro JM, Borojevic R: Platelet-rich plasma preparation for regenerative medicine: optimization and quantification of cytokines and growth factors. *Stem Cell Res Ther*, 4:67, 2013. DOI: 10.1186/scrt218

25. Bielecki TM, Gazdzik TS, Arendt J, Szczepanski T, Krol W, Wielkoszynski T: Antibacterial effect of autologous platelet gel enriched with growth factors and other active substances. *J Bone Joint Surg*, 89-B, 417-420, 2007.

26. Gürbulak K, Canoğlu E, Abay M, Atabay Ö, Bekyürek T: İneklerde subklinik mastitisin farklı yöntemlerle saptanması. *Kafkas Univ Vet Fak Derg*, 15 (5): 765-770, 2009.

27. Kaşıkçı G, Çetin Ö, Bingöl EB, Gündüz MC: Relations between electrical conductivity, somatic cell count, California Mastitis Test and some quality parameters in the diagnosis of subclinical mastitis in dairy cows. *Turk J Vet Anim Sci*, 36 (1): 49-55, 2012. DOI: 10.3906/vet-1103-4

28. Darbaz İ, Ergene O: Sürü meme sağlığı yönetiminde somatik hücre sayısının önemi. *Erciyes Üniv Vet Fak Derg*, 12 (3): 203-210, 2015.

29. Rişvanlı A, Kalkan C: Sütçü ineklerde yaş ve ırkın subklinik mastitisli memelerin sütlerindeki somatik hücre sayıları ile mikrobiyolojik izolasyon oranlarına etkisi. *Y Y Ü Vet Fak Derg*, 13 (1-2): 84-87, 2002.

30. Gerardi G, Bernardini D, Elia CA, Ferrari V, Iob L, Segato S: Use of serum amyloid A and milk amyloid A in the diagnosis of subclinical mastitis in dairy cows. *J Dairy Res*, 76, 411-417, 2009. DOI: 10.1017/S0022029909990057

31. Jaeger S, Virchow F, Torgerson PR, Bischoff M, Biner B, Harnack S, Rüegg SR: Test characteristics of milk amyloid A ELISA, somatic cell count, and bacteriological culture for detection of intramammary pathogens that cause subclinical mastitis. *J Dairy Sci*, 100, 7419-7426, 2017. DOI: 10.3168/jds.2016-12446

32. Sheffield LG: Mastitis increases growth factor messenger ribonucleic acid in bovine mammary glands. *J Dairy Sci*, 80, 2020-2024, 1997. DOI: 10.3168/jds.S0022-0302(97)76146-0

Kafkas Univ Vet Fak Derg
25 (3): 365-372, 2019
DOI: 10.9775/kvfd.2018.21007

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

The Efficacy of Conjunctiva Coverage in Combination with Amnion Liquid Supernatant Eye Drop on Deep Layer Corneal Ulcer in Canine Caused by Alkali Burn Combined with Mechanical Injury

Jiasan ZHENG [1] Renyue WEI [1] Jiaren ZHANG [1] Zheng WANG [1]
Tingting ZHU [1] Hongri RUAN [1] Jun SONG [1]

[1] College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, 163319, CHINA

Article Code: KVFD-2018-21007 Received: 20.09.2018 Accepted: 30.12.2018 Published Online: 30.12.2018

How to Cite This Article

Zheng J, Wei R, Zhang J, Wang Z, Zhu T, Ruan H, Song J: The efficacy of conjunctiva coverage in combination with amnion liquid supernatant eye drop on deep layer corneal ulcer in canine caused by alkali burn combined with mechanical injury. *Kafkas Univ Vet Fak Derg,* 25 (3): 365-372, 2019. DOI: 10.9775/kvfd.2018.21007

Abstract

The purpose of this study was to establish the model of canine deep layer corneal ulcer by means of alkali burn combined with mechanical injury and evaluate the efficacy of conjunctival covering combined with amnion liquid supernatant deep layer corneal ulcer in beagles. By using alkali burnand mechanical injury establishd the beagle deep layer corneal ulcer model (n=15) , randomly divided into 3 groups: deep layer corneal ulcer (C group), conjunctival covering group (T1 group), conjunctival covering combined amnion liquid supernatant eyedrop group (T2 group). On -3, 1, 7, 14, 21, 45 days, examine the levels of Interleukin -1 (IL-1), Interleukin-8 (IL-8), Vascular Endothelial Growth Factor (VEGF), Matrix metalloproteinase-1 (MMP-1), Matrix metalloproteinase-2 (MMP-2) and Matrix metalloproteinase-9 (MMP-9) in canine aqueous humor; On the 45th day, two experimental canines were randomly selected from each group to collect corneal tissue for histopathological observation. C group compared to groups T1 group and T2 group , the levels of IL-1, IL-8, VEGF, MMP-1, MMP-2, MMP-9 in canine aqueous humor significantly decreased ($P<0.05$). Histopathology revealed that in the T2 group corneal epithelial blood vessels were less, corneal thickness was moderate, the cornea was more complete, the cornea had fewer blood vessels, and the inflammatory cell infiltration was lower. Conjunctival coverage combined with amnion liquid supernatant eyedrop can effectively reduce keratitis cell infiltration of canine deep layer corneal ulcer, reduce corneal damage, and improve corneal transparency, the therapeutic effect is better than conjunctival coverage individually.

Keywords: *Canine, Alkali burn combined with mechanical injury, Amnion liquid supernatant, Conjunctival cover, Corneal ulcer*

Köpeklerde Mekanik Hasar ve Alkali Yakma İle Oluşturulan Derin Korneal Ülserde Amniyon Sıvısı Süpernatantı Göz Damlası İle Birlikte Kullanılan Konjuktiva Örtüsünün Etkinliği

Öz

Bu çalışmanın amacı, alkali yakma ile birlikte mekanik hasar oluşturularak Beagle köpeklerde derin korneal ülser meydana getirmek suretiyle bir model oluşturmak ve oluşturulan bu derin korneal ülsere amniyon sıvısı süpernatantı ile birlikte korneal örtmenin etkisini araştırmaktır. Alkali yakma ve mekanik hasar ile derin korneal ülser oluşturmak amacıyla Beagle köpekler (n=15) rastgele olarak 3 gruba ayrıldı: Derin korneal ülser grubu (C grubu), konjuktival örtü grubu (T1 grubu) ve amniyon sıvısı süpernatantı göz damlası ile birlikte konjuktival örtü grubu (T2 grubu). Çalışmanın -3, 1, 7, 14, 21 ve 45. günlerinde, köpeklerin akuöz humorlarında İnterlökin-1 (IL-1), İnterlökin-8 (IL-8), Vasküler Endotelyal Büyüme Faktörü (VEGF), Matriks metalloproteinaz-1 (MMP-1), Matriks metalloproteinaz-2 (MMP-2) ve Matriks metalloproteinaz-9 (MMP-9) seviyeleri incelendi. Çalışmanın 45. gününde, her bir gruptan rastgele iki köpek seçilerek histopatolojik inceleme amacıyla korneal dokuları elde edildi. T1 ve T2 grubu ile karşılaştırıldığında C grubu köpek akuöz humorlarında IL-1, IL-8, VEGF, MMP-1, MMP-2, MMP-9 seviyelerinin anlamlı derecede azaldığı gözlemlendi ($P<0.05$). Histopatolojik incelemede, T2 grubundaki hayvanlarda korneal epitelyal kan damarları daha az, korneal kalınlık orta derecede, kornea daha fazla tamam, korneada daha az kan damarı ile daha az yangısal hücre infiltrasyonu gözlemlendi. Amniyon sıvısı süpernatantı göz damlası ile birlikte konjuktival örtü uygulaması köpeklerde derin korneal ülsere ilişkin keratite bağplı hücre infiltrasyonunu ve korneal hasarı etkili bir şekilde azaltmış ve korneal transparanlığı artırmıştır. Bu bulgular, Amniyon sıvısı süpernatantı göz damlası ile birlikte konjuktival örtü uygulamasının tek başına konjuktival örtü uygulamasından daha iyi tedavi edici etkisinin olduğunu göstermiştir..

Anahtar sözcükler: *Köpek, Alkali yakma ile birlikte mekanik hasar, Amniyon sıvısı süpernatantı, Konjuktival örtü, Korneal ülser*

İletişim (Correspondence)
+86 1577 6501082
songjun_2005@126.com

INTRODUCTION

Deep corneal ulcers are severe corneal diseases caused by microbial infection, chemical burns, or trauma [1,2]. In the absence of timely and effective treatment, it is possible to cause blindness in corneal perforation [3]. The purpose of this study was to establish a model of deep corneal ulcer in canine by alkali burn combined with mechanical injury. Conjunctival coverage combined with supernatant of canine amnion was used to treat deep corneal ulcer in canine. We evaluate the clinical efficacy of treatment. This experiment provides reliable data support and new treatment ideas for deep corneal ulcer in canine.

MATERIAL and METHODS

Ethics

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC). All beagle experimental procedures were performed in accordance with the regulations for the Administration of Affairs Concerning Experimental Animals approved by the school Council of Heilongjiang Bayi Agricultural University of China Daqing. The study protocol was approved by the Ethics Committee on the Use and Care of Animals of Heilongjiang Bayi Agricultural University (Daqing, China).

Preparation of Clear Liquid on Amniotic Membrane

Fresh sterile amniotic membrane was extracted by caesarean section in healthy immunized female dogs that reached the expected delivery date. Add appropriate amount of liquid nitrogen to the aseptic fresh amniotic membrane and grind it quickly until it becomes powdery. Powder was added into the centrifugal tube, and PBS phosphate buffer was added in a 1:1 mass ratio for dilution. On the shaking table full range 20 min, put in 4°C high speed centrifuge, 8000 r/min, the centrifugal 10 min, remove the liquid supernatant and set aside.

Establishment of Experimental Animal Model

Choose healthy beagles (n=15), weight 4-5 kg, aging1-2 years old. The model of deep corneal ulcer was established by alkali burn combined with mechanical injury. General anesthesia was performed on the experimental dogs, Bupivacaine was applied to the eyes of experimental dogs, and then sterilization filter paper was used to dry the eye surface. The circular filter paper with a diameter of 6 mm was fully infiltrated in the NaOH solution. The filter paper was attached to the corneal surface for 90 s, and then the filter paper was removed. Then the normal saline was used to rinse thoroughly until the pH value of the ocular surface was about 7.0. Negative pressure corneal trephine was used to cut the cornea of alkali burns with a cutting depth of 0.4 mm and a cutting diameter of 6 mm. After cutting, the cornea was peeled off and then washed with physiological saline to complete the modeling. After modeling, all experimental animals wore Elizabeth collars to prevent scratching and biting.

Grouping of Experimental Animals

The successful model beagles were randomly divided into three groups. Deep corneal ulcer group (C group, without surgical treatment, gatifloxacin eye drops were given 2 drops/time and 5 times/day), Conjunctiva coverage group (T1 group, only with gatifloxacin eye drop after conjunctiva coverage, 2 drops/time and 5 times/day), The conjunctiva coverage in combination with amnion liquid supernatant group (T2 group, after the conjunctiva coverage was given to Gatifloxacin Eye Drop 2 drops/time and 5 times/day and amnion liquid supernatant for 2 drops/time and 5 times/day). During the experiment, all experimental animals were given butorphanol (0.02 mg/kg) analgesic management.

Sample Collection

Three days before the experiment, 1, 7, 14, 21, 45 days after the beginning of the experiment, Bupivacaine was applied twice to the eyes of experimental dogs, and 10% iodov solution was used to disinfect the skin and conjunctival sac of eyelid of experimental dogs. Use your left hand to hold the upper and lower eyelids open, the assistant illuminates with a hand-held light source, 1 mL syringe needle was used for anterior chamber puncture. The puncture position was 1 mm inside the Angle sclera margin, and the needle was inserted in the direction of parallel iris, and the tip inclined plane was upward. After the tip enters the anterior chamber, collect the aqueous humor 0.2-0.3 mL. Use 10 times dilution sample diluent, in -80°C refrigerators save, waiting for inspection.

Corneal tissue samples were collected 45 days after treatment. Experimental dogs general anesthesia, after conventional disinfection of the surgery department, eyelid opener was used to expand the eyelids. Corneal tissue was collected with a corneal knife and placed in a solution of 10% formaldehyde. Routine care was given to experimental animals after surgery.

Aqueous Humor Testing Indicators

Interleukin-1, IL-8, VEGF, MMP-1, MMP-2 and MMP-9 were tested by ELISA kit. All aqueous humor samples and reagents were placed in the room for complete temperature recovery. First, test samples and standard substances of different concentrations were added to the orifice plate. Horseradish peroxidase was added into each hole and incubated in a incubator for 60 min. Get rid of the orifice plate liquid, rinse thoroughly with cleaning fluid, then add substrates A and B, 37°C avoid light incubation 15 min. Finally, add the termination fluid. The absorbance (OD) value in each hole was measured by enzyme-labeled instrument, and the standard curve

was drawn. Calculate the content of each sample.

Corneal Histopathological Examination Results

The corneal tissue was placed in 10% formaldehyde for 24 h. dehydration treatment, paraffin embedding, 4 μm thick continuous slices, HE staining and observation with an optical microscope.

Data Analysis

SPSS 19.0 software was used for statistical analysis of the test data using one-way anova.

RESULTS

The content of VEGF was very significant differences between T1 group and T2 group in 7 days ($P<0.01$) and it is significant differences between T1 group and T2 group in 14 and 21 days ($P<0.05$). The content of VEGF was very significant differences between T2 group and group C in 7, 14, 21, 45 days ($P<0.01$). On the 14th day after treatment, VEGF content in the aqueous humor of both the experimental group and the C group reached the maximum at the same time. The maximum values of C group were 673.3914±12.4211 pg/mL, the maximum values of T1 group was 462.3956±5.6017 pg/mL and the maximum values of T2 group was 446.925±3.4829 pg/mL. The specific test results are shown in *Fig. 1*.

The content of IL-1 in aqueous humor was very significant differences between T1 group and T2 group in 7 days and 14 days ($P<0.01$) and it is significant differences between T1 group and T2 group in 21 days ($P<0.05$). There was no significant difference at other time points ($P>0.05$). The content of IL-1 in aqueous humor was very significant differences between T2 group and group C in 7, 14, 21, 45 days ($P<0.01$). IL-1 content in canine eye aqueous humor in the experimental group reached the maximum on the 7th day after treatment. The maximum values of T1 group were 166.1077±4.1936 pg/mL, the maximum values of T1 group were 146.8177±2.1139 pg/mL. IL-1 content in canine eye aqueous humor in the C group reached the maximum on the 14th day after treatment. The maximum values of C group were 193.2093±4.2624 pg/mL. The specific test results are shown in *Fig. 2*.

The content of IL-8 in aqueous humor was significant differences between T1 group and T2 group in 7, 14, 45 days ($P<0.05$). The content of IL-8 in aqueous humor was very significant differences between T2 group and group C in 7, 14, 21, 45 days ($P<0.01$). IL-8 content in the aqueous humor of canine eyes in both the experimental group and the C group reached the maximum on the 7th day after treatment. The maximum values of C group were 168.1898±4.8832 pg/mL, the maximum values of T1 group were 145.6043±6.9070 pg/mL, the maximum values of T2 group were 137.4154±4.3391 pg/mL. The specific test results are shown in *Fig. 3*.

The content of MMP-1 in aqueous humor was very significant differences between T1 group and T2 group in 7, 14 days ($P<0.01$) and it is significant differences between T1 group and T2 group in 21, 45 days ($P<0.05$). The content of MMP-1 in aqueous humor was very significant differences between T2 group and group C in 7, 14, 21, 45 days ($P<0.01$). MMP-1 content in the aqueous humor of canine eyes in both the experimental group and the C group reached the maximum on the 7th day after treatment. The maximum values of C group were 115.8756±4.2794 ng/mL, the maximum values of T1 group were 104.6135±3.8075 ng/mL, the maximum values of T2 group were 92.0798±2.0476 ng/mL. The specific test results are shown in *Fig. 4*.

The content of MMP-2 in aqueous humor was significant differences between T1 group and T2 group in 7, 14 days ($P<0.05$). There was no significant difference at other time points ($P>0.05$). The content of MMP-2 in aqueous humor

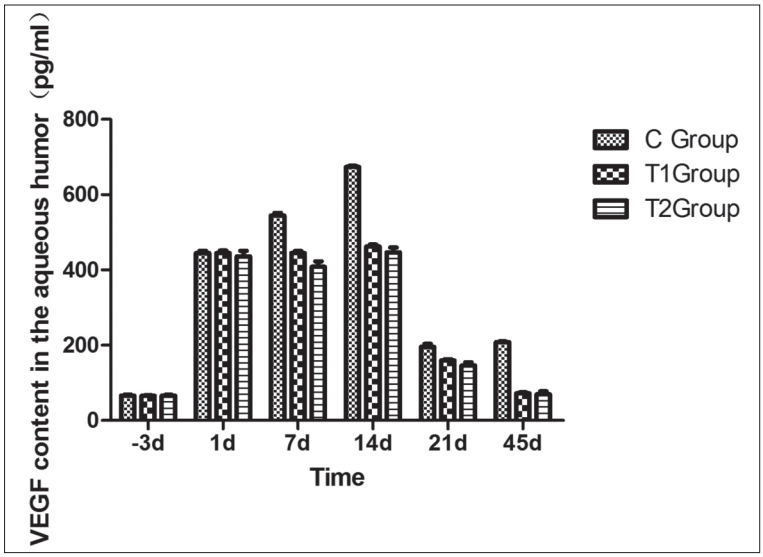


Fig 1. The content of VEGF was very significant differences between T1 group and T2 group in 7 days *(P<0.01)* and it is significant differences between T1 group and T2 group in 14 and 21 days *(P<0.05)*. The content of VEGF was very significant differences between T2 group and group C in 7, 14, 21, 45 days *(P<0.01)*

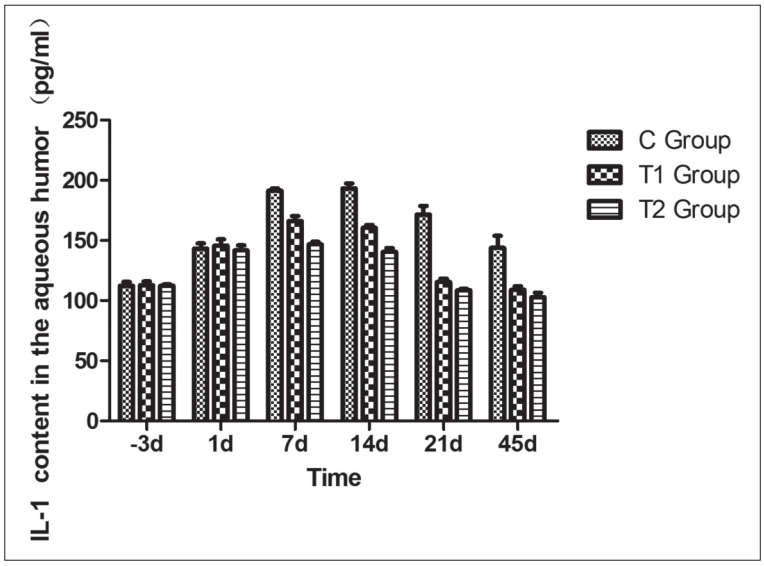
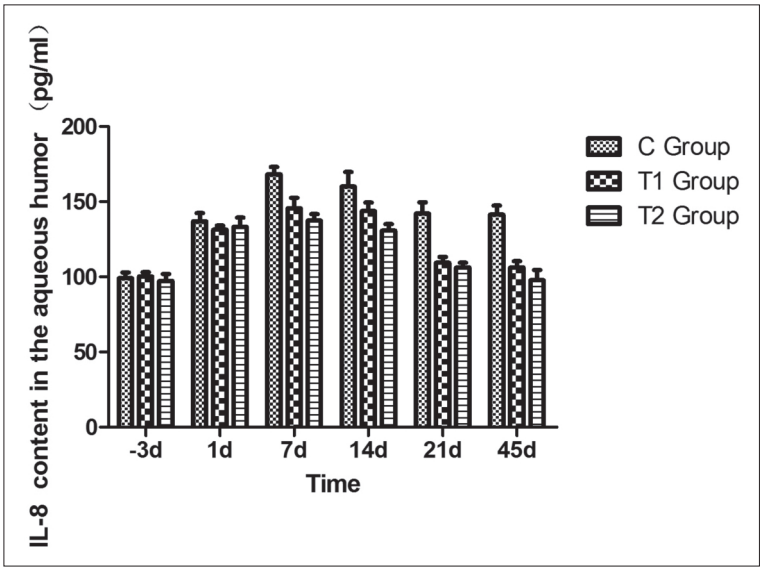


Fig 2. the content of IL-1 in aqueous humor was very significant differences between T1 group and T2 group in 7 days and 14 days *(P<0.01)* and it is significant differences between T1 group and T2 group in 21 days *(P<0.05)*. There was no significant difference at other time points *(P>0.05)*. The content of IL-1 in aqueous humor was very significant differences between T2 group and group C in 7, 14, 21, 45 days *(P<0.01*)

Fig 3. The content of IL-8 in aqueous humor was significant differences between T1 group and T2 group in 7, 14, 45 days *(P<0.05)*. The content of IL-8 in aqueous humor was very significant differences between T2 group and group C in 7, 14, 21, 45 days *(P<0.01)*



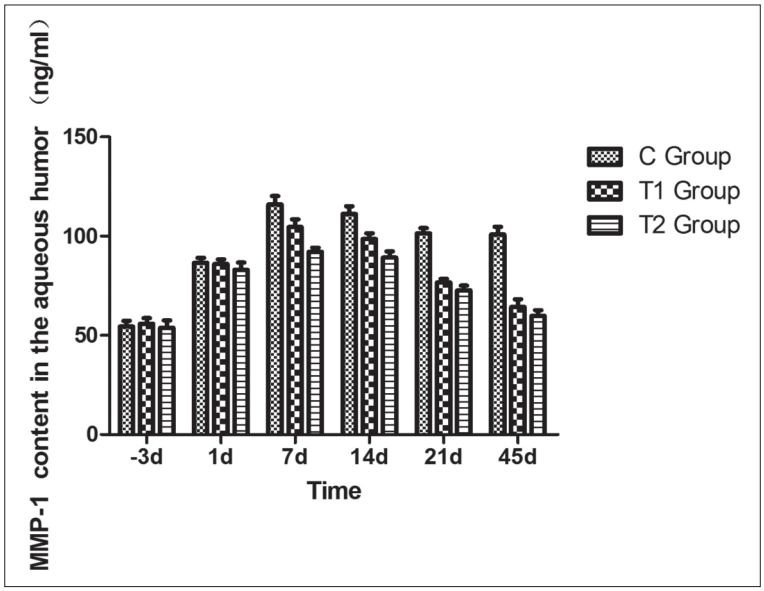


Fig 4. The content of MMP-1 in aqueous humor was very significant differences between T1 group and T2 group in 7, 14 days *(P<0.01)* and it is significant differences between T1 group and T2 group in 21, 45 days *(P<0.05)*. The content of MMP-1 in aqueous humor was very significant differences between T2 group and group C in 7, 14, 21, 45 days *(P<0.01)*

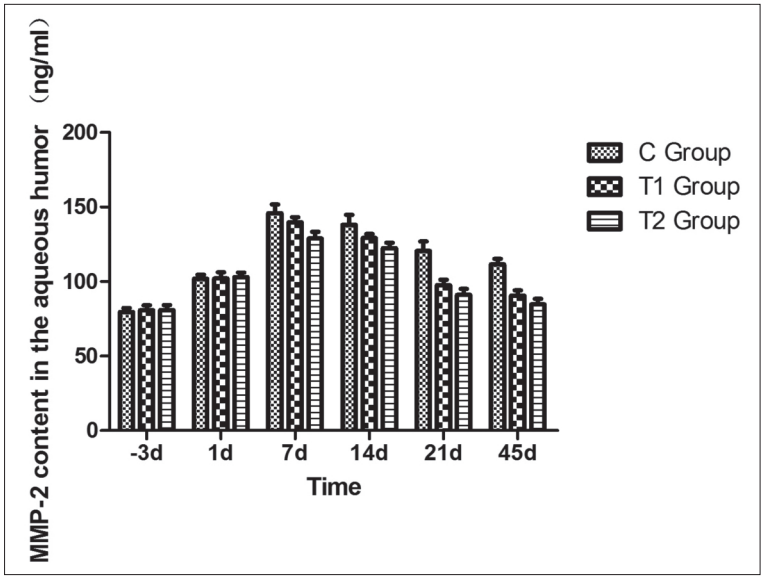
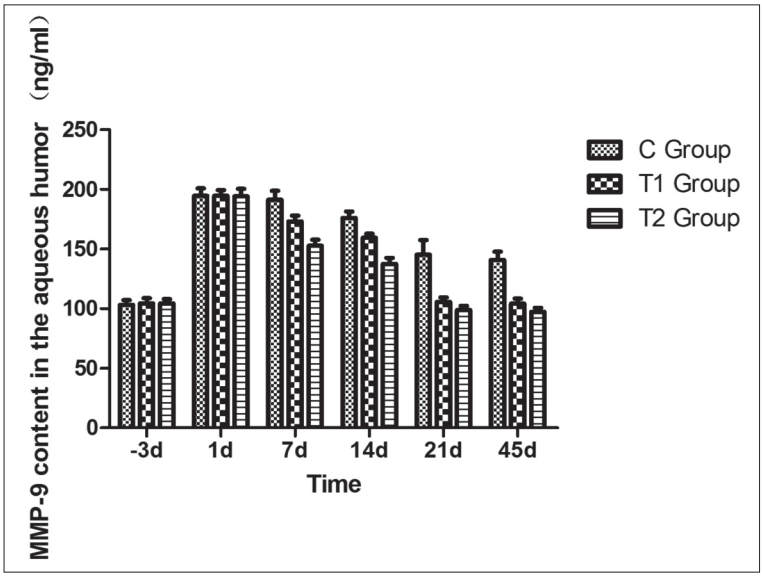


Fig 5. The content of MMP-2 in aqueous humor was significant differences between T1 group and T2 group in 7, 14 days *(P<0.05)*. There was no significant difference at other time points *(P>0.05)*. The content of MMP-2 in aqueous humor was very significant differences between T2 group and group C in 7, 14, 21, 45 days *(P<0.01)*

Fig 6. The content of MMP-9 in aqueous humor was very significant differences between T1 group and T2 group in 7, 14 days *(P<0.05)*. There was no significant difference at other time points *(P>0.05)*. The content of MMP-9 in aqueous humor was very significant differences between T2 group and group C in 7, 14, 21, 45 days *(P<0.01)*



was very significant differences between T2 group and group C in 7, 14, 21, 45 days (P<0.01). MMP-2 content in the aqueous humor of canine eyes in both the experimental group and the C group reached the maximum on the 7th day after treatment. The maximum values of C group were 145.8769±5.9418 ng/mL, the maximum values of T1 group were 139.6685±3.6469 ng/mL, the maximum values of T2 group were 128.8777±4.6122 ng/mL. The specific test results are shown in *Fig. 5*.

The content of MMP-9 in aqueous humor was very significant differences between T1 group and T2 group in 7, 14 days (P<0.05). There was no significant difference at other time points (P>0.05). The content of MMP-9 in aqueous humor was very significant differences between T2 group and group C in 7, 14, 21, 45 days (P<0.01). MMP-9 content in the aqueous humor of canine eyes in both the experimental group and the C group reached the maximum on the first day after treatment. The maximum values of C group were 194.6672±6.1742 ng/mL, the maximum values of T1 group were 194.7671±4.6571 ng/mL, the maximum values of T2 group were 194.2776±6.2889 ng/mL. The specific test results are shown in *Fig. 6*.

On the 45th day, the histopathological examination showed that the corneal epithelium was relatively intact and the corneal epithelium was thickened and healed. Under the epithelium, inflammation is very visible, the corpus ciliare is ruptured and the dilated and hyperemic vessels are seen in the mesenchyme below the epithelium, mainly with capillaries *(Fig. 7)*. T1 group corneal epithelium is complete, partial thickness corneal thickness, corneal fiber hyperplasia, and the junction of conjunctiva blood vessels are less, neutrophils is relatively rare, corneal epithelium no inflammatory cell infiltration phenomenon *(Fig. 8)*; In T2 group, the corneal epithelium was intact, the cornea was healed, the cornea thickness was moderate, some tissues had hyperplasia, and the corneal epithelium had few blood

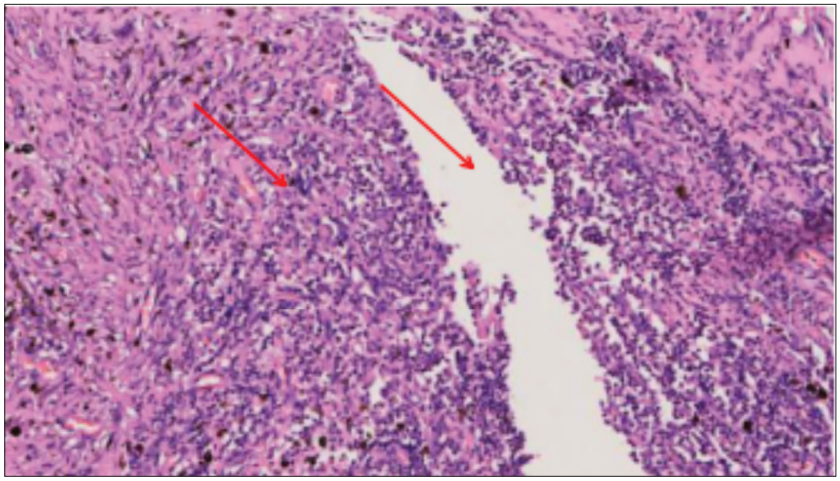
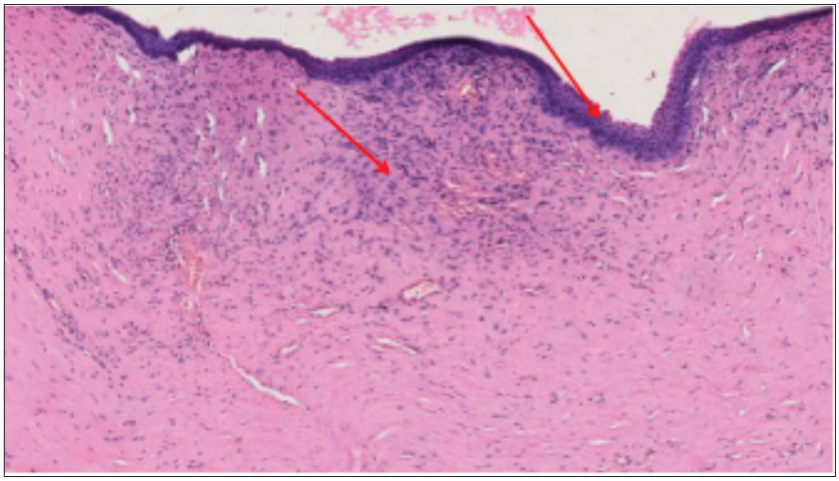


Fig 7. Large amounts of blood capillary and ciliary body fracture in group C (HE staining 10 x 20)

Fig 8. Thick corneal epithelium and subcorneal fibroplasia in group T1 (HE staining 10 x 10)



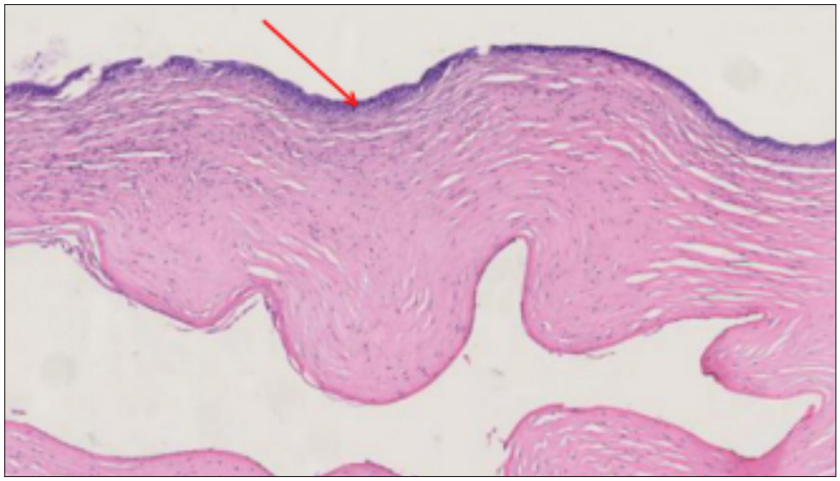


Fig 9. Corneal epithelium thickness is moderate and corneal healing is good in group T2 (HE staining is 10 x 10)

vessels, few inflammatory cells, and no inflammatory cell infiltration *(Fig. 9)*.

DISCUSSION

After corneal ulcer, the blood vessels of the conjunctiva membrane are rapidly growing in the direction of corneal damage from the Angle of the sclera. Causes the cornea to appear the blood vessel, VEGF plays an important role in promoting vascular endothelial cell proliferation and angiogenesis [4]. Studies have also shown that VEGF overexpression exists in corneal neovascularization caused by various causes to varying degrees [5]. IL-1 and IL-8 are two important cytokines in the development of inflammatory corneal disease [6]. Inflammatory cell infiltration plays an important role, and its expression level is closely related

to the occurrence and development of inflammation and the degree of injury of corneal tissue [7]. In this study, VEGF, IL-1 and IL-8 in group C were sustained at a high level, it's consistent with previous research, it may be related to the infiltration of inflammatory cells after corneal ulcer. Since IL-1 and other inflammatory factors can induce neutrophil to chemotaxis to the cornea and aqueous humor. In addition, the increase of neutrophils can stimulate the release of local secondary inflammatory factors, aggravate corneal injury, and a large number of inflammatory cells infiltrate and cause local hypoxia. Hypoxia inducible factor-1 is one of the important promoters of VEGF, which provides support for the continuous high level expression of VEGF. In the T2 group, the levels of VEGF, IL-1 and IL-8 in the aqueous chamber were significantly reduced, which may be related to the TIMPs, IL-1ra, PEDF and other cytokines in the amniotic epithelial cells and mesenchyme cells [8,9]. At the same time, the amniotic membrane can achieve anti-inflammatory effect by reducing the chemokine's of neutrophils, improve the surrounding environment of corneal tissue, reduce the occurrence of hypoxic environment, and further inhibit the expression of VEGF. It is indicated that the amniotic fluid can synergize the corneal neovascularization and reduce the inflammatory infiltration.

Matrix metalloproteinases are important proteases that affect the structure of collagen fibers, and are related to various tissue damage and repair. More than 90% of corneal tissue is the matrix layer, and collagen fiber is an important component to maintain the stable structure of stromal layer, and it is of great significance to ensure the properties and structure of collagen fibers. The changes of MMP content directly affect the pathological changes of corneal tissue. The stability of collagen fibers in corneal stromal cells plays an important role in maintaining hydrophobicity of corneal epithelium and corneal transparency.

Studies have confirmed that amniotic membrane may inhibit the activity of matrix metalloproteinase (MMP) by increasing the expression level of tissue inhibitor of metalloproteinase-1 (TIMP-1) in the cornea, thus inhibiting the action of corneal ulcer. Paterson et al.[10] confirmed that TIMP-1 plays an important role in inhibiting the occurrence and development of corneal ulcer. In this study, the contents of MMP-1, 2 and 9 in the T2 group were significantly lower than that in T1 group, and very significantly lower than group C. This is consistent with previous research results. This may be associated with MMP hydrolyzed substrate, because deep corneal ulcer will cause massive damage corneal stromal layer collagen fiber, in the case of inflammation, MMP enzymes are activated, release, involved in cell migration, the removal of the necrotic tissue and wound healing process [11]. In group C, the corneal healing was slow, and the cornea was in a high state of traction, while the high amplitude of traction caused MMP-2 to significantly increase, while inhibiting the expression of TIMP, affecting the corneal healing. After the conjunctiva coverage, the corneal tension was relieved, and the bFGF and other growth factors in the amniotic membrane accelerated the fusion of cornea and conjunctiva. Meanwhile, the anti-inflammatory action of amniotic membrane can improve the corneal environment, further reduce the activation of MMP enzyme, and achieve the important role of inhibiting corneal ulcer and accelerating corneal healing.

In the case of deep corneal ulcer, the integrity of corneal tissue is destroyed, and Herretes S [12] research shows that amniotic fluid can effectively reduce inflammatory cell infiltration in corneal tissue and inhibit the formation of new blood vessels. Corneal histopathological observations showed that the inflammatory cells in group C were significantly infiltrated, and the corneal stromal layer was disordered and the capillaries with more hyperemia were scattered. This was consistent with the results of significantly increased IL, VEGF and MMP in group C. There were capillaries between the conjunctiva flap and cornea in the T1 group, indicating that the healing was incomplete and the conjunctiva was not corneal. The hyperplasia of the corneal stroma indicates that the collagen fibers have not yet completed the repair of the corneal epithelium and corneal stromal layer, and the cornea debridement has not been completed. T2 group corneal thickness is moderate, vascular hyperplasia of only a small amount. Results show that amniotic membrane eye accelerated the supernatant points on the cornea of speed, reducing the inflammatory cell infiltration, at the same time by reducing the content of VEGF inhibition new angiogenesis, and to promote healing of corneal transparency has played a positive role.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgements

This research was supported by grants from the Heilongjiang Bayi Agricultural university campus cultivation project (Grant no. XZR2017-05) and the National Key Research and Development Program (Grant no. 2016YFD0501008)

REFERENCES

1. Ramani C, Rambabu K, D'Souza NJ, Vairamuthu S, Subapriya S, William BJ: Surgical bacteriology and grading of corneal ulcers in dogs a retrospective study in 24 dogs. *Indian J Canine Practice,* 5 (1): 136-138, 2013.

2. Ito S, Terakado K, Ichikawa Y, Zama T, Minami T, Kudo S, Kanemaki N: Repair of a traumatic corneal laceration in a cat using a tectonic heterograft. *e-Polish J Vet Ophthalmol*, (2): 1-6, 2014.

3. Chanie M, Bogale B: Thelaziasis: Biology, species affected and pathology (conjunctivitis): A Review. *Acta Parasitol Globalis*, 5, 65-68, 2014.

4. Morabito A, De Maio E, Di Maio M, Normanno N, Perrone F: Tyrosine kinase inhibitors of vascular endothelial growth factor receptors in clinical trials: Current status and future directions. *Oncologist*, 11 (7): 753-764, 2006. DOI: 10.1634/theoncologist.11-7-753

5. **Lai CM, Spilsbury K, Brankov M, Zaknich T, Rakoczy PE:** Inhibition of corneal neovascularization by recombinant adenovirus mediated antisense VEGF RNA. *Exp Eye Res*, 75 (6): 625-634, 2002. DOI: 10.1006/exer.2002.2075

6. Stapleton WM, Chaurasia SS, Medeiros FW, Mohan RR, Sinha S, Wilson SE: Topical interleukin-1 receptor antagonist inhibits inflammatory cell infiltration into the cornea. *Exp Eye Res*, 86 (5): 753-757, 2008. DOI**:** 10.1016/j.exer.2008.02.001

7. Matsumoto K, Ikema K, Tanihara H: Role of cytokines and chemokines in pseudomonal keratitis. *Cornea*, 24 (8): S43-S49, 2005. DOI**:** 10.1097/01.ico.0000178737.35297.d4

8. Fine HF, Biscette O, Chang S: Ocular hypotony: A review. *Compr Ophthalmol Update*, 8 (1): 29-37, 2007.

9. Volpert OV, Zaichuk T, Zhou W, Reiher F, Ferguson TA, Stuart PM, Amin M, Bouck NP: Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. *Nat Med*, 8 (4): 349-357, 2002. DOI**:** 10.1038/nm0402-349

10. Paterson CA, Wells JG, Koklitis PA, Higgs GA, Docherty AJ: Recombinant tissue inhibitor of metalloproteinases type 1 suppresses alkali-burn-induced corneal ulceration in rabbits. *Invest Ophthalmol Vis Sci*, 35 (2): 677-684, 1994.

11. Sivak JM, Fini ME: MMPs in the eye: Emerging roles for matrix metalloproteinases in ocular physiology. *Prog Retin Eye Res*, 21 (1): 1-14, 2002. DOI**:** 10.1016/S1350-9462(01)00015-5

12. Herretes S, Suwan-Apichon O, Pirouzmanesh A, Reyes JMG, Broman AT, Cano M, Gehlbach PL, Gurewitsch ED, Duh EJ, Behrens A: Use of topical human amniotic fluid in the treatment of acute ocular alkali injuries in mice. *Am J Ophthalmol*, 142 (2): 271-278, 2006. DOI**:** 10.1016/j.ajo.2006.03.025

Kafkas Univ Vet Fak Derg
25 (3): 373-378, 2019
DOI: 10.9775/kvfd.2018.21009

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Monitoring of Some Anthelmintics Against Gastrointestinal Nematodes in Sheep and Implications of Resistance in Barani Region, Pakistan

Ali MUHAMMAD [1,a] Haroon AHMED [2,b] Shahzad ALI [3] Muhammad SAQLAIN [4]
Mazhar QAYYUM [5] Sami SIMSEK [6,c]

[1] Department of Zoology, University of Poonch Rawalakot, 12350, Azad Kashmir, PAKISTAN
[2] Department of Biosciences, COMSATS Institute of Information Technology (CIIT), Islamabad, PAKISTAN
[3] Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, TR-54000 Lahore - PAKISTAN
[4] Department of Biochemistry, PMAS Arid Agriculture University, TR-46000 Rawalpindi - PAKISTAN
[5] Department of Zoology, PMAS Arid Agriculture University, TR-46000 Rawalpindi - PAKISTAN
[6] Department of Parasitology, Faculty of Veterinary Medicine, University of Firat, TR-23119 Elazig - TURKEY
[a] ORCID: 0000-0002-4797-5302; [b] ORCID: 0000-0002-0382-3569; [c] ORCID: 0000-0002-3567-326X

Article ID: KVFD-2018-21009 Received: 21.09.2018 Accepted: 18.02.2019 Published Online: 21.02.2019

How to Cite This Article

Muhammad A, Ahmed H, Ali S, Saqlain M, Qayyum M, Simsek S: Monitoring of some anthelmintics against gastrointestinal nematodes in sheep and implications of resistance in Barani region, Pakistan. *Kafkas Univ Vet Fak Derg,* 25 (3): 373-378, 2019. DOI: 10.9775/kvfd.2018.21009

Abstract

The widespread prevalence of gastrointestinal nematode (GIN) infections has an important impact on livestock affecting of meat, milk, traction and manure in tropical and sub-tropical areas. The present study was aimed to monitor the efficacy of commonly available anthelmintic drugs at publicly owned sheep farms. The sheep naturally infected with nematodes were selected, aged between 10 to 18 months, for field efficacy trial. Selected sheep were divided into five groups (15 animals each group) based on their equal egg per gram (EPG) and body weight. Group-I served as control (untreated), while Group-II was treated with albendazole at the recommended rate of 8.8 mL/100 kg of body weight by oral drench, Group-III was treated with levamisole at the rate of 4.4 mL per 100 kg body weight, Group-IV was treated with ivermectin, 200 µg of ivermectin per kilogram of body weight and Group-V was treated with mixture of levamisole and ivermectin at the rate of 10 mg/kg by injecting subcutaneous, respectively. The results revealed that the highest efficacy rate (88.25%) was observed in sheep treated with mixture of levamisole and ivermectin, followed by ivermectin (86.43%), levamisole (81.32%), and albendazole (51.11%), respectively. The data has indicated that nematodes *(Haemonchus contortus* as the predominant species followed by *Trichostrongylus colubriformis, Trichostrongylus axei* and *Oesophagostomum columbianum)* might have developed resistance against the major anthelmintic drugs, encountered throughout this trial. Overall results showed that anthelmintic resistance resulted because non-judicial use anthelmintic drugs without considering the epidemiological knowledge related to occurrence of commonly nematode parasites.

Keywords: *Anthelmintic efficacy, Gastrointestinal nematodes, resistance, Ivermectin, Levamisole, Albendazole*

Pakistan'ın Barani Bölgesi'nde Koyunlarda Mide-Bağırsak Nematodlarına Karşı Bazı Antelmintiklerin Etkisi ve Direncin İzlenmesi

Öz

Gastrointestinal nematod (GIN) enfeksiyonlarının yaygın olması, tropik ve subtropikal bölgelerdeki çiftlik hayvanlarında et, süt, iş gücü ve gübre kayıplarına yol açması bakımından önemlidir. Bu çalışmada, halka elindeki koyun çiftliklerinde yaygın olarak kullanılan antelmintik ilaçların etkinliğinin izlenmesi amaçlandı. Nematodlarla doğal enfekte 10 ile 18 aylık yaştaki koyunlar, bu çalışma için seçildi. Seçilen koyunlar gram başına çıkardıkları yumurtalar (EPG) ve vücut ağırlığına göre beş gruba (her grupta 15 hayvan) ayrıldı. Grup-I kontrol olarak ayrıldı (tedavi edilmedi), Grup-II oral sonda ile önerilen dozu olan 8.8 mL/100 kg vücut ağırlığı oranında albendazol ile tedavi edilirken, Grup-III 100 kg vücut ağırlığı başına 4.4 mL oranında levamizol ile tedavi edildi, Grup-IV, kilogram başına 200 µg ivermektin ve Grup-V ise levamizol ile ivermectin karışımı ile 10 mg/kg dozda subkutan olarak uygulandı. Sonuçlar, en yüksek etkinlik oranının (%88.25), levamizol ve ivermektin karışımı ile muamele edilen koyunlarda, ardından da ivermektin (%86.43), levamizol (%81.32) ve albendazol (%51.11) uygulanan grupların takip ettiğini gösterdi. Veriler, nematodların *Haemonchus conttortus,* dominant tür, *Trichostrongylus colubriformis, Trichostrongylus axei* ve *Oesophagostomum columbianum* ana anthelmintic ilaçlara karşı direnç geliştirebileceğini göstermiştir. Genel sonuçlar, antelmintik direncin, epidemiyolojik bilgileri dikkate almadan rastgele kullanılan antelmintik ilaçların kullanılmasından kaynaklandığını göstermiştir.

Anahtar sözcükler: *Antelmentik etki, Mide bağırsak nematodları, Direnç, İvermektin, Levamizol, Albendazol*

İletişim (Correspondence)
+424 2370000 ext: 3967 Fax: +90 424 2388173
ssimsek@firat.edu.tr

INTRODUCTION

Livestock, particularly sheep and goats help to beat economic losses in case of crop deterioration [1]. It contributes 11.8 percent to national Gross Domestic Product (GDP) and 56.3 percent to Agriculture GDP of Pakistan [2]. One of the paramount factors that influence adequate feed conversion is gastrointestinal nematode (GIN) infections. The widespread prevalence of GIN infections has infested many livestock development programs by reducing the level of output of meat, milk, traction and manure in tropical and sub-tropical areas. Same also reduces their asset value due to an increase in mortality rate, especially of young stock [3].

Although anthelmintic treatment can be used to minimize the losses by ensuring the sustainability of sheep production, other approaches, such as pasture management, could be worth mentioning. There are reports about the increasing rates of parasite resistance against chemotherapeutic agents across the globe [4-9]. When parasitic populations are not killed by the therapeutically recommended dosages of previously effective drugs, the resistance is said to have been developed [10]. Moreover, anthelmintic resistance causes great damage to both agricultural revenue and animal well-being. The loss of anthelmintic activity was established to be one of the important factors of high occurrence of GIN infections in small ruminants in Pakistan [9,11-14]. Comprehensive drugs screening studies are required to know exactly about position of currently available anthelmintic drugs. The main objectives of the present study were to find the accuracy of treatment with broad-spectrum anthelmintic using fecal egg count reduction test (FECRT) and determine the sensitivity of gastrointestinal nematodes (GINs) to benzimidazole, levamisole, ivermectin and a mixture (levamisole + ivermectin). The objective of the study was also determination of some haematological parameters in groups during post treatment period.

MATERIAL and METHODS

Animal's Selection: The present study was carried out at the Small Ruminants Research Program's farm located in the National Agricultural Research Center in Islamabad which is present in Barani region, Pakistan. The sheep breed used was Bulkhi commonly known as Afghani sheep. Sheep were reared in a semi-intensive system, where they grazed on permanent pasture during the day and were housed in brick sheds with concrete-floored pens at night mixed husbandry with goats, cattle, and buffalo. The sheep were supplemented fed with pelleted concentrate (PARC Feed Technology Brand) at the rate of 0.54/kg/animal/day. While green fodder and water were provided *ad libitum* during day and night.

Experimental Design: A total of 75 Bulkhi sheep naturally infected with nematodes were selected, aged between 10 to 18 months, for field efficacy trial. Body weights and egg per gram (EPG) of all sheep were recorded. Animals were individually weighed before treatment on a Tru-Test scale. The accuracy of scale was checked by comparing with certified weights. Selected sheep were divided into five groups (15 animals each group) based on their equal EPG and body weight. All sheep in one group were approximately the same weight and had similar excretion rates to ensure the correct therapeutic dose. Group-I served as control (untreated), while Group-II was treated with albendazole (Albazen®) at the recommended rate of 8.8 mL/100 kg of body weight by oral drench, Group-III was treated with levamisole (Levasole®) at the rate of 4.4 mL per 100 kg body weight, Group-IV was treated with ivermectin (Ivomec®) 200 µg of ivermectin per kilogram of body weight and Group-V was treated with mixture of levamisole and ivermectin (Primisol®) at the rate of 10 mg/kg by injecting subcutaneous, respectively. The dose of each anthelmenthic was calculated (dose rate per kg) as provided by the manufacturers. Fecal sampling schedule for experimental trial was 7 and 3 days pre-treatment, 0 day and 7, 14, 21, 28 and 35 days post treatment.

Faecal Collection and Analysis: On the above mentioned days, 5 g faecal sample of each sheep was taken directly from rectum for faecal egg count reduction test (FECRT). Faecal egg counts (FEC) were performed by using the modified McMaster method [15] with saturated sodium chloride as the flotation fluid.

Faecal Egg Count Reduction Test: Mean FEC, percentage reduction and 95% confidence interval (CI) was determined by using the formulae recommended by the World Association for the Advancement of Veterinary Parasitology guidelines for detecting anthelmintic resistant nematodes of sheep [16].

$$\text{Anthelmintic Efficacy} = \frac{\text{(Pre-treatment mean - Post-treatment mean)}}{\text{Pre-treatment mean}} \times 100$$

An efficacy of less than 90% and 95% upper confidence levels of less than 90% was taken as indicative of the presence of anthelmintic resistant for nematodes in the sheep flock.

Evaluation of Haematological Parameters: Subsequently, on the above mentioned days, along with faecal samples, blood samples was also collected via jugular vein puncture into 5 mL ethylene di-amine tetra acetic acid (EDTA) coated and without (EDTA) coated vacutainer tubes for assessment of hematological parameters i.e. haemoglobin level, packed cell volume, total protein level, while total erythrocytes count, total leucocytes count and differential leucocytes count were analyzed on days 0, 7, 14, 21, 28 and 35, respectively [17].

Coproculture Analysis: The larvae were recovered through Baermann procedure to determine the relative composition

of specific nematode species. The identification larvae (L3) were carried out by following the keys and description given by [18].

Statistical Analysis

Data was analyzed by statistical package POST HOC TEST (univariate analysis of variance) using SPSS version 16.0. FEC and larval culture records were transformed [log10 (n+1)] before analysis to stabilize the variance. The values of blood parameters and body weight were measured in respective units. No transformation was applied to blood parameters.

RESULTS

The results illustrated a significant difference of FECRT on 7th, 14th, 21st, 28th and 35th days post-treatment with mixture of levamisole and ivermectin in Group V *(Fig. 1)* compared to control (Group I) ($P<0.05$) at 95% CI. The mean minimum FEC of mixture (66.72±3.3) was noted at 7th day while that of ivermectin (81.47±4.0) and levamisole (143.50±7.1) showed the same at 14th day. No significant effects were found on the total FEC regarding albendazole *(Fig. 1)*. Moreover, the Group-V and Group-IV were found to be proficient enough with greatest efficacy (88.25%) and (86.43%), respectively followed by Group-III which showed moderate effectiveness with value (81.32%), while Group-II had lowest effectiveness status with low efficiency (51.11%) *(Table 1)*.

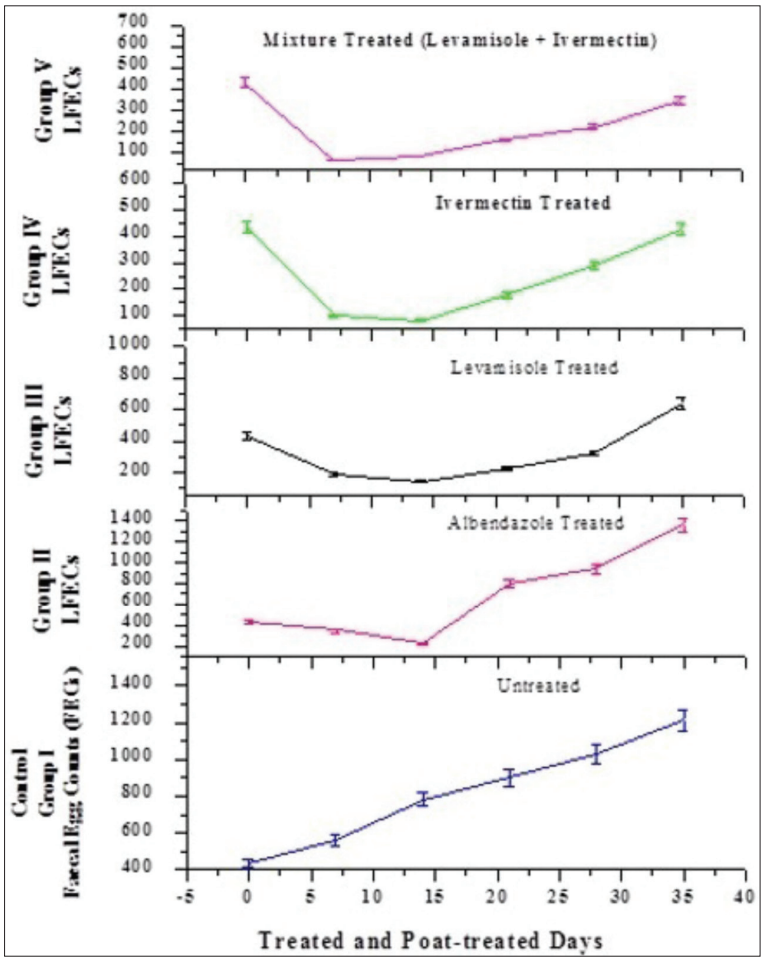


Fig 1. Group-wise anthelmintic efficacy of broad spectrum anthelmintic drugs in Bulkhi sheep

Our findings regarding the individual faecal cultures of trichostrongyles larvae (L3) pointed towards the frequencies of generic composition describing the existence of *Haemonchus contortus* as the predominant species followed by *Trichostrongylus colubriformis*, *Trichostrongylus axei* and *Oesophagostomum columbianum* throughout the study trail *(Table 2)*.

The results of current study pointed towards an increase in live body weight in Group-V and Group-IV, while a slight body weight regain was noticed in Group III. Whereas, no significant change in body weight was observed in Group-II and Group-I (control) seemed to be losing weight continuously, being untreated *(Fig. 2)*. A significant ($P<0.05$) increase in the haematological parameters was observed viz.; packed cell volume (PCV), haemoglobin level (Hb) and protein level (PL) was occurred in Group-IV and V as compared to the Group-I and II, in the post treatment period. Highest mean values of PCV, Hb and PL were recorded at days 7 and 14 in Group IV (25.70±2.1; 8.32±1.2 and 7.21±1.0) and V (26.83±2.64; 8.40±1.4 and 7.30±1.1), respectively. Furthermore, lowest mean values at day 35 in Group-I (19.20±1.9; 6.71±1.4 and 6.0±1.0) and Group-II (22.91±2.2; 7.23±1.2 and 6.40±1.1) were recorded *(Fig. 3a, 3b, 3c)*.

Our data revealed a significant ($P<0.05$) increase in mean values of Total Erythrocyte Count (TEC) *(Fig. 4a)* and decrease in TLC within the Group IV and V after anthelmintic treatments *(Fig. 4b)*. While in Group II, the mean values of TEC showed no significant changes followed by Group I. Reasons for the above results for all the haematological parameters were found to be the same. Furthermore, a significant decrease in differential counts ($P<0.05$) after anthelmintic treatment with mixture and ivermectin was monitored in Group IV and V but not in Group II *(Table 3)*.

DISCUSSION

The efficacy of various synthetic broad spectrum anthelmintic products checked by using FECRT against the GINs within the sheep flock revealed a significant difference ($P<0.05$) of FECRT on pre-treatment (0) and post-treatment (7th) days post-treatment with mixture of levamisole and ivermectin in Group V compared to control,

Table 1. *Faecal egg count (epg) reduction (FECR) in the Bulkhi sheep before and after anthelmintic treatments*

Groups	Host (Bulkhi Sheep)				
	No of Animals Examined	Anthelmintic Drugs	Mean FEC	Mean Faecal Egg Count Reduction	
			Pre-treatments Mean±SEM	Post-treatments Mean±SEM	FECR (%)
I	15	Control (untreated)	493.2±26.3	774.2±13.5	-
II	15	Albendazole (0.75 mL/11.34 kg)	503.4±25.1	246.1±12.3	51.11
III	15	Levamisole (2 mL/45.35 kg)	796.3±39.8	148.8±7.4	81.32
IV	15	Ivermectin (1 mL/34 kg)	765.92±38.2	104.11±5.2	86.43
V	15	Levamisole + Ivermectin (1 mL/22.67 kg + 0.5 mL/17.23 kg)	859±42.9	100.9±5.0	88.25

Table 2. *Post-treatment generic composition of trichostrongyles larvae (L3) recovered from faecal cultures in Bulkhi sheep*

Parasite	Prevalence (%)
Haemonchus contortus	62
Trichostrongylus colubriformis	15
Trichostrongylus axei	12
Oesophagostomum columbianum	11

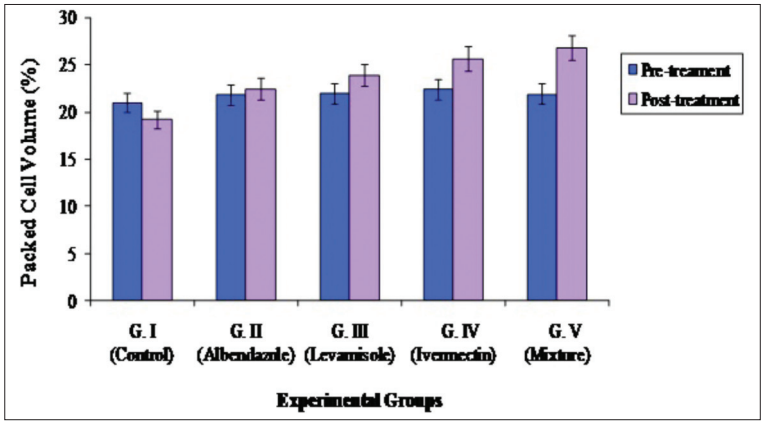


Fig 2. Effect of broad spectrum anthelmintic drugs on live body weight in Bulkhi sheep

Group I. The findings in our study are in accordance with Arslan and Muhammed [19], Islam et al.[20] and Muhammad et al.[21] as they also reported similar trend in albendazole treated sheep. The results about anthelmintic efficacy among various groups are similar to findings of Nari et al.[22] and Zajac and Gipson [23] . Uppal et al.[24] which are about 80 to 88% verified similar results in India. Many factors like genetic, biological or operational contribute in the emergence of anthelmintic resistance Raza et al.[13]. Similar observations were found in the experimental sheep flock where frequent, (6-7/annum), long term use of the broad spectrum anthelmintic drug especially the albendazole, was responsible for the development of anthelmintic resistance the continual use of the said drug might be due to its low price, availability and easily administrable by the local farmers. Similar findings and outcome was reported by Prichard [25] and Jackson [10].

Presently, a trend of the degree of resistance has been noted against the levamisole, ivermectin and mixture of both among the Bulkhi flock reared at NARC, Islamabad, which is alarming for veterinarians and farmers. This compels us to think about alternatives and control strategies against GINs, particularly in Barani region, Pakistan. Other approaches, such as pasture management, could be better alternaive. The probable reason for the development of anthelmintic resistance might be the fact that the climate of Barani region, being humid and warm, highly supports the development and survival of free living stages of trichostrongyles and represent a reservoir of infective larvae throughout the year. The higher occurrence of GINs, might favor the development of anthelmintic resistance. Our findings of anthelmintic resistance are in accordance with Chandrawathani et al.[3] and Muhammad et al.[21]. Farmers carry on using drugs without the basic knowledge of their dosage and administration hence producing a stern anthelmintic resistance as demonstrated in our results.

The development of resistance on this study farm for instance could be facilitated by continuous grazing on permanent pasture and mixed growing with goats, cattle's and buffaloes throughout the study year by Muhammad et al.[21] According to Coles and Roush [16], the optimal proposition is to use anthelmintic from various families one by one according to the demand of the host. However, during the past few decades there have been escalating rates of resistance of parasites to chemotherapeutic agents all over the world [6]. Correspondingly, in Pakistan, one of the important factors of high prevalence of gastrointestinal nematode infections in small ruminants

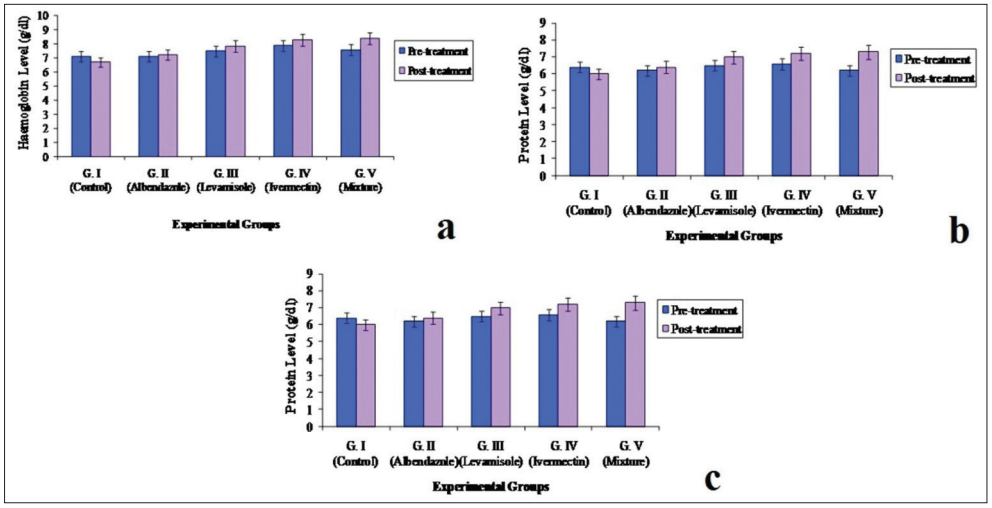


Fig 3. Effect of broad spectrum anthelmintic drugs on some blood parameters in Bulkhi sheep **a-** Post-treatment packed cell volume profiles in Bulkhi sheep; **b-** Post-treatment haemoglobin levels in Bulkhi sheep; **c-** Post-treatment elevation in protein levels in Bulkhi sheep

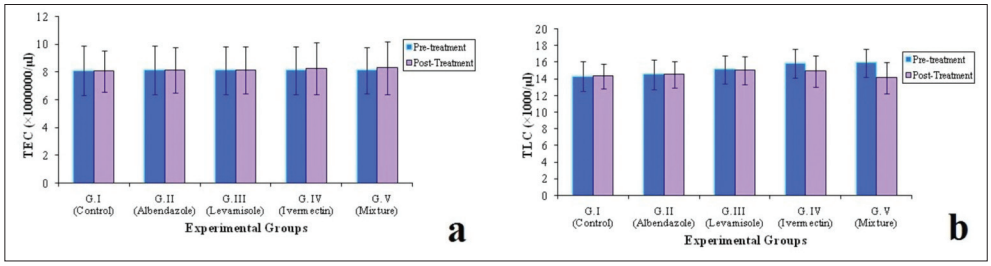


Fig 4. Effect of broad spectrum anthelmintic drugs on Total Erythrocyte and Leucocyte Counts in Bulkhi sheep **a-** Post-treatment elevation in Total Erythrocyte Count (TEC) **b-** Post-treatment decrease in Total Leucocyte Count (TLC)

Table 3. *Differential leucocyte count (%) in Bulkhi sheep*

Groups	Differential Leucocyte Count (%)									
	Lymphocytes		Neutrophils		Eosinophils		Monocytes		Basophils	
	Day 0	Day 35	Day 0	Day 35	Day 0	Day 35	Day 0	Day35	Day 0	Day35
I	55.57	55.45	38.55	38.23	47.42	47.57	3.53	3.47	0.53	0.54
II	54.86	54.78	37.97	37.56	48.43	48.25	3.86	3.81	0.51	0.50
III	56.54	56.32	38.52	38.11	47.89	48.31	4.01	3.97	0.49	0.50
IV	55.64	53.97	38.31	35.85	48.57	47.12	3.62	3.21	0.53	0.45
V	55.12	54.23	38.43	36.67	48.26	46.87	3.59	3.12	0.54	0.43

may be failure of efficacy of anthelmintics [13]. Suggested meaningful study can provide valuable information's that may help in devising strategic guidance for the health and management in small ruminants.

The body weight regain might be a result of parasitic load removal, as parasitic free gastro-intestinal tract promotes proper digestion, absorption and metabolism of feed nutrients which make a sound base for proper weight regain. Similar findings were reported by Hussein [26] and Kenyon et al.[27]. The logical explanation for current observations might be the parasitic infection, responsible for the arrested growth.

Our results revealed a significant ($P<0.05$) increase in packed cell volume (PCV), haemoglobin level (Hb) and protein level (PL) was occurred in Group-IV and V as compared to the Group-I and II, in the post treatment period. The reason for the significant decrease in PCV, Hb and PL in Group-I and Group-II might be due to heavy nematode burden with *Haemonchus contortus* resulting in anemia and hypoproteinemia. Whereas, the high efficacy of mixture in Group V and ivermectin in Group IV might be responsible for a significant increase in PCV, HB and PL values for their action against blood sucking parasites. Similar findings have been reported by Chaichisemsari et al.[28] and Akanda et al.[29].

A significant ($P<0.05$) increase in mean values of TEC and decrease in TLC within the Group IV and V after anthelmintic treatments was observed. The results regarding change in TEC and TLC are according to Akanda et al.[29]. Differences in leukocytes count might be a result of different levels of nematode parasites present within the host, type of sampling site, utilization of techniques used for leukocytes count and concentration of anticoagulants. In these results, again mixture and ivermectin showed the maximum ability as compared to albendazole and levamisole. Overall, results indicated that all the hematological parameters viz, PCV, Hb, PL, and TECs showed positive significant

correlation with each other in Bulkhi sheep, while inverse correlation within the host worm burden was detected, throughout study trail.

The present findings strongly suggest planning further studies on resistant nematode worms prevalent within the gastrointestinal tract among different host breeds, in different agro-ecological regions of Pakistan. Appropriate use of anthelmintic treatments concerning therapeutic dose recommended by manufactures is required, as overdose uphold homozygous and under dose promote heterozygous population of resistant worms Shalaby [30] so, it is suggested that the animals from the same sex, breed and age class must be weighed precisely for therapeutic dosage.

Acknowledgements

The authors are highly thankful to staff members in NARC for their technical support during this research work. We are very thankful to TUBITAK (2216-research fellowship program for international researchers) to provide an opportunity and funding. This study has been supported by a grant from Pakistan Science Foundation.

REFERENCES

1. Khajuria JK, Katoch R, Yadav A, Godara R, Gupta SK, Singh A: Seasonal prevalence of gastrointestinal helminths in sheep and goats of middle agro-climatic zone of Jammu province. *J Parasit Dis,* 37, 21-25, 2013. DOI: 10.1007/s12639-012-0122-3

2. Anonymus: Economic Survey of Pakistan 2016-17. Finance Division, Islamabad. Government of Pakistan, pp.29-31. http://www.finance.gov.pk/survey_1617.html. *Accessed:* 01.02.2019.

3. Chandawathani P, WallerPJ, Adnan M, Hoglund J: Evaluation of high-level multiple anthelmintic resistance on a sheep farm in Malaysia. *Trop Anim Health Prod,* 35, 17-25, 2003.

4. Dargatz DA, Traub-Dargatz JL, Sangster NC: Antimicrobial and anthelmintic resistance. *Vet Clin North Am Equine Pract,* 16, 515-535, 2000.

5. Gabrie S, Phiri IK, Dorny P, Vercruysse J: A survey on anthelmintic resistance in nematode parasites of sheep in Lusaka, Zambia. *Onderstepoort J Vet Res*, 68, 271-274, 2001.

6. Atanasio A, Boomker J, Sitoe C: A survey on the occurrence of resistance to anthelmintics of gastrointestinal nematodes of goats in Mozambique. *Onderstepoort J Vet Res,* 69, 215-220, 2002.

7. Kaplan RM: Drug resistance in nematodes of veterinary importance: A status report. *Trends Parasitol*, 20, 477-481, 2004. DOI: 10.1016/j.pt.2004.08.001

8. Coles GC, Jackson F, Pomroy WE, Prichard RK, Samson-Himmelstjerna VG, Silvestre A, Taylor MA, Vercruysse J: The detection of anthelmintic resistance in nematodes of veterinary importance. *Vet Parasitol*, 136, 167-185, 2006. DOI: 10.1016/j.vetpar.2005.11.019

9. Jabbar A, Iqbal Z, Kerboeuf D, Muhammad G, Khan MN, Afaq M: Anthelmintic resistance: The state of play revisited. *Life Sci*, 79, 2413-2431, 2006. DOI: 10.1016/j.lfs.2006.08.010

10. Jackson F: Anthelmintic resistance the state of play. *Brit Vet J*, 149, 123-138, 1993.

11. Iqbal Z, Lateef M, Khan MN, Muhammad G, Jabbar A: Temporal density of trichostrongylid larvae on a communal pasture in a sub-tropical region of Pakistan. *Pak Vet J,* 25, 87-91, 2005.

12. Lateef M, Iqbal Z, Jabbar A, Khan MN, Akhtar MS: Epidemiology of trichostrongylid nematode infections in sheep under traditional husbandry system in Pakistan. *Int J Agric Biol,* 7, 596-600, 2005.

13. Raza MA, Iqbal Z, Jabbar A, Yaseen M: Point prevalence of gastrointestinal helminthosis in ruminants in southern Punjab (Pakistan). *J Helminthol,* 81 (3): 323-328, 2007. DOI: 10.1017/S0022149X07818554

14. Saddiqi HA, Jabbar A, Iqbal Z, Babar W, Sindhu ZD, Abbas RZ: Comparative efficacy of five anthelmintics against trichostrongylid nematodes in sheep. *Can J Anim Sci,* 86, 471-477, 2006. DOI: 10.4141/A06-036

15. Coles GC, Bauer C, Borgsteede FHM, Geerts S, Klei TR, Taylor MA, Waller PJ: World Association for the Advancement of Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Vet Parasitol,* 44, 35-44, 1992. DOI: 10.1016/0304-4017(92)90141-U

16. Coles GC, Roush RT: Slowing the spread of anthelmintic resistant nematodes of sheep and goats in the United Kingdom. *Vet Rec,* 130, 505-510, 1992.

17. Linne J, Ringsrud K: Basic Techniques in Clinical Laboratory Science. 3rd ed., 112-113, Mosby Year Book, 1999.

18. Coffin DL: Manual of Veterinary Clinical Pathology. 3rd ed., 89-92, Coinstock Publishing, New York, USA, 1961.

19. Arslan SH, Mohammed BA: The efficacy of albendazole and ivermectin in the control of parasitic helminths in sheep in Ninevah Province. *Iraqi J Vet Sci,* 14 (1): 9-17, 2001.

20. Islam MS, Begum F, Alam MS: Comparative efficacy of Aldazole®, Fenvet® and Ivomec® injection against natural infection of gastrointestinal nematodes in goats. *J Anim Vet Adv*, 2 (7): 382-384, 2003.

21. Muhammad A, Ahmed H, Iqbal MN, Qayyum M: Detection of multiple anthelmintic resistance of *Haemonchus contortus* and *Teladorsagia circumcincta* in sheep and goats of Northern Punjab, Pakistan. *Kafkas Univ Vet Fak Derg*, 21 (3): 389-395, 2015. DOI: 10.9775/kvfd.2014.12581

22. Nari A, Salles J, Gil A, Waller PJ, Hansen JW: The prevalence of anthelmintic resistance in nematode parasites of sheep in southern Latin America: Uruguay. *Vet Parasitol,* 62, 213-222, 1996. DOI: 10.1016/0304-4017(95)00908-6

23. Zajac AM, Gipson TA: Multiple anthelmintic resistances in a goat herd. *Vet Parasitol*, 87, 163-172, 2000. DOI: 10.1016/S0304-4017(99)00174-0

24. Uppal RP, Yadav CL, Bhushan C: Efficacy of closantel against fenbendazole and levamisole resistant *Haemonchus contortus* in small ruminants. *Trop Anim Health Prod,* 25, 30-32, 1993.

25. Prichard RK: Anthelmintic resistance in nematodes: Extent, recent understanding and future directions for control and research. *Int J Parasitol*, 20, 515-523, 1990. DOI: 10.1016/0020-7519(90)90199-W

26. Hussein AF: Effect of biological additives on growth indices and physiological responses of weaned najdi ram lambs. *J Exp Biol Agric Sci*, 2 (6): 597-607, 2014.

27. Kenyon F, McBean D, Greer AW, Burgess CGS, Morrison AA, Bartley DJ, Bartley Y, Devin L, Nath M, Jackson F: A comparative study of the effects of four treatment regimens on ivermectin efficacy, body weight and pasture contamination in lambs naturally infected with gastrointestinal nematodes in Scotland. *Int J Parasitol Drugs Drug Resist*, 3, 77-84, 2013. DOI: 10.1016/j.ijpddr.2013.02.001

28. Chaichisemsari M, Eshratkhah B, Maherisis N, Sadaghian M, Hassanpour S: Evaluation of total protein, albumin, globulin and blood urea nitrogen concentrations in gastrointestinal nematodes infected sheep. *Global Vet,* 6, 433-437, 2011.

29. Akanda MR, Hossain FMA, Ashad FA, Kabir MG, Howlader MMR: Anthelmintics against gastrointestinal nematodiasis in black Bengal goat inducing live weight and hematological indices. *Pharmacologia*, 3 (12): 700-706, 2012.

30. Shalaby HA: Anthelmintics resistance; How to overcome it? *Iran J Parasitol*, 8 (1): 18-32, 2013.

Kafkas Univ Vet Fak Derg
25 (3): 379-385, 2019
DOI: 10.9775/kvfd.2018.21021

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Influence of Anticoccidials on Oxidative Stress, Production Performance and Faecal Oocyst Counts in Broiler Chickens Infected with *Eimeria* Species

Marko PAJIĆ [1] Nevenka ALEKSIĆ [2] Branislav VEJNOVIĆ [3] Vladimir POLAČEK [1]
Nikolina NOVAKOV [4] Dušica Ostojić ANDRIĆ [5] Zoran STANIMIROVIĆ [2]

[1] Department for Epizootiology, Clinical Diagnostic, Pathology and DDD, Scientific Veterinary Institute "Novi Sad", Rumenački Put 20, 21000 Novi Sad, SERBIA
[2] Department of Biology, Faculty of Veterinary Medicine, University of Belgrade, Bulevar Oslobodjenja 18, 11000 Belgrade, SERBIA
[3] Department of Economics and Statistics, Faculty of Veterinary Medicine, University of Belgrade, Bulevar Oslobodjenja 18, 11000 Belgrade, SERBIA
[4] Department for Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, SERBIA
[5] Department of Animal Breeding and Genetics, Institute for Animal Husbandry, Autoput 16, 11080 Zemun-Belgrade, SERBIA

Article Code: KVFD-2018-21021 Received: 24.09.2018 Accepted: 31.12.2018 Published Online: 31.12.2018

How to Cite This Article

Pajić M, Aleksić N, Vejnović B, Polaček V, Novakov N, Andrić DO, Stanimirović Z: Influence of anticoccidials on oxidative stress, production performance and faecal oocyst counts in broiler chickens infected with *Eimeria* species. *Kafkas Univ Vet Fak Derg,* 25 (3): 379-385, 2019. DOI: 10.9775/kvfd.2018.21021

Abstract

The influence of certain anticoccidial drugs on oxidative stress in broiler chickens infected with *Eimeria* species was assessed. There were two untreated (uninfected and infected), and three groups infected and treated with anticoccidials. The first treated group (Ro) was given robenidine, the 2nd a herbal anticoccidial (Herb) and the 3rd the combination of robenidine and the herbal anticoccidial (Ro+Herb). All infected groups were on day 14 challenged with oral inoculation of oocysts. The activities of catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST), and the concentration of malondialdehyde (MDA) were estimated in blood taken on days 21 and 40. The oocyst numbers were calculated per gram, and chicken body weight and feed conversion ratio (FCR) measured. The activities of CAT, GST and the level of MDA were significantly lower ($P<0.05$), whilst the activity of SOD was higher in infected chickens treated with anticoccidials ($P<0.05$) in comparison to those untreated. The most prominent change in the parameters of oxidative stress was recorded in the Ro+Herb group. In chickens treated with anticoccidials body weight was significantly higher ($P<0.05$), and the FCR and the oocyst counts significantly lower ($P<0.05$) than in untreated chickens. Oocyst counts were lower in the Ro and Ro+Herb groups than in the Herb group. Our study demonstrated that both anticoccidial substances exerted antioxidant and anticoccidial effects.

Keywords: *Broilers, ROS, Coccidia, Robenidine, Herbal anticoccidial*

Antikoksidiyal Maddelerin *Eimeria* Türleri İle Enfekte Etlik Piliçlerde Oksidatif Stres, Üretim Performansı ve Dışkı Oosit Sayıları Üzerine Etkisi

Öz

Eimeria türleri ile enfekte Broilerlerde bazı antikoksidiyal ilaçların oksidatif stress üzerine etkisi araştırılmıştır. Çalışmada, enfekte edilmeyen ve enfekte edilen olmak üzere iki, ayrıca enfekte edilerek uygulama yapılan üç grup vardı. Uygulama yapılan gruplardan ilkine robenidin (Ro), ikincisine bitkisel antikoksidiyal (Herb) ve üçüncüsüne robenidin ile birlikte bitkisel antikoksidiyal (Ro+Herb) uygulandı. Tüm enfekte edilen gruplara 14. günde oral oosit inookulasyonu yapıldı. Çalışmanın 21 ve 40. günlerinde alınan kan örneklerinde katalaz (CAT), süperoksit dismutaz (SOD) ve glutatyon S-transferaz (GST) aktiviteleri ile malondialdehit (MDA) konsantrasyonu incelendi. Her bir gramdaki oosit sayıları hesaplandı ve tavukların vücut ağırlıkları ile yem konversiyon oranları ölçüldü. Uygulama yapılmayan grupla karşılaştırıldığında antikoksidiyal uygulanan gruplarda CAT, GST aktivitileri ve MDA seviyeleri anlamlı olarak daha düşük ($P<0.05$), SOD aktivitesi ise daha yüksekti ($P<0.05$). Oksidatif stress parametrelerindeki en belirgin değişim Ro+Herb grubunda kayıt edildi. Antikoksidiyal uygulanan etlik piliçlerde vücut ağırlığı uygulanmayanlara göre anlamlı olarak daha yüksek ($P<0.05$), yem konversiyon oranı ile oosit sayıları ise daha düşüktü ($P<0.05$). Ro ve Ro+Herb gruplarında oosit sayıları Herb grubundan daha düşüktü. Çalışmamız, kullanılan her iki antikoksidiyal maddenin de antioksidan ve antikoksidiyal etkileri olduğunu göztermiştir.

Anahtar sözcükler: *Broiler, ROS, Koksidiya, Robenidin, Bitkisel antikoksidiyal*

İletişim (Correspondence)
+381 21 4895 385 Mobile: +381 64 8185 426 Fax: +381 21 518 544
markopajic@niv.ns.ac.rs

INTRODUCTION

Coccidiosis is one of the economically most important diseases threatening intensive broiler production. The annual world losses are estimated to reach 2.3 billion euros [1]. Like some other pathogens [2-5], coccidia may also cause oxidative stress [2,6,7]. Reactive oxygen species (ROS) react spontaneously, targeting membrane lipids. The oxidative destruction of unsaturated fatty acids causes cell membrane damages, its decreased function and increases its permeability. These damages lead to a series of reactions which result in permanent consequences on chicken health, or even death [2,6].

Modern broiler production renders fattening almost unimaginable without anticoccidials. To avoid residues in meat, synthetic and ionophore anticoccidials are withdrawn 3-7 days before slaughter [8]. By contrast, herbal anticoccidials are administered until the end of the fattening period [9]. Leaving no residues, herbal medicines in the prevention of certain animal diseases have been arousing interest [10,11].

This research was aimed at the investigation into the influence of certain anticoccidials on oxidative stress, production performance and faecal oocyst counts in broiler chickens infected with *Eimeria*. Thus, the activities of antioxidative enzymes (catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST)) and the concentrations of malondialdehyde (MDA) were measured, and feed conversion ratio (FCR) and shed oocyst counts were assessed.

MATERIAL and METHODS

The research was conducted on 250 clinically healthy, one-day-old, unsexed broiler chickens (Ross 308 hybrid), average body weight of 39.6±1.5 g. They were randomly placed into 25 boxes, each containing 10 birds. Five boxes chosen at random were assigned to each experimental group: two control and three treated groups. Thus, each group had five replicates of 10 birds, which were kept in a separate place, unable to maintain any mutual contact. All chickens were housed on deep litter, except the negative control, which was kept in a box with meshed floor. The chickens were fed on standard commercial feed. The control groups were not treated with anticoccidials. The negative control remained uninfected, whilst the positive was infected on day 14 [12]. The Ro group was administered robenidine. The Ro+Herb group received in the first two weeks robenidine, which was followed by the administration of the herbal anticoccidial from day 15 until slaughter. The Herb group was given the herbal anticoccidial during the whole fattening period. All treated groups were infected on day 14 in the same way as the positive control.

The experiment was done at the Institute for animal husbandry (Belgrade), approved of by its Ethical committee (Decision no. 323-07-2340/2017-05) and performed in accordance with the recommendations of the European Commission (Directive 2010/63 EEC) [13] and the law on animal welfare [14].

The synthetic anticoccidial robenidine (Robenz® 66G, Zoetis Ltd) was mixed in feed (450-550 g/t), given to chickens from day 1 and withdrawn 5 days before slaughter.

The phytogenic feed additive (Herbakoks, Essentico DOO, Kula, Serbia), a mixture of essential oils (mainly derived from *Thymus vulgaris*, *Origanum vulgare* and *Coriandrum sativum*), organic acid salts, dextrose, sodium chloride was applied as recommended by the manufacturer. The details of the products' recipe are proprietary.

The oocysts of *Eimeria* species were obtained from naturally infected farm chickens, isolated by flotation and preserved in 2.5% potassium dichromate solution [15]. The experimental chickens were orally infected with sporulated oocysts: 1.5 mL of suspension containing $5x10^5$ sporulated oocysts administered with a disposable syringe.

Venous blood was taken on days 21 and 40 in heparinized tubes (BD Vacutainer®) and centrifuged to separate the plasma. The red blood cells were rinsed three times in physiological saline solution and stored at -20°C until analysis.

The activities of CAT [16] and SOD [17] were analysed in the hemolisates and expressed in units/g of haemoglobin. GST activity (mmol of GSH-CDNB conjugate formed/min/mg of haemoglobin) was determined [18]. The MDA levels (nmol MDA/g of haemoglobin) were estimated spectrophotometrically [19].

Haemoglobin concentrations were estimated as described in Tentori and Salvati [20]. All biochemical analyses were done simultaneously in triplicate for each sample using the Biobase UV/VIS spectrophotometer.

The chickens were measured individually at the beginning and at the end of the experiment. Body weight gain, feed consumption and FCR were calculated pen wise.

For FCR and oocyst counts each pen was the experimental unit, and for the biochemical and body weight assessment it was each animal. Given that the data on FCR were heterogeneous, the groups were compared using Kruskal-Wallis ANOVA followed by Dunn's multiple-comparison test. Data on oocyst counts were heterogeneous, and the transformation $\log_{10}$(value+1) was applied to all data. Data on biochemical analyses and live body weight were normally distributed (Shapiro-Wilk's test, $P>0.05$), and along with oocyst yields compared using the two-way ANOVA with repeated measures in one factor followed by Tukey´s test. All analyses were performed with GraphPad Prism 6 (GraphPad, USA).

RESULTS

Superoxide dismutase activities in the blood of 21- and 40-day-old chickens are shown in *Fig. 1*. A significant decrease in its activities were detected in the positive control in comparison to the negative one (P<0.05) at both time points. The activities of SOD were significantly higher in infected chickens treated with both anticoccidials in comparison with the infected but untreated broilers (P<0.05). The differences in SOD activities between the treated groups at both time points did not differ significanty (P>0.05).

The activities of the antioxidative enzyme CAT in 21- and 40-day-old chickens in all experimental groups are presented in *Fig. 2*. In infected untreated broilers the activities were significantly increased in comparison to uninfected ones (P<0.05). By comparison with the infected but not treated group, a significant decrease was noticed in the CAT activity in groups treated with anticoccidials (P<0.05) in both 21- and 40-day-old broilers. At the first time point the activity of CAT in the Herb group did not differ significantly from the one in the Ro group. However, at the second time point the herbal anticoccidial led to a significant decrease in the

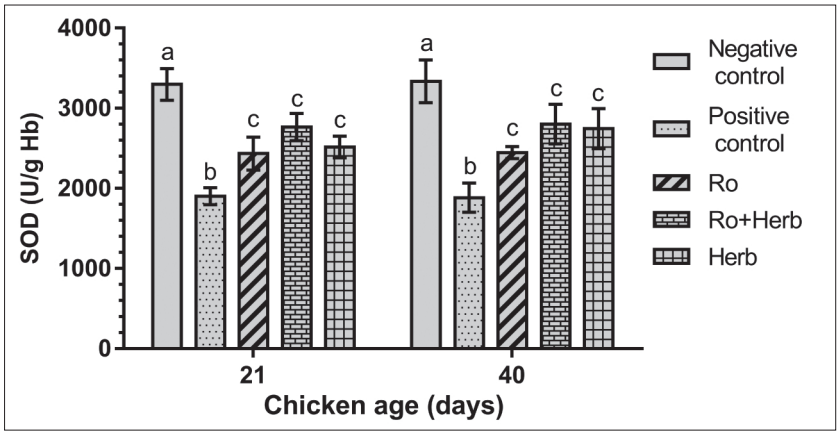
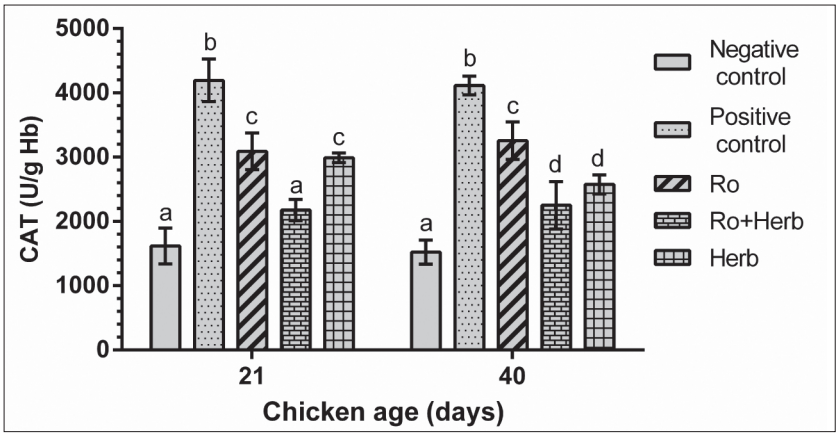


Fig 1. SOD activities in blood in 21- and 40-day-old chickens (mean ± SD). Different letters in chickens of the same age indicate significant differences between the groups (P<0.05). Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine + herbal anticoccidial

Fig 2. CAT activities in blood in 21- and 40-day-old chickens (mean ± SD). Different letters in chickens of the same age indicate significant differences between the groups (P<0.05). Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine + herbal anticoccidial



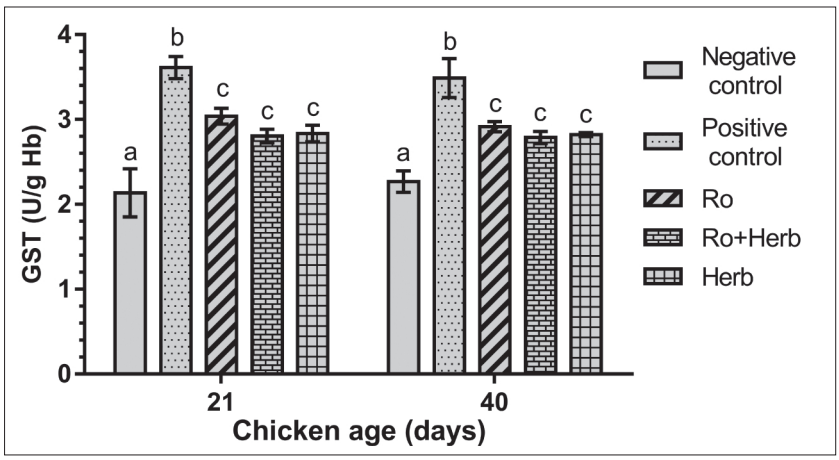


Fig 3. GST activities in blood in 21- and 40-day-old chickens (mean ± SD). Different letters in chickens of the same age indicate significant differences between the groups (P<0.05). Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine + herbal anticoccidial

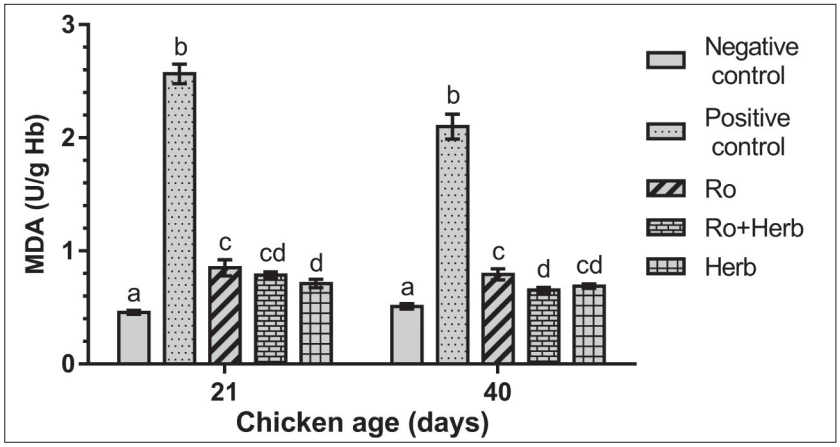


Fig 4. Concentration MDA in blood in 21- and 40-day-old chickens (mean ± SD). Different letters in chickens of the same age indicate significant differences between the groups (P<0.05). Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine + herbal anticoccidial

Table 1. *Body weight and FCR in control and treated chicken groups*

Groups	Initial Body Weight (g) Mean±SD	Final Body Weight (g) Mean±SD	FCR Median (IQR)
Negative control	39.84±1.18[a]	2821.22±531.82[a]	1.61 (1.45-1.85)[a]
Positive control	39.87±0.99[a]	1858.68±533.93[b]	2.76 (2.10-3.62)[b]
Ro	39.67±1.06[a]	2618.70±494.79[ac]	1.69 (1.57-2.08)[ac]
Ro+Herb	39.62±0.91[a]	2440.35±391.35[c]	1.91 (1.76-2.07)[c]
Herb	39.72±0.96[a]	2523.00±521.94[cd]	1.82 (1.60-2.28)[ac]

Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine+herbal anticoccidial
[a-d] Values within a column with different superscripts are significantly different (P<0.05)

CAT activity in comparison to robenidine (P<0.05). The most pronounced decrease of CAT activity among treated groups was observed in Ro+Herb group in 21-day-old broilers (P<0.05).

In the positive control there were significant increases in the GST activity (P<0.05) in comparison to the negative control *(Fig. 3)*. Moreover, in the groups treated with anticoccidials the average activities of this enzyme were significantly lower than that in the infected but untreated group (P<0.05). By comparison of the GST activity between the treated groups it was revealed that there were no significant differences between groups of 21-day-old and 40-day-old chickens (P>0.05).

In *Fig. 4* the average concentrations of MDA in the experimental broilers can be seen. The concentrations of this lipid oxidation marker were significantly higher in the positive control in comparison to the negative one (P<0.05), as well as comparison with infected broilers which were treated with anticoccidials (P<0.05). These changes apply for both 21- and 40-day-old broilers. As for the treated groups, in 21-day-old chickens the lowest average MDA concentration was measured in Herb group (P<0.05). However, in 40-day-old broilers MDA level was lowest in the Ro+Herb group (P<0.05). By comparison of the MDA activity between the treated groups it was revealed that there were significant differences between Ro and Herb groups in 21-day-old chickens as well as between Ro and Ro+Herb in 40-day-old chickens (P<0.05).

The average body weight and FCR were monitored throughout the experiment *(Table 1)*. In the beginning, on day 0, the average body weight was uniform in all experimental groups. However, in the end it was significantly lower in the positive control than in all others (P<0.05). FCR in the positive control was significantly higher (P<0.05) than in uninfected and infected but treated groups (P<0.05).

In uninfected broilers in faecal samples taken on days 14, 21, 28, 35 and 42 oocysts were not detected *(Fig. 5)*. In infected untreated chickens eimerian oocysts were first detected on day 21 and were continually on increase. In Ro group the oocyst counts were significantly lower than in the positive control. Similar tendency was observed in Ro+Herb group, where a reduction of oocyst counts was noticed in comparison to the positive control (P<0.05). In Herb group the numbers of oocysts per gram were higher than in chickens treated with robenidine and those which were treated with both anticoccidials, but remained significantly lower than in the positive control.

DISCUSSION

In spite of plenty of literature data which indicate that there are significant differences in the parameters of oxidative stress in chickens infected with coccidia, those obtained on broilers treated with anticoccidials are scarce. Monitoring of the parameters of oxidative stress following their application can provide useful information on the

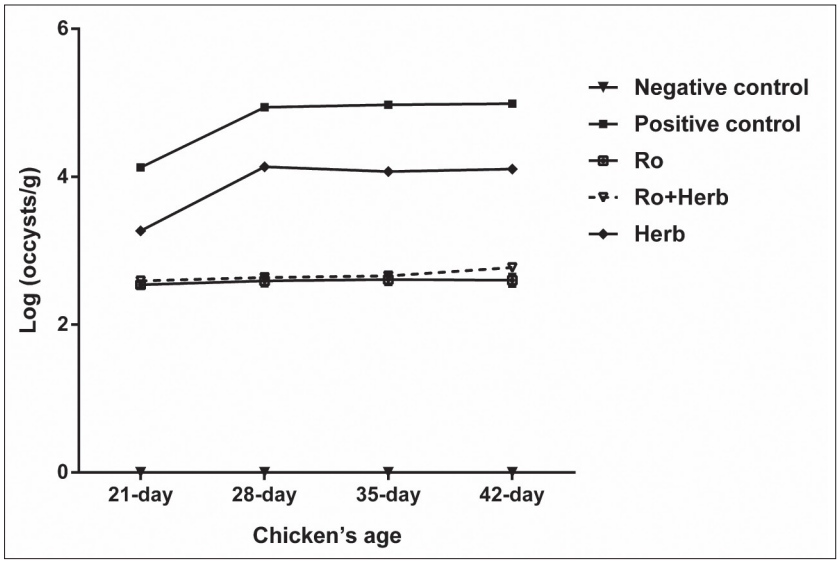


Fig 5. Faecal oocyst counts (o.p.g.) in control and treated chicken groups. Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine + herbal anticoccidial

possible antioxidative effects of anticoccidial drugs. In the current research the synthetic anticoccidial robenidine, a herbal anticoccidial preparation, and their combination were administered to broilers infected with *Eimeria*. The results indicated that the anticoccidials applied significantly influenced the changes in the oxidative stress parameters (SOD, CAT, GST and MDA) in infected chickens.

The results of this research detected lower activity of SOD in the blood of infected but untreated broilers (positive control). SOD is involved in the antioxidative defence system, the first line of defence against ROS [4]. Lower SOD activity in the blood of infected poultry than in uninfected probably results from the increased production of H_2O_2. Decreased SOD activity in broilers infected with coccidia has also been detected by some other researchers [2,21]. The administration of anticoccidials in infected chickens resulted in increased activity of SOD, most prominent in the Ro+Herb group, which means diminished the effect of oxidative stress. It is supposed that the anticoccidials prevented the inactivation of SOD by H_2O_2, which results from the dismutation of superoxide anion [22]. Bozkurt et al.[12] were the ones who also noticed significant increase in the activity of SOD in infected chickens fed with feed supplemented with the anticoccidial based on oregano oil.

Besides SOD, the activity of CAT was monitored following the treatment of infected broilers with anticoccidials. There are no published data on the influence of the combination of herbal and synthetic anticoccidials on CAT activity. In our experiment the largest increase in the activity of this enzyme was detected in the positive control, which is in accordance with the findings published by Georgieva et al.[2]. Similar results were obtained by other authors, who pointed to the increase in the activity of CAT in broilers infected with *E. acervulina* and *E. tenella* [21,23]. CAT activity is on the increase in oxidative stress, which is considered a compensatory mechanism in poultry infected with coccidia. In infected chicken groups which were administered anticoccidials in feed lower CAT activity was detected than in infected but untreated broilers. In the treated groups the largest decrease in the activity of CAT was noticed in the Ro+Herb group, which means that the combination of the two anticoccidials significantly influenced the decrease in the oxido-reduction disbalance which resulted from the response to the presence of the parasites. CAT plays an important role in preserving the cellular integrity by degrading the reactive hydrogen peroxide, which can lead to the emergence of reactive hydroxyl radical. Hydroxyl radical is highly unstable and can produce cellular damage via lipid peroxidation, and the oxidation of DNA and proteins [24].

The analysis of GST in this research detected its increased activity in infected chickens. The same phenomenon in broilers, due to some environmental factors, was described by Ismail et al.[25] GST is involved in the protection of cells from the negative effects of ROS, to which it bonds directly, covalently and renders it less reactive [26]. In this research the GST activity was lower in treated groups in comparison to the positive control, which leads to a hypothesis that the anticoccidials influenced the decrease in the substrate (ROS) production resulting in decreased activity of the detoxication enzyme. The largest decrease in the GST activity was observed in the Ro+Herb group. In the research conducted by Giannenas et al.[27] it was revealed that preparations based on fungi can increase the activity of GST and positively influence growth and feed utilization, and stimulate the secretion of digestive enzymes. Similarly, it was proven that a herbal preparation based on rosemary exerts antioxidative effects by increasing the activity of GST [28].

Malondialdehyde is produced in the process of lipid peroxidation due to the influence of ROS on the poly-unsaturated lipids. Its concentrations in blood and tissues are directly proportional to cellular damages caused by ROS and is, for this reason, a useful marker in the analysis

of oxidative stress [2]. The results of the current research revealed increased concentrations of MDA in infected chickens, resulting from increased lipid peroxidation. Similar data on the changes in the concentrations of MDA were reported by some other researchers [2,21,29]. Significant decrease in the levels of MDA was detected in broilers in all treated groups, which leads to conclusion that anticoccidials influenced the decrease in the production of ROS and thus led to reduced lipid peroxidation. In the research undertaken by Bozkurt et al.[12] it was found that herbal anticoccidials mitigate oxidative stress by decreasing the concentrations of MDA. Giannenas et al.[27] noticed that preparations based on fungi have anti-oxidative properties and decrease the concentrations of MDA.

The analysis of production performance of the broilers in the experiment showed that the lowest average body mass was in infected untreated chickens, which had the highest FCR. This lower weight gain can be attributed to infection with coccidia. Developing in the intestines *Eimeria* produce mucosal disruptions resulting in malabsorption and direct negative effect on growth, and facilitate infections with other pathogens [30]. It is obvious from the performance data that the broilers treated with robenidine had highest average body mass and lowest FCR. The lowest average body mass was recorded in chickens treated with both anticoccidials (Ro+Herb). Our results of increased weight gain and body weight in broilers treated with both the synthetic and herbal anticoccidials are in line with some previous data and resulted from their beneficial effects [29,31]. Positive effects on production performance resulting from the use of chemical and herbal anticoccidials have already been described [32]. Herbal anticoccidials, unlike synthetic ones, do not leave residues in broiler meat, which is why they are gaining increasing interest [33,34]. They contribute to weight gain and decrease FCR, which can be explained by increased absorption area of the intestines and better enzyme activities resulting from healing [12,35,36]. Data showed that herbal anticoccidials containing oregano, thyme, coriander, carvacrol, thymol and some other active ingredients exert anticoccidial and antioxidative effects [9,12,35].

The results obtained in this work showed that infected broilers shed significantly lower oocyst counts following the treatment with anticoccidials in comparison to those untreated. The least average number of oocysts was detected in chickens treated with robenidine, proving its satisfactory anticoccidial effect. Its mechanism of action is based on the inhibition of oxidative phosphorylation in the parasite mitochondria, which prevents their development. Chickens treated with robenidine and the herbal anticoccidial shed small numbers oocysts in comparison with the infected untreated group, which indicates that this combination of anticoccidials produced strong anticoccidial effect. This corresponds to the data obtained with the use of combinations of diclazuril and oregano essential oil [9] and, amprolium and garlic [32]. The analysis of our results obtained by spectrophotometry showed that the combination of the two anticoccidials significantly influenced the decrease in the oxidative stress, given that the values of biochemical parameters were closest to those in uninfected broilers. By this mechanism, robenidine influenced the decrease of oocyst counts in broilers, whilst the use of the herbal anticoccidial influenced the increased level of antioxidative defence, which resulted in the obtained values of oxidative stress parameters. In the current research it was proven that herbal extracts (oregano, thyme and coriander) in the tested herbal anticoccidial exert antioxidative properties. Herbal anticoccidials mainly consist of bioactive compounds such as polyphenols, kinins, flavonoids, alkaloids and polypeptides. Phenol compounds of aromatic herbs and their essential oils are potent sources of natural antioxidants. Flavonoids can act as powerful antioxidants by scavenging free radicals and stop oxidative reactions [36]. In broilers which were administered the herbal anticoccidial faecal oocysts shedding was higher than in the other two treated groups (Ro, Ro+Herb). Nevertheless, the applied herbal preparation exerted powerful anticoccidial effect.

The synthetic and herbal anticoccidials significantly influenced the parameters of oxidative stress: the activity of CAT and GST and the concentration of MDA were lower, whilst the activity of SOD was higher in treated groups than in untreated infected broilers, which points to the decrease in oxidative stress. Moreover, the anticoccidials led to the decrease in the oocysts production. Oocyst counts were lower in Ro and Ro+Herb groups in comparison to Herb group, which means that the synthetic anticoccidial alone and in combination with the herbal one resulted in better effect in the control of coccidiosis than the herbal applied exclusively. However, it was proven that the tested herbal preparation can be used in coccidiosis control and the prevention of oxidative stress. These results can help in the selection of anticoccidial drugs and influence directly the decrease in the economic losses attributed to coccidiosis.

Acknowledgements

This work was supported by the grant provided by the Ministry of Education, Science and Technological Development of the Republic of Serbia for the Projects Nos. III 46002 and TR 31071.

REFERENCES

1. De Gussem M: Coccidiosis in poultry: Review on diagnosis, control, prevention and interaction with overall gut health. *Proceedings of the 16th European Symposium on Poultry Nutrition,* Strasbourg, 253-261, 2007.

2. Georgieva NV, Koinarski V, Gadjeva V: Antioxidant status during the course of *Eimeria tenella* infection in broiler chickens. *Vet J*, 172 (3): 488-492, 2006. DOI: 10.1016/j.tvjl.2005.07.016

3. Del Vesco AP, Gasparino E: Production of reactive oxygen species,

gene expression, and enzymatic activity in quail subjected to acute heat stress. *J Anim Sci*, 91 (2): 582-587, 2013. DOI: 10.2527/jas.2012-5498

4. Surai PF: Antioxidant systems in poultry biology : Superoxide dismutase. *J Anim Res Nutr*, 1 (1): 1-17, 2015. DOI: 10.21767/2572-5459.100008

5. Radakovic M, Davitkov D, Borozan S, Stojanovic S, Stevanovic J, Krstic V, Stanimirovic Z: Oxidative stress and DNA damage in horses naturally infected with *Theileria equi*. *Vet J*, 217, 112-118, 2016. DOI: 10.1016/j.tvjl.2016.10.003

6. Koinarski V, Georgieva N, Gadjeva V, Petkov P: Antioxidant status of broiler chickens, infected with *Eimeria acervulina*. *Rev Med Vet,* 10, 498-502, 2005.

7. El-Maksoud A, Afaf HA, Abdel-Magid D, El-Badry MA: Biochemical effect of coccidia infestation in laying hen. *Benha Vet Med J,* 26 (1): 127-133, 2014.

8. Łowicki D, Huczyński A: Structure and antimicrobial properties of monensin a and its derivatives: Summary of the Achievements. *BioMed Res Int,* 2013:742149, 2013. DOI: 10.1155/2013/742149

9. Mohiti-Asli M, Ghanaatparast-Rashti M: Dietary oregano essential oil alleviates experimentally induced coccidiosis in broilers. *Prev Vet Med*, 120 (2): 195-202, 2015. DOI: 10.1016/j.prevetmed.2015.03.014

10. Stanimirović Z, Glavinić U, Lakić N, Radović D, Ristanić M, Tarić E, Stevanović J: Efficacy of plant-derived formulation 'Argus ras' in *Varroa destructor* control. *Acta Vet-Beograd,* 67 (2): 191-200, 2017. DOI: 10.1515/acve-2017-0017

11. Khaliq K, Akhtar M, Awais MM, Anwar MI: Evaluation of immuno-therapeutic effects of *aloe vera* polysaccharides against coccidiosis in chicken. *Kafkas Univ Vet Fak Derg,* 23 (6): 895-901, 2017. DOI: 10.9775/kvfd.2017.17957

12. Bozkurt M, Ege G, Aysul N, Akşit H, Tüzün AE, Küçükyılmaz K, Borum AE, Uygun M, Akşit D, Aypak S, Şimşek E, Seyrek K, Koçer B, Bintaş E, Orojpour A: Effect of anticoccidial monensin with oregano essential oil on broilers experimentally challenged with mixed *Eimeria* spp. *Poult Sci*, 95 (8): 1858-1868, 2016. DOI: 10.3382/ps/pew077

13. European Commission: Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union,* 2010.

14. Zakon o Dobrobiti Životinja: Službeni Glasnik RS, 41/2009, 2009.

15. Carvalho FS, Wenceslau AA, Teixeira M, Carneiro JAM, Melo ADB, Albuquerque GR: Diagnosis of *Eimeria* species using traditional and molecular methods in field studies. *Vet Parasitol*, 176 (2-3): 95-100, 2011. DOI: 10.1016/j.vetpar.2010.11.015

16. Aebi H: Catalase *in vitro*. *Methods Enzymol*, 105, 121-126, 1984. DOI: 10.1016/S0076-6879(84)05016-3

17. Misra HP, Fridovich I: The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem,* 247 (10): 3170-3175, 1972.

18. Habig WH, Pabst MJ, Jakoby WB: Glutathione S transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem,* 249 (22): 7130-7139, 1974.

19. Girotti MJ, Khan N, McLellan BA: Early measurement of systemic lipid peroxidation products in the plasma of major blunt trauma patients. *J Traum,* 31 (1): 32-35, 1991.

20. Tentori L, Salvati AM: Hemoglobinometry in human blood. *Methods Enzymol,* 76, 707-715, 1981. DOI: 10.1016/0076-6879(81)76152-4

21. Liu YX, Liu YL, Yang JP, Li WT: Effects of dietary conjugated linoleic acid on the duodenal mucosal immunity response and redox status of broiler chicks infected with *Eimeria acervulina*. *Czech J Anim Sci,* 61 (4): 186-191, 2016. DOI: 10.17221/8850-CJAS

22. Nikolić-Kokić A, Blagojević D, Spasić MB: Complexity of free radical metabolism in human erythrocytes. *J Med Biochem,* 29 (3): 189-195, 2010. DOI: 10.2478/v10011-010-0018-7

23. Georgieva NV, Koinarski V, Gabrashanska M: Combined effect of Cygro® and vitamin E on oxidative stress status of broiler chickens infected with *Eimeria tenella*. *J Agric Sci Tech,* 2 (4): 191-196, 2010.

24. Aprioku JS: Pharmacology of free radicals and the impact of reactive oxygen species on the testis. *J Reprod Infertil*, 14 (4): 158-172, 2013.

25. Ismail IB, Al-Busadah KA, El-Bahr SM: Oxidative stress biomarkers and biochemical profile in broilers chicken fed zinc bacitracin and ascorbic acid under hot climate. *Am J Biochem Mol Biol*, 3, 202-214, 2013. DOI: 10.3923/ajbmb.2013.202.214

26. Chikezie PC: Glutathione S-transferase activity in diagnostic pathology. *Metabolomics,* 5 (4):153, 2015. DOI: 10.4172/2153-0769.1000153

27. Giannenas I, Pappas IS, Mavridis S, Kontopidis G, Skoufos J, Kyriazakis I: Performance and antioxidant status of broiler chickens supplemented with dried mushrooms *(Agaricus bisporus)* in their diet. *Poult Sci*, 89 (2): 303-311, 2010. DOI: 10.3382/ps.2009-00207

28. Ghozlan SA, El-Far AH, Sadek KM, Abourawash AA, Abdel-Latif MA: Effect of rosemary *(Rosmarinus officinalis)* dietary supplementation in broiler chickens concerning immunity, antioxidant status, and performance. *Alexandria J Vet Sci*, 55 (1): 152-161, 2017. DOI: 10.5455/ajvs.275350

29. Pourali M, Kermanshahi H, Golian A, Razmi GR, Soukhtanloo M: Antioxidant and anticoccidial effects of garlic powder and sulfur amino acids on *Eimeria*-infected and uninfected broiler chickens. *Iran J Vet Res,* 15 (3): 227-232, 2014.

30. Yegani M, Korver DR: Factors affecting intestinal health in poultry. *Poult Sci,* 87 (10): 2052-2063, 2008. DOI: 10.3382/ps.2008-00091

31. Assis RCL, Cury MC, Luns FD, Assis RL: Anticoccidial efficacy of drinking dater soluble diclazuril in the control of *Eimeria Acervulina* oocysts on experimentally-infected broiler chickens. *Arq Bras Med Vet Zootec,* 64 (5): 1188-1193, 2012. DOI: 10.1590/S0102-09352012000500016

32. Adulugba IA, Goselle ON, Ajayi OO, Tanko JT: Development of a potent anticoccidial drug: A phyto-synthetic approach. *Am J Phytomed Clin Ther,* 5 (1): 1-7, 2017.

33. Abou-Elkhair R, Gaafar KM, Elbahy NM, Helal MA, Mahboub HDH, Sameh G: Bioactive effect of dietary supplementation with essential oils blend of oregano, thyme and garlic oils on performance of broilers infected with *Eimeria* species. *Glob Vet,* 13 (6): 977-985, 2014.

34. Muthamilselvan T, Kuo TF, Wu YC, Yang WC: Herbal remedies for coccidiosis control: A review of plants, compounds, and anticoccidial actions. *Evid Based Complement Alternat Med,* 2016:2657981, 2016. DOI: 10.1155/2016/2657981

35. Peek HW, Landman WJM: Coccidiosis in poultry: Anticoccidial products, vaccines and other prevention strategies. *Vet Q,* 31 (3): 143-161, 2011. DOI: 10.1080/01652176.2011.605247

36. Masood S, Abbas RZ, Iqbal Z, Mansoor MK, Sindhu ZD, Zia MA, Khan JA: Role of natural antioxidants for the control of coccidiosis in poultry. *Pak Vet J,* 33 (4): 401-407, 2013.

Kafkas Univ Vet Fak Derg
25 (3): 387-396, 2019
DOI: 10.9775/kvfd.2018.21035

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Interrelationships of Serum and Colostral IgG (Passive Immunity) with Total Protein Concentrations and Health Status in Lambs [1]

Erhan GÖKÇE [1] Onur ATAKİŞİ [2]

[1] *This project supported by TUBITAK (Project Code: TOVAG 108 O 847) and Scientific Research Projects Coordination Unit of Kafkas University*

[1] Departments of Internal Diseases, Faculty of Veterinary Medicine, Kafkas University, TR-36300 Kars - TURKEY
[2] Department of Chemistry, Faculty of Art and Science, Kafkas University, TR-36300 Kars - TURKEY

Article Code: KVFD-2018-21035 Received: 25.09.2018 Accepted: 31.12.2018 Published Online: 31.12.2018

How to Cite This Article

Gökçe E, Atakişi O: Interrelationships of serum and colostral IgG (passive immunity) with total protein concentrations and health in lambs. *Kafkas Univ Vet Fak Derg,* 25 (3): 387-396, 2019. DOI: 10.9775/kvfd.2018.21035

Abstract

This study was designed to determine relationship between sheep serum before lambing, colostrum and 1-day-old lamb serum total protein (TP) and immunoglobulin-G (IgG) concentration and their effect on neonatal diseases and also the linear relationship between serum TP and IgG concentration (STPC and SIgGC, respectively) in different days of the neonatal period thereby determining the feasibility of TP concentration in the prediction of colostrum quality and passive immunity and to define a cut-off point for STPC and SIgGCat 24 h after birth (STPC-24 and SIgGC-24, respectively) associated with increased risk of illness or death in lambs. For this purpose, blood was obtained from the lambs and ewes at day 1 (n=325), at day 0 (before colostrum intake) and on different days in neonatal periods (n=50) and blood (10-15 days before lambing) and colostrum were obtained from the respective ewes related to the lambs. Mean serum TP and IgG concentrations on days 1, 2, 4, 7, 14 and 28 were significantly higher than values on day 0 (before colostrum intake) from the lambs remained healthy in neonatal period. The STPC-24 was significantly lower in diseased and dead lambs when compared to healthy lambs in the neonatal period (P<0.001 and P<0.001 respectively). The STPC-24 in lambs that died or became ill was 62% to 67% in SIgGC-24, respectively. Mean colostral TPC was significantly (P<0.05) higher in dams (n=254) of healthy lambs when compared to those of sick lambs in neonatal period. There was a significant correlation between the dams' STPC and both the SIgGC in dams and SIgGC-24 in their lambs (R=0.454, R=0.342, respectively). The study revealed that STPC-24 >55 g/L and SIgGC-24>600 mg/dL is probably consistent with adequate level of passive transfer. It was also noted that in addition to determining colostrum quality, STPC plays an essential role in the prediction and prevention of neonatal diseases in lambs. In conclusion, immediate and inexpensive determination of TP concentrations is beneficial in making timely management and treatment decisions related to failure of passive transfer (FPT).

Keywords: *Neonatal lamb, Colostrum, Total protein, IgG, Passive transfer failure*

Kuzularda Serum ve Kolostral IgG (Pasif İmmünite) Konsantrasyonlarının Total Protein ve Sağlık İle İlişkisi

Öz

Bu çalışma kuzulamadan önce koyun serum, kolostrum ve 1. günde kuzularının total protein (TP) ve immunoglobulin-G (IgG) seviyeleri arasındaki ilişkinin ve bu parametlerin neonatal hastalıklar üzerindeki etkisinin ve ayrıca neonatal periyodun farklı günlerinde serum TP ve IgG konsantrasyonları (STPK ve SIgGK) arasındaki linear ilişkinin belirlenmesi, böylece TP konsantrasyonunun pasif immunite ve kolostrumun kalitesinin belirlenmesinde kullanılabilirliğinin belirlenmesi ve doğumdan sonra 24. saat STPK ve SIgGK pasif immunite ve kuzularda hastalık ve ölüm riskini artıran eşik değerinin (STPK-24 ve SIgGK-24, sırasıyla) belirlenmesi için dizayn edildi. Bu amaçla tüm kuzulardan 1. gün (n=325), kolostrum almadan önce (0. saat/gün) ve sonraki günlerde (n=50) ve annelerinden kuzulamadan 10-15 gün önce kan ve ayrıca kolostrum örnekleri alındı. Neonatal periyotta sağlıklı olduğu belirlenen kuzulardan yaşamın 1, 2, 4, 7, 14 ve 28. günlerinde ortalama serum TP ve IgG seviyelerinin 0. güne göre (kolostrum almadan önce) önemli seviyede yüksek olduğu belirlendi. Neonatal dönemde hastalanan ve ölen kuzuların STPC-24'ları sağlıklı olanlara göre önemli seviyede düşük olduğu saptandı (sırasıyla P<0.001 ve P<0.001). Neonatal sağlıklı kuzuların annelerinin (n=254) ortalama kolostral TPK hastalanan kuzularınkine göre önemli seviyede (P<0.05) yüksek bulundu. Annelerin STPK'nun hem anne SIgGK'u hem de kuzularının SIgGK-24 ile arasında önemli bir korelasyon olduğu belirlendi (sırasıyla R=0.454 ve R=0.342). Bu çalışma STPK-24'nun >55 g/L ve SIgGK-24'nun >600 mg/dLolması yeterli konsantrasyonda pasif transferini işaret etmektedir. Bu çalışma ile ayrıca KTPK'larının kolostrumun kalitesinin belirlenmesinin yanında, neonatal hastalıkların belirlenmesi ve önlenmesinde önemli bir rolü olduğu da tespit edildi. Sonuç olarak total protein konsantrasyonlarının hızlı ve ucuz bir şekilde belirlenmesi pasif transfer yetmezliği ile ilgili sevk-idare ve tedavi kararlarının zamanında alınması açısından faydalıdır.

Anahtar sözcükler: *Neonatal kuzu, Kolostrum, Total protein, IgG, Pasif transfer yetmezliği*

İletişim (Correspondence)
+90 474 2426807/5237 Fax: +90 474 2426853
erhangokce36@hotmail.com

INTRODUCTION

Neonatal morbidity and mortality are important causes of economic loss for sheep farms [1-4], thus making this period the most critical [5-9]. The syndesmochorial placenta in ruminants does not allow the transfer of maternal antibodies, also known as immunoglobulins (Ig), to the fetus during pregnancy. Thus, lambs are born hypogammaglobulinemic. Therefore, neonatal lambs depend on ingestion and absorption of maternal antibodies in colostrum to provide humoral immunity during the neonatal period. This process is termed passive transfer and is determined by measuring serum IgG concentrations. To ensure adequate passive transfer of immunity, lambs should receive a sufficient volume and quality of colostrum within the first 12 h of life [5,8-11]. Inability of neonatal lambs to obtain and absorb sufficient amount of colostral IgG is a secondary immunodeficiency disorder termed as Failure of Passive Transfer (FPT). FPT results in hypogammaglobulinemia which in turn predisposes neonates to develop diseases and death [1,2,5,8-16]. Therefore, passive immunity plays a critical determining role in the short-term health and survival for lambs until their own immune system begins functioning fully. Numerous studies in the past three decades correlated neonatal diseases with inadequate serum IgG, in other words, FPT in animals and thus suggested the importance of IgG in the prevention of infections and enhancing growth performance in neonates [1,2,5,8,9,14,16-18].

Currently, the incidence of FPT in lambs ranges from 3.4% to 20%, with mortality rates fluctuating between 45% and 50% in the first 2 weeks of life, particularly the first week [3,5,11-13].

Major economic losses may occur in the farms experiencing FPT frequently. Thus, FPT is a major economic concern for producers. Therefore, it is prerequisite for sheep producers to prevent lamb sickness and losses by monitoring immune status of lambs [3,8,9].

Several methods are currently being used to detect FPT in newborn ruminants. SRID [19] and ELISA [20] are the most accurate tests for direct measurement of serum IgG concentration (SIgGC). However, these tests require significant diffusion time and are expensive [20]. Therefore, for the detection of FPT in individual herds, screening with indirect methods and confirmatory diagnosis with SRID or ELISA may be more appropriate [20]. Other tests, including the determination of serum total protein concentrations (STPC) [5,12,15], serum GGT activity [5,21,22] and serum γ-globulin concentrations [5,14], zinc sulfate turbidity (ZST) test [12,15,23] and the serum glutaraldehyde coagulation test [3], provide an approximate assessment of SIgGC based on the concentration of total globulins or other proteins associated with IgG during passive transfer in lambs [8,9,22]. The ability to obtain fast and accurate test results on the farm is imperative in making timely management and treatment decisions. Furthermore, the accurate and rapid availability of test results is important in terms of clinical practice, for example in the evaluation of prognosis and determination of alternative treatments of neonatal diseases [5,24].

Different studies were carried out in calves to quantify increased risk of death associated with low SIgGC. However, there is a scarcity of data concerning this association in lambs [5,20]. In neonatal calves, an increased risk of death and illness was associated with SIgGC below 1,000 mg/dL as determined by single radial immunodiffusion (SRID) [5,8,9]. However, a dividing line between hypogammaglobulinemia and normal SIgGC in neonatal lambs has not yet universally been accepted. Studies investigating association between risk of developing death and SIgGC [1,5,13,22]. Nevertheless, there is a lack of universally accepted threshold for the SIgGC below which FPT develops in lambs [5,22]. Furthermore, studies investigating relationship between passive immunity and lamb death beyond neonatal period where ZST test, an indirect method, was used and extended the results to cover neonatal period [14,15]. Studies evaluated passive immunity using ZST test revealed that ZST test units below 10 [12] and/or 20 [15] had increased of contracting disease in neonatal period. Therefore, the association between neonatal lamb death and SIgGC is not yet fully understood [25]. Furthermore, association between passive immunity and diseases encountered beyond neonatal period has not been fully elucidated in lambs. For this reason, the direct role of SIgGC for the prevention of diseases and data regarding the SIgGC or FPT threshold values that increases risk of sickness and death in the lambs is not yet clear.

Different methods are presently available for detecting STPC in newborn ruminants. These tests are practical, quick and inexpensive, as well as suitable for the field use. However, the accuracy of STPC to calculate IgG concentrations has only been evaluated in healthy lambs [5]. Furthermore, data regarding the serum TP threshold values associated with lamb health during the neonatal and subsequent periods and the accuracy of these threshold values as well as the availability of using TP concentration to assess the quality of sheep colostrum are either insufficient or lacking entirely.

The objectives of this study as follows; 1- to determine the relationship between the level of TP and IgG in the ewe's serum, colostrum and that in the one day-old lamb; 2- to determine whether survival or illness of the newborn lamb is correlated with concentration of passively acquired TP byone day-old lamb, thereby defining a cut-off point for STPC and SIgGC associated with increased risk of illness or death in lambs; 3- to identify a relationship between serum or colostral IgG and TP concentrations, thereby determining the accuracy of TP concentrations in the prediction of passive immunity (IgG) and colostrum quality; 4- to determine a relationship between serum IgG and TP concentrations in healthy lambs during the neonatal period, thereby demonstrating the feasibility of using STPC to identify the status of passive immunity in this period.

MATERIAL and METHODS

Animals: The study was conducted after obtaining ethical

approval from the Kafkas University Institutional Ethical Committee for Animal Care and Use (KAÜ-HADYEK, 2008-23). Details of animals, farm selection, farm management practices, clinical examination and blood and colostrums sampling method were given elsewhere [26]. Briefly, 301 ewes and their 347 lambs on two neighboring and similar management practices farms were included in the study.

IgG and Total Protein Assays: Serum IgG concentrations were measured using a commercial ELISA kit (Bio-X Competitive ELISA kit for Ovine blood serum IgG Assay-BIO K 350, Bio-X Diagnostics, Belgium). Colostrum IgG concentration was also tested with the same kit using bovine colostrum calibrator (Bio-X Elisa Kit for Bovine Immunoglobulin Assays-BIO K 165). Serum and colostral total protein (TP) concentrations were measured by using spectrophotometry with a commercial kit (TML, Total Protein, Code; A1279, Tani Medical, Turkey).

Statistical Analysis: The lambs were categorized based on the clinical examination as healthy or sick. In addition, sick lambs were also categorized as dead and recovered. The results of clinical examination were categorized in terms of life period as neonatal period and the period from 5 to 12 weeks of life (postneonatal period) to compare morbidity, mortality and their relations with serum IgG and TP concentrations in lambs. Animals whose serum or colostrum TP or IgG concentrations could not be measured for any reason excluded (n=22, only serum TP were not measured) from the analyses. Data was collected and entered into a database (Microsoft access).

Independent samples T test was used to compare serum or colostral IgG concentrations in different period of life. Time-dependent differences were localized by use of the Tukey HSD test. The relationship between serum IgG and TP concentrations were explored by Pearson correlation and simple/multiple regression analysis (SPSS).

The accuracy of STPC for estimating IgGC was established by using standard linear regression analysis previously described in detail [5,22,26]. Calculations were performed by use of SPSS. Origin 6 program was used to obtain scatter diagrams illustrations. For all analyses, values of $P<0.05$ were considered significant. Morbidity and mortality risk according to different SIgGC-24 and STPC-24 levels were calculated according to X^2 for trend (X^2, Odds ratios).

RESULTS

The morbidity and mortality rates in neonatal and post-neonatal periods, disease reasons were given elsewere (26). The majority of neonatal deaths occurred (84.6%, 11/13) in the first week of life.

The mean± SD (min-max) serum IgG (n=347) and TP (n=325) concentrations at the 24th h after birth were, 2198±1162 mg/dL (19-5302) mg/dL and 73±13 (21-117) g/L, respectively and were significantly (R^2=0.671, P=0.000) correlated. Serum TP and IgG concentrations were significantly ($P<0.001$) higher on days 1, 2, 4, 7, 14 and 28 of the neonatal periods compared to day 0 (before colostrum intake) in healthy lambs *(Table 1, Table 2)*.

Simple and multiple regression models were calculated between variables which had linear correlations *(Table 3)*. Multiple regression models were developed to predict SIgGC based on lamb's age and STPC. STPC was linearly and significantly ($P<0.001$) correlated with SIgGC on different days during the neonatal period *(Table 3)*.

The most accurate result for predicting SIgGC was on day 1 (R^2= 0.562) compared to other days. Additionally, in healthy neonatal lambs, multiple regression model established between variables with data obtained on days 1, 2, 4, 7, 14 and 28 before and after taking colostrum was determined as the most accurate model for calculation of SIgGC from STPC (R^2= 0.701) *(Table 3)*.

The SIgGC and STPC at 24 h after birth was lower in clinically ill, recovered and dead lambs compared to healthy lambs in the first week of life and the neonatal period *(Table 4)*.

Table 1. *Serum TP (g/L) and IgG (mg/dL) concentrations [mean±SD, (Min-Max)] in neonatal healthy lambs*

Parameter	Day						
	0	1 (±1th)	2	4	7	14	28
TP (n=41)	40±6^a (21-47)	73±11^b (52-107)	73±13^b (47-117)	71±9^b (49-86)	70±10^b (46-88)	63±7^c (48-78)	59±7^c (46-71)
IgG (n=50)	26±16^a (8-62)	2666±1316^b (781-5302)	2743±1359^b (805-5308)	2295±1110^b (709-5029)	1714±816^c (493-3518)	1013±401^d (295-1893)	935±357^d (301-1707)

Different letters refer to significant differences between the values in the same rows (P<0.05). Tukey HSD test was used to detect differences

Table 2. *The correlation between mean serum TP and IgG concentrations in neonatal healthy lambs (n=41)*

Parameter	Day						
	0	1	2	4	7	14	28
Pearson Correlation	0.157	0.749**	0.735**	0.680**	0.620**	0.729**	0.634**
Sig. (2-tailed)	0.327	0.000	0.000	0.000	0.000	0.000	0.000

*Statistically significant (**P<0.01 and *P<0.01). The relationship was determined by Pearson correlation test*

Table 3. *Simple and multiple regression analyses between IgG and TP of the clinical status of the lambs*

Parameter		Days	n	Formulas	R^2	P
Regression analysis in neonatal healthy lambs on different days	Simple Regression Analysis	0	41	IgG= 8.043+(0.449 x TP)	0.025	0.327
		1	41	IgG= (92.16 x TP)-3960.9	0.562	<0.0001
		2	41	IgG= (77.42 x TP)-2826.3	0.540	<0.0001
		4	41	IgG= (84.36 x TP)-3562.2	0.462	<0.0001
		7	41	IgG= (50.46 x TP)-1723.2	0.385	<0.0001
		14	41	IgG= (37.75 x TP)-1294.7	0.532	<0.0001
		28	41	IgG= (33.75 x TP)-1032.5	0.402	<0.0001
	Multiple Regression Analysis	0,1,2,4,7,14,28	287	IgG= [(73.4 x TP) - (29.74 x day)] - 2755.6	0.701	<0.0001
		1,2,4,7,14,28	246	IgG= [(72.4 x TP) - (31.75 x day)] - 2657.5	0.617	<0.0001
		1,2,4,7,14	205	IgG= [(72.1 x TP) - (79.2 x day)] - 2419.9	0.610	<0.0001
		1,2,4,7	164	IgG= [(76.7 x TP) - (125.2 x day)] - 2608.1	0.540	<0.0001
		1,2,4	123	IgG= [(83.8 x TP) - (64.4 x day)] - 3239.3	0.531	<0.0001
		1,2	82	IgG= [(83.4 x TP) + (67.1 x day)] - 3398.1	0.547	<0.0001
Simple regression analysis according to the results of clinical examination	Neonatal period	1	325[a]	IgG= (57.84 x TP) - 2047.9	0.451	<0.0001
		1	268[b]	IgG= (57.25 x TP) - 2000.5	0.327	<0.0001
		1	57[c]	IgG= (58.12 x TP) - 2049.4	0.673	<0.0001
		1	44[d]	IgG= (58.29 x TP) - 2074.8	0.567	<0.0001
		1	13[e]	IgG= (47.24 x TP) - 1649.2	0.616	<0.0001
	Postneonatal period	1	312[a]	IgG= (57.52 x TP) - 1988.6	0.381	<0.0001
		1	208[b]	IgG= (60.79 x TP) - 2181.9	0.383	<0.0001
		1	104[c]	IgG= (48 x TP) - 1429.2	0.322	<0.0001
		1	89[d]	IgG= (43.17 x TP) - 1156.5	0.289	<0.0001
		1	15[e]	IgG= (67.15 x TP) - 2423.8	0.483	0.0004

[a] *General (without categories based on clinical examination),* [b] *healthy,* [c] *ill,* [d] *recovered,* [e] *died*

A significant (P<0.0001) linear relationship was noted between SIgGC-24 and STPC-24 in healty, diseased, recovered and dead lambs (R^2=0.327, R^2=0.673, R^2=0.567 and R^2=0.616, respectively, *Table 3)* in neonatal period. STPC and SIgGC measured at 24 h after birth were associated with developing diseases in the first week of life; but, this was not evident during the last 3 weeks of neonatal life *(Table 4)*.

A significant linear relationship was found between SIgGC-24 and STPC-24 in health, ill, dead and recovered lambs (R^2=0.383, P< .0001; R^2=0.322, P< .0001; R^2=0.483, P< .004 and R^2=0.289, P< .0001, respectively, *Table 3)* in post-neonata period..

SIgGC-24 and/or STPC-24 markedly differed between sick, dead and recovered lambs in the neonatal and postneonatal periods of life. This difference was most obvious between the first week of life and the last three weeks of the neonatal period. Additionally, SIgGC-24 or STPC-24 was lower in clinically ill and dead lambs in the neonatal period compared to the same groups in the postneonatal period *(Table 4)*.

The levels of SIgGC-24 were allocated into various categories *(Table 5)*. As SIgGC-24 concentrations increased morbidity and mortality rate decreased in neonatal period. The critical threshold of SIgGC-24 for increased risk of mortality and morbidity in the neonatal period was <200 mg/dL (OR=Undefined x^2=293, P=0.0000) and ≤600 mg/dL (OR=107.7 x^2=76.5, P=0.0000), respectively. The morbidity risk of lambs with SIgGC-24 <800 mg/dLwas approximately 4.4 times higher in postneonatal period when compared with lambs having SIgGC-24 above 800 ng/mL (OR=4.37 x^2=6.5, P=0.024). However, no specific SIgGC-24 was determined for mortality in this period *(Table 5)*.

The STPC-24 was also categorised *(Table 6)*. As STPC-24 concentrations increased the morbidity and mortality rate decreased during the neonatal period. The critical threshold of STPC-24 for mortality and morbidity in the neonatal period was ≤45 g/L (OR=51.56 x^2=103.8, P=0.000) and ≤55 mg/dL (OR=256.6 x^2=173.2, P=0.000), respectively. The morbidity risk of lambs with STPC-24 <70 g/L was 2.5 times higher in postneonatal period when compared with lambs having STPC-24 above 70 g/L (OR=2.54 x^2=14.5, P=0.00001). However, no specific TP level that increased the risk of death in this period was identified *(Table 6)*.

Colostral IgG concentrations (CIgGC) (n=169) ranged from 1337 to 12877 mg/dL (mean±SD, 6078 ± 2526 mg/dL) and colostral TP concentrations (CTPC) (n=254) were between 14 and 98 g/dL (42±18 g/dL). There were significant correlations (R^2=0.683, P=0.000) between these parameters *(Table 7)*. Furthermore, a significant linear relationship (R^2=0.460, P<0.0001) was determined between CTPC and CIgGC (Regression model; CIgGC=1941.6+91.35*CTPC, *Fig. 2)*. However, there was no significant correlation between STPC orSIgGCin lambs and CTPC or CIgGC *(Table 8)*.

CTPC and CIgGC were significantly (P<0.05) higher in dams

Table 4. *Effect of STPC(g/L) and SIgGC(mg/dl) determined at 24 h of birth on health status of lambs in neonatal and postneonatal periods*[a]

Periods		Health Status			
		Healthy	Ill	Outcome	
				Dead	Recovered
1st week	IgG	2319±1097 (271-5302)	475±508[b***] (19-1601)	54±32[b***] (19-113)	861±419[b***, e***] (271-1601)
	n	324	23	11	12
	TP	74±11 (44-117)	46±11[b***] (21-74)	38±8[b***] (21-46)	52±10[b***, e**] (35-74)
	n	302	23	11	12
2nd to 4th week	IgG	2337±1087 (527-5302)	2179±1174[c***] (271-4837)	1768±687[c***] (1283-2254)	2203±1198[c***] (271-4837)
	n	287	37	2	35
	TP	75±11 (44-117)	72±14[c***] (46-99)	62±4[c**] (60-65)	72±14[c***] (46-99)
	n	268	34	2	32
Whole Neonatal	IgG	2337±1087 (527-5302)	1526±1279[b***, d*] (19-4837)	318±674[b***, d***] (19-2254)	1860±1205[b**, e***] (271-4837)
	n	287	60	13	47
	TP	75±11 (44-117)	61±18[b***, d***] (21-99)	42±11[b***, d***] (21-65)	66±16 [b***, e***] (35-99)
	n	268	57	13	44
Postneonatal	IgG	2409±1113 (371-5302)	1982±1067[b**] (271-5017)	2311±1307 (571-4837)	1925±1018[b***] (271-5017)
	n	225	109	16	93
	TP	76±11 (46-117)	70±12[b***] (35-99)	72±14 (53-99)	70±12[b***] (35-92)
	n	208	104	15	89

[a] *Data are presented as the mean±SD. Numbers in parentheses represent the range of values in that group. Independent samples T-test was used to detect differences in the STPC-24 or SIgGC-24 among healthy, sick, dead and recovered lambs;* [b] *Significantly different from healthy lambs (b***= P<.0001, b**= P<0.01, b*=P<0.05);* [c] *Significantly different in the same group between the first week and the last three weeks in the neonatal period within the same parameters (i.e. comparison of STPC-24 in sick lambs between the two different periods, c***= P<0.001, c**= P<0.01, c*= P<0.05);* [d] *Significantly different in the same group between in neonatal and postneonatal periods within the same parameters (d***= P<.0001, d**= P<.001, d*= P<0.05);* [e] *Significantly different from deceased lambs (e***=P<0.001, e**=P<0.01, e*=P<0.05)*

of healthy neonates compared to those of sick and recovered lambs (P<0.05) but these values did not significantly differ in neonatal death (P=0.054 and P=0.125, respectively). Similarly, CTPC and CIgGC were not associated with diseases or death in the postneonatal period *(Table 7)*.

Maternal STPC and SIgGC did not significantly differ between healthy and sick and recovered lambs in the neonatal as well as postneonatal period *(Table 7)*.

The mean concentration of maternal STPC and SIgGC(n=41) 10-15 days prior to lambing was 62.4±6.9 g/L (range 52.9-78.3) and 5.463±1.870 mg/dL (range 1.846-11.766), respectively, and there were significant (R=0.454) correlations between these parameters. A significant positive correlation (P=0.01, R=0.388) existed between maternal SIgGC and at of lamb born to them. A significant (R=0.314, P=0.045) positive correlation existed between the SIgGC of dams and STPC of their lambs. Additionally, the correlations between maternal STPC and SIgGC of their lambs was positive and significant (R=0.342, P=0.029) *(Table 8)*.

DISCUSSION

Veterinarians often use tests for passive transfer to guide their decisions in diagnostic, treatment and protection in neonatal diseases. Determination of passive transfer status (PTS) results in two important consequences; either sufficiency which suggests better health and flock management system or insufficiency which requires diagnosis and treatment of such individuals and taking maximum preventive measures [5,8,9]. PTS determination of newborns is also valuable in routine herd health programs, observation during disease investigations and in the assessment of individual neonates with questionable colostral intake [8,9,24]. Passive transfer of immunity prevents disease and enhances growth performance not only in the short term, such as in neonatal period, but also in the long term, such as the post-weaning period in lambs [5,8,9,18,27]. Inadequate PTS may not be effect health provided that sanitation is excellent, but adequate PTS may be sufficient even if poor sanitation and extreme infectious pressure exist [8,9,27]. FPT in sick neonatal ruminants suggests a poor prognosis [5,8,9,24]. Therefore, quick and accurate tests for determining FPT applied on farm are ofparamount importance.

No currently available assay procedure is entirely satisfactory [5,8,9,20,27]. Being capable of directly measuring IgG concentration, ELISA has become available for use in calves

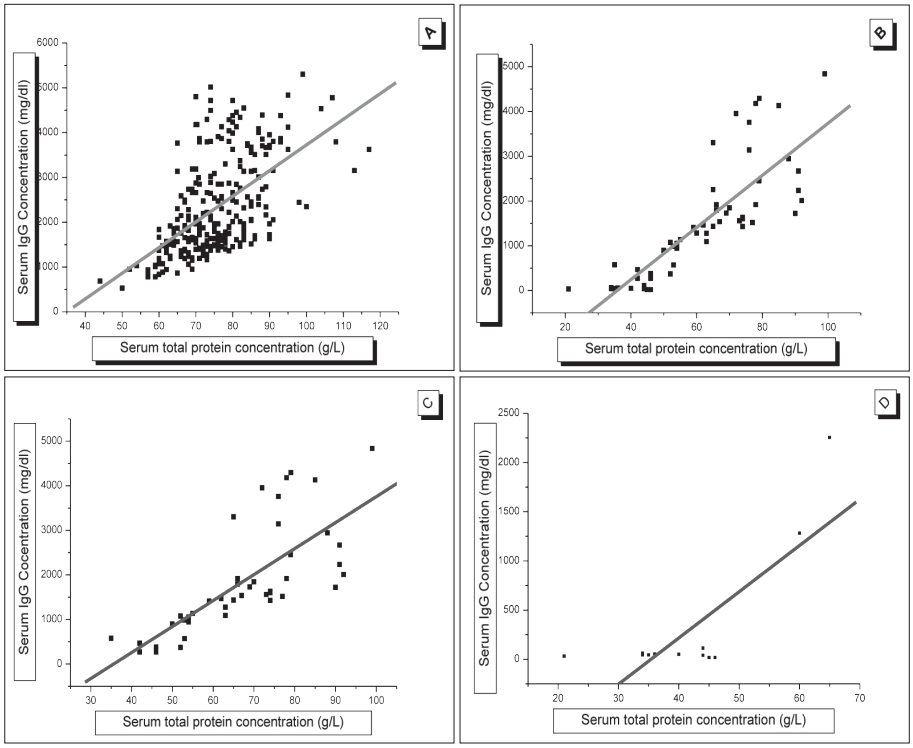


Fig 1. Scatter diagrams illustrating the associations between SIgGC-24 and STPC-24, observed in 268 healthy (A), 57 sick (B), 44 recovered (C) and 13 diseased (D) lambs in the neonatal period. Each data point represents a value for 1 lamb, and each regression line represents the best fit for the data

Table 5. *Lamb morbidity and mortality associated with various categories of SIgGC at 24th h after the birth*

IgG (mg/dL)	Neonatal Period				Postneonatal Period			
	Morbidity		Mortality		Morbidity		Mortality	
Categories	n^1/n^2	%	n^1/n^2	%	n^1/n^2	%	n^1/n^2	%
1-200	11/11	100	11/11	100	0/0	0	0/0	0
201-500	6/6	100	0/6	0	4/6	66.7	0	0
501-600	2/3	66.7	0/3	0	2/3	66.7	1/3	33.3
601-800	0/3	0	0	0	2/3	66.7	0	0
801-1000	3/14	21.4	0	0	6/14	42.9	2/14	14.3
1001-1500	11/67	16.4	1/67	1.5	29/66	43.9	3/64	4.5
1501-2000	11/87	12.6	0	0	25/87	28.7	2/87	2.3
2001-2500	5/42	11.9	1/42	2.4	15/41	36.6	2/41	4.9
>2501	11/114	9.6	0	0	26/114	22.8	3/108	5.3

n^1= number of sick or dead lambs, n^2= total number of lambs in this category

and there is only one study evaluating passive immunity in healthy neonatal lambs [5,25] and there is no such study conducted in diseased lambs. In the current study we used ELISA procedure designed to directly determine serum or colostral IgG concentrations in dams and their lambs. ELISA seems to have some advantages in terms of cost, time, and capacity for measuring large number of samples at once, better diagnostic performance over SRID, a gold standard test [20].

The optimum period to determine passive transfer is 24 h of life because neonatal ruminants are capable of absorbing many proteins, including macromolecular substances due to nonselective absorption by intestines within the first 24 h [8-10]. In this study mean SIgGC-24 determined before colostrums intake was in agreement with previous reports [5,21] and increased significantly after colostrum ingestion. The IgG concentration of day 1 was similar to that reported previously by researchers [5,22,27] but lower than that of others [16,21,28]. This variation might be related to the number of subjects investigated as previous studies used small number of animals, controlled colostrum intake and farm management system (vaccination, good hygiene and feeding practices etc.) [13,28].

Our study found that STPC (40±6 g/L) prior to colostrum intake (hour 0), similar to levels expected in severe passive immune deficiency, were lower than the levels (58-69 g/L) reported by Oztabak and Ozpinar [29], but similar to data from Pauli [30], 40.7 g/L. To the best of our knowledge, there are no other studies in which STPC is determined prior to colostrum intake. The mean STPC on different days of the first week of life (70-73 g/L) in our study was similar to figures reported by Brujeni et al.[16], but comparatively higher than

Table 6. *Lamb morbidity and mortality rates associated with various categories of STPC at 24 h after the birth*

TP (g/L)	Neonatal Period				Postneonatal Period			
	Morbidity		Mortality		Morbidity		Mortality	
Categories	n^1/n^2	%	n^1/n^2	%	n^1/n^2	%.	n^1/n^2	%
1-40	7/7	100	6/7	85.7	1/1	100	0/1	0
41-45	6/7	85.7	4/7	57.1	3/3	100	0/3	0
46-50	5/6	83.3	1/6	16.6	2/5	40	0/5	0
51-55	7/9	77.8	0/9	0	5/9	55.6	2/9	22.2
56-60	2/18	11.1	1/18	5.5	8/17	47.1	2/17	11.8
61-65	6/30	20	1/30	3.3	14/29	48.3	2/29	6.9
66-70	5/52	9.6	0/52	0	21/52	40.4	2/52	3.8
71-75	5/59	8.5	0/59	0	17/59	28,8	2/59	3.4
76-80	7/55	12.7	0/55	0	12/55	21,8	1/55	1.8
81-117	7/82	8.5	0/82	0	21/82	25.6	4/82	4.9

n^1= number of sick or dead lambs in this category, n^2= total number of lambs in this category

Table 7. *Relationship between maternal serum or colostral TP and/or IgG concentrations [mean±SD, (Min-Max)] and lamb health status*

Period	Sample		Clinical Examination			
			Healthy	Ill	Outcome After the Illness	
					Died	Survived
Neonatal	Serum	TP (g/L)	62±7 (53-78)	62±6 (56-73)	-	62±6 (56-73)
		IgG (mg/dL)	5578±2060 (1846-11766)	5054.8±890 (3192-5938)	-	5054.8±890 (3192-5938)
		N	32	9	0	9
	Colostrum	TP (g/dL)	43±18 (15-98)	35± 14[a] (14-82)	41±18 (25-82)	27±33[a] (14-55)
		N	219	35	8	27
		IgG (mg/dL)	6327±2392 (1337-12594)	5123.5±282[a] (1800-12877)	4702±2930 (1887-11127)	5269.4±2829[a] (1800-12877)
		N	134	35	9	26

[a] Significantly lower than healthy lambs (P<0.05)

Table 8. *Correlations between maternal serum or colostral TP and IgG concentration and serum TP and IgG concentration at 24 h after birth in lambs*

Correlations		TP[l]	IgG[l]	TP[c]	IgG[c]	IgG[d]	TP[d]
TP[c]	Pearson Correlation	-0.018	0.018	1.000	.683**	-0.113	-0.160
	Sig. (2-tailed)	0.777	0.781		0.000	0.599	0.457
	N	238	254	254	154	24	24
IgG[c]	Pearson Correlation	0.073	-0.009	0.683**	1.000	0.041	-0.241
	Sig. (2-tailed)	0.353	0.911	0.000		0.881	0.368
	N	162	169	154	169	16	16
IgG[d]	Pearson Correlation	0.314*	0.388*	-0.113	0.041	1.000	0.454**
	Sig. (2-tailed)	0.045	0.012	0.599	0.881		0.003
	N	41	41	24	16	41	41
TP[d]	Pearson Correlation	0.230	0.342*	-0.160	-0.241	0.454**	1.000
	Sig. (2-tailed)	0.148	0.029	0.457	0.368	0.003	
	N	41	41	24	16	41	41

[l] Lamb, [c] Colostrum, [d] Dam

that reported by Loste et al.[28], Massimini et al.[5], Pauli [30] and lower than that of Bekele et al.[2] and Oztabak and Ozpinar [29]. In our study, the mean STPC on day 14 (63 g/L) was lower than that reported by Brujeni et al.[16], Oztabak and Ozpinar [29], but comparatively higher than that of Loste et al.[28]. The mean STPC at day 28 (59 g/L), was lower than that reported by Brujeni et al.[16], but comparatively higher than that reported by Loste et al.[28]. Differences in the course

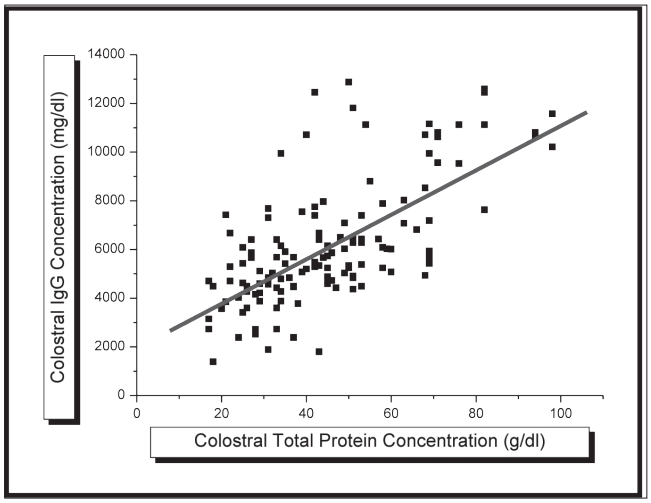


Fig 2. Scatter plot of colostral IgG and TP concentrations observed in 154 sheep. In the graph, the solid line represents the best fit for the data, as determined by means of simple linear regression

of STPC during the neonatal period might be explained based on methods used for measuring STPC such as refractometry [5,23,27], the biuret method [15,27], serum protein electrophoresis [16], spectrophotometric analysis also used in our study adapted to the biuret method [29] and automated biochemical analyzer [28] and management practices such as colostrum quality, the amount of colostrum taken, feeding program implemented. In our study STPC and SIgGC peaked within 24 h after colostrum intake in healthy lambs, generally remained stable during the first week and then declined significantly on day 14 and 28 of the neonatal period. Other studies have reported similar findings [16,28,29]. However, some studies have shown that STPC and SIgGC on day 30 were slightly higher than that of day 15 and have claimed that this could be associated with the balance between resecretion mechanisms and new IgG production or early activation of the immune system in lambs [5,16]. In addition, production of indigenous IgG following antigenic stimulation of the lambs' immune system may cause the slight increase in IgG concentration around day 30 [16]. However, STPC and SIgGC on day 28 were lower than those of day 14 in this study.

In the present study, neonatal losses were mainly encountered in the first week of life (84.6%) as reported earlier [3,11,13-15]. Accordingly, SIgGC were significantly lower in both ill and dead lambs in the first week of life when compared with other periods (last three weeks of neonatal life and postneonatal period) and thus making passive immunity a key factor in the first week of neonatal life. On the other hand, a study by Bekele et al.[2], reported that passive immunity had no effect on neonatal lamb mortality, but the threshold value of serum Ig set above 2300 mg/dL for adequate passive immune status was quite high compared to other studies [5,29]. Universally accepted optimal threshold value of IgG by the veterinary community, below which FPT occurs in lambs, does not exist. Information regarding the risk of illness or death associated with varying categories of SIgGC is limited for lambs [5,22]. Studies, using various threshold values of different indicators, have revealed that 24 to 36 hour-old lambs had an increased risk of death when SIgGC were below 800 mg/dL [11], 1.500 mg/dL [8], 600 mg/dL(IgG_1) [13] and 500 mg/dL(γ-globulin) [14]. The lamb mortality rates of aforementioned studies were 46% (27/59), 18% (2/11), 45% (9/20) and %60 (3/5), respectively. In our study, the mortality rate in lambs having SIgGC-24 below 500 mg/dL, 600 mg/dL, 800 mg/dL and 1500 mg/dL was 64.7% (11/17), 52.4% (11/20), 47.8%(11/23) and 11.5% (12/104), respectively. This finding is in accordance with many studies but slightly differs from some [10,13]. However, use of different strata of SIgGC may be misleading as all lambs having SIgGC-24 concentrations below 200 mg/dL died in our study. Therefore SIgGC-24 below 200 mg/dL may be considered as a significant threshold value for lamb mortality. A study in which the same categorization criteria as ours was used disclosed that the mortality rate in 36-hour-old lambs with serum IgG_1 concentrations below 1.000 mg/dL was 3 to 4 times greater when compared with higher concentrations [1]. However, the mortality rates greatly differed between our study and that of Gilbert et al.[1]. The reasons for this difference may be the cut off value used by Gilbert et al.[1] as the value was much greater and thus variation in mortality might have been wider and also the method used for measurement of IgG concentration was SRID. Studies disclosed that the threshold value of IgG below which passive immunity develops is <1000 mg/dL when SRID used while it was <500 mg/dL when ELISA used and it is known that SRID is prone to overestimation and ELISA is considered more specific [20,26]. It may be concluded that SIgGC-24 >200 mg/dL for 1-day old lambs may be a reasonable goal for producers to decrease the risk of death associated with FPT as all lambs below this figure died in the present study.

The STPC-24 was significantly lower in lambs that died compared to lambs which were healthy or recovered during the first week and neonatal period. This was accordance with previous studies [14,15]. Some studies considered that lambs with STPC-24 of less than 50 g/L [15] or 58 g/L [17] to be hypogammaglobulinemic and claimed that the risk of death in those lambs was high. Our study indicated that STPC-24 of 45 g/L or less could be a threshold that increases the risk of mortality in the neonatal period as 10 of the 13 lambs died in the neonatal period had STPC-24 ≤45 g/L. A close and linear relationship was found between SIgGC and STPC on day 1 in lambs died in the neonatal period, and 62% of the variation in SIgGC could be explained in association with STPC *(Fig. 1 D)*. Furthermore, all lambs with STPC-24 ≤45 and died had SIgGC-24 values of less than 200 mg/dL *(Table 2)*. This level was established as the SIgGC threshold that increases the risk of mortality in the neonatal period in the present study. Therefore, STPC-24 of 45 g/L or less and SIgGC-24≤600 mg/dL could be used as a threshold for passive immunity that indicates a high risk of death in the neonatal period.

In contrast to neonatal calves [31], the relationship between healthy neonatal lamb and SIgGC has not yet been studied in detail and no widely accepted threshold value of SIgGC associated with risk of developing illness is available. The present study revealed that the risk of morbidity was much greater in lambs having SIgGC-24 below 600 mg/dL than those having SIgGC-24 above 600 mg/dL. This figure may be a candidate for threshold concentration for disease development in neonatal lambs. There islimited number of studies where passive transfer deficiency is indicated by cut off values obtained using indirect methods in lambs. Zinc sulfate turbidity (ZST) test was utilized and the results were designated by several researchers as <12 [12] and <20 units [15] in 1- to 2-day-old lambs. The model predicting STPC based on ZST units for 1-day-old lambs revealed that STPC of 5.2 and 5.4 g/dL were equivalent to 10 and 12 ZST units, respectively [18]. This was the only study exist in the literature relating STPC with neonatal diseases in lambs and no STPC threshold was used for morbidity risk. However, this issue has extensively been researched in calves [5,8,9,32]. In our study, the sick lambs had significantly lower STPC-24 than healthy lambs. The morbidity rates in lambs with STPC-24 ≤55 g/L was 3.9 to 11.8 times higher than those with STPC-24>55g/L and close and linear correlations noted between SIgGC-24 and STPC-24 in sick neonatal lambs. Additionally, SIgGC-24 of 23 lambs out of 29 lambs with STPC-24 ≤55 g/L was below the IgG cut-off value (≤600 mg/dL), raising risk of morbidity. These data may indicate that STPC-24 ≤55 g/L could be cut off value predicting illness in advance.

Our study revealed that STPC-24 and SIgGC-24 were not associated on mortality rate of lambs in the neonatal period. However, other studies conducted in calves and lambs [8,9,14,15,31] showed that TP and IgG concentrations or passive immunity were associated with deaths in post-neonatal period and can be used to predict outcomes. In our study, STPC-24 and SIgGC-24 ranges had no significant prevention effects on death of lambs in terms of post-neonatal period. This may be attributed to inappropriate management regimens (poor sanitation, overcrowded housing, absence of vaccination), environmental conditions (temperature, season) as reportedcalves up to 12 weeks [15,31,33].

In the present study, the mean CIgGC was close to that of Maden et al.[21], comparatively higher than that of Zarilli et al.[34] and lower than other studies [1,10,28]. Dams'CIgGC was 2-3 times higher than SIgGC of respective lambs at 24-72 h of birth and did not correlate which each other as reported previously [1]. Lambs born to ewes with low CIgGC and CTPC were more likely to be exposed to disease in our study as reported by Khan et al.[15], but opposite results were also disclosed [13]. No correlation was detected between CIgGC or CTPC and lamb serum TP or IgG concentrations. High CIgGC could not always be protective in lambs due to delayed lactogenesis, malnutrition or under nutrition during pregnancy, poor colostral management (delay in colostrum intake, inadequate amount of colostrum, colostrum quality etc.), infections, prematurity, mismothering, dams' health, low birth weight and weakness of neonates, cold exposure, crowded housing [1,13,28,32]. These factors may have played a role in the present study. Quigley et al.[32] reported a significant linear correlation between colostral protein and IgG (R^2=0.510) in cows. Similarly, a significant correlation (R^2=0.683) and a linear relationship (R^2=0.460) was found between colostral TP and IgG concentrations in our study indicating that TP levels could be beneficial in evaluating colostrum quality.

A positive correlation was found between STPC prior to lambing in ewes and SIgGC-24 in lambs. Similarly, a positive correlation was found between SIgGC in ewes and STPC-24 or SIgGC-24 in their lambs. In addition, positive correlations were found between the individual values of IgG and TP in ewes before lambing. These results were in agreement with that of Andres et al.[17]. Therefore, measuring serum IgG or TP in ewes before lambing would be a valuable indicator of the risk of lamb diseases or passive immunity. Andres et al.[17] suggested that low immunity in lambs may be caused by a lower level of gamma globulin (<1.3 g/dL) and TP (<5.9 g/dL) in the dams. In the present study, mortality rates in the lambs of dams with STPC ≥59 g/L were low. However, dam's serum IgG or TP concentration seemed to have no effect on lamb's health [28] that maternal STPC measured 10-15 days before birth in the first two months had no effect on lamb mortality as reported in the present study.

Passive immunity and growth performance are critical for lambs in the neonatal period [18]. Determining passive immune status accurately and in a timely fashion is important for taking preventive measures [5,22]. In the present study, the accuracy of STPC, a test that can be adapted to the field, for determining passive immunity was demonstrated. The linear models that were used to determine SIgG-24 from STPC-24 in lambs that were healthy (R^2=0.33 to 0.70), diseased (R^2=0.67) or died (R^2=0.62) in the neonatal period were sufficient. However, the calculation of IgG concentrations based on TP concentrations was not clearly specific according to the linear regression formulas. Our study provides useful information for practicing veterinarians in terms of validating an alternative way that is measurement of STPC for FPT in individual lambs and colostrum quality could contribute to the development of passive transfer monitoring programs. Measurement of STPC for FPT in individual lambs and colostrum quality, compared to other tests such as IgG measurement (SRID or ELISA), does not require expensive instrumentation, provides an immediate result, and is also adaptable to field use. These advantages should be beneficial for timely management and therapeutic decisions [5,27]. Furthermore, in our model SIgGC could be calculated from STPC with an accuracy of 70% at any day of neonatal period of lambs.

Overall the present study disclosed that the first week of neonatal life was critical for lamb's health and passive transfer of immunity was of paramount importance for maintenance of health. The study also revealed critical cut off point of SIgGC and STPC at 24 h after birth for increased risk of disease and death in the both periods. STPC using a

<55 g/L and SIgG-24 ≤600 mg/dL threshold value resulted in correct classification of the highest percentage of lambs with regard to their passive transfer status. It was also noted that CIgGC and CTPC have essential role in prevention of diseases in lambs. Furthermore, there was a significant linear relationship between CIgGC and CTPC. Therefore, serum TP concentration was an important consideration in determining passive transfer of immunity and colostrum quality in sheep farms.

Acknowledgements

The authors are thankful to TUBITAK (Project Code; TOVAG 108 O 847) and Scientific Research Projects Coordination Unit of Kafkas University (Project No; 2010-VF-04) for financial support.

REFERENCES

1. Gilbert RP, Gaskins CT, Hillers JK, Parker CF, McGuire TC: Genetic and environmental factors affecting immunoglobulin G_1 concentrations in ewe colostrum and lamb serum. *J Anim Sci*, 66, 855-863, 1988.

2. Bekele T, Kasali OB, Woldeab T: Causes of lamb morbidity and mortality in the Ethiopian highlands. *Vet Res Commun*, 16, 415-424, 1992. DOI: 10.1007/BF01839018

3. Gökçe E, Erdoğan HM: An epidemiological study on neonatal lamb health. *KafkasUniv Vet Fak Derg*, 15 (2): 225-236, 2009. DOI: 10.9775/kvfd.2008.104-A

4. Gökçe E, Kırmızıgül AH, Erdoğan HM, Çitil M: Risk factors associated with passive immunity, health, birth weight and growth performance in lambs: I. Effect of dam health status and parity, birth weight, gender, type of birth and lambing season on morbidity and mortality. *Kafkas Univ Vet Fak Derg,* 19 (Suppl.-A): A153-A160, 2013. DOI: 10.9775/kvfd.2012.8440

5. Massimini G, Britti D, Peli A, Cinotti S: Effect of passive transfer status on preweaning growth performance in dairy lambs. *J Am Vet Med Assoc,* 229, 111-115, 2006. DOI: 10.2460/javma.229.1.111

6. Gökçe E, Erdoğan HM: Pneumonia in neonatal lambs: Frequency and some associated risk factors. *Kafkas Univ Vet Fak Derg,* 14 (2): 223-228, 2008. DOI: 10.9775/kvfd.2008.58-A

7. Gökçe E, Ünver A, Erdoğan HM: İshalli neonatal kuzularda enterik patojenlerin belirlenmesi. *Kafkas Univ Vet Fak Derg,* 16 (5): 717-722, 2010. DOI: 10.9775/kvfd.2009.1514

8. Elitok B: Indicators of passive immunity failure in neonatal calves. *Oncol Res Rev,* 1 (3): 1-2, 2018. DOI: 10.15761/ORR.1000113

9. Pekcan M, Fidancı UR, Yüceer B, Özbeyaz C: Estimation of passive immunity in newborn calves with routine clinical chemistry measurements. *Ankara Univ Vet Fak Derg,* 60, 85-88, 2013.

10. Hunter AG, Reneau JK, Williams JB: Factors affecting IgG concentration in day-old lambs. *J Anim Sci,* 45 (5): 1146-1151, 1977.

11. Sawyer M, Willadsen CH, Osburn BI, McGuire TC: Passive transfer of colostral immunoglobulins from ewe to lamb and its influence on neonatal lamb mortality. *J Am Vet Med Assoc,* 171 (12): 1255-1259, 1977.

12. Harker DB: Serum immune globulin levels in artificially reared lambs. *Vet Rec*, 95, 229-231, 1974.

13. McGuire TC, Regnier J, Kellom T, Gates NL: Failure in passive transfer of immunoglobulin G_1 to lambs: Measurement of immunoglobulin G_1 in ewe colostrums. *Am J Vet Res,* 44, 1064-1067, 1983.

14. Vihan VS: Immunoglobulin levels and their effect on neonatal survival in sheep and goats. *Small Ruminant Res,* 1, 135-144, 1988. DOI: 10.1016/0921-4488(88)90029-6

15. Khan A, Sultan MA, Jalvi MA, Hussain I: Risk factors of lamb mortality in Pakistan. *Anim Res,* 55, 301-311, 2006.DOI: 10.1051/animres:2006017

16. Brujeni GN, Jani SS, Alidadi N, Tabatabaei S, Sharific H, Mohri M: Passive immune transfer in fat-tailed sheep: Evaluation with different methods. *Small Ruminant Res*, 90, 146-149, 2010. DOI: 10.1016/j.smallrumres.2009.12.024

17. Andres S, Jimenez A, Sanchez J, Alonso JM, Gomez L, Lopez F, Rey J: Evaluation of some etiological factors predisposing to diarrhoea in lambs in "La Serena" (Southwest Spain). *Small Ruminant Res,* 70, 272-275, 2007. DOI: 10.1016/j.smallrumres.2006.04.004

18. Gökçe E, Atakişi O, Kırmızıgül AH, Erdoğan HM: Risk factors associated with passive immunity, health, birth weight and growth performance in lambs: II. Effects of passive immune status and some risk factors on growth performance during the first 12 weeks of life. *Kafkas Univ Vet Fak Derg,* 19 (4): 619-627, 2013. DOI: 10.9775/kvfd.2013.8442

19. Fahey JL, McKelvey EM: Quantitative determination of serum immunoglobulins in antibody-agar plates. *J Immunol*, 94, 84-90, 1965.

20. Lee SH, Jaekal J, Bae CS, Chung BH, Yun SC, Gwak MJ, Noh GJ, Lee DH: Enzyme-Linked immunosorbent assay, single radial immunodiffusion and indirect methods for the detection of failure of transfer of passive immunity in dairy calves. *J Vet Intern Med,* 22, 212-218, 2008. DOI: 10.1111/j.1939-1676.2007.0013.x

21. Maden M, Altunok V, Birdane FM, Aslan V, Nizamlıoglu M: Blood and colostrum/milk serum γ-glutamyltransferase activity as a predictor of passive transfer status in lambs. *J Vet Med B Infect Dis Vet Public Health,* 50, 128-131, 2003. DOI: 10.1046/j.1439-0450.2003.00629.x

22. Britti D, Massimini G, Peli A, Luciani A, Boari A: Evaluation of serum enzyme activities as predictors of passive transfer status in lambs. *J Am Vet Med Assoc,* 226, 951-955, 2005. DOI: 10.2460/javma.2005.226.951

23. Reid JF, Martinez AA: A modified refractometer method as a practical aid to the epidemiological investigation of disease in the neonatal ruminant. *Vet Rec,* 22, 177-179, 1975.

24. Wilson LK, Tyler JW, Besser TE, Parish SM, Gant R: Prediction of serum IgG_1 concentration in beef calves based on age and serum gamma-glutamyl-transferase activity. *J Vet Intern Med,* 13, 123-125, 1999. DOI: 10.1111/j.1939-1676.1999.tb01139.x

25. Boucher Z: Breed and diet effects on ewe colostrum quality, lamb birthweight and the transfer of passive immunity. School of Animal and Veterinary SciencesFaculty of Science, Charles Sturt University, WaggaWagga, *Thesis*, 2014.

26. Gökçe E, Atakişi O, Kırmızıgül AH, Ünver A, Erdoğan HM: Passive immunity in lambs: Serum lactoferrin concentrations as a predictor of IgG concentration and its relation to health status from birth to 12 weeks of life. *Small Ruminant Res,* 116 (2-3): 219-228, 2014. DOI: 10.1016/j.smallrumres.2013.11.006

27. Massimini G, Peli A, Boari A, Britti D: Evaluation of assay procedures for prediction of passive transfer status in lambs. *Am J Vet Res*, 67 (4): 593-598, 2006. DOI: 10.2460/ajvr.67.4.593

28. Loste A, Ramos JJ, Fernández A, Ferrer LM, Lacasta D Verde MT, Marca MC, Ortín A: Effect of colostrum treated by heat on immunological parameters in newborn lambs. *Livest Sci*, 117, 176-183, 2008. DOI: 10.1016/j.livsci.2007.12.012

29. Öztabak K, Özpınar A: Growth performance and metabolic profile of chios lambs prevented from colostrum intake and artificially reared on a calf milk replacer. *Turk J Vet Anim Sci*, 30, 319-324, 2006.

30. Pauli JV: Colostral transfer of gamma glutamyl transferase in lambs. *N Z Vet J,* 31, 150-151, 1983. DOI: 10.1080/00480169.1983.35004

31. Dewell RD, Hungerford LL, Keen JE, Laegreid WW, Griffin DD, Rupp GP, Grotelueschen DM: Association of neonatal serum immunoglobulin G_1 concentration with health and performance in beef calves. *J Am Vet Med Assoc,* 228, 914-921, 2006. DOI: 10.2460/javma.228.6.914

32. Quigley JD, Kost CJ, Wolfe TM: Absorption of protein and IgG in calves fed a colostrum supplement or replacer. *J Dairy Sci,* 85, 1243-1248, 2002. DOI: 10.3168/jds.S0022-0302(02)74188-X

33. Gökçe E, Kırmızıgül AH, Atakişi O, Erdoğan HM: Risk factors associated with passive immunity, health, birth weight and growth performance in lambs: III. The relationship among passive immunity, birth weight, gender, birth type, parity, dam's health and lambing season. *Kafkas Univ Vet Fak Derg*, 19 (5): 741-747, 2013. DOI: 10.9775/kvfd.2013.8441

34. Zarilli A, Micera E, Lacarpıa N, Lombordi P, Pero ME, Pelagallii A, d'Angelo D, Mattia M, Avallone I: Evaluation of ewe colostrum quality by estimation of enzyme activity levels. *Revue Med Vet,* 154 (8-9): 521-523, 2003.

Kafkas Univ Vet Fak Derg
25 (3): 397-404, 2019
DOI: 10.9775/kvfd.2018.21097

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Protective Effect of Ozone Against Gentamicin-Induced Neprotoxicity and Neutrophil Gelatinase-Associated Lipocalin (NGAL) Levels: An Experimental Study

Sefer ÜSTEBAY [1,a] Döndü Ülker ÜSTEBAY [1,b] Ömür ÖZTÜRK [2,c] Ömer ERTEKİN [1,d] Y asemen ADALI [3,e]

[1] Kafkas University, Faculty of Medicine, Department of Pediatrics, TR-36300 Kars - TURKEY
[2] Çanakkale Onsekiz Mart University, Faculty of Medicine, Department of Anesthesiology and Reanimation, TR-17000 Çanakkale - TURKEY
[3] Çanakkale Onsekiz Mart University, Faculty of Medicine, Department of Pathology, TR-17000 Çanakkale - TURKEY
[a] ORCID: 0000-0003-1507-5921; [b] ORCID: 0000-0003-3270-8305; [c] ORCID: 0000-0003-2270-2778; [d] ORCID: 0000-0002-7846-7634
e ORCID:0000-0002-8004-7364

Article ID: KVFD-2018-21097 Received: 02.10.2018 Accepted: 10.02.2019 Published Online: 15.02.2019

How to Cite This Article

Üstebay S, Üstebay DÜ, Öztürk Ö, Ertekin Ö, Adalı Y: Protective effect of ozone against gentamicin-induced neprotoxicity and neutrophil gelatinase-associated lipocalin (NGAL) levels: An experimental study. *Kafkas Univ Vet Fak Derg,* 25 (3): 397-404, 2019. DOI: 10.9775/kvfd.2018.21097

Abstract

Our aim was to investigate the protective role of ozone treatment against gentamicin-induced nephrotoxicity in an experimental rat model. In this study, a total of 30 rats were allocated in 5 groups (n=6 in each group). The control group (Group 1) received isotonic saline only, while Groups 2 and 3 received gentamicin at doses of 15 mg/kg/day and 50 mg/kg/day, respectively. In Group 4, intraperitoneal ozone treatment (1 mg/kg, 5% O_3-95% O_2) was performed after administration of gentamicin at a dose of 15 mg/kg/day. Group 5 underwent ozone treatment intraperitoneally following the application of gentamicin (50 mg/kg/day). Nephrotoxicity was formed by administration of glycerol. Serum levels of urea, creatinine, neutrophil-gelatinase-associated lipocalin (NGAL), lactate dehydrogenase (LDH), total antioxidant capacity (TAC) and protein carbonyl were measured, and kidneys were histopathologically examined after the sacrifice of animals on the 5th day. Group 4 displayed more favorable outcomes regarding biochemical markers of oxidative stress such as NGAL, LDH, creatinine, urea, TAC and protein carbonyl. Similarly, histopathological alterations indicating gentamicin-induced nephrotoxicity such as hemorrhage, the presence of protein casts and epithelial injury in renal tubules were less evident in Groups 4 and 5 which received ozone treatment. To conclude, results of this experimental study demonstrated that ozone treatment might ameliorate biochemical disturbances and histopathological alterations linked with gentamicin-induced nephrotoxicity. However, further trials are warranted to document the actual therapeutic potential of ozone treatment in the clinical setting.

Keywords: *Gentamicin-induced nephrotoxicity, Ozone, Oxidative stress, NGAL, Antioxidant defense*

Gentamisinin İndüklediği Nefrotoksisitede Ozonun Koruyucu Etkisi ve Neutrophil Gelatinase-Associated Lipocalin (NGAL) Düzeyleri: Deneysel Çalışma

Öz

Deneysel sıçan modelinde, ozon tedavisinin gentamisin kaynaklı nefrotoksisiteye karşı koruyucu rolünü araştırmak amaçlanmıştır. Bu çalışmaya 5 grup olacak şekilde toplamda 30 rat dahil edildi (her grupta n = 6). Kontrol grubu (Grup 1) sadece izotonik salin alırken, Grup 2 ve 3, sırasıyla 15 mg/kg/gün ve 50 mg/kg/gün dozlarında gentamisin aldı. Grup 4'e 15 mg/kg/gün dozunda gentamisin uygulamasından sonra intraperitoneal ozon tedavisi (1 mg/kg, %5 O_3-%95 O_2) uygulandı. Grup 5'e 50 mg/kg/ gün dozunda gentamisin uygulamasından sonra intraperitoneal ozon tedavisi (1 mg/kg, %5 O_3-%95 O_2) uygulandı. Gliserol uygulanması ile nefrotoksisite oluşturuldu. Serum düzeyleri üre, kreatinin, nötrofil-jelatinaz ilişkili lipokalin (NGAL), laktat dehidrojenaz (LDH), total antioksidan kapasite (TAC) ve protein karbonil ölçüldü ve 5. gün hayvanların sakrifiye edilmesinden sonra böbrekler histopatolojik olarak incelendi. Grup 4'ün; NGAL, LDH, kreatinin, üre, TAC ve protein karbonil gibi oksidatif stresin biyokimyasal belirleyicileri ile daha olumlu sonuçlar verdiği gözlemlendi. Benzer şekilde, kanama, protein döküntüleri ve renal tüplerde epitel hasarı gibi gentamisin ile indüklenen nefrotoksisiteyi gösteren histopatolojik değişiklikler, ozon tedavisi alan Grup 4 ve 5'te daha az belirgindi. Bu deneysel çalışmanın sonuçları ozon tedavisinin, gentamisin kaynaklı nefrotoksisite ile bağlantılı biyokimyasal bozuklukları ve histopatolojik değişiklikleri iyileştirebileceğini göstermiştir. Bununla birlikte, klinik ortamda ozon tedavisinin gerçek terapötik potansiyelini belgelemek için daha fazla deneme yapılması gerekmektedir.

Anahtar sözcükler: *Gentamisin kaynaklı nefrotoksisite, Ozon, Oksidatif stres, NGAL, Antioksidan savunma*

İletişim (Correspondence)
+90 536 4326293
ustabay_dr@hotmail.com

INTRODUCTION

Kidneys are frequently vulnerable to the deleterious effects of various drugs. Among these, antibiotics are the most common medications resulting in nephrotoxicity. The reason for this is excretion of antibiotics or their metabolites and high concentration of these substances in renal tissue. Nephrotoxicity due to antibiotics may occur due to several mechanisms including direct cellular injury, immunological mechanisms, hypersensitivity reactions and intratubular obstruction attributed to precipitation of drugs. Acute renal failure (ARF) linked with acute tubular necrosis, or acute interstitial nephritis may be encountered as a consequence of these pathological processes. Moreover, disturbances of acid-base and electrolyte balances may be detected following the use of antibiotics [1-5].

Nephrotoxicity is diagnosed in 10-25% of patients treated with aminoglycosides. Nephrotoxicity may develop due to the toxic accumulation in the renal cortex and diminution of glomerular filtration and renal blood flow. Risk factors involve advanced age, former renal dysfunction, dehydration, pregnancy, hypothyroidism, metabolic acidosis, use of other nephrotoxic agents (amphotericin B, vancomycin, diuretics, non-steroidal anti-inflammatory drugs, cisplatin, cyclosporin) and long-term administration of antibiotics [6].

In current practice, monitorization of serum levels of creatinine and aminoglycosides, the omission of simultaneous use of other nephrotoxic agents and limitation of the administration of aminoglycosides in short-term are recommended to decrease the risk of nephrotoxicity. Gentamicin is an important aminoglycoside used against Gram (-) bacterial infections. Acute renal failure is detected in up to 20% of patients even after the use of aminoglycosides at therapeutic doses [7-10].

Neutrophil gelatinase-associated lipocalin (NGAL) gene was recently shown to be one of the maximally induced genes early phase in the postischemic and nephrotoxic kidney injury. NGAL production is rapidly increasing in response to renal epithelium damage or inflammation. Animal models of NGAL protein have been shown to increase in renal tubule cells in ischemic and nephrotoxic akute kidney injury, as well as in plasma and urine levels. Protein carbonyl content is the most general and well-used biomarker of severe oxidative protein damage [11-15].

Glycerol administration is an established model for ARF in rats [16]. In the present study, we aimed to investigate the alterations in biochemical markers, and histopathological indicators of renal injury in an experimental model of ARF triggered with the administration of glycerol in rats receiving aminoglycosides at two different doses high doses. Furthermore, we sought whether ozone exhibited a protective effect against the hazardous impacts of gentamicin on kidneys.

MATERIAL and METHODS

Study Design

In this experimental study, ARF was elicited with the use of 50% glycerol at a dose of 9 mL/kg intramuscularly in all groups else than the control group. Five groups (n=6 for each group) consisting of male Sprague-Dawley rats were constituted. The animals were kept under a 12 h light/dark cycle with room temperature maintained at 25°C, humidity at 60% and food and water available ad libitum. The experiments were performed in compliance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee for Animal Experimentation (The study was approved from the Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK/2018-076).

After the administration of glycerol, rats were followed-up for macroscopic hematuria. Gentamicin was parenterally given at doses of 15 mg/kg/day and 50 mg/kg/day consecutively for 3 to 5 days. In the control group (Group 1), isotonic saline (0.1 mL) was administered intraperitoneally for five days. Second and third groups received gentamicin at doses of 15 mg/kg/day and 50 mg/kg/day, respectively. In the fourth group, intraperitoneal ozone treatment (1 mg/kg, 5% O_3-95% O_2) was performed after administration of gentamicin at a dose of 15 mg/kg/day. The fifth group underwent ozone treatment intraperitoneally (1 mg/kg, 5% O_3-95% O_2) following application of gentamicin (50 mg/kg/day). After the treatment protocols were complete, intracardiac blood samples were obtained from rats that were kept fasting for 8 h. Body weights of all animals were recorded in 5 groups. Prior to the intervention, thiopental sodium was intraperitoneally given at a dose of 75 mg/kg. Animals were sacrificed on the 5th day, while those with the deterioration of general condition, poor feeding, significant macroscopic hematuria were sacrificed on the 3rd day. Kidneys were rapidly dissected, removed and fixed in 10% formaldehyde. Tissues were embedded in paraffin, sectioned at 3 mm, stained with hematoxylin and eosin (H/E) and evaluated under light microscopy.

Outcome Parameters

Histopathological examination involved assessment of intratubular hemorrhage, intratubular protein leakage, tubular epithelial injury, interstitial hemorrhage and inflammation, glomerular injury and edema in kidney tissue.

Biochemical indicators under investigation were neutrophil gelatinase-associated lipocalin (NGAL) (ng/mL), antioxidant (Mm), protein carbonyl (nmol/mL), urea (mg/dL), lactate dehydrogenase (LDH) (U/L), and creatinine (mg/dL).

The laboratory investigators were blinded to the sample sources and clinical outcomes until the end of the study.

Measurement of Biochemical Indicators

Neutrophil Gelatinase-Associated Lipocalin (NGAL): For measurement of serum NGAL levels, ELISA was performed in accordance with the previous literature [17]. Microtiter plates pre-coated with a mouse monoclonal antibody raised against human NGAL (*HYB211-05, AntibodyShop, Gentofte, Denmark*) were blocked with buffer containing 1% bovine serum albumin, coated with 100 µL of serum samples or standards (NGAL concentrations ranging from 1-1000 ng/mL, Randox Laboratories, Crumlin, UK), and incubated with a biotinylated monoclonal antibody against human NGAL (HYB211-01B, AntibodyShop) followed by avidin-conjugated HRP (Dako, Carpinteria, CA, USA). TMB substrate (BD Biosciences, San Jose, CA) was added for color development, which was read after 30 min at 450 nm with a microplate reader (Benchmark Plus, BioRad, Hercules, CA, USA). All measurements were made in triplicate. The inter- and intra-assay coefficient variations ranged between 5-10%.

Total Antioxidant Capacity (TAC): Total antioxidant capacity (TAC) was measured in serum via a commercially available kit (Randox Co, England). The assay is based on the incubation of 2, 2'-azinodi-(3-ethylbenzthiazoline sulphonate) (ABTS) with methmyoglobin and hydrogen peroxide to produce the radical cation ABTS+, which has a relatively stable blue-green color, measured at 600 nm. The suppression of the color is compared with that of the Trolox, which is widely used as a traditional standard for TAS measurement assays, and the assay results are expressed as Trolox equivalent (mmol/L) [18].

Protein Carbonyl: Serum protein levels were measured, and the concentration was brought to 0.5-2.0 mg protein in the test sample by diluting with high purity water (TKA MicroMed, TKA Wasseraufbereitungssysteme GmbH, Germany). Protein carbonyls (damaged proteins) in the serum were measured using a commercially available protein carbonyl content kit (BioVision Inc., USA) according to the manufacturer's instructions [19].

Urea and Creatinine: Serum urea and creatinine were determined using the standard assay kit following back titration, diacetyl monoxime, and alkaline picrate methods, respectively [20].

Lactate Dehydrogenase (LDH): Following serum preparation, level of LDH was measured using the method described by Buhl and Jackson [21].

Statistical Analysis

Biochemical variables were compared using one way ANOVA, and post hoc comparisons were carried out by Tukey HSD test under the assumption of equal variances. The assumption of normality is assessed with the Shapiro-Wilk test for each group ($P>0.05$). The pathological variables were compared using the Fisher's exact tests.

RESULTS

A comparative analysis of biochemical variables including NGAL, protein carbonyl, urea, creatinine, and LDH in 5 experimental groups is shown in *Table 1* and *Fig. 1*. Our results yielded that Group 4 displayed the most favorable results in terms of markers of antioxidant defense and oxidative stress. Interestingly, an increased dose of gentamicin seemed to result in deterioration of oxidative system balance in spite of administration of ozone treatment. An increased dose of gentamicin without administration of ozone treatment was associated with a more obvious increase in biochemical variables, whereas rats receiving the therapeutic dose of gentamicin together with the application of ozone treatment displayed the most favorable outcomes.

In *Table 2*, an overview of frequencies of histopathological alterations is presented. Accordingly, hazardous histopathological effects of gentamicin-induced nephropathy such as intratubular hemorrhage, tubular protein cast formation and tubular epithelial injury were alleviated by administration of ozone treatment in Group 4. However, histopathological changes such as interstitial hemorrhage and interstitial inflammation did not display any significant differences between any groups. *Fig. 2-5* demonstrate various histopathological alterations that occur secondary to gentamicin-induced nephrotoxicity.

DISCUSSION

The purpose of the present study was to determine the effect of ozone treatment against gentamicin-induced nephrotoxicity in an experimental rat model. Histo-pathological and biochemical evaluations of tissue and serum samples obtained from subjects demonstrated that ozone treatment alleviated the nephrotoxic effects, particularly in rats receiving therapeutic doses of gentamicin. These effects were evident both at the tissue level and in terms of biochemical oxidative stress marker levels.

The mechanism underlying aminoglycoside-induced nephrotoxicity is not well elucidated. The increase in oxidative stress markers and pathological alterations in renal tissue may provide important hints on the underlying pathophysiology. Histopathological examination of the kidneys of rats yielded that intratubular hemorrhage, tubular epithelial injury, glomerular injury and edema might reflect the renal injury secondary to aminoglycosides. As a novel mode of treatment, ozone may have remarkable potential as a protective agent against gentamicin-induced nephrotoxicity. As shown in relevant publications, the basis for this potential may be related to antioxidative mechanisms [22].

Borrego et al.[23] reported that oxidative preconditioning with ozone (O_3) displayed protective effects against drug-induced acute nephrotoxicity in rats. They noted that

Table 1. *Comparison of biochemical variables between groups*

Variable	Groups					
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**P⁺**
NGAL (ng/mL) Min-Max (Mean)	4.2-10.0 (6.4)	24.7-51.7 (41.52)	45.6-81.3 (68.6)	10.1-22.5 (16.2)	20.5-67.1 (43.1)	<0.001
Tukey HSD Test/F value	$(2.1)^{b,c,e}$	$(10.2)^{a,c,d}$	$(14.3)^{a,b,d,e}$	$(4.2)^{b,c,e}$	$(18.0)^{a,c,d}$	
TAC (Mm) Min-Max (Mean)	2.0-3.8 (2.8)	3.2-3.8 (3.6)	2.1-3.7 (3.1)	3.8-4.6 (4.2)	3.9-4.7 (4.3)	<0.001
Tukey HSD Test/F value	$(0.7)^{d,e}$	(0.2)	$(0.6)^{d,e}$	$(0.3)^{a,c}$	$(0.4)^{a,c}$	
Protein carbonyl (nmol/mL) Min-Max (Mean)	43.4-80.4 (64.5)	75.0-108.8 (87.9)	94.2-115.9 (101.2)	68.8-89.5 (79.3)	68.1-83.6 (77.7)	<0.001
Tukey HSD Test/F value	$(12.8)^{b,c}$	$(12.2)^{a}$	$(8.6)^{a,d,e}$	$(7.9)^{c}$	$(7.4)^{c}$	
Urea (mg/dL) Min-Max (Mean)	34-58 (41.3)	364-632 (488.3)	505-771 (624.0)	58-352 (198.3)	398-771 (498.2)	<0.001
Tukey HSD Test/F value	$(8.8)^{b,c,e}$	$(102.7)^{a,d}$	$(115.9)^{a,d}$	$(112.8)^{b,c,e}$	$(155.1)^{a,d}$	
LDH (U/L) Min-Max (Mean)	736-913 (834.5)	953-1182 (1100.5)	1471-2204 (1769.0)	354-1380 (839.5)	763-1123 (962.8)	<0.001
Tukey HSD Test/F value	$(71.6)^{c}$	$(80.0)^{c}$	$(319.5)^{a,b,d,e}$	$(350.8)^{c}$	$(145.0)^{c}$	
Creatinine (mg/dL) Min-Max (Mean)	0.45-0.59 (0.50)	4.73-7.98 (6.23)	9.43-11.7 (10.35)	2.85-5.75 (4.05)	3.04-9.48 (6.11)	<0.001
Tukey HSD Test/F value	$(0.05)^{b,c,d,e}$	$(1.26)^{a,c}$	$(0.88)^{a,b,d,e}$	$(1.30)^{a,c}$	$(3.05)^{a,c}$	

⁺One-way ANOVA is performed; TAC: total antioxidant capacity; NGAL: neutrophil gelatinase-associated lipocalin; LDH: lactate dehydrogenase; Post hoc comparisons are conducted for further investigation of the differences between individual groups using the Tukey HSD test which assumes equal variances for the groups. Statistically significant differences are presented. ^{a} P<0.05 is observed for comparison with Group 1; ^{b} P<0.05 is observed for comparison with Group 2; ^{c} P<0.05 is observed for comparison with Group 3; ^{d} P<0.05 is observed for comparison with Group 4; ^{e} P<0.05 is observed for comparison with Group 5; The letters ^{a,b,c,d,e} in superscripts indicate Groups 1, 2, 3, 4, 5, respectively. They indicate the groups with which the group displays significant difference in terms of variable under investigation

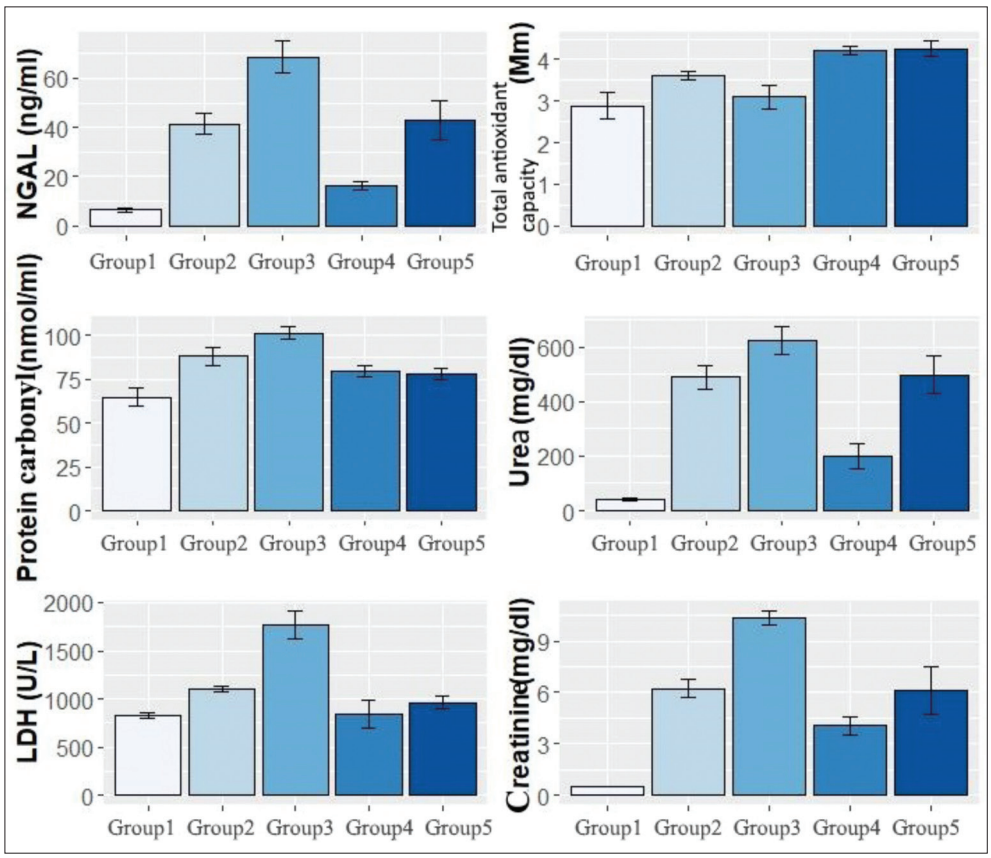


Fig 1. Demonstration of alteration of neutrophil gelatinase associated lipocalin, antioxidant, protein carbonyl, urea, lactate dehydrogenase and creatinine levels in 5 experimental groups

Table 2. *Comparison of frequencies for pathological variables*

Pathological Variables	Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=6)	Group 4 (n=6)	Group 5 (n=6)	P+
Intratubular hemorrhage						
None	$6^{b,c,d,e}$	$0^{a,d,e}$	$0^{a,d,e}$	$0^{a,b,c}$	$0^{a,b,c}$	<0.001
Moderate	$0^{b,c,d,e}$	$0^{a,d,e}$	$0^{a,d,e}$	$4^{a,b,c}$	$3^{a,b,c}$	
Mild	$0^{b,c,d,e}$	$1^{a,d,e}$	$0^{a,d,e}$	$1^{a,b,c}$	$3^{a,b,c}$	
Severe	$0^{b,c,d,e}$	$5^{a,d,e}$	$6^{a,d,e}$	$1^{a,b,c}$	$0^{a,b,c}$	
Intratubular protein casts						
None	$6^{b,c,d,e}$	0^{a}	$0^{a,d}$	$0^{a,c}$	0^{a}	<0.001
Moderate	$0^{b,c,d,e}$	0^{a}	$0^{a,d}$	$4^{a,c}$	3^{a}	
Mild	$0^{b,c,d,e}$	5^{a}	$3^{a,d}$	$2^{a,c}$	3^{a}	
Severe	$0^{b,c,d,e}$	1^{a}	$3^{a,d}$	$0^{a,c}$	0^{a}	
Intratubular epithelial injury						
None	$6^{b,c,d,e}$	$1^{a,d}$	$0^{a,d}$	$0^{a,b,c}$	0^{a}	<0.001
Mild	$0^{b,c,d,e}$	$0^{a,d}$	$0^{a,d}$	$5^{a,b,c}$	4^{a}	
Moderate	$0^{b,c,d,e}$	$5^{a,d}$	$6^{a,d}$	$1^{a,b,c}$	2^{a}	
Severe	$0^{b,c,d,e}$	$0^{a,d}$	$0^{a,d}$	$0^{a,b,c}$	0^{a}	
Interstitial hemorrhage	0	0	0	0	0	-
Interstitial inflammation	0	0	0	0	0	-
Glomerular injury						
None	$6^{b,c}$	0^{a}	0^{a}	3	2	<0.01
Minimal	$0^{b,c}$	6^{a}	6^{a}	3	4	
Edema						
None	6	5	5	3	3	N/A
Yes	0	1	1	3	3	

+Fisher's Exact test is performed
[a] P<0.05 is observed for comparison with Group 1; [b] P<0.05 is observed for comparison with Group 2; [c] P<0.05 is observed for comparison with Group 3; [d] P<0.05 is observed for comparison with Group 4; [e] P<0.05 is observed for comparison with Group 5
The letters a, b, c, d, e in the table indicate Groups 1, 2, 3, 4, and 5, respectively. They show the groups with which the group displays significant difference in terms of variable under investigation

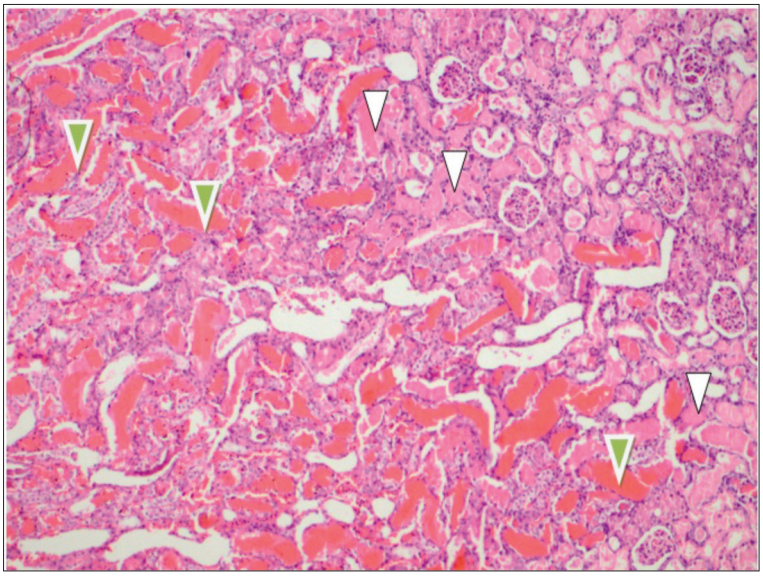


Fig 2. Extensive presence of protein casts and fibrin (H&E; 100x magnification); *green arrowheads* denote fibrin and *white arrowheads* indicate protein casts

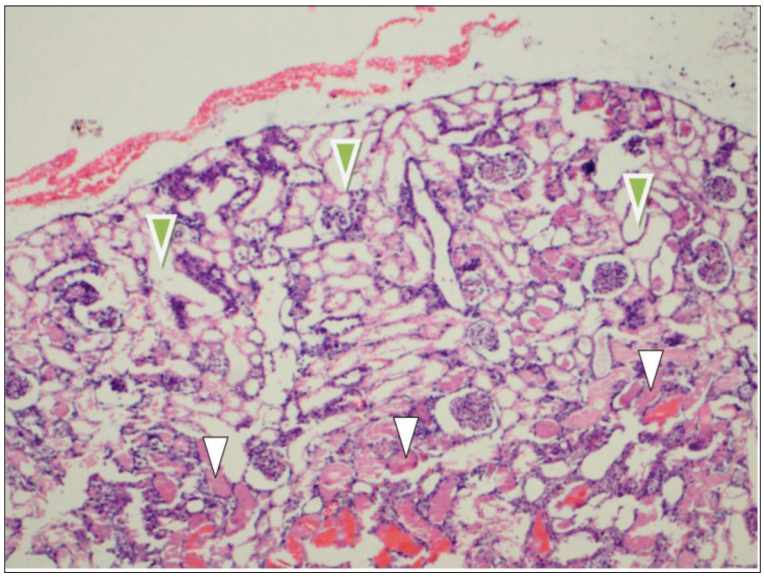
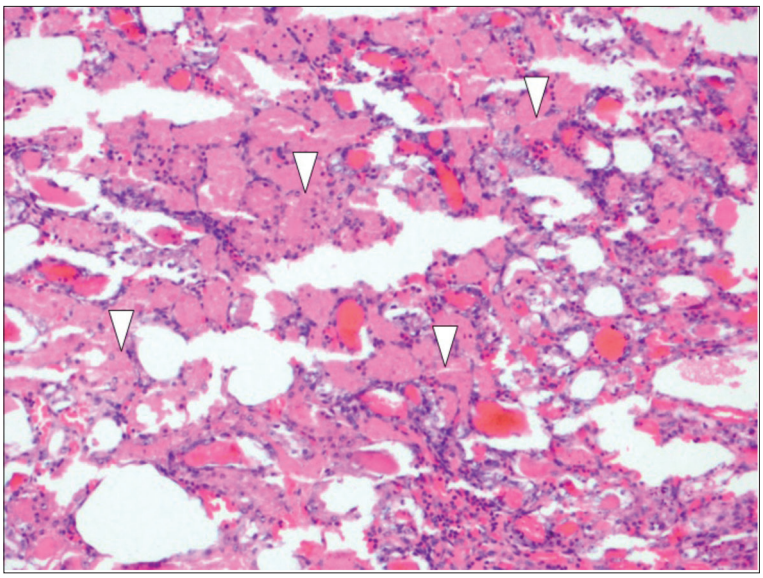


Fig 3. Decreased protein casrs in cortical tubules (H&E; 100x magnification), *green arrowheads* show tubules without protein casts, while *white arrowheads* indicate protein casts

Fig 4. Extensive intratubular casts (H&E; 200x magnification), intratubular protein casts are demonstrated with *white arrowheads*



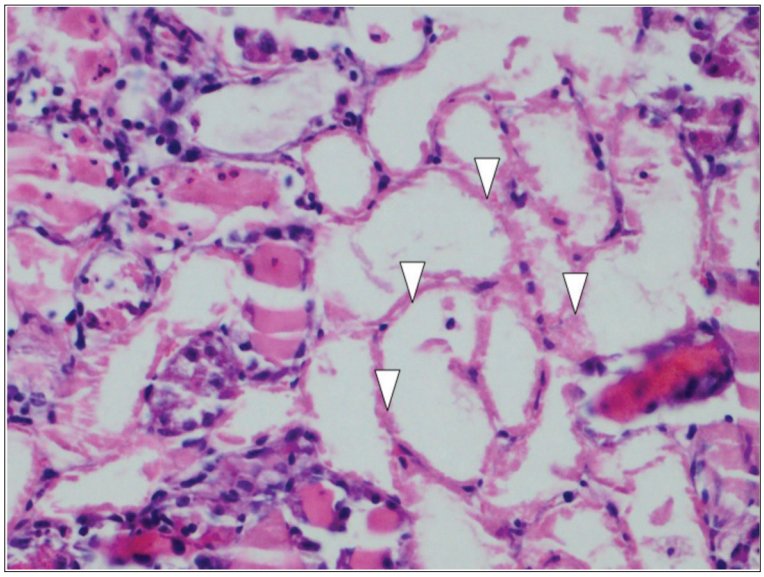


Fig 5. Dilatation and epithelial degenration in tubules (H&E; 400x magnification), *white arrowheads* indicate degenerated tubular epithelium

ozone treatment remarkably avoided the decrease in renal antioxidant defense mechanisms and thereby avoided the deleterious impacts of drugs [23,24]. They implied that the beneficial effects of ozone treatment were dose-dependent. Ozone treatment was supposed not only to enhance the biosynthesis of antioxidant enzymes but also it could inactivate deleterious pathways of oxidative stress injury [23,24]. In conjunction with our results, ozone treatment may attenuate renal tubular damage and facilitate the regenerative response of damaged renal tubular cells. The renal damage caused by aminoglycosides may be a consequence of depletion and inhibition of antioxidant systems.

Aminoglycosides have been commonly used due to their powerful effects particularly against life-threatening Gram (-) infections. However, nephrotoxicity may occur in up to 10-15% of patients and may influence the rates of morbidity, mortality, cost-effectivity as well as the duration of hospitalization [1,2,25]. Aminoglycosides are polycationic drugs that are water-soluble, and they scarcely bind to plasma proteins. They are excreted from kidneys without any significant metabolic change. Some amount of drug may bind to the anionic phospholipids on the apical membrane of tubular cells, and it may be transferred intracellularly to lysosomes using pinocytosis. The subsequent release of lysosomal content to the cytoplasm may cause deterioration of respiratory functions of mitochondria, which leads to the formation of reactive oxygen species. Accumulation of drug in renal cortex causes a high level of drug at tissue compared to the serum drug levels. Binding of aminoglycosides on the double phospholipid layer of cell membrane adversely affects the transport processes and receptor functions. Thereby, the damaged cellular membrane cannot be repaired, and necrosis can be evident. Clinically, aminoglycoside-induced nephrotoxicity may lead to various renal findings. Deterioration of proximal tubular transport may result in glucosuria, aminoaciduria, and tubular proteinuria. Loss of potassium and magnesium may be attributed to an adverse effect on membrane transport and cellular permeability. Disturbance of the effect of adenylate cyclase on renal collecting ducts may cause failure of the concentrating function of kidneys in case of aminoglycoside-induced nephrotoxicity. Aminoglycoside nephrotoxicity is diagnosed clinically as a non-oliguric acute renal failure after administration of aminoglycoside treatment for about one week. Monitorization of drug levels to foresee nephrotoxicity is a controversial issue since the increase of threshold value is mostly due to the decrease of excretion linked with diminution of glomerular filtration rate. In other words, the increased threshold already indicates the presence of nephrotoxicity. Furthermore, there has been no relationship between peak aminoglycoside levels and occurrence of renal failure. Thus, follow-up of aminoglycoside levels has very limited value for early identification of nephrotoxicity [1,2,26-28].

In the literature, there are publications that support the beneficial effects of ozone treatment against cisplatin -induced nephrotoxicity [23,24]. To the best of our knowledge, this study is the first trial that demonstrated the protective effects of ozone against gentamicin-induced nephrotoxicity. We hope that our promising results for the use of ozone to ameliorate the hazardous effects of aminoglycosides on kidneys may encourage implementation of further experimental and clinical trials on this topic. The utility of ozone against gentamicin-induced nephrotoxicity may not only improve the rates of mortality and morbidity, but it may also contribute to achieving a more favorable cost-effectivity.

The oxidative features of ozone function as a double-edged sword. The generation of reactive oxygen species (ROS) may trigger either cell-activation or impairment depending on the amount. Usually, an optimal amount of ROS may activate phosphokinases and enhance the intracellular calcium levels; exerting a cytoprotective effect. On the other hand, a large amount of ROS is associated with hyperoxidation of DNA lipids and proteins, which in turn causes further impairment of cellular metabolism [23]. Hence, determination of the optimal dose in ozone treatment is a key point that remains to be investigated in further studies.

Moreover, possible protective roles of other antioxidant agents such as vitamin E, ascorbic acid, lipoic acid and glutathione against aminoglycoside-induced nephrotoxicity must be elucidated. Borrego et al.[23] have reported that ozone pretreatment had eliminated the increase in serum creatinine levels and had inhibited the acute tubular necrosis induced by cisplatin in renal tissue. Our data demonstrated that ozone might display these beneficial effects similarly versus gentamicin-induced nephrotoxicity.

Evaluation of both biochemical and histopathological aspects of ozone was a strength of the present study. On the other hand, lack of investigation of the dose-related effects of ozone treatment on gentamicin-induced nephrotoxicity was a weakness of our study.

To conclude, results of this experimental study demonstrated that ozone treatment might ameliorate biochemical disturbances and histopathological alterations due to gentamicin-induced nephrotoxicity. However, further trials are warranted to document the actual therapeutic potential of ozone treatment in the clinical setting.

Acknowledgments

The authors declare no competing interests.

Funding Statement

No financial support or funding was received for this paper.

REFERENCES

1. Kaloyanides GJ: Metabolic interactions between drugs and renal tubulointerstitial cells: Role in nephrotoxicity. *Kidney Int,* 39, 531-540, 1991. DOI: 10.1038/ki.1991.61

2. Kaloyanides GJ: Antibiotic-related nephrotoxicity. *Nephrol Dial Transplant*, 9 (Suppl.4): 130-134, 1994.

3. Atamer Şimşek Ş**:** Antimikrobiklerin böbreğe istenmeyen etkileri. *Klinik Derg,* 4, 114-119, 1991.

4. Ecder ST: Antibiyotik nefrotoksisitesi. *ANKEM Derg,* 12, 366-368, 1998.

5. Parlakpınar H, Örüm MH, Acet A: Free oxygen radicals in drug-ınduced nephrotoxicity. *F Ü Sağ Bil Tıp Derg*, 27 (1): 51-56, 2013.

6. Radigan EA, Gilchrist NA, Miller MA: Management of aminoglycosides in the intensive care unit. *J Intensive Care Med,* 25 (6): 327-342, 2010. DOI: 10.1177/0885066610377968

7. Wargo KA, Edwards JD: Aminoclycoside-induced nephrotoxicity. *J Pharm Pract*, 27, 573-577, 2014. DOI: 10.1177/0897190014546836

8. Ateşşahin A, Karahan I, Yılmaz S, Çeribaşı AO, Pirincci I: The effect of manganese chloride on gentamicin-induced nephrotoxicity in rats. *Pharmacol Res,* 48 (6): 637-642, 2003. DOI: 10.1016/S1043-6618(03)00227-5

9. Ali BH, Al-Qarawi AA, Mousa HM: The effect of calcium load and the calcium channel blocker verapamil on gentamicin nephrotoxicity in rats. *Food Chem Toxicol*, 40 (12): 1843-1847, 2002. DOI: 10.1016/S0278-6915(02)00167-9

10. Walker PD, Shah SV: Evidence suggesting a role for hydroxyl radical in gentamicin-induced acute renal failure in rats. *J Clin Invest,* 814, 334-341, 1988.

11. Mishra J, Mori K, Ma Q, Kelly C, Yang J, Mitsnefes M, Barasch J, Devarajan P: Amelioration of ischemic acute renal injury by neutrophil gelatinase-associated lipocalin. *J Am Soc Nephrol,* 15, 3073-3082, 2004. DOI: 10.1097/01.ASN.0000145013.44578.45

12. Karadeniz Z, Tuncel Z, Yapıcı N, Kudsioğlu T, İzgi Çoşkun F, Nuraç H, Karaci AR, Karabulut S, Ukil F, Öğütmen B, Aykaç Z: Akut böbrek hasarının erken tanısında plazma ngal (neutrophil gelatinase-associated lipocalin) etkinliğinin off-pump ve on-pump kalp cerrahisinde karşılaştırılması. *GKDA Derg*, 19 (4): 168-174, 2013. DOI: 10.5222/GKDAD.2013.168

13. Yuen PST, Jo SK, Holly MK, Hu X, Star RA: Ischemic and nephrotoxic acute renal failure are distinguished by their broad transcriptomic responses. *Physiol Genomics*; 25 (3): 375-386, 2006. DOI: 10.1152/physiolgenomics.00223.2005

14. Altekin E, Kenesarı Y: NGAL as a potential diagnostic biomarker. *Turk Klinik Biyokimya Derg,* 11 (1): 37-41, 2013.

15. Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A: Protein carbonylation in human diseases. *Trends Mol Med*; 9 (4): 169-176, 2003. DOI: 10.1016/S1471-4914(03)00031-5

16. Medhat Hegazy A, Hafez AS, Eid RM: Protective and antioxidant effects of copper-nicotinate complex against glycerol-induced nephrotoxicity in rats. *Drug Chem Toxicol,* 26, 1-6, 2018. DOI: 10.1080/01480545.2018.1481084

17. Schmidt-Ott KM, Mori K, Kalandadze A, Li JY, Paragas N, Nicholas T, Devarajan P, Barasch J: Neutrophil gelatinase-associated lipocalin-mediated iron traffic in kidney epithelia. *Curr Opin Nephrol Hypertens*, 15 (4): 442-449, 2006. DOI: 10.1097/01.mnh.0000232886.81142.58

18. Hajhosseini L, Khaki A, Merat E, Ainehchi N: Effect of rosmarinic acid on sertoli cells apoptosis and serum antioxidant levels in rats after exposure to electromagnetic fields. *Afr J Tradit Complement Altern Med*, 10 (6): 477-480, 2013.

19. Fernando N, Wickremesinghe S, Niloofa R, Rodrigo C, Karunanayake L, de Silva HJ, Wickremesinghe AR, Premawansa S, Rajapakse S, Handunnetti SM: Protein carbonyl as a biomarker of oxidative stress in severe leptospirosis, and its usefulness in differentiating leptospirosis from dengue infections. *PLoS One*, 11 (6): e0156085, 2016. DOI: 10.1371/journal.pone.0156085

20. Saka WA, Akhigbe RE, Popoola OT, Oyekunle OS: Changes in serum electrolytes, urea, and creatinine in aloe vera-treated rats. *J Young Pharm,* 4, 78-81, 2012. DOI: 10.4103/0975-1483.96620

21. Buhl SN, Jackson KY: Optimal conditions and comparison of lactate dehydrogenase catalysis of the lactate to pyruvate and pyruvate to lactate reactions in human serum at 25, 30 and 37°C. *Clin Chem,* 24, 828-831, 1978.

22. Borrego A, Zamora ZB, González R, Romay C, Menéndez S, Hernández F, Montero T, Rojas E: Protection by ozone preconditioning is mediated by the antioxidant system in cisplatin-induced nephrotoxicity in rats. *Mediators Inflamm,* 13, 13-19, 2004. DOI: 10.1080/09629350410001664806

23. Borrego A, Zamora ZB, González R, Romay C, Menéndez S, Hernández F, Berlanga J, Montero T: Ozone/oxygen mixture modifies the subcellular redistribution of Bax protein in renal tissue from rats treated with cisplatin. *Arch Med Res,* 37 (6): 717-722, 2006. DOI: 10.1016/j.arcmed.2006.02.008

24. González R, Borrego A, Zamora Z, Romay C, Hernández F, Menéndez S, Montero T, Rojas E: Reversion by ozone treatment of acute nephrotoxicity induced by cisplatin in rats. *Mediators Inflamm,* 13, 307-312, 2004. DOI: 10.1080/09629350400008836

25. Appel GB, Neu HC: Gentamicin in 1978. *Ann Intern Med*, 89, 528-538, 1978.

26. Maden M, Aslan V: Deneysel gentamisin nefrotoksisitesinde üriner enzim aktivitelerinin önemi. *T*ürk J Vet Anim Sci, 23, 29-42, 1999.

27. Hottendorf GH: Comparative ototoxicity (cats) and nephrotoxicity (rats) of amikacin and gentamicin. *Am J Aled,* 61, 97-104, 1977.

28. Pedraza-Chaverri J, Maldonado PD, Mediana-Campos ON, Olivares-Corichi IM, Granados-Silvestre MA, Hernandez-Pando R, Ibarra-Rubio ME: Garlic ameliorates gentamicin nephrotoxicity: Relation to antioxidant enzymes. *Free Radic Biol Med,* 29, 602 611, 2000. DOI: 10.1016/S0891-5849(00)00354-3

Kafkas Univ Vet Fak Derg
25 (3): 405-414, 2019
DOI: 10.9775/kvfd.2018.21137

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Establishment and Application of a Real-time, Duplex PCR Method for Simultaneous Detection of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*

Yuzi WU [1,2] Hassan Z. A. ISHAG [3] Lizhong HUA [1] Lei ZHANG [1] Beibei LIU [1] Zhenzhen ZHANG [1]
Haiyan WANG [1] Yanna WEI [1] Zhixin FENG [1] Hafizah Yousuf CHENIA [2] Guoqing SHAO [1] Qiyan XIONG [1]

[1] Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences· Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture· National Center for Engineering Research of Veterinary Bio-Products, Nanjing 210014 CHINA
[2] Discipline: Microbiology, School of Life Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, SOUTH AFRICA
[3] College of Veterinary Science, University of Nyala, Nyala, SUDAN

Article Code: KVFD-2018-21137 Received: 08.10.2018 Accepted: 21.02.2019 Published Online: 22.02.2019

How to Cite This Article

Wu Y, Ishag HZA, Hua L, Zhang L, Liu B, Zhang Z, Wang H, Wei Y, Feng Z, Chenia HY, Guoqing Shao G, Xiong Q: Establishment and application of a real-time, duplex PCR method for simultaneous detection of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*. *Kafkas Univ Vet Fak Derg*, 25 (3): 405-414, 2019. DOI: 10.9775/kvfd.2018.21137

Abstract

The objective of this study was to develop a TaqMan probe-based, sensitive, specific duplex real-time PCR assay for simultaneous detection of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*. The specific primers and probes, labeled with FAM and Texas Red, respectively, were designed to amplify the *p97* gene of *M. hyopneumoniae* and *p37*gene of *M. hyorhinis*. The duplex real-time PCR reaction mixtures were established and optimized and the sensitivity, specificity and reproducibility of the assay were assessed. The sensitivity of the duplex real-time PCR was found to be 10 copies/µL for both *M. hyopneumoniae* and *M. hyorhinis*, respectively. There was no cross reaction with other common viral and bacterial pathogens. The concentration of standard coefficient of variation of Ct values was less than 5%, indicating a good reproducibility. Clinical samples (n = 937) were tested by the duplex real-time PCR assay, including broncho-alveolar lavage fluids, nasal swabs, tissues and cell culture supernatant. Duplex real-time PCR for simultaneous detection of *M. hyopneumoniae* and *M. hyorhinis* was highly sensitive and can be utilized for diagnosing clinical samples. It is time-efficient and economic, thereby providing a new approach to control both *M. hyopneumoniae* and *M. hyorhinis*.

Keywords: *Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, duplex real-time PCR, Swine, Detection*

Mycoplasma hyopneumoniae ve *Mycoplasma hyorhinis*'in Aynı Anda Tespitinde Gerçek Zamanlı, Dubleks PCR Metodunun Uygulanması

Öz

Bu çalışmanın amacı, *Mycoplasma hyopneumoniae* ve *Mycoplasma hyorhinis*'in aynı anda tespitinde TaqMan prob temelli, hassas, spesifik dubleks gerçek zamanlı PCR yönteminin geliştirilmesidir. FAM ve Teksas Kırmızısı ile işaretli spesifik primer ve problar *M. hyopneumoniae p97* geni ile *M. hyorhinis p37* geninin amplifikasyonu amacıyla dizayn edildi. Dubleks gerçek zamanlı PCR reaksiyon karışımları oluşturularak optimize edildi ve yöntemin hassasiyetliği, özgüllüğü ve tekrarlanabilirliği hesaplandı. Dubleks gerçek zamanlı PCR'ın hassasiyetliği hem *M. hyopneumoniae* hem de *M. hyorhinis* için 10 kopya/µL olarak bulundu. Diğer yaygın viral ve bakteriyel patojenler ile çapraz reaksiyon yoktu. Ct değerlerinin varyasyonlarının standart katsayısının konsantrasyonu %5'ten az olup iyi bir tekrarlanabilirliğe işaret etmekteydi. Bronkoalveoler lavaj sıvısı, nazal svablar, dokular ve hücre kültürü süpernatantlarını içeren klinik örnekler (n = 937) dubleks gerçek zamanlı PCR ile test edildi. *Mycoplasma hyopneumoniae* ve *Mycoplasma hyorhinis*'in aynı anda tespitinde dubleks gerçek zamanlı PCR oldukça yüksek hassasiyetliğe sahip olup klinik örneklerde tanı amacıyla kullanılabilir. Yöntem kısa zamanda uygulanabilmesi ve ekonomik olması sebebiyle hem *M. hyopneumoniae* hem de *M. hyorhinis*'in kontrolünde yeni bir yaklaşım olarak kullanılabilir.

Anahtar sözcükler: *Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, Dubleks gerçek zamanlı PCR, Domuz, Tespit*

İletişim (Correspondence)
+86 025 84390880 Fax: +86 025 84391913
zzzizi24@163.com

INTRODUCTION

Mycoplasma hyopneumoniae and *Mycoplasma hyorhinis* are members of the Mycoplasmatales family that affect swine health and production in worldwide [1]. *M hyopneumoniae* is the etiological agent of enzootic pneumonia in swine, a chronic respiratory disease characterized by highly infectious, high morbidity and low mortality rates [2]. In the acute phase of the disease, catarrhal pneumonia is observed, with exudates in the airways. The bronchial and mediastinal lymph nodes are often enlarged. In the chronic stage of the disease, recovering lesions, consisting of fissures of collapsed alveoli adjoining areas of alveolar emphysema, are observed [3]. *M. hyopneumoniae* is a very contagious bacterium and may be transmitted *via* direct contact between pigs [4] or *via* the environment [5,6].

M. hyorhinis is a common pollutant in cell culture and is associated with the development of certain human tumor diseases *in vitro* [7], with unknown the mechanisms. It may cause arthritis, polyserositis, ear infections, pneumonia, pleurisy, peritonitis, pericarditis, pharyngeal tube inflammation and otitis media [8-10], with high morbidity and low mortality rates. The mixed bacterial infection with porcine enzootic pneumonia and porcine reproductive and respiratory syndrome was thought to facilitate the development of disease. *M. hyorhinis* generally occurs in 3 to 10-week-old pigs and is generally transmitted through nasal secretions by sows to piglets. It has been isolated from the nasal secretions of about 30-40% of weaning pigs or from lung tissue with typical lesions.

The establishment of detection methods for *M. hyopneumoniae* and *M. hyorhinis* is crucial for epidemiological and pathogenesis studies. Many methods are mainly based on clinical diagnosis (slaughterhouse monitoring), bacterial culture, serology and molecular biology diagnostic methods [11-15]. The culture isolation detection method is often regarded as the gold standard method for *M. hyopneumoniae* detection. Molecular detection systems have the potential to provide a higher degree of sensitivity and time-saving compared to culture isolation. PCR methods have been applied to lung tissue [16-18], aerosol samples [19], nasal swabs [20-23], broncho-alveolar lavage fluids and cell culture. Fluorescent, quantitative PCR technology is a method of choice to diagnose diseases because of its high sensitivity/specificity as well as being rapid, quantitative and accurate [24]. This study established a method for simultaneous detection of *M. hyopneumoniae* and *M. hyorhinis*. The double fluorescent quantitative PCR method of *M. hyopneumoniae* is helpful for rapid qualitative and quantitative monitoring of *M. hyopneumoniae* and *M. hyorhinis* infections, providing a useful technology for the prevention and control of animal diseases caused by these organisms. It is simpler, faster, more accurate and has wide application prospect when compared to conventional PCR, nested PCR and singleplex real-time PCR.

MATERIAL and METHODS

The laboratory in which this study was conducted practices strict physical separation of all the various steps involved in PCR, and a unidirectional workflow was employed to reduce risk of contamination.

Bacterial Strains, Virus and Cells

Fourteen bacterial and viral strains were detected. Bacterial strains: *Actinobacillus pleuropneumoniae*, *Escherichia coli*, *Haemophilus parasuis*, *M. hyopneumoniae*, *M. hyorhinis*, *M. flocculare*, *M. gallisepticum*, and *Staphylococcus aureus,* as well as viruses: Porcine circovirus type 2 (PCV2), Porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus infection (PPI), classical swine fever virus (CSFV) and Swine influenza virus were isolated, identified and provided by the Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, China. *M. hyosynoviae* (M60, ATCC® 27720™) was obtained from the American Type Culture Collection (Rockville, Md.).

Twelve cell lines, including the parental porcine mono-myeloid cell line (3D4/21; ATCC CRL-2843), St. Jude porcine lung cells (SJPL; ATCC PTA-3256), porcine kidney cell (PK15; ATCC PTA-8244) and swine tracheal epithelial cells (STEC) were provided by the Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, China.

DNA and RNA Extraction

Processing of the lung tissue: The dead swine to be tested were euthanatized and fresh lung tissues were taken and rinsed with sterile phosphate buffered saline (PBS) solution. The junctions of normal and diseased tissue were cut, and DNA was extracted from the tissue using Column Animal DNA_{OUT} Kit (Tiandz Inc., Beijing, China) following the manufacturer's instructions [19].

Processing of bronchial alveolar lavage fluids: The trachea was filled with sterile PBS solution and gently kneaded to ensure full immersion of PBS solution into the lung tissues and BALF samples were collected [25]. DNA was extracted using Column Bacterial DNA_{OUT} (Tiandz Inc.).

Processing of aerosol samples: Aerosol samples were collected using an electromagnetic air pump [26] in pig herds, injected into Erlenmeyer flask, and centrifuged at 12000 rpm/min. The precipitate was collected and used to extract DNA using the phenol-chloroform method [27].

Processing of nasal swabs sample: Pigs were tethered and a cotton swab was gently touched to the nasal septum to stimulate swine sneezing 3 times. The swab was pulled and placed into sterile PBS solution at 4°C for 12 h. Following centrifugation at 10000 rpm/min for 5 min, the precipitate was collected and used to extract DNA using Column Swab DNA_{OUT} Kit (Tiandz Inc.) according to the manufacturer's instructions.

Processing of bacterial and viral strains: DNA of *A. pleuropneumoniae*, *E. coli*, *H. parasuis*, *M. flocculare*, *M. gallisepticum*, *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae*, *S. aureus*, and PPI was extracted using the Column Bacterial DNA_{OUT} kit (Tiandz Inc.). RNA of PCV2, PRRSV, CSFV, Swine influenza virus was extracted using the One-Tube Viral DNA-RNA_{OUT} kit (Tiandz Inc.).

Primers and Probes

The real-time PCR method for *M. hyopneumoniae p97* assay has been described previously by Strait et al.[28]. The *M. hyorhinis*-specific real-time PCR assay developed according to our previous studies [29] was modified slightly. The difference was reflected on the labeling of the probe. Optimization included using *M. hyorhinis p37* sequence as the probe instead of the previously described labeling with 5′-6-carboxyfluorescein (FAM) and a 3′ minor grove binder (MGB) non-fluorescent quencher, a Texas Red-labeled probe was used *(Table 1)*. All oligonucleotides were synthesized by TaKaRa (Dalian, China).

Optimization of Duplex Real-Time PCR Assay

The concentrations of the primers and the probe were optimized to establish the optimum duplex real-time PCR reaction system. DNA of *M. hyopneumoniae* and *M. hyorhinis* were used as template, the primers concentration range (3 μM to 10 μM), a probe concentration range (0.5 μM to 5 μM), and an annealing temperature (50°C to 60°C). The duplex real-time PCR reaction system (20 μL) was composed as follows: 10 μL AceQ qPCR probe Master Mix (Vazyme Biotech Co., Ltd), 1 μL template (approximately 0.1 ng/μL), 1 μL ddH_2O, the primers and probes (concentrations described in *Table 1)* were merged as a master mix. Each run included a positive control (the gradient dilution of recombinant plasmid), a negative control (ddH_2O). The reaction conditions were as follows: 40 cycles of 50°C for 2 min, 95°C for 10 min; 95°C for 15 s, 60°C for 60 s). The reaction was carried out in Quant Studio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Templates were tested in triplicate and the Cycle threshold (CT) values were plotted against the copy number in order to verify the reproducibility.

Testing Inter- and Intra-Detection Specific of Assay

Positive plasmid of *M. hyopneumoniae* and *M. hyorhinis* was prepared as described by Strait et al.[28] and Bai et al.[29]. The plasmids were diluted 10 times as standard template, and optimized reaction mixtures and conditions were utilized to detect the sensitivity of the method. DNA and RNA extracted from 14 bacterial and viral strains were used to confirm the specificity of the assay. These strains included *A. pleuropneumoniae*, *E. coli*, *H. parasuis*, *S. aureus*, *M. flocculare*, *M. gallisepticum*, *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae*, PCV2, PRRSV, PPI, CSFV, and Swine influenza virus *(Fig. 1)*.

Table 1. *The primers and probes selected for singleplex and duplex real-time PCR*

Primers or Probes	Sequence 5′-3′	Genomic Target	Concentration (pmol/reaction)		References
			Singleplex Real-Time PCR	Duplex Real-Time PCR	
*p97*F	CCAGAACCAAATTCCTTCGCTG	*p97*	1	0.5	[28]
*p97*R	ACTGGCTGAACTTCATCTGGGCTA		1	0.5	
*p97*P	FAM[a]-AGCAGATCTTAGTCAAAGTGCCCGTG–TAMRA[b]		0.5	0.5	
*p37*F	AGAAGGTTCTTTTGCTTGAACACA	*p37*	1	0.5	[29]
*p37*R	TGCTTCCATCTTTTCATTTGCTT		1	0.5	
*p37*P	TXR[c]-ATCAGCAACAAAACCTT-BHQ[d]		0.5	1.5	

[a] FAM, 6-carboxyfluorescein, fluorescence reporter dye; [b] TAMRA, Carboxytetramethylrhodamine; [c] TXR, texas-red, fluorescence reporter dye; [d] BHQ, Black Hole Quencher

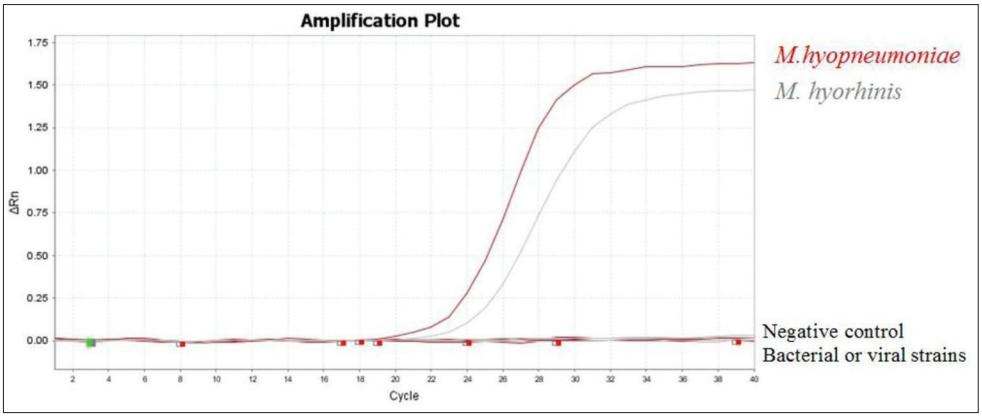


Fig 1. The amplification curve of specific experiments: *M. hyopneumoniae, M. hyorhinis* and other strains were tested using the duplex real-time PCR. *M. hyopneumoniae* and *M. hyorhinis* tested positive, while the other samples (i.e., *M. flocculare, M. gallisepticum, M. hyosynoviae, A. pleuropneumoniae, E. coli, H. parasuis, S. aureus,* PCV2, PRRSV, PPI, CSFV, and Swine influenza virus) tested negative. The negative control and other common bacterial or viral pathogens did not amplify, were straight lines. There was no cross reaction with other common bacterial or viral pathogens

Varying concentrations of *M. hyopneumoniae* and *M. hyorhinis* plasmid DNA (1×10^6, 1×10^5, 1×10^4 copies/µL respectively), were incorporated into three reaction mixtures. Three batches of intra- and inter-assay testing were performed in order to calculate the Coefficient of Variation (CV) and reproducibility was also measured.

Evaluation of Clinical Samples

The duplex real-time PCR was evaluated for the detection of different clinical samples. Clinical samples tested included broncho-alveolar lavage fluids, nasal swabs and tissues.

One hundred negative samples from known mycoplasma-negative pigs (15 lung tissues, 65 BALF, 20 nasal swabs) were frozen at -70°C by the Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, China. Nasal swabs (*n*=583) were obtained from different eleven pig herds in Jiangsu province, China.

Twelve pigs were used in animal experiments to obtain different samples. Seven of them were experimentally infected with *M. hyopneumoniae* [7], while the remaining five pigs were not inoculated with *M. hyopneumoniae*. The different samples including BALF, blood and tissue samples (hilar lymph nodes, lung tissue, muscle, kidney, heart, spleen, liver, stomach, brain, pancreas, duodenum, jejunum, ileum, colon, rectum and cecum) were from these twelve pigs. All experimental procedures were approved by the Ethical and Animal Welfare Committee of the Jiangsu Academy of Agricultural Sciences (No.161028).

RESULTS

Analytical Specificity, Sensitivity and Reproducibility of the Duplex Real-Time PCR

Singleplex assays integrated in the newly developed duplex real-time PCR assay have been assessed previously with respect to sensitivity and specificity. The sequences of all primers and probes included in the duplex real-time PCR *(Table 1)* were aligned with publically available sequence information (NCBI GenBank) with a special focus on porcine viruses. There was no indication of possible cross-reactions.

The specific detection: The duplex real-time PCR approach has been established to exclude non-specific reactions. Nucleic acids extracted from lung tissue, BALF and nasal swabs collected from healthy pigs were tested. All samples scored negative in assays included in the duplex real-time PCR *(Table 2,* sample ID 01-100). *M. hyopneumoniae*, *M. hyorhinis* and other strains were tested using the duplex real-time PCR. *M. hyopneumoniae* and *M. hyorhinis* tested positive, while the other samples (i.e., *M. flocculare*, *M. gallisepticum*, *M. hyosynoviae*, *A. pleuropneumoniae*, *E. coli, H. parasuis*, *S. aureus,* PCV2, PRRSV, PPI, CSFV, and Swine influenza virus) tested negative *(Table 2,* sample ID 101 to 114). There was no cross reaction with other common bacterial or viral pathogens *(Fig. 1)*.

Establishment of the standard curve: The recombinant plasmid of *M. hyopneumoniae* and *M. hyorhinis* was diluted 10 times with 1×10^9 copies/µL to 1×10^4 copies/µL dilution as a template for duplex real-time PCR. The concentration of the amplification results was the abscissa, and the corresponding Ct value was the ordinate, and two standard curves were obtained *(Fig. 2)*. The linear correlation, coefficient R^2 and the amplification efficiency E of *M. hyopneumoniae* and *M. hyorhinis* were -3.207, 1, and 104.68%; -3.215, 1 and 105.04% respectively. The linear relationship of the amplified product was good between the Ct value and the concentration.

The sensitivity test: The analytical sensitivity in the duplex real-time PCR was evaluated using a series of 10-fold dilutions of recombination plasmid of *M. hyopneumoniae* and *M. hyorhinis* in three replicates per run on three different days. The results indicated that the sensitivity was 10 copies/µL for both *M. hyopneumoniae* and *M. hyorhinis (Fig. 3)*.

The reproducibility test: To test the reproducibility of the duplex real-time PCR, standard plasmids of *M. hyopneumoniae* and *M. hyorhinis* at three different concentrations, 1.0×10^7 - 1.0×10^5 copies/µL were used *(Table 3)*. The variations were assessed by three replicates per run on three different days. The results demonstrated that the duplex TaqMan Ct values are easily achieved at the end of the process with a CV of Ct values between the intra-assay test and the inter-assay test being less than 5% *(Table 3)*. The study showed that the reproducibility were good.

Clinical and Experimentally Infected Sample Detection Using Duplex Real-Time PCR

In total, 126 individual samples were tested by the duplex real-time PCR, and in the respective singleplex assays, simultaneously *(Table 2,* sample ID 1 to 126). Overall, a high agreement could be observed between the Ct values obtained in the duplex real-time PCR and each single-target PCR assay for the clinical samples.

Twelve cell lines of STEC, PK15, SJPL and 3D4/21 were examined *(Table 2,* sample ID 115 to 126). Only a single STEC cell line was positive for *M. hyorhinis*. The detection result was accordant to that of the above Single-target real-time PCR. It appeared to be contaminated with *M. hyorhinis (Table 2,* sample ID 115). .

Following collection of nasal swabs from 11 pig farms *(Table 2,* sample ID 127 to 709), *M. hyopneumoniae* and *M. hyorhinis* could be detected, although the Ct values were relatively low. The positive rate of *M. hyorhinis* was higher than *M. hyopneumoniae*, with only a single pig farm where the positive rate of *M. hyorhinis* was lower than *M.*

Table 2. *Assessment of diagnosis of M. hyoneumoniae and M. hyorhinis in clinical samples using real-time PCR*

No.	Sample Material	Duplex Real-Time PCR (Ct*)		Single-Target Real-Time PCR (Ct)	
		M. hyopneumoniae	***M. hyorhinis***	***M. hyopneumoniae***	***M. hyorhinis***
Negative samples					
1-15	lung tissue	no Ct	no Ct	no Ct	no Ct
16-80	BALF	no Ct	no Ct	no Ct	no Ct
81-100	nasal swabs	no Ct	no Ct	no Ct	no Ct
Bacterial or viral strains					
101	*M. hyopneumoniae*	20.26	no Ct	20.94	no Ct
102	*M. hyorhinis*	no Ct	22.58	no Ct	23.01
103	*M. hyosynoviae*	no Ct	no Ct	no Ct	no Ct
104	*M. flocculare*	no Ct	no Ct	no Ct	no Ct
105	*M. gallisepticum*	no Ct	no Ct	no Ct	no Ct
106	*H. parasuis*	no Ct	no Ct	no Ct	no Ct
107	PCV2	no Ct	no Ct	no Ct	no Ct
108	pleuropneumoniae	no Ct	no Ct	no Ct	no Ct
109	PRRSV	no Ct	no Ct	no Ct	no Ct
110	PPI	no Ct	no Ct	no Ct	no Ct
111	*S. aureus*	no Ct	no Ct	no Ct	no Ct
112	CSFV	no Ct	no Ct	no Ct	no Ct
113	Swine influenza virus	no Ct	no Ct	no Ct	no Ct
114	*E. coli*	no Ct	no Ct	no Ct	no Ct
Cell culture supernatant					
115	STEC	no Ct	37.3	no Ct	37.5
116	STEC	no Ct	no Ct	no Ct	no Ct
117	STEC	no Ct	no Ct	no Ct	no Ct
118	STEC	no Ct	no Ct	no Ct	no Ct
119	PK15	no Ct	no Ct	no Ct	no Ct
120	PK15	no Ct	no Ct	no Ct	no Ct
121	SJPL	no Ct	no Ct	no Ct	no Ct
122	SJPL	no Ct	no Ct	no Ct	no Ct
123	SJPL	no Ct	no Ct	no Ct	no Ct
124	3D4/21	no Ct	no Ct	no Ct	no Ct
125	3D4/21	no Ct	no Ct	no Ct	no Ct
126	3D4/21	no Ct	no Ct	no Ct	no Ct
Clinical samples from different pig herds					
127-181	nasal swabs	11/55	39/55	n.t#	n.t
182-211	nasal swabs	13/30	9/30	n.t	n.t
212-311	nasal swabs	18/100	31/100	n.t	n.t
312-359	nasal swabs	19/47	34/47	n.t	n.t
360-417	nasal swabs	18/58	25/58	n.t	n.t
418-452	nasal swabs	17/35	18/35	n.t	n.t
453-494	nasal swabs	18/42	31/42	n.t	n.t
495-552	nasal swabs	30/58	37/58	n.t	n.t
553-591	nasal swabs	2/39	27/39	n.t	n.t
592-649	nasal swabs	20/58	26/58	n.t	n.t
650-709	nasal swabs	21/60	23/60	n.t	n.t

**Ct –cycle threshold; # n.t.-not tested*

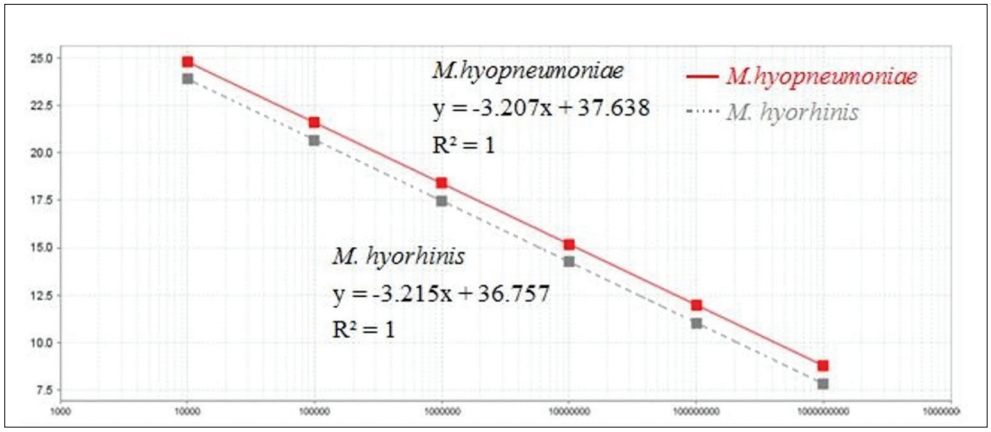


Fig 2. Standard curve of duplex real-time PCR: The linear correlation, coefficient R2 and the amplification efficiency E of *M. hyopneumoniae* were -3.207, 1, and 104.68%, respectively. The linear correlation, coefficient R2 and the amplification efficiency E of *M. hyorhinis* were -3.215, 1 and 105.04%, respectively. The standard curve of *M. hyopneumoniae* and *M. hyorhinis* were y = -3.207x + 37.638, y = -3.215x + 36.757. The linear relationship of the amplified product was good between the Ct value and the concentration

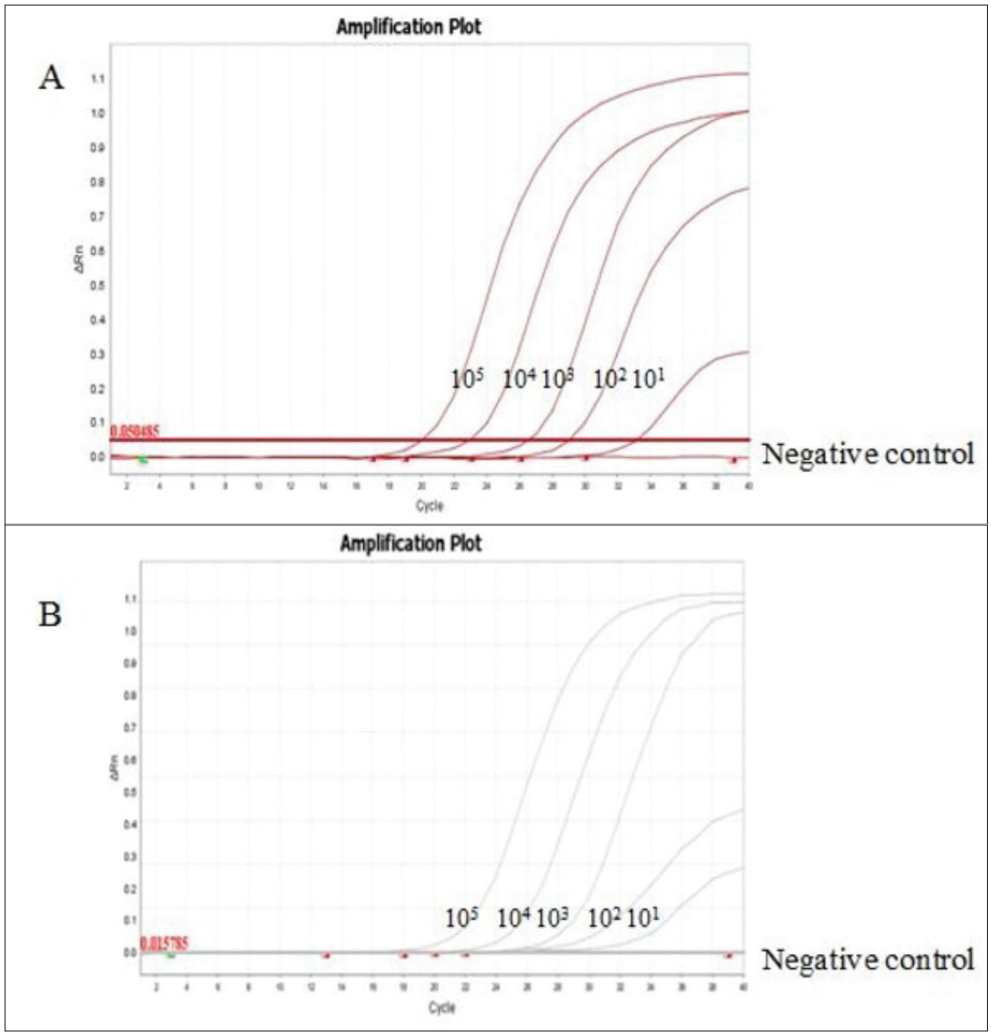


Fig 3. Sensitivity test of the real-time PCR. A: Amplification curve of *M. hyopneumoniae*, B: Amplification curve of *M. hyorhinis*, The sensitivity of both *M. hyopneumoniae* and *M. hyorhinis* was 10 copies/μL, respectively

hyopneumoniae (*Table 2*, sample ID 182 to 211).

Seven animals were inoculated with *M. hyopneumoniae*, while other five animals were not inoculated. The clinical samples from twelve pigs, including nasal swabs, BALF, blood, lung tissue, hilar lymph nodes, muscle, kidney, heart, spleen, liver, stomach, brain, pancreas, duodenum, jejunum, ileum, colon, rectum and cecum *(Table 4)* were detected by using the established duplex real-time PCR assay. All the samples of hilar lymph nodes, lung tissue from seven pigs inoculated with *M. hyopneumoniae*, BALF were positive for *M. hyopneumoniae* (*Table 4* Pig No.1, 2, 3, 4, 5, 6, and 7). In a few nasal swabs samples of the experimentally infected animals with *M. hyopneumoniae*, *M. hyorhinis* was detected, although the Ct was relatively low *(Table 4)*. Pig No.4, 6, 9 and 10, these four nasal swabs were positive for *M. hyorhinis*, whether or not to be challenged *M. hyopneumoniae.*

Table 3. *The intra- and inter-detection result of duplex real-time PCR*

Agent	Concentration of Standard (copies/μL)*	Intra-Assay	Inter-Assay
		CV (%)#	CV (%)#
M. hyopneumoniae	1×10^7	0.03	0.23
	1×10^6	0.11	2.27
	1×10^5	0.15	1.62
M. hyorhinis	1×10^7	0.08	1.32
	1×10^6	0.22	1.77
	1×10^5	0.17	2.33

** **Copies/μL:** the DNA copy numbers per microliter, # **CV (%):** Ct coefficients of variations*

Table 4. *Detection of M. hyopneumoniae and M. hyorhinis in experimentally infected tissues using a duplex Real-time PCR assay*

Specimen	Ct# Values for Detection of *M. hyopneumoniae* (A) and *M. hyorhinis* (B)															
	Pig															
	1		2		3		4		5		6		7		8-12	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Nasal Swabs	33.7	no Ct	35.1	no Ct	33.8	no Ct	34.1	35.7	34.1	no Ct	33.8	36.5	31.7	no Ct	no Ct	no Ct 37.2 38.2 no Ct no Ct
Hilar lymph nodes	33.4	no Ct	31.8	no Ct	33.2	no Ct	33.4	no Ct	32.7	no Ct	32.9	no Ct	36.1	no Ct	no Ct	no Ct
Lung tissue	33.0	no Ct	31.5	no Ct	28.5	no Ct	33.5	no Ct	34.6	no Ct	32.1	no Ct	32.8	no Ct	no Ct	no Ct
BALF	31.8	no Ct	28.5	no Ct	31.9	no Ct	33.3	no Ct	33.1	no Ct	29.2	no Ct	32.2	no Ct	no Ct	no Ct
Blood	32.3	no Ct	no Ct	no Ct	no Ct	no Ct	36.4	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Muscle	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Kidney	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Heart	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Spleen	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Liver	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Stomach	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Brain	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Pancreas	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Duodenum	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Jejunum	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Ileum	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Colon	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Rectum	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Cecum	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct

***A:** M. hyopneumoniae; **B:** M. hyorhinis; Ct –cycle threshold*

DISCISSION

Diseases associated with *M. hyopneumoniae* and *M. hyorhinis* are difficult to control because of the long survival of the organism in the environment, shedding by apparently healthy but infected animals and the unreliability of diagnostic tests [26,27]. Therefore, a rapid diagnosis of the causative agent is crucial [30]. A variety of detection methods for swine viruses have been developed during recent years, for instance, multiplex PCR [31,32], real-time PCR [26,33]. Wu et al.[32] established a duplex PCR detection method based on Hps *p2* protein gene and *M.*

hyorhinis p37 protein gene of *Haemophilus parasuis* and *M. hyorhinis*. The results showed that the sensitivity was 100 copies/reaction. This method could determine the pathogenicity of *Haemophilus parasuis,* according to the size of amplification products.

For detection of several pathogens, or for multiple genetic tests of the same pathogen, singleplex PCR is severely

limited, because it will lead to waste of time, human resources and detergent. By using multiplex PCR systems, several infectious agents can be detected and differentiated simultaneously in a single reaction, reducing costs and efforts as well as the amount of sample material and time required [34]. Duplex real-time PCR has several advantages, combining a reduced risk of cross-contamination with a high sensitivity and the possibility of quantitative analysis. Oligonucleotide probes labeled with different fluorophores permit multiplexing in a qPCR format, enabling the detection of different target sequences as well as the co-amplification of internal controls. The duplex real-time PCR assay for the simultaneous detection of *M. hyopneumoniae* and *M. hyorhinis* was developed and validated in this study. The probes specific for genome detection of the two notifiable bacteria were labeled with the different fluorophores, i.e., Texas Red and FAM. A rapid (time to completion, <4 h, including DNA extraction), convenient, quantitative and reliable screening system is beneficial for monitoring the clinical course of *M. hyopneumoniae* and *M. hyorhinis* and enhances the clinical utility of molecular testing. Duplex real-time PCR assay labelling with FAM and Texas Red for detection can yield results within 2 h. It does not require post-PCR processing, reduces sample handling, minimizes the risks of contamination [35], and is beneficial for monitoring the clinical course of *M. hyopneumoniae* and *M. hyorhinis*, which will enhance the clinical utility of molecular testing. It is simple, rapid and particularly useful for clinic detection, including BALF, nasal swabs, blood, and tissues. The test revealed a specificity of 100%, has higher sensitivity than Normal PCR [36-38], and equal as Real-time PCR described by Dubosson et al.[39], Marois et al.[18] Fourour et al.[26] increased detection of *M. flocculare*, established a multiplex real-time PCR targets the *p102, p37* and *fruA* genes for *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare*. The detection limits reached 14, 146, and 16 genome equivalents μl^{-1}, respectively, the sensitivity is more than five times lower than this study.

One of the main problems caused by a large number of oligonucleotides in the same reaction tube is a possible interaction of those molecules with each other, resulting in inhibition of the amplification reactions and a subsequent reduced sensitivity [5,34,40]. In our assays, only two pairs of primers and probes were used in the same reaction tube, for each of the large number of clinical samples tested. Similar Ct results were achieved in both the single and duplex approaches, which is consistent with results of Wernike et al.[41].

The newly developed duplex real-time PCR is suitable for use with diverse sample materials, such as BALF, nasal swabs, blood, tissues (lung tissue, hilar lymph nodes, muscle, kidney, heart, spleen, liver, stomach, brain, pancreas, duodenum, jejunum, ileum, colon, rectum cecum) and cell culture supernatant. The application of real-time PCR in diverse clinical samples has been replicated many times [18,29,42,43]. *M. hyopneumoniae* was the persistent organism in the trachea and bronchial lymph nodes, and could be re-isolated from inner organs like liver, spleen and kidneys of experimentally infected pigs. The observed persistence cannot be explained by dissemination of *Mycoplasma* spp. in internal organs, as this phenomenon seems to be transient with no *Mycoplasma* spp. being re-isolated from internal organs at the end of the studies. This suggests that *M. hyopneumoniae* can ephemerally colonize the internal organs of the host, indicating that *M. hyopneumoniae* exists in these tissues without causing disease, and maybe spread through the lymph circulation or blood circulation. Friis [44] isolated *M. hyopneumoniae* from brains of infected pigs. Jin [45] detected *M. hyopneumoniae* in heart, liver, brain, and muscles, indicating that *M. hyopneumoniae* could colonize the internal organs of the host. Wang et al.[46] detected *M. hyorhinis* in blood with the positive rate of 20% (16/80). In this study, it was observed that *M. hyopneumoniae* could be detected in a blood sample of two pigs experimentally infected with *M. hyopneumoniae (Table 4, 1A, 4A)*. Whether *Mycoplasma* spp. spreads through lymphatic circulation or blood circulation remains a problem needed for further research.

The STEC cell line, derived from tracheal epithelial, is more susceptible to contamination from *M. hyorhinis* than other cells, therefore, it is easier to do *M. hyopneumoniae* infestation experiment. The availability of accurate, sensitive and reliable detection duplex real-time PCR and the application of robust and successful elimination methods provides a powerful means for overcoming the problem of mycoplasma contamination in cell cultures. The contamination of cell cultures by *Mycoplasma* spp., especially *M. hyorhinis,* remains a major problem in cell culture. Ideal detection methods for contaminating mycoplasma should be highly sensitive and specific, but also simple, rapid, efficient and cost effective.

In conclusion, the newly developed duplex real-time PCR allows the simultaneous detection of *M. hyopneumoniae* and *M. hyorhinis* combined in a single tube assay with a rapid, convenient, and reliable screening system. The new system could therefore significantly improve the early detection of diseases of swine and could lead to a new approach in syndromic surveillance. Our study indicates that the reported duplex Real-Time PCR could be an accurate diagnostic tool for assessing infection *M. hyopneumoniae* and *M. hyorhinis*. Future detailed studies in diverse geographical locations are warranted to investigate the clinical value of this technique.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This work was supported by grants from The National Key Research and Development Program of China (2016YFD0500702), The National Natural and Science Foundation of China (31400164), and The Jiangsu Province Natural Sciences Foundation (BK20140754).

REFERENCES

1. Holst S, Yeske P, Pieters M: Elimination of *Mycoplasma hyopneumoniae* from breed-to-wean farms: A review of current protocols with emphasis on herd closure and medication. *J Swine Health Prod*, 23 (6): 321-330, 2015.

2. Otake S, Dee S, Corzo C, Oliveira S, Deen J: Long-distance airborne transport of infectious PRRSV and *Mycoplasma hyopneumoniae* from a swine population infected with multiple viral variants. *Vet Microbiol*, 145 (3-4): 198-208, 2010. DOI: 10.1016/j.vetmic.2010.03.028

3. Scudamore JM, Harris DM: Control of foot and mouth disease: lessons from the experience of the outbreak in Great Britain in 2001. *Rev Sci Tech*, 21 (3): 699-710, 2002.

4. Jiang Y, Shang H, Xu H, Zhu L, Chen W, Zhao L, Fang L: Simultaneous detection of porcine circovirus type 2, classical swine fever virus, porcine parvovirus and porcine reproductive and respiratory syndrome virus in pigs by multiplex polymerase chain reaction. *Vet J*, 183 (2): 172-175, 2010. DOI: 10.1016/j.tvjl.2008.11.016

5. Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE: Multiplex PCR: optimization and application in diagnostic virology. *Clin Microbiol Rev*, 13 (4): 559-570, 2000. DOI: 10.1128/CMR.13.4.559

6. Pomeranz LE, Reynolds AE, Hengartner CJ: Molecular biology of pseudorabies virus: Impact on neurovirology and veterinary medicine. *Microbiol Mol Biol R*, 69 (3): 462-500, 2005. DOI: 10.1128/MMBR.69.3.462-500.2005

7. Yang H, Qu L, Ma H, Chen L, Liu W, Liu C, Meng L, Wu J, Shou C: *Mycoplasma hyorhinis* infection in gastric carcinoma and its effects on the malignant phenotypes of gastric cancer cells. *BMC Gastroenterol*, 10:132, 2010. DOI: 10.1186/1471-230X-10-132

8. Bongtae K, Kichan L, Kiwon H, Duyeol K, Yooncheol H, Chung HK, Yeonsu O, Ikjae K, Jeehoon L, Chanhee C: Development of in situ hybridization for the detection of *Mycoplasma hyorhinis* in formalin-fixed paraffin-embedded tissues from naturally infected pigs with polyserositis. *J Vet Med Sci*, 72 (9): 1225-1227, 2010. DOI: 10.1292/jvms.10-0062

9. Chen DJ, Wei YW, Huang LP, Wang YP, Sun JH, Du WJ, Wu HL, Liu CM: Synergistic pathogenicity in sequential coinfection with *Mycoplasma hyorhinis* and porcine circovirus type 2. *Vet Microbiol*, 182, 123-130, 2016. DOI: 10.1016/j.vetmic.2015.11.003

10. Palzer A, Haedke K, Heinritzi K, Zoels S, Ladinig A, Ritzmann M: Associations among *Haemophilus parasuis*, *Mycoplasma hyorhinis*, and porcine reproductive and respiratory syndrome virus infections in pigs with polyserositis. *Can Vet J*, 56 (3): 285-287, 2015.

11. Du GM, Liu MJ, Wu YZ, Xiong QY, Bai FF, Feng ZX, Shao GQ: Development of a loop-mediated isothermal amplification assay for rapid detection of *Mycoplasma hyorhinis*. *Clin Lab*, 59 (11-12): 1363-1371, 2013. DOI: 10.7754/Clin.Lab.2013.121223

12. Kang I, Kim D, Han K, Seo HW, Oh Y, Park C, Lee J, Gottschalk M, Chae C: Optimized protocol for multiplex nested polymerase chain reaction to detect and differentiate *Haemophilus parasuis*, *Streptococcus Suis*, and *Mycoplasma hyorhinis* in formalin-fixed, paraffin-embedded tissues from pigs with polyserositis. *Can J Vet Res*, 76 (3): 195-200, 2012.

13. Liu MJ, Du GM, Bai FF, Wu YZ, Xiong QY, Feng ZX, Li B, Shao GQ: A rapid and sensitive loop-mediated isothermal amplification procedure (LAMP) for *Mycoplasma hyopneumoniae* detection based on the *p36* gene. *Genet Mol Res*, 14 (2): 4677-4686, 2015. DOI: 10.4238/2015.May.4.27

14. Tocqueville V, Ferre S, Nguyen NHP, Kempf I, Marois-Créhan C: Multilocus sequence typing of *Mycoplasma hyorhinis* strains identified by a real-time Taq Man PCR assay. *J Clin Microbiol*, 52 (5): 1664-1671, 2014. DOI: 10.1128/JCM.03437-13

15. Wu YZ, Zhang X, Bai FF, Liu MJ, Hua LZ, Du GM, Shao GQ: Evaluation on clinical application of three nucleic acid amplification assays for the detection of *Mycoplasma hyorhinis*. *Chinese J Prev Vet Med*, 35, 985-988, 2013.

16. Charlebois A, Marois-Créhan C, Hélie P, Gagnon CA, Gottschalk M, Archambault M: Genetic diversity of *Mycoplasma hyopneumoniae* isolates of abattoir pigs. *Vet Microbiol*, 168 (2-4): 348-356, 2014. DOI: 10.1016/j.vetmic.2013.11.006

17. Hillen S, Von Berg S, Köhler K, Reinacher M, Willems H, Reiner G: Occurrence and severity of lung lesions in slaughter pigs vaccinated against *Mycoplasma hyopneumoniae*, with different strategies. *Prev Vet Med*, 113 (4): 580-588, 2014. DOI: 10.1016/j.prevetmed.2013.12.012

18. Marois C, Dory D, Fablet C, Madec F, Kobisch M: Development of a quantitative Real-Time TaqMan PCR assay for determination of the minimal dose of *Mycoplasma hyopneumoniae*, strain 116 required to induce pneumonia in SPF pigs. *J Appl Microbiol*, 108 (5): 1523-1533, 2010. DOI: 10.1111/j.1365-2672.2009.04556.x

19. Zhang X, Bai FF, Wu YZ, Liu MJ, Feng ZX, Xiong QY, Zhang Y, Shao GQ: Research advances on the detection of *Mycoplasma hyopneumoniae* by PCR. *Biotechnol Bullet*, 5, 54-60, 2012.

20. Cho JG, Dee SA, Deen J, Guedes A, Trincado C, Fano E, Jiang Y, Faaberg K, Collins JE, Murtaugh MP, Joo HS: Evaluation of the effects of animal age, concurrent bacterial infection, and pathogenicity of porcine reproductive and respiratory syndrome virus on virus concentration in pigs. *Am J Vet Res*, 67 (3): 489-493, 2006. DOI: 10.2460/ajvr.67.3.489

21. Hua LZ, Wu YZ, Bai FF, William KK, Feng ZX, Liu MJ, Yao JT, Zhang X, Shao GQ: Comparative analysis of mucosal immunity to *Mycoplasma hyopneumoniae* in Jiangquhai porcine lean strain and DLY piglets. *Genet Mol Res*, 13 (3): 5199-5206, 2014. DOI: 10.4238/2014.July.7.13

22. Nathues H, Woeste H, Doehring S, Fahrion AS, Doherr MG, Beilage E: Herd specific risk factors for *Mycoplasma hyopneumoniae* infections in suckling pigs at the age of weaning. *Acta Vet Scand*, 55:30, 2013. DOI: 10.1186/1751-0147-55-30

23. Pieters M, Cline GS, Payne BJ, Prado C, Ertl JR, Rendahl AK: Intra-farm risk factors for *Mycoplasma hyopneumoniae* colonization at weaning age. *Vet Microbiol*, 172 (3-4): 575-580, 2014. DOI: 10.1016/j.vetmic.2014.05.027

24. Ginzinger DG: Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Exp Hematol*, 30 (6): 503-512, 2002. DOI: 10.1016/S0301-472X(02)00806-8

25. Hu WX, Zhou WY, Zhu XL, Wen Z, Wu LH, Wu XM, Wei HP, Wang WD, He D, Xiang Q, Hu GZ: Anti-interleukin-1 beta/tumor necrosis factor-alpha IgY antibodies reduce pathological allergic responses in guinea pigs with allergic rhinitis. *Mediat Inflamm*, 2016:3128182, 2016.

26. Fourour S, Fablet C, Tocqueville V, Dorenlor V, Eono F, Eveno E, Kempf I, Marois-Créhan C: A new multiplex real-time TaqMan® PCR for quantification of *Mycoplasma hyopneumoniae*, *M. hyorhinis* and *M. flocculare*: Exploratory epidemiological investigations to research mycoplasmal association in enzootic pneumonia-like lesions in slaughtered pigs. *J Appl Microbiol*, 125 (2): 345-355, 2018. DOI: 10.1111/jam.13770

27. Rebaque F, Camacho P, Parada J, Lucchesi P, Ambrogi A, Tamiozzo P: Persistence of the same genetic type of *Mycoplasma hyopneumoniae* in a closed herd for at least two years. *Rev Argent Microbiol*, 50 (2): 147-150, 2018. DOI: 10.1016/j.ram.2017.05.002

28. Strait EL, Madsen ML, Minion FC, Christopher-Hennings J, Dammen M, Jones KR, Thacker EL: Real-time PCR assays to address genetic diversity among strains of *Mycoplasma hyopneumoniae*. *J Clin Microbiol*, 46 (8): 2491-2498, 2008. DOI: 10.1128/JCM.02366-07

29. Bai FF, Wu YZ, Liu MJ, Feng ZX, Xiong QY, Wei YN, Ma QH, Shao GQ: Development of TaqMan MGB probe real-time PCR for detection of *Mycoplasma hyorhinis*. *Chinese J Prev Vet Med*, 35, 833-836, 2013.

30. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*, 55 (4): 611-622, 2009. DOI: 10.1373/clinchem.2008.112797

31. Lung O, Ohene-Adjei S, Buchanan C, Joseph T, King R, Erickson A, Detmer S, Ambagala A: Multiplex PCR and microarray for detection of swine respiratory pathogens. *Transbound Emerg Dis*, 64 (3):834-848, 2017. DOI: 10.1111/tbed.12449

32. Wu JB, Nan WJ, Huang JQ, Hu HH, Peng GL: Establishment and application of duplex PCR assay for detection of *Haemophilus parasuis* and *Mycoplasma hyorhinis*. *Chinese Vet Sci*, 9, 1094-1101, 2016.

33. Wu YZ, Xiong QY, Bai Y, Wei YN, Zhang ZZ, Wang HY, Feng ZX, Chenia HY, Shao GQ: Standardization study of Real-time PCR method for the quantitative detection of *Mycoplasma hyopneumoniae* Culture. *Agric Sci Technol*, 18, 2479-2484, 2017.

34. Markoulatos P, Siafakas N, Moncany M: Multiplex polymerase chain reaction: A practical approach. *J Clin Lab Anal*, 16 (1): 47-51, 2002. DOI: 10.1002/jcla.2058

35. Wu YZ, Zhang X, Hua LZ, Liu MJ, Shao GQ: Introduction on *Mycoplasma* contamination in cell culture. *Prog Vet Med*, 34, 112-117, 2013.

36. Jiangsu Province Quality and Technical Supervision Bureau: DB32/ T 1461-2009 Method of PCR for detecting *Mycoplasma hyopneumoniae*. Agricultural local standard of Jiangsu province, 2009.

37. Cai HY, Van Dreumel T, Mcewen B, Hornby G, Bell-Rogers P, Mcraild P, McRaild P, Josephson G, Maxie G: Application and field validation of a PCR assay for the detection of *Mycoplasma hyopneumoniae* from swine lung tissue samples. *J Vet Diagn Invest,* 19 (1): 91-95, 2007. DOI: 10.1177/104063870701900115

38. Ministry of Agriculture of the People's Republic of China: NY/T 1186-2017. Diagnostic Criteria for Mycoplasmal Pneumonia of Swine. National Standard of the People's Republic of China, 2017.

39. Dubosson CR, Conzelmann C, Miserez R, Boerlin P, Frey J, Zimmermann W, Hani H, Kuhnert P: Development of two real-time PCR assays for the detection of *Mycoplasma hyopneumoniae* in clinical samples. *Vet Microbiol,* 102 (1-2): 55-65, 2004. DOI: 10.1016/j.vetmic.2004.05.007

40. Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH: Multiplex PCR: critical parameters and step-by-step protocol. *Biotechniques*, 23 (3): 504-511, 1997. DOI: 10.2144/97233rr01

41. Wernike K, Hoffmann B, Beer M: Single-tube multiplexed molecular detection of endemic porcine viruses in combination with background screening for transboundary diseases. *J Clin Microbiol*, 51 (3): 938-944, 2013. DOI: 10.1128/JCM.02947-12

42. Kurth KT, Hsu T, Snook ER, Thacker EL, Thacker BJ, Minion FC: Use of a *Mycoplasma hyopneumoniae* nested polymerase chain reaction test to determine the optimal sampling sites in swine. *J Vet Diagn Invest*, 14 (6): 463-469, 2002. DOI: 10.1177/104063870201400603

43. Verdin E, Saillard C, Labbé A, Bové JM, Kobisch M: A nested PCR assay for the detection of *Mycoplasma hyopneumoniae* in tracheo-bronchiolar washings from pigs. *Vet Microbiol*, 76 (1):31-40, 2000. DOI: 10.1016/S0378-1135(00)00228-5

44. Friis NF: *Mycoplasma suipneumoniae* and *Mycoplasma flocculare* in comparative pathogenicity studies. *Acta Vet Scand*, 15 (4): 507-518, 1974.

45. Jin MM: Development and application of the real-time fluorescent quantitative PCR detection of *Mycoplasma hyopneumoniae*. *MSc Thesis*, Nanjing Agriculture University, 2012.

46. Wang GP, Li JP, Wang YN, Luo G, Hu SF, Li XY: Distribution of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* in the respiratory tract and blood of slaughter pigs. *Swine Prod*, 1, 119-120, 2016.

Kafkas Univ Vet Fak Derg
25 (3): 415-420, 2019
DOI: 10.9775/kvfd.2018.21141

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Evaluation of VEGF, Cytokeratin-19 and Caspase 3 Immunolocalization in the Lung Tissue of Rat with Experimentally Induced Diabetes [1]

Zeynep Deniz ŞAHİN İNAN [1,a] Serpil ÜNVER SARAYDIN [1,b]

[1] *The preliminary result of the study was presented at "IMCOFE V. International Multidisciplinary Congress of Eurasia Congress, 24-26 July 2018, Barcelona, Spain"*

[1] Cumhuriyet University, Faculty of Medicine, Department of Histology Embryology, TR-58140 Sivas - TURKEY
[a] ORCID: 0000-0002-0292-4448; [b] ORCID: 0000-0001-7639-7487

Article Code: KVFD-2018-21141 Received: 12.05.2018 Accepted: 16.01.2019 Published Online: 16.01.2019

How to Cite This Article

Şahin İnan ZD, Ünver Saraydin S: Evaluation of VEGF, cytokeratin-19 and caspase 3 immunolocalization in the lung tissue of rat with experimentally induced diabetes. *Kafkas Univ Vet Fak Derg,* 25 (3): 415-420, 2019. DOI: 10.9775/kvfd.2018.21141

Abstract

Diabetes Mellitus (DM) manifests itself with changes in the functional structure of the lungs and impairments in gas exchange. These changes in diabetic lung tissue may be due to various factors. Our aim in this study is to correlate the damage of diabetes with lung tissue in terms of VEGF, CK19, caspase 3 immunolocalizations. In this study, animals were divided into 4 groups, 60 mg/kg streptozotocin was given to each of the groups with experimental diabetes and the physiological saline solution was given intraperitoneally to the control group. On days 7 and 14 of the experiment, diabetic and control groups were euthanized, and lung tissues were removed. Tissue samples were evaluated histochemically and immunohistochemically by monitoring with standard light microscopy. In the diabetic group, the localization of CK19 and Caspase 3 increased on the 7th and 14th days compared to the control group, but the immunolocalization of VEGF decreased. Based on our findings, it was determined that lung tissue was one of the target organs of diabetes. The increase in pulmonary parenchyma due to hyperglycemia is accepted as a source of fibrosis. We concluded that due to increased CK19 localization of fibrosis source, decreased VEGF localization has increased apoptosis in the pulmonary capillary endothelium, which has a significant role in the blood-air barrier in the lung parenchyma, especially in endothelial cells.

Keywords: *Experimental diabetes, Lung, CK19, VEGF, Caspase 3*

Deneysel Olarak Diyabet Oluşturulan Ratların Akciğer Dokusunun Caspase 3, Cytokeratin 19 ve VEGF İmmünolokalizasyonunun Değerlendirilmesi

Öz

Diabetes Mellitus (DM), akciğerlerin fonksiyonel yapısındaki değişikliklerle ve gaz değişimlerindeki bozukluklarla kendini gösterir. Diyabetik akciğer dokusunda bu değişiklikler çeşitli faktörlere bağlı olabilir. Bu çalışmada amacımız VEGF, CK19, kaspaz 3 immunolokalizasyonları ile ilgili olarak diyabetin akciğer dokusuna verdiği zararı ilişkilendirmektir. Çalışmada hayvanlar 4 gruba ayrıldı, deneysel diyabetli gruplara 60 mg/kg streptozotosin verildi ve kontrol grubuna serum fizyolojik solüsyonu intraperitoneal olarak verildi. Deneyin 7 ve 14. günlerinde diyabetik ve kontrol grupları sakrifiye edildi ve akciğer dokuları çıkarıldı. Doku örnekleri, standart ışık mikroskobu ile izlenerek histokimyasal ve immünhistokimyasal olarak değerlendirildi. Diyabetik grupta 7. ve 14. günlerde kontrol grubu ile kıyaslandığında CK19 ve Kaspaz 3 lokalizasyonu artmış, ancak VEGF immunolokalizasyonu azalmıştır. Bulgularımıza göre, akciğer dokusu diyabetin hedef organlarından biri olduğunu göstermektedir. Hiperglisemiye bağlı pulmoner parankimde artış fibrozis kaynağı olarak kabul edildi. Sonuç olarak, pulmoner fibrozis kaynağının CK19 immünolokalizasyonunun artmasına bağlı olarak, azalmış VEGF lokalizasyonunun, özellikle endotelyal hücrelerde, akciğer parankimindeki kan-hava bariyerinde önemli bir rolü olan, pulmoner kapiler endotelyumda apoptosisi arttırdığı sonucuna varıldı.

Anahtar sözcükler: *Deneysel diabetes, Akciğer, CK19, VEGF, Caspase 3*

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease with an increase in blood glucose levels, and its prevalence has increased rapidly in the last ten years. Some of the causes of this disease can be expressed as a lack of insulin secretion,

İletişim (Correspondence)
+90 505 7136677
zinan@cumhuriyet.edu.tr

decreased insulin action or decreased insulin receptor sensitivity [1]. Hyperglycemia and lack of insulin cause various organ dysfunction in patients with DM. DM has negative effects on the lung as well as in many organs. Some of the complications with DM in the lungs can be infections, pulmonary function abnormalities, pleural effusion, and obstructive sleep apnea. DM can also cause lung cancer [2]. Biochemical changes in the lungs in diabetics include decreased glutathione peroxidase activity, NO-induced endothelial dysfunction, and increased heparan sulfate level of the vascular endothelial basement membrane. Biochemical changes in the diabetic lung cause structural changes in the lung parenchyma. Some of these may be expressed as narrowing and interstitial involvement in alveolar areas. Pulmonary vessels, alveolar epithelial basement membrane, bronchial epithelium, and pulmonary capillaries are also affected by diabetes. The most common pathology of this disease is the deterioration in the vascular structure [3,4]. It is very difficult to examine the damages on diabetic lung tissue on the human. Therefore, experimental diabetes models have been developed. Streptozotocin (STZ) is used as an agent to induce experimental hyperglycemia in rats [5]. STZ reduces insulin biosynthesis and secretion and induces hyperglycemia as β-cells cause excessive free radical production [6]. Therefore, STZ is a widely used model to investigate the effects of DM on cells and tissues in experimental studies. High blood glucose levels can damage the blood vessels and cause endothelial dysfunction. Therefore, DM is a risk factor for cardiovascular diseases [7]. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is an angiogenic factor that causes the proliferation and permeability of vascular wall endothelial cells [8,9].

Oxidative stress plays a major role in the development of micro- and macrovascular complications. Accumulation of free radicals in the vasculature of diabetic patients is responsible for the activation of detrimental biochemical pathways, miRs deregulation, disruption of apoptosis mechanisms, and epigenetic changes contributing to vascular inflammation and reactive oxygen species (ROS) generation [10]. So oxidative stress induced by hyperglycemia in lung endothelial cells due to DM induces apoptosis [11]. Apoptosis is regulated by specific functional genes and their protein products. Caspases are vital mediators of programmed cell death (apoptosis) in lung tissue as well as in many cells. Among these, caspase-3 is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types and helps in determining the initial stage of apoptosis. Hyperglycemia induces apoptosis and causes damage to many organs and systems including the reproductive system [12]. Diabetes mellitus induces apoptosis by regulating signal molecules such as Bcl-2/Bax/Caspase-3-9 in the apoptosis pathway [13]. Diabetes mellitus causes pulmonary fibrosis by increasing collagen fibers in the lung parenchyma [14,15]. Cytokeratin 19 (CK19) is a kind of cytoskeleton element for many epithelia including bronchial epithelium [14]. In addition, it has been reported that CK19 which is a kind of cytoskeleton element for many epithelia including bronchial epithelium is expressed due to lung cancer, especially from type 2 pneumocytes [16,17]. However, no studies are reporting the localization of CK19 in lung tissue. In this study, we hope to provide the first understanding of the mechanism of damage in lung tissue due to diabetes and the expressions of Caspase3, CK19, and VEGF in the literature and we hope to benefit from the development of treatment methods with the studies which are planned to be carried out.

MATERIAL and METHODS

Animals

The animals used in the study were obtained from the Experimental Animal Research Laboratory of Cumhuriyet University Faculty of Medicine. The rats were housed at 26-28°C with a 12 h light:12 h dark cycle and free access to standard diet and water stainless steel cages. All procedures were approved by the Ethical Committee (Cumhuriyet University, 65202830-050.04.04-225)

Twenty Wistar albino male rats were used in this study. The control group was divided into 4 equal groups on the 7th day (n = 5), the control group on the 14th day (n = 5) and the DM group on the 7th day (n = 5) and the DM on the 14th day (n = 5). One dose of STZ (60 mg/kg) dissolved in citrate buffer was given i.p. to an animal in DM group. Citrate buffer (vehicle) was given i.p. to the animals of the control group. Blood samples were collected after 48 h in the diabetic group, and those with glucose levels higher than 140 mg/dL were included in the study. On days 7 and 14 of the experiment, rats were anesthetized with a cocktail of ketaminehydrochloride (50 mg/kg) and xylazine (5 mg/kg) which were administered i.p. before the animals were killed [18].

Histology

Lung tissues were fixed in 10% buffered neutral formalin for 24-48 h at room temperature, then washed with tap water, dehydrated through 70, 80, 95 and 100% alcohol, cleared in two baths of xylene, embedded in paraffin and sectioned at 4-6 µm. Paraffin sections were stained with Hematoxylin-Eosin (H&E), Van Gieson, Silver precipitation methods. Lung sectioning and staining are essential methods for studying lung development or lung pathology. H&E staining is most widely used in histology studies [19,20] and medical diagnosis Verhoess's Van Gieson staining detects elastic fibers and collagen deposition in tissues [21]. Silver impregnation lends itself especially well to the demonstration of the reticular connective tissue [22].

The stained sections were evaluated according to Zhou and Moore [23] methods under the microscope.

Immunohistochemistry

Briefly, after deparaffinization in xylene and rehydration, antigen retrieval was performed by microwaving sections in Citrate Buffer, pH 6.0 for 3x5 min. After cooling at room temperature, the sections were washed with phosphate buffer solution then they were treated with 3% hydrogen peroxide (Thermo, Rockford, USA) 10 min. The sections were washed three times with phosphate-buffered saline (PBS) (pH 7.6) (Sigma, Darmstadt, Germany). The sections were treated with blocking reagent for 20 min and incubated (90 min) at (37°C) with VEGF Ab1 (RB-222-R7; Neomarkers, Fremont, California), Caspase 3 Ab4 (RB-1197-R7, Neomarkers, Fremont, USA), Cytokeratin 19 (A53-B/A2.26 (Ks 19.1), ScyTec, Logan, USA)

Sections then were washed three times in PBS and incubated with biotinylated Goat anti-mouse secondary antibody for 10 min at room temperature. Sections were washed three times in PBS and incubated in streptavidin-HRP conjugate (TP-125-HL, Lab Vision, Fremont, USA) for 10 min at room temperature. After rinsing in PBS, the sections were incubated in DAB (3,3 'diamino-benzidine, TA-XXX-QHCX, Lab Vision, Fremont, USA) for 5 min for visualization. Sections were washed with distilled water and observed under the light microscope (BX51, Olympus, Japan) and photographed. The specificity of the antibody was previously confirmed. Negative control experiments were performed by omitting primary antibodies and were also used for comparison in case of residual expression.

RESULTS

In the diabetic group, the alveolar epithelium was gradually thinner than the control group, and the basal lamina of the pulmonary capillaries increased, and the alveolar parenchyma was steadily increased in the HE staining. Van Gieson staining was performed to see the density of collagen fiber, and it was determined that diabetic groups had more intense collagen life than the control group. In the case of silver precipitated preparations regarding reticular fiber density, the amount of reticular fiber is much higher in diabetic groups than in control *(Fig. 1)*. In immunohistochemical studies, the localization of VEGF was gradually decreased in DM groups at 7 and 14 days compared to the control group, but the immunolocalization of CK19 and Caspase 3 increased on DM 7th and 14th days compared to the control group *(Fig. 2)*.

DISCUSSION

Diabetes develops some chronic complications, including pulmonary dysfunction. Little is known about the effects of pulmonary dysfunction on diabetes. Findings in human diabetic subjects and experiments with diabetic rats thickened alveolar epithelium, pulmonary capillary basal lamina, centrilobular emphysema, and pulmonary microangiopathy. Other authors describe ultrastructural changes in pneumocytes, bronchiolar epithelium and connective tissue proteins in streptozotocin-induced diabetic rats. These anatomical changes may result from

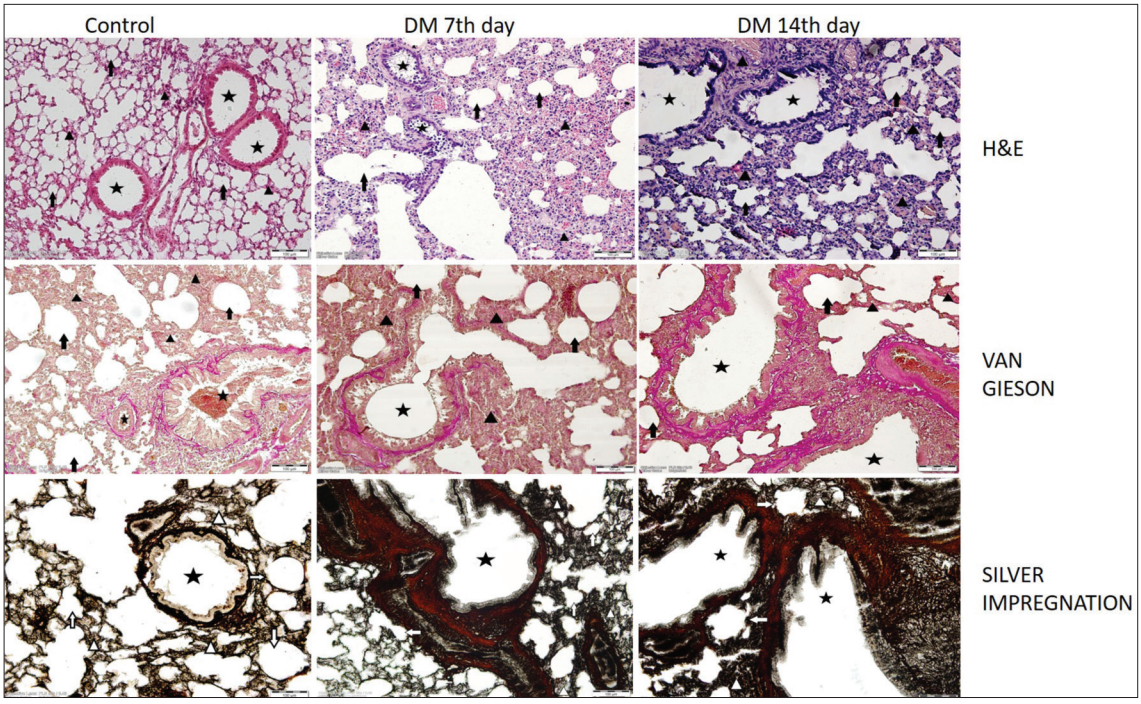


Fig 1. Comparison between H&E, Van Gieson and Silver impregnation staining between the control group, the DM 7th-day group, and the DM 14th-day group (20X Magnification). In the control and experimental groups, alveoli (→), bronchioles (*), and interalveolar septum (▲) are shown

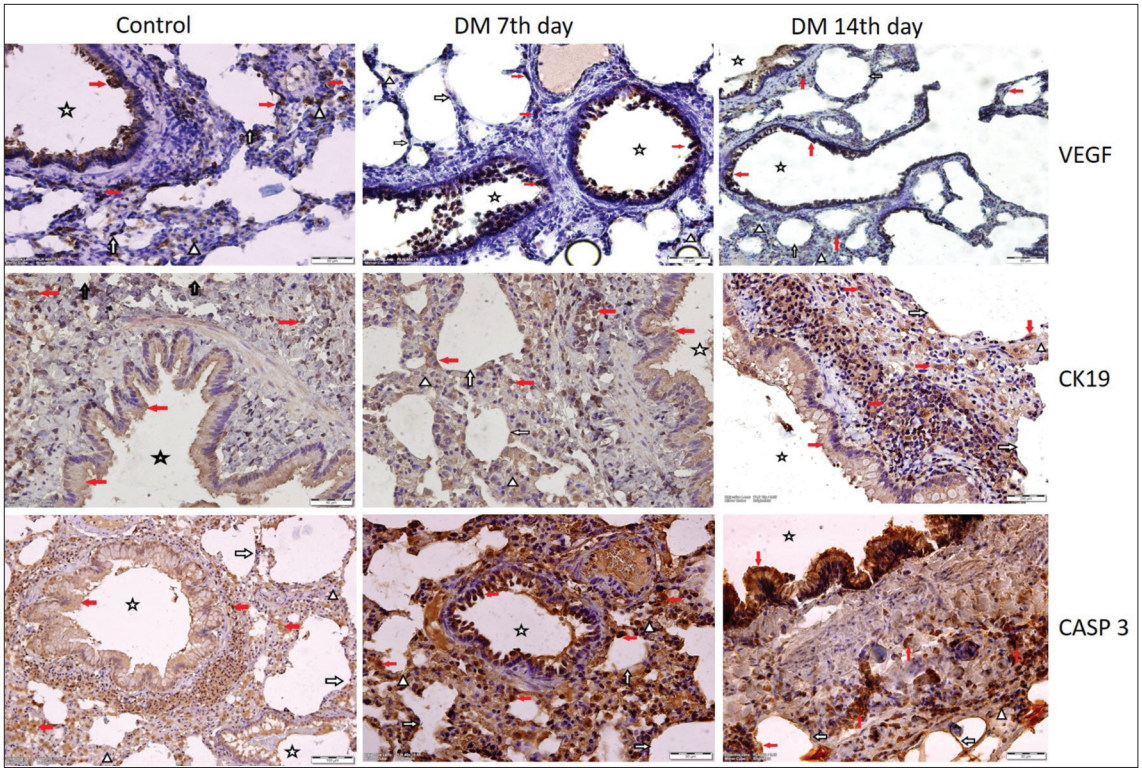


Fig 2. Control and DM groups immunostaining of VEGF, CK19 and Caspase 3 in the lung tissue bronchiolar, parenchymal and alveolar areas of DM groups (40X magnification). In the control and experimental groups, alveoli (→), bronchioles (*), interalveolar septum (▲) and VEGF, CK 19, CASP3 immunopositive cells (red →) in alveoli, bronchioles, interalveolar septum are shown

biochemical changes of the connective tissue components caused by proteins and non-enzymatic glycosylation of peptides caused by chronic high circulating glucose [24-26]. Histological evaluations were done on slides stained with H&E, Von Gieson, and silver impregnation. In the lung tissue of the diabetic groups, it was determined that the alveolar epithelium was progressively thinner than the control group, the basal lamina of the pulmonary capillaries decreased, and the alveolar parenchyma was gradually increased. However, it was seen that the amount of collagen fiber and reticular fiber in the diabetic group showed much more accumulation on day 14 than on day 7 *(Fig. 1)*. Researchers have shown that [24,25], diabetes causes lung fibrosis by increasing the amount of collagen fiber in the lung tissue parenchyma. In this study, an increase in collagen accumulation was observed in the lung tissue of hyperglycemic rats treated with STZ. In previous studies, it is stated that collagen accumulation increases as a result of high glucose, high fat and high oxidative stress caused by diabetes [26].

According to the immunohistochemical findings in our study, CK19 localization was observed in 7 and 14 days in the diabetic group *(Fig. 2)*. Any study that determines the immunolocalization of CK19 in diabetic lung tissue, is no found. CK19 is expressed in epithelial cells [16]. In some studies, however, CK19 has been reported to be profoundly expressed in lung injury [15] or lung fibrosis [15] by hyperblastic type II cells. It was emphasized that excessive collagen accumulation occurred in the lung parenchyma and this resulted in fibrosis. In our study, CK19 immunostaining of the bronchiolar epithelium and alveolar type II cells were found to be more than 7 days on the 14th day with diabetes *(Fig. 2)*.

However, immunostaining of Caspase 3 significantly increased in diabetic group compared to that of control groups. In particular, diabetes was observed to be more intense localization than 14 days at 7 days *(Fig. 2)*. Hyperglycemia induces apoptosis and causes damage to many organs and systems [11,13]. Oxidative stress resulting from hyperglycemia has been reported to play a major role in the initiation of apoptosis [27,28].

However, when compared to the control group, VEGF immunolocalization was observed to be decreased gradually on the 7th and 14th days *(Fig. 2)*. In diabetics, reduced glutathione peroxidase activity due to biochemical changes in lungs, NO-induced endothelial dysfunction, increased heparan sulfate level of the vascular endothelial basement membrane, structural changes in lung parenchyma, contraction of alveolar areas, and interstitial involvement. Pulmonary vessels, alveolar epithelial basement membrane, bronchial epithelium, and pulmonary capillaries are affected by diabetes. The pathology that is always associated with the disease is microangio-

pathy [3,4,29]. Microangiopathy is the cause of the multiorgan complication of diabetes [29].

In the pathogenesis, elevated glucose levels in the serum and extra enzymatic glycosylation of proteins and peptides in the extracellular matrix play a major role. As a result of nonenzymatic glycosylation occurring in the extracellular area of all organs, end products (adversely glycation end products, AGEels) are formed. These end products are highly concentrated in the vessel walls due to high blood pressure. By immunohistochemical methods, these end products can be shown in vascular tissue. Microangiopathy has been shown in renal, retinal and many other organs [4]. Studies on diabetic rats and hamsters indicated that the target organ in the lung, thickening of the alveolar walls, increased collagen and elastin fiber in the basal lamina [29]. It has a dense and extensive capillary system network in the lung. However, in the literature, there are very few studies investigating the effect of diabetes on lung capillaries. In particular, there are few studies that express VEGF expression in the diabetic lung [30]. However, there is no study on immunolocalization of it. In a few studies, it was reported that testicular VEGF decreased in diabetic rats and the decrease in VEGF was associated with increased apoptosis and testicular damage [9,31]. According to the studies, it is known that the pathogenesis of the complex biological processes involved in diabetic pulmonary dysfunction can make lung tissue one of the target organs of diabetes. An increase in lung parenchyma due to hyperglycemia is accepted as the source of fibrosis. Although this relationship has not been fully elucidated, our findings suggest that CK19 immunolocalization increases and VEGF immunolocalization decreases hyperglycemia-induced fibrosis. The increase in the number of apoptotic cells may be due to oxidative stress associated with hyperglycemia. In this study, it was determined that fibrosis increased with the change of reticular structure and collagen accumulation in diabetic lung parenchyma. However, the localization of VEGF in the endothelium of lung capillaries decreased, and vascular pathology developed. Diabetes-induced vascular pathology is caused by an increase in apoptosis of the vascular endothelium. Thus, immunolocalization of CK19 in diabetic lung tissue also increased and triggered lung fibrosis, leading to diabetic lung pathology. In conclusion, we believe that this study will be useful in understanding the mechanism of damage in the lung tissue due to diabetes and the development of treatment methods.

REFERENCES

1. Bahadır Erdoğan B, Uzaslan E: Diabet ve akciğer. *Uludağ Üniv* Tıp Fak Derg, 31 (1): 71-74, 2005.

2. Liao YF, Yin S, Chen ZQ, Li F, Zhao B: High glucose promotes tumor cell proliferation and migration in lung adenocarcinoma via the RAGE-NOXs pathway. *Mol Med Rep,* 17 (6): 8536-8541, 2018. DOI: 10.3892/mmr.2018.8914

3. Nicolaie T, Zavoianu C, Nuta P: Pulmonary involvement in diabetes mellitus. *Rom J Intern Med*, 41, 365-374, 2003.

4. Hsia CCW, Raskin P: The diabetic lung: Relevance of alveolar microangiopathy for the use of inhaled insulin. *Am J Med*, 118, 205-211, 2005. DOI: 10.1016/j.amjmed.2004.09.019

5. Masiello P, Broca C, Gross R, Roye M, Manteghetti M, Hillaire-Buys D, Novelli M, Ribes G: Experimental NIDDM: Development of a new model in adult rats administered streptozotocin and nicotinamide. *Diabetes*, 47, 224-229, 1998. DOI: 10.2337/diab.47.2.224

6. Szkudelski T: The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiol Res*, 50, 537-546, 2001.

7. Kehler DS, Stammers AN, Susser SE, Hamm NC, Kimber DE, Hlynsky MW, Duhamel TA: Cardiovascular complications of type 2 diabetes in youth. *Biochem Cell Biol*, 93 (5): 496-510, 2015. DOI: 10.1139/bcb-2014-0118

8. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z: Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J*, 13, 9-22, 1999.

9. Kolluru GK, Bir SC, Kevil CG: Endothelial dysfunction and diabetes: Effects on angiogenesis, vascular remodeling, and wound healing. *Int J Vasc Med,* 2012:918267, 2012. DOI: 10.1155/2012/918267

10. Paneni F, Bekman JA, Creager MA, Cosentino F: Diabetes and vascular disease: Pathophysiology, clinical consequences, and medical therapy: Part I. *Eur Heart J,* 34 (31): 2436-2443, 2013. DOI: 10.1093/eurheartj/eht149

11. İrtegün S, Deveci E: Diyabetik sıçanların testis dokusunda VEGF ve Bcl-2 ekspresyon düzeylerinin immünohistokimya ve western blot yöntemleri ile incelenmesi. *Dicle Med J*, 43 (4): 527-533, 2016.

12. Adler A: Obesity and target organ damage: Diabetes. *Int J Obes*, 26, 11-14, 2002. DOI: 10.1038/sj.ijo.0802212

13. Yilmaz BO, Yildizbayrak N, Aydin Y, Erkan M: Evidence of acrylamide- and glycidamide-induced oxidative stress and apoptosis in Leydig and Sertoli cells. *Hum Exp Toxicol*, 36 (12): 1225-1235, 2017. DOI: 10.1177/0960327116686818

14. Dobashi N, Fujita J, Ohtsuki Y, Yamadori I, Yoshinouchi T, Kamei T, Takahara J: Elevated serum and BAL cytokeratin 19 fragment in pulmonary fibrosis and acute interstitial pneumonia. *Eur Respir J*, 14 (3): 574-578, 1999. DOI: 10.1034/j.1399-3003.1999.14c15.x

15. Fujita J, Dobashi N, Ohtsuki Y, Yamadori I, Yoshinouchi T, Kamei T, Tokuda M, Hojo S, Okada H, Takahara J: Elevation of anti-cytokeratin 19 antibody in sera of the patients with idiopathic pulmonary fibrosis and pulmonary fibrosis associated with collagen vascular disorders. *Lung*, 177 (5): 311-319, 1999.

16. Iyonaga K, Miyafuji M, Suga M, Saita N, Ando M: Alterations in cytokeratin expression by the alveolar lining epithelial cells in lung tissue from patients with idiopathic pulmonary fibrosis. *J Pathol*, 182, 217-224, 1997. DOI: 10.1002/(SICI)1096-9896(199706)182:2<217::AID-PATH833>3.0.CO;2-A

17. Nakamura H, Abe S, Shibata Y, Yuki H, Suzuki H, Saito H, Sata M, Kato S, Tomoike H: Elevated levels of cytokeratin 19 in the bronchoalveolar lavage fluid iof patients with chronic airway inflammatory disease: A specific marker for bronchial epithelial injury. *Am J Respir Crit Care Med,* 155, 1217-1221, 1997. DOI: 10.1164/ajrccm.155.4.9105057

18. Iraz M, Ozerol E, Gulec M, Tasdemir S, Idiz N, Fadıllıoğlu E, Naziroglu M, Akyol O: Protective effect of caffeic acid phenethyl ester (CAPE) administration on cisplatin-induced oxidative damage to liver in rat. *Cell Biochem Funct,* 24, 357-361, 2006. DOI: 10.1002/cbf.1232

19. Oliveira TL, Candeia-Medeiros N, Cavalcante-Araújo PM, Melo IS, Fávaro-Pípi E, Fátima LA, Rocha AA, Goulart LR, Machado UF, Campos RR, Sabino-Silva R: SGLT1 activity in lung alveolar cells of diabetic rats modulates airway surface liquid glucose concentration and bacterial proliferation. *Sci Rep,* 6:21752, 2016. DOI: 10.1038/srep21752

20. Aktaş MS, Kandemir FM, Özkaraca M, Hanedan B, Kırbaş A: Protective effects of rutin on acute lung injury induced by oleic acid in rats. *Kafkas Univ Vet Fak Derg,* 23 (3): 443-451, 2017. DOI: 10.9775/kvfd.2016.16977

21. Sime PJ, Marr RA, Gauldie D, Xing Z, Hewlett BR, Graham FL, Gauldie J: Transfer of tumor necrosis factor-α to rat lung induces severe pulmonary inflammation and patchy interstitial fibrogenesis with

induction of transforming growth factor-β1 and myofibroblasts. *Am J Pathol,* 153 (3): 825-832, 1998. DOI: 10.1016/S0002-9440(10)65624-6

22. Huang TW: Chemical and histochemical studies of human alveolar collagen fibers. *Am J Pathol,* 86 (1): 81-98, 1977.

23. Zhou X, Moore BB: Lung section staining and microscopy. *Bio Protoc*, 7 (10):e2286, 2017. DOI: 10.21769/BioProtoc.2286

24. Mo A: Pulmonary complications in diabetes mellitus. *Mymensingh Med J*, 23 (3): 603-605, 2014.

25. Marvisi M, Marani G, Brianti M, Della Porta R: Pulmonary complications in diabetes mellitus. *Recenti Prog Med*, 87 (12): 623-627, 1996.

26. Guo S, Meng XW, Yang XS, Liu XF, Ou-Yang CH, Liu C: Curcumin administration suppresses collagen synthesis in the hearts of rats with experimental diabetes. *Acta Pharmacol Sin,* 39, 195-204, 2018. DOI: 10.1038/aps.2017.92

27. Waisundara VY, Hsu A, Huang D, Tan BKH: Scutellaria baicalensis: Enhances the anti-diabetic activity of metformin in streptozotocin-induced diabetic Wistar rats. *Am J Chinese Med*, 36, 517-540, 2008. DOI: 10.1142/S0192415X08005953

28. Komoike Y, Matsuoka M: Endoplasmic reticulum stress-mediated neuronal apoptosis by acrylamide exposure. *Toxicol Appl Pharmacol*, 310, 68-77, 2016. DOI: 10.1016/j.taap.2016.09.005

29. Dalquen P: The lung in diabetes mellitus. *Respiration*, 66, 12-13, 1999. DOI: 10.1159/000029330

30. Braun L, Kardon T, Reisz-Porszasz Z, Banhegyi G, Mandl J: The regulation of the induction of vascular endothelial growth factor at the onset of diabetes in spontaneously diabetic rats. *Life Sci,* 69 (21): 2533-2542, 2001. DOI: 10.1016/s0024-3205(01)01327-3

31. Sisman AR, Kiray M, Camsari UM, **Evren M, Ateş M, Baykara B, Aksu I, Güvendi G, Uysal N:** Potential novel biomarkers for diabetic testicular damage in streptozotocin induced diabetic rats: Nerve growth factor beta and vascular endothelial growth factor. *Dis Markers*, 2014:108106, 2014. DOI: 10.1155/2014/108106

Kafkas Univ Vet Fak Derg
25 (3): 421-425, 2019
DOI: 10.9775/kvfd.2018.21174

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

The Aggrecan Expression Post Platelet Rich Fibrin Administration in Gingival Medicinal Signaling Cells in Wistar Rats *(Rattus novergicus)* During the Early Osteogenic Differentiation *(In Vitro)*

Alexander Patera NUGRAHA [1,2,3,a] Ida Bagus NARMADA [1,b] Diah Savitri ERNAWATI [4,c] Aristika DINARYANTI [3,d]
Eryk HENDRIANTO [3,e] Igo Syaiful IHSAN [3,f] Wibi RIAWAN [5,g] Fedik Abdul RANTAM [3,6,h]

[1] Orthodontic Department, Faculty of Dental Medicine, Universitas Airlangga, Surabaya - INDONESIA
[2] Doctoral Student of Medical Science, Faculty of Medicine, Universitas Airlangga, Surabaya - INDONESIA
[3] Stem Cell Research and Development Center, Universitas Airlangga, Surabaya - INDONESIA
[4] Oral Medicine Department, Faculty of Dental Medicine, Universitas Airlangga, Surabaya - INDONESIA
[5] Biochemistry Biomolecular Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang - INDONESIA
[6] Virology and Immunology Laboratory, Microbiology Department, Faculty of Veterinary Medicine, Universitas Airlangga, INDONESIA
[a] ORCID: 0000-0001-7427-7561; [b] ORCID: 0000-0003-2453-9601; [c] ORCID: 0000-0002-4288-3222; [d] ORCID: 0000-0001-7114-5846
[e] ORCID: 0000-0001-9723-8098; [f] ORCID: 0000-0001-9038-0358; [g] ORCID: 0000-0001-5027-5051; [h] ORCID: 0000-0001-8182-1465

Article Code: KVFD-2018-21174 Received: 12.05.2018 Accepted: 22.01.2019 Published Online: 11.01.2019

How to Cite This Article

Nugraha AP, Narmada IB, Ernawatı DS, Dınaryantı A, Hendrıanto E, Ihsan IS, Riawan W, Rantam FA: The aggrecan expression post platelet rich fibrin administration in gingival medicinal signaling cells in Wistar rats *(Rattus novergicus)* during the early osteogenic differentiation *(in vitro)*. *Kafkas Univ Vet Fak Derg,* 25 (3): 421-425, 2019. DOI: 10.9775/kvfd.2018.21174

Abstract

Platelet Rich Fibrin (PRF) is rich for growth factors which can improve the Gingival Medicinal Signaling Cells' (GMSCs) osteogenic differentiation. Aggrecan is chondrogenic differentiation marker which has a significant role in the early stage of GMSCs' osteogenic differentiation. This study aimed to analyze the expression of Aggrecan post PRF administration on the osteogenic differentiation in vitro. This research is a true experimental study using the post-test only control group design with a simple random sampling. GMSCs were isolated from the lower gingival tissue of healthy male Wistar rats (Rattus novergicus) (n=4), weighted around 250 g, a month old, then cultured for 2 weeks and passaged for 4-5 days. GMSCs in the passage 3-5 were cultured in five M24 plates (N=54; n=6/group) for 7 days, 14 days, and 21 days in three different culture mediums, they were negative control group which included α Modified Eagle Medium; positive control group which contained High Glucose-Dulbecco's Modified Eagle Medium (DMEM-HG) combined with osteogenic medium; and at last, treatment group which were DMEM-HG combined with both osteogenic medium and PRF. A one-way Analysis of Variance (ANOVA) test ($P<0.05$) was performed. The treatment group showed the highest Aggrecan expression of 16.15±2.15 on the 7th day. The lowest Aggrecan expression with a value of 3.67±0.76 on the 21th day occurred in the negative control group. There was a significant difference of Aggrecan expression between groups ($P<0.05$). PRF administration unexpectedly stimulates Aggrecan expression of GMSCs during the osteogenic differentiation that useful to accelerate the bone remodeling or neo-cartilage formation.

Keywords: *Aggrecan, Gingival medicinal signaling cells, Osteogenic differentiation, Platelet rich fibrin*

Erken Osteojenik Farklılaşma *(In Vitro)* Süresince Wistar Sıçanlarda *(Rattus novergicus)* Gingival Medicinal Signaling Hücrelere Post Trombositten Zengin Fibrin Uygulamasının Agrekan Ekspresyonuna Etkisi

Öz

Trombositten Zengin Fibrin (TZF) büyüme faktörlerince zengin olup Gingival Medicinal Signaling Hücreleri (GMSH)'nin (GMSH) osteojenik farklılaşmasını geliştirebilir. Agrekan, kondrojenik farklılaşma markırı olup GMSH'nin osteojenik farklılaşmasının erken evrelerinde önemli bir rol oynamaktadır. Bu çalışma, TZF uygulaması sonrasında in vitro osteojenik farklılaşmada Agrekan ekspresyonunu araştırmayı amaçlamaktadır. Bu araştırma, post-test sadece kontrol grup dizayn kullanan rastgele örneklemeli gerçek bir deneysel çalışmadır. Yaklaşık 250 gram gelen bir aylık sağlıklı erkek Wistar sıçanların (Rattus novergicus) (n=4) alt gingival dokularından GMSH izole edildi, sonrasında 2 hafta kültüre edildi ve 4-5 gün pasajlandı. 3-5. pasajlarda GMSH, beş M24 besiyeri içerisinde 7, 14 ve 21 gün boyunca üç farklı kültür medyumunda (α Modified Eagle Medyum içeren negatif kontrol grubu, osteojenik medyum katkılı High Glucose-Dulbecco's Modified Eagle Medyum (DMEM-HG) içeren pozitif kontrol grubu, hem osteojenik medyum hem de TZF katkılı DMEM-HG içeren uygulama grubu) kültüre edildi. Tek yönlü varyans analizi (ANOVA) testi uygulandı ($P<0.05$). Uygulama grubu 16.15±2.15 ile 7. günde en yüksek Agrekan ekspresyonu gösterdi. En düşük Agrekan ekspresyonu, 3.67±0.76 ile 21. günde negatif kontrol grubunda meydana geldi. Gruplar arasında Agrekan ekspresyonu bakımından anlamlı derecede farklar bulunmaktaydı ($P<0.05$). TZF uygulaması osteojenik farklılaşma süresince GMSH'nin Agrekan ekspresyonunu umulmadık bir şekilde uyarmaktadır ve bu nedenle kemik remodelleme veya neo-kartilaj oluşumunun hızlandırılmasında faydalı olabilir.

Anahtar sözcükler: *Agrekan, Gingival medicinal signaling hücreler, Osteojenik farklılaşma, Trombositten zengin fibrin*

İletişim (Correspondence)
+62 31 5992785
fedik-a-r@fkh.unair.ac.id

INTRODUCTION

Mesenchymal Stem Cells (MSCs) are immature and unspecialized cells which possess a potential ability to differentiate the various cell lineages [1]. MSCs based on their regenerative secretome and capacity for differentiation toward multiple mesenchymal lineage show a promise for a wide range of regenerative medical applications and tissue engineering [2]. In 2017, Caplan changed the name of MSCs into Medicinal Signaling Cells because it more accurately reflects the endogenous stem cells in defect, injury or disease area. These cells secrete the bioactive factors which are immunomodulatory and regenerative tropically, thus, make MSC as therapeutic and medicinal drugs. Site-specific and tissue-specific endogenous stem cells that revive the new tissue formation as stimulated by the bioactive tropical factors are secreted by MSCs exogenously [3].

Mesenchymal Stem Cells have an important role to improve innovative technologies for tissue engineering such as to regenerate or replace damaged, defect or missing tissues by in vitro cell manipulation and extracellular niche design [4]. Stem cell and tissue engineering therapies are expected to be the regenerative medicine strategies in dentistry that provide a novel capability to restore various tissues in orofacial region such as alveolar bone or condylar cartilage of temporomandibular joint [5]. The oral tissues, which are easily accessed by dentists, are a rich source of MSCs. MSCs from the oral cavity such as Gingival Medicinal Signaling Cells (GMSCs) possess an ability to induce the endogenous stem cell to differentiate into various types of cells; for example, osteoblast and chondroblast [3,4].

The ability of GMSCs' osteogenic differentiation can be accelerated by Platelets Rich Fibrin (PRF) administration in the osteogenic culture medium [5]. PRF contains with abundant and various beneficial growth factor for GMSCs to differentiate and proliferate optimally. PRF as a natural biomaterial also serves and acts as a bio-scaffold to support GMSCs. PRF increases the early indicator of osteogenic differentiation such as Bone Alkaline Phosphatase (BALP) and Runt-related Transcription Factor 2 (RUNX2) /Core-Binding Factor Subunit Alpha-1 (CBF-alpha-1) in 7th day and late marker of osteogenic differentiation such as Osteocalcin in 21st day [5,6]. Our previous study showed that PRF administration in GMSCs' osteogenic culture medium decreases Sox9 expression which is the master gene of chondrogenic differentiation [6]. During the early stage of osteogenic differentiation and pre-osteoblast, some chondrogenic differentiation markers play have important role. Even though the osteogenic and chondrogenic differentiation are considered as two separate processes during endochondral bone formation. The previous study mentioned that there is a correlation between them as a continuous developmental lineage which defines the biological process [6,7]. Furthermore, Aggrecan as the early chondrogenic differentiation marker also has an important role during the osteogenic differentiation for osteoblast formation. It is because RUNX2 as the master gene transcription for osteogenic differentiation is not sufficient enough to mature osteoblasts which cannot be induced by activation of RUNX2 alone. The other transcription factors are needed to activate the genetic pathways controlling GMSCs osteogenic differentiation [8]. The Aggrecan expression is used to evaluate the chondrogenesis for any potential endochondral ossification. In the previous study, the aggrecan expression between MSCs osteogenic culture medium and control medium did not alter significantly different [9].

Thus, the hypothesis of this study is that the PRF administration in GMSCs osteogenic culture medium can increase the Aggrecan expression during the early osteogenic differentiation. Furthermore, this study aimed to analyze the Aggrecan expression post PRF administration to GMSCs culture *in vitro* during an osteogenic differentiation.

MATERIAL and METHODS

Ethical Clearance

This research has been granted an approval of animal research ethical clearance with the reference number 289/HRECC.FODM/XII/2017 from the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia.

Study Design and Animal Model

This study was a true experimental research with a post-test only control group design. Sample groups were selected by using a simple random sampling. The minimum sample size was determined using Lameshow's formula, which was (n=4) for GMSCs isolation. The subjects consisted of male Wistar rats *(Rattus norvegicus)*. GMSCs were isolated from the lower gingival tissue of four male rats aged a month old, healthy, with the weight of 250 g each. In minimizing the suffering of animal model used rodent anesthesia with an intramuscular (IM) injection at the dosage of 0.05-0.1 mL/10 g body weight, they were ketamine, xylazine, acepromazine, and a sterile isotonic saline from Sigma Aldrich, USA. It followed the method of Nugraha et al.[5], GMSCs was passaged every 4-5 days also based on the culture method of Nugraha et al.[5] in Gingival Mesenchymal Stem Cells (MSCs) [6]. The GMSCs in passage 3-5 were cultured in five M24 plates from Sigma-Aldrich with (N=54) and (n=6) per group until 7th, 14th, an 21st day in three different culture mediums, which were control negative group, control positive group and treatment group (see below for details) [5,6]. The study was conducted at an experimental laboratory within the Stem Cell Research and Development Centre in Universitas Airlangga, Surabaya, Indonesia for stem cell culture and animal model.

Platelet Rich Fibrin Preparation

Platelet Rich Fibrin was isolated and extracted from whole blood of different rat population. PRF isolation was done with (n= 36), 36 months-old; with the mean weight of 250 g each. The rats terminated using the rodent anesthesia with the dosages of 60 mg/body weight of ketamine and a 3 mg/body weight of xylazine from Sigma Aldrich intraperitoneally (IP). Next, the whole blood (6 mL) was aspirated using a 10 mL disposable syringe and inserted in a non-coagulant vacutainer tube then centrifuged at 3000 rpm/min for 10 min (Kubota, Tokyo, Japan). Thus, PRF obtained mince and it was inserted into each culture plate of the treatment group [5,6].

Osteogenic Differentiation in a Combination of Platelet Rich Fibrin and Gingival Medicinal Signaling Cells

The analysis was conducted for three groups which consisted of two control groups and anexperimental group. In the treatment group, GMSCs were cultured with PRF which contained with 2 mM L-glutamine, 100 µg/mL sodium pyruvate, 0.2 mM ascorbic acid-2 phosphate, dexamethasone 10-7 M 10 ng/mL TGF-β3 and a high dosage of glucose-Dulbecco's Modified Eagle Medium (DMEM-HG) from Sigma Aldrich, USA. While, in the positive control group, GMSCs were placed on an osteogenic culture plate medium with 2 mM L-glutamine, 100 µg/mL sodium pyruvate 0.2 mM ascorbic acid-2 phosphate, dexamethasone 10-7 M from Sigma Aldrich, USA. Furthermore, in the negative control group, GMSCs were cultured with α Modified Eagle Medium (αMEM) also from Sigma Aldrich, USA.

Osteogenic differentiation was analyzed on the 7th, 14th, 21st day of culture cells groups. It employed an immunocytochemical staining by indirect technique using a 3.3'-diaminobenzidine stain kit by Pierce DAB Substrate Paint Kit 34002 from Sigma Aldrich, USA and monoclonal antibodies by Abcam, Cambridge, MA, USA. An anti-Aggrecan (mouse monoclonal; ab-3773) was performed to analyze all samples. The Aggrecan expression was read using a light microscope using the CX22 Binocular from Olympus at 200x magnification. Every cell expressing Aggrecan in five field was examined three times by three experts which were WR, EH and FAR; and then, the mean was then calculated.

Data Analysis

All data were recapitulated and then Statisitical Package for Social Science (SPSS) version 20.0 by IBM SPSS, Chicago, USA was used to analyze the data. The experiments were replicated three times with (n=54). The data was then duplicated for (n=108) using an estimation formula and SPSS. Saphiro-Wilk normality test and a Levene's variance of homogeneity test (P>0.05) were performed then Analysis of Variance (ANOVA) test (P<0.05) was conducted to analyze the different between groups.

RESULTS

All data were normally distributed and homogeny (P>0.05). The positive expression of Aggrecan was detected in all groups *(Fig. 1)*. The treatment group showed the highest Aggrecan expression with the value of (16.15±2.15) on the 7th day. While, the lowest Aggrecan expression with the value of (3.67±0.76) on the 21st day was seen in the

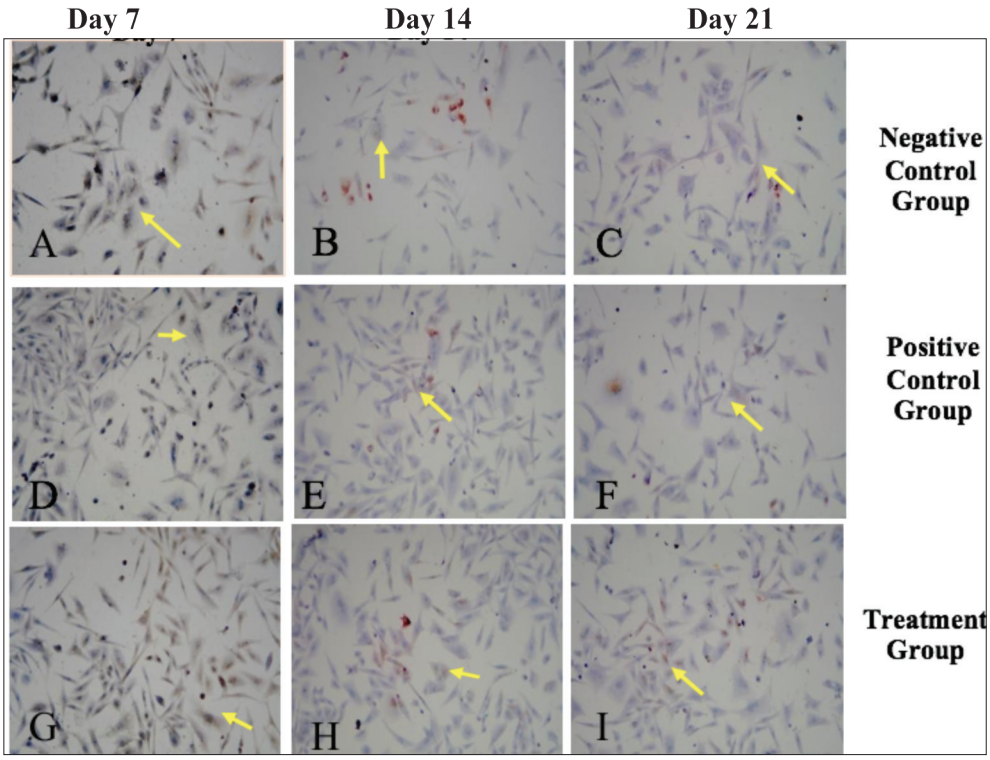


Fig 1. Aggrecan expression in GMSCs of Wistar rats *(Rattus novergicus)*. A, B, and C show the Aggrecan expression in the negative control group; D, E, and F exhibit the Aggrecan expression in the positive control group; and G, H, and I present the Aggrecan expression in the treatment group. Aggrecan expression in GMSCs is observed on the 7th, 14th and 21st day. A positive Aggrecan expression is marked by a yellow arrow in the cell and examined through an Olympus CX22 Binocular, at 200x magnification

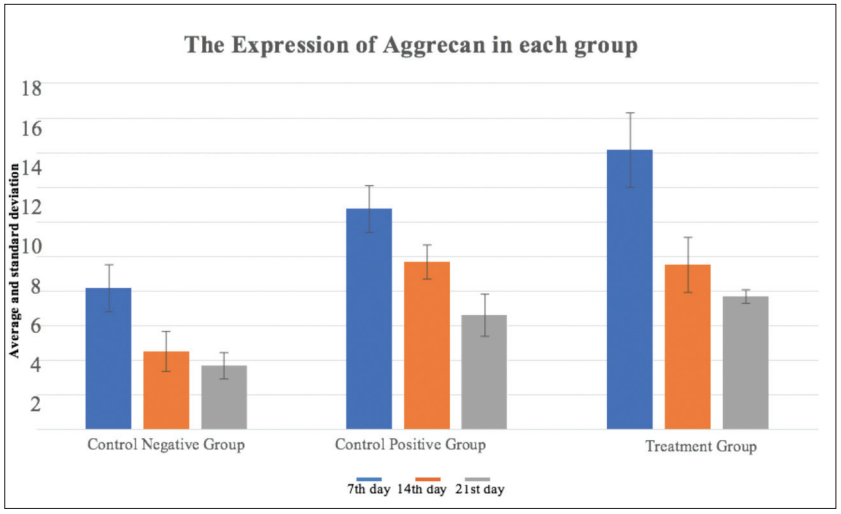


Fig 2. The average and standard deviation of Aggrecan expression on the 7th, 14th and 21st day in each group

Table 1. *Mean±Standard deviation (SD) and one-way anova test result of aggrecan*

Time	Aggrecan Expression Negative Control Group	Aggrecan Expression Positive Control Group	Aggrecan Expression Treatment Group	One-way ANOVA P–value
Day 7	8.17±1.37	12.74±1.35	16.15±2.15	
Day 14	4.5±1.16	9.67±0.99	9.49±1.59	0.001*
Day 21	3.67±0.76	6.59±1.22	7.67±0.39	

** Information: significant at P<0.05*

negative control group *(Fig. 2)*. There was a significant difference Aggrecan expression between groups with the value of (P< 0.05) *(Table 1)*.

DISCUSSION

The large Chondroitin Sulphated Proteoglycan Aggrecan or Aggrecan (ACAN) is the most plentiful non-collagenous protein in cartilage and essential for its structure and function [10]. Aggrecan is the founding member of lectican protein family. Aggrecan includes versican, brevican and neurocan. Aggrecan consists of a 250 kDa protein core with around 100 chondroitin sulphate glycosaminoglycan and also 30 keratan sulphate chains attached to a large domain and located between three globular domains. Aggrecan comprises an N-terminal domain, two globular domains (G1 and G2), two inter-globular domains, a selectin-like domain (G3) and a C-terminal domain [11]. The aggrecan expression in the osteogenic differentiation or bone is lower than in the chondrogenic differentiation or cartilage. The function of aggrecan in bone is to help endochondral ossification. Aggrecan relatively exists in low concentration, but it has an effect on growth plate (cartilage) calcification, rather than having a direct effect on bone. Thus, it is very substantive and important for growth plates [12,13].

Endochondral ossification is a fundamental biology process in forming hard tissue when bone replaces the cartilage. During endochondral ossification, abundant bones are formed, for example it is the primary way that long bones increase in length. The cartilage and the underlying bone are linked through the deepest layers of the hypertrophic chondrocytes, which are surrounded by a mineralized matrix [10,11,13]. Aggrecan expression increases during the endochondral ossification. In line with that, the previous study conducted by Namkoong et al.[9] showed that Aggrecan does not show any expression differences between the control and the osteogenic mediums. In this study, the Aggrecan expression increases on the 7th day in the treatment group with a significant different between group. PRF administration in osteogenic culture medium unexpectedly stimulates the Aggrecan expression during the early osteogenic differentiation of GMSCs. This result of study is consistent with the research of Sumarta et al.[14] which showed that Aggrecan expression increases significantly with PRF administration in culture medium. Furthermore, the triad tissue engineering consists of 3 elements, they are MSCs, natural scaffold and niche. Growth factor contained in PRF plays an important role to enhance MSCs differentiation capability and acts as advantageous bio-scaffold. Biodegradable polymerized fibrin matrix forms networks that support and stimulate the beneficial MSCs secretome [5,6,14].

Platelets Rich Fibrin is abundant with growth factor such as Insulin Growth Factor (IGF-I), Transforming Growth Factor-β1 (TGFβ-1), Vascular Endothelial Growth Factor (VEGF), Insulin Growth Factor (IGF-I), and Platelet Derived Growth Factor-β (PDGF-β) [6,14]. IGF stimulates aggrecan

proteoglycan synthesis and suppresses catabolism of proteoglycans. While, TGF-β increases Aggrecan expression in MSCs. PDGF is a strong mitogen and plays an important role in the proliferation and maintenance of MSCs. Moreover, FGF-2 enhances the Aggrecan expression from MSCs [15]. Ten percent concentration of PRF stimulates the synthesis of proteoglycan (ACAN) [16]. The highest result is shown in the treatment group due to PRF administration in GMSC. GMSCs combined with PRF will fulfill all mandatory factors that complete the key of tissue engineering, for example cells, GFs, and scaffold [17]. In addition, it is unexpected that in the treatment group shows the highest Aggrecan expression in MSCs osteogenic culture medium compared to the other groups. The beneficial result of this study surprisingly can be useful as the references for the further research to accelerate bone remodeling or neo-cartilage formation especially PRF administration as a new generation of platelet-derived concentration for bone or cartilage healing in human.

Platelets Rich Fibrin unexpectedly stimulates and increases the Aggrecan expression of GMSCs during osteogenic differentiation. With this the beneficial result, the combination of PRF and GMSCs is recommended to be an alternative to accelerate bone remodeling or neo-cartilage formation as this will support all important substance of tissue engineering which are the scaffold, the growth factor and the cells. We would like to suggest that further research is needed to study combined GMSC and PRF on *in vivo* model.

Acknowledgement

The authors would like to thank the Doctoral Medical Science, Faculty of Medicine and Faculty of Dental Medicine, Faculty of Medicine, Stem Cell Research and Development Centre, Universitas Airlangga (UNAIR), Surabaya, East Java, Indonesia for supporting our research. The research grant was provided by Program Menuju Doktor Sarjana Unggul (PMDSU) Batch III of the Ministry of Research, Technology and Higher Education, Republic of Indonesia (Kemenristekdikti RI) 2018 with a Letter of Appointment Agreement 218 Number 1035/D3/PG/2017 and Number 2146/D3/PG/2017.

Conflict of Interest

No conflict of interest was associated with this work.

REFERENCES

1. Nugraha AP, Rantam FA, Ernawati DS, Narmada IB, Widodo ADW, Lestari P, Dinaryanti A, Hendrianto E, Ihsan IS, Susilowati H, Karsari D: Gingival mesenchymal stem cells from wistar rat's gingiva *(rattus novergicus)* isolation and characterization (in vitro study). *J Int Dent Med Res,* 11 (2): 694-699, 2018.

2. Fitzsimmons REB, Mazurek MS, Soos A, and Simmons CA: Mesenchymal stromal/stem cells in regenerative medicine and tissue engineering. *Stem Cells Int*, 2018:8031718, 2018 DOI: 10.1155/2018/8031718

3. Caplan AI: Mesenchymal stem cells: Time to change the name! *Stem Cells Transl Med,* 6 (6): 1445-1451, 2017. DOI: 10.1002/sctm.17-0051

4. Egusa H, Sonoyama W, Nishimura M, Atsuta I, Akiyama K: Stem cells in dentisry - Part I: Stem cells sources, *J Prosthodont Res,* 56 (3): 151-165, 2012. DOI: 10.1016/j.jpor.2012.06.001

5. Nugraha AP, Narmada IB, Ernawati DS, Dinaryanti A, Hendrianto E, Riawan W, Rantam FA: Bone alkaline phosphatase and osteocalcin expression of rat's gingival mesenchymal stem cells cultured in platelet-rich fibrin for bone remodeling *(in vitro* study). *Eur J Dent,* 12 (4): 566-573, 2018. DOI: 10.4103/ejd.ejd_261_18

6. Nugraha AP, Narmada IB, Ernawati DS, Dinaryanti A, Hendrianto E, Ihsan IS, Rantam FA: Osteogenic potential of gingival stromal progenitor cells cultured in platelet rich fibrin is predicted by core-binding factor subunit-α1/sox9 expression ratio *(in vitro)*. *F1000Res,* 7:1134, 2018. DOI: 10.12688/f1000research.15423.1

7. Jing Y, Jing J, Ye L, Liu X, Harris SE, Hinton RJ, Feng JQ: Chondrogenesis and osteogenesis are one continuous developmental and lineage defined biological process. *Sci Rep,* 7:10020, 2017. DOI: 10.1038/s41598-017-10048-z

8. Qi H, Aguiar DJ, Williams SM, LA Pean A, Pan W, Verfaillie CM: Identification of genes responsible for osteoblast differentiation from human mesodermal progenitor cells. *Proc Natl Acad Sci U S A,* 100 (6): 3305-3310, 2003. DOI: 10.1073/pnas.0532693100

9. Namkoong B, Güven S, Ramesan S, Liaudanskaya V, Abzhanov A, Demirci U: Recapitulating cranial osteogenesis with neural crest cells in 3-d microenvironments. *Acta Biomater,* 31, 301-311, 2016 DOI: 10.1016/j.actbio.2015.12.004

10. Lauing KL, Cortes M, Domowicz MS, Henry JG, Baria AT, Schwartz NB: Aggrecan is required for growth plate cytoarchitecture and differentiation. *Dev Biol,* 396 (2): 224-236, 2014. DOI: 10.1016/j.ydbio.2014.10.005

11. Gibson BG, Briggs MD: The aggrecanopathies; an evolving phenotypic spectrum of human genetic skeletal diseases. *Orphanet J Rare Dis,* 11:86, 2016. DOI: 10.1186/s13023-016-0459-2

12. Sato T, Kudo T, Ikehara Y, Ogawa H, Hirano T, Kiyohara K, Hagiwara K, Togayachi A, Ema M, Takahashi S, Kimata K, Watanabe H, Narimatsu H: Chondroitin sulfate N-acetylgalactosaminyltransferase 1 is necessary for normal endochondral ossification and aggrecan metabolism. *J Biol Chem,* 286 (7): 5803-5812, 2011. DOI: 10.1074/jbc.M110.159244

13. Brunetti G, Mori G, D'Amelio P, Faccio R: The crosstalk between the bone and the immune system: Osteoimmunology. *Clin Dev Immunol,* 2013:617319, 2013. DOI: 10.1155/2013/617319

14. Sumarta NPM, Pramono DC, Hendrianto E, Susilowati H, Karsari D, Rantam FA: Chondrogenic differentiation capacity of human umbilical cord mesenchymal stem cells with platelet rich fibrin scaffold in cartilage regeneration *(in vitro* study). *Bali Med J,* 5 (3): 420-426, 2016. DOI: 10.15562/bmj.v5i3.295

15. Nugraha AP, Narmada IB, Ernawati DS, Dinaryanti A, Hendrianto E, Ihsan IS, Riawan W, Rantam FA: *In vitro* bone sialoprotein-I expression in combined gingival stromal cells and platelet rich fibrin during osteogenic differentiation. *Trop J Pharm Res*, 17 (12): 2341-2345, 2018.

16. Sakata R, Iwakura T, Reddi AH: Regeneration of articular cartilage surface: Morphogens, cells, and extracellular matrix scaffolds. *Tissue Eng Part B Rev,* 21 (5): 461-473, 2015. DOI: 10.1089/ten.TEB.2014.0661

17. Utomo DN, Mahyudin F, Hernugrahantoa KD, Suroto H, Chilmia MZ, Rantam FA: Implantation of platelet rich fibrin and allogenic mesenchymal stem cells facilitate the healing of muscle injury: An experimental study on animal. *Int J Surg,* 11, 4-9, 2018. DOI: 10.1016/j.ijso.2018.03.001

Kafkas Univ Vet Fak Derg
25 (3): 427-433, 2019
DOI: 10.9775/kvfd.2018.21175

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Immunohistochemical Distribution of Somatostatin in Gastric Tissue of Diabetic Rats Treated with *Cinnamon* Extract [1]

Sevda ELİŞ YILDIZ [1] Buket BAKIR [2,b] Şükran YEDİEL ARAS [1,c] Serpil DAĞ [3,d] Ebru KARADAĞ SARI [4,e]

[1] *This research was presented at 15th International Congress of Histochemistry and Cytochemistry OP:29, 18-21 May, 2017, Antalya, Turkey*

[1] Department of Midwifery, Faculty of Health Sciences, Kafkas University, TR-36100 Kars - TURKEY
[2] Department of Histology and Embryology, Faculty of Veterinary Medicine, Namik Kemal University, TR-59030 Tekirdag - TURKEY
[3] Department of Patology, Faculty of Veterinary Medicine, Kafkas University, TR-36040 Kars - TURKEY
[4] Department of Histology and Embryology, Faculty of Veterinary Medicine, Kafkas University, TR-36040 Kars - TURKEY
[a] ORCID: 0000-0002-3585-6648; [b] ORCID:0000-0003-3637-3688; [c] ORCID:0000-0002-3267-5251; [d] ORCID: 0000-0001-7667-689X
[e] ORCID:0000-0001-7581-6109

Article Code: KVFD-2018-21175 Received: 15.10.2018 Accepted: 23.01.2019 Published Online: 23.01.2019

How to Cite This Article

Eliş Yıldız S, Bakır B, Yediel Aras Ş, Dağ S, Karadağ Sarı E: Immunohistochemical distribution of somatostatin in stomach tissue of diabetic rats treated with *Cinnamon* extract. *Kafkas Univ Vet Fak Derg,* 25 (3): 427-433, 2019. DOI: 10.9775/kvfd.2018.21175

Abstract

Diabetes is a chronic metabolic disorder, as well as a situation of increased oxidative stress. We examined the distribution of somatostatin in gastric tissues of *cinnamon* extract treated streptozotocin-induced diabetic rats using the immunohistochemistry technique. A total of 30 male *Sprague Dawley* rats were used in the study. The rats were assigned to five groups as control, sham, *cinnamon,* diabetes and diabetes + *cinnamon*. No application was made to the control group, the sham group received intraperitoneally (i.p.) 50 mg/kg sodium citrate, and diabetes was induced by i.p. injection of 50 mg/kg STZ in diabetes and diabetes + *cinnamon* groups. *Cinnamon* extracts were then given to cinnamon and diabetes + *cinnamon* groups by oral gavage at a dose of 200 mg/kg for 14 days. The streptavidin-biotin-peroxidase method was used to determine the immunoreactivity of somatostatin. Gastric tissue sections were prepared and stained by Crossman's triple and Hematoxylin-Eosin staining in order to examine histological structure of the gastric tissue. We determined that somatostatin immunoreactivity of the control, sham and *cinnamon* groups was stronger than for the diabetes, and diabetes + *cinnamon* groups. While a weak immunoreactivity was found in the cardia, fundus and pyloric mucosa of the gastric tissue in the diabetes and diabetes + *cinnamon* groups, a strong immunoreactivity was found in the *cinnamon*, sham, and control groups. Also, a statistically significant was observed when all groups compared in terms of count of parietal and principal cells ($P<0.001$). It was determined that there was a statistically significant difference between diabetes, diabetes + *cinnamon* groups and control, sham, *cinnamon* groups in terms of fasting blood glucose levels ($P<0.05$). In conclusion, somatostatin, which plays an important role in gastroduodenal diseases, was found to be lower in the diabetes and *cinnamon* + diabetes groups.

Keywords: *Cinnamon, Diabetes, Gastric, Immunohistochemistry, Somatostatin*

Tarçın Ekstraktı İle Tedavi Edilen Diyabetik Sıçanların Mide Dokusunda Somatostatinin İmmunohistokimyasal Dağılımı

Öz

Diyabet, kronik metabolik bir bozukluk olduğu gibi aynı zamanda da artmış bir oksidatif stres durumudur. Çalışmamızda immunohistokimyasal teknik kullanarak tarçın uygulanan streptozotosin ile diabet oluşturulan ratların mide dokusundaki somatostatinin salınımını inceledik. Çalışmada 30 adet *Sprague Dawley* cinsi erkek rat kullanıldı. Deney grupları kontrol, sham, tarçın, diyabet ve diyabet + tarçın olarak 5 gruba ayrıldı. Kontrol grubuna herhangi bir uygulama yapılmadı, sham grubuna intraperitoneal (i.p.) olarak 50 mg/kg sodyum sitrate uygulandı. Diyabet ve diyabet + tarçın gruplarına i.p. 50 mg/kg STZ enjeksiyonu yapılarak diabet oluşturuldu. Tarçın ve diyabet + tarçın gruplarına tarçın ekstraktı 200 mg/kg olacak şekilde oral gavaj yolu ile 14 gün verildi. Somatostatinin immunoreaktivitesini belirlemek için streptavidin-biotin-peroxidase metotu uygulandı. Mide dokularının normal histolojik yapısını incelemek için Crossman'ın üçlü boyama yöntemi ve Hematoksilen-Eosin boyaması uygulandı. Kontrol, sham ve tarçın gruplarındaki somotostatin immunoreaktivitesi, diyabet ve diyabet + tarçın gruplarından daha güçlü olduğu tesbit edildi. Diyabet ve diyabet + tarçın gruplarında mide dokusunun kardia, fundus ve pilor mukozasında zayıf immunoreaktivite bulunurken tarçın, sham ve kontrol gruplarında güçlü immunoreaktivite bulundu. Ayrıca tüm gruplar parietal ve prensipal hücre sayıları bakımından karşılaştırıldığında istatiksel olarak anlamlı bulundu ($P<0.001$). Açlık kan glikoz değerleri karşılaştırıldığında diyabet, diyabet+tarçın grupları ile kontrol, sham ve tarçın grupları arasında istatistiksel olarak anlamlı farklılık olduğu belirlendi ($P<0.05$). Sonuç olarak; gastroduodenal hastalıklarda önemli rol oynayan somatostatinin diyabet ve diyabet+ tarçın grubunda daha az olduğu tespit edilmiştir.

Anahtar sözcükler: *Tarçın, Diyabet, Mide, İmmunohistokimya, Somatostatin*

İletişim (Correspondence)
+90 474 2251567 Fax : +90 474 2251265
sevdaelis36@hotmail.com

INTRODUCTION

Diabetes mellitus (DM) is a systemic disease which is characterized by hyperglycemia and causes other disorders in the body, because of insufficient level or lack of insulin production or incomplete usage of insulin [1].

Studies showed that most of the diabetic patients use herbal medicines more than the other supplemental therapies because they believe that herbal medicines are natural, and healthy, whereas in poor quality and with improper use, they can be harmful and cause adverse effects [2-4]. *Cinnamon* has been reported to have positive effects on serum lipids and blood glucose. The active component cinnamaldehyde found in *Cinnamon* expresses its effect on blood glucose can be attributed to it [5]. *Cinnamon* is suggested to reduce high blood glucose levels, repair the damaged β cells and have positive effects on diabetes mellitus [6,7]. Mechanism of action for *cinnamon* was suggested to be increased glycogen storage by acting on glycogen synthesis activity through its polyphenols, and strengthened antioxidant and insulin effects through polyphenol type A; *cinnamon* is thus stated to be benefial in glucose tolerence and treatment of diabetes [8-10].

Being a 14-aminoacid peptide hormone that is secreted from hypothalamus and D-cells of islets of Langerhans of the pancreas; somatostatin is known as the factor inhibiting the secretion of growth hormone from hypothalamus [11]. Somatostatin is an inhibitory peptide with a wide-spectral biological activities [12]. It is included in pancreatic, gastric and intestinal mucosa or gastrointestinal system [12,13] and myenteric neurons. It reduces hepatic biliary, pancreatic and gastric acid secretions and decelerates intestinal passage [12].

The aim of this study was to investigate the effect of *cinnamon* on the immunohistochemical distribution of somatostatin which exists in many areas of the body and whose mechanisms of action differ among organs in the gastric tissue of streptozotocin (STZ)-induced experimental diabetic rats, and the changes caused by diabetes in the gastric structure. This study is based on the view indicating that antioxidant properties and pharmacological effects of *cinnamon* in diabetes mellitus, as well as its protective effects against possible harms of diabetes would lead to alternative ways in fields of medicine and pharmacology.

MATERIAL and METHODS

Animals

Ethical approval of Kafkas University Experimental Animals Local Ethical Committee (No: KAÜ-HADYEK/2017-041) was obtained to conduct the study.

A total of 30 male *Sprague-Dawley* rats were used in the study. The rats were kept at 22±2°C, in standard cages under 12-h light-12-h dark conditions and fed *ad libitum* using standard rodent chow and tap water. The rats were divided into 5 groups including 6 animals in each one: control, sham, *cinnamon*, diabetes and diabetes + *cinnamon* groups. No application was made the control group, the sham group received intraperitoneally (i.p.) 50 mg/kg sodium citrate, diabetes group was administered i.p. 50 mg/kg STZ (50 mL citric acid solved in 40 mL disodium hydrogen phosphate buffer solution, pH 4.5) [14]. Diabetes + *cinnamon* group was administered i.p. 50 mg/kg STZ (50 mL citric acid solved in 40 mL disodium hydrogen phosphate buffer solution, pH 4.5) The animals were considered as diabetic, if their blood glucose values were above 250 mg/dL on the third day after STZ injection [15]. And after then *Cinnamon* extracts were then given to *cinnamon* and diabetes + *cinnamon* groups by oral gavage at a dose of 200 mg/kg for 14 days [6]. At the end of the 14th day, body weights of the rats were measured, they were sacrificed under diethyl ether anesthesia, and gastric tissue samples were obtained subsequently.

Histological Examination

Gastric tissue samples obtained were fixed within 10% formalin solution. Following routine procedures, they were embedded into paraffin blocks, and 5 µm sections were obtained. In order to demonstrate histological structure of gastric tissue, the sections were performed Crossman's Triple Staining and Hematoxylin-Eosin (HE) staining [16] methods and examined under light microscope (Olympus BX51; Olympus Optical Co. Osaka, Japan).

Immunohistochemical Examination

The sections obtained from paraffin blocks after de-paraffinization and rehydration procedures, and incubated in 3% H_20_2 prepared in 0.1 M phosphate buffered saline PBS for 15 min, in order to inhibit endogenous peroxidase activity. Then sections were washed in PBS solution. The samples were exposed to maximum temperature in citrate buffer solution, pH 6.0, in an 800-watt microwave oven for 10 min to release the antigens. Afterwards, they were washed again with PBS. In order to inhibit non-specific bindings, Blocking solution A was dropped (Invitrogen Histostain Plus Broad Spectrum Ref. 85.9943). Somatostatin primary antibody (abcam ab183855, diluted at a rate of 1/500) was administered on the sections for 1 h at room temperature and humidity. Rabbit serum without primer antibody served as the negative control. Following incubation of primary antibodies, streptavidin-biotin method [17] was used, which is one of the indirect methods. For this purpose, Broad Spectrum Antibody (Invitrogen Histostain Plus Broad Spectrum (AEC) Ref. 85.9943), towards the species for which primary antibody was produced, was added on the sections and they were incubated at room temperature for 15 min. Subsequently, HRP streptavidin (Invitrogen Histostain Plus Broad Spectrum Ref. 85.9943) was dropped on the sections and incubated at

room temperature for 15 min. For chromogen incubation, 3,3′-Diaminobenzidine tetrahydrochloride (DAB, Dako Corp) Substrate Solution was added [18]. The sections were immersed into hematoxylin for counterstaining. The slides were examined under light microscope and their images were obtained. Percentage and degree of staining in stained cells were scored by using the semi-quantitative method. Degree of the staining was expressed as 0 (no staining), +1 (weak staining), +2 (moderate staining), and +3 (strong staining) [19,20].

Somatostatin positive cells were counted by 100 square ocular micrometer (eye piece graticule) at 40X magnification under Olympus microscope (BX51). All the obtained data was converted to number of somatostatin positive cells per 1 mm^2 unit area [21,22]. Numerical distribution of somatostatin positive cells were observed in six different sections chosen from ten unit area of parietal and principle cells of each animals.

Statistical Analysis

SPSS (20.0) package software was used to evaluate the data obtained in the study. One Way ANOVA test was performed to determine differences between groups (control, sham, *cinnamon*, diabetes, diabetes + *cinnamon)*. The Duncan test was used to compare the differences between the significant groups.

RESULTS

Blood Glucose Levels

Intra-group and inter-group statistical evaluation of fasting blood glucose levels of rats was carried out and the results obtained were given in the *Table1*. There was no statistically significant difference between the 3rd and 17th days in terms of the mean fasting blood glucoselevels of the rats in the diabetes group. However, it was determined that the diabetes + *cinnamon* group had a statistically significant decrease in the mean fasting blood glucose levels on the 17th day (P<0.05). Control, sham and *cinnamon* groups was not statistically significant difference in terms of fasting blood glucose level between the days 3rd and 17th *(Table 1)*.

Histological Results for the Gastric Tissue

Histologically, normal cardia, fundus and pylorus tissue structures were observed in rats of all groups (control, sham, *cinnamon,* diabetes and diabetes + *cinnamon* groups) *(Fig. 1)*.

Immunohistochemical Results

Somatostatin immunolocalization was determined in similar area in the gastric tissue of rats in control, sham, *cinnamon*, diabetes and diabetes+*cinnamon* groups *(Table 2)*. Strong (+3) somatostatin immunoreactivity was found in the cardia, fundus and pyloric mucosa of control, sham and *cinnamon* groups *(Fig. 2a,b,c)* and weak (+1) immunoreactivity in the diabates and diabetes+*cinnamon* groups *(Fig. 2d,e)*.

A weak (+1) cytoplasmic somatostatin immunoreactivity was found in the parietal and principal cells of fundus in the diabetes *(Fig. 3a)*, and diabetes + *cinnamon* groups while a strong (+3) cytoplasmic somatostatin immunoreactivity in the control *(Fig. 3b)*, sham and *cinnamon* groups. Somatostatin immunoreactivity of parietal and principal cells was statistically significant in the control, sham, *cinnamon,* diabetes and diabetes + *cinnamon* groups (P<0.001). Count of somatostatin positive parietal and principal cells in among groups were summarized in *Table 3* and *Table 4*.

DISCUSSION

In the present study, we evaluated the antioxidant which *cinnamon* on distribution of somatostatin in gastric tissue in streptozotocin diabetic rats. Diabetes is a metabolic problem which is increased by oxidative stress. It is concluded that 14 days of *cinnamon* administration increases somatostatin secretion, which has different roles at different stages of life processes such as cell proliferation,

Table 1. *Statistical evaluation of fasting blood glucose levels of rats according to groups*

Group	Day			F
	1st day	3rd day	17th day	
Control	76.67±2.45 ^{bA}	76±2.76 ^{cA}	80.33±2.21 ^{cA}	0.87
Sham	76.67±1.40 ^{bA}	78.33±1.72 ^{cA}	77.83±2.19 ^{cA}	0.22
Cinnamon	87.83±3.59 ^{aA}	77.33±1.85 ^{cA}	78±1.31 ^{cA}	5.74
Diabetes	84.50±1.33 ^{aB}	373.33±6.31 ^{aA}	363.67±6.83 ^{aA}	913.13
Diabetes + *Cinnamon*	88.83±2.98 ^{aC}	331.16±10.27 ^{bA}	245.16±34.55 ^{bB}	34.6
F	5.32	718.67	68.02	
P	0.00	0.00	0.00	

$^{A, B, C}$ The differences between the mean values indicated by different letters on the same line are statistically significant (P<0.05)
^{a,b,c} differences in the values with different letters in the same column were statistically significant (P<0.05)

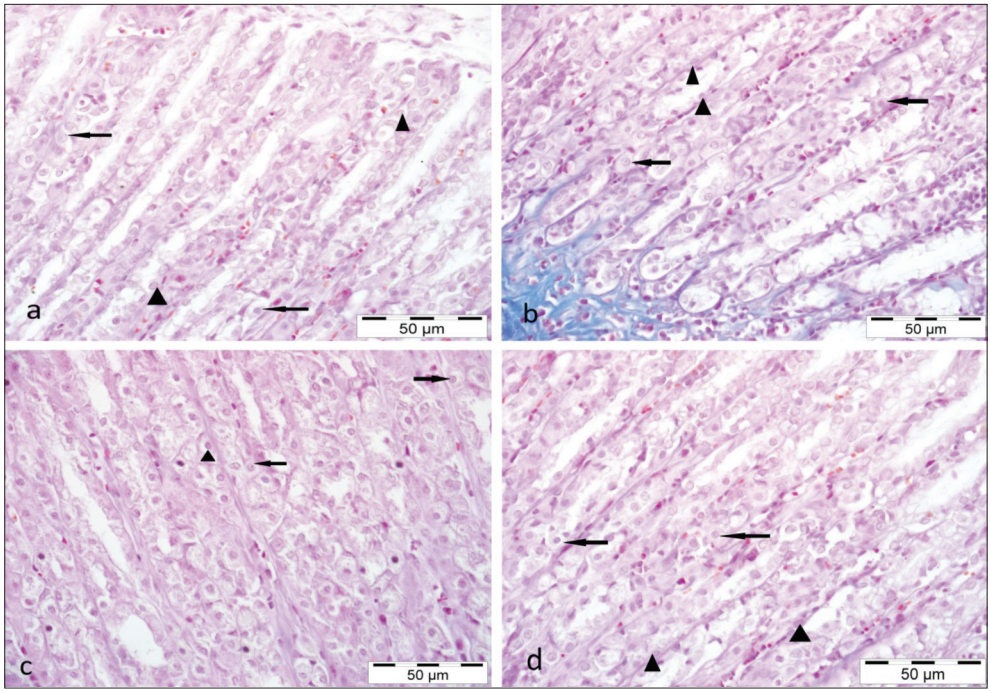


Fig 1. Rat gastric tissue. **a-** Control Group, **b-** *Cinnamon* Group, **c-** Diabetes Group, **d-** Diabetes + *Cinnamon* Group; *arrow:* parietal cells, *arrowhead:* principal cells, Triple, Bar = 50 µm

Table 2. *Comparison of somatostatin immunoreactivity among groups*

Gastric Structures	Diabetes Group	Diabetes + *Cinnamon* Group	*Cinnamon*, Sham and Control Groups
Parietal cells	Weak (+1)	Weak (+1)	Strong (+3)
Principal cells	Weak (+1)	Weak (+1)	Strong (+3)
Pyloric mucosa	Weak (+1)	Weak (+1)	Strong (+3)
Cardia mucosa	Weak (+1)	Weak (+1)	Strong (+3)

cell differentiation, cell migration, tumor growth and apoptosis in rat gastric.

Cinnamon has been shown to lower blood glucose levels, regulate lipid metabolism, suppresses the blood sugar levels by slowing the absorption of carbohydrates from the intestines and have a healing role in type 2 diabetes mellitus with an insulin-like effect [23,24]. Shokri et al.[25] studied three groups 50, 100 and 200 mg/kg doses of *cinnamon* extract daily by gavages for 6 weeks. They determined every doses reduced blood glucose levels. But the dose of 200 mg/kg *cinnamon* extract was the most effective other doses. Kumar et al.[26] in their study investigating the effects of *cinnamon* on blood glucose levels in rats, have administered 150 mg/kg of *cinnamon* extract for 21 days and observed that *cinnamon* had a decreasing effect on blood glucose levels. The decrease in high blood glucose levels and the absence of any toxic effect on the histochemical examination of kidney and pancreatic tissues after a single daily dose of 120 mg/kg *cinnamon* extract in diabetic female and male rats have been considered as positive effects of *cinnamon*. In this case, it was suggested that the *cinnamon* dose is insignificant [27]. In our study, a single dose of 200 mg/kg *cinnamon* extract was administered via oral gavage for 14 days in diabetic rats in the light of literature [6,28]. In our study, the decrease in high blood glucose levels especially in diabetes + *cinnamon* group male rats were similar to some literature studies [6,26-28]. In conclusion, we have determined in our study, which statistically evaluated the effects of *cinnamon* administration on fasting blood glucose that *cinnamon* administration in diabetic male rats may be effective in lowering blood glucose levels.

Diabetes mellitus has been reported to manifest many different pathological situations and damage gastrointestinal system in the long term [29,30]. It has been stated that gastrointestinal symptoms were common in diabetes mellitus which were generally associated with autonomic neuropathy [31]. It was revealed in the study by Bastaki et al.[32] to investigate the morphological alterations in the gastric tissues and parietal cells of streptozotocin-induced experimental diabetic rats with long-term (6 months) that parietal cells were irregularly distributed in diabetic rats compared to those in normal rats, and they reduced acide secretion. The present study revealed no pathological finding in *cinnamon*, diabetes and diabetes + *cinnamon* groups in microscopic evaluation of tissues obtained from STZ-induced diabetic rats. This may be related to the duration of exposure to STZ, which might have changed if STZ was administered to the rats for longer than 14 days.

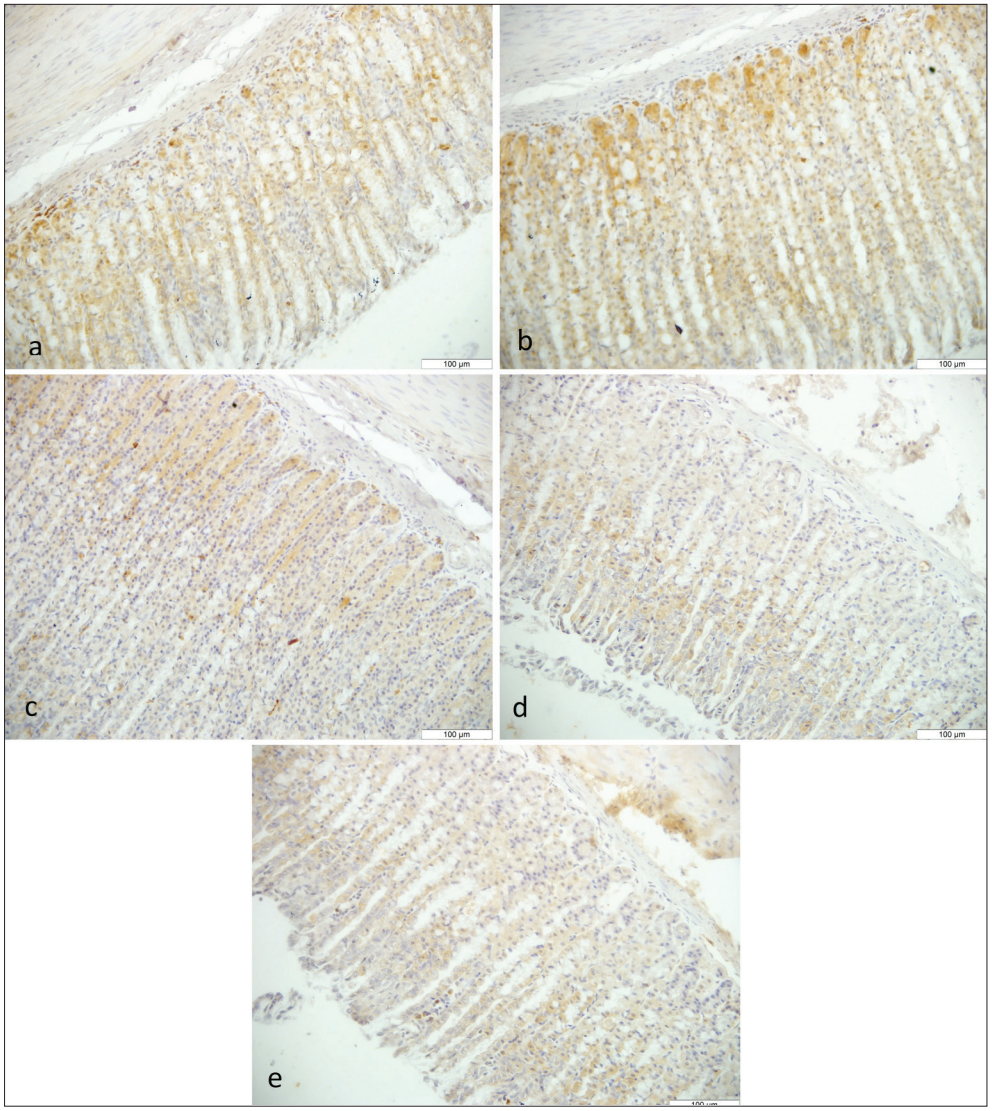


Fig 2. Rat gastric tissue. Intense somatostatin immunoreactivity in control group (a), sham group (b), *cinnamon* group (c); weak somatostatin immunoreactivitiy in diabetes group (d) and diabetes + *cinnamon* group (e). Bar: 100 µm, IHC

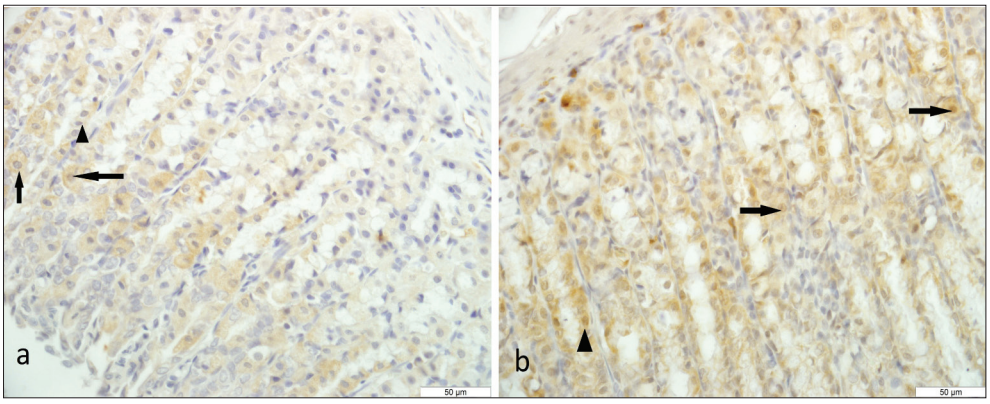


Fig 3. Rat gastric tissue. Weak somatostatin immunoteactivity in principal and parietal cells in diabetes group (a), Intense somatostatin immunoractivity in principal and parietal cells in control group (b), *arrow:* parietal cells, *arrowhead:* principal cells, Bar: 50 µm, IHC

Cinnamon was stated to have an effect to decrease high blood glucose, regulate lipid metabolism, suppress blood glucose by slowing down intestinal absorption of carbohydrates in rats and to likely have a therapeutic role in diabetes mellitus by displaying an insulin-like effect [3,23,24]. Cinnamaldehyde, which is one of the components of *cinnamon*, was determined to reduce blood glucose level in diabetic rats, to increase plasma insulin levels and to regenerate pancreatic β cells damaged by STZ. It has been reported that *cinnamon* releases insulin from β cells and results in a reduction in glucose level, and protects and regenerates β cells via its antioxidant effect [33]. In parallel with the literature review [33-36], somatostatin was determined to display immunolocalization in similar zones in

Table 3. *Comparison of count of somatostatin positive cells in parietal cells among groups*		
Groups	**Number (unit area)**	**M±SD**
Diabetes Group	60	0.96±0.58[a]
Diabetes + *Cinnamon* Group	60	0.80±0.38[a]
Control Group	60	4.10±0.85[b]
Sham Group	60	4.16±0.05[b]
Cinnamon Group	60	4.36±0.67[b]
M: *mean;* ***SD:*** *standard deviation;* [a,b] *Different superscripts in the same column indicate significant differences between groups (P<0.001)*		

Table 4. *Comparison of count of somatostatin positive cells in principal cells among groups*		
Groups	**Number (unit area)**	**M±SD**
Diabetic Group	60	0.96±0.40[a]
Diabetes + *Cinnamon* Group	60	0.70±0.23[a]
Control Group	60	4.10±0.85[b]
Sham Group	60	4.23±0.13[b]
Cinnamon Group	60	4.43±0.68[b]
M: *mean;* ***SD:*** *standard deviation;* [a,b] *Different superscripts in the same column indicate significant differences between groups (P<0.001)*		

control, sham, *cinnamon,* diabetes, and diabetes + *cinnamon* groups in immunohistochemical examinations in the present study. Cytoplasmic and nuclear somatostatin immunoreactivity was observed in parietal and principal cells in fundus area. We determined that on day 14 somatostatin immunoreactivity of the diabetes and diabetes + *cinnamon* groups was weaker than for the control, sham and *cinnamon* groups. Weak immunoreactivity was found in the cardia mucosa and pyloric mucosa of the gastric in the diabates and diabetes+*cinnamon* groups and strong immunoreactivity was found in the control, sham and *cinnamon* groups. It was reported in previous studies that diabetes caused irregular distribution of parietal cells in fundus area [28] In the present study, on the other hand, diabetes was identified to decrease somatostatin immunoreactivity in parietal and principal cells. As a result of these results, the present study revealed that diabetes negatively influenced somatostatin immunoreactivity in fundus area of gastric tissue.

In conclusion, when compared to diabetes groups, *cinnamon* extract administration was determined to increase the secretion of somatostatin which is somatostatin are important regulators of gastric acid secretion. Because we did not found any study on somatostatin immunoreactivity we mentioned in parietal and principal cells, we think that this issue needs to be investigated in more details. This study evaluated whether or not *cinnamon* extract which is reported to be effective in reducing the level of high blood glucose and somatostatin which is reported to be secreted from enteroendocrine cells and to have an inhibiting role on insulin and glucose metabolisms were effective on gastric tissue. We believe that since there is no immunohistochemical study explaining the relationship between somatostatin, *cinnamon,* diabetes and gastric tissue so far, the present study would contribute to literature and further studies should be conducted on the subject.

Declaration of Interest

The authors report no conflicts of interest.

REFERENCES

1. Robertson RP, Harmon JS: Diabetes, glucose toxicity, and oxidative stress: A case of double jeopardy for the pancreatic islet beta cell. *Free Radic Biol Med*, 41 (2): 177-184, 2006. DOI: 10.1016/j.freeradbiomed.2005.04.030

2. Hlebowicz J, Darwiche G, Björgell O, Almér LO: Effect of cinnamon on postprandial blood glucose, gastric emptying, and satiety in healthy subjects. *Am J Clin Nutr,* 85 (6): 1552-1556, 2007. DOI: 10.1093/ajcn/85.6.1552

3. Kardiatun T, Dibua UM, Badger-Emeka L, Ugonabo JA, Tirwomwe JF, Agwu E, Ssamula M: The effect of cinnamon on glucose control in patients with type 2 diabetes mellitus in Pontianak, Indonesia. *Int J Med Med Sci*, 5 (10): 434-437, 2013.

4. Alanazi AS, Khan MU: Cinnamon use ın type 2 diabetes: An updated meta-analysıs. *WJPPS,* 4 (5): 1838-1852, 2015.

5. Ulbricht C, Seamon E, Windsor RC, Armbruester N, Bryan JK, Costa D, Grimes Serrano, JM, Tanguay-Colucci S, Weissner W, Yoon H, Zhang J: An evidence-based systematic review of *cinnamon (Cinnamomum* spp.) by the Natural Standard Research Collaboration. *J Diet Suppl,* 8 (4): 378-454, 2011. DOI: 10.3109/19390211.2011.627783

6. Jia Q, Liu X, Wu X, Wang R, Hu X, Li Y, Huang C: Hypoglycemic activity of a polyphenolic oligomer-rich extract of cinnamomumparthenoxylon bark in normal and streptozotocin induced diabetic rats. *Pythomedicine,* 16, 744-750, 2009. DOI: 10.1016/j.phymed.2008.12.012

7. Kumar SS, Mukkadan JK: Anti diabetic effect of oral administration of cinnamon in wistar albino rats. *BMJ,* 2 (3): 97-99, 2013.

8. Broadhurst CL, Polansky MM, Anderson RA: Insulin-like biological activity of culinary and medicinal plant aqueous extracts *in vitro*. *J Agric Food Chem*, 48, 849-852, 2000. **DOI:** 10.1021/jf9904517

9. Anderson RA, Broadhurst CL, Polansky MM, Schmidt WF, Kahan A, Flanagan VP, Schoene NW, Graves DJ: Isolation and characterization of polyphenol type-a polymers from cinnamon with ınsulin-like biological activity. *J Agric Food Chem,* 52, 65-70, 2004. DOI: 10.1021/jf034916b

10. Şimşek ÜG, Çiftçi M, Doğan G, Özçelik M: Antioxidant activity of cinnamon bark oil *(Cinnamomum zeylanicum L.)* in japanese quails under thermo neutral and heat stressed conditions. *Kafkas Univ Vet Fak Derg,* 19 (5): 889-894, 2013. DOI: 10.9775/kvfd.2013.9049

11. Narin S, Piskin İE, Üstündag G: 2014'te Somatostatin'in tıp'ta kullanımı (Oktreotid). *Güncel Gastroenterol*, 18 (2): 272-276, 2014.

12.Demir can C, Kapıcıoğlu S, Kuşkonmaz İ, Taşkın A, Günaydın M, Kaya N: Mekanik intestinal obstruksiyonlu ratlarda somatostatin analogu SMS 201- 995 (Octreotide) ve omeprazolun etkileri. *Uludağ Üniv Tıp Fak Derg,* 29 (1): 11-14, 2003.

13. Timurkaan S, Timurkaan N, Ozkan E, Girgin M: Immuno-histochemical distribution of somatostatin, glucagon and gastrin in the gastric fundus of the citellus *(Spermophilus xanthoprymnus). J Anim Vet Adv,* 8 (11): 2210-2214, 2009.

14. Gajdosik A, Gajdosikova A, Stefek M, Navarova J, Hozova R: Streptozotocin-induced experimental diabetes in male wistar rats. *Gen Physiol Biophys,* 18, 54-62, 1999.

15. Kanitkar M, Bhonde R: Existence of islet regenerating factors within in pancreas. *Rev Diabet Stud,* 1 (4): 185-192, 2004. DOI: 10.1900/RDS.2004.1.185

16. Luna LG: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed., 72-100, Mc Graw-Hill Book Comp, 1968.

17. Tru LD: Principles of immunohistochemistry. **In,** Tru LD (Ed): Atlas of Diagnostic Immunohistopathology. 1-31, New York Press. New York. 1990.

18. Shu S, Ju G, Fan L: The glucose oxidase-dab-nickel in peroxidase histochemistry of the nervous system. *Neurosci Lett*, 85, 169-171, 1988.

19. Zhu QY: Analysis of blood vessel invasion by cells of thyroid follicular carcinoma using image processing combined with immuno-histochemistry. *Zhonghua Yi Xue Za Zhi,* 69, 573-575, 1989.

20. Seidal T, Balaton AJ, Battifora H: Interpretation and quantification of immunostains. *Am J Surg Pathol*, 25, 1204-1207, 2001.

21. Sun FP, Song Y G, Cheng W, Zhao T, Yao YL: Gastrin, somatostatin, G and D cells of gastric ulcer in rats. *World J Gastroenterol*, 8 (2): 375-378, 2002.

22. Bakır B, Karadağ Sarı E, Eliş Yıldız S, Asker H: Effcts of thymoquinone supplementation on somatostatin secretion in pancreas tissue of rats. *Kafkas Univ Vet Fak Derg,* 23 (3): 409-413, 2017. DOI: 10.9775/kvfd.2016.16893

23. Cheng D, M Kuhn P, Poulev A, Rojo LE, Lila MA, Raskin I: *In vivo* and *in vitro* antidiabetic effects of aqueous cinnamon extract and cinnamon polyphenol-enhanced food matrix. *Food Chem,* 135 (4): 2994-3002, 2012. DOI: 10.1016/j.foodchem.2012.06.117

24. Kim SH, Hyun SH, Choung SY: Anti-diabetic effect of cinnamon extract on blood glucose in db/db mice. *J Ethnopharmacol*, 104 (1): 119-123, 2006. DOI: 10.1016/j.jep.2005.08.059

25. Shokri G, Fathi H, Sabet MJ, Nasrabadi NN, Ataee R: Evaluation of anti-diabetic effects of hydroalcoholic extract of green tea and cinnamon on streptozotocin-induced diabetic rats. *Pharm Biomed Res,* 1 (2): 20-29, 2015. DOI: 10.18869/acadpub.pbr.1.2.20

26. Kumar S, Vasudeva N, Sharma S: GC-MS analysis and screening of antidiabetic, antioxidant and hypolipidemic potential of cinnamomum tamala oil in streptozotocin induced diabetes mellitus in rats. *Cardiovasc Diabetol*, 11:95, 2012. DOI: 10.1186/1475-2840-11-95

27. Ranasinghe P, Perera S, Gunatilake M, Abeywardene E, Gunapala N, Premakumara S, Perera K, Lokuhetty D, Katulanda P: Effects of cinnamon zeylanicum on blood glucose and lipids in a diabetic and healthy rat model. *Pharmacognosy Res,* 4 (2): 73-79. 97, 2012. DOI: 10.4103/0974-8490.94719

28. Rahman EANS, Abdel-Haleem AMH, Al Mudhaffar HM: Anti diabetic effects of cinnamon powder and cinnamon aqueous extract on serum glucose of rats. *IJFSNPH,* 3 (2): 183-197, 2010.

29. O'Reilly D, Long RG: Diabetes and the gastrointestinal tract. *Dig Dis*, 5, 57-64, 1987. DOI: 10.1159/000171163

30. Takehara K, Tashima K, Takeuchi K: Alterations in duodenal bicarbonate secretion and mucosal susceptibility to acid in diabetic rats. *Gastroenterology,* 112, 418-428, 1997. DOI: 10.1053/gast.1997.v112.pm9024295

31. Weber JR, Ryan JC: Effects on the gut of systemic disease and other extraintestinal conditions. **In,** Scharschmidt BF, Slei-singer MH, Feldman M (Ed): Gastrointestinal and liver disease. 6th ed., 413-416, WB Saunders Co, Philadelphia, 1998.

32. Bastaki SMA, Adeghate E, Chandranath IS, Amir N, Tariq S, Hameed RS, Adem A: Effects of streptozotocin-induced long-term diabetes on parietal cell function and morphology in rats. *Mol Cell Biochem*, 341 (1-2): 43-50, 2010. DOI: 10.1007/s11010-010-0435-4

33. Subash-Babu P, Alshatwi AA, Ignacimuthu S: Beneficial anti-oxidative and antiperoxidative effect of cinnamaldehyde protect streptozotocin-induced pancreatic β-cells damage in wistar rats. *Biomol Ther,* 22 (1): 47-54, 2014. DOI: 10.4062/biomolther.2013.100

34. Kasacka I, Majewski M: An immunohıstochemıcal study of endocrine cells in the stomach of hypertensive rats. *J Physiol Pharmacol*, 58 (3): 469-478, 2007.

35. Kasacka I, Łebkowski W, Janiuk I, Łapińska J, Lewandowska A: Immunohistochemical identification and localisation of gastrin and somatostatin in endocrine cells of human pyloric gastric mucosa. *Folia Morphol*, 71 (1): 39-44, 2012.

36. Chen M, He M, Peng K, Liu T, Jin C, Cao W, Wang L, Xiao K: An immunohistochemical study of somatostatin in the stomach and the small intestine of the African ostrich *(Struthio camelus)*. *Tissue Cell*, 45 (6): 363-366, 2013. DOI: 10.1016/j.tice.2013.06.002

INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given in the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal cowers all aspects of veterinary medicine and animal science.

In the interests of brevity and standalone readability, **Kafkas Universitesi Veteriner Fakultesi Dergisi** strongly discourages the submission of multi-part manuscripts. Authors who feel that their topic requires an exception should obtain approval from the editor before submission of a multi-part manuscript. If submitted, multipart papers can be assigned to different editorial board members and independent outside expert reviewers. It is necessary to load all parts of manuscript are required to be loaded into the online system at the same time.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an *Open Access* journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

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 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://submit.vetdergikafkas.org/*

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- Types of Manuscripts

Original (full-length) Manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit not include tables and illustrations. Abstract 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 abstracts 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 in total. The page limit not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary Scientific Reports are short description of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

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. The length of the text should be no longer than 4 pages in total. The page limit not include tables and illustrations.

Letters to the Editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 2 pages in total. The page limit includes tables and illustrations.

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 (with appropriate titles), conclusion, and references. The length of the text should be no longer than 15 pages in total. *Invited reviews* will be considered for priority publication.

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: **Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z:** Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg,* 23 (5): 767-772, 2017.

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: **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 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.

Follow the link below for EndNote style of Kafkas Universitesi Veteriner Fakultesi Dergisi; *https://researchsoftware.com/downloads/journal-faculty-veterinary-medicine-kafkas-university*

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 field editor and two referees specialized in the particular field.

9- All responsibilities from published articles merely belong to the authors. According to ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with broad database of academic publications.

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://vetdergikafkas.org/*

12- Reprints (in multiples of 50) of the article are sent to the authors for free.

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- **Cover Letter (without author/authors name)**

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction)

• Other information has been added that should be known by the editorial board (e.g.; list of other journal or conference papers (if any) published or submitted by you or any co-author)

• Authors should add the necessary clarifications about editor//adviser/reviewer's comments to the cover letter section for each revision.

All necessary files have been uploaded

- **Title page**

• Include title, running title (no more than 5 words)

• The author's name, institutional affiliation

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- **Manuscript**

• Include title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

- **Supplemental files (where applicable)**

Further considerations

• Journal policies detailed in this guide have been reviewed

• Manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Acknowledgement and conflicts of interest statement provided