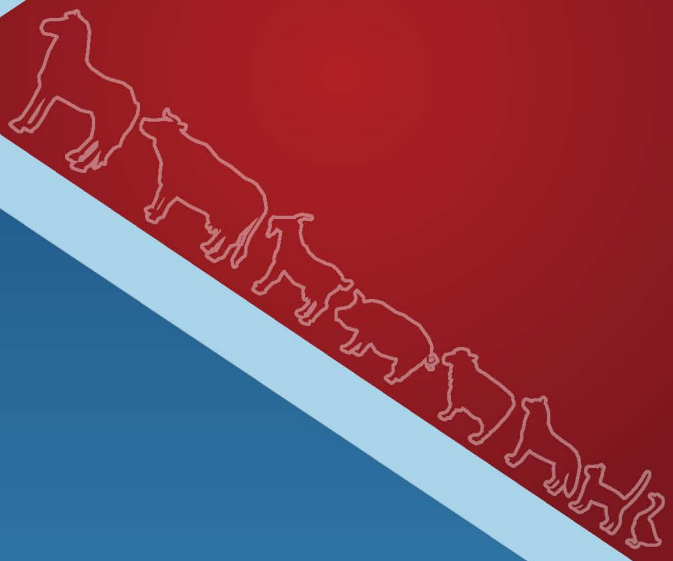
ISSN 1300 - 6045
e-ISSN 1309 - 2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University



http://vetdergi.kafkas.edu.tr

Online Submission
http://submit.vetdergikafkas.org

Volume: 27
Issue: 2 (March - April)
Year: 2021

ISSN 1300 - 6045
e-ISSN 1309 - 2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University

Published Bi-monthly

Volume: 27
Issue: 2 (March - April)
Year: 2021

ISSN (Print): 1300-6045 **ISSN (Electronic): 1309-2251**

This journal is published bi-monthly, by the Faculty of Veterinary Medicine, University of Kafkas, Kars - Turkey

This journal is indexed and abstracted in:

- Web of Science Core Collection: Science Citation Index Expanded (since 2007)
- Additional Web of Science Indexes: Essential Science Indicators - Zoological Record
- CABI - Veterinary Science Database
- DOAJ
- EBSCO - Academic Search Premier
- Elsevier - SCOPUS
- Elsevier - EMBASE
- Index Copernicus
- SOBİAD Atıf Dizini
- TÜBİTAK/ULAKBİM TR-Dizin
- Türkiye Atıf Dizini

Print

ESER OFSET MATBAACILIK
BOSNAHERSEK CAD. ALTUNALEM YAPI KOOP. ZEMİN KAT - ERZURUM
Tel: +90 442 2334667 E-mail: eserofset25@hotmail.com

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

Electronic Edition http://vetdergikafkas.org

Online Submission http://submit.vetdergikafkas.org

The Referees List of This Issue *(in alphabetical order)*

Ahmet AYDOĞAN	Çukurova Üniversitesi Ceyhan Veteriner Fakültesi
Ahrar KHAN	Shandong Animal Husbandry and Veterinary Vocational College, China
Ahu DEMİRTAŞ	Burdur Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
Ali Tümay GÜRLER	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
Alireza LOTFİ	Islamic Azad Unversity, Ilkchi Branch, Iran
Aydın GÜREL	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
Birten EMRE	Harran Üniversitesi Veteriner Fakültesi
Cansel Güzin ÖZGÜDEN AKKOÇ	Bursa Uludağ Üniversitesi Veteriner Fakültesi
Çiğdem SEZER	Kafkas Üniversitesi Veteriner Fakültesi
Ece KOLDAŞ ÜRER	Hatay Mustafa Kemal Üniversitesi Veteriner Fakültesi
Elif ÇELİK	Kafkas Üniversitesi Veteriner Fakültesi
Ender ERDOĞAN	Selçuk Üniversitesi Tıp Fakültesi
Fatma Ceren ANLAŞ	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
Figen KIRKPINAR	Ege Üniversitesi Ziraat Fakültesi
Gencay Taşkın TAŞÇI	Kafkas Üniversitesi Veteriner Fakültesi
Ghazanfar ABBAS	University of Melbourne, Faculty of Veterinary Science, Australia
Gürdal DAĞOĞLU	Fırat Üniversitesi Veteriner Fakültesi
Gürsel SÖNMEZ	Bursa Uludağ Üniversitesi Veteriner Fakültesi
Güzin KABAN	Atatürk Üniversitesi Ziraat Fakültesi
Hasan Hüseyin HADİMLİ	Selçuk Üniversitesi Veteriner Fakültesi
Hasan Tarık ATMACA	Kırıkkale Üniversitesi Veteriner Fakültesi
Ijaz AHMAD	The University of Agriculture, Peshawar, Pakistan
İ. İsmet TÜRKMEN	Bursa Uludağ Üniversitesi Veteriner Fakültesi
İlhami ÇELİK	Selçuk Üniversitesi Veteriner Fakültesi
İsmail Hakkı EKİN	Van Yüzüncü Yıl Üniversitesi Veteriner Fakültesi
Korhan ARSLAN	Erciyes Üniversitesi Veteriner Fakültesi
Kutlay GÜRBULAK	Erciyes Üniversitesi Veteriner Fakültesi
M. Yavuz GÜLBAHAR	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
Marko PAJIĆ	Scientific Veterinary Institute, Serbia
Meryem AYDEMİR ATASEVER	Atatürk Üniversitesi Veteriner Fakültesi
Mithat EVECEN	İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi
Murat KARA	İnönü Üniversitesi Tıp Fakültesi
Musa TATAR	Burdur Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
Mustafa HİTİT	Kastamonu Üniversitesi Veteriner Fakültesi
Mustafa Necati MUZ	Tekirdağ Namık Kemal Üniversitesi Veteriner Fakültesi
Mükremin Özkan ARSLAN	Kafkas Üniversitesi Tıp Fakültesi
Neriman MOR	Kafkas Üniversitesi Tıp Fakültesi
Nesimi AKTAŞ	Nevşehir Hacı Bektaş Veli Üniversitesi Mühendislik Fakültesi
Nikola ČOBANOVIĆ	University of Belgrade, Faculty of Veterinary Medicine, Serbia
Nurhan ERTAŞ ONMAZ	Erciyes Üniversitesi Veteriner Fakültesi
Özgür ÇELEBİ	Kafkas Üniversitesi Veteriner Fakültesi
Özgür ÖZYİĞİT	Bursa Uludağ Üniversitesi Veteriner Fakültesi
Recai KULAKSIZ	Balıkesir Üniversitesi Veteriner Fakültesi
Rıfkı HAZIROĞLU	Ankara Üniversitesi Veteriner Fakültesi
Serap SAVAŞAN	Aydın Adnan Menderes Üniversitesi Veteriner Fakültesi
Seyit Ali BİNGÖL	Kafkas Üniversitesi Tıp Fakültesi
Zati VATANSEVER	Kafkas Üniversitesi Veteriner Fakültesi
Zuhal ÖNDER	Erciyes Üniversitesi Veteriner Fakültesi

İÇİNDEKİLER (Contents)

OLGU SUNUMU (Case Report)

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

Kafkas Univ Vet Fak Derg
27 (2): 135-140, 2021
DOI: 10.9775/kvfd.2020.24832

RESEARCH ARTICLE

Effects of Different Degrees of Cold Stress on FIAF Expression in Pigs

Hong JI [1,a,†] Ziyi SHAO [1,b,†] Ying LIU [2,c] Xu ZHANG [1,d] Chunyang NIU [1,e]
Jingru GUO [1,f] Bin XU [1,g] Xuelong ZHAN [1,h] Juxiong LIU [2,i (*)] Jianfa WANG [1,j (*)]

[†] *These authors contributed equally to the work*

[1] College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang, 163319, CHINA

[2] College of Animal Science and Veterinary Medicine, Jilin University, Changchun, Jilin, 130062, CHINA

ORCIDs: [a] 0000-0001-7550-1457; [b] 0000-0003-4871-4814; [c] 0000-0002-4872-7511; [d] 0000-0002-2520-0354; [e] 0000-0002-5457-753X
[f] 0000-0001-9333-2773; [g] 0000-0002-7628-1772; [h] 0000-0002-5328-9456; [i] 0000-0003-0764-811X; [j] 0000-0002-6897-7526

Article ID: KVFD-2020-24832 Received: 09.08.2020 Accepted: 25.01.2021 Published Online: 26.01.2021

Abstract

Cold stress is the main stressor restricting the development of animal husbandry in cold regions. Fasting-induced adipose factor (FIAF), also known as angiopoietin-like protein 4 (ANGPTL4), plays an important regulatory role in the metabolism of lipids. Its functions include inhibiting lipoprotein lipase (LPL) to eliminate triglycerides and free fatty acids in blood, reducing fat deposition and promoting adipose tissue degradation. This experiment was designed to investigate the effects of different degrees of cold stimulation on fat metabolism in finishing pigs. Growing and fattening pigs were randomly divided into different groups and exposed to temperatures of -10±2°C, -5±2°C, 0±2°C, 5±2°C and 21±2°C for 2 h. Serum, liver, neck, abdominal subcutaneous and mesenteric adipose tissues were collected and analyzed by Real-Time quantitative PCR (qRT-PCR), western blotting and enzyme-linked immunosorbent assay (ELISA) to examine FIAF expression. The results showed that a gradual increase in cold stress intensity resulted in a gradual increase in FIAF mRNA and protein expression levels in liver, neck, abdomen and mesenteric adipose tissues and FIAF concentration also gradually increased in the blood. It indicated that FIAF is involved in energy and fat metabolism in response to cold stress and may be regulated by the activation of peroxisome proliferator-activated receptor (PPAR) by free fatty acids in the blood induced by cold stress.

Keywords: *Cold stress, Fat tissue, Fasting-induced adipose factor, FIAF, Pig*

Farklı Derecelerde Soğuk Stresinin Domuzlarda FIAF Ekspresyonuna Etkileri

Öz

Soğuk stresi, soğuk bölgelerde hayvancılığın gelişmesini kısıtlayan ana stres faktörüdür. Anjiopoietin benzeri protein 4 (ANGPTL4) olarak da bilinen Fasting-induced adipose faktör (FIAF), yağların metabolizmasında önemli bir düzenleyici rol oynar. FIAF'ın fonksiyonları arasında, kandaki trigliseritleri ve serbest yağ asitlerini uzaklaştırmak için lipoprotein lipazı (LPL) inhibe etmek, yağ birikimini azaltmak ve yağ dokunun bozulmasını teşvik etmek bulunur. Bu çalışma, farklı derecelerde soğuk uyarımın, besili domuzlarda karaciğerin FIAF ve yağ metabolizması üzerine etkilerini araştırmak için tasarlanmıştır. Ergin ve besili domuzlar rastgele farklı gruplara ayrıldı ve 2 saat boyunca -10±2°C, -5±2°C, 0±2°C, 5±2°C ve 21±2°C sıcaklıklara maruz bırakıldı. Serum, karaciğer, boyun yağ dokusu, abdominal subkutanöz yağ doku ve mezenterik yağ doku toplandı ve FIAF ekspresyon analizi, kantitatif real-time PCR (qRT-PCR), Western Blot ve Enzim-işaretli immünosorbent test (ELISA) ile gerçekleştirildi. Sonuçlar, soğuk stres yoğunluğundaki kademeli artışın, karaciğer, boyun yağ dokusu, karın yağ dokusu ve mezenterik yağ dokusunda FIAF'ın mRNA ve protein ekspresyon seviyelerinde kademeli bir artışa neden olduğunu ve ayrıca kanda FIAF konsantrasyonunun da kademeli olarak arttığını gösterdi. FIAF'ın soğuk stresine yanıt olarak enerji ve yağ metabolizmasında rol oynadığını ve soğuk stresi ile indüklenen kandaki serbest yağ asitleri tarafından Peroksizom proliferatör-aktive reseptör (PPAR) aktivasyonu ile düzenlenebilir olduğunu gösterdi.

Anahtar sözcükler: *Domuz, FIAF, Soğuk stresi, Yağ doku*

INTRODUCTION

Cold stress is the main stress factor in the agricultural production process in cold regions. Low environmental temperatures in animal housing cause a series of physiological changes, including accelerated breathing, vasoconstriction, enhanced endocrine activities and accelerated nutrient and energy metabolism to maintain

How to cite this article?

Ji H, Shao Z, Liu Y, Zhang X, Niu C, Guo J, Xu B, Zhan X, Liu J, Wang J: Effects of different degrees of cold stress on FIAF expression in pigs. *Kafkas Univ Vet Fak Derg,* 27 (2): 135-140, 2021.
DOI: 10.9775/kvfd.2020.24832

(*) Corresponding Author

Tel: +86-0459-6819201 (J. Liu), 86-0459-6819200 (J. Wang)
E-mail: 274623375@qq.com (J. Liu), wjflw@sina.com (J. Wang)

a normal body temperature [1]. Cold stress also causes accelerated fat mobilization and weight loss, decreased meat quality and impaired growth in animals, affecting animal welfare and resulting in significant impairments to the healthy development of the agriculture industry. Thermogenesis is required to maintain euthermia in changing external temperatures and is a physiological process performed predominantly by adipose tissue [2]. In addition, adaptive heat production mediated by adipose tissue is essential for chronic adaptation to cold. Studies have shown that long-term cold exposure can increase the rate of lipolysis in adipose tissue. Therefore, the study of fat metabolism in livestock and poultry subjected to cold stress would have theoretical and practical significance for animal production and fat metabolism in cold environmental conditions.

Adipose tissue acts as an endocrine organ and produces numerous bioactive factors, such as adipokines that communicate with other organs and modulate a range of metabolic pathways [3]. For example, adipocytokines are substances secreted by adipose tissue that have a variety of biological functions. In the process of fat metabolism, they are directly involved in the hydrolysis of triglycerides, the oxidation of free fatty acids and free fatty acid mobilization in non-adipose tissues. Adipocytokines in animals mainly include leptin, adiponectin, resistin and fasting-induced adipose factor (FIAF) [4]. FIAF, also known as angiopoietin-like protein 4 (ANGPTL4), plays an important regulatory role in the metabolism of lipids, and its functions include the inhibition of lipoprotein lipase (LPL) to eliminate triglycerides and free fatty acids in the blood, the reduction of fat deposition and the promotion of adipose tissue degradation [5]. The action of adipokines is mainly mediated by binding to their respective receptors on the membrane of target cells, which triggers intracellular signaling pathways [3]. Previous studies have found that some food nutrients, fasting, endurance training and gut microbes can affect FIAF concentrations in blood [6]. The aim of this study was to assess FIAF expression levels in various tissues and in blood to identify the role of FIAF in fat metabolism of pigs under cold stress.

Material and Methods

Sample Collection

Pig breed: Junmu No. 1 pig, provided by: Jida original pig farm (Changchun, China). The environment of the pig house is so clean that the pigs are well fed with feed and drinking water every day. A total of 30 healthy Junmu No. 1 pigs with an average body weight of 60±5 kg was randomly divided into five groups, with six pigs in each group. The control group of pigs were kept at 21±2°C prior to slaughter. The test groups of pigs were exposed to -10±2°C, -5±2°C, 0±2°C or 5±2°C for 2 h prior to slaughter. Both the control group and the test groups were slaughtered in the Jida original pig farm. The blood was collected from the femoral artery of the pig to separate serum before slaughter. And the liver tissue, abdominal fat tissue, neck fat tissue, mesenteric fat tissue was collected after slaughter. All samples were labeled and immediately stored in liquid nitrogen. The protocol was approved by the Animal Ethics Committee of Jilin University.

qRT-PCR

Approximately 100 mg tissue samples (liver tissue, abdominal fat tissue, neck fat tissue, and mesenteric fat tissue) were separately collected and placed in liquid nitrogen. Total RNA was then extracted using the one-step TRIZOL method (Invitrogen Corp., Carlsbad, CA, USA). A PrimeScript^{T}M RT-PCR Kit (Takara Biotechnology Co., Dalian, China) was used to reverse transcribe total RNA samples. The primer and probe sequences of FIAF and the reference gene, GAPDH, are shown in *Table 1* (synthesized by GeneCore Biotechnology Co., Shanghai, China). The PMD18-T vector was used to construct the recombinant gene plasmid (Takara), which was transformed into Escherichia coli DH5α host bacteria. After screening for ampicillin resistance, a BioTeke Plasmid DNA miniprep Kit (BioTeke Corp., DP1001 Beijing, China) was used to extract standard plasmid to create a standard curve. A Mastercycler® ep realplex Real-time PCR instrument (Eppendorf, Hamburg, Germany) was used to measure gene expression. The reaction system included 12.5 μL Premix Ex Taq^{TM} (2×) (Takara), 10 μM upstream primer, 0.5 μL downstream primer, 1 μL probe, 2 μL cDNA and 8.5 μL ddH_2O. The template-free negative control (NTC) was set as the control. The obtained standard curve indicated that the amplification efficiency of the two genes was 100%. Therefore, the $2^{-\triangle\triangle Ct}$ method was used to calculate the relative expression levels of genes [7]. Each sample was measured in triplicate.

Western Blotting

Protein samples were ground into homogenates after being weighed, and a Bicinchoninic acid (BCA) protein assay kit (BioTeke., Beijing, China) was used to measure total protein concentration. Proteins were separated on 5% and 12% polyacrylamide gels (Bio-Rad Laboratories Inc., Hercules, CA, USA), and a Trans-Blot SD semi-dry transfer tank (Bio-Rad) was used to transfer separated proteins onto a polyvinylidene difluoride membrane (Millipore Corp., Burlington, MA, USA). The membrane was blocked, incubated with detection antibody (1: 2000 dilution) followed by the horseradish peroxidase-conjugated secondary antibody (1: 2000 dilution) for 2 h and developed in the dark-room. Antibody information was shown in *Table 2*.

ELISA

The concentration of FIAF in serum was measured with a Porcine of Angiopoietin-Like Protein 4 ELISA Kit (R&D Systems Inc., Shanghai, China) according to the manufacturer's instructions.

Table 1. *List of primers and probes used for quantitative real-time PCR*

Target	Accession Number	Primers and Probe	Amplicon Length (bp)
FIAF	AY751522	F: 5′-CTGGTGGTTGGTGGTTTGG-3′ R: 5′-GCTGCCGAGGGATGGAAT-3′ P: 5′-(FAM) TGACCTCCGCGCCCTGGC (Eclipse) -3′	75
GAPDH	NM_001206359.1	F: 5′-CTGACCTGCCGTCTGGAGAA-3′ R: 5′-TAGCCCAGGATGCCCTTGAG-3′ P: 5′-(FAM) CCTCGGACGCCTGCTTCACCACCT (Eclipse)-3′	95

Table 2. *List of antibodies used in this study*

Antibody Name		Species in which the Antibody was Raised	Dilution used in Western Blotting	Manufacturer of the Antibody
Primary antibody	FIAF	Rabbit	1: 500	Bioss
	β-actin	Goat	1: 2000	Santa cruz
Secondary antibody	FIAF	Goat Anti-Rabbit IgG	1: 1000	Santa cruz
	β-actin	Rabbit Anti-Goat IgG	1: 1000	Santa cruz

Statistical Analysis

Statistical analysis and comparisons between groups were performed using one-way ANOVA. All data were expressed as mean ± standard deviation (mean±SD). P≤0.05 was considered statistically significant. All analyses were performed by SPSS 17.0 software and Graphpad Prism (version 7.0).

Results

Expression of FIAF mRNA in Different Tissues

Fig. 1, 2, 3, 4 showed that a decrease in ambient temperature resulted in a gradual increase in levels of FIAF mRNA in different tissues. In pigs exposed to 5°C for 2 h, FIAF mRNA levels in neck fat tissue were significantly higher than those of the control group (21°C) (P<0.01). In pigs exposed to 0°C for 2 h, FIAF mRNA levels in liver tissue (P<0.05) and neck fat tissue (P<0.01) were significantly higher than those of the control group. In pigs exposed to -5°C for 2 h, FIAF mRNA levels in mesenteric fat tissue (P<0.01), abdominal fat tissue (P<0.05), liver tissue (P<0.01) and neck fat tissue (P<0.001) were significantly higher than those of the control group. In pigs exposed to -10°C for 2 h, FIAF mRNA levels were significantly higher in liver tissue (P<0.01), abdominal fat tissue (P<0.01), mesenteric fat tissue (P<0.01) and neck fat tissue (P<0.001) than those of the control group.

Moreover, there were significant differences between the -5°C group and other three groups (5°C, 0°C and -10°C) in liver tissue (P<0.05). There were significant differences between the -10°C group and other two groups (5°C and 0°C) in liver tissue (P<0.01). For the abdominal fat tissue, there were significant differences between the -10°C group and other groups (P<0.01), and there were significant differences between the -5°C group and other two groups (5°C and 0°C) (P<0.05). For the neck fat tissue, there were significant differences in FIAF mRNA levels among all groups (P<0.001). As for the mesenteric fat tissue, there were significant differences in FIAF mRNA levels between -5°C group and other two groups (5°C and 0°C) (P<0.05), and there were significant differences between other groups (P<0.01).

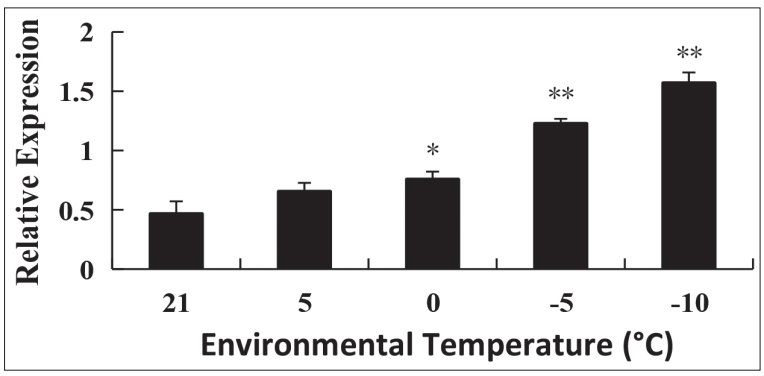


Fig 1. The effect of different environmental temperatures on FIAF mRNA levels in liver tissue. Compared to control * P<0.05; ** P<0.01

Expression of FIAF Protein in Different Tissues

As shown in *Fig. 5*, it was found that FIAF was expressed in liver tissue, abdominal fat tissue, neck fat adipose tissue and mesenteric fat tissue, and the protein molecular weight was consistent with the actual size. The molecular weight of FIAF protein was about 50 KD and β-actin was 42 KD.

FIAF Concentrations in Serum

As shown in *Table 3*, FIAF concentrations in the blood of pigs were low at 21°C (approximately 32.78 pg/L). As the ambient temperature decreased,

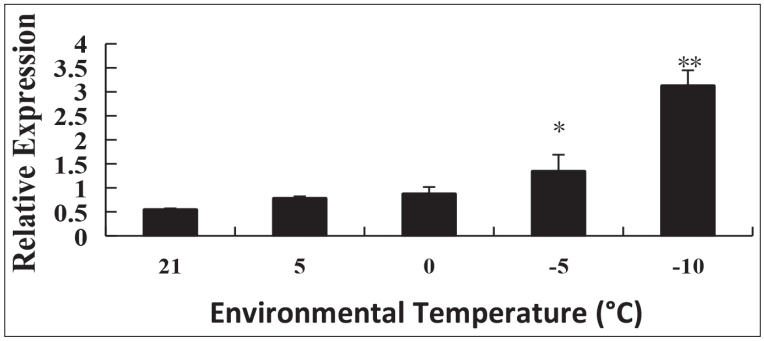


Fig 2. The effect of different environmental temperatures on FIAF mRNA levels in abdominal fat tissue

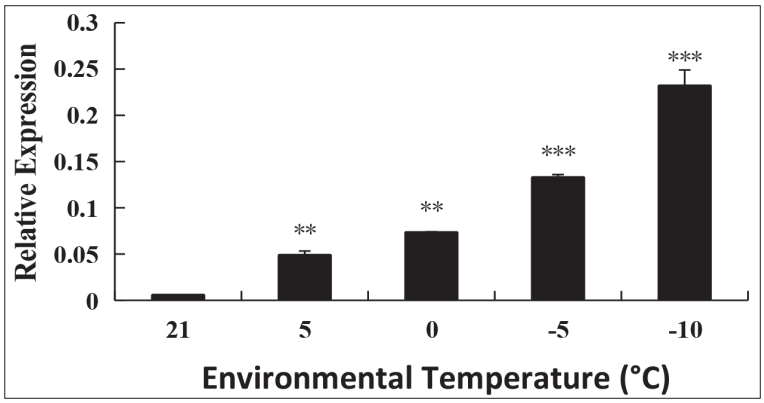


Fig 3. The effect of different environmental temperatures on FIAF mRNA levels in neck fat tissue. Compared to control *** P<0.001

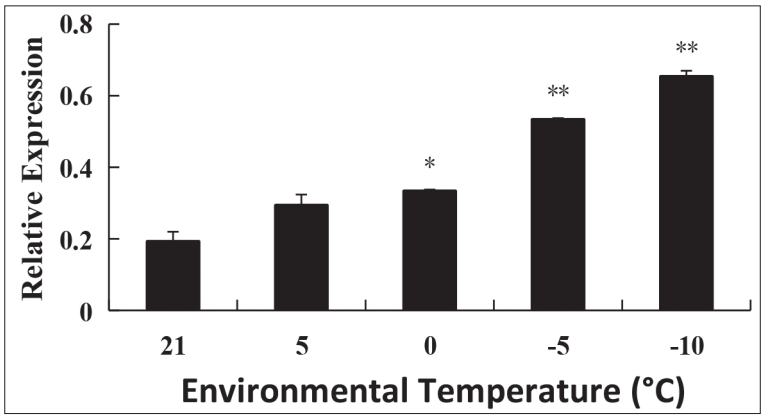


Fig 4. The effect of different environmental temperatures on FIAF mRNA levels in mesenteric fat tissue

FIAF concentrations in the blood increased. In pigs exposed to 5°C for 2 h, FIAF concentrations in blood were significantly higher than in the control group (P<0.05). In pigs exposed to 0°C, -5°C and -10°C for 2 h, FIAF concentrations in blood were significantly higher than in the control group (P<0.01). At an ambient temperature of -10°C, FIAF concentrations in blood reached 104.68 pg/L. Besides, in pigs exposed to -10°C for 2 h, FIAF concentrations in blood were significantly higher than in the -5°C group (P<0.05), the 0°C group (P<0.01) and the 5°C group (P<0.01). There were significant differences between other groups (P<0.01).

Discussion

Cold is a common stress factor for animals, especially newborn animals, at high altitudes and in cold regions. Cold stress can slow down animal growth, impair disease resistance and even cause death in severe cases, resulting in major losses to the farming industry. It is also one of the important limiting factors restricting agricultural development in regions at high latitudes. A previous study showed that acute cold stress increased the metabolic rate of animals, enhanced sugar, protein and fat catabolism and raised peripheral blood metabolites, including free fatty acids (FFA), glucose and arginine [8]. Under cold stress conditions, the metabolic activity of adipose tissue is regulated by neural and endocrine pathways. Adipocytokines were produced and secreted by adipose tissue also play important roles in maintaining energy metabolism stability and regulating lipid and carbohydrate metabolism *in vivo*. The previous research showed that the expression of leptin and adiponectin mRNA in the neck, back and mesenteric adipose tissue of pigs gradually decreased with the increase of cold stress intensity, but it had little effect on the expression of resistin mRNA [9]. It has also been confirmed that adipocytokines play a regulatory role in the fat metabolism process under cold stress.

Table 3. *The effect of different environmental temperatures on the concentration of FIAF in serum (pg/L)*

Group	Temperature	The Concentration of FIAF
Control	21±2°C	32.78±5.49
Test 1	5±2°C	52.44±4.34*
Test 2	0±2°C	76.30±2.57**
Test 3	-5±2°C	97.12±9.11**
Test 4	-10±2°C	104.68±10.92**

*Significant labeling based on the comparison between the control group and the four test groups. Compared to control * P<0.05; ** P<0.01*

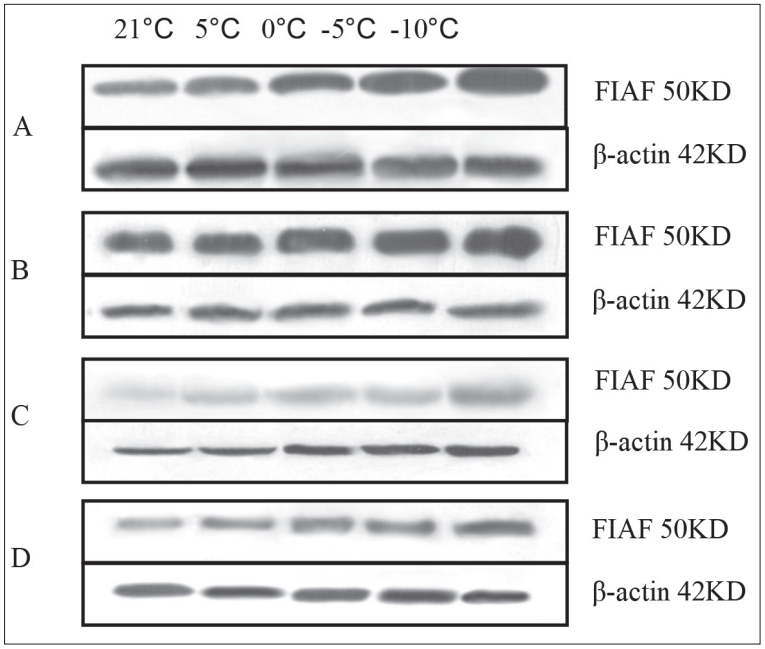


Fig 5. The effect of different environmental temperatures on FIAF protein levels in different tissues. **A:** liver tissue, **B:** abdominal fat tissue, **C:** neck fat tissue, **D:** mesenteric fat tissue

FIAF is a target gene of peroxisome proliferator-activated receptor (PPAR) and an endocrine signal that regulates energy metabolism. Fasting can cause an increase in FIAF concentrations in serum, whereas a high-fat diet may lead to decreased FIAF concentrations in serum. PPAR agonist can also cause elevated FIAF concentrations in serum [10]. The angiopoietin-like proteins (ANGPTLs) have emerged as key regulators of plasma lipid metabolism by serving as potent inhibitors of the enzyme, LPL [11]. Injection of FIAF has been shown to increase monomeric triglyceride fatty acids, glycerol, total cholesterol and high-density lipoprotein concentrations, inhibit the activity of apolipoprotein enzyme, enhance fatty acid oxidation and promote the expression of the fat uncoupling protein, UCP, in rat plasma, resulting in reduced adipose tissue weight [12].

Zhang et al.[13] found that the FIAF gene was expressed in the liver, fat, heart, small intestine and large intestine of pigs, and was most abundant in adipose tissue, followed by liver, large intestine, heart and small intestine. They also confirmed that *Bacteroides thetaiotaomicron* inhibited FIAF gene expression in ileal epithelial cells in the intestinal canal of pigs. In the present study, FIAF mRNA and protein expression levels were high in liver and abdominal fat tissues, whereas levels were low in neck fat. Exposure to low temperatures enhanced FIAF mRNA and protein expression levels in the liver and adipose tissue of various organs and elevated FIAF concentrations in serum. As the ambient temperature decreased, changes in FIAF expression levels increased. Elevated FIAF concentrations promote the degradation of adipose tissue, increasing free fatty acid and triglyceride levels in blood and enhance fatty acid oxidation, all of which are beneficial for adaptation to the high demands of energy metabolism in cold environments.

Under cold stress, changes in leptin and adipo-nectin expression are regulated by the sympathetic nervous system. Injection of β-adrenergic receptor agonist imitates the effects of acute cold exposure and inhibits leptin and adiponectin gene transcription levels in adipose tissue [14,15]. Intravenous injection of fat emulsion, other PPAR agonists (e.g., fenofibrate, pioglitazone, rosiglitazone, etc.) or β-adrenergic receptor agonist (albuterol) increases FIAF expression levels in blood, and significantly increases free fatty acids in blood. However, injection of salbutamol, a β-adrenergic receptor agonist, and acipimox, a fat degradation inhibitor, significantly reduces FIAF levels in blood [6]. Under cold stress, the sympathetic nerve is stimulated, resulting in increased secretion of adrenal medullary catecholamines, which mobilize adipose tissue to promote catabolism, and changes in free fatty acid concentrations in the blood. A recent study found that cold stress induces rat mesenteric fat cells to synthesize norepinephrine and epinephrine, and increase catecholamine synthetic enzyme content in stromal vascular fraction cells, indicating that an independent catecholamine synthesis system exists in fat cells and plays an important role in adipose tissue mobilization induced by cold stress [16]. Free fatty acids in the blood activate PPAR, and FIAF is a downstream target gene of PPAR. Previous studies have found that cold stress elevates PPARγ2 mRNA and protein levels in liver and adipose tissue [17]. Therefore, it was likely that the changes in FIAF concentrations in liver, adipose tissue and blood observed in pigs under cold stress were caused by the free fatty acid concentrations in the blood, which might be subject to joint control by the sympathetic nerve in the catecholamine sympathetic system of fat tissues and activation of PPAR.

This study confirmed that cold stress increased the levels of FIAF mRNA and protein in liver, neck, abdomen and mesenteric fat tissues of pigs, as well as FIAF concentrations in blood. This study also found that FIAF expression levels in liver and fat tissues were related to the intensity of the cold stress, and lower ambient temperatures resulted in higher FIAF expression. The results of this study would be beneficial for deeper understanding of the impact of cold stress on fat metabolism and for studying the neuroendocrine regulation of fat metabolism in pigs.

Acknowledgements

This research was supported by the Natural Science Foundation of Heilongjiang Province (Grant No. C2017051), the Scientific Research Project of Heilongjiang Provincial

Agricultural Reclamation Administration (Grant Number: HKKYZD190308), the National Key R&D Program of China (Grant No. 2016YFD0501210), and the Doctoral Research Startup Fund of Heilongjiang Bayi Agricultural University (Grant No. XDB-2016-09).

Conflicts of Interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Author Contributions

Hong JI designed the experiment. Ying LIU and Ziyi SHAO performed the experiment and analyzed the data. Hong JI and Ziyi SHAO made pictures or tables, and wrote the paper. Chunyang NIU revised the manuscript. All authors reviewed and approved the final manuscript.

References

1. Scharf I, Wertheimer K, Xin JL, Gilad T, Goldenberg I, Subach A: Context-dependent effects of cold stress on behavioral, physiological, and life-history traits of the red flour beetle. *Insect Sci*, 26 (1): 142-153, 2019. DOI: 10.1111/1744-7917.12497

2. Cline DL, Short LI, Forster MAM, Gray SL: Adipose tissue expression of PACAP, VIP, and their receptors in response to cold stress. *J Mol Neurosci,* 68 (3): 427-438, 2019. DOI: 10.1007/s12031-018-1099-x

3. Luo L, Liu M: Adipose tissue in control of metabolism. *J Endocrinol*, 231 (3): R77-R99, 2016. DOI: 10.1530/JOE-16-0211

4. Harwood Jr HJ: The adipocyte as an endocrine organ in the regulation of metabolic homeostasis. *Neuropharmacology*, 63 (1): 57-75, 2012. DOI: 10.1016/j.neuropharm.2011.12.010

5. Yang J, Li X, Xu D: Research progress on the involvement of ANGPTL4 and loss-of-function variants in lipid metabolism and coronary heart disease: Is the „prime time" of ANGPTL4-targeted therapy for coronary heart disease approaching? *Cardiovasc Drugs Ther*, 2020. DOI: 10.1007/s10557-020-07001-0

6. Kersten S, Lichtenstein L, Steenbergen E, Mudde K, Hendriks HFJ, Hesselink MK, Schrauwen P, Müller M: Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids. *Arterioscler Thromb Vasc Biol*, 29 (6): 969-974, 2009. DOI: 10.1161/ATVBAHA.108.182147

7. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, 25 (4): 402-408, 2001. DOI: 10.1006/meth.2001.1262

8. Li SZ, Yang YY, Yang HM, Ni J: Study on comparative-physiology of some endocrinal activities on cold exposure and cold acclimation in rats and chicks. *Chin J Appl Physiol*, 24 (1): 23, 2008. DOI: 10.13459/j.cnki.cjap.2008.01.002

9. Fu SP, Ren BY, Liu Y, Li SN, Li Y, Wang MP, Yang ZQ, Wei LB, Li ZQ, Liu JX, Wang W, Guo WB, Hu ZM: Adiponectin and its receptors mRNA levels in swine adipose tissue in the condition of cold stress. *Chin Anim Husb Vet Med,* 39 (3): 28-32, 2012.

10. Kersten S, Mandard S, Tan NS, Escher P, Metzger D, Chambon P, Gonzalez FJ, Desvergne B, Wahli W: Characterization of the fasting-induced adipose factor FIAF, a novel peroxisome proliferator-activated receptor target gene. *J Biol Chem*, 275 (37): 28488-28493, 2000. DOI: 10.1074/jbc.M004029200

11. Dijk W, Kersten S: Regulation of lipid metabolism by angiopoietin-like proteins. *Curr Opin Lipidol*, 27 (3): 249-256, 2016. DOI: 10.1097/MOL.0000000000000290

12. Desai U, Lee EC, Chung K, Gao CH, Gay J, Key B, Hansen G, Machajewski D, Platt KA, Sands AT, Schneider M, Sligtenhorst IV, Suwanichkul A, Vogel P, Wilganowski N, Wingert J, Zambrowicz BP, Landes G, Powell DR: Lipid-lowering effects of anti-angiopoietin-like 4 antibody recapitulate the lipid phenotype found in angiopoietin-like 4 knockout mice. *Proc Natl Acad Sci U S A,* 104 (28): 11766-11771, 2007. DOI: 10.1073/pnas.0705041104

13. Zhang LP: Study on the relationship of gut microbiota with body fat and ANGPTL4 gene in different breeds of pigs. *Sichuan Agricultural University*, 2007.

14. Gettys TW, Harkness PJ, Watson PM: The beta 3-adrenergic receptor inhibits insulin-stimulated leptin secretion from isolated rat adipocytes. *Endocrinology*, 137 (9): 4054-4057, 1996. DOI: 10.1210/endo.137.9.8756584

15. Delporte ML, Funahashi T, Takahashi M, Matsuzawa Y, Brichard SM: Pre- and post-translational negative effect of beta-adrenoceptor agonists on adiponectin secretion: *In vitro* and *in vivo* studies. *Biochem J,* 367 (3): 677-685, 2002. DOI: 10.1042/BJ20020610

16. Kvetnansky R, Ukropec J, Laukova M, Manz B, Pacak K, Vargovic P: Stress stimulates production of catecholamines in rat adipocytes. *Cell Mol Neurobiol,* 32 (5): 801-813, 2012. DOI: 10.1007/s10571-012-9822-6

17. Liu Y, Ren WZ, Li SN, Li WH, Fu SP, Wu YK, Wang K, Hu ZM: PPARγ2 gene expression of swine adipose tissue liver heart and kidney in cold stress. *Chin J Vet Sci,* 32 (4): 592-597, 2012. DOI: 10.16303/j.cnki.1005-4545.2012.04.022

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

Kafkas Univ Vet Fak Derg
27 (2): 141-149, 2021
DOI: 10.9775/kvfd.2020.24858

RESEARCH ARTICLE

Effects of Different Zinc Sources on Growth Performance, Serum Biochemical Indexes and Zinc Metabolism of Pregnant Goats and Their Offspring

Mengli ZHENG [1,a] Xilin LI [2,b] Qiongxian YAN [2,c (*)]
Chuanshe ZHOU [2,d] Zhiliang TAN [2,e] Peihua ZHANG [1,f (*)]

[1] College of Animal Science and Technology, Hunan Agricultural University, Changsha 410128, CHINA
[2] Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha 410125, CHINA
ORCIDs: [a] 0000-0001-6253-3724; [b] 0000-0002-1184-3551; [c] 0000-0003-0035-6819; [d] 0000-0000-0000-0000; [e] 0000-0002-7483-1478
[f] 0000-0003-1408-122X;

Article ID: KVFD-2020-24858 Received: 17.08.2020 Accepted: 20.01.2021 Published Online: 02.02.2021

Abstract

This study investigated the effects of different zinc (Zn) sources on body weight, body size, serum biochemical indexes and mineral concentrations in Liuyang pregnant goats and their offspring. Twenty-four two-years-old Liuyang Black goats were randomly allocated to three groups and fed a basal corn-soybean meal diet supplemented respectively with Zn-sulfate (ZnS), Zn-methionine (ZnM), Zn-glycinate (ZnG) at 60 mg/kg Zn for the last 52 days of gestation. The weight and chest circumference of kids in the ZnG group were significantly higher than those in the ZnS group. The concentrations of CHOL and HDL in the serum were significantly higher in the ZnG group than that in the ZnM group 10 days before gestation, but CRPL levels were significantly higher in the ZnS group than in other two groups. The serum ALP activity of 60-day old kid-goats was significantly higher than that of 30-day old and 100-day old ones. The concentrations of potassium and Zn in serum of ZnG group were significantly higher than that of ZnS group. These findings revealed that ZnG as dietary supplementation can improve the performance of kid-goats, while ZnG and ZnM can enhance the immunity of pregnant and postpartum goats, and ZnG supplement is better than ZnM.

Keywords: *Capra, Dietary zinc sources, Growth, Pregnancy, Serum biochemical indices, Zinc*

Farklı Çinko Kaynaklarının Gebe Keçi ve Yavrularında Büyüme Performansı, Serum Biyokimyasal İndeksleri ve Çinko Metabolizması Üzerine Etkileri

Öz

Bu çalışmada, farklı çinko (Zn) kaynaklarının Liuyang gebe keçilerinde ve yavrularında vücut ağırlığı, vücut büyüklüğü, serum biyokimyasal indeksleri ve mineral konsantrasyonları üzerine etkileri araştırıldı. Yirmi dört adet iki yaşlı Liuyang Karası keçi rastgele üç gruba ayrıldı ve gruplar gebeliklerinin son 52 gününde, Zn konsantrasyonu 60 mg/kg olacak şekilde hazırlanan sırasıyla Zn-sülfat (ZnS), Zn-metiyonin (ZnM), Zn-glisinat (ZnG) ilaveli bazal mısır-soya küspesi diyeti ile beslendi. ZnG grubundaki keçi yavrularının ağırlıkları ve göğüs çevresi, ZnS grubundakilerden önemli ölçüde daha yüksek saptandı. Serum CHOL ve HDL konsantrasyonları gebelikten 10 gün önce, ZnG grubunda ZnM grubundakilerden anlamlı derecede daha yüksekti, ancak CRPL seviyeleri diğer iki gruba göre ZnS grubunda anlamlı derecede daha yüksekti. 60 günlük oğlakların serum ALP aktivitesi, 30 günlük ve 100 günlük oğlakların serum ALP aktivitesinden anlamlı derecede daha yüksekti. ZnG grubunun serum potasyum ve çinko konsantrasyonları, ZnS grubuna göre anlamlı derecede daha yüksekti. Bu bulgular, besin takviyesi olarak ZnG'nin oğlakların performansını artırabileceğini, ZnG ve ZnM'nin gebe ve doğum sonrası keçilerin bağışıklığını artırabileceğini ve ZnG takviyesinin ZnM'den daha iyi olduğunu ortaya koydu.

Anahtar sözcükler: *Keçi, Besinsel çinko kaynakları, Büyüme, Gebelik, Serum biyokimyasal indeksleri, Çinko metabolizması*

INTRODUCTION

Zinc (Zn) has been demonstrated to be an essential element for ruminants [1,2], and it promotes growth development [3], improves animal's immunity [4] and reproductive performance [5]. The deficiency of Zn is associated with growth

How to cite this article?

Zheng M, Li X, Yan Q, Zhou C, Tan Z, Zhang P: Effects of different zinc sources on growth performance, serum biochemical indexes and zinc metabolism of pregnant goats and their offspring. *Kafkas Univ Vet Fak Derg,* 27 (2): 141-149, 2021.
DOI: 10.9775/kvfd.2020.24858

(*) **Corresponding Author**

Tel: +86-1354 8638664
E-mail: zhang1970@hunau.edu.cn (P. Zhang), yanqx14@isa.ac.cn (Q. Yan)

retardation [6] and immune dysfunction [7]. In animal production, inorganic Zn (in the form of Zn sulfate), organic Zn (methionine chelated Zn, glycine chelated zinc, Zn gluconate, etc.) and nano-Zn are widely used in diet [8,9]. Studies have shown that organic Zn and nano-Zn could improve the growth performance and immune performance of animals [10-13]. Nano-Zn is superior to organic Zn, but its products are costly. Therefore, organic Zn is widely used in dietary supplementation to improve the productive efficiency of farming animals.

The effects of Zn on the growth performance of ruminants were controversial. It was reported that supplementing lambs, goats and calves with Zn improved growth performance and blood antioxidants regardless of the sources [9,14]. Fadayifar et al.[15] and Garg et al.[16] found that Zn supplementation or methionine chelated zinc (ZnM) improved performance of lambs. Mattioli et al.[17] noted that Zn supplementation could improve daily weight gain of pre-weaning calves. Alimohamady et al.[18] also showed that supplementation of Zn in growing lambs improved growth performance. In addition, Salama et al.[19] reported that ZnM reduced the contents of whey protein and non-protein nitrogen, and increased the apparent absorption of nitrogen and the retention rate of Zn in goats. However, Liu et al.[20] found that Zn sources did not affect the body weight gain in male Liaoning Cashmere goats. Therefore, efficacy of Zn supplementation on ruminants growth performance may be affected by animal species and ages, and the sources of Zn.

Studies on the effects of Zn supplementation on serum biochemical indexes in ruminants are scarce. Previous studies showed that supplementation of Zn improved blood antioxidants and thyroid hormone levels in growing lambs and increased serum alkaline phosphatase activity [18,20]. Mandal et al.[21] indicated that bulls supplemented with Zn propionate had higher cell mediated and humoral immune responses, while there was no alteration in immune response by zinc sulfate (ZnS) supplementation when compared with the unsupplemented control. The serum concentrations of urea and triglycerides increased and total cholesterol and high-density lipoprotein (HDL) cholesterol decreased in pre-partum dairy cows supplemented with Zn [22]. Therefore, the effects of Zn sources on serum biochemical indexes are related to animal species and Zn sources.

Zinc maintains homeostasis through Zn circulation in the blood, excretion of feces and urine, and deposition of bones and organs. The serum Zn concentration increased in cows fed organic Zn [9]. However, Solaiman et al.[23] found that serum Zn did not change and stayed relatively stable regardless of dietary Zn levels in growing Boer-cross goat kids. Zn can interact with other mineral elements, so its effect can vary depending on the statuses of other elements. Copper supplementation causes a significant decrease in serum and tissue Zn [24]. Serum concentration of Ca, Cu, Fe, K, P, Mg, Mo, Ps, Pt, Se, and Zn varied according to days of lactation in dairy cows [25].

In pregnant goats, mineral nutrients including Zn can be transferred to kids through the placenta. Therefore, the objective of the current study was to examine the effects of supplementation of different Zn sources on growth performance of pregnant goats, serum biochemical indexes and Zn metabolism of both the mother and offspring.

Material and Methods

Animal Ethics

The experiments were conducted according to the Animal Care and Use Guidelines of the Animal Care Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China.

Animal Management and Dietary Treatments

Oestrus synchronization of a flock of Liuyang Black goats (a local breed) was carried out with the administration of progestogen followed by equine chorionic gonadotropin, and then artificial insemination was performed. The goats underwent ultrasound test at 40 days after the insemination to ensure pregnancy. Pregnant goats were grazed until about 90 days of gestation. Then, goats were selected (with an initial body weight [BW] of 38.1±9.7 kg and similar pregnancy dates [90-100]), transported into the animal house, and individually fed with corn and soybean meal as basic diet (the concentrate) and fresh awn feed.

Miscanthus forage was harvested and cut into small pieces and then mixed with the concentrate before feeding. The diet prepared with reference to the NRC (2007) nutritional requirements of the diets are presented in *Table1*. Goats were divided into three groups with eight goats in each group. Three forms of Zn in the premix were used as the Zn treatments: Zn sulfate (ZnS), Zn methionine chelates (ZnM) and Zn glycine chelates (ZnG), and the dose was controlled at 60 mg/kg Zn dry matter for each animals. ZnS was purchased from Tanke Co. (China); ZnM was purchased from Noves Co. (China) contained 16% Zn; and ZnG was purchased from Tanke Co. (China) contained 29% Zn.

The selected goats were transported into an animal house at about 90 days of pregnancy, kept in individual pens, and fed the basal diet. After two weeks acclimation, three groups of goats were fed the diet supplemented with the different Zn sources from day 105 of gestation until the kidding. Feed consumption was recorded daily, and body weight was recorded at the beginning and end of the experimental period to calculate the body weight change of the goats over the period. The basal diets for both pregnancy goats and their offspring *(Table 1, Table 3)* were prepared to meet the nutrient requirements of pregnant goats in the late gestation period as recommended by NRC

Table 1. *Composition and nutrient levels of the experimental diets (DM basis) **

Item	ZnS	ZnM	ZnG
Ingredients, %			
Miscanthus	40.00	40.00	40.00
Corn	34.66	34.66	34.66
Soybean meal	11.74	11.74	11.74
Fat powder	4.49	4.49	4.49
Soy protein concentrate	5.03	5.03	5.03
$Ca(HCO_3)_2$	0.50	0.50	0.50
$CaCO_3$	0.84	0.84	0.84
Premix[1)]	2.34	2.34	2.34
NaCl	0.40	0.40	0.40
Nutrient levels			
DM	91.79	92.36	92.17
CP	17.76	16.74	18.72
EE	2.50	3.04	3.14
ADF	29.30	27.85	31.24
NDF	45.42	43.78	47.64
Ash	10.47	10.58	10.65
Basic dietary zinc (mg/kg)	22.00	22.00	22.00
The total content of zinc (mg/kg)	82.00	82.00	82.00

** **ZnS,** Zinc-sulfate; **ZnM,** Zinc-methionine; **ZnG,** Zinc-glycinate chalates; **ADF,** Acid detergent fiber; **ADFI,** Average daily feed intake; **ADG,** Average daily gain; **CP,** Crude protein; **DM,** Dry matter; **EE,** Ether extract; **NDF,** Neutral detergent fiber*

(2007). All goats were fed in two equal portions of the diet at 08:00 and 18:00, and had free access to fresh drinking water. Samples of the ration were collected weekly, pooled at the end of the experimental period, and sub-sampled for the determination of nutrients levels.

After giving birth, all the lactating goats were fed the same basal diet without adding Zn to address the carry-over effect of Zn supplements during the pregnancy on the growth and development of the offspring. Lactating goats and their offspring were fed separately, the offspring were only placed in the parent house when they are breastfed. The kids were weaned at 60 days of age. Their birth weights (before suckling), and body weights at 30 days, 60 days and 100 days of age were recorded. At 100 day of age all kids, after overnight fasting, were slaughtered for taking liver samples. The kids did not eat the feed of the goats before weaning and the weaning diet of the kids after weaning.

Sampling and Laboratory Analysis

Blood samples were collected by the jugular vein into tubes with heparin sodium from each of the pregnant goats 10 days before kidding, and the kids at 30 days, 60 days and 100 days of age. Blood samples were kept for 2 h at room temperature and then centrifuged at 3000xg for 10 min at 4ºC to harvest serum for determination of the biochemical indexes. After blood sampling, the kids were weighed, euthanized with over-dosed barbital, and bled. The liver was immediately dissected for taking samples. The liver samplers were stored at -20ºC for mineral elements determination later on.

Goat feces were collected twice a day before feeding (08:00 and 18:00) for 7 days. Subsamples (2% of total weight) of fecal samples were acidified with 10% H_2SO_4 and stored at -20ºC for determination of dry matter and mineral elements. Fecal samples were dried at 65ºC for 48 h and stored in plastic bags until laboratory analysis.

Dietary samples were collected in the trial period, and the contents of dry matter, Ash, crude protein, crude fat and crude fiber were determined after mixing using the procedures of Jackson et al.[26]. In short, the samples of diets and feces were dried at 105ºC overnight and ignited at 550ºC for 6 h for measuring dry matter and Ash. Crude protein content was determined as 6.25xN. The concentration of serum biochemical indexes, including total protein (TP), albumin (ALB), alanine transaminase (ALT), AST, alkaline phosphatase (ALP), blood urea nitrogen (BUN), CREA, glucose (GLU), total glycerides (TG), CHOL, HDL, LDL, C-reactive protein (CRPL3) were determined by automatic biochemical analyzer (Cobas c311 Switzerland). The concentrations of P, Zn, Fe, Cu and K in feed, serum, feces and liver samples were determined by inductively coupled serum spectrometer (Agilent Technologies 5110 ICP-OES, American) as described by Salama et al.[19]. The samples were digested with nitric acid-perchloric acid (4:1) and filtered to volume, and then sampled on ICP-OES.

Statistical Analysis

The data were analyzed by SAS software, version 9.2 of the SAS.1, using the General Linear Model (GLM) procedure. For the pregnant goats, Zn source was considered as a factor. For data from kids, Zn source, age and sex of the kids were included as the factors, and the interactions between Zn source and age and between Zn source and sex were also considered. However, the kid sex was not attribute from the Zn treatment (supplemented during the late stage of gestation), the statistical effect of sex on the measurements are not shown in this paper. The least squares means are presented and Duncan's multiple range tests were used to compare the differences between the means. P values <0.05 are declared as statistically significant.

Results

Effects of different Zn sources on body weight (BW) and body size of the offspring are presented in *Table 2*. The BW of kid goats was affected by Zn sources, with greater BW in the ZnG group than that of the ZnS and ZnM groups ($P<0.05$). BW was also affected by the age ($P<0.01$) and sex ($P<0.01$) of kid goats. The BW was increased from birth to weaning ($P<0.05$), meanwhile BW of female kid goats

Table 2. *Body weight and body size of kid goats**

Item	Treatment	Age				SEM	P Value				
		Birth	d30	d60	d100		Treatment	Age	Sex	Treatment x Age	Treatment x Sex
Weight (kg)	ZnS	1.58	3.90	6.09	6.63	0.32	<0.001	<0.001	0.007	0.837	0.020
	ZnM	2.16	4.08	5.86	6.37	0.40					
	ZnG	2.60	5.08	7.15	7.72	0.35					
Body slanting length (cm)	ZnS	—	28.63	30.85	32.85	0.97	0.015	0.001	0.026	0.916	0.092
	ZnM	—	30.83	31.97	33.54	1.26					
	ZnG	—	31.90	33.83	34.65	1.07					
Height at withers (cm)	ZnS	—	23.42	25.86	29.97	0.82	0.055	<0.001	0.419	0.216	0.519
	ZnM	—	23.80	25.66	27.37	1.05					
	ZnG	—	25.94	28.19	29.19	0.89					
Chest circumference (cm)	ZnS	—	39.64	43.44	45.61	0.97	0.003	<0.001	0.058	0.643	0.175
	ZnM	—	42.48	44.95	47.38	1.25					
	ZnG	—	44.33	46.36	47.34	1.06					

** **ZnS,** Zinc-sulfate; **ZnM,** Zinc-methionine; **ZnG,** Zinc-glycinate chalates*

Table 3. *The blood biochemical indexes of pregnant goats**

Item	ZnS	ZnM	ZnG	SEM	P Value
TP, g/L	65.93	69.74	77.88	4.466	0.274
ALB, g/L	24.32	23.92	26.48	1.828	0.672
ALT, U/L	15.05	19.22	14.90	1.767	0.243
AST, U/L	66.00	81.40	72.50	9.348	0.556
ALP, U/L	61.67	57.00	78.00	12.673	0.828
BUN, mmol/L	6.98	7.68	7.33	0.611	0.749
CREA, μmol/L	48.17	48.00	58.50	3.411	0.145
GLU, mmol/L	1.68	1.01	1.53	0.260	0.286
TG, mmol/L	0.56	0.53	0.54	0.083	0.976
CHOL, mmol/L	3.22	2.79	3.78	0.207	0.041
HDL, mmol/L	1.53	1.28	1.84	0.118	0.043
LDL, mmol/l	1.51	1.29	1.87	0.138	0.072
CRPL3, mg/L	4.02	3.99	3.97	0.007	0.006

** **ZnS,** Zinc-sulfate; **ZnM,** Zinc-methionine; **ZnG,** Zinc-glycinate chalates*

was higher than that of males (P<0.05). And there was an interaction of Zn source and sex on the BW of kid goats, and male BW was higher than that of female under the same zinc source (P<0.05).

At 30 and 60 days of age, body slanting length of the ZnG group was greater than that of the ZnS group (P<0.05), but the difference was not significant at 100 days of age among groups (P>0.05), and the sex did not affect the body slanting length (P>0.05). Height at withers and chest circumference at 100 days were higher than that at 60 days and 30 days, and these two indexes at 60 days were higher than that at 30 days (P<0.05). The chest circumference of the ZnG group was higher than that of the ZnS group, while the difference between the ZnM group and the other two groups was not significant (P>0.05).

As shown in *Table 3*, the serum CHOL concentration of the ZnG group of pregnant goats was higher than that of the ZnM group (P<0.05). Compared to other groups, the concentration of HDL of the ZnG group increased (P<0.05). However, there were no differences between ZnM and ZnS groups (P>0.05). Moreover, the concentration of CRPL3 in ZnG and ZnM groups were higher than that of ZnS group (P<0.05). No significant differences in TP, ALB, ALT, AST, ALP, BUN, CREA, GLU, TG and LDL-C3 concentrations were observed among treatment groups (P>0.05).

The effects of the Zn sources on the serum biochemical indexes of the offspring are shown in *Table 4*. On 30 days, 60 days and 100 days of offspring in this experiment, the activities of ALT in the ZnG group were higher than that of the ZnS group (P<0.05). However, there was no

Table 4. *The blood biochemical indexes of kid goats**

Item	Treatment	Age				SEM	P Value				
		Birth	d30	d60	d100		Treatment	Age	Sex	Treatment × Age	Treatment × Sex
TP, g/L	ZnS	—	67.89	64.85	66.45	4.57	0.127	0.769	0.460	0.389	0.979
	ZnM	—	68.02	78.42	72.73	5.90					
	ZnG	—	80.58	73.69	69.88	5.02					
ALB, g/L	ZnS	—	23.90	24.86	26.19	1.87	0.029	0.293	0.703	0.80	0.648
	ZnM	—	24.39	24.86	29.22	2.41					
	ZnG	—	29.19	30.17	29.72	2.05					
ALT U/L	ZnS	—	7.69	12.86	12.85	1.30	0.058	0.664	0.713	0.003	0.080
	ZnM	—	10.46	12.43	9.28	1.67					
	ZnG	—	16.42	12.09	12.77	1.43					
AST, U/L	ZnS	—	57.67	86.44	92.22	9.57	0.227	0.532	0.918	0.131	0.687
	ZnM	—	56.49	68.06	58.20	12.34					
	ZnG	—	79.26	63.04	68.37	11.72					
ALP, U/L	ZnS	—	161.16	210.82	96.95	36.04	0.325	0.040	0.532	0.157	0.314
	ZnM	—	150.73	110.30	113.08	46.51					
	ZnG	—	138.29	287.50	143.30	39.87					
BUN, mmol/L	ZnS	—	4.60	5.27	10.16	0.84	0.318	0.010	0.232	<0.001	0.568
	ZnM	—	4.88	5.70	5.20	1.09					
	ZnG	—	8.39	4.93	5.43	0.93					
CREA, µmol/L	ZnS	—	39.38	45.27	49.94	4.59	0.010	0.236	0.354	0.849	0.324
	ZnM	—	44.37	52.79	49.51	5.91					
	ZnG	—	56.90	57.13	61.10	5.04					
GLU, mmol/L	ZnS	—	4.01	3.14	2.34	0.45	0.021	0.181	0.843	0.005	0.883
	ZnM	—	5.48	3.26	5.04	0.59					
	ZnG	—	3.07	4.10	3.47	0.50					
TG, mmol/L	ZnS	—	0.62	0.49	0.57	0.08	0.686	0.223	0.233	0.160	0.776
	ZnM	—	0.70	0.53	0.43	0.10					
	ZnG	—	0.58	0.74	0.53	0.08					
CHOL, mmol/L	ZnS	—	4.63	5.80	2.96	0.59	0.882	0.126	0.756	0.029	0.936
	ZnM	—	5.03	4.53	4.72	0.76					
	ZnG	—	3.23	5.19	4.88	0.65					
HDL, mmol/L	ZnS	—	2.12	2.29	1.47	0.23	0.222	0.485	0.770	0.022	0.930
	ZnM	—	2.31	2.08	2.23	0.30					
	ZnG	—	1.80	2.55	2.65	0.25					
LDL, mmol/L	ZnS	—	2.42	3.46	1.26	0.42	0.889	0.081	0.531	0.025	0.954
	ZnM	—	2.67	2.46	2.45	0.55					
	ZnG	—	1.41	2.74	2.69	0.47					
CRPL3, mg/L	ZnS	—	3.99	3.98	3.98	0.01	0.504	0.965	0.547	0.751	0.863
	ZnM	—	3.99	3.99	4.00	0.01					
	ZnG	—	3.99	4.00	3.99	0.01					

** **ZnS,** Zinc-sulfate; **ZnM,** Zinc-methionine; **ZnG,** Zinc-glycinate chalates*

difference in the activity of ALT between ZnM and ZnS groups (P>0.05). Moreover, the concentration of ALP on 60 days was higher than that of the 30 days and the 100 days (P<0.05). The concentration of BUN in ZnG group was higher than ZnS group on 30 days, while ZnG group was lower than that of the ZnS group on 100 days (P<0.05). The levels of BUN on ZnM were higher than ZnS group on 30 days. The concentration of CREA in the ZnG group

Table 5. *Mineral concentrations in the serum of pregnant goats**

Item	ZnS	ZnM	ZnG	SEM	P Value
Cu (mg/kg)	0.17	0.17	0.20	0.017	0.439
Fe (mg/kg)	0.25	0.32	0.15	0.048	0.109
K (mg/kg)	23.63	24.06	26.20	2.729	0.784
P (mg/kg)	15.00	12.88	17.74	2.382	0.407
Zn (mg/kg)	0.20	0.78	0.05	0.286	0.240

* ***ZnS,*** *Zinc-sulfate;* ***ZnM,*** *Zinc-methionine;* ***ZnG,*** *Zinc-glycinate chalates*

Table 6. *Mineral concentrations in the feces of pregnant goats**

Item	ZnS	ZnM	ZnG	SEM	P Value
Cu (mg/kg)	6.33	5.63	6.55	0.557	0.373
Fe (mg/kg)	127.93	94.80	119.97	5.299	0.144
K (mg/kg)	523.23	463.20	543.50	25.436	0.141
P (mg/kg)	812.70 ab	506.77b	1079.52 a	86.638	0.023
Zn (mg/kg)	15.04	15.32	15.60	1.358	0.978

* ***ZnS,*** *Zinc-sulfate;* ***ZnM,*** *Zinc-methionine;* ***ZnG,*** *Zinc-glycinate chalates*

was higher than that of the ZnS group (P<0.05), however, there was no difference between ZnM and ZnS groups (P>0.05). The concentration of GLU in the ZnM group was higher than that of the ZnS group on 30 days and 100 days (P<0.05). The concentrations of HDL and LDL in the ZnG group were higher than that of the ZnS group (P<0.05). However, the serum biochemical indexes of TP, ALB, AST, TG and CRPL3 were not different among these three groups (P>0.05). There was an interactive effect of Zn sources and age on the ALT, BUN, GLU, CHOL, HDL and LDL of offspring (P<0.05), and the concentrations of these indicators at d100 were higher than that of d30 and d60 under the same Zn sources (P<0.05).

The effects of the different Zn sources on the mineral (copper, iron, potassium, phosphorus and Zn) concentrations in the serum and feces of the pregnant goats are shown in *Table 5* and *Table 6*, respectively. The source of Zn had no effect on the concentrations of Zn, Cu, K, P in the serum of the pregnant goats (P>0.05). The concentration of phosphorus in the feces of the ZnG group was higher than that of the ZnM group (P<0.05), but there was no difference between the ZnG and ZnS groups (P>0.05). The source of Zn had no effect on the concentrations of copper, iron, potassium and Zn in the feces of pregnant goats (P>0.05).

The concentrations of copper, iron, potassium, phosphorus and Zn in the offspring serum and livers are shown in *Table 7*. The potassium and Zn concentrations in the serum of ZnG group were higher than that of the ZnS group (P<0.05), but there was no difference between the ZnM and ZnS groups (P>0.05). The concentration of phosphorus on 30 days was higher than that of 100 days (P<0.05), but there was no difference in serum phosphorus concentration between 60 days and 100 days (P>0.05). The concentration of iron in male goats was higher than that in female goats. There was an interaction of Zn source and age on the serum copper concentration of offspring (P<0.05). In ZnS and ZnM groups, the serum copper content of 30-day goats was significantly higher than that of 60 and 100-day, while in ZnG group, the serum copper content of 100-day goats was significantly higher than that of 30 and 60-day. The concentration of phosphorus in the liver of the female kid goats was higher than that of the males at 100 days (P<0.05), but other mineral elements were not changed by treatment, age and sex of kid goats (P>0.05).

Discussion

The aim of this experiment was to compare the different Zn sources on the productive performances, blood biochemical indexes and mineral concentrations in pregnant goats and their offspring, with a particular focus on the carry-over effects of the offspring. The umbilical cord and placenta are important tissues connecting the mother and the fetus. Nutrients digested and absorbed by the pregnant goats can be transferred to the fetus through the placenta, so theoretically, Zn supplemented to pregnant goats can be transferred to their fetuses to maintain the Zn status. However, the Zn utilization efficiency does differ among Zn sources, which can affect the Zn status of the offspring. This was supported by significant differences in the Zn concentration in the kids aged 30 days, where ZnG and ZnM had the high serum Zn concentration in the present study. Previous studies have shown that Zn sources, especially organic Zn, can increase daily weight gain of ruminants [6,20]. Our previous results also shown that the total and average daily weight gain of pregnant goats were increased by supplementing organic Zn at 42 days before delivery. In contrast, Niu et al.[27], and Mandal et al.[28], found that there were no significant differences

Table 7. *Mineral concentrations (mg/kg) in the serum and liver of kid goats**

Item	Treatment	Age				SEM	P Value				
		Birth	d30	d60	d100		Treatment	Age	Sex	Treatment x Age	Treatment x Sex
Serum											
Cu	ZnS	—	0.17	0.14	0.15	0.021	0.359	0.200	0.942	0.026	0.909
	ZnM	—	0.21	0.14	0.18	0.020					
	ZnG	—	0.11	0.15	0.18	0.020					
Fe	ZnS	—	0.17	0.19	0.23	0.088	0.148	0.911	0.027	0.301	0.325
	ZnM	—	0.28	0.13	0.34	0.085					
	ZnG	—	0.35	0.50	0.31	0.093					
K	ZnS	—	21.54	17.83	15.45	2.709	0.029	0.619	0.332	0.264	0.472
	ZnM	—	22.68	19.61	22.40	2.746					
	ZnG	—	24.72	30.77	25.40	2.833					
P	ZnS	—	21.01	20.55	14.38	2.010	0.279	0.027	0.163	0.580	0.473
	ZnM	—	19.66	16.40	17.10	2.038					
	ZnG	—	22.67	21.85	19.00	2.102					
Zn	ZnS	—	0.14	0.23	0.23	0.102	0.002	0.367	0.987	0.460	0.485
	ZnM	—	0.32	0.17	0.09	0.120					
	ZnG	—	0.63	0.73	0.45	0.102					
Liver											
Cu	ZnS	—	—	—	10.49	1.475	0.186	-	0.557	-	0.209
	ZnM	—	—	—	5.90	1.904					
	ZnG	—	—	—	9.85	1.971					
Fe	ZnS	—	—	—	6.08	0.574	0.554	-	0.232	-	0.261
	ZnM	—	—	—	6.18	0.741					
	ZnG	—	—	—	5.13	0.767					
K	ZnS	—	—	—	297.52	8.769	0.774	-	0.338	-	0.605
	ZnM	—	—	—	288.26	11.320					
	ZnG	—	—	—	289.66	11.718					
P	ZnS	—	—	—	370.45	7.475	0.470	-	0.045	-	0.594
	ZnM	—	—	—	376.64	9.650					
	ZnG	—	—	—	386.22	9.988					
Zn	ZnS	—	—	—	5.25	1.072	0.344	-	0.882	-	0.689
	ZnM	—	—	—	7.60	1.403					
	ZnG	—	—	—	4.81	1.479					

** **ZnS,** Zinc-sulfate; **ZnM,** Zinc-methionine; **ZnG,** Zinc-glycinate chalates*

on average daily gain weight, average daily feed intake of growing-finishing pigs with different Zn sources.

Blood indices were used to evaluate the physiological, nutritional and pathological status of livestock. Serum TP and ALB concentrations are indicators of ruminant protein intake, and inadequate dietary protein intake leads to a decrease in TP and ALB [29]. Our results showed that ALB concentration increased in the blood of goats at the age of weaning may be due to the change from breast-feeding to feeding miscanthus and feeds, which increased the protein intake of goats. The main function of HDL was to transport excess cholesterol from the extra hepatic tissues to the liver for metabolism, so as to prevent cholesterol accumulation in these tissues. And we found that the blood total cholesterol and HDL levels of pregnant goats increased in ZnG group, and the blood contents of high density and low density lipoprotein were also increased in kid goats. The reason is that goats feeding organic Zn increased maternal or energy intake, which is conducive to the increase of body weight and body measurements. Urea is a good indicator of energy or protein imbalance and a sensitive indicator of protein utilization efficiency [30]. CRPL3 is an important inflammatory marker. It is produced in the liver under the

transcriptional control of interleukin-6. CRP is produced in response to inflammation, infection, and tissue damage. It is produced in the liver and under the transcriptional control of interleukin-6 CRP. The concentrations of ALT and creatinine in blood of glycine and Zn goats increased, which indicated that liver and kidney function of goats were enhanced. Conversely, El-Hack [31] established that Zn supplementation could significantly affect serum triglyceride, total cholesterol, and LDL cholesterol (low-density lipoprotein), while HDL is not affected. Consistent with our results, Váradyová [32] demonstrated that serum concentration of CRP was significantly higher in ZnS group compared with ZnG group.

Animal body's digestion and metabolism of Zn affects the other mineral elements of digestion and metabolism of maternal intakes. Zinc can be passed to the offspring through the placenta and breast milk. Replacing ZnM with ZnS increased the concentration of potassium and zinc in the blood and liver of offspring. Pal et al.[33] found that goats fed ZnM increased the concentration of Zn in liver, which was inconsistent with our results. Váradyová et al.[32], also found that the serum concentration of Zn was higher fed a diet supplemented with organic Zn. We also found that the iron concentration in male goats was higher than that in female. The concentration of phosphorus in liver of female kid goats was higher than that of male kid goats at the 100th day, but other mineral elements did not change in treatment age and gender. In conclusion, the digestion and metabolism of Zn affect the changes of phosphorus, Zn, potassium and iron in the maternal body and its offspring.

The findings in the present study indicated that using ZnM and ZnG supplements in diets could improve the performance, and serum biochemical indexes in pregnant goats and the offspring. Moreover, based on the results of this trial, we recommend a dietary supplementation of 60 mg/kg Zn from ZnM and ZnG as practical nutrition for pregnant goats. ZnM and ZnG can be used as suitable sources Zn to replace inorganic Zn in diet to improve the production performance and immune function of gestation goats. In a nutshell, ZnG supplement is better than ZnM in this experiment.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was jointly supported the National Key Research and Development Project (2018YFD0501604), Science and Technology Major Project of Hunan Province, China (2017NK1020), the National Natural Science Foundation of China (2018YFD0501604, 31730092), Youth Innovation Team Project of ISA, CAS (2017QNCXTD_ZCS). The authors declare that they have no conflicts of interests.

Author Contributions

Peihua ZHANG designed the study**.** Mengli ZHENG drafted and wrote the manuscript. Xilin LI collected and analyzed the data. Mengli ZHENG and Xilin LI performed the animal trial and laboratory analysis. Qiongxian YAN, Chuanshe ZHOU and Zhiliang TAN revised the manuscript. All authors gave intellectual input to the study and approved the final version of the manuscript.

References

1. Maares M, Haase H: Zinc and immunity: An essential interrelation. *Arch Biochem Biophys,* 611, 58-65, 2016. DOI: 10.1016/j.abb.2016.03.022

2. Baltaci AK, Yuce K, Mogulkoc R: Zinc metabolism and metallothioneins. *Biol Trace Elem Res,* 183, 22-31, 2018. DOI: 10.1007/s12011-017-1119-7

3. Ianni A, Innosa D, Martino C, Grotta L, Bennato F, Martino G: Zinc supplementation of Friesian cows: Effect on chemical-nutritional composition and aromatic profile of dairy products. *J Dairy Sci*, 102, 2918-2927, 2019. DOI: 10.3168/jds.2018-15868

4. Swain P, Das R, Das A, Padhi SK, Das KC: Effects of dietary zinc oxide and selenium nanoparticles on growth performance, immune responses and enzyme activity in rohu, *Labeo rohita* (Hamilton). *Aquac Nutr*, 25, 486-494. 2019. DOI: 10.1111/anu.12874

5. Hemalatha K, Arangasamy A, Selvaraju S, Krishnaiah MV, Rani GP, Mishra A, Soren NM, Reddy IJ, Ravindra JP: Effect of dietary supplementation of organic zinc and copper on *in vitro* semen fertility in goat. *Small Ruminant Res,* 161, 68-72, 2018. DOI: 10.1016/j.smallrumres.2018.02.003

6. Jia W, Jia Z, Zhang W, Wang R, Zhang S, Zhu X: Effects of dietary zinc on performance, nutrient digestibility and serum zinc status in Cashmere goats. *Small Ruminant Res,* 80, 68-72, 2008. DOI: 10.1016/j.smallrumres.2008.09.009

7. Hirano T, Murakami M, Fukada T, Nishida K, Yamasaki S, Suzuki T: Roles of zinc and zinc signaling in immunity: Zinc as an intracellular signaling molecule. *Adv Immunol,* 97, 149-176, 2008. DOI: 10.1016/S0065-2776(08)00003-5

8. Wrightt CL, Spears JW: Effect of zinc source and dietary level on zinc metabolism in Holstein calves. *J Dairy Sci,* 87, 1085-1091, 2004. DOI: 10.3168/jds.S0022-0302(04)73254-3

9. Sobhanirad S, Naserian AA: Effects of high dietary zinc concentration and zinc sources on hematology and biochemistry of blood serum in Holstein dairy cows. *Anim Feed Sci Technol,* 177, 242-246, 2012. DOI: 10.1016/j.anifeedsci.2012.06.007

10. Narasimhaiah M, Arunachalam A, Sellappan S, Mayasula V, Guvvala PR, Ghosh SK, Chandra V, Ghosh J, Kumar H: Organic zinc and copper supplementation on antioxidant protective mechanism and their correlation with sperm functional characteristics in goats. *Reprod Domest Anim,* 53, 644-654, 2018. DOI: 10.1111/rda.13154

11. Spears JW: Zinc methionine for ruminants: Relative bioavailability of zinc in lambs and effects of growth and performance of growing heifers. *J Anim Sci,* 67, 835-843, 1989. DOI: 10.2527/jas1989.673835x

12. Spears JW, Harvey RW, Brown TT: Effects of zinc methionine and zinc oxide on performance, blood characteristics, and antibody titer response to viral vaccination in stressed feeder calves. *J Am Vet Med Assoc,* 199, 1731-1733, 1991

13. Swain PS, Rao SBN, Rajendran D, Dominic G, Selvaraju S: Nano zinc, an alternative to conventional zinc as animal feed supplement: A review. *Anim Nutr,* 2, 134-141, 2016. DOI:10.1016/j.aninu.2016.06.003

14. Berrie RA, Hallford DM, Galyean ML: Effects of zinc source and level on performance and metabolic hormone concentrations of growing and finishing lambs. *Prof Anim Scientist,* 11, 149-156, 1995. DOI: 10.15232/S1080-7446(15)32579-1

15. Fadayifar A, Aliarabi H, Tabatabaei MM, Zamani P, Bahari A, Malecki M, Dezfoulian AH: Improvement in lamb performance on barley based

diet supplemented with zinc. *Livest Sci,* 144, 285-289, 2012. DOI: 10.1016/j.livsci.2011.12.002

16. Garg AK, Mudgal V, Dass RS: Effect of organic zinc supplementation on growth, nutrient utilization and mineral profile in lambs. *Anim Feed Sci Technol,* 144, 82-96, 2008. DOI: 10.1016/j.anifeedsci.2007.10.003

17. Mattioli GA, Rosa DE, Turic E, Testa JA, Lizarraga RM, Fazzio LE: Effect of injectable copper and zinc supplementation on weight, hematological parameters, and immune response in pre-weaning beef calves. *Biol Trace Elem Res*, 189, 456-462, 2019. DOI: 10.1007/s12011-018-1493-9

18. Alimohamady R, Aliarabi H, Bruckmaier RM, Christensen RG: Effect of different sources of supplemental zinc on performance, nutrient digestibility, and antioxidant enzyme activities in lambs. *Biol Trace Elem Res,* 189, 75-84, 2019. DOI: 10.1007/s12011-018-1448-1

19. Salama AA, Caja G, Albanell E, Such X, Casals R, Plaixats J: Effects of dietary supplements of zinc-methionine on milk production, udder health and zinc metabolism in dairy goats. *J Dairy Res,* 70, 9-17, 2003. DOI: 10.1017/s0022029902005708

20. Liu HY, Sun MH, Yang GQ, Jia CL, Zhang M, Zhu YJ, Zhang Y: Influence of different dietary zinc levels on cashmere growth, serum testosterone level and zinc status in male Liaoning Cashmere goats. *J Anim Physiol Anim Nutr,* 99, 880-886, 2015. DOI: 10.1111/jpn.12292

21. Mandal GP, Dass RS, Isore DP, Garg AK, Ram GC: Effect of zinc supplementation from two sources on growth, nutrient utilization and immune response in male crossbred cattle *(Bos indicus×Bos taurus)* bulls. *Anim Feed Sci Technol,* 138, 1-12, 2007. DOI: 10.1016/j.anifeedsci.2006.09.014

22. Chandra G, Aggarwal A, Kumar M, Singh AK: Effect of zinc and vitamin E supplementation on hormones and blood biochemicals in peri-partum Sahiwal cows. *J Trace Elem Med Biol,* 50, 489-497, 2018. DOI: 10.1016/j.jtemb.2018.02.015

23. Solaiman SG, Min BR: The effect of high levels of dietary zinc on growth performance, carcass characteristics, blood parameters, immune response and tissue minerals in growing Boer-cross goat kids. *Small Ruminant Res,*177, 167-174,2019. DOI: 10.1016/j.smallrumres.2019.05.018

24. Derouiche S, Kechrid Z: Zinc supplementation overcomes effects of copper on zinc status, carbohydrate metabolism and some enzyme activities in diabetic and nondiabetic rats. *Can J Diabetes,* 40, 342-347, 2016. DOI: 10.1016/j.jcjd.2016.02.005

25. Bicalho MLS, Lima FS, Ganda EK, Foditsch C, Meira EBS, Machado VS, Teixeira AVG, Oikonomou G, Gilbert RO, Bicalho RC: Effect of trace mineral supplementation on selected minerals, energy metabolites, oxidative stress, and immune parameters and its association with uterine diseases in dairy cattle. *J Dairy Sci,* 97, 4281-4295, 2014. DOI: 10.3168/jds.2013-7832

26. Jackson MA, Readman RJ, Huntington JA, Sinclair LA: The effects of processing at harvest and cutting height of urea-treated whole-crop wheat on performance and digestibility in dairy cows. *Anim Sci,* 78, 467-476, 2004. DOI: 10.1017/S1357729800058860

27. Niu X, Yang W, Huang L, Zhang C, Feng X, Sun J: Effects of different zinc sources and levels on serum enzyme activities, tissue and organ zinc accumulations of growing-finishing pigs. *Chin J Anim Nutr*, 30, 910-917, 2018. DOI: 10.3969/j.issn.1006-267x.2018.03.014

28. Mandal GP, Dass RS, Garg AK, Varshney VP, Mondal AB: Effect of zinc supplementation from inorganic and organic sources on growth and blood biochemical profile in crossbred calves. *J Anim Feed Sci,* 17, 147-156, 2008.

29. Parnian Khajehdizaj F, Taghizadeh A, Baghbanzadeh Nobari B: Effect of feeding microwave irradiated sorghum grain on nutrient utilization, rumen fermentation and serum metabolites in sheep. *Livest Sci,* 167, 161-170, 2014. DOI: 10.1016/j.livsci.2014.06.004

30. Kenny DA, Humpherson PG, Leese HJ, Morris DG, Tomos AD, Diskin MG, Sreenan JM: Effect of elevated systemic concentrations of ammonia and urea on the metabolite and ionic composition of oviductal fluid in cattle. *Biol Reprod,* 66, 1797-1804, 2002. DOI: 10.1095/biolreprod66.6.1797

31. El-Hack MEA, Alagawany M, Salah AS, Abdel-Latif MA, Farghly MFA: Effects of dietary supplementation of zinc oxide and zinc methionine on layer performance, egg quality, and blood serum indices. *Biol Trace Elem Res,* 184, 456-462, 2018. DOI: 10.1007/s12011-017-1190-0

32. Váradyová Z, Mravčáková D, Holodová M, Grešáková L, Pisarčíková J, Barszcz M, Taciak M, Tuśnio A, Kišidayová S, Čobanová K: Modulation of ruminal and intestinal fermentation by medicinal plants and zinc from different sources. *J Anim Physiol Anim Nutr,* 102, 1131-1145, 2018. DOI: 10.1111/jpn.12940

33. Pal DT, Gowda NKS, Prasad CS, Amarnath R, Bharadwaj U, Babu GS, Sampath KT: Effect of copper- and zinc-methionine supplementation on bioavailability, mineral status and tissue concentrations of copper and zinc in ewes. *J Trace Elem Med Biol,* 24, 89-94, 2010. DOI: 10.1016/j.jtemb.2009.11.007

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

Kafkas Univ Vet Fak Derg
27 (2): 151-158, 2021
DOI: 10.9775/kvfd.2020.24902

RESEARCH ARTICLE

OVGP1 Expression in BOEC and Oviduct: An Immunohistochemical and Immunocytochemical Study [1][2]

Aytül KÜRÜM [1,a (*)] Hakan KOCAMIŞ [1,b] Siyami KARAHAN [1,c] Mustafa TÜRK [2,d] Yasin ÖZKABADAYI [1,e]

[1] *This study was supported by Kirikkale University Scientific Research Coordination Unit (Project no. 2015/053)*
[2] *A Part of some datas of this study was presented in the Mediterranean Veterinary Congress coupled with 7th REEV-Med General Assembly, 13-14 December 2018 as an oral presentation and reletad abstract was published in the abstract book of the Mediterranean Veterinary Congress coupled with 7th REEV-Med General Assembly, Kırıkkale - Turkey*

[1] Kırıkkale University, Faculty of Veterinary Medicine, Department of Histology and Embryology, TR-71450 Yahsihan, Kirikkale - TURKEY
[2] Kırıkkale University, Faculty of Engineering, Department of Bioengineering, TR-71450 Yahsihan, Kirikkale - TURKEY
ORCIDs: [a] 0000-0002-0170-0231; [b] 0000-0001-5202-3229; [c] 0000-0002-2744-1717; [d] 0000-0001-8202-090X; [e] 0000-0003-0326-3407

Article ID: KVFD-2020-24902 Received: 27.08.2020 Accepted: 02.01.2021 Published Online: 03.01.2021

Abstract

Oviduct is an important tubular organ fostering critical physiological processes such as transport of gametes and embryos, capacitation, fertilization, early embryo development, and maturation of gametes. The aim of the present study was to evaluate oviduct specific glycoprotein-1 (OVGP1) expression in the oviduct regions at different phases of the sexual cycle and bovine oviduct epithelial cells (BOEC). In the study, oviduct samples collected from 6 cows in estral and luteal phases were used. The oviduct samples were collected from the ampulla, isthmus and fimbria and evaluated through routine histology and immunohistochemical studies for OVGP1. The primary BOEC were obtained from the ampulla region and characterized by cytokeratin expression. The immunohistochemistry assay indicated that OVGP1 is expressed in secretory cells of the bovine oviduct. OVGP1 expression varies by the oviduct regions and phases of the sexual cycle. Changes in OVGP1 expression during the sexual cycle suggestively indicates a hormonal influence. Regional difference in OVGP1 expression is most likely related to the physiological events that occur in different regions of the oviduct. BOEC isolated from the oviduct of estral and luteal phases also expresses OVGP1. Further studies should focus on possible role of OVGP1 in adaption of BOEC to very tedious condition like cell culture.

Keywords: *BOEC, OVGP1, Oviduct, Cow, Sexual cycle*

BOEC ve Ovidukt'ta OVGP1 Ekspresyonu: İmmunohistokimyasal ve İmmunositokimyasal Çalışma

Öz

Ovidukt, gametlerin olgunlaşması, erken dönemde embriyo gelişimi fertilizasyon, kapasitasyon embriyo ve gametlerin taşınması gibi kritik fizyolojik süreçleri destekleyen önemli bir tubuler organdır. Bu çalışmanın amacı, seksüel siklusun farklı evrelerinde oviduktun bölgelerinde ve sığır ovidukt epitel hücrelerinde (BOEC) ovidukta özgü glikoprotein-1 (OVGP1) ekspresyonunu değerlendirmektir. Bu çalışmada östral ve luteal dönemdeki 6'şar inekten alınan ovidukt örnekleri kullanıldı. Oviduktun ampulla, istmus ve fimbriya bölgelerinden alınan örneklerde, OVGP1 immünohistokimyasal çalışmalarla değerlendirildi. BOEC ampulla bölgesinden elde edildi ve sitokeratin ekspresyonu ile karakterize edildi. İmmünohistokimya ile, OVGP1'in sığır oviduktunda özellikle sekretorik hücrelerde bulunduğu gösterildi. OVGP1 ekspresyonu, oviduktun bölgelerine ve seksüel siklusun dönemlerine göre değişmektedir. Seksüel siklus sırasında OVGP1 ekspresyonundaki değişiklikler muhtemel bir hormonal bir etkiyi düşündürür. OVGP1 ekspresyonundaki bölgesel farklılık, büyük olasılıkla yumurta kanalının farklı bölgelerinde meydana gelen fizyolojik olaylarla ilgilidir. Estral ve luteal dönemlerde oviduktan izole edilen BOEC'te OVGP1'i ekspresse etmektedir. Bundan sonraki çalışmalar, OVGP1'in BOEC'in zorlu hücre kültürü şartlarına, adaptasyonundaki rolüne odaklanmalıdır.

Anahtar sözcükler: *BOEC, OVGP1, Oviduk, Sığır, Seksüel siklus*

How to cite this article?

Kürüm A, Kocamış H, Karahan S, Türk M, Özkabadayı Y: OVGP1 expression in BOEC and oviduct: An immunohistochemical and immunocytochemical study. *Kafkas Univ Vet Fak Derg,* 27 (2): 151-158, 2021.
DOI: 10.9775/kvfd.2020.24902

(*) Corresponding Author

Tel: +90 318 357 3301/6381 Fax: +90 318 357 3304
E-mail: aytululum@hotmail.com (A. Kürüm)

Introduction

Oviduct is a tubular organ and known as the fertilization site, which provides optimal conditions for sperm capacitation, fertilization and early embryonic development as a consequence connection between oocyte and spermatozoon coming in opposite directions [1]. Oviduct is divided into three parts: fimbria (infundibulum), ampulla and isthmus [2,3]. The oviduct epithelial lining consists of ciliated and secretory cells, the proportions and morphologies of which change during the sexual cycle [4,5]. Following cell culturing, the morphological properties of these cells are defined electron microscopically and immunohistochemically [6]. Secretory activities of the secretory cells are important for gametes and embryos. It is also reported that the secretory modalities of these cells differ by the phases of the cycle [7]. The oviduct is under the influence of ovarian hormones (estrogen and progesterone). Estrogen is undoubtedly active in the differentiation of the epithelium during the follicular (estral) phase and the addition of various macromolecules to the development and secretion of secretory cells [8]. Impairment of oviduct function or deficiency may result in infertility or develop conditions such as ectopic pregnancy with vital consequences [9].

Oviduct-specific glycoproteins have been reported to have positive effects on sperm capacitation, sperm-ovum binding, ovum penetration and embryo development *in vitro* [10]. It has been suggested that they contribute to sperm viability and motility as well as sperm capacitation [11]. Oviduct-specific glycoproteins have been reported to increase in the estral phase [8-12]. One of them is oviduct glycoprotein 1 (OVGP1). OVGP1, also known as oviductin in some species, is a high molecular weight protein similar to chitinase. Synthesized in secretory cells of the oviduct during estrous cycle [13], OVGP1 has been reported to have positive effects on sperm capacitation, ovum-sperm binding, sperm penetration to the ovum [14] and prevention of polyspermia [15] and early embryonic development [14]. In a study conducted in buffaloes, both recombinant and natural buffalo OVGP1 had a significant effect on sperm characteristics and *in vitro* embryo development [16]. It has been proposed that as an embryotrophic protein OVGP1 causes molecular changes in the zona pellucida of oocyte, especially during fertilization [15]. Oviduct-specific glycoprotein 1 is reported to increase monospermia in pigs in addition to its embryotrophic activities [17,18].

In vitro fertilization and embryo production have been an important tool in determining the effects of oviduct secretions in the presence of gametes, fertilization and embryo development [10]. Early embryonic deaths is one of the well-known fertility problems especially in cattle that causes significant economic losses [19]. Assisted reproductive techniques have provided a very useful tool for studying early embryonic development. Examining the relationship between the mother and the embryo or gametes is very valuable in terms of fertilization and early embryonic deaths. However, *in vivo* studies are particularly expensive in cattle [20]. In this regard, *in vitro* systems represent an alternative tool for explaining *in vivo* pathways and mechanisms, especially in farm animals [21]. The low success rate in the production of bovine embryos using *in vitro* techniques has led researchers to examine *in vitro* conditions in more detail. Various somatic cells were used as co-cultures in bovine embryos. Bovine oviduct epithelial cells (BOEC) are the most popular choice among them [22]. Thus, there is a continuous effort to characterize BOEC.

In this study, we aimed to investigate the OVGP1 expression in different parts of the oviduct tissues during estral and luteal phases as well as in BOEC.

Material and Methods

BOEC Isolation

For BOEC cultures, the oviduct samples were collected from 6 estral and 6 luteal cows slaughtered in a regional slaughterhouse. To determine the phase of the sexual cycle in a slaughtered animal, both ovaries were macro-scopically examined [23]. The ampulla region of the oviduct was separated and brought to the laboratory in sterile conditions in a transport solution, DMEM (Dulbecco's Modified Eagle Medium) medium containing penicillin-streptomycin and Fetal Bovine Serum (FBS). In a laminar flow cabin, the ampulla region was cleaned from the surrounding tissues, the oviduct lumen was cut open, and then the epithelial tissue was scraped mechanically into a flask. The scrapped epithelial tissue was gently homogenized in DMEM containing 10% FBS, 1% Penicillin-Streptomycin and transferred into sterile chambered coverslip with 6-wells and then incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2-3 days. Cell cultures exhibiting a 90-95% confluence were used for immunocytochemistry. Furthermore, BOEC were characterized by cytokeratin expression based on their epithelial origin [24].

Immunocytochemistry for Cytokeratin-5 and OVGP-1 in BOEC

Cytokeratin-5 and OVGP1 immunocytochemistry procedures were conducted using the routine immunocytochemistry. The cells on the cell culture coverslip were fixed in 4% paraformaldehyde for 30 min. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide solution prepared in methanol. Upon treating cells with normal goat serum to prevent nonspecific binding, either a monoclonal cytokeratin-5 antiserum (ab194135 at a dilution of 1/200) or a monoclonal anti-OVGP1 antibody (ab118590, 1 µg/mL) were applied to cells and then treated with a horse radish peroxidase (HRP) labeled secondary antibody (Thermo Scientific TP-125-HL) followed by AEC

chromogen (Thermo Scientific TA-125-HA) incubation, and then covered with water-based adhesive (Abcam Mounting Medium ab64230).

OVGP1 Immunohistochemistry in Oviduct Tissues

The bovine oviduct samples were collected during slaughter. The oviducts were then separated into three parts: ampulla, isthmus and fimbria and processed for routine immunohistochemistry. For antigen retrieval, sections were incubated in citrate buffer for 4 times with an interval of 5 min at 700-800 W in a microwave oven. Sections were placed in 0.3% triton-X in PBS for permeabilization for 15 min. Endogenous peroxidase activity was quenched in 3% H_2O_2 for 20 min. Following blocking solution steps, sections were incubated with the primary antibody (anti-OVGP1 antibody ab118590, 5 µg/mL) for an hour, biotinylated secondary antibody (Thermo Scientific TP-125-HL) and streptavidin peroxidase solution for 30 min each at room temperature. Sections were then treated with chromogen (Thermo Scientific TA-125-HA), counterstained with Gill's hematoxylin for 20 sec, washed in tap water and then coverslipped using a water based mounting solution (Abcam Mounting Medium ab64230). Sections were examined using a light microscope (Nicon Eclipsi 50i, Tokyo, JAPAN). The intensity and localization of OVGP1 expressions were assessed with a semiquantitative scoring system: (-) negative, (+) weak, (++) moderate, (+++) strong [25].

Results

Immunocytochemistry for Cytokeratin-5 and OVGP-1 in BOEC OVGP-1 Immunohistochemistry in Oviduct Tissues

The cultured BOEC cells were cytokeratin immunopositive to a significant degree in both estral and luteal phases *(Fig. 1-A,B)*. The OVGP1 expression was observed in some BOEC cells in both phases of the sexual cycle *(Fig. 2-A,B)*.

Immunohistochemistry for OVGP1

In oviduct samples, OVGP1 immunoreactivity was observed in the epithelial lining during both phases of sexual cycle. It has been determined that OVGP1 immunoreactivity was limited to the secretory cells as ciliated cells expressed no immunoreactivity. The immunoreactivity was observed especially in the fimbria and ampulla of the oviduct. In samples of the luteal phase, the immunoreactivity was common in the cytoplasm of secretory cells in all oviduct regions *(Fig. 3-A,B,C,D,E)*, except in the isthmus *(Fig. 3-F)*. The cytoplasmic immunoreactivity was especially seen at the apical site of the cells and there even were immuno-

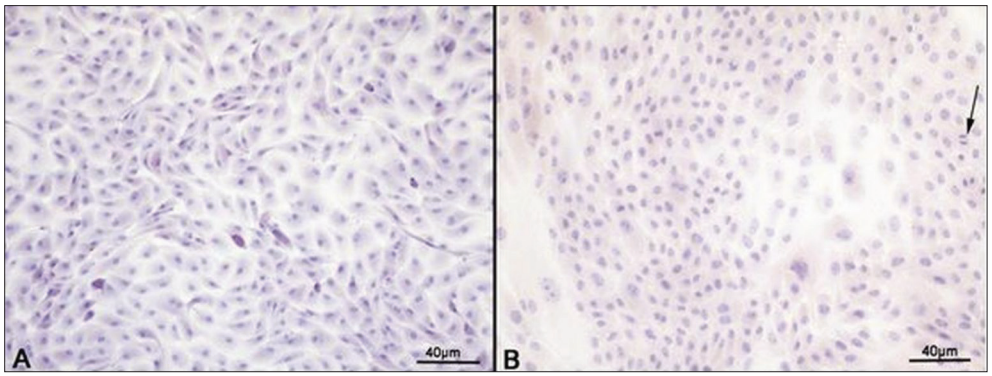


Fig 1. Cytokeratin immunohistochemistry in BOEC cell culture. Cytokeratin immunoreactivity was observed in both estral **(A)** and luteal **(B)** phases. Immunoperoxidase staining, hematoxylin counterstaining

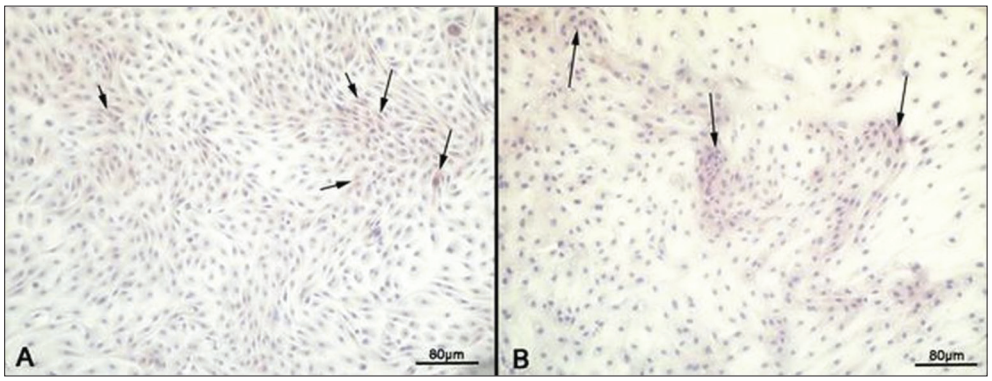


Fig 2. OVGP-1 immunocytochemistry in BOEC. The OVGP-1 immunoreactivity *(arrows)* was seen in some cells of both the luteal **(A)** and estral **(B)** phases of the sexual cycle. Immunoperoxidase staining, hematoxylin counter staining

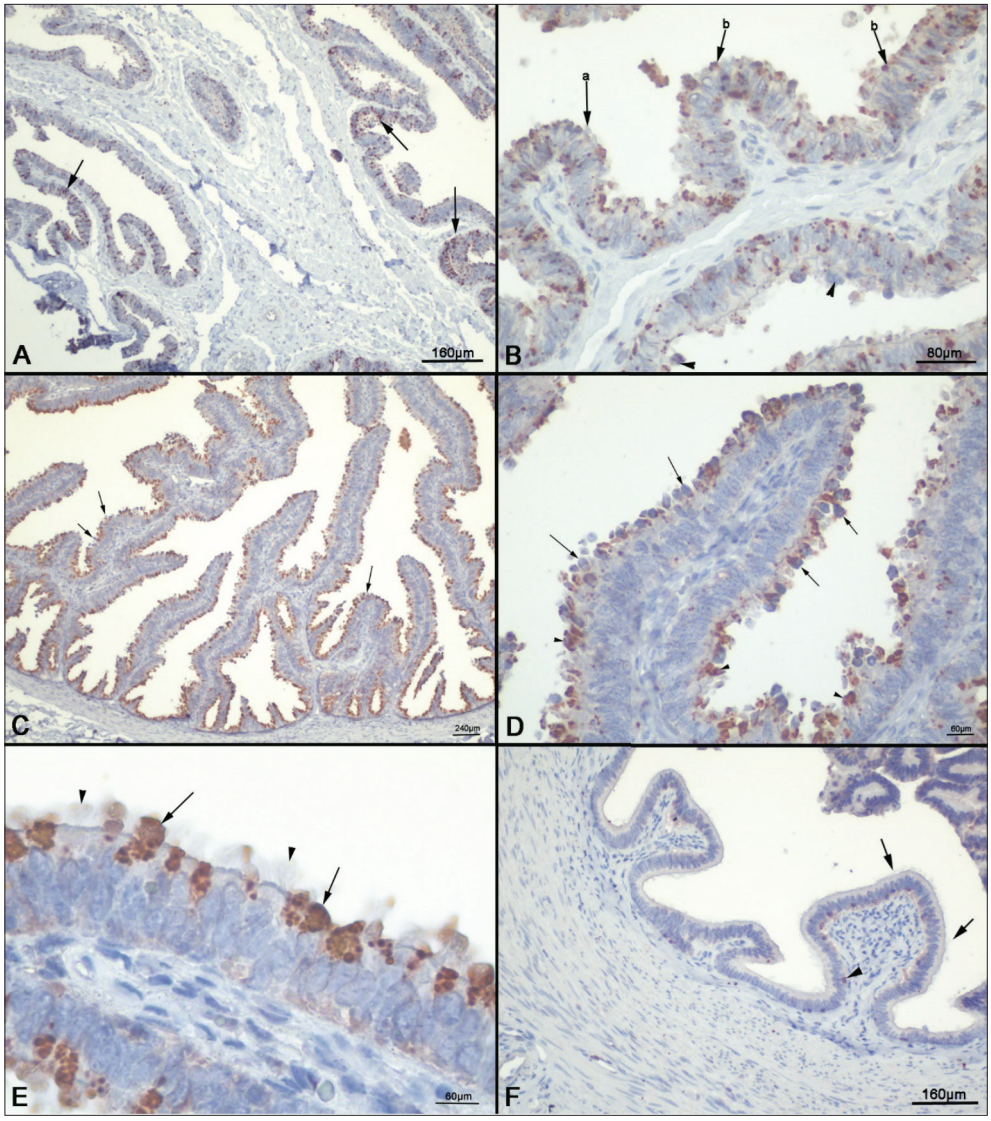


Fig 3. OVGP1 immunoreactivity in the fimbria, ampulla and isthmus regions of the bovine oviduct at the luteal phase. **A)** In the fimbria, immunoreactivity *(arrow)* was present in the epithelial lining, **B)** Ciliated cells are immunonegative (a) while secretory cells are immune positive (b). Nuclear extrusion *(arrowheads)* can also be seen. **C)** Immunoreactivity *(arrows)* is also present in the ampulla in a similar manner, **D)** In the ampulla at the late luteal phase, the immunoreactivity (arrowheads) is localized in the apical sites of the secretory cells, characterized by numerous nucleus extrusions *(arrows),* **E)** Immunoreactive secretory cells *(arrows)* can be seen, **F)** Immunoreactivity was usually absent in the epithelial lining (arrows) of the isthmus; however, it can rarely be seen in a very limited number of cells. Immunoperoxidase staining and hematoxylin counterstain

reactive secretory protrusions towards the lumen along with the nucleus *(Fig. 3-E)*. In the samples of the estral phase, OVGP-1 immunoreactivity distribution was similar to those the luteal phase *(Fig. 4-A,B,C,D,E)*; however, there was a limited immunoreactivity in a very short region of the isthmus, neighboring to the ampulla *(Fig. 4-F)*. The intensity of immunoreactivity by regions and periods of the sexual cycle is given in *Table 1*.

Discussion

Oviduct is a dynamic organ and influenced by the sexual cycle. It undergoes through some physiological and biochemical changes while creating an optimal micro-environment required for the early phase of the embryo cleavage, fertilization and ovulation. These changes are hormonally controlled by ovarian steroids, especially estrogen [26] as the oviduct expresses hormone receptors that were also up/down regulated during the sexual cycle [27] Oviduct epithelial cells and oviduct fluid originating from blood plasma contains various proteins and enzymes. These are of great importance for the maturation of gametes, fertilization and embryo development in the oviduct [24]. Cattle embryos are in close contact with the oviduct fluid in the first 4-5 days of pregnancy [28]. It has been reported that the effects of oviduct fluid on gamete functions and embryo development vary according to the cycle phase and region of the oviduct [10]. Boice et

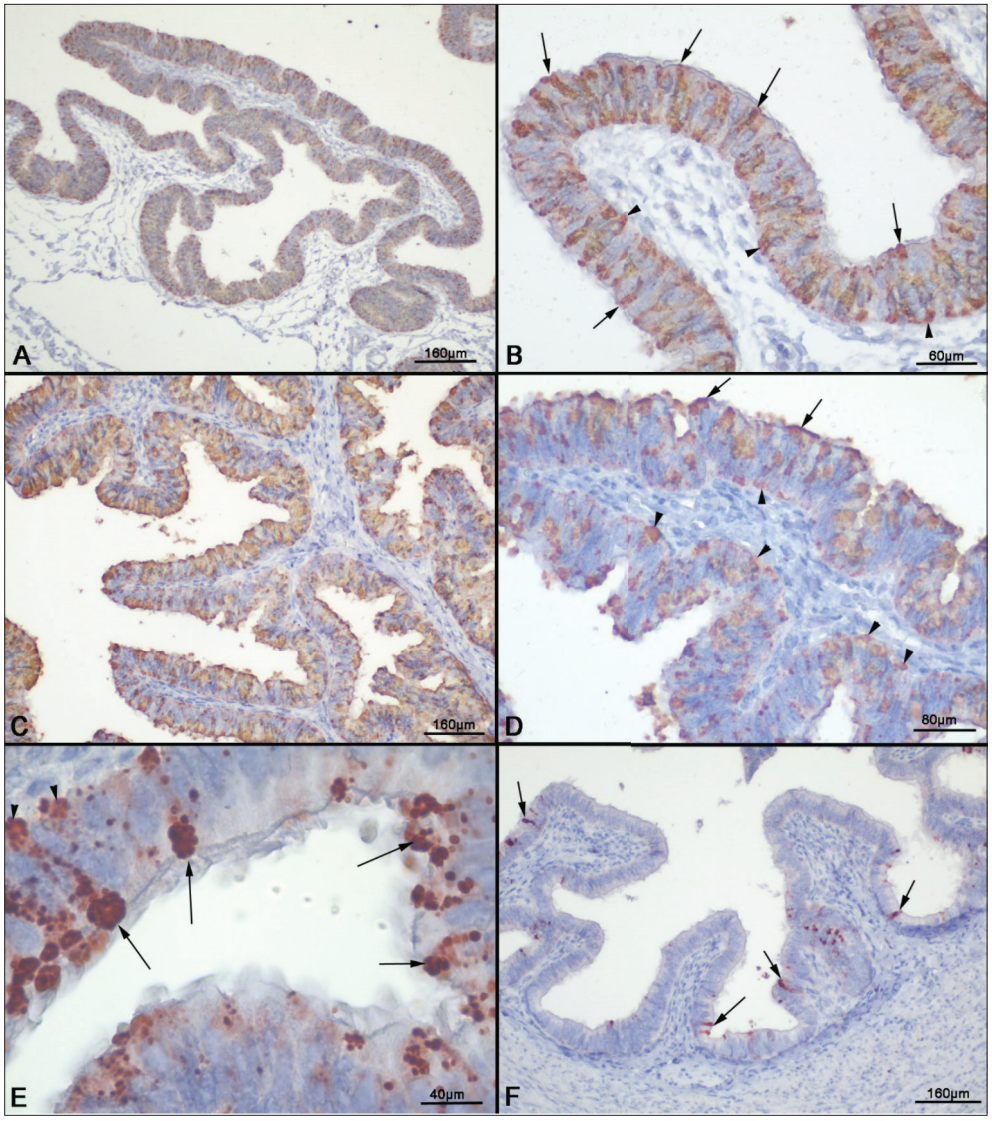


Fig 4. OVGP1 immunoreactivity in fimbria, ampulla and isthmus regions at the estral phase. **A)** Immunoreactivity is present in epithelial lining of the fimbria, **B)** Immunoreactivity can be seen not only in the apical region *(arrows)* but also in the basal region *(arrowheads),* **C-D)** Similarly, immunoreactivity is seen in secretory cells (arrow) of the epithelial layer of the ampulla, filling the whole cytoplasm *(arrowheads),* **E)** In addition, the apical *(arrows)* and basal sites (arrowheads) of the secretory cells of the ampulla had intense immunoreactivity, **F)** In the isthmus, immunoreactivity *(arrows)* is very limited to the regions adjacent to the ampulla. Immunoperosidase staining, hematoxylin counterstaining

al.[29] reported that a group of oviduct-specific glycoproteins including OVGP1 synthesized from bovine oviduct epithelium are present in the oviduct fluid, especially during fertilization. They also reported that these glycoproteins are oviduct specific, not present in other reproductive organs [29]. OVGP1 is reported to be intensely secreted from secretory cells in cattle, especially during the phase of estrogen predominance [30] and supports fertilization and embryonic development [9]. It has also been suggested that OVGP1 prevents polyspermia by preserving the zona pellucida stability [31]. In support to previous studies, the present study also found the OVGP1 immunoreactivity in bovine oviduct epithelial cells, specifically in the secretory cells. Therefore, the present study strongly supports the OVGP1 involvement in various physiological processes in the oviduct during the sexual cycle. The sexual cycle is a continuous and repeating dynamic process, and the oviduct is one of the organ sites influenced by the cycle [10]. Likewise, expression of secreted molecules by the oviduct cells can be influenced as a consequence of hormonal changes [32]. Abe et al.[33] reported that oviduct-specific glycoproteins expressions were concentrated in the follicular phase, but the expressions were weaker in the luteal phase. The present study also found that OVGP1 immunoreactivity exhibited some regional and periodical variation. The OVGP1 immunoreactivity was intense especially in samples of the estral phase, especially in the fimbria and ampulla, and it was weaker in samples of the

Table 1. *Intensity distribution of OVGP-1 immunoreactivity in the oviduct by regions and phases of the sexual cycle*

Oviduct Region	The Phase of the Sexual Cycle			
	Luteal		Oestral	
	Ciliated Cell	Secretory Cell	Ciliated Cell	Secretory Cell
Fimbria	-	+	-	++
Ampulla	-	+	-	+++
İsthmus	-	-	-	-

Semiquantitative scoring of immunostaining intensity; - negative, + weak, ++ moderate, +++ strong

luteal phase. There was also some difference in the mode of expression. The immunoreactivity was seen throughout the cytoplasm of the secretory cells in samples of the estral phase while it was generally limited to the apical site of the same cells in the samples of the luteal phase. Furthermore, there were immunoreactive protrusions at the luteal phase, suggesting secretory cells are emptying OVGP1 along with nucleus and other substances. The Abe et al.[33] study also found a regional variation for OVGP1 immunoreactivity such that the immunoreactivity was intense in the fimbria and ampulla of the estral phases, but weaker in the isthmus of both phases. The present study found also found some regional difference by means OVGP1 immunoreactivity, similar to Abe et al.[33] study with some differences such that the immunoreactivity was absent in the isthmus of the luteal phase, but present at the estral phase, especially in the isthmus sites near the ampulla. Thus, not only the Abe et al.[33] study but also the present study present suggest that synthesis of OVGP1 by secretory cells changes regionally and periodically. In particular, the OVGP1 immunoreactivity is intense in samples of the estral phase, during which estrogen is the dominant sexual hormone. Thus, further studies should focus on how sexual hormones affect gene expression, proteome and secretion in the oviduct epithelium by managing a series of changes through genomic or non-genomic means. As a general concept, not specifically for OVGP1, such a notion has been previously argued by Coy et al.[34] for the oviduct epithelial cells.

In vitro methods of fertilization and embryo production provide a very favorable experimental environment for investigation of biological effects of oviduct secretions on gametes, fertilization and embryo development [10]. In particular, the oviduct and uterine epithelial cell cultures have gained importance in terms of evaluating the paracrine effects [35]. Short-term BOEC culture systems are instruments for studies examining embryo-mother interaction by mimicking the *in vivo* oviduct environment [36]. Co-culture of embryo with BOEC is a method to support the development of bovine embryos derived from IVM/IVF procedures. In this concept, BOEC have been reported to stimulate embryos to produce IFNT, which is in turn influence immune cells to promote an anti-inflammatory response in the oviduct [37]. In this respect, BOEC creates a favorable environment for embryonic development [38]. In the present study, the presence of OVGP1 expression in some BOEC cells suggests that OVGP1 may contribute to the adaptation of BOEC to *in vitro* environments or cell survival.

The OVGP1 expression in the oviduct is influenced by hormonal changes. Progesterone addition suppresses the OVGP1 transcription in BOEC [5]. Expression of oviductin increases in human mucosal cells in response to estrogen *in vitro* [39]. In the present study, cytoplasmic expression of OVGP1 in secretory cells is more prominent and present throughout cytoplasm in samples of the estral phase. OVGP1 immunoreactivity in the luteal phase was limited to the apical site of the secretory cells. Furthermore, secretory cells seemed to empty OVGP1 probably along with other others. Thus, our study also suggests that OVGP1 expression is the highest in estrogen predominance.

In BOEC culture, cytokeratin expression is generally evaluated for fibroblast contamination and BOEC purity or ratio [24]. Similarly, we evaluated cytokeratin immuno-expression in the present study which resulted in intensive positive immune reaction in BOEC cultures of both phases. The appearance of cytokeratin expression without any addition of hormones is consisted with the findings of Comer et al.[40].

It has been proposed that exosomes and microvesicles found in the oviduct fluid may module the relationship between embryo and mother [41]. The researchers claimed that exosomes were the key components of the oviduct secretion *in vitro* and *in vivo*. The exosomes contained OVGP1 *in vivo*. In the present study, secretory cells expressed strong immunoreactivity at the apical site especially at the estral period. Thus, a further study may focus on presence of OVGP1 exosomes and their extrusions from the cells.

As a result, OVGP1 is expressed in secretory cells of the bovine oviduct. It varies by regions and phases of the sexual cycle. Changes in OVGP1 expression during the sexual cycle suggestively indicates a hormonal influence. Regional difference in OVGP1 expression is most likely related to the physiological events that occur in different regions of the oviduct. BOEC isolated from the oviduct

of estral and luteal phases also expresses OVGP1. Further studies should focus on a possible involvement of OVGP1 in BOEC adaption to very tedious condition like cell culture.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

We would like to thank Prof. Dr. Hasan Ceyhun Macun of the Department of Obstetrics and Gynecology, for his contribution to determination of the period of the cycle based on the macroscopic examination of the ovaries.

Author Contributions

The design of the study and evaluation of the results were executed by the contribution of A. KÜRÜM, S. KARAHAN, H. KOCAMIŞ and M. TÜRK. The oviduct samples were collected by A. KÜRÜM and Y. ÖZKABADAYI. Cell culture studies were implemented by M. TÜRK, A. KÜRÜM and Y. ÖZKABADAYI. The Immunohistochemistry and immunocytochemistry studies were executed by A. KÜRÜM, S. KARAHAN, H. KOCAMIŞ and Y. ÖZKABADAYI. All authors also contributed to the preparation of the manuscript.

References

1. Besenfelder U, Havlicek V, Brem G: Role of the oviduct in early embryo development. *Reprod Domest Anim*, 47 (4): 156-163, 2012. DOI: 10.1111/j.1439-0531.2012.02070.x

2. Perez-Cerezales S, Ramos-Ibeas P, Acuna OS, Aviles M, Coy P, Rizos D, Gutierez-Adan A: The oviduct: From sperm selection to the epigenetic landscape of the embryo. *Biol Reprod*, 98 (3): 262-276, 2018. DOI: 10.1093/biolre/iox173

3. Martinez HR: Role of the oviduct in sperm capacitation. *Theriogenology*, 68, S138-S146, 2007. DOI: 10.1016/j.theriogenology.2007.03.018

4. Ito S, Kobayaski Y, Yamamoto Y, Kimura K, Okuda K: Remodeling of bovine oviductal epithelium by mitosis of secretory cells. *Cell Tissue Res*, 366, 403-410, 2016. DOI: 10.1007/s00441-016-2432-8

5. Rottmayer R, Ulbrich SE, Kölle S, Prelle K, Neumueller C, Sinowatz F, Meyer HHD, Wolf E, Hiendleder S: A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interaction: Morphological and functional characterization. *Reproduction*, 132, 637-648, 2006. DOI: 10.1530/rep.1.01136

6. Joshi MS: Isolation, cell culture and immunocytochemical characterization of oviduct epithelial cells of the cow. *J Reprod Fertil*, 83, 249-261, 1988. DOI: 10.1530/jrf.0.0830249

7. Grippo AA, Way AL, Killian GJ: Effect of bovine ampullary and isthmic oviductal fluid on motility acrosome reaction and fertility of bull spermatozoa. *J Reprod Fertil*, 105, 57-64, 1995. DOI: 10.1530/jrf.0.1050057

8. Buhi WC: Characterization and biological roles of oviduct-specific, oestrogen dependent glycoprotein. *Reproduction*, 123, 355-362, 2002. DOI: 10.1530/rep.0.1230355

9. Li S, Winuthayanon W: Oviduct: Roles in fertilization and early embryo development. *J Endocrinol*, 232, R1-R26, 2017. DOI: 10.1530/JOE-16-0302

10. Killian GJ: Evidence for the role of oviduct secretions in sperm function, fertilization and embryo development. *Anim Reprod Sci*, 82-83, 141-153, 2004. DOI: 10.1016/j.anireprosci.2004.04.028

11. Zhang N, Mao W, Zhang Y, Huang N, Liu B, Gao L, Zhang S, Cao J: The prostaglandin E_2 receptor PTGER2 and prostaglandin $F_{2\alpha}$ receptor PTGFR mediate oviductal glycoprotein 1 expression in bovine oviductal epithelial cells. *J Reprod Dev*, 64, 101-108, 2018. DOI: 10.1262/jrd.2017-076

12. Bhatt P, Kadam K, Saxena A, Natraj U: Fertilization, embryonic development and oviductal environment: Role of esrogen induced oviductal glycoprotein. *Indian J Exp Biol*, 42, 1043-1055, 2004.

13. Choudhary S, Janjanam J, Kumar S, Kaushik JK, Mohanty AK: Structural and functional characterization of buffalo oviduct-specific glycoprotein (OVGP1) expressed during estrous cycle. *Biosci Rep,* 39 (12): BSR20191501, 2019. DOI: 10.1042/BSR20191501

14. Yang X, Zhao Y, Yang X, Kan FWK: Recombinant hamster oviductin is biologically active and exerts positive effects on sperm functions and sperm-oocyte binding. *Plos One,* 10 (4): e0123003, 2015. DOI: 10.1371/journal.pone.0123003

15. Rapala L, Starzynski RR, Trzeciak PZ, Dabrowski S, Gajewska M, Jurka P, Smolarczyk R, Duszewska AM: Influence of elevated temperature on bovine oviduct epithelial cells (BOECs). *Plos One,* 13 (6): e0198843, 2018. DOI: 10.1371/journal.pone.0198843

16. Choudhary S, Kumaresan A, Kumar M, Chhillar S, Malik H, Kumar S, Kaushik JK, Datta TK, Mohanty AK: Effect of recombinant and native buffalo OVGP1 on sperm functions and *in vitro* embryo development: A comparative study. *J Anim Sci Biotechnol*, 8:69, 2017. DOI: 10.1186/s40104-017-0201-5

17. Mondéjar I, Martínez-Martínez I, Avilés M, Coy P: Identification of potential oviductal factors responsible for zona pellucida hardening and monospermy during fertilization in mammals. *Biol Reprod,* 89 (3): 67, 1-8. 2013. DOI: 10.1095/biolreprod.113.111385

18. Coy P, Cánovas S, Mondéjar I, Saavedra MD, Romar R, Grullón L, Matás C, Avilés M: Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proc Natl Acad Sci U S A,* 105 (41): 15809-15814, 2008. DOI: 10.1073/pnas.0804422105

19. Diskin MG, Par MH, Morris DG: Embryo death in cattle: An update. *Reprod Fertil Dev*, 24, 244-251, 2011. DOI: 10.1071/RD11914

20. Maillo V, Lopera-Vasquez R, Hamdi M, Gutierez-Adan A, Lonergan P, Rizos D: Maternal-embryo interaction in the bovine oviduct: Evidence from *in vivo* and *in vitro* studies. *Theriogenology*, 86, 443-450, 2016. DOI: 10.1016/j.theriogenology.2016.04.060

21. Maillo V, Sanchez-Calabuig MJ, Lopera-Vasquez R, Hamdi M, Gutierez-Adan A, Lonergan P, Rizos D: Oviductal response to gametes and early embryos in mammals. *Reproduction*, 152, R127-R141, 2016. DOI: 10.1530/REP-16-0120

22. Jang HY, Jung YS, Cheong HT, Kim JT, Park CK, Kong HS, Lee HK, Yang BK: Effects of cell status bovine oviduct epithelial cell (BOEC) on the development of bovine IVM/IVF embryos and gene expression in the BOEC used or not used for the embryo culture. *Asian-Australas J Anim Sci*, 21, 980-987, 2008. DOI: 10.5713/ajas.2008.70413

23. Abe H, Hoshi H: Bovine oviductal epithelial cells: Their cell culture and applications in studies for reproductive biology. *Cytotechnology,* 23, 171-183, 1997. DOI: 10.1023/A:1007929826186

24. Pillai VV, Weber DM, Phinney BS, Selvaraj V: Profiling of proteins secreted in the bovine oviduct reveals diverse functions of this luminal microenvironment. *Plos One*, 12 (11): e0188105, 2017. DOI: 10.1371/journal.pone.0188105

25. Özen A, Ergun E, Oztas E, Ergun L, Özcan Z, Alabay B, Bayraktaroglu AG, Kürüm A, Erdogan E: Immunohistochemical expression of nitric oxide synthase enzymes (iNOS, eNOS, nNOS) in the estrual and luteal phases of the sexual cycle in the cow oviduct. *Anat Histol Embryol*, 42, 384-393, 2013. DOI: 10.1111/ahe.12027

26. Fernandez-Fuertes B, Rodriguez-Alonso B, Sanchez JM, Simintiras CA, Lonergan P, Rizos D: Looking at the big picture: Understanding how the oviduct's dialogue with gametes and the embryo shapes reproductive success. *Anim Reprod*, 15, 751-764, 2018. DOI: 10.21451/1984-3143-AR2018-0036

27. Yang L, Yao X, Chen X, Zang S, Sun H, Liu Y, Dun W: Expression of follicle-stimulating hormone, luteotropic hormone, estrogen and progesterone receptors in ovary, oviduct and endometrium after estrus

induction in ewe. *Kafkas Univ Vet Fak Derg*, 26 (1): 65-70, 2020. DOI: 10.9775/kvfd.2019.22274

28. Banliat C, Tsikis G, Labas V, Teixeira-Gomes AP, Com E, Lavigne R, Pineau C, Guyonnet B, Mermillod P, Saint-Dizier M: Identification of 56 proteins involved in embryo-maternal interactions in the bovine oviduct. *Int J Mol Sci,* 21:466, 2020. DOI: 10.3390/ijms21020466

29. Boice ML, Geisert RD, Blair RM, Verhage HG: Identification and characterization of bovine oviductal glycoproteins synthesized at estrus. *Biol Reprod,* 43, 457-465, 1990.

30. Binelli M, Gonella-Diaza AM, Mesquita FS, Membrive CMB: Sex steroid-mediated control of oviductal function in cattle. *Biology,* 7 (1): 15, 2018. DOI: 10.3390/biology7010015

31. Pradeep MA, Jagadeesh J, De AK, Kaushik JK, Malakar D, Kumar S, Dang AK, Das SK, Mohanty AK: Purification, sequence characterization and effect of goat oviduct-specific glycoprotein on *in vitro* embryo development. *Theriogenology,* 75, 1005-1015, 2011. DOI: 10.1016/j.theriogenology.2010.11.007

32. Barton BE, Herrera GG, Anamthathmakula P, Rock JK, Willie AM, Harris EA, Takemaru KI, Winuthayanon W: Roles of steroid hormones in oviductal function. *Reproduction*, 159, R125-R137,2020. DOI: 10.1530/REP-19-0189

33. Abe H, Numazawa C, Abe M, Onodera M, Katsumi A: Immunocytochemical localization of oviduct-specific glycoproteins in the oviductal epithelium from cows at follicular and luteal phases. *Cell Tissue Res*, 274, 41-47, 1993.

34. Coy P, Garcia-Vazquez FA, Visconti PE, Aviles M: Roles of the oviduct in mammalian fertilization. *Reproduction*, 144, 649-660, 2012. DOI: 10.1530/REP-12-0279

35. Ménézo YJR, Servy E, Veiga A, Hazout A, Elder K: Culture systems: Embryo co-culture. *Methods Mol Biol*, 912, 231-247, 2012. DOI: 10.1007/978-1-61779-971-6_14

36. Ulbrich SE, Zitta K, Hiendleder S, Wolf E: *In vitro* systems for intercepting early embryo-maternal cross-talk in the bovine oviduct. *Theriogenology*, 73, 802-816, 2010. DOI: 10.1016/j.theriogenology.2009.09.036

37. Talukder AK, Rashid MB, Yousef MS, Kusama K, Shimizu T, Shimada M, Suarez SS, Imakawa K, Miyamoto A: Oviduct epithelium induces interferon-tau in bovine day-4 embryos, which generates an anti-inflammatory response in immune cells. *Sci Rep,* 8:7850, 2018. DOI: 10.1038/s41598-018-26224-8

38. Edwards LJ, Batt PA, Gandolfi F, Gardner DK: Modifications made to culture medium by bovine oviduct *epithelial* cells: Changes to carbohydrates stimulate bovine embryo development. *Mol Reprod Dev,* 46, 146-154, 1997.

39. Briton-Jones C, Lok IH, Cheung CK, Chiu TTY, Cheung LP, Haines C: Estradiol regulation of oviductin/oviduct-specific glycoprotein messenger ribonucleic acid expression in human oviduct mucosal cells *in vitro*. *Fertil Steril,* 81, 749-756, 2004. DOI: 10.1016/j.fertnstert.2003.08.016

40. Comer MT, Leese HJ, Southgate J: Induction of a differentiated ciliated cell phenotype in primary cultures of fallopian tube epithelium. *Hum Reprod,* 13 (11): 3114-3120, 1998. DOI: 10.1093/humrep/13.11.3114

41. AlminanA C, Corbin E, Tsikis G, Alcantara-Neto AS, Labas V, Reynaud K, Galio L, Uzbekov R, Garanina AS, Druart X, Mermillod P: Oviduct extracellular vesicles protein content and their role during oviduct-embryo cross-talk. *Reproduction*, 154, 253-268, 2017. DOI: 10.1530/REP-17-0054

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

Kafkas Univ Vet Fak Derg
27 (2): 159-164, 2021
DOI: 10.9775/kvfd.2020.24917

RESEARCH ARTICLE

Comparative Efficacy of Synthetic Acaricides Against Tick Infestations in Goats

Ayesha MALIK [1,a] Kiran AFSHAN [1,b (*)] Abdul RAZZAQ [2,c]
Zahida FATIMA [2,d] Munib HUSSAIN [3,e] Sabika FIRASAT [1,f]

[1] Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, 45320, PAKISTAN
[2] Animal Sciences Division, Pakistan Agricultural Research Council, Islamabad, PAKISTAN
[3] Animal Health Program, National Agriculture Research Centre, Islamabad, PAKISTAN
ORCIDs: [a] 0000-0003-0124-7112; [b] 0000-0002-3979-7606; [c] 0000-0001-8094-5335; [d] 0000-0001-8196-8782; [e] 0000-0001-9329-6303
[f] 0000-0003-4959-7832

Article ID: KVFD-2020-24917 Received: 02.09.2020 Accepted: 16.02.2021 Published Online: 18.02.2021

Abstract

Four commercial synthetic compounds, pyrethroid, organophosphates, macrocyclic lactones and phenylpyrazole have been used for tick control worldwide. However, periodic monitoring of the effectiveness of acaricides has not been fully explored, although such information could contribute to a more effective application, economic analysis and harmful impact on other organisms and environmental contamination. This study investigates the effect of cypermethrine (CYM), deltamethrin, trichlorphon + dimethylester, ivermectin (IVM) and fipronil on natural infestations of ticks in goats. The *in vivo* quantitative assessment of four tick genera i.e. *Hyalomma, Rhipicephalus, Ixodes* and *Haemaphysalis* revealed that both CYM and IVM treated groups resulted in significantly lower ($P<0.05$) tick counts relative to other compounds and controls on all post-treated days. The maximum reduction in the mean number of ticks in the CYM and IVM treated group was recorded from days 3 to 4, followed by complete shedding of ticks on day 5. However, deltamethrin, trichlorphon + dimethylester and fipronil showed 100% efficacy on the sixth day. *In-vitro* efficacy trials showed a 100% tick's mortality based upon the use of fipronil (0.25 g/100 mL) within the 18th h in the post-treated group, while deltamethrin, trichlorphon + dimethylester and CYM were ranked 2nd, 3rd and 5th based on their 100% efficacy within 24-33 h, 33-42 h and 39-48 h, respectively. The investigation has shown that tested acaricides varied in their efficacy to reduce the tick infestation and further experiments on different formulations of the other members of the major acaricidal classes need to be standardized.

Keywords: *Acaricides, Efficacy trials, Goats, Tick*

Keçilerde Kene Enfestasyonuna Karşı Sentetik Akarisitlerin Karşılaştırmalı Etkinliği

Öz

Dünya genelinde kene kontrolü için piretroid, organofosfatlar, makrosiklik laktonlar ve fenilpirazol olmak üzere dört ticari sentetik bileşik kullanılmıştır. Fakat, akarisitlerin etkinliği periyodik olarak tam olarak izlenmemiştir ki bu tür bilgiler daha etkili bir uygulamaya, ekonomik analize ve diğer organizmalar ve çevresel kontaminasyon üzerindeki zararlı etkilere katkıda bulunabilir. Bu çalışmada, sipermetrin (CYM), deltametrin, triklorfon + dimetilester, ivermektin (IVM) ve fipronil'in keçilerde doğal kene enfestasyonu üzerine etkisi araştırılmıştır. *Hyalomma, Rhipicephalus, Ixodes* ve *Haemaphysalis* gibi dört kene cinsi üzerinde yapılan *in vivo* kantitatif değerlendirmede hem CYM hem de IVM ile sağaltılan grupların, tedavi sonrası tüm günlerde diğer bileşiklere ve kontrollere oranla önemli ölçüde daha düşük kene popülasyonuna sahip olduğu saptanmıştır ($P<0.05$). CYM ve IVM ile tedavi edilen gruplardaki ortalama kene sayısındaki maksimum azalma, 3 ile 4. günler arasında kaydedilmiş, takiben 5. günde kenelerin tamamen döküldüğü izlenmiştir. Bununla birlikte, deltametrin, triklorfon + dimetilester ve fipronil, uygulamanın 6. gününde %100 etkinlik göstermiştir. *In vitro* etkinlik denemelerinde, tedavi sonrası grupta fipronil'in (0.25 /100 mL) oranında kullanımına bağlı olarak 18. saatte %100 kene ölüm oranı saptanırken, deltametrin, triklorfon + dimetilester ve sipermetrin'in %100 etkinlikleri sırasıyla 24-33. saat, 33-42. saat ve 39-48. saatler içerisinde saptanmış ve buna göre etkinlik açısından 2., 3. ve 5. sıralarda yer almışlardır. Bu çalışma, test edilen akarisitlerin kene enfestasyonunu azaltmada etkinliklerinde farklılıklar olduğunu ve temel akarisit sınıflarının diğer üyelerinin farklı formülasyonları üzerinde daha fazla deneylerin standartlaştırılması gerektiğini göstermiştir.

Anahtar sözcükler: *Akarisit, Etkinlik denemeleri, Keçi, Kene*

How to cite this article?

Malik A, Afshan K, Razzaq A, Fatima Z, Hussain M, Firasat S: Comparative efficacy of synthetic acaricides against tick infestations in goats. *Kafkas Univ Vet Fak Derg,* 27 (2): 159-164, 2021.
DOI: 10.9775/kvfd.2020.24917

(*) Corresponding Author

Tel: +92 51 90643252
E-mail: kafshan@qau.edu.pk (K. Afshan)

Introduction

Ticks are one of the leading vectors of diseases of economic importance to the livestock industry in tropical and sub-tropical countries of the world. In tropical country like Pakistan, the warm-humid climate favors perpetuation and propagation of ticks. Tick fauna of Pakistan is rich in number of genera and species [1]. In Pakistan, the overall rate of tick infestation has been detected about 50%. Economic losses creating food insecurity [2], reduced growth and milk production and causes estimated global cost of control and productivity losses of 7 billion USD per year [3]. The adverse effects include paralysis/toxicosis and tick-transmitted haemoparasites that reduce production or cause mortality [4].

In Pakistan tick fauna comprises of at least 40 species belonging to mainly three genera i.e. *Hyalomma*, *Haemaphysalis* and *Rhipicephalus* [5]. The prevalence of tick infestation in small ruminants was estimated as 27.85%. Tick infestation was apparently found higher in goats (30.67%) than sheep (23%). A significant variation in the prevalence (22.2%-70.5%) of bovine ticks i.e. *Hy. anatolicum*, *Hy. hussaini*, *Hy. scupense*, *Rh. annulatus* and *Rh. microplus* was recorded across five agroecological zones of Pakistan [6].

Ticks are the major constraints to small ruminant production, and worldwide its control is based mainly on the repeated use of acaricides [7,8]. Number of methods exists to suppress tick's population i.e. dusting, hand spraying, mechanical spray race, hand dressing, systemic and dipping [9]; However, chemotherapeutic control remains the most extensively applied method in the developing world. Acaricides such as synthetic pyrethroids and organophosphates, macro-cyclic lactones, organochlorines, carbamates, and insect growth regulators have been found with significant efficacy for tick control [10-12]. However, populations of several tick species mainly in tropical and subtropical countries have developed resistance to all major classes of these compounds due to the high intensity of their use in tick management [13,14].

In Pakistan the main tick control methods in small and large ruminants are periodic application of acaricides i.e. macrocyclic lactones, trichlorfon and cypermethrin [1,15,16]. However, studies on assessment of the in vivo efficacy of acaricidal drugs are limited and to date few in vivo efficacy testing studies on coumaphos, cypermethrin, diazinon and ivermectin were performed in both sheep and goats in Pakistan [17,18]. Therefore, it is necessary to undertake periodic monitoring of effectiveness of acaricides to provide updated information on the efficacy of commercial acaricides for effective control against tick infestations on animals. Here, current study presented *in vivo* and *in vitro* efficacy testing to establish the current level of acaricidal resistance for five products representing every major acaricidal class (cypermethrine and deltamethrin representing the synthetic pyrethroid; trichlorphon + dimethyl ester representing organophosphates; ivermectin representing the macrocyclic lactones and fipronil representing phenylpyrazole compound) in controlling natural infestations with ticks of goats.

Material and Methods

Site/Experimental Animal's Selection

The present research was carried out at Livestock Research Station (LRS), National Agricultural Research Centre (NARC), Islamabad (33.6844° N, 73.0479° E) involving female goats between 2-5 years aged naturally and heavily infested with ticks. Ticks were collected from infested goats with the help of forceps avoid damage to mouth parts. Identification of ticks was performed through observation of morphological characteristic under stereomicroscope following the taxonomic keys [19,20].

In vivo Acaricidal Efficacy Trials

The *in vivo* acaricidal efficacy trials were conducted per guideline of WAAVP [21]. Briefly, 60 adult female goats, age between 2-5 years, with semi-intensive management, no history of acaricidal treatment and tick infestation rate of 100-120 ticks per animal, are used. Five compounds were subjected to acaricidal treatment viz., cypermethrine, deltamethrin, trichlorphon + dimethylester, ivermectin and fipronil. These compounds were selected based on their extensive usage in livestock farms for tick control.

The animals were divided into six equal groups named A through F *(Table 1)*. Groups A to E were treated with acaricidal compounds as per manufactory instructions, while group F served as control. After treatment with either of the above mentioned acaricides, the animals were examined quantitatively through "finger counting" [22], the number of ticks shed after the first 24 h and the duration for which the treatment remained effective that calculated from the data. The data were expressed as post-treatment tick burden on days 0, 1, 2, 3, 4, 5, and 6.

In vitro Acaricidal Efficacy Trials

The fully engorged ticks (4-5 mm in size) were collected from naturally infected goats managed at livestock research stations. Two different dilutions of each acaricidal compounds were prepared *(Table 2)*, and 30 adult ticks were used in each *in vitro* test dilutions, while one group served as control treated with distilled water. The petri dishes were kept at 25±2°C and 80±5% relative humidity in an incubator for 24 h. The mortality of ticks in all groups was evaluated after different time intervals.

Statistical Analysis

Descriptive analyses were performed according to the

Table 1. *In vivo therapeutic trial against tick's infestation in goats*

Experimental Groups	Composition and Packing	Dilution of Medicines	Dose Rate/Mode of Application
A (10 goats)	Cypermethrine 25%	5 mL /liter	Spray on animals 20 mL/animal
B (10 goats)	Deltamethrin 2.5% W/V (100 mL)	4 mL/liter	Spray on animals 20 mL/animal
C (10 goats)	Trichlorphon 98% W/W, Dimethylester of (2,2,2- trichloro-1 hydroxy-ethyl phosphoric acid) (100gm)	2 g/liter	Spray on animals 20 mL/animal
D (10 goats)	Ivermectin-1gm Vit-A-2500,000U Vit-D-375000U Vit-E-2.5gm	As such	Sub-cut administration 1 mL/50 kg live-body weight
E (10 goats)	Fipronil 0.25g in each 100 mL	As such	Spray on animals 20 mL/animal
F (10 goats)	Control (Water)	-	-

Table 2. *In-vitro acaricide efficacy trial against ticks collected from goats*

Medicine	Petri Dish #	Concentration
Cypermethrine 25%	A1	5 mL/liter (0.125 g/mL)
	A2	4 mL/liter (1 g/m)
Deltamethrin 2.5% W/V (100 mL)	B1	4 mL/liter (0.1 mg/mL)
	B2	3 mL/liter (0.075 mg/mL)
Trichlorphon 98% W/W, Dimethylester of (2,2,2- trichloro-1 hydroxy-ethyl phosphoric acid) (100 gm)	C1	2 mg/mL
	C2	1.5 mg/mL
Fibronil 0.25g in each 100 mL	D 1	0.0025g /100 mL
	D2	0.002 g/100 mL
Control	E	Water

scale of infestation as recommended [23]. The raw data of the ticks count were transformed in a natural logarithm of 10 (count +1). The data were analyzed using the analysis of variance test (ANOVA) followed by least significant difference (LSD) test for means comparisons. The level of significance used was $P \leq 0.05$. The threshold of 90% reduction in the counts of ticks in treated goats compared to untreated ones was considered as of acceptable efficacy for tick control agents, as recommended [23].

The data of five acaricides efficacy in *in vivo* trail were initially analyzed by descriptive statistics (mean, standard error) using Statistix 8.1 program. The efficacy was determined as follows:

Efficacy (%) = C-T/C X 100

Where: C = present mean number of ticks per animal in the control group and T = mean number of ticks per animal in the treatment group.

The data of acaricides efficacy in *in vitro* trail were as follows:

Efficacy (%) = N_0- N/N_0 100*

Where N_0 is the number of ticks prior to acaricidal treatment and N is the number of ticks recorded post-treatment [21].

Results

In vivo Experiment

The experimental goats were infested with four tick genera i.e. *Hyalomma*, *Rhipicephalus*, *Ixodes* and *Haemaphysalis*. The *in vivo* post-treatment quantitative assessment of tick burden revealed that both cypermethrine and ivermectin treated groups resulted in significantly lower ($P<0.05$) tick counts relative to other medicines and controls on all post-treated days. The finger counts were significantly higher ($P=0.00$) in group A (cypermethrine -treated group) than in group D (ivermectin treated), as shown in *Table 3*. From day 0 (pre-treatment) to day 1 (post-treatment), the reduction in the mean number of ticks was not significant ($P>0.05$) in all treatments. The maximum reduction in mean number of ticks in the CYM and IVM treated group was recorded from day 3 to 4, followed by complete shedding of ticks on day 5. However, deltamethrin, trichlorphon-dimethylester and fipronil showed 100% efficacy on the sixth day. The ticks in control group almost remained the same with no significant ($P>0.05$) changes during the experimental period.

In vitro Experiment

The results of *in vitro* efficacy trail showed that fipronil recorded 100% tick's mortality within 18th h post-treatment

Table 3. *Mean (±SD) and percentage reduction (%) in tick burden against different acaricidal treatments in goats*

Acaricide	Pre-treatment	Post-treatment Days					
		1st	2nd	3rd	4th	5th	6th
Cypermethrine	117.7±4.2 (0.4)	117.3±4.1^{a} (33.6)	75.9±1^{c} (73.3)	18.9±3.4^{c} (81.91)	4.4±1.4^{c} (87.12)	0.9±0.3^{b} (100)	
Deltamethrin	118.9±6.12 (0.7)	118.1±6.16^{a} (21)	92.1±3.2^{b} (67.4)	29.8±4.8^{bc} (66.7)	8.2±0.9^{c} (72.1)	2.1±1.1^{b} (95.8)	0.1±0.0^{b} (100)
Trichlorphon Dimethylester	116.7±1.81 (0.3)	116.3±1.94^{a} (22.1)	91±9.2^{bc} (65.6)	29.4±2.3^{bc} (69.2)	8.5±0.83^{c} (78.6)	1.9±0.6^{b} (95.5)	0.2±0.0^{b} (100)
Ivermectin	113.9±2.67 (0.3)	113.5±2.3^{a} (22.3)	87.9±6.6^{b} (70.8)	25.5±4.2^{bc} (73.3)	6±1^{c} (96.2)	0.2±0.6^{b} (100)	
Fibronil	118±3.46 (0.8)	117±3.17^{a} (14.6)	99.9±4^{b} (63.6)	36±5.5^{b} (59.4)	15.6±8.5^{b} (90.8)	1.3±0.4^{b} (56.7)	0.5±0.4^{b} (100)
Control	118.1±0.63 (0.9)	117.2±1.13^{a} (0.1)	116.3±1.42^{a} (-0.6)	116.9±1.4^{a} (0.2)	117.6±0.8^{a} (0.2)	117.5±0.8^{a} (-1.4)	118.8±0.4^{a} (0.2)
P- Value		0.90	0.00	0.00	0.00	0.00	0.00

Parenthesis indicates the tick's mortality percentage; Mean with different letters are significantly different (P<0.05)

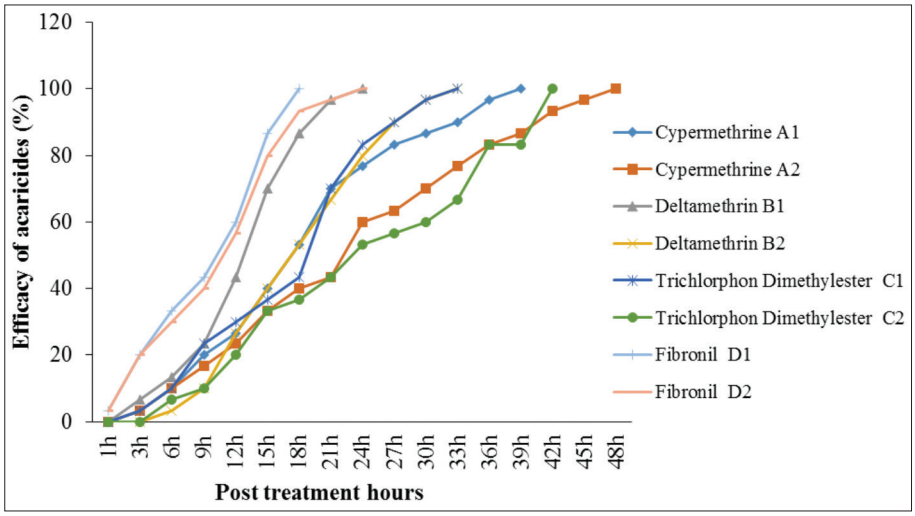


Fig 1. *In-vitro* percentage reduction of ticks on goats against two different concentrations of five commercial acaricides

with higher concentration (0.25 g/100 mL) and 24 h with lower concentration (0.2 g/100 mL). Deltamethrin, trichlorphon + dimethylester and cypermethrine were on 2nd, 3rd and 5th ranked based of their 100% efficacy within 24-33 h, 33-42 h and 39-48 h, respectively *(Fig. 1)*. However, all the four acaricides i.e., trichlorphon+ dimethylester, deltamethrin and cypermethrine showed 100% tick mortality within 48 h of post application. The cypermethrine and trichlorphon+ dimethylester treated group (A2 and C2) showed lowest efficacy, as 83.3% after 36th h.

Discussion

The application of acaricides may significantly reduce the abundance of the tick species [24] and help to mitigate the risk of tick-borne diseases [25]. However, application of acaricides may lead to development of tick resistance to several chemical compounds [26], which needs regular monitoring of acaricides. The present investigation was designed to measure the comparative efficacy of five different formulations of acaricides. The current *in vivo* trials showed 100% tick mortality with cypermethrin and ivermectin on the 5th day of post-treatment. Similar findings were recorded on larval stages of different species of ticks *Hyalomma*, *Haemaphysalis* and *Rhipicephalus* with cypermethrin [27]. *Ixodes ricinus* showed 100% mortality at the 9th day of ivermectin post-treatment [28], while another study reported even longer period of 21 days against *R. microplus* [29]. The resistance of ivermectin against *I. ricinus* was also reported [30,31]. Comparative to the present study, a higher efficacy of cypermethrine as 50% tick's mortality was recorded within 10 min and 100% in 30 min with the dose rate of 1.0 mg/mL or 10.0 mg/m [32]. In contrast to these results, lower mortality (92% and 96.7%) was recorded with cypermethrin application on unfed female of *R. sanguineus* and engorged females, respectively [33]. The differences among the mortality rates may dependent on the dose formulation, mode of application and the type of tick species.

In current research, trichlorphon showed complete reduction of ticks on the 6th day of post-treatment. Several studies recorded lower efficacy, resistance, and reinfection to tick populations after trichlorphon treatment [29,34,35]. *In vitro* trichlorphon concentrations 2 mg/mL and 1.5 mg/mL resulted 50% ticks' mortality within 9th and 24th h and 100% at 18th and 24th h, respectively [36].

The post treatment efficacy of deltamethrin was 100% at the 6th day in the present investigation, which is not consistent with the previous findings [35,37]. Lower efficacy of 13.2%, 12.3% and 16.2% was observed at 3, 7, and 14 days of post-treatment for immature ticks, respectively [38]. Deltamethrin produced about 52.8% reduction of semi-engorged females at 3 days post-treatment and lower percentages were observed at 7 and 14-days post-treatment. The present deltamethrin trials with two concentrations i.e., 0.1 mg/mL and 0.075 mg/mL caused 50% tick's mortality in 12th and 18th h and 100% in 24th and 33rd h, respectively. A previous study on *R. microplus* and *H. anatolicum* ticks showed both susceptibility and resistance to deltamethrin [39]. The deltamethrin (0.0025) tested for *R. sanguineus* engorged female showed low sensitivity [40]. However, resistance with deltamethrin concentration of 0.1 mg/mL was 86.7% (26/30) [34] and for commercial preparation of 1.25% against *R. microplus* was 63% [41]. The possible reasons for differences in results are inconsistent experimental conditions, route of administration, formulation, sampling and analytical methods.

The fipronil *in vivo* formulation presented acaricidal efficacy of 90.8% and 100% on day 4 and 6, respectively in the current study, which agrees with the study recorded maximum efficacy (99.39%) against *R. microplus* female after nine days post-treatment [41]. The mean efficacy of fipronil at a dose of 1 mg/kg in cattle on adults, nymphs and larvae of *R. microplus* female was 74.96%, 92.24% and 80.13%, respectively [41]. Longer period of 17 days of 100% effectiveness was also recorded for fipronil against *R. sanguineus* [42]. However, a study on tick's counts of dogs calculated efficacy of fipronil on weekly basis (2nd, 4th, 8th, 12th) and were 97.6%, 93.8%, 100% respectively [43]. These difference in the effectiveness of fipronil may be dose dependent, as higher dosage caused mortality of both adults and larva of *R. microplus* [42].

The study concluded that application of the tested compounds can reduce the abundance of successive generations of four tick genera namely: *Hyalomma, Rhipicephalus, Ixodes and Haemaphysalis*, which may contribute to reduction of population of tick species. Further-more, experimentation on acaricidal efficacy testing with different formulations of other members of major acaricidal classes needs to be standardized.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author's contribution

K.A. and A.R. designed the study. A.M. performed the experiment. A.R., Z.F., M.H. advised on methods, experimentation and interpretation of findings. A.M., K.A., S.F. and A.R. conducted literature search, data analysis and manuscript preparation. K.A. and S.F. reviewed the manuscript. All authors participated in the study and concur with the submission and subsequent revisions submitted by the corresponding author.

References

1. Jabbar A, Abbas T, Sandhu Z, Saddiqi HA, Qamar MF, Gasser RB: Tick-borne diseases of bovines in Pakistan: Major scope for future research and improved control. *Parasit Vectors,* 8:283, 2015. DOI: 10.1186/s13071-015-0894-2

2. Habeeb SM: Ethno-veterinary and medical knowledge of crude plant extracts and its method of application (traditional and modern) for tick control. *World Appl Sci J*, 11 (9): 1047-1054, 2010.

3. Nchu F, Magano SR, Eloff JN: *In vitro* anti-tick properties of the essential oil of *Tagetesminuta L.* (Asteraceae) on *Hyalomma rufipes* (Acari: Ixodidae). *Onderstepoort J Vet Res*, 79 (1): 358, 2012. DOI: 10.4102/ojvr.v79i1.358

4. Walker A, Bouatour A, Camicas JL: Ticks of Domestic Animals in Africa: A Guide to Identification Species. The University of Edinburgh, UK, 2003.

5. Ghafar A, Abbas T, Rehman A, Sandhu ZU, Cabezas-Cruz A, Jabbar A: Systematic review of ticks and tick-borne pathogens of small ruminants in Pakistan. *Pathogens,* 9 (11): 937, 2020. DOI: 10.3390/pathogens9110937

6. Ghafar A, Gasser RB, Rashid I, Ghafoor A, Jabbar A: Exploring the prevalence and diversity of bovine ticks in five agro-ecological zones of Pakistan using phenetic and genetic tools. *Ticks Tick Borne Dis*, 11 (5): 101472, 2020. DOI: 10.1016/j.ttbdis.2020.101472

7. Pirali-Kheirabadi KH, da Silva JAT: *In-vitro* assessment of the acaricidal properties of *Artemisia annua* and *Zataria multiflora* essential oils to control cattle ticks. *Iran J Parasitol*, 6 (1): 58-65, 2011.

8. Shyma KP, Kumar S, Sangwan AK, Sharma AK, Nagar G, Ray DD, Ghosh S: Acaricide resistance status of *Rhipicephalus (Boophilus) microplus* and *Hyalomma anatolicum* collected from Haryana. *Indian J Anim Sci,* 83 (6): 591-594, 2013.

9. Koney EBM: Livestock Production and Health in Ghana. 2nd ed., 4-21, Advent Press, Accra, 2004.

10. Whitnall ABM, Mchardy WM, Whitehead GB, Meerholz F: Some observations on the control of bont tick, *Amblyomma hebraeum* Koch. *Bull Entomol Res,* 41, 577-591, 1951. DOI: 10.1017/S0007485300027838

11. Ware GW: The Pesticide Book. 5th ed., Thomson Publication, Fresno, California 2000.

12. Davey RB, Ahrens EH: Control of *Boophilus* ticks on heifers with two pyrethroids applied as sprays. *Am J Vet Res*, 45, 1008-1010, 1984.

13. Abbas RZ, Zaman MA, Colwell DD, Gilleard J, Iqbal Z: Acaricide resistance in cattle ticks and approaches to its management: The state of play. *Vet Parasitol,* 203, 6-20, 2014. DOI: 10.1016/j.vetpar.2014.03.006

14. Lenka DR, Ravindran R, Jyothimol G, ,Udaykumar M,Reddy PMT, Sathish N,Palla I, Chandramohan B, Ajithkumar KG, Nair SN, Chandrasekhar L, Priya MN, Deepa CK, Sabu L, Juliet S, Ghosh S: Deltamethrin resistance in south Indian isolates of *Rhipicephalus (Boophilus) microplus. Vet Parasitol Reg Stud Reports*, 5, 37-41, 2016. DOI: 10.1016/j.vprsr.2016.09.002

15. Iqbal A, Usman M, Abubakar M: Mini review: Current tick control strategies in Pakistan are possible environmental risks. *Iraqi J Vet Sci,* 31, 81-86, 2017. DOI: 10.33899/ijvs.2017.145601

16. Graf JF, Gogolewski R, Leach-Bing N, Sabatini GA, Molento MB, Bordin EL, Arantes GJ: Tick control: An industry point of view. *Parasitology,* 129, S427-S442, 2004. DOI: 10.1017/S0031182004006079

17. Khan M: Taxonomical Study of Ticks of Genus *Rhipicephalus* and Their Relation to the Incidence of Haemoparasites and Comparative Efficacy of Different Acaricides on Ticks in Sheep and Goats in Kaghan Valley. *Master's Thesis,* College of Veterinary Sciences, Lahore, University of Agriculture, Faisalabad, Pakistan, 1993.

18. Sajid MS, Iqbal Z, Khan MN, Muhammad G, Needham G, Khan MK: Prevalence, associated determinants, and *in vivo* chemotherapeutic control of hard ticks (Acari: Ixodidae) infesting domestic goats (*Capra hircus*) of lower Punjab, Pakistan. *Parasitol Res,* 108, 601-609, 2011. DOI: 10.1007/s00436-010-2103-8

19. Walker AR: Ticks of domestic animals in Africa: A guide to identification species. The University of Edinburgh, UK, *Bioscience Reports,* 3-210, 2014.

20. Barker SC, Walker AR: Ticks of Australia. The species that infest domestic animals and humans. *Zootaxa,* 3816, 1-144, 2014. DOI: 10.11646/zootaxa.3816.1.1

21. Holdsworth PA, Kemp D, Green P, Peter RJ, De Bruin C, Jonsson NN, Letonja T, Rehbein S, Vercruysse J: World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of acaricides against ticks (Ixodidae) on ruminants. *Vet Parasitol,* 136 (1): 29-43, 2006. DOI: 10.1016/j.vetpar.2005.11.011

22. Rugg D, Hair JA: Dose determination of a novel formulation of metaflumizone plus amitraz for control of cat fleas *(Ctenocephalides felis felis)* and brown dog ticks *(Rhipicephalus sanguineus)* on dogs. *Vet Parasitol*, 150 (3): 203-208, 2007. DOI: 10.1016/j.vetpar.2007.08.036

23. Marchiondo AA, Holdsworth PA, Green P, Blagburn BL, Jacobs DE: World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of parasiticides for the treatment, prevention and control of flea and tick infestation on dogs and cats. *Vet Parasitol,* 145, 332-344, 2007.

24. Benelli G, Maggi F, Romano D, Stefanini C, Vaseeharan B, Kumar S, Higuchi A, Alarfaj AA, Mehlhorn H, Canale A: Nanoparticles as effective acaricides against ticks - A review. *Ticks Tick Borne Dis,* 8 (6): 821-826, 2017. DOI: 10.1016/j.ttbdis.2017.08.004

25. Otranto D, De Caprariis D, Lia RP, Tarallo V, Lorusso V, Testini G, Dantas-Torres F, Latrofa S, Diniz PPVP, Mencke N, Maggi RG, Breitschwerdt E, Capelli G, Stanneck D: Prevention of endemic canine vector-borne diseases using imidacloprid 10% and permethrin 50% in young dogs: A longitudinal field study. *Vet Parasitol,* 172, 323-332, 2010. DOI: 10.1016/j.vetpar.2010.05.017

26. Abbas RZ, Zaman MA, Colwell DD, Gilleard J, Iqbal Z: Acaricide resistance in cattle ticks and approaches to its management: The state of play. *Vet Parasitol*, 203, 6-20, 2014. DOI: 10.1016/j.vetpar.2014.03.006

27. Rani RS, ED'Souza P, Byregowda SM, Veeregowda BM, Sengupta PP, Chandranaik BM, Thimmareddy PM: *In vitro* acaricidal efficacy of deltamethrin, cypermethrin and amitraz against sheep ticks in Karnataka. *JEZS,* 6 (3): 758-762, 2018.

28. Kröber T, Guerin PM: An *in vitro* feeding assay to test acaricides for control of hard ticks. *Pest Manag Sci,* 63, 17-22, 2007, DOI: 10.1002/ps.1293

29. Muhammad G, Naureen A, Firyal S, Saqib M: Tick control strategies in dairy production medicine. *Pak Vet J,* 28 (1): 43-50, 2008.

30. Martins JR, Furlong J: Avermectin resistance of the cattle tick *Boophilus microplus* in Brazil. *Vet Rec*, 149:64, 2001.

31. Lopez-Arias A, Villar-Argaiz D, Chaparro-Gutierrez J, Miller RJ, Perez de Leon AA: Reduced efficacy of commercial acaricides against populations of resistant cattle tick *Rhipicephalus microplus* from two municipalities of Antioquia, Colombia. *Environ Health Insights*, 8 (S2): 71-80, 2014. DOI: 10.4137/ehi.s16006

32. Myung-Jo Y: Resistance and control of cypermethrin and chlorpyrifos as acaricide for control of hard tick *Haemaphysalis longicornis* (Acari: Ixodidae). *Korean J Vet Res*, 54 (2): 117-120, 2014. DOI: 10.14405/kjvr.2014.54.2.117

33. Bicalho KA, Ferreira F, Borges LMF, Ribeiro MFB: *In-vitro* evaluation of the effects of some acaricides on life stages of *Rhipicephalus sanguineus* (Acari: Ixodidae). *Arq Bras Med Vet Zootec,* 53 (5): 548-552, 2001.

34. Vudriko P, Okwee-Acai P, Tayebwa DS, Byaruhanga J, Kakooza S, Wampande E, Omara R, Muhindo JB, Tweyongyere R, Owiny DO, Hatta T, Tsuji N, Umemiya-Shirafuji R, Xuan X, Kanameda M, Fujisaki K, Suzuki H: Emergence of multi-acaricide resistant *Rhipicephalus* ticks and its implication on chemical tick control in Uganda. *Parasit Vectors*, 9:4, 2016. DOI: 10.1186/s13071-015-1278-3

35. Katuri RN, Das G, Singh AK, Chalhotra SK, Nath S: Comparative efficacy of deltamethrin and chlorpyriphos in bovine ticks in and around Jabalpur. *J Parasit Dis,* 41 (3): 713-715, 2017. DOI: 10.1007/s12639-016-0872-4

36. Rodriguez-Vivas RI, Grisi L, Pérez de León AA, Villela HS, Torres-Acosta JFJ, Sánchez FH, Salas RD, Cruz RR, Saldierna F, Carrasco DG: Potential economic impact assessment for cattle parasites in Mexico. Review. *Rev Mex Cienc Pecu*, 8 (1): 61-74, 2017.

37. Brito LG, Barbieri FS, Rocha RB, Oliveira MCS, Ribeiro ES: Evaluation of the efficacy of acaricides used to control the cattle tick, *Rhipicephalus microplus*, in dairy herds raised in the Brazilian Southwestern Amazon. *Vet Med Int*, 2011:806093, 2011. DOI: 10.4061/2011/806093

38. Barre N, Andrew YL, Miller RJ, Gaïa H, Delathière JM, Davey RB, George JE: *In-vitro* and *in-vivo* evaluation of Deltamethrine and Amitraz mixtures for the control of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodiadae) in New Caledonia. *Vet Parasitol*, 155, 110-119, 2008. DOI: 10.1016/j.vetpar.2008.04.016

39. Gaur RS, Kumar SA, Sangwan N. Ghosh M, Kumar S: Comparative study of esterases in deltamethrin and diazinon resistant *Rhipicephalus microplus* and *Hyalomma anatolicum* ticks collected from the Trans-Gangetic plains of India. *Exp Appl Acarol,* 73, 115-127, 2017.

40. Shyma KP, Gupta JP, Singh V, Patel KK: *In-vitro* detection of acaricidal resistance status of *Rhipicephalus (Boophilus) microplus* against commercial preparation of deltamethrin, flumethrin, and fipronil from North Gujarat, India. *J Parasitol Res,* 2015:506586, 2015. DOI: 10.1155/2015/506586

41. Cid YP, Ferreira TP, Magalhaes VS, Correia TR , Scott FB: Injectable fipronil for cattle: Plasma disposition and efficacy against *Rhipicephalus microplus*. *Vet Parasitol*, 220, 4-8, 2016. DOI: 10.1016/j.vetpar.2016.02.008

42. Tiawsirisup S, Thiansirikhun K, Thanadumkerng K, Pastarapatee N, Trirattananuwong N, Rattanatayarom W: Antiparasitic efficacy of 10% w/v fipronil spot-on (Fiproline Spot-on) against experimental tick *(Rhipicephalus sanguineus)* infestations on dogs. *Thai J Vet Med*, 43 (2): 279-284, 2013.

43. Rohdich N, Roepke RKA, Eva Z: A randomized, blinded, controlled and multi-centered field study comparing the efficacy and safety of Bravecto™ (fluralaner) against Frontline™ (fipronil) in flea- and tick-infested dogs. *Parasit Vectors*, 7:83, 2014. DOI: 10.1186/1756-3305-7-83

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

Kafkas Univ Vet Fak Derg
27 (2): 165-171, 2021
DOI: 10.9775/kvfd.2020.24949

RESEARCH ARTICLE

Effect of Gelatin/Chitosan Coating on Chicken Patty Quality During Frozen Storage: A Response Surface Methodology Application [1]

Aydın ERGE [1,a (*)] Ömer EREN [2,b]

[1] *This study was supported by Bolu Abant Izzet Baysal University, Turkey (Grant no: 2016.09.04.1000)*

[1] Bolu Abant İzzet Baysal University, Faculty of Agriculture, Poultry Science Department, Gölköy Campus, TR-14030 Bolu - TURKEY
[2] Bolu Abant İzzet Baysal University, Faculty of Engineering, Food Engineering Department, Gölköy Campus, TR-14030 Bolu - TURKEY
ORCIDs: [a] 0000-0001-7419-4221; [b] 0000-0001-5650-1320

Article ID: KVFD-2020-24949 Received: 10.09.2020 Accepted: 10.02.2021 Published Online: 14.02.2021

Abstract

Quality loss of food products is an important problem for food producers and consumers. Edible coating application is an alternative method to preserve food quality and to extend shelf life. In this study, a coating solution composed of chicken gelatin (0-6%), chitosan (0-2%) and sorbitol (0-1.5%), was practiced to preserve chicken patty during frozen storage. Gelatin was extracted from chicken MSM (Mechanically Separated Meat) residue and chicken patties were prepared from spent hen. The physicochemical properties (moisture, pH, thiobarbituric acid value, shrinkage value, texture and color) of chicken patties were evaluated using response surface methodology (RSM) by 15 different coating combinations. The increase in gelatin and chitosan concentrations reduced significantly lipid oxidation. The application of chitosan decreased hardness of chicken patties and improved texture properties. Shrinkage decreased by increasing sorbitol concentration. Overall, an optimal coating blend formed by chicken gelatin (6%), chitosan (1.5-2.0%) and sorbitol (1.0-1.5%) showed the best effect on preserving quality of chicken patties during frozen storage.

Keywords: *Chicken gelatin, Chicken patty, Chitosan, Edible coating, Lipid oxidation*

Jelatin/Kitosan Kaplamanın Dondurarak Muhafaza Edilen Tavuk Köftesi Üzerindeki Etkisi: Bir Yanıt Yüzey Metodu Uygulaması

Öz

Gıda ürünlerindeki kalite kayıpları, gıda üreticileri ve tüketiciler açısından önemli bir sorundur. Yenilebilen kaplama işlemi, gıda kalitesini muhafaza ve raf ömrünü uzatmak amacıyla uygulanan bir metoddur. Bu çalışmada tavuk köftelerine (%0-6) tavuk jelatini, (%0-2) kitosan ve sorbitoldan (%0-1.5) oluşan kaplama işlemi uygulanmış ve dondurarak muhafaza edilmiştir. Jelatin, mekanik ayrılmış kanatlı eti posasından; tavuk köfteleri ise anaç tavuklardan üretilmiştir. Tavuk köftelerinin fizikokimyasal özellikleri (nem, pH, tiyobarbütirik asit değeri, büzüşme oranı, tekstür ve renk) yanıt yüzey metoduna göre 15 farklı kombinasyonda değerlendirilmiştir. Kaplama solüsyonunda jelatin ve kitosan oranlarının artışı, lipid oksidasyonunu belirgin şekilde azaltmıştır. Kitosan uygulaması tavuk köftelerinin sertliğini düşürmüş ve tekstürel özelliklerini geliştirmiştir. Büzüşme oranı, solüsyondaki sorbitol oranı arttıkça azalmıştır. Sonuçta tavuk köftelerinin dondurarak depolama sürecinde kalite özelliklerinin korunmasında optimum etkiyi gösteren kaplama formülasyonunun %6 tavuk jelatini, %1.5-2.0 kitozan ve %1.0-1.5 sorbitol olduğu tespit edilmiştir.

Anahtar sözcükler: *Kitosan, Lipit oksidasyonu, Tavuk jelatini, Tavuk köftesi, Yenilebilir kaplama*

INTRODUCTION

Recently, there is a big awareness of healthy diet according to the consumers for their lifestyle and new natural techniques need to be developed instead of some conventional methods that used for extending shelf life of foods. For this reason, using some edible coating and film materials became important alternatives. Edible film or coating material is a primary packaging material prepared from some edible materials. This material can cover the food

How to cite this article?

Erge A, Eren Ö: Effect of gelatin/chitosan coating on chicken patty quality during frozen storage: a response surface methodology application. *Kafkas Univ Vet Fak Derg,* 27 (2): 165-171, 2021.
DOI: 10.9775/kvfd.2020.24949

(*) **Corresponding Author**

Tel: +90 374 254 1000-2621 Fax: +90 374 253 4346
E-mail: aydin.erge@ibu.edu.tr (A. Erge)

without having any effect to its content or process. This method is used for many types of foods in order to make a gas/water vapor barrier, to enhance some sensory and mechanical properties and to prolong the shelf life preventing form some oxidative and microbiological factors [1]. Edible coating or film materials can be prepared by some proteins, polysaccharides, lipids or their composites because of their potential advantage of being biodegradable. Most prevalent proteins used for this purpose are collagen, gelatin, casein, whey protein, corn zein, wheat gluten, soy protein, egg white protein, myofibrillar proteins and keratin. Most prevalent polysaccharides are starch, cellulose and its derivatives, pectin, chitosan, alginate, carrageenan, pullulan and gellan gum [2]. At the other hand, glycerol, sorbitol, monoglyceride, polyethylene glycol and glucose can be used as a plasticizer to make more flexible the material. Some lipid compounds can also be used to make an emulsion based edible films such as waxes, vegetative oils and fatty acids [1].

Gelatin is a food additive having a characteristic of protein derived from collagen which is one of the major proteins in animal tissues. Gelatin is an effective hydrocolloid used prevalently in food industry through its gelling and thickening properties. The surface properties of gelatin comes from the charged groups in protein chain and the hydrophobic/hydrophilic amino acids repeating at collagen molecule [3]. For this reason, gelatin may be used for the manufacture of edible films or coating materials because of its some properties such as being cheaper, being biodegradable and being capable to interact with many types of materials [4-8].

Chitosan, an animal origin fiber, is one of important materials used as a coating or film forming material. It is derived from chitin which is a polysaccharide material consisted of *N*-acetylglucosamine and glucosamine units. The anti-microbial activity and film forming property [9], texturizing property [10,11] and antioxidant property [11,12] of chitosan have been reported. The use of chitosan for preserving different types of meat, fish and poultry products are reported [9,11-14].

Some studies were performed by formulating chitosan and gelatin [7-9,12,13]; however there is no available information on optimization of an edible coating material prepared by a combination of chicken gelatin, chitosan and sorbitol. Therefore, the aim of this study was to investigate the effects of three factors (chicken gelatin, chitosan and sorbitol in a coating solution) on some physicochemical, textural and industrial quality properties of chicken patties and to propose an optimal coating blend.

Material and Methods

Materials

Mechanically separated chicken meat residues and spent hens were collected from a chicken slaughterhouse (Beypiliç, Turkey) and kept at -18°C till use. All chemicals and reagents used were analytical grade. Cleaning the material and preparation of the samples was carried out as described by Erge and Zorba [15].

Gelatin Extraction

Washed material was subjected to 1 g/100 mL NaCl for 30 min at ambient temperature and the material was washed and filtered. After that, the pretreatment process was performed in 6.73 g/100 mL HCl solution for 24 h and extraction process was performed at 86.8°C for 2 h in water bath (Memmert WNB-45, MEMMERT, Germany). After the extraction, the slurry was filtered using double folded of cheese-cloth to get gelatinous extract. Lastly, gelatin solution was dried at 42°C and stored at at 4°C described as Erge and Zorba [15].

Preparation of Coating Solutions

The different combinations of coating solutions composed of three independent factors (gelatin, chitosan and sorbitol concentrations) prepared according to the central composite design *(Table 1)*. Gelatin was put in water at 25°C for 1 h and heated to 55°C for 30 min. Chitosan was solubilized in 1g/100 mL acetic acid solution. At the end, gelatin solution, chitosan solution and sorbitol were mixed in order to make a final ratio according to the experimental design *(Table 1)*.

Preparation of Chicken Patties and Coating Process

Deboned and defatted spent hen meat prepared from breast fillets and legs was ground with a chopper to 3 mm. 1.5 g/100 g Salt, 5 g/100 g onion powder, 5 g/100 g bread crumbs, 0.3 g/100 g sodium tripolyphospate and 0.7 g/100 g spices were added homogeneously to the grounded chicken meat and standard chicken patties (70 mm diameter and 15 mm thickness) were prepared before the coating process [16]. Chicken patties were kept at refrigerator overnight and were coated by dipping in different coating solutions for 5 min. Coated patties were kept in ventilated oven for 2 min at room temperature in order to lose over solution. The patties were put at refrigerator at 4°C for 4 h in order to make dry partially their surface. Lastly, chicken patties were put in plastic trays closed and kept in freezer for 4 months at -18°C. After this period, frozen patties were thawed at refrigerator overnight and were cooked at 185°C for 25 min.

Biochemical Analysis

The pH analysis was performed by homogenizing a mixture of 10 g sample in 100 mL of distilled water using a digital pH meter (Schott Instruments, Lab 860, Germany). The pH analysis of coating solutions were measured directly dipping in the solution [17]. Moisture content was determined by drying 10 g of chicken patty sample to a stable weight in an air oven at 105°C for 16 h [18]. Crude fat content

Table 1. *Central composite design of three independent variables*

Run Order	Gelatin Concentration (g/100 mL) X_1	Chitosan Concentration (g/100 mL) X_2	Sorbitol Concentration (g/100 mL) X_3
1	0.00	0.00	0.75
2	0.00	1.00	0.00
3	0.00	1.00	1.50
4	0.00	2.00	0.75
5	3.00	0.00	0.00
6	3.00	0.00	1.50
7	3.00	1.00	0.75
8	3.00	1.00	0.75
9	3.00	1.00	0.75
10	3.00	2.00	0.00
11	3.00	2.00	1.50
12	6.00	0.00	0.75
13	6.00	1.00	0.00
14	6.00	1.00	1.50
15	6.00	2.00	0.75

was determined by the extraction with hexane [18]. After the storage period, lipid oxidation of the samples was evaluated spectrophotometrically as thiobarbituric acid (TBA) value in duplicate as described by Raharjo et al.[19].

Physical Analysis

- Technological quality evaluation

The shrinkage analysis was performed according to Serdaroğlu and Değirmencioğlu [20].

Shrinkage (%) ={[uncooked (g) - cooked (g)] + [diameter of uncooked - diameter of cooked sample]}/[thickness of uncooked sample + diameter of uncooked sample] x100 (1)

- Textural analysis

In this context, texture profile analysis (TPA) and Warner Bratzler Shear Force (WBSF) analysis were performed using a texture analyzer (TA-XT2 Stable Micro Systems, Surrey, England) equipped with a load cell of 5 kg. Patty samples were prepared cutting into standard cubes with (5x2 cm) length and width. For TPA analysis, a cylindrical plunger (58 mm in diameter) was used. The samples were compressed to 50% of height. The parameter values determined were hardness (g), springiness (g/100 g), cohesiveness (adimensional), chewiness (g.cm), gumminess (g), and adhesiveness (g/cm) [21]. For Warner Bratzler Shear Force (WBSF) analysis, cutting measurements were performed on coated surface of chicken patties using Warner-Bratzler shear blade (crosshead speed of 1 mm s^{-1}). The units of WBSF and the cutting work values were defined with *g* and *g.second,* respectively. Each sample was tested two times, and the average of the two measurement was used [16].

- Color evaluation

The colorimetric evaluations were performed according to Du et al.[22] on the surface of chicken patties using a Chroma Meter (Konica Minolta CR-400, Japan) in duplicate. The color values were evaluated by using L*, a*, and b* values showing lightness, red (+)/green (-) and yellow (+)/blue (-) color respectively.

Statistical Analysis

The analysis was performed using Response Surface Methodology (RSM) with 15 combinations. Box - Behnken design was used including three replicates of the centre point. The effect of three factor [gelatin concentration (X1), chitosan concentration (X2) and sorbitol concentration (X3)] were analyzed *(Table 1)*. This study was expressed by using a second order polynomial equation. The equation is:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j \quad (2)$$

where Y is the dependent variables (moisture and lipid contents, pH and TBA values, color properties, technological and texture properties of chicken patties), β0, βi, βii, and βij are regression coefficients, k (3) is the number of factor variables, and Xi, Xii, and Xij are levels of independent variables. The (SAS 6.12) was used to carry out the statistical analysis [15].

Results

The results of variance analyses were indicated at the *Table 2*.

Table 2. *Variance analyses results of the effects of independent variables on the physicochemical properties of chicken patties*

Sources of Variation	DF	Moisture (g/100 g) F-value	TBA (mg/kg) F-value	Peroxide (meq/kg) F-value	L* F-value	WBSF (g) F-value	WB Cutting Work (g.sec) F-value	Hardness (g) F-value	Cohesiveness F-value	Chewiness (g*cm) F-value	Gumminess (g) F-value	Adhesiveness (g/cm) F-value
Model	9	1.6541	2.7732	1.4304	2.6271	2.2027	2.2895	1.8928	2.737	2.2027	2.2316	1.7573
X_1 (Gelatin g/100 mL)	1	0.0453	13.0634**	1.0792	1.1331	2.7457	7.9359*	0.2310	1.0182	2.7457	0.3349	3.3442
X_2 (Chitosan g/100 mL)	1	0.2044	7.6494**	3.8891	15.8829**	8.6354**	8.8244**	8.2598**	14.0732**	8.6354**	11.1544**	9.3222**
X_3 (Sorbitol g/100 mL)	1	0.0861	0.0800	0.4862	1.1989	1.6843	0.1130	0.4166	2.3459	1.6843	0.6704	0.0679
X_1*X_1	1	3.6023	0.2554	5.2510*	0.3666	0.4364	0.8719	0.1517	0.0871	0.4364	0.1540	0.0062
X_1*X_2	1	9.3923**	0.1976	0.9988	2.7211	0.3065	0.4137	7.0296*	0.6573	0.3065*	6.8520*	0.7538
X_2*X_2	1	0.0174	2.0060	0.6783	0.1524	2.5521	1.6268	0.0008	0.0148	2.5521	0.0020	1.7074
X_1*X_3	1	0.2597	0.8712	0.0675	1.9698	2.8584	0.5665	0.0415	0.0550	2.8584	0.0661	0.0137
X_2*X_3	1	1.1865	0.0711	0.0350	0.2360	0.2865	0.0402	0.7064	3.5550	0.2865	0.4555	0.4835
X_3*X_3	1	0.0340	0.7416	0.1235	0.0035	0.0911	0.0201	0.1712	2.9053	0.0911	0.3540	0.1501
Lack of fit	5	0.2466	1.3717	1.1028	2.8108	1.8992	0.8142	0.1927	0.1600	1.8992	0.2418	0.3642
General	31	1.6541	2.7732	1.4304	2.6271	2.2027	2.2895	1.8928	2.737	2.2027	2.2316	1.7573

*** P<0.01; * P<0.05*

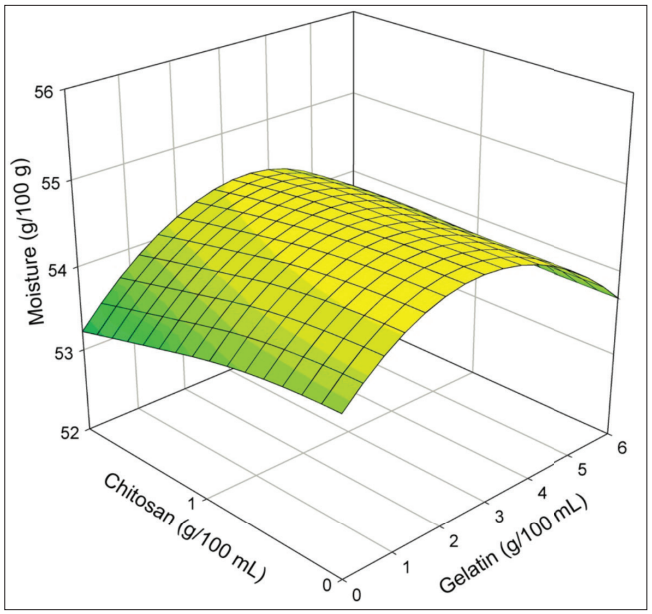


Fig 1. The effect of gelatin and chitosan concentration on the moisture of chicken patties

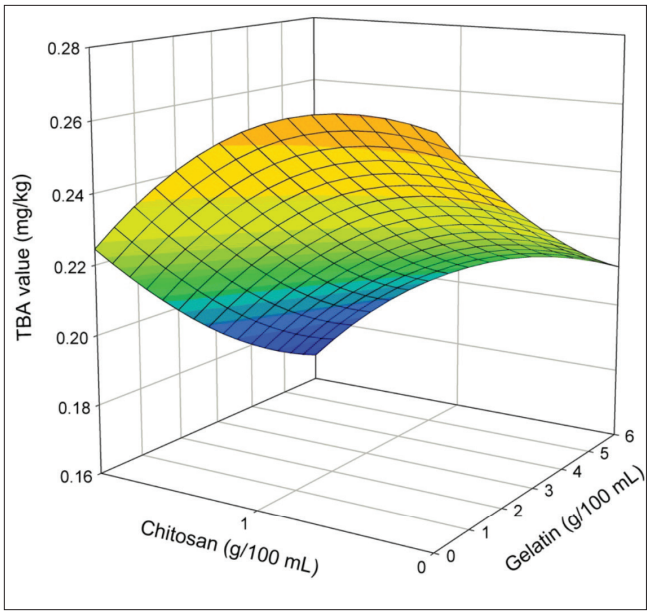


Fig 2. The effect of gelatin and chitosan concentration on the TBA value of chicken patties

Physicochemical Properties of Chicken Patties

The interaction between gelatin concentration and chitosan concentration had significant (P<0.01; *Table 2)* effects on the moisture of chicken patties. The maximum moisture content was determined at 3.5 g/100 mL gelatin concentration approximately *(Fig. 1)*. The effects of independent variables on the lipid content and pH values were insignificant (P>0.05; *Table 2)*.

A significant linear effect of gelatin (P<0.01) and chitosan (P<0.05) concentrations on TBA values were viewed *(Table 2)*. Results showed that gelatin concentration was effective in decreasing TBA values *(Fig. 2)*. Minimum TBA value was observed at 6 g/100 mL gelatin concentration.

The color is another quality characteristic of meat products because of its direct effect on consumer choice. A significant (P<0.01) quadratic effect of chitosan concentration on L* value of cooked chicken patties was viewed *(Table 2)*. L* value increased by increasing of chitosan concentration at cooked patties *(Fig. 3)*. Coating with chitosan decreased the redness and increased the lightness of chicken patties after a freezing storage.

Technological Properties of Chicken Patties

A significant (P<0.05) interaction effect between chitosan and sorbitol concentration on the shrinkage of chicken patties was viewed *(Table 2)*. The shrinkage value decreased by increasing of sorbitol concentration *(Fig. 4)*.

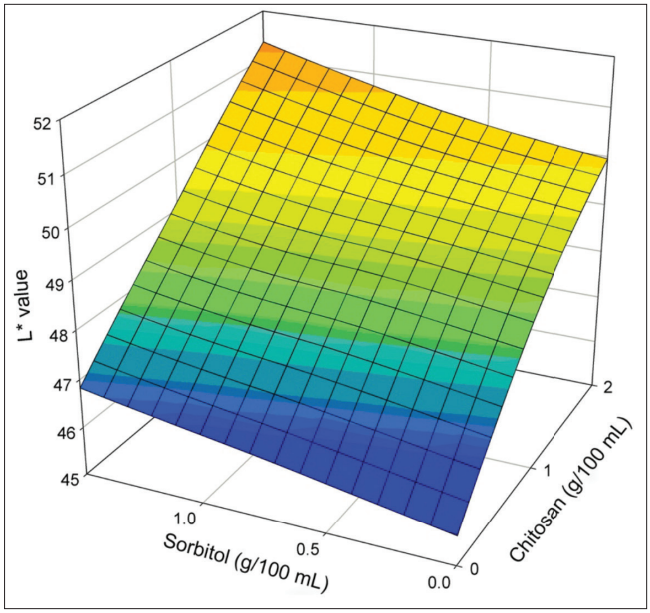


Fig 3. The effect of chitosan and sorbitol concentration on the L* value of chicken patties

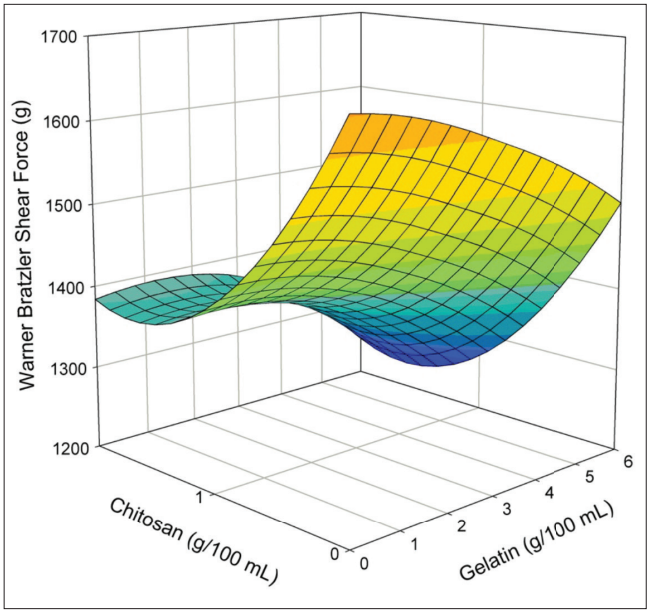


Fig 5. The effect of gelatin and chitosan concentration on the Warner Bratzler Shear Force value of chicken patties

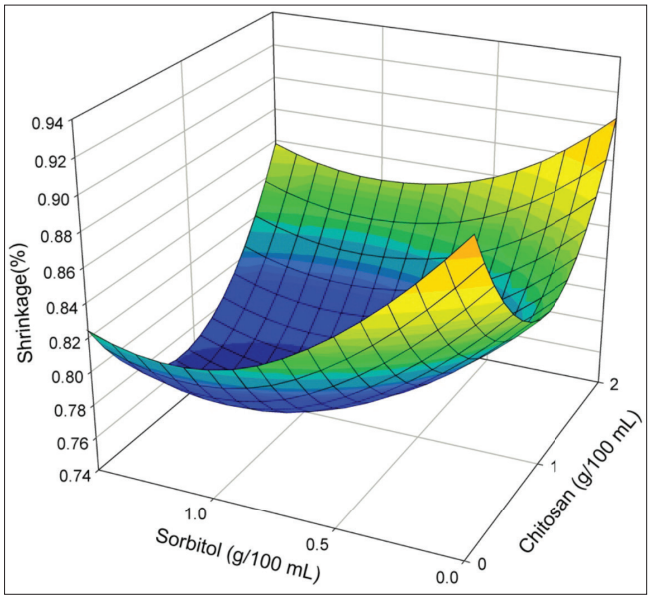


Fig 4. The effect of chitosan and sorbitol concentration on the shrinkage of chicken patties

Texture Properties of Chicken Patties

A significant (P<0.01) linear effect of chitosan concentration on the Warner Bratzler Shear Force (WBSF) of chicken patties was viewed *(Table 2)*. As observed *(Fig. 5)*, the increase of chitosan concentration reduced the WBSF of patties. This reduction was clearer until 1 g/100 mL chitosan concentration. The linear effects of gelatin (P<0.05) and chitosan concentration (P<0.01) on the Warner Bratzler cutting work of patties were found to be significant *(Table 2)*. Warner Bratzler cutting work decreased with increasing of chitosan.

In point of TPA parameters, a significant (P<0.01) linear effect of chitosan concentration on the hardness value of chicken patties was viewed *(Table 2)*. As observed *(Fig. 6)*, the increase of chitosan concentration reduced the hardness of chicken patties. The linear effects of chitosan on the adhesiveness, cohesiveness and chewiness values of patties were found to be significant (P<0.01; *Table 2*). The effects of independent variables are stated mathematically in *Table 3*. These predicted model equations are useful for understanding the effects of studied factors.

Discussion

The increase in the moisture of chicken patties can be referred to the positive effect of gelatin preventing the water loss from chicken patties. Cardoso et al.[12] also observed a reduction in weight loss in chitosan-gelatin coated beef steaks. Similar results have been stated also by Yu et al.[11] who reported that chitosan coating reduced the water loss of grass carp fillets.

The biggest effect on TBA values was found to be gelatin concentration. The decrease in TBA values could be explained by the positive effect of gelatin preventing the lipid oxidation by covering chicken patties. The minimum TBA value (0.18 mg/kg) was observed at the 15th treatment (6 g/100 mL gelatin, 2 g/100 mL chitosan, 0.75 g/100 mL sorbitol). The results showed us that the lipid oxidation reduced when the gelatin ratio is over 3 g/100 mL. During the frozen storage, lipid oxidation is one the most important factor resulting to the quality loss in meat products [23]. Similarly to our study, Morachis-Valdez et al.[23] reported a decrease of TBA value in chitosan coated carb. Farajzadeh et al.[24] stated also a decrease of TBA value in

Table 3. *Predicted model equations for the effects of independent variables on the physicochemical, technological and textural properties of chicken patties*

Parameters	Equations	P-Values
Moisture (g/100 g)	$Y = 54.54 + 0.07X_1 - 0.15X_2 - 0.09X^3 - 0.90X_1^2 + 1.39X_2X_1 - 0.06X_2^2 - 0.23X_3X_1 - 0.50X_3X_2 + 0.087X_3^2$	0.1668
TBA value (mg/kg)	$Y = 0.23 - 0.03X_1 - 0.02X_2 + 0.002X_3 + 0.006X_1^2 - 0.005X_2X_1 - 0.02X_2^2 + 0.01X_3X_1 - 0.003X_3X_2 - 0.01X_3^2$	0.0275
Peroxide value (mEq/kg)	$Y = 4.41 - 0.24X_1 + 0.46X_2 + 0.16X_3 - 0.79X_1^2 - 0.33X_2X_1 + 0.29X_2^2 + 0.09X_3X_1 + 0.06X_3X_2 - 0.12X_3^2$	0.2406
L* Value	$Y = 48.94 + 0.59X_1 + 2.19X_2 + 0.60X_3 - 0.49X_1^2 + 1.28X_2X_1 - 0.32X_2^2 - 1.09X_3X_1 + 0.38X_3X_2 + 0.05X_3^2$	0.0345
Shrinkage	$Y = 0.77 + 0.03X_1 + 0.007X_2 - 0.03X_3 - 0.01X_1^2 + 0.01X_2X_1 + 0.08X_2^2 - 0.01X_3X_1 + 0.09X_3X_2 + 0.04X_3^2$	0.3782
Warner Bratzler Shear Force (g)	$Y = 1397.08 - 72.42X_1 - 128.43X_2 + 56.72X_3 - 42.50X_1^2 + 34.22X_2X_1 + 102.77X_2^2 - 104.49X_3X_1 - 33.08X_3X_2 - 19.42X_3^2$	0.0679
WB Cutting Work (g.sec)	$Y = 15875.78 - 1315.96X_1 - 1387.68X_2 + 156.10X_3 - 642.08X_1^2 + 424.9X_2X_1 + 877.02X_2^2 - 497.25X_3X_1 - 132.49X_3X_2 + 97.54X_3^2$	0.0590
Hardness (g)	$Y = 2892.95 - 45.05X_1 - 269.35X_2 - 60.49X_3 + 53.73X_1^2 - 351.40X_2X_1 + 3.91X_2^2 + 27.008X_3X_1 + 111.39X_3X_2 - 57.07X_3^2$	0.1126

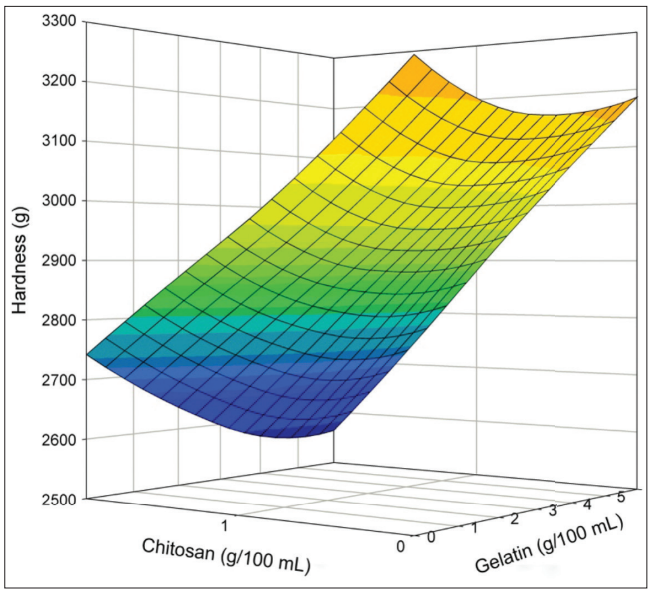


Fig 6. The effect of gelatin and chitosan concentration on the hardness of chicken patties

chitosan-gelatin coated shrimp. The function of gelatin coating as a barrier to oxygen could be explained by the hydrogen bonds in gelatin gel preventing against lipid oxidation [25]. Cardoso et al.[12] observed minimum TBARS values in beef steaks coated with a combination of gelatin and chitosan at higher gelatin concentration (>2%). Jeon et al.[26] reported also a decrease in TBA value of herring as a result of chitosan coating. Another work performed by Ojagh et al.[27], who observed that chitosan coated rainbow trout exhibited lower TBA value than untreated samples.

The positive effect of chitosan coating on the lightness of products was reported in some previous studies [13,24,26,28]. The color protection effect of chitosan on beef and pork meat was reported by Antoniewski et al.[29] and Herring et al.[25], respectively. The reduction in a* value with the increase of chitosan concentration could be related to the thickness of the film less translucent composed by higher polymer concentration [30].

The decreasing influence of sorbitol on the shrinkage value might be related to the plasticizer effect of sorbitol and to the high-moisture characteristic of chitosan coating. So, the loss of moisture from patties could be prevented, as well.

The decrease in hardness value of chicken patties might be due to the higher water retention of chicken patty with the increase of chitosan. The minimum hardness value (2095.68 g) was observed at the 15th treatment (6 g/100 mL gelatin, 2 g/100 mL chitosan, 0.75 g/100 mL sorbitol). Results about the texture properties showed that coating with chitosan can improve the texture properties such as hardness, adhesiveness, cohesiveness and chewiness in chicken patties during frozen storage. Similar findings were stated in the literature. Fang et al.[31] reported that chitosan coating of pork loins was capable to preserve the meat tenderness decreasing the shear force values during MAP storage. Chamanara et al.[32] showed that the hardness and springiness values of rainbow trout were decreased by chitosan coating. Benjakul et al.[33] reported also the decreasing effect of chitosan on the gel strength of surimi.

Overall, edible coating prepared by chicken gelatin, chitosan and sorbitol decreased effectively lipid oxidation, improved textural and technological properties for chicken patties during 4 months of frozen storage. RSM was used in order to optimize the coating solution formulation, and based on the predicted models, the best concentrations were determined as (6 g/100 mL) chicken gelatin, (1.5-2 g/100 mL) chitosan and (1-1.5 g/100 mL) sorbitol. On the other hand, with this study, two important poultry industry by-product such as chicken MDM residue and spent hen have been evaluated to some added value products.

Conflicts of Interest

There is no conflict of interest between authors.

Statement of Authors' Contributions

AE and ÖE designed the project. AE provided samples, performed gelatin extraction, prepared coating solution, prepared chicken patties and performed the analysis. ÖE performed statistical analysis of data and AE wrote the article. All authors read and approved the final manuscript.

References

1. Galus S, Kadzińska J: Food applications of emulsion-based edible films and coatings. *Trends Food Sci Technol*, 45 (2): 273-283, 2015. DOI: 10.1016/j.tifs.2015.07.011

2. Falguera V, Quintero JP, Jiménez A, Muñoz JA, Ibarz A: Edible films and coatings: Structures, active functions and trends in their use. *Trends Food Sci Technol*, 22 (6): 292-303, 2011, DOI: 10.1016/j.tifs.2011.02.004

3. Gomez-Guillen MC, Gimenez B, Lopez-Caballero ME, Montero MP: Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocoll*, 25 (8): 1813-1827, 2011. DOI: 10.1016/j.foodhyd.2011.02.007

4. Ramos M, Valdés A, Beltrán A, Garrigós MC: Gelatin-based films and coatings for food packaging applications. *Coatings*, 6 (4): 41, 2016. DOI: 10.3390/coatings6040041

5. López D, Márquez A, Gutiérrez-Cutiño M, Venegas-Yazigi D, Bustos R, Matiacevich S: Edible film with antioxidant capacity based on salmon gelatin and boldine. *LWT - Food Sci Technol*, 77, 160-169, 2017. DOI: 10.1016/j.lwt.2016.11.039

6. Nazmi NN, Isa MIN, Sarbon NM: Preparation and characterization of chicken skin gelatin/CMC composite film as compared to bovine gelatin film. *Food Biosci*, 19, 149-155, 2017. DOI: 10.1016/j.fbio.2017.07.002

7. Uranga J, Puertas AI, Etxabide A, Dueñas MT, Guerrero P, de la Caba K: Citric acid-incorporated fish gelatin/chitosan composite films. *Food Hydrocoll*, 86, 95-103, 2019. DOI: 10.1016/j.foodhyd.2018.02.018

8. Yeddes W, Djebali K, Wannes WA, Horchani-Naifer K, Hammami M, Younes I, Tounsi MS: Gelatin-chitosan-pectin films incorporated with rosemary essential oil: Optimized formulation using mixture design and response surface methodology. *Int J Biol Macromol*, 154, 92-103, 2020. DOI: 10.1016/j.ijbiomac.2020.03.092

9. Mohebi E, Shahbazi Y: Application of chitosan and gelatin based active packaging films for peeled shrimp preservation: A novel functional wrapping design. *LWT - Food Sci Technol*, 76, 108-116, 2017. DOI: 10.1016/j.lwt.2016.10.062

10. Elsabee MZ, Abdou ES: Chitosan based edible films and coatings: A review. *Mater Sci Eng C*, 33 (4): 1819-1841, 2013. DOI: 10.1016/j.msec.2013.01.010

11. Yu D, Li P, Xu Y, Jiang Q, Xia W: Physicochemical, microbiological, and sensory attributes of chitosan-coated grass carp *(Ctenopharyngodon idellus)* fillets stored at 4°C. *Int J Food Prop*, 20 (2): 390-401, 2017. DOI: 10.1080/10942912.2016.1163267

12. Cardoso GP, Dutra MP, Fontes PR, Ramos ALS, Gomide LAM, Ramos EM: Selection of a chitosan gelatin-based edible coating for color preservation of beef in retail display. *Meat Sci*, 114, 85-94, 2016. DOI: 10.1016/j.meatsci.2015.12.012

13. Feng X, Bansal N, Yang H: Fish gelatin combined with chitosan coating inhibits myofibril degradation of golden pomfret *(Trachinotus blochii)* fillet during cold storage. *Food Chem*, 200, 283-292, 2016. DOI: 10.1016/j.foodchem.2016.01.030

14. Thaker M, Hanjabam MD, Gudipati V, Kannuchamy N: Protective effect of fish gelatin-based natural antimicrobial coatings on quality of Indian salmon fillets during refrigerated storage. *J Food Process Eng*, 40 (1): e12270, 2017. DOI: 10.1111/jfpe.12270

15. Erge A, Zorba Ö: Optimization of gelatin extraction from chicken mechanically deboned meat residue using alkaline pre-treatment. *LWT*, 97, 205-212, 2018. DOI: 10.1016/j.lwt.2018.06.057

16. Parlak Ö, Zorba Ö, Kurt Ş: Modelling with response surface methodology of the effects of egg yolk, egg white and sodium carbonate on some textural properties of beef patties. *J Food Sci Technol*, 51 (4): 780-784, 2014. DOI: 10.1007/s13197-011-0552-4

17. Gökalp HY, Kaya M, Tülek Y, Zorba Ö: Quality Control of Meat and Meat Products. Laboratory Workbook, 5th ed., Erzurum, Turkey: Atatürk University, 2010.

18. AOAC: Official Methods of Analysis of the Association of Official Analytical Chemists'. Arlington, 2005.

19. Raharjo S, Sofos JN, Schmidt GR: Improved speed, specificity, and limit of determination of an aqueous acid extraction thiobarbituric acid-C18 method for measuring lipid peroxidation in beef. *J Agric Food Chem*, 40 (11): 2182-2185, 1992. DOI: 10.1021/jf00023a027

20. Serdaroğlu M, Değirmencioglu Ö: Effects of fat level (5%, 10%, 20%) and corn flour (0%, 2%, 4%) on some properties of Turkish type meatballs (koefte). *Meat Sci*, 68 (2): 291-296, 2004. DOI: 10.1016/j.meatsci.2004.03.010

21. García ML, Dominguez R, Galvez MD, Casas C, Selgas MD: Utilization of cereal and fruit fibres in low fat dry fermented sausages. *Meat Sci*, 60 (3): 227-236, 2002. DOI: 10.1016/S0309-1740(01)00125-5

22. Du L, Khiari Z, Pietrasik Z, Betti M: Physicochemical and functional properties of gelatins extracted from turkey and chicken heads. *Poult Sci*, 92 (9): 2463-2474, 2013. DOI: 10.3382/ps.2013-03161

23. Morachis-Valdez AG, Gómez-Oliván LM, García-Argueta I, Hernández-Navarro MD, Díaz-Bandera D, Dublán-García O: Effect of chitosan edible coating on the biochemical and physical characteristics of carp fillet *(Cyprinus carpio)* stored at -18°C. *Int J Food Sci*, 2017:2812483, 2017. DOI: 10.1155/2017/2812483

24. Farajzadeh F, Motamedzadegan A, Shahidi SA, Hamzeh S: The effect of chitosan-gelatin coating on the quality of shrimp *(Litopenaeus vannamei)* under refrigerated condition. *Food Control*, 67, 163-170, 2016. DOI: 10.1016/j.foodcont.2016.02.040

25. Herring JL, Jonnalongadda SC, Narayanan VC, Coleman SM: Oxidative stability of gelatin coated pork at refrigerated storage. *Meat Sci*, 85 (4): 651-656, 2010. DOI: 10.1016/j.meatsci.2010.03.019

26. Jeon YJ, Kamil JYVA, Shahidi F: Chitosan as an edible invisible film for quality preservation of herring and Atlantic cod. *J Agric Food Chem*, 50 (18): 5167-5178, 2002. DOI: 10.1021/jf011693l

27. Ojagh SM, Rezaei M, Razavi SH, Hosseini SMH: Effect of chitosan coatings enriched with cinnamon oil on the quality of refrigerated rainbow trout. *Food Chem*, 120 (1): 193-198, 2010. DOI: 10.1016/j.foodchem.2009.10.006

28. Arancibia MY, López-Caballero ME, Gómez-Guillén MC, Montero P: Chitosan coatings enriched with active shrimp waste for shrimp preservation. *Food Control*, 54, 259-266, 2015. DOI: 10.1016/j.foodcont.2015.02.004

29. Antoniewski MN, Barringer SA, Knipe CL, Zerby HN: Effect of a gelatin coating on the shelf life of fresh meat. *J Food Sci*, 72 (6): E382-E387, 2007. DOI: 10.1111/j.1750-3841.2007.00430.x

30. Petrou S, Tsiraki M, Giatrakou V, Savvaidis IN: Chitosan dipping or oregano oil treatments, singly or combined on modified atmosphere packaged chicken breast meat. *Int J Food Microbiol*, 156 (3), 264-271, 2012. DOI: 10.1016/j.ijfoodmicro.2012.04.002

31. Fang Z, Lin D, Warner RD, Ha M: Effect of gallic acid/chitosan coating on fresh pork quality in modified atmosphere packaging. *Food Chem*, 260, 90-96, 2018. DOI: 10.1016/j.foodchem.2018.04.005

32. Chamanara V, Shabanpour B, Gorgin S, Khomeiri M: An investigation on characteristics of rainbow trout coated using chitosan assisted with thyme essential oil. *Int J Biol Macromol*, 50 (3): 540-544, 2012. DOI: 10.1016/j.ijbiomac.2012.01.016

33. Benjakul S, Visessanguan W, Phatchrat S, Tanaka M: Chitosan affects transglutaminase-induced surimi gelation. *J Food Biochem*, 27, 53-66, 2003. DOI: 10.1111/j.1745-4514.2003.tb00266.x

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

Kafkas Univ Vet Fak Derg
27 (2): 173-181, 2021
DOI: 10.9775/kvfd.2020.25019

RESEARCH ARTICLE

Bm86 Genetic Diversity of Indigenous Tick Population from Punjab Province Pakistan

Muhammad Faiz RASOOL [1,a] Nisar AHMAD [1,b (*)] Haroon AKBAR [1,c] Tahir YAQUB [2,d] Kamran ASHRAF [1,e]
Aftab Ahmad ANJUM [2,f] Mian Abdul HAFEEZ [1,g] Muhammad Muddassir ALI [3,h]

[1] Department of Parasitology, University of Veterinary & Animal Sciences, Lahore, PAKISTAN
[2] Institute of Microbiology, University of Veterinary & Animal Sciences, Lahore, PAKISTAN
[3] Institute of Biochemistry and Biotechnology, University of Veterinary & Animal, Sciences, Lahore, PAKISTAN

ORCIDs: [a] 0000-0001-6818-7244; [b] 0000-0002-1614-0142; [c] 0000-0002-8294-5862; [d] 0000-0001-8698-3818; [e] 0000-0002-7619-429X
[f] 0000-0001-8262-8720; [g] 0000-0001-7796-9352; [h] 0000-0001-7930-7782

Article ID: KVFD-2020-25019 Received: 25.09.2020 Accepted: 25.01.2021 Published Online: 25.01.2021

Abstract

Ticks transmit a number of bacterial, protozoal and viral pathogens that cause many diseases like ehrlichiosis, hemorrhagic fever, theileriosis, babesiosis and anaplasmosis in livestock. This study was designed for molecular characterization of BM86 gene of *Rhipicephalus (Boophilus) microplus* tick. In this study, the BM86 gene was amplified, using primers flanked by restriction enzyme sites. The molecular detection of *R. microplus* was studied in three districts belonging to different ecological zones in the province of the Punjab, Pakistan. Tick samples were collected and initially screened through microscopy and further analyzed by PCR and sequencing. The phylogenetic tree was generated by using the MEGA 7 through Neighbor Joining method employing best model through the phylogenetic analysis of *R. microplus*. Pairwise comparisons of nucleotide sequences showed nucleotide differences ranging between 0.007 and 0.01%. Haplotype and nucleotide diversity in Bm86 gene was found among different districts. Six single nucleotide polymorphisms were seen in sequences of BM 86 from indigenous tick populations collected from the Punjab province. More interestingly, out of these 6 polymorphisms, we got 2 from district Okara, 3 from district Sahiwal, and 1 in Mandi Bahauddin. Conserved regions were observed among the local strains for BM86 gene. A common convergence in similar clade was with the local Pakistan *R. microplus*. Local mean diversity was 0.005 and overall mean diversity was 0.038. Field strain has been isolated as candidate specie for local tick vaccine, which in turn will increase the efficacy of future tick vaccine including reduction of economic burden on the farmer.

Keywords: *Rhipicephalus (Boophilus) microplus, BM86 gene, Molecular characterization*

Pakistan'ın Punjab Eyaletine Özgü Kene Popülasyonunun Bm86 Genetik Çeşitliliği

Öz

Keneler, çiftlik hayvanlarında erlişiyozis, hemorajik ateş, theileriozis, babeziozis ve anaplazmozis gibi birçok hastalığa neden olan bir dizi bakteriyel, protozoal ve viral patojeni bulaştırırlar. Bu çalışma, *Rhipicephalus (Boophilus) microplus* kenesinin BM86 geninin moleküler karakterizasyonu için tasarlanmıştır. Bu çalışmada, BM86 geni restriksiyon enzim bölgeleri ile çevrili primerler kullanılarak amplifiye edildi. *R. microplus*'un moleküler tespiti, Pakistan'ın Punjab eyaletindeki farklı ekolojik bölgelere ait üç yörede gerçekleştirildi. Kene örnekleri toplanıp ilk mikroskopik tarama yapıldıktan sonra, PCR ve sekans analizleri gerçekleştirildi. Filogenetik ağaç, *R. microplus*'un filogenetik analizi için en iyi model olan komşu birleştirme yöntemi kullanılarak MEGA 7 ile oluşturuldu. Nükleotid dizilerinin ikili karşılaştırmaları %0.007 ile %0.01 arasında değişen nükleotid farklılıkları olduğunu saptadı. Çalışılan farklı bölgelerde Bm86 geninde haplotip ve nükleotid çeşitliliği saptandı. Punjab eyaletinden toplanan lokal kene popülasyonunda BM86 geninde altı adet tek nükleotid polimorfizmi belirlendi. Daha da ilginci, bu 6 polimorfizmden 2'si Okara, 3'ü Sahiwal ve 1'i Mandi Bahauddin bölgesine aitti. Yerel suşlara ait BM86 geninde korunaklı bölgeler saptandı. Pakistan'ın lokal *R. microplus* suşlarında benzer sınıfta ortak bir kümelenme gözlendi. Lokal ortalama çeşitlilik 0.005 ve genel ortalama çeşitlilik 0.038 olarak saptandı. Lokal kene aşı aday türü olarak saha suşunun izolasyonu, çiftçiler üzerindeki ekonomik yükün azaltılması da dahil olmak üzere gelecekteki kene aşısının etkinliğini artıracaktır.

Anahtar sözcükler: *Rhipicephalus (Boophilus) microplus, BM86 geni, Moleküler karakterizasyon*

How to cite this article?

Rasool MF, Ahmad N, Akbar H, Yaqub T, Ashraf K, Anjum AA, Hafeez MA, Ali MM: Bm86 genetic diversity of indigenous tick population from Punjab Province Pakistan. *Kafkas Univ Vet Fak Derg,* 27 (2): 173-181, 2021.
DOI: 10.9775/kvfd.2020.25019

(*) Corresponding Author

Tel: +92 313 4408972
E-mail: drnisar65@uvas.edu.pk (N. Ahmad)

Introduction

Tick infestation not only causes pathogen transmission but also restlessness, stress induction and damage to hide quality [1,2]. Ticks transmit bacterial, protozoal and viral pathogens that cause many diseases like ehrlichiosis, hemorrhagic fever, theileriosis, babesiosis and anaplasmosis in livestock [3] likewise mediterranean spotted fever, lyme disease (borreliosis), Q fever, rocky mountain spotted fever, relapsing fever, babesiosis, congo fever and tick-borne encephalitis in humans [4]. Two major families of ticks are prevalent around the globe; first is Argasidae (soft ticks) having about 193 species, second is Ixodidae (hard ticks) with approximately 702 species [5]. Infestation with hard tick *R. microplus* economically impacts the livestock and causes huge losses [6]. Tick infestation significantly reduces milk and meat production [7] which is major contributor in overall cost associated with tick borne diseases [8-10]. Kivaria [11] reported an annual loss of $364M with approximate 1.3 M mortality in cattle due to tick borne diseases.

During 2019-20, Pakistan exported Rs. 13644 M leather and leather products, 18,139M hides [12].

The cattle and buffalo population in Pakistan are at risk of tick infestation throughout the year, although very little is known about the biology, diversity and distribution of tick species across different agro-ecological zones (AEZ) of the country [13]. The occurrence and prevalence of tick-borne pathogens (TBPs) in bovines have been reported from different parts of Pakistan [14-16]. In north east Khyber Pakhtunkhwa (KPK) and Gilgit Baltistan, the overall prevalence of tick infestation in livestock was 75.03% [17] while in northwest of KPK it was 77.9% [18]. The overall prevalence in Punjab (farm animals) was 36.52% [19], point prevalence in south Punjab was 75.1% [20] while in Sargodha, Khushab and Rawalpindi districts it was 54.76% [21].

In Pakistan, ticks are found frequently during mid-April to September, principally the summer season. Ticks prefer to bind on animals at neck, around and inside ear, udder region and inner side of thighs [22]. Traditionally many practices have been observed in Pakistan to control the ticks including burning of grass and temporary sheds but mostly it is through use of injectable ivermectins and use of acaricides [23]. Acaricides are being used in the form of spray and dip but this activity is not feasible due to presence of residual effects in food and water, and of high cost [24]. In recent studies, resistance to acaricides has also been reported [25-30]. Therefore vaccination may be considered as best alternate to overcome this problem. In this regard, BM 86 is considered as candidate gene for vaccine production against *Rhipicephalus (Boophilus) microplus.* BM 86 derived vaccines cause reduction in weight of blood engorged female ticks, reduction in egg mass weight, reduction in tick population in the field over one generation, a significant declined reproductive efficacy of *R. microplus* females, reduced rate of treatments with acaricides and a helpful addition for integrated control programs [31]. In silico analysis of BM86 gut glycoprotein showed that it has antigenic epitopes, one of which has showed more than 80% efficacy in vaccinated cattle against *R. microplus* [32]. Advanced and well planned strategies will be beneficial to achieve the goals for controlling ticks in meat and dairy animals [33].

Keeping in view the economic importance of tick born infestations, present study was designed for molecular characterization, phylogenetic association, haplotype diversity and nucleotide diversity of BM86 gene of *R. microplus* tick, a local strain for future vaccine production to reduce the cost of tick control measures in livestock.

Material and Methods

Study Plan

The three different ecological regions from where ticks were sampled are from district Okara (representative district of Northern irrigated agro-ecological zone), district Sahiwal (representative district of Northern irrigated agro-ecological zone) and district Mandi Bahauddin (representative district at junction of barani lands and Northern irrigated agro-ecological zone) in the Punjab province, Pakistan. These areas were selected due to presence of favorable climate condition for ticks and high population of dairy animals. Total livestock population in these three districts is estimated to be 1.09 million cattle (local, cross bred and exotic breeds), 0.36 million buffaloes, 0.36 million sheep and 1.4 million goats.

Collection of Ticks

Tick collection was performed systematically according to the recommended procedures [20]. Ticks collected were placed in falcon tubes. The tick samples were dispatched to Parasitology laboratory in clean and properly labeled plastic containers. The outer covering of these containers was covered by cheese cloth. The collected ticks were characterized microscopically on the basis of morphology with the help of key described by [34] and [35].

RNA Extraction, cDNA Synthesis & Polymerase Chain Reaction

Total RNA was extracted from the mid gut of female ticks, by using a Trizol reagent (Sigma) according to the given instructions. The extracted RNA was amplified by reverse transcription polymerase chain reaction (RTPCR). The cDNA strand synthesis reaction was performed using a cDNA Synthesis Kit (Amersham, UK), following the manufacturer's instructions. All RNA samples were quantified by nano drop method, 2 µL distilled water was used to calibrate blank then 2 µL sample loaded and reading was noted.

Reverse Transcriptase PCR was performed for synthesis of cDNA with the following method.

Total extracted RNA (5 µg) was taken and cDNA was prepared by adding 1 µL oligo dT and 1 µL 10 mMdNTPs to RNA. Total volume of 10 µL was obtained by adding distilled water. After proper mixing, it was heated at 65°C for five minutes. The sample was chilled hurriedly in ice for 2 min. Then it was micro centrifuged to get the solution to the bottom. RNA mixture was placed on ice while preparing the reaction mixture. In reaction mixture 2 µL 10X RT, 4 µL 25 mM $MgCl_2$, 2 µL 0.1M DTT and 1 µL RNase were added to the reaction mixture. It was softly mixed; a quick spin was applied to collect the mixture in the tube. Sample was incubated at 42°C for 2 min. 1 µL SS II Reverse Transcriptase (was kept on ice for the entire time) was added. Sample was incubated at 42°C for 50 min. Heat inactivation of the enzyme at 65°C to 70°C for 15 min was performed. The sample was kept on ice for 5 min and mixed gently. After that 1 µL RNase H was added to the reaction. The sample was incubated at 37°C for 20 min. The sample was micro centrifuged and obtained PCR products were examined through electrophoresis in a 1.0% agarose. PCR reaction was carried out by using BM 86 primers *(Table 1)* in a 25 µL volume in a thermal cycle(PCR-G-Storm Thermocycler–AG1972).

Conditions of PCR

- Heated Lid at 112°C
- Initial temperature at 94°C for 4 min
- Start Cycle
- Denaturation at 94°C for 30 sec
- Annealing at 59.4°C for 30 sec
- Extension at 72°C for 45 sec

} X 35 cycles

- End Cycle
- Last extension at 72°C for 10 min
- Store

Isolation of cDNA Product by Gel DNA Extraction Kit

cDNA band was separated from agarose gel with the help of sharp scalpel. It was transferred in a tube. For experiment, 300 µL of the agarose was used (as per manufacturer`s instructions). Re-suspended the Silica Suspension (Vial 1) until a homogeneous suspension was obtained. Then silica suspension amounting 10 µL was added in the sample. Ten min incubation was observed at 60°C along with vortexing after every 3 min. Centrifugation at 15000 rpm was applied for 40 seconds to samples and upper layer was discarded. cDNA containing matrix was taken in 500 µL Nucleic Acid Binding Buffer (Vial 3, green cap) and it was vortexed. Again, centrifuged and upper layer was discarded. The obtained pellet was briefly washed with washing buffer. For elution of cDNA, distilled water with pH 8 was used. To increase the elution effectiveness, more volume of elution buffer was taken. Then it was vortexed and 10 min incubation was given at 56°C then transferred the cDNA-containing solution to a new reaction. PCR products of *R. microplus* were sequenced along with the primers.

Table 1. *Primers used to target BM86 gene of R. microplus ticks*

BM 86 Primers	Sequence (5'->3')
F	ACGAGTGTTCTAGGGAGCCT
R	TGCGGTGACTGAAGTAGCTG

Primers were designed with bioinformatics tools by using reference sequence: KJ995910.1 Accession Number

Sequencing and Phylogenetic Analysis of Rhipicephalus (Boophilus) microplus BM86

One hundred and ten samples were analyzed by PCR. 18 PCR products of *Rhipicephalus (Boophilus) microplus* were sequenced along with the primers used in PCR. The Accession numbers are BankIt2333962 Seq1, MT344675 and BankIt2333962 Seq2 MT344676 *(https://www.ncbi.nlm.nih.gov/Genbank/update.html)*. Applied biosystems Genetic analyzer 3130 was used for sequencing at Centre of Excellence in Molecular Biology Lahore, Pakistan. The quality of the sequences was analyzed through Geneious software. Consensus sequences obtained from Geneious software were aligned with MUSCLE software and further confirmed manually by using MESQUITE software. Finally, the phylogenetic tree was generated by using the MEGA 7 *(Table 2)* through Neighbor Joining method. For phylogenetic tree, best model was selected showing the phylogenetic analysis of *R. microplus* through MEGA 7.0.

Analysis of Haplotype and Nucleotide Diversity

Genetic variability of Bm86 gene sequences was further analyzed in *R. microplus* through nucleotide and haplotype diversity. The number and the values for these diversities for each district were further calculated through software DnaSP 5.10 [36] *(http://www.ub.edu/dnasp/DnaSP_OS.html)*.

Results

Molecular Characterization of BM86

For the confirmation of 248 bp of BM86 gene, PCR products were run in 1.5% agarose gel marked with SYBER green safe dye and observed under the Ultra-violet light to declare positive against 100 bp ladder, as shown in *Fig. 1*.

Pairwise comparisons of nucleotide sequences showed nucleotide differences ranging between 0.007 and 0.01% *(Table 3)*. Furthermore, a comparison of BM 86 gene sequences determined herein and our sequences revealed that sequences as mentioned in *Table 2* from other parts of world were identical to BM 86 gene sequences.

Phylogenetic Analysis of Rhipicephalus (Boophilus) microplus Based on Maximum Likelihood Method

The presented phylogenetic analysis were carried out by

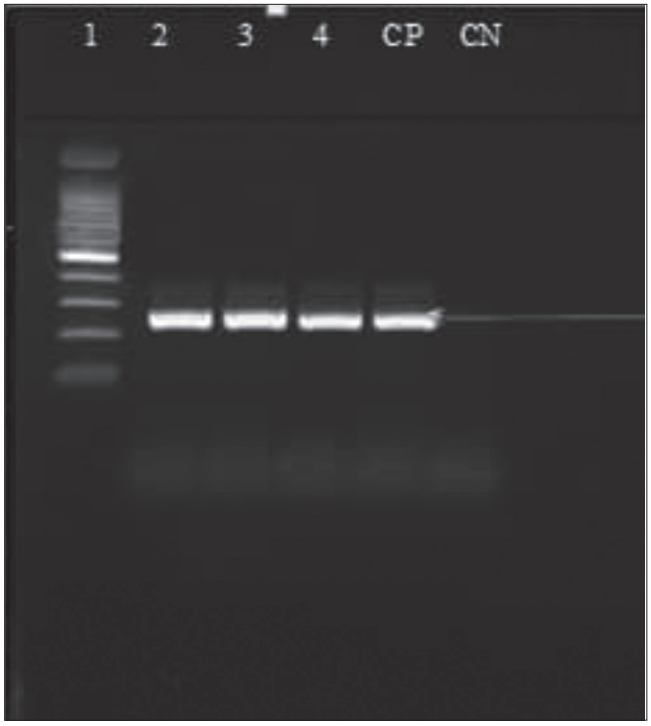


Fig 1. PCR results show the amplification of 248 bp. Lane 1 indicates 100bp ladder, lane 2-4 positive sample of *Rhipicephalus (Boophilus) microplus*. Lane CP is positive control while Lane CN negative control

Maximum likelihood method [37]. Preliminary tree for experimental search were got spontaneously by Neighbor join and BioNJ algorithms to a matrix of pairwise distances projected using the Maximum Composite Likelihood approach, trophy was selected with higher log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Twenty nine nucleotide sequences were analyzed. Gap positions and missing nucleotides were eradicated. There were a total of 1757 positions in the final dataset. Phylogenetic association showed the close resemblance of studied samples with the local strains of Pakistan, whereas it has also showed association with strains of Thailand. A common divergence point was observed with the local Pakistan *R. microplus* population with that of *R. annulatus* BA86 Israel. Other clades had more convergence for *R. microplus* BM86 gene from the USA, Spain, Australia, South Africa *(Fig. 2)*. Sequence alignment of current study revealed the conserved regions of studied gene with polymorphism among local population.

Evolutionary Divergence Between Sequences Estimates

The base replacements numbers per site from amongst sequences is presented. Twenty-nine nucleotides were part of analysis. All gaps containing positions including missing data were rejected. A total of 1757 positions were shown in the final dataset. And evolutionary analysis was carried out in MEGA7.

Local mean diversity = 0.005
Overall mean diversity = 0.038

Table 2. *Sequence identical to other parts of world*

Sr. No	Country	Accession Number	Gene
1	Thailand	GenBankKJ995910.1	*R. microplus*_NE14
2	Thailand	GenBank KJ995907.1	*R. microplus*_NE11
3	USA	HQ014394.1	*R. microplus* _ Zapata
4	USA	HQ014392.1	*R. microplu s*_ Zapata
5	USA	KX786647.1	*R. microplus*_Bm86
6	USA	M29321.1	*B. microplus*_BM86
7	South Africa	FJ809946.1	*R. microplus*_Bm86
8	Spain	FJ456928.1	*R. microplus*_bm86
9	Spain	EU191620.1	*R. microplus*_BM86
10	Spain	EU191620.1	*R. microplus*_BM86
11	Brazil	EU352677.1	*R. microplus*_BM86
12	Brazil	EU352677.1	*R. microplus*_BM86
13	Australia	MG002399.1	*R. microplus*_Bm86
14	Australia	MG002403.1	*R. microplus*_Bm86
15	Australia	MG002401.1	*R. microplus*_Bm86
16	Spain	EU191620.1	*R. microplus*_BM86
17	Spain	EU191620.1	*R. microplus*_BM86
18	Netherland	FJ809946.1	*R. microplus*_Bm86
19	Netherland	FJ809946.1	*R. microplus*_Bm86
20	Netherland	GU144589.1	*R. microplus*_bm86
21	India	DQ131539.1	*R. microplus*_BM86
22	India	DQ131539.1	*R. microplus*_BM86

Haplotype and Nucleotide Diversity

Haplotype and nucleotide diversities were found among different districts in Bm86 sequenced gene of *R. microplus*. Lowest haplotype diversity (0.88) was found in Okara district and highest (0.91) was found in Mandi Bahaudin district. Whereas, highest (0.012) nucleotide diversity was observed in Sahiwal district and lowest (0.010) was revealed in Mandi Bahaudin district *(Table 4)*.

Discussion

The present study provides the insights for the presence of *R. microplus* from bovines in three districts of Punjab, Pakistan and it confirms the already reported studies from Pakistan [16,38-44]. The tick-borne parasites i.e. *B. bovis* and *B. bigemina* are known to cause bovine babesiosis posing serious threat to the livestock health around the world [45-49]. In Indo-Pakistan, bovine ticks are frequently being diagnosed through classical methods i.e. microscopic observation of morphological features [22].

Genetic data shows that *R. microplus* is found in Thailand, USA, South Africa, Spain, Brazil, Australia, Netherland, India and many other parts of the world; this specie is assumed to be evolved from Asia and have been distributed mainly with cattle in all continents [50]. Previous studies on Phylogenetic

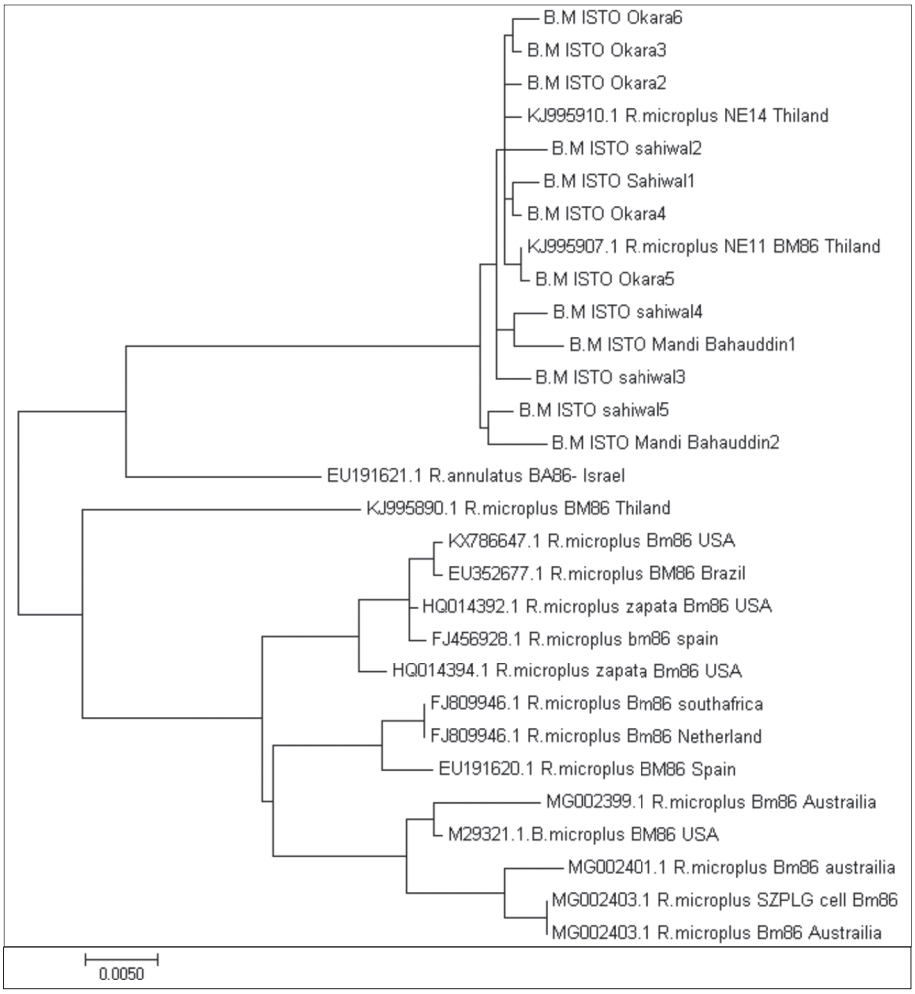


Fig 2. Phylogenetic analysis of *R. microplus* based on Maximum Likelihood method, comparison of BM 86 of local strain with other parts of world, identical to BM 86 gene sequences

analysis [51] show that *R. microplus* is present in every country of the Neotropical region apart from Chile [52] and has been reported to West Africa recently, probably from Brazil with Girolando cattle where it is supposed to be transferring local species of the alike subgenus [53]. It is supposed that East and Southern Africa got *R. microplus* from Asia, probably using the route of Madagascar [34]. Dispersed in savanna, including southern coastal strip of Kenya to the Cape Province of South Africa, as well as in Madagascar [35]. However all records for *R. microplus* including genetic, phenotypic and crossing studies with other *R. microplus* complicated taxa are desirable to check the findings [51].

The molecular-phylogenetic analysis revealed that *R. microplus* sequences are grouped into different clades with local mean diversity 0.005 and overall means diversity 0.038. Similar comments have been given in many renowned studies [54-60]. This study identified highly conserved regions with the help of alignment of sequences of BM86 gene from different isolates. Based on these findings, fully engorged female *R. microplus* ticks can be used to isolate cDNA encoding using transcriptomic studies on gut tissues [61]. Tick infestation has been significantly controlled by using *R. microplus* Bm86 antigen. But additional work related to tick-protective antigens is required to see the variable performance of BM 86 based vaccine due to local strain variation. Candidate protective antigens can be regarded as potential priority in vaccination studies based on gene knockdown on tick mortality and fertility. Though the evaluation of vaccine is subject to large scale field trials of recombinant antigens [62].

The current study confirmed that built on Bm86, R. microplus populations were homogeneous and showed high convergence among the different isolates studied by De la Fuente et al.[63], Sossai et al.[64], Guerro et al.[61], Canales et al.[65], Nijhof et al.[66], Freeman et al.[67]; strains of *R. microplus*-Thailand [68]; and Bm86 orthologs of *R. microplus,* such as *R. annulatus* (ABY58969) and *R. decoloratus* (ABY58970, ABG21130, ABG21131). Consequently, these results polymorphic sites peptides are more conserved whey they are compared with the Bm86 protein sequence.

Kamau et al.[69] described that cDNA of 4 *R. appendiculatus*

Table 3. *Estimates of Evolutionary Divergence between sequences*

Sr. No.	BM 86 Gene Sequences	Pairwise Comparison																											
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	MG002403.1_R.microplus_SZPLG_cell_Bm86																												
2	MG002403.1_R.microplus_Bm86_Austrailia	0																											
3	MG002401.1_R.microplus_Bm86_austrailia	0.007	0.007																										
4	MG002399.1_R.microplus_Bm86_Austrailia	0.018	0.018	0.019																									
5	M29321.1.B.microplus_BM86_USA	0.012	0.012	0.013	0.008																								
6	KX786647.1_R.microplus_Bm86_USA	0.029	0.029	0.03	0.029	0.022																							
7	KJ995910.1_R.microplus_NE14_Thiland	0.058	0.058	0.059	0.062	0.055	0.059																						
8	KJ995907.1_R.microplus_NE11_BM86_Thiland	0.06	0.06	0.061	0.064	0.057	0.061	0.002																					
9	KJ995890.1_R.microplus_BM86_Thiland	0.05	0.05	0.051	0.049	0.042	0.043	0.047	0.048																				
10	HQ014394.1_R.microplus_zapata_Bm86_USA	0.028	0.028	0.029	0.028	0.021	0.007	0.054	0.056	0.036																			
11	HQ014392.1_R.microplus_zapata_Bm86_USA	0.026	0.026	0.027	0.026	0.019	0.003	0.056	0.058	0.04	0.006																		
12	FJ809946.1_R.microplus_Bm86_southafrica	0.028	0.028	0.029	0.029	0.022	0.018	0.059	0.061	0.042	0.02	0.019																	
13	FJ809946.1_R.microplus_Bm86_Netherland	0.028	0.028	0.029	0.029	0.022	0.018	0.059	0.061	0.042	0.02	0.019	0																
14	FJ456928.1_R.microplus_bm86_spain	0.027	0.027	0.028	0.027	0.02	0.003	0.056	0.058	0.04	0.006	0.002	0.021	0.021															
15	EU352677.1_R.microplus_BM86_Brazil	0.029	0.029	0.03	0.029	0.022	0.001	0.059	0.061	0.043	0.007	0.003	0.018	0.018	0.003														
16	EU191621.1_R.annulatus_BA86-_Israel	0.045	0.045	0.046	0.051	0.043	0.042	0.04	0.04	0.043	0.037	0.039	0.043	0.043	0.041	0.042													
17	EU191620.1_R.microplus_BM86_Spain	0.027	0.027	0.027	0.03	0.023	0.019	0.059	0.061	0.043	0.02	0.02	0.006	0.006	0.021	0.019	0.043												
18	B.M_ISTO_sahiwal5	0.06	0.06	0.061	0.064	0.057	0.061	0.005	0.005	0.048	0.056	0.058	0.061	0.061	0.058	0.061	0.04	0.061											
19	B.M_ISTO_sahiwal4	0.063	0.063	0.064	0.067	0.06	0.063	0.005	0.004	0.05	0.059	0.06	0.064	0.064	0.061	0.063	0.043	0.064	0.007										
20	B.M_ISTO_sahiwal3	0.06	0.06	0.061	0.064	0.057	0.061	0.004	0.004	0.049	0.056	0.058	0.061	0.061	0.058	0.061	0.04	0.061	0.006	0.006									
21	B.M_ISTO_sahiwal2	0.062	0.062	0.063	0.066	0.059	0.063	0.004	0.004	0.05	0.058	0.06	0.063	0.063	0.06	0.063	0.042	0.063	0.005	0.007	0.006								
22	B.M_ISTO_Sahiwal1	0.061	0.061	0.062	0.066	0.058	0.062	0.003	0.003	0.049	0.058	0.059	0.062	0.062	0.059	0.062	0.041	0.063	0.006	0.006	0.005	0.004							
23	B.M_ISTO_Okara6	0.061	0.061	0.062	0.066	0.058	0.062	0.003	0.003	0.05	0.058	0.059	0.062	0.062	0.059	0.062	0.041	0.063	0.006	0.006	0.005	0.005	0.005						
24	B.M_ISTO_Okara5	0.061	0.061	0.062	0.065	0.058	0.061	0.003	0.001	0.049	0.057	0.058	0.061	0.061	0.059	0.061	0.04	0.062	0.005	0.005	0.005	0.005	0.004	0.004					
25	B.M_ISTO_Okara4	0.06	0.06	0.061	0.064	0.057	0.061	0.002	0.002	0.048	0.056	0.058	0.061	0.061	0.058	0.061	0.04	0.061	0.005	0.005	0.004	0.004	0.002	0.003	0.003				
26	B.M_ISTO_Okara3	0.06	0.06	0.061	0.064	0.057	0.061	0.002	0.002	0.048	0.056	0.058	0.061	0.061	0.058	0.061	0.04	0.061	0.005	0.005	0.004	0.004	0.003	0.002	0.003	0.002			
27	B.M_ISTO_Okara2	0.06	0.06	0.061	0.064	0.057	0.061	0.002	0.002	0.048	0.056	0.058	0.061	0.061	0.058	0.061	0.04	0.061	0.005	0.005	0.004	0.004	0.003	0.003	0.003	0.002	0.002		
28	B.M_ISTO_Mandi_Bahauddin2	0.063	0.063	0.064	0.067	0.06	0.064	0.007	0.007	0.05	0.059	0.061	0.064	0.064	0.061	0.064	0.043	0.064	0.006	0.009	0.008	0.009	0.009	0.009	0.008	0.007	0.007	0.007	
29	B.M_ISTO_Mandi_Bahauddin1	0.063	0.063	0.064	0.069	0.061	0.065	0.006	0.006	0.053	0.061	0.062	0.064	0.064	0.063	0.065	0.041	0.064	0.008	0.006	0.007	0.008	0.006	0.007	0.007	0.005	0.006	0.006	0.01

The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 29 nucleotide sequences out of which 13 were from current study. All positions containing gaps and missing data were eliminated. There were a total of 1757 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

Table 4. *Nucleotide and haplotype diversity of BM86 gene sequence analysis in R. microplus from different districts of Pakistan*

Districts	Observed Haplotypes in Number	Unique Haplotypes in Number	Diversity Haplotype (Bm86 gene)	Diversity of Nucleotide
Okara	5	2	0.88	0.011
Sahiwal	4	3	0.89	0.012
Mandi Bahauddin	3	1	0.92	0.010

field strains depicted genotypic polymorphisms and recommended that additional aspects like exposure during blood meal with innate immune components may be responsible for selection pressure which led to the observed polymorphism in these samples [64,70].

Various other studies reported the genotypic variations at molecular level between *B. microplus* isolates. To characterize habitat adoption and variant speciation in tick require more information following biogeographical separation among these species. Besides molecular approaches, morphological and physiological studies provide important information to achieve such goals which leads to the selection of useful tick antigens for anti-tick vaccine [63].

Considering the extensive sequence and functional polymorphism observed among strains of *R. microplus* from different geographical regions, we can conclude that it may be possible to achieve effective vaccination against these cattle ticks using a single universal Bm86-based antigen. With the advancement in genomic technologies in vaccine development sequencing of tick genome may help in identification of candidate tick strains for global application of anti-tick vaccine useful against different species of ticks [70].

Six single nucleotide polymorphisms were seen in sequences of BM 86 from indigenous tick population collected from the Punjab province. More interestingly out of these 6 polymorphism, we got 2 from district Okara (representative district of Northern irrigated Agro-ecological zone), 3 from Sahiwal (representative district of Northern irrigated Agro-ecological zone), and 1 in Mandi Bahauddin (representative district of junction of barani lands and Northern irrigated Agro ecological zone). Association of unique sequences to a particular geographical region supports the hypothesis of phylogeography proposed by different authors [71-73].

To our best knowledge this is only type of such study conducted in the area which identified the field strain as candidate specie for local tick vaccine, which in turn will increase the efficacy of tick control including reduction of economic burden on the farmer. New studies must concentrate on analyzing ticks in domestic and wild ruminants, covering diverse environmental areas of Pakistan and assessing the impact of diverse factors on its occurrence. Further research employing latest tools of genomics, proteomics and transcriptomics are necessary to depict the tick vector(s) involved in the spread of protozoal infections in Pakistan.

Author's Contribution

Higher Education Commission of Pakistan provided the financial assistance to Dr. M. Faiz Rasool for this research study, Dr. Nisar Ahmad, Dr. Haroon Akbar, Prof. Dr. Tahir Yaqub and Prof. Dr. Kamran Ashraf supervised this research study and Dr. M. Faiz Rasool is responsible for the provision of integration and writing of the article data. Acknowledgments to Prof. Dr. Aftab Ahmad Anjum, Dr. Muhammad Muddassir Ali and Dr. Mian Abdul Hafeez for their help in optimization of PCR, sequence analysis and estimation of genetic diversity.

Conflict of Interest

Authors declare no conflict of interest.

REFERENCES

1. Chaudhry ZI, Saiddain A, Sabir N, Malik NA, Azeem S, Sajid A: Prevalence of pathological conditions causing skin damage and consequently reducing its market value in domestic ruminants of Punjab, Pakistan. *Vet Sci Dev,* 1:e4, 2011. DOI: 10.4081/vsd.2011.2444

2. Saleem MZ, Akhtar R, Aslam A, Rashid MI: Histopathological investigation of skin and hides damage of small and large ruminants due to naturally infested ticks. *Trop Biomed,* 36 (4): 1081-1086, 2019.

3. de La Fuente J, Antunes S, Bonnet S, Cabezas-Cruz A, Domingos AG, Estrada-Peña A, Johnson N, Kocan KM, Mansfield KL, Nijhof AM, Papa A, Rudenko N, Villar M, Alberdi P, Torina A, Ayllon N, Vancova M, Golovchenko M, Grubhoffer L, Caracappa S, Fooks AR, Gortazar C, Rego ROM: Tick-pathogen interactions and vector competence: Identification of molecular drivers for tick-borne diseases. *Front Cell Infect Microbiol,* 7:114, 2017. DOI: 10.3389/fcimb.2017.00114

4. Dantas-Torres F, Chomel BB, Otranto D: Ticks and tick-borne diseases: A one health perspective. *Trends Parasitol,* 28 (10): 437-446, 2012. DOI: 10.1016/j.pt.2012.07.003

5. Guglielmone AA, Robbins RG, Apanaskevich DA, Petney TN, Estrada-Pena A, Horak IG, Shao R, Barker SC: The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: A list of valid species names. *Zootaxa*, 2528, 1-28, 2010. DOI: 10.5281/zenodo.196488

6. Jabbar A, Abbas T, Sandhu ZD, Saddiqi HA, Qamar MF, Gasser RB: Tick-borne diseases of bovines in Pakistan: major scope for future research and improved control. *Parasit Vectors,* 8:283, 2015. DOI: 10.1186/s13071-015-0894-2

7. Jonsson NN, Mayer DG, Matschoss AL, Green PE, Ansell J: Production effects of cattle tick *(Boophilus microplus)* infestation of high yielding dairy cows. *Vet Parasitol,* 78 (1): 65-77, 1998. DOI: 10.1016/S0304-4017(98)00118-6

8. Jonsson N, Davis R, De Witt M: An estimate of the economic effects of cattle tick *(Boophilus microplus)* infestation on Queensland dairy

farms. *Aust Vet J,* 79 (12): 826-831, 2001. DOI: 10.1111/j.1751-0813.2001.tb10929.x

9. Narladkar BW: Projected economic losses due to vector and vector-borne parasitic diseases in livestock of India and its significance in implementing the concept of integrated practices for vector management. *Vet World,* 11 (2): 151-160, 2018. DOI: 10.14202/vetworld.2018.151-160

10. Pratap B, Jha A: Economic losses due to various constraints in dairy production in India. *Indian J Anim Sci,* 75 (12): 1470-1475, 2005.

11. Kivaria FM: Estimated direct economic costs associated with tick-borne diseases on cattle in Tanzania. *Trop Anim Health Prod,* 38 (4): 291-299, 2006. DOI: 10.1007/s11250-006-4181-2

12. Finance Division GoP: Economic Survey of Pakistan. 2019-2020 2020. Report No. Retrieved from: https://download1.fbr.gov.pk/Docs/20206121964516543PakistanEconomicSurvey2019-20.pdf; *Accessed:* 20.12.2020

13. Ghafar A, Gasser RB, Rashid I, Ghafoor A, Jabbar A: Exploring the prevalence and diversity of bovine ticks in five agro-ecological zones of Pakistan using phenetic and genetic tools. *Ticks Tick Borne Dis,* 11 (5): 101472, 2020. DOI: 10.1016/j.ttbdis.2020.101472

14. Ghafar A, Cabezas-Cruz A, Galon C, Obregon D, Gasser RB, Moutailler S, Jabbar A: Bovine ticks harbour a diverse array of microorganisms in Pakistan. *Parasit Vectors,* 13:1, 2020. DOI: 10.1186/s13071-019-3862-4

15. Akhtar K, Anees R, Karim T, Gul SU, Rehman HU, Ali A, Wazir MI, Khan F, Achakzai SK: Prevalence of tick infestation in cows of various Regions of district Karak, Pakistan. *J Entomol Zool Stud*, 7 (2): 791-795, 2019.

16. Karim S, Budachetri K, Mukherjee N, Williams J, Kausar A, Hassan MJ, Adamson S, Dowd SE, Apanskevich D, Arijo A, Sindhu ZU, Kakar MA, Khan RMD, Ullah S, Sajid MS, Ali A, Iqbal Z: A study of ticks and tick-borne livestock pathogens in Pakistan. *PLoS Negl Trop Dis,* 11 (6): e0005681, 2017. DOI: 10.1371/journal.pntd.0005681

17. Sajid MS, Iqbal Z, Shamim A, Siddique RM, HASSAN MJU, Rizwan HM: Distribution and abundance of ticks infesting livestock population along Karakorum highway from Mansehra to Gilgit, Pakistan. *J Hellenic Vet Med Soc,* 68 (1): 51-58, 2018. DOI: 10.12681/jhvms.15556

18. Zeb J, Shams S, Ayaz S, Din IU, Khan A, Adil N, Ullah H, Raza A: Epidemiology of ticks and molecular characterization of *Rhipicephalus microplus* in cattle population in North-Western Pakistan. *Int J Acarol,* 46 (5): 335-343, 2020. DOI: 10.1080/01647954.2020.1775294

19. Batool M, Nasir S, Rafique A, Yousaf I, Yousaf M: Prevalence of tick infestation in farm animals from Punjab, Pakistan. *Pak Vet J,* 39 (3): 406-410, 2019. DOI: 10.29261/pakvetj/2019.089

20. Sajid MS, Iqbal Z, Khan MN, Muhammad G: Point prevalence of hard ticks (Ixodids) infesting domestic ruminants of lower Punjab, Pakistan. *Int J Agric Biol,* 10 (3): 349-351, 2008.

21. Atif F, Khan M, Iqbal H, Ali Z, Ullah S: Prevalence of cattle tick infestation in three districts of the Punjab, Pakistan. *Pak J Sci,* 64 (1): 49-53, 2012.

22. Ali Z, Maqbool A, Muhammad K, Ashraf K, Khan MS: Prevalence and distribution of hard ticks in cattle and buffaloes. *J Vet Parasitol,* 24 (1): 67-70, 2010.

23. Jabbar A, Abbas T, Sandhu ZD, Saddiqi HA, Qamar MF, Gasser RB: Tick-borne diseases of bovines in Pakistan: major scope for future research and improved control. *Parasit Vectors,* 8:283, 2015. DOI: 10.1186/s13071-015-0894-2

24. Benelli G, Pavela R, Canale A, Mehlhorn H: Tick repellents and acaricides of botanical origin: A green roadmap to control tick-borne diseases? *Parasitol Res,* 115 (7): 2545-2560, 2016. DOI: 10.1007/s00436-016-5095-1

25. George J, Pound J, Davey RB: Chemical control of ticks on cattle and the resistance of these parasites to acaricides. *Parasitology,* 129, S353-S366, 2004. DOI: 10.1017/s0031182003004682

26. Abbas RZ, Zaman MA, Colwell DD, Gilleard J, Iqbal Z: Acaricide resistance in cattle ticks and approaches to its management: The state of play. *Vet Parasitol,* 203 (1-2): 6-20, 2014. DOI: 10.1016/j.vetpar.2014.03.006

27. George JE, Pound JM, Davey R: Acaricides for controlling ticks on cattle and the problem of acaricide resistance. *Ticks Biol Dis Cont*, 408-423, 2008. DOI: 10.1017/CBO9780511551802.019

28. Coles TB, Dryden MW: Insecticide/acaricide resistance in fleas and ticks infesting dogs and cats. *Parasit Vectors,* 7:8, 2014. DOI: 10.1186/1756-3305-7-8

29. Guerrero FD, Lovis L, Martins JR: Acaricide resistance mechanisms in *Rhipicephalus (Boophilus) microplus*. *Rev Bras Parasitol Vet,* 21 (1): 1-6, 2012. DOI: 10.1590/s1984-29612012000100002

30. Nolan J: Acaricide resistance in single and multi-host ticks and strategies for control. *Parassitologia.* 32 (1): 145-153, 1990.

31. Vargas M, Montero C, Sánchez D, Pérez D, Valdés M, Alfonso A, Joglar M, Machado H, Rodríguez E, Méndez L, Lleonart R, Suarez M, Fernandez E, Estrada MP, Rodriguez-Mallon A, Farnos O: Two initial vaccinations with the Bm86-based Gavac plus vaccine against *Rhipicephalus (Boophilus) microplusinduce* similar reproductive suppression to three initial vaccinations under production conditions. *BMC Vet Res,* 6:43, 2010. DOI: 10.1186/1746-6148-6-43

32. Blecha IMZ, Csordas BG, Aguirre AdAR, Cunha RC, Garcia MV, Andreotti R: Analysis of Bm86 conserved epitopes: Is a global vaccine against cattle tick *Rhipicephalus microplus* possible? *Rev Bras Parasitol Vet,* 27, 267-279, 2018. DOI: 10.1590/S1984-296120180056

33. Willadsen P: Perspectives for subunit vaccines for the control of ticks. *Parassitologia,* 32 (1): 195-200, 1990.

34. Hoogstraal H: African Ixodoidea. Vol. I. Ticks of the Sudan (with special reference to Equatoria Province and with Preliminary Reviews of the Genera Boophilus, Margaropus, and Hyalomma), 1956. https://www.cabdirect.org/cabdirect/abstract/19572901469; *Accessed:* 10-09-2020

35. Walker AR: Ticks of Domestic Animals in Africa: A Guide to Identification of Species. Bioscience Reports Edinburgh, 2003.

36. Librado P, Rozas J: DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics,* 25 (11): 1451-1452, 2009. DOI: 10.1093/bioinformatics/btp187

37. Tamura K, Nei M, Kumar S: Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Nat Acad Sci USA,* 101 (30): 11030-11035, 2004. DOI: 10.1073/pnas.0404206101

38. Rehman A, Nijhof AM, Sauter-Louis C, Schauer B, Staubach C, Conraths FJ: Distribution of ticks infesting ruminants and risk factors associated with high tick prevalence in livestock farms in the semi-arid and arid agro-ecological zones of Pakistan. *Parasit Vectors,* 10:190, 2017. DOI: 10.1186/s13071-017-2138-0

39. Ali A, Parizi LF, Ferreira BR, da Silva Vaz Junior I: A revision of two distinct species of Rhipicephalus: *R. microplus* and *R. australis. Cienc Rural,* 46 (7): 1240-1248, 2016. DOI: 10.1590/0103-8478cr20151416

40. Ghafar A, Khan A, Cabezas-Cruz A, Gauci CG, Niaz S, Ayaz S, Mateos-Hernández L, Galon C, Nasreen N, Moutailler S, Gasser RB, Jabbar A: An assessment of the molecular diversity of ticks and tick-borne microorganisms of small ruminants in Pakistan. *Microorganisms,* 8 (9): 1428, 2020. DOI: 10.3390/microorganisms8091428

41. Ramzan M, Naeem-Ullah U, Saba S, Iqbal N, Saeed S: Prevalence and identification of tick species (Ixodidae) on domestic animals in district Multan, Punjab Pakistan. *Int J Acarol,* 46 (2): 83-87, 2020. DOI: 10.1080/01647954.2020.1711803

42. Nasreen N, Niaz S, Khan A, Ayaz S, Rashid M, Khattak I, Yu Z, Wang T, Al Sarraf M, Ali A: Molecular characterization of ticks infesting livestock in Khyber Pakhtunkhwa Province, Pakistan. *Inter J Acarol,* 46 (3): 165-170, 2020. DOI: 10.1080/01647954.2020.1734082

43. Bibi S, Rafique N, Kareem A, Taj MK, Iqbl K, Bibi A, Shafiq M, Ghafoor G, Ghafoor A, Ijaz A: Prevalence and taxonomic identification of hard ticks (Ixodidea) found in livestock of Harnai District, Balochistan, Pakistan. *Pure App Biol,* 9 (4): 2330-2338, 2020. DOI: 10.19045/bspab.2020.90247

44. Siddique RM, Sajid MS, Iqbal Z, Saqib M: Association of different risk factors with the prevalence of babesiosis in cattle and buffalos. *Pak J Agri Sci,* 57 (2): 517-524, 2020. DOI: 10.21162/PAKJAS/19.8626

45. Ghosh S, Bansal GC, Gupta SC, Ray D, Khan MQ, Irshad H, Shahiduzzaman M, Seitzer U, Ahmed JS: Status of tick distribution in Bangladesh, India and Pakistan. *Parasitol Res,* 101 (Suppl. 2): S207-S216, 2007. DOI: 10.1007/s00436-007-0684-7

46. Khan A, Noushin R, Attaullah M, Khan SN, Hussain R, Dawar FU, Rehman FU, Ijaz M, Ullah K: Prevalence of tick born *Babesia* infection in domestic cattle of Khyber Pakhtunkhwa, Pakistan. *Pak J Zool,* 52 (6): 2401-2403, 2020.

47. Farooqi SH, Ijaz M, Rashid MI, Aqib AI, Ahmad Z, Saleem MH, Hussain K, Islam S, Naeem H, Khan A: Molecular epidemiology of *Babesia bovis* in bovine of Khyber Pakhtunkhwa, Pakistan. *Pak Vet J,* 37 (3): 275-280, 2017.

48. Hassan MA, Liu J, Rashid M, Iqbal N, Guan G, Yin H, Luo J: Molecular survey of piroplasm species from selected areas of China and Pakistan. *Parasit Vectors,* 11:457, 2018. DOI: 10.1186/s13071-018-3035-x

49. Ahmad AS, Rashid I, Ashraf K, Shehzad W, Khan M, Hussain K, Farooqi SH, Khan A, Sohail ML: Molecular occurrence of canine babesiosis in rural dog population in Pakistan. *Trop Biomed,* 35 (3): 593-603, 2018.

50. Evans DE: Ticks, tick-borne diseases and insect pests of cattle in the Southern Cone countries of South America, 1992.

51. Roy BC, Estrada-Peña A, Krücken J, Rehman A, Nijhof AM: Morphological and phylogenetic analyses of *Rhipicephalus microplus* ticks from Bangladesh, Pakistan and Myanmar. *Ticks Tick Borne Dis,* 9 (5): 1069-1079, 2018. DOI: 10.1016/j.ttbdis.2018.03.035

52. Guglielmone AA, Robbins RG, Apanaskevich DA, Petney TN, Estrada-Peña A, Horak IG: Comments on controversial tick (Acari: Ixodida) species names and species described or resurrected from 2003 to 2008. *Exp Appl Acarol,* 48, 311-327, 2009. DOI: 10.1007/s10493-009-9246-2

53. De Clercq EM, Vanwambeke SO, Sungirai M, Adehan S, Lokossou R, Madder M: Geographic distribution of the invasive cattle tick *Rhipicephalus microplus,* a country-wide survey in Benin. *Exp Appl Acarol,* 58, 441-452, 2012. DOI: 10.1007/s10493-012-9587-0

54. Muhanguzi D, Byaruhanga J, Amanyire W, Ndekezi C, Ochwo S, Nkamwesiga J, Mwiine FN, Tweyongyere R, Fourie J, Madder M, Schetters T, Horak I, Juleff N, Jongejan F: Invasive cattle ticks in East Africa: Morphological and molecular confirmation of the presence of *Rhipicephalus microplus* in south-eastern Uganda. *Parasit Vectors,* 13:165, 2020. DOI: 10.1186/s13071-020-04043-z

55. Sungirai M, Moyo DZ, De Clercq P, Madder M, Vanwambeke SO, De Clercq EM: Modelling the distribution of *Rhipicephalus microplus* and *R. decoloratus* in Zimbabwe. *Vet Parasitol Reg Stud Rep,* 14, 41-49, 2018. DOI: 10.1016/j.vprsr.2018.08.006

56. De Clercq EM, Estrada-Peña A, Adehan S, Madder M, Vanwambeke SO: An update on distribution models for *Rhipicephalus microplus* in West Africa. *Geospat Health,* 8 (1): 301-308, 2013. DOI: 10.4081/gh.2013.75

57. Kanduma EG, Emery D, Githaka NW, Nguu EK, Bishop RP, Šlapeta J: Molecular evidence confirms occurrence of *Rhipicephalus microplus* Clade A in Kenya and sub-Saharan Africa. *Parasit Vectors,* 13:432, 2020. DOI: 10.1186/s13071-020-04266-0

58. Martínez-Arzate SG, Sánchez-Bermúdez JC, Sotelo-Gómez S, Diaz-Albiter HM, Hegazy-Hassan W, Tenorio-Borroto E, Barbabosa-Pliego A, Vázquez-Chagoyán JC: Genetic diversity of Bm86 sequences in *Rhipicephalus (Boophilus) microplus* ticks from Mexico: Analysis of haplotype distribution patterns. *BMC Genet,* 20:56, 2019. DOI: 10.1186/s12863-019-0754-8

59. De Clercq EM, Leta S, Estrada-Peña A, Madder M, Adehan S, Vanwambeke SO: Species distribution modelling for *Rhipicephalus microplus* (Acari: Ixodidae) in Benin, West Africa: Comparing datasets and modelling algorithms. *Prev Vet Med,* 118 (1): 8-21, 2015. DOI: 10.1016/j.prevetmed.2014.10.015

60. Robbertse L, Baron S, van der Merwe NA, Madder M, Stoltsz WH, Maritz-Olivier C: Genetic diversity, acaricide resistance status and evolutionary potential of a *Rhipicephalus microplus* population from a disease-controlled cattle farming area in South Africa. *Ticks Tick Borne Dis,* 7 (4): 595-603, 2016. DOI: 10.1016/j.ttbdis.2016.02.018

61. Guerrero FD, Andreotti R, Bendele KG, Cunha RC, Miller RJ, Yeater K, Pérez de León AA: *Rhipicephalus (Boophilus) microplus* aquaporin as an effective vaccine antigen to protect against cattle tick infestations. *Parasit Vectors,* 7:475, 2014. DOI: 10.1186/s13071-014-0475-9

62. Almazán C, Lagunes R, Villar M, Canales M, Rosario-Cruz R, Jongejan F, de la Fuente J: Identification and characterization of *Rhipicephalus (Boophilus) microplus* candidate protective antigens for the control of cattle tick infestations. *Parasitol Res,* 106 (2): 471-479, 2010. DOI: 10.1007/s00436-009-1689-1

63. de la Fuente J, Garcia-Garcia JC, González DM, Izquierdo G, Ochagavia ME: Molecular analysis of *Boophilus* spp. (Acari: Ixodidae) tick strains. *Vet Parasitol,* 92 (3): 209-222, 2000. DOI: 10.1016/s0304-4017(00)00308-3

64. Sossai S, Peconick AP, Sales-Junior PA, Marcelino FC, Vargas MI, Neves ES, Patarroyo JH: Polymorphism of the bm86 gene in South American strains of the cattle tick *Boophilus microplus. Exp Appl Acarol,* 37, 199-214, 2005. DOI: 10.1007/s10493-005-3262-7

65. Canales M, Almazán C, Naranjo V, Jongejan F, De la Fuente J: Vaccination with recombinant *Boophilus annulatus* Bm86 ortholog protein, Ba86, protects cattle against *B. annulatus* and *B. microplus* infestations. *BMC Biotechnol,* 9:29, 2009. DOI: 10.1186/1472-6750-9-29

66. Nijhof AM, Balk JA, Postigo M, Jongejan F: Selection of reference genes for quantitative RT-PCR studies in *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus appendiculatus* ticks and determination of the expression profile of Bm86. *BMC Mol Biol,* 10:112, 2009. DOI: 10.1186/1471-2199-10-112

67. Freeman JM, Davey RB, Kappmeyer LS, Kammlah DM, Olafson PU: Bm86 midgut protein sequence variation in South Texas cattle fever ticks. *Parasit Vectors,* 3:101 (2010), 2010. DOI: 10.1186/1756-3305-3-101

68. Kaewmongkol S, Kaewmongkol G, Inthong N, Lakkitjaroen N, Sirinarumitr T, Berry C, Jonsson N, Stich R, Jittapalapong S: Variation among Bm86 sequences in *Rhipicephalus (Boophilus) microplus* ticks collected from cattle across Thailand. *Exp Appl Acarol,* 66 (2): 247-256, 2015. DOI: 10.1007/s10493-015-9897-0

69. Kamau LM, Skilton RA, Githaka N, Kiara H, Kabiru E, Shah T, Musoke AJ, Bishop RP: Extensive polymorphism of Ra86 genes in field populations of *Rhipicephalus appendiculatus* from Kenya. *Ticks Tick Borne Dis,* 7 (5): 772-781, 2016. DOI: 10.1016/j.ttbdis.2016.03.011

70. Liu YH, Li KR, He B, Li F, Zhang LY, Pan JJ, Wang QR, Zhao L: Preliminary study of high efficiency vaccine of *Rhipicephalus (Boophilus)* microplus in South Xinjiang, China. *Kafkas Univ Vet Fak Derg,* 25 (4): 483-490, 2019. DOI: 10.9775/kvfd.2018.21125

71. Derouiche S, Deville M, Taylor M, Akbar H, Guillot J, Carreto-Binaghi L, Pottier M, Aliouat E, Aliouat-Denis C, Dei-Cas E, Demanche C: Pneumocystis diversity as a phylogeographic tool. *Mem Inst Oswaldo Cruz,* 104, 112-117, 2009. DOI: 10.1590/s0074-02762009000100017

72. McCormack JE, Hird SM, Zellmer AJ, Carstens BC, Brumfield RT: Applications of next-generation sequencing to phylogeography and phylogenetics. *Mol Phylogenet Evol,* 66 (2): 526-538, 2013. DOI: 10.1016/j.ympev.2011.12.007

73. Hare MP: Prospects for nuclear gene phylogeography. *Trends Ecol Evol,* 16 (12): 700-706, 2001. DOI: 10.1016/S0169-5347(01)02326-6

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

Kafkas Univ Vet Fak Derg
27 (2): 183-189, 2021
DOI: 10.9775/kvfd.2020.25058

RESEARCH ARTICLE

Protective Effects of Chitosan and Chitosan Oligosaccharide on Sodium Fluoride-Induced Testicular Damage in Male Rats: A Stereological and Histopathological Study

Fikret ALTINDAĞ [1,a (*)] Uğur ÖZDEK [2,b]

[1] Van Yüzüncü Yıl University, Faculty of Medicine, Department of Histology and Embryology, TR-65080 Van - TURKEY
[2] Van Yüzüncü Yıl University, Vocational Higher School of Healthcare Studies, TR-65080 Van - TURKEY
ORCIDs: [a] 0000-0002-7085-623X; [b] 0000-0002-0709-1545

Article ID: KVFD-2020-25058 Received: 10.10.2020 Accepted: 22.02.2021 Published Online: 22.02.2021

Abstract

The aim of the present study was to investigate the potential protective effects of Chitosan (C) and Chitosan Oligosaccharide (COS) on sodium fluoride (NaF) induced testicular damage. Forty-two male Wistar rats were separated into six groups (n=7). The Control group was given drinking water without Fluoride. NaF group was given NaF (100 mg/L) in their drinking water. NaF+C group was given NaF (100 mg/L) and C (250 mg/kg/day). NaF+COS group was given NaF (100 mg/L) and COS (250 mg/kg/day). C group was given C (250 mg/kg/day). COS group was given COS (250 mg/kg/day). Rats were given C and COS by gastric gavage. As a result, the total number of spermatogonia, Leydig cells, the total volume of the testis, the total volume of germinal epithelium, the total volume of seminiferous tubules, the total length of seminiferous tubules, and the height of germinal epithelium significantly decreased in the NaF group compared to the control group ($P<0.05$). These parameters significantly increased in the NaF + C and NaF + COS groups compared to the NaF group ($P<0.05$). Also, GSH and CAT significantly decreased, while MDA significantly increased in the NaF group. C and COS alleviate these changes. These findings indicate that NaF can cause testis damage. C and COS can have cytoprotective effects against testis damage.

Keywords: *Chitosan, Chitosan oligosaccharide, Rat, Sodium fluoride, Stereology, Testis*

Kitosan ve Kitosan Oligosakkaridin Erkek Sıçanlarda Sodyum Florür Kaynaklı Testis Hasarı Üzerindeki Koruyucu Etkileri: Stereolojik ve Histopatolojik Bir Çalışma

Öz

Bu çalışmanın amacı, Kitosan (K) ve Kitosan Oligosakkarit (KOS) sodyum florür (NaF) kaynaklı testis hasarı üzerindeki potansiyel koruyucu etkilerini araştırmaktır. Kırk iki erkek Wistar sıçanı altı gruba ayrıldı (n=7). Kontrol grubuna florsuz içme suyu verildi. NaF grubuna içme suyunda NaF (100 mg/L) verildi. NaF+K grubuna NaF (100 mg/L) ve K (250 mg/kg/gün) verildi. NaF + KOS grubuna NaF (100 mg/L) ve KOS (250 mg/kg/gün) verildi. K grubuna K (250 mg/kg/gün) verildi. KOS grubuna KOS verildi (250 mg/kg/gün). Sıçanlara gastrik gavaj ile K ve KOS verildi. NaF grubunda, toplam spermatogonyum sayısı, Leydig hücresi sayısı, toplam testis hacmi, toplam germinal epitel hacmi, toplam seminifer tübül hacmi, seminifer tübüllerin toplam uzunluğu ve germinal epitel yüksekliği kontrol grubuna göre anlamlı olarak azalmıştır ($P<0.05$). Bu parametreler NaF + K ve NaF + KOS gruplarında NaF grubu ile karşılaştırıldığında istatistiksel açıdan önemli ($P<0.05$) biçimde artmıştır. Ayrıca NaF grubunda GSH ve CAT önemli derecede ($P<0.05$) azalırken, MDA önemli biçimde artmıştır. K ve KOS bu değişiklikleri hafiflettiği gözlenmiştir. Bu bulgular, verilen dozdaki NaF'nin testis hasarına neden olabileceğini, K ve KOS'un sitoprotektif etkilerinin olabileceğini göstermektedir.

Anahtar sözcükler: *Kitosan, Kitosan oligosakkarit, Sıçan, Sodyum florür, Stereoloji, Testis*

INTRODUCTION

Fluoride is an essential molecule for the growth and development of living. It is found naturally in soil, water, and nutrients [1]. The recommended concentration of fluoride must be between 0.5-1.5 mgL^{-1} in drinking water according to the guideline of The World Health Organization [2]. However, excessive and prolonged consumption causes

How to cite this article?

Altındağ F, Özdek U: Protective effects of chitosan and chitosan oligosaccharide on sodium fluoride-induced testicular damage in male rats: A stereological and histopathological study. *Kafkas Univ Vet Fak Derg,* 27 (2): 183-189, 2021.
DOI: 10.9775/kvfd.2020.25058

(*) **Corresponding Author**

Tel: +90 505 576 7940
E-mail: altindagfikret@gmail.com (F. Altındağ)

fluorosis, which is defined as fluoride toxicity in organisms [3]. Fluorosis can damage soft tissues such as brain [4], testis [1], duodenum [5], liver, and kidney [6]. In recent studies, special attention has been drawn to the side effects of fluorosis on the male reproductive system [7]. Although fluorosis has been reported to reduce the effectiveness of the antioxidant enzyme system and cause oxidative damage by inducing the formation of free oxygen species, its mechanism of cellular damage on the male reproductive system is not fully disclosed.

The use of herbal medicines for therapeutic purposes is increasing in the world. In some countries, herbal medicines are even more used than prescribed medicines [8]. Chitosan (C), a naturally abundant polysaccharide, has been the center of attention of scientists for more than 40 years due to its potential biomedical applications [9]. Chitosan is a natural substance used in medical applications for therapeutic purposes. It has the property of excellent biocompatibility, biodegradability, antioxidant, and accelerating wound healing [10]. Chitosan oligosaccharide (COS) is formed from the deacetylation and hydrolysis of chitin. COS has anti-inflammatory [11], anti-tumoral [12], and antioxidant [13,14] effects.

The testes have high amounts of antioxidants, and these antioxidants play important roles in the protection of testis against oxidative stress. But, if the injury is prolonged for a long time or is exposed to the severe toxic agent, the testes can become irreversibly damaged [15,16]. Therefore, in the present study, we aimed to examine whether C and COS have protective effects against the possible adverse effects induced by fluorosis on rat testes by stereological, histopathological, and biochemical methods.

Material and Methods

Chemicals

Chitosan (CAS number: 9012-76-4, Sigma-Aldrich), chitosan oligosaccharide (CAS number: 148411-57-8, Sigma-Aldrich), sodium fluoride (CAS number: 7681-49-4, Sigma-Aldrich), ketamine HCl (Ketalar 50 mg/mL, Inj., Pfizer, Istanbul, Turkey) and xylazine HCl (Alfazyne 2% Inj., Interhas A.S., Ankara, Turkey) were purchased from commercial companies.

Animals and Experimental Design

The present study was approved by Van Yuzuncu Yil University Animal Experiments Local Ethics Committee (decision number: 2019/09). In this study, a total of 42 adult male Wistar rats weighing 200-300 g were used, and the animals were purchased from the Van Yüzüncü Yıl University Experimental Animals Research and Application Center. Rats were randomly divided into six groups (n=7). The Control group was given drinking water. NaF group received 100 mg/L NaF in their drinking water. NaF+C group received NaF (100 mg/L) and C (250 mg/kg/day). NaF+COS group was exposed to NaF (100 mg/L) and also COS was given at 250 mg/kg/day. C group was given C (250 mg/kg/day), and COS group received COS (250 mg/kg/day). C and COS were given by gastric gavage. Rats were housed under standard conditions of temperature (25±2°C), relative humidity (50±10%), and 12 h light/12 h dark cycle. The animals were fed with a standard pellet diet *(ad libitum)*. The experimental period continued for 12 weeks. At the end of the experiment, rats were sacrificed by ketamine overdose. The testis tissue was removed by an incision made from the scrotal region and fixed in 10% buffered formalin.

Stereology

Isotropic identical random sections were obtained using the orientator method [17]. Thus, on average, 8-10 slabs were selected from each testis. The selected slabs were embedded in the same paraffin block. 10-15 consecutive sections were taken in 4-µm in thickness. The sections were stained with Hematoxylin-Eosin (H-E) and examined under a light microscope.

Total Number of Cells

The physical dissector counting method was used to calculate the total number of spermatogonia, Sertoli, and Leydig cells. In the area limited by an unbiased counting frame, cells were calculated that existing in the reference section but not existing in the look-up section *(Fig. 1-A)*. The following formula was used to calculate the cell number: $N = N_v \times V_{ref}$. Where N_v is the numerical density of the cell of interest (cells/unit volume) and V_{ref} is the total reference volume of the testis [15,18].

Estimation of Total Volume

Cavalieri principle was followed to estimate the total volume of the testis, seminiferous tubule, germinal epithelium, and interstitial tissue. For this, the point grid was used *(Fig. 1-B)*. The following formula was used to calculate the volume: $V = \sum P \times a/p \times t$. Where "V" is the volume of the structure, "∑P" is the total number of points hitting the structure, "a/p" is the area covered by one point, and "t" is the section thickness [15].

After the histological tissue processing stages (Fixation, processing, sectioning and staining), the volume of tissue is usually shrunk. For estimation of testis final volume, the tissue shrinkage value must be determined. Thus, the degree of tissue shrinkage ($d_{(shr)}$) is calculated by the following formula:

$$d_{(shr)} = 1 - (AA/AB)^{1.5}$$

Where $d_{(shr)}$ is the degree of tissue shrinkage, AA is the area of the circular piece after histological processing stages, AB is the area of the circular piece before histological processing stages. Coefficient (3/2=1.5) is required to calculate

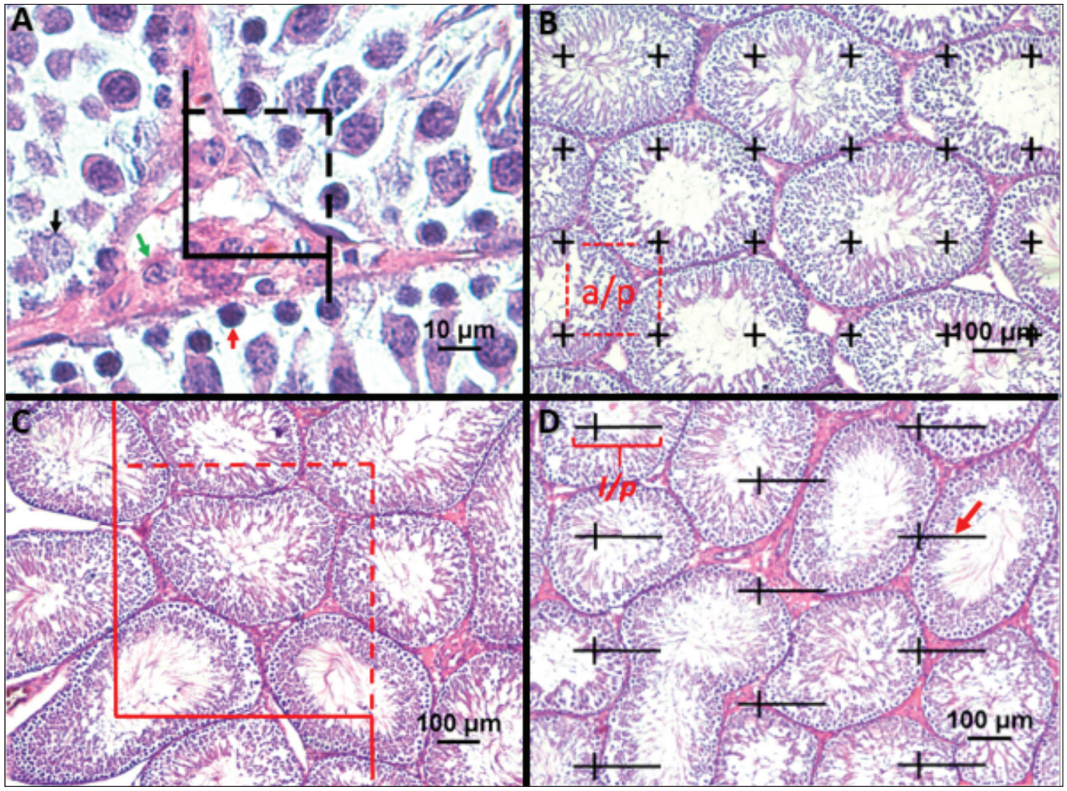


Fig 1. A. Application of physical disector counting method. An unbiased counting frame has been used. Black arrow: Sertoli cell, green arrow: Leydig cell, red arrow: Spermatogonia; **B.** The superimposed of a point grid on the image of section; **C.** The superimposed of an unbiased counting frame on the image of section; **D.** The superimposed of a grid of test lines on the image of the section

the tissue shrinkage degree in three dimensions (3) from two dimensional (2) areas. Accordingly, the final volume of the testis was calculated using the following formula:

$$V_{(shrunken)} = V_{(unshrunken)} \times [1 - d_{(shr)}]$$

Where $V_{(shrunken)}$ is the volume after the histological processing steps, $V_{(unshrunken)}$ is the volume before the histological processing steps, $d_{(shr)}$ is the degree of tissue shrinkage [15].

Estimation of Length Density and Total Length of Seminiferous Tubule

The length density of seminiferous tubules was calculated using an unbiased counting frame *(Fig. 1-C)*. The unbiased counting frame with prohibited and independent lines is put on the images. The profiles of the seminiferous tubules, which are either totally or partially inside the counting frame and do not contact prohibited lines and their extensions are counted [19]. The length density (L_v) of seminiferous tubules is calculated using the following formula:

$$L_v = 2 \times \Sigma Q/(\Sigma F \times a/f)$$

"∑Q" is the total number of the profiles of seminiferous tubules counted per testis, "∑F" is the total number of frames and "a/f" is the frame area. The total length of seminiferous tubules (L) is obtained by multiplying the length density (Lv) value with the total volume value.

Estimation of Height of Germinal Epithelium

To estimate the height of germinal epithelium, primarily the surface density of epithelium must be estimated. The surface density was calculated using a grid of test lines. A grid of test lines was put on the images of the sections. Each test line is accommodated with one point *(Fig. 1-D)*. The surface density of the epithelium (S_v) was calculated using the following formula:

$$S_{V(epithelium/ref)} = 2 \times \Sigma I/(\Sigma P \times l/p)$$

$$\text{The height of germinal epithelium} = V_{V(epithelium/ref)}/S_{V(epithelium/ref)}$$

"∑I" is the total number of intersection points of the luminal surface of the epithelium and the test lines, "∑P" is the total number of points hitting testis and "l/p" is the length of a test line. The total surface area is calculated by multiplying the surface density by the total volume [19].

Histopathological Observations

Four µm thick sections taken from paraffin blocks of testis were stained with H-E and examined by light microscope (Nikon Y-IM 7551012, Japan). For histopathological evaluation, an average of 15-20 areas was evaluated by random sampling for each animal in the groups. The findings were semiquantitatively evaluated according to the number of lesions observed in the examined areas.

Biochemical Analysis

The testis samples were homogenized in phosphate buffer (pH: 7.4). Homogenates were centrifuged at 10.000 G for 15 min at +4°C. The obtained supernatants were used for biochemical analysis. Analysis of GSH, MDA, and CAT was evaluated by spectrophotometric method.

Statistical Analysis

Statistical analyses were accomplished using SPSS 21.0 software. Differences between groups were evaluated by the Kruskal-Wallis test followed by Mann-Whitney U test. P-value ≤0.05 was accepted as statistically significant. All data were expressed as means ± standard deviations.

Results

The Total Number of Spermatogonia, Sertoli, and Leydig Cells

The results indicated that there was a significant reduction in the total number of spermatogonia and Leydig cells in the NaF group compared to the control group (P<0.05). However, this reduction was prevented in the NaF+C and NaF+COS groups compared to the NaF group (P<0.05) *(Table 1)*.

The Total Testicular Volume

As shown in *Table 2*, when compared to the control group, the total volume of the testis, the total volume of germinal epithelium, and the total volume of seminiferous tubule significantly decreased (P<0.05) in the NaF group. But, no significant change was observed in the total volume of interstitial tissue. Also, the total volume of the testis, the total volume of germinal epithelium, and the total volume of seminiferous tubule significantly increased in NaF+C and NaF+COS groups compared to NaF group (P<0.05) *(Table2)*.

The Total Length of Seminiferous Tubule

The data of the present study showed that the total length of seminiferous tubules significantly decreased in the NaF group compared to the control group (P<0.05). The total length of seminiferous tubule was significantly increased in NaF+C and NaF+COS groups compared to NaF group (P<0.05) *(Table 2)*.

Height of Germinal Epithelium

As the results in *Table 2* showed that NaF caused a significant decrease in the height of germinal epithelium (P<0.05). However, the height of germinal epithelium significantly increased in NaF+C and NaF+COS groups compared to NaF group (P<0.05) *(Table 2)*.

Results of the Histopathological Evaluations

The control group displayed normal histological architecture. The number of germinal epithelial layers was between 5-9. The Spermatogenic cells and Sertoli cells were seen in the germinal epithelium. There were numerous morphologically mature sperm cells in the lumina of the seminiferous

Table 1. *Effects of C and COS on the total testicular cell numbers (×10⁶) in fluoride-induced testicular damage*

Groups	Spermatogonia	Sertoli Cell	Leydig Cell
Control	15013534.13±693584.13[b]	8021941.12±479449.01	5987347.25±722449.09[b]
NaF	12800925.05±697264.84[a]	7336928.54±668999.64	4437461.49±775097.53[a]
NaF+C	13854743.58±592270.19[a,b]	7232094.41±734270.39	5411601.51±390257.57[b]
NaF+COS	14761358.87±743880.32[b]	7435793.68±689778.15	5571682.93±554982.46[b]
C	14942930.47±1025808.47[b]	7761246.84±100584.08	6013985.48±859375.71[b]
COS	15135822.09±831371.61[b]	8245787.11±435083.26	5748381.14±768643.24[b]

Values are expressed as means ± SD. [a] P<0.05, compared with the control group, [b] P<0.05, compared with the NaF group

Table 2. *Effects of C and COS on the total volume (mm³) of testes, the total length of seminiferous tubules (m), and the height of germinal epithelium (μm) in testis damage induced by fluoride*

Groups	The Total Volume of Testis	The Total Volume of Interstitial Tissue	The Total Volume of Germinal Epithelium	The Total Volume of Seminiferous Tubules	The Total Length of Seminiferous Tubules	The Height of Germinal Epithelium
Control	1063.33±98.74[b]	194.33±15.35	508.50±55.02[b]	846.00±43.89[b]	10.28±0.59[b]	69.17±3.31[b]
NaF	816.16±64.26[a]	179.50±13.72	393.50±49.39[a]	644.17±59.15[a]	8.71±0.66[a]	51.50±1.87[a]
NaF +C	930.50±56.31[a,b]	186.67±14.84	450.17±31.33[a,b]	715.01±27.19[a,b]	9.37±0.42[a,b]	60.33±2.15[a,b]
NaF +COS	981.00±77.11[b]	185.71±16.03	469.29±43.16[b]	773.71±65.67[a,b]	9.51±0.48[a,b]	63.00±2.16[a,b]
C	1119.00±60.85a[b]	192.67±20.60	493.33±33.17[b]	804.00±58.51[b]	10.01±0.65[b]	71.67±2.52[b]
COS	1148.67±77.67[b]	202.00±15.53	529.00±32.23[b]	873.01±47.00[b]	10.49±1.15[b]	72.33±1.53[b]

Values are expressed as means ± SD. [a] Significant differences as compared with the control group at P<0.05, [b] Significant differences as compared with the NaF group at P<0.05

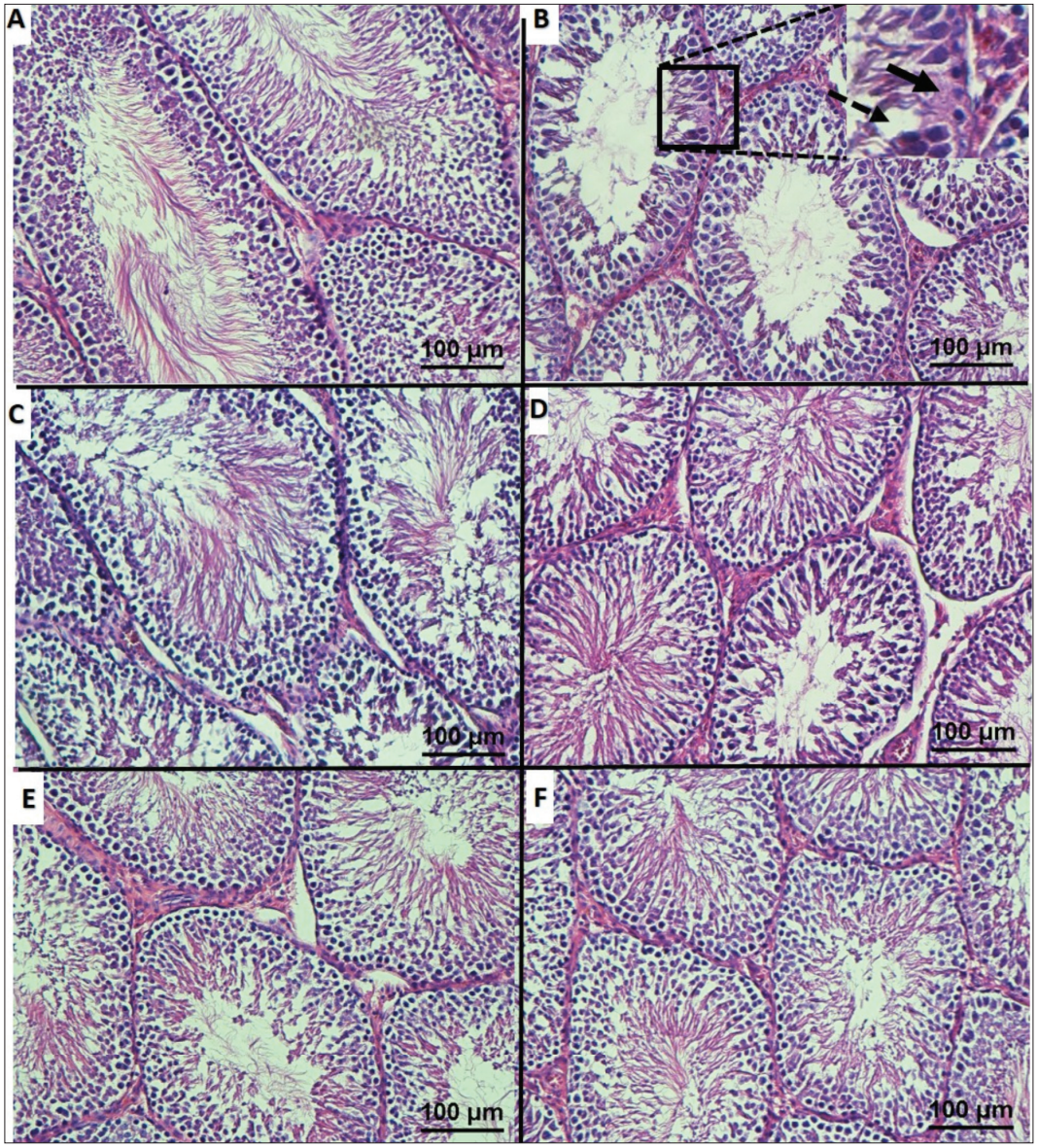


Fig 2. Photomicrographs of testicular sections. Control (A), a normal histological structure of testis. NaF group (B), atrophy and necrosis in seminiferous tubule (dashed arrow: atrophy, arrow: necrotic cell), a decrease of the number of germinal epithelial layers. NaF+C (C) and NaF+COS (D) groups were restored according to the NaF group. C (E) and COS (F) groups were similar to the control

tubules. Leydig cells are located in the interstitial regions *(Fig. 2-A)*. In the NaF group; severe atrophy, degenerate and necrotic cells were observed in the germinal epithelium. The germinal epithelial layers were between 3-6. There were very few mature sperm cells in the tubular lumina *(Fig 2-B)*. Less atrophy and degeneration were observed in the germinal epithelium of the NaF+C *(Fig. 2-C)* and NaF+COS *(Fig. 2-D)* groups. The histological structure of testes in the C *(Fig. 2-E)* and COS *(Fig. 2-F)* groups was similar to the control.

Biochemical Analysis

The level of MDA, a marker of oxidative stress, was significantly higher in the NaF group compared to the control group. In the NaF+COS group, a significant decrease in MDA level was observed compared to the NaF group ($P<0.05$). Furthermore, the levels of GSH and CAT significantly decreased in the NaF group compared to the control group. However, compared to the NaF group, the levels of GSH and CAT were significantly higher in the NaF+C and NaF+COS groups ($P<0.05$). In C and COS groups, the levels of MDA, GSH, and CAT were similar to the control group *(Fig. 3)*.

Discussion

Infertility affects about 15% of couples who want to have children. About half of these cases are of male origin. Oxidative stress induced by toxic substances is considered one of the most important causes of male infertility. Oxidative

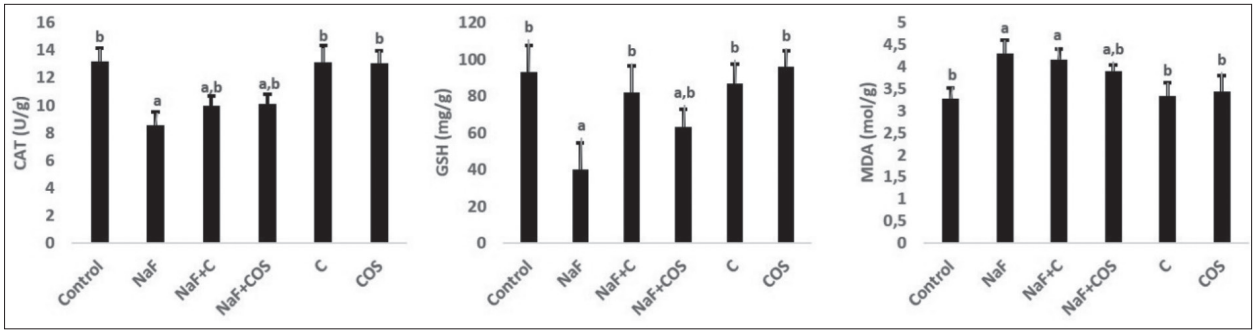


Fig 3. Effect of C and COS on the activity of CAT and GSH and content of MDA in NaF-induced testis damage. Values are expressed as means ± SD. [a] Significant differences as compared with the control group at P<0.05, [b] Significant differences as compared with the NaF group at P<0.05

stress is a pathological condition associated with cellular damage caused by free oxygen species (ROS). ROS can cause cell death by disrupting DNA, lipids, proteins, and enzymes. Thus, it may cause significant deteriorations in the semen parameters associated with male infertility [19].

Fluoride, which is found in many sources on earth, plays an important role in bone development, growth and remodeling. However, taking it too much can cause fluorosis [1]. In the previous studies, it was reported that fluorosis has harmful effects on the male reproductive system [7]. In our study, exposure to NaF increased MDA content and significantly decreased GSH, CAT activity which is an indicator of enhanced oxidative stress in the rats. A significant decrease in the stereological parameters of the testes is an indicator of the toxic effect of fluoride on the testis [20].

Spermatogonia are the precursor cells of sperm cells which are mature male reproductive cells. Spermatogonia are easily affected by toxic substances. In a previous study, it was reported that fluoride increased the number of apoptotic spermatogonia [21]. Also, fluoride decreased sperm count and sperm viability [7]. In the present study performed using stereological methods, it was estimated that the decrease in the total number of spermatogonia as a result of cell death is caused by the cytotoxic effect of NaF (by inducing oxidative stress). The significant decrease in total seminiferous tubule volume, total seminiferous tubule length, total seminiferous tubule germinal epithelium height, and volume may occur due to the decrease in the number of spermatogonia. These quantitative data support each other. It is estimated that these decreased parameters may also result in a decrease in the number of mature sperm cells, thus causing male infertility [7,21].

Leydig cells secrete testosterone which is the male sex hormone playing very important androgenic effects in the maturation of male sexual organs and the development of secondary sex characters. Testosterone is also a necessary hormone for normal sperm production. It has been reported that fluorosis reduces nerve growth factor (NGF) expression, which stimulates sperm motility, induces Leydig cell differentiation and proliferation, and testosterone production [8]. Also, it was demonstrated that NaF caused a significant decrease in levels of testosterone in rats [22] and mice Leydig cells. In our study, the decreased total number of Leydig cells caused by fluoride may result in decreased testosterone level, thereby spermatogenesis may be impaired [7,23].

Chitosan has been reported to have a protective effect against reproductive toxicity [22]. In the present study, chitosan exhibited antioxidant properties by preventing the decrease in CAT and GSH levels caused by fluorine. In addition, it was observed that chitosan alleviated the decrease in spermatogonia and Leydig cell numbers, total testicular volume, the total volume of the seminiferous tubule, the total volume of germinal epithelium, seminiferous tubule length, and germinal epithelium height.

The previous studies have shown that COS exhibited anti-oxidative effects in pancreatic β cells [24] and endothelial cells [25]. In the present study, COS reversed the increase of MDA content, a decrease of GSH and CAT activity induced by NaF. Thus, it was concluded that COS exhibited an antioxidative effect. We found that COS prevents the decrease in mean spermatogonia and Leydig cell numbers induced by the cytotoxic effect of fluorosis. Also, COS protected the total volume of the testis, the total volume of interstitial tissue, total volume and the total length of seminiferous tubules, and height of germinal epithelium from cytotoxic effects of fluorosis.

As a result, NaF caused a decrease in the number of spermatogonia, Leydig cells, and testis volume. Also, NaF was observed to cause oxidative stress in rat testis. Thus, the results of our study indicated that NaF may cause testis damage. C and COS may have a protective effect against the toxic effects of NaF on the testis.

Statement of Author Contributions

FA planned the study, performed experiments, commented on the data, edited the manuscript, analyzed the data, approved the final version of the manuscript to be

responsible for all of the study. UÖ performed the biochemical analyses and interpreted them, approved the final version of the manuscript, and agreed to be responsible for all of the study.

Acknowledgments

We would like to thank Sadi ELASAN for contributing to the statistical evaluation of our data.

Conflicts of Interest Statement

The authors declare that they have no conflict of interest.

References

1. Barbier O, Arreola-Mendoza L, Del Razo LM: Molecular mechanisms of fluoride toxicity. *Chem Biol Interact*, 188, 319-333, 2010. DOI: 10.1016/j.cbi.2010.07.011

2. WHO G: Guidelines for drinking-water quality. World Health Organization. 216, 303-304, 2011.

3. Yu Y, Han Y, Niu R, Wang J, Manthari RK, Ommati MM, Sun Z: Ameliorative effect of VE, IGF-I, and hCG on the fluoride-induced testosterone release suppression in mice leydig cells. *Biol Trace Elem Res*, 181, 95-103, 2018. DOI: 10.1007/s12011-017-1023-1

4. Tu, W, Zhang Q, Liu Y, Han L, Wang Q, Chen P, Zhang S, Wang A, Zhou X: Fluoride induces apoptosis via inhibiting SIRT1 activity to activate mitochondrial p53 pathway in human neuroblastoma SH-SY5Y cells. *Toxicol Appl Pharmacol*, 347, 60-69, 2018. DOI: 10.1016/j.taap.2018.03.030

5. Aydogan I, Yildirim E, Kabakci R, Sumer T: The Effect of fluoride toxication on duodenum, jejunum and ileum of broilers. *J Poult Res*, 15, 34-39, 2018.

6. Kaya I, Karapehlivan M: The investigation of biochemical and histological effects of ellagic acid in experimental chronic fluorosis induced mice. *Int J Health Sci Res*, 7, 396-406, 2017.

7. Zhang J, Li Z, Qie M, Zheng R, Shetty J, Wang J: Sodium fluoride and sulfur dioxide affected male reproduction by disturbing blood-testis barrier in mice. *Food Chem Toxicol*, 94, 103-111, 2016. 103-111, 2016. DOI: 10.1016/j.fct.2016.05.017

8. Heber D: Herbal preparations for obesity: Are they useful? *Prim Care*, 30, 441-463, 2003.

9. Kumar MNVR: A review of chitin and chitosan applications. *React Funct Polym*, 46, 1-27, 2000. DOI: 10.1016/S1381-5148(00)00038-9

10. Lin HY, Chou CC: Antioxidative activities of water-soluble disaccharide chitosan derivatives. *Food Res Int*, 37, 883-889, 2004. DOI: 10.1016/j.foodres.2004.04.007

11. Yoon HJ, Moon ME, Park HS, Im SY, Km YH: Chitosan oligosaccharide (COS) inhibits LPS-induced inflammatory effects in RAW 264.7 macrophage cells. *Biochem Biophys Res Commun*, 358, 954-959, 2007. DOI: 10.1016/j.bbrc.2007.05.042

12. Muanprasat C, Chatsudthipong V: Chitosan oligosaccharide: Biological activities and potential therapeutic applications. *Pharmacol Ther*, 170, 80-97, 2017. DOI: 10.1016/j.pharmthera.2016.10.013

13. Park PJ, Je JY, Kim SK: Free radical scavenging activity of chitooligosaccharides by electron spin resonance spectrometry. *J Agric Food Chem*, 51, 4624-4627, 2003. DOI: 10.1021/jf034039

14. Li X, Ding X, Peng X, Chi, X, Cui H, Zuo Z, Fang J: Effect of chitosan oligosaccharides on antioxidant function, lymphocyte cycle and apoptosis in ileum mucosa of broiler. *Kafkas Univ Vet Fak Derg*, 23, 571-577, 2017. DOI: 10.9775/kvfd.2016.17329

15. Mayhew TM, Gundersen HJG: If you assume, you can make an ass out of u and me: A decade of the disector for stereological counting of particles in 3D space. *J Anat*, 188, 1-15, 1996.

16. Chiang C, Mahalingam S, Flaws JA: Environmental contaminants affecting fertility and somatic health. *Semin Reprod Med*, 35, 241-249, 2017. DOI: 10.1055/s-0037-1603569

17. Noorafshan A: Stereology as a valuable tool in the toolbox of testicular research. *Ann Anat*, 196, 57-66, 2014. DOI: 10.1016/j.aanat.2012.07.008

18. Altindağ F, Rağbetli MÇ: The effect of maternal treatment with diclofenac sodium and thymoquinone on testicular parameters in rat offspring. *Rev Int Androl*, 19, 34-40, 2021. DOI: 10.1016/j.androl.2019.07.001

19. Agarwal A, Virk G, Ong C, Du Plesis SS: Effect of oxidative stress on male reproduction. *World J Mens Health*, 32, 1-17, 2014. DOI: 10.5534/wjmh.2014.32.1.1

20. Aitken RJ, Roman SD: Antioxidant systems and oxidative stress in the testes. *Oxid Med Cell Longev*, 1, 15-24, 2008.

21. Zhang S, Niu Q, Gao H, Ma R, Lei R, Zhang C, Xia T, Li P, Xu C, Wang C, Chen J, Dong L, Zhao Q, Wang A: Excessive apoptosis and defective autophagy contribute to developmental testicular toxicity induced by fluoride. *Environ Pollut*, 212, 97-104, 2016.

22. Tharanathan RN, Kittur FS: Chitin-the undisputed biomolecule of great potential. *Crit Rev Food Sci Nutr*, 43, 61–87, 2003. DOI: 10.1080/10408690390826455

23. Das S, Maiti R, Ghosh D: Management of fluoride induced testicular disorders by calcium and vitamin-E co-administration in the albino rat. *Reprod Toxicol*, 22, 606-612, 2006. DOI: 10.1016/j.reprotox.2006.05.001

24. Karadeniz F, Artan M, Kong CS, Kim SK: Chitooligosaccharides protect pancreatic β-cells from hydrogen peroxide-induced deterioration. *Carbohydr Polym*, 82, 143-147, 2010. DOI: 10.1016/j.carbpol.2010.04.046

25. Liu HT, Li WM, Xu G, Li XY, Bai XF, Wei P, Yu C, Du, YG: Chitosan oligosaccharides attenuate hydrogen peroxide-induced stress injury in human umbilical vein endothelial cells. *Pharmacol Res*, 59, 167-175, 2009. DOI: 10.1016/j.phrs.2008.12.001

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

Kafkas Univ Vet Fak Derg
27 (2): 191-201, 2021
DOI: 10.9775/kvfd.2020.25060

RESEARCH ARTICLE

Detection of Botulinum Neurotoxin Serotypes C and D, and Their Effects on Expressions of SNAP-25 and Synaptobrevin in Ruminants: An Immunohistochemical Study [1,2]

Mehmet Burak ATEŞ [1,a (*)] Funda TERZİ [2,b] Mustafa Kemal ÇİFTÇİ [1,c]
Mustafa ORTATATLI [1,d] Zeynep ÇELİK [1,e]

[1] *This article was presented as abstract at X. National and I. International Veterinary Pathology Congress (Online), 27-31 October 2020, Burdur, Turkey*

[2] *This study was supported by Selçuk University, Coordination Office of Scientific Research Projects (Project No: 17401054)*

[1] Selçuk University, Faculty of Veterinary Medicine, Department of Pathology, TR-42130 Konya - TURKEY

[2] Kastamonu University, Faculty of Veterinary Medicine, Department of Pathology, TR-37200, Kastamonu - TURKEY

ORCIDs: [a] 0000-0003-1297-426X; [b] 0000-0002-6184-5408; [c] 0000-0003-4476-2114; [d] 0000-0002-3713-813X; [e] 0000-0002-9667-5728

Article ID: KVFD-2020-25060 Received: 11.10.2020 Accepted: 04.02.2021 Published Online: 08.02.2021

Abstract

In humans and animals, botulism is a disease characterized by generalized and progressive paralysis caused by *Clostridium botulinum* neurotoxins (BoNT). BoNTs, defined in seven different antigenic types (A to G), proteolyze SNAREs (synaptosomal-associated protein/SNAP-25 and synaptobrevin) responsible for acetylcholine release in peripheral cholinergic neurons, and thus cause flaccid paralysis and death. Currently, mouse experiments are considered the reference method for definitive diagnosis. However, new diagnostic methods that are fast and accurate and would not raise ethical issues need to be developed. Therefore, using antibodies specific to the toxoid forms of BoNTs, the presence of BoNT-C and/or BoNT-D was investigated by immunohistochemical method (IHC) in the study. The tissues of thirty ruminants (twenty cattle, seven sheep, three goats), which had the clinical and pathological findings of botulism and a herd history of the disease, were used as material. BoNTs were detected with IHC in sixteen of the thirty ruminants as three BoNT-C, eleven BoNT-D, and two BoNT C+D. In the mouse experiments, BoNT was isolated in only three cases (two BoNT-D, one BoNT-C). Additionally, being responsible for the clinical findings of botulism, the interaction of BoNTs with SNAP-25 and synaptobrevin was investigated using IHC. It was determined that BoNT-C specifically reduces the expression of SNAP-25, and BoNT-D reduces the expression of synaptobrevin and partially SNAP-25. It was concluded that additional studies may be valuable to investigate the use of IHC in the diagnosis of botulism.

Keywords: *BoNT-C, BoNT-D, SNAP-25, Synaptobrevin, Immunohistochemistry*

Ruminantlarda Botulinum Nörotoksin Serotip C ve D'nin Saptanması ve Bunların SNAP-25 ve Sinaptobrevin Ekspresyonları Üzerindeki Etkileri: İmmunohistokimyasal Bir Çalışma

Öz

Botulizm insan ve hayvanlarda *Clostridium botulinum* nörotoksinlerinin (BoNT) neden olduğu, generalize ve ilerleyici paraliz ile karakterize bir hastalıktır. Yedi farklı antijenik tipi (A dan G'ye) tanımlanan BoNT periferik kolinerjik nöronlardaki asetilkolin salınımından sorumlu olan SNARE proteinlerini (SNAP-25 ve sinaptobrevin) proteolize ederek flasid paralize ve ölüme neden olur. Günümüzde fare deneyleri hastalığın kesin teşhisi için referans yöntem olarak kabul edilmektedir. Ancak, hızlı, güvenilir ve etik problemlere yol açmayan yeni teşhis metotlarının geliştirilmesi gerekmektedir. Yapılan çalışma ile botulismus sürü öyküsü, klinik ve patolojik bulguları bulunan 30 adet ruminanta (20 adet sığır, 7 adet koyun ve 3 adet keçi) ait çeşitli dokular BoNT-C ve BoNT-D yönünden immunohistokimyasal (IHC) yöntemle BoNT'un toksoid formuna spesifik antikorlar kullanılarak incelenmiştir. Bu kapsamda 30 ruminantın 16 (3 BoNT-C, 11 BoNT-D ve 2 BoNT C+D)'sında IHC metodu ile BoNT tespit edildi. Fare deneylerinde ise yalnızca 3 vakada BoNT (2; BoNT-D, 1; BoNT-C) izole edildi. Ayrıca, botulizm klinik bulgularından sorumlu olan SNAP-25 ve sinaptobrevinin BoNT ile olan etkileşimi IHC ile araştırılmış ve BoNT-C'nin ruminantlarda spesifik olarak SNAP-25'i, BoNT-D'nin ise spesifik olarak sinaptobrevini kısmi olarak da SNAP-25'i proteolize ettiği saptanmıştır. Botulizm tanısında IHC kullanımını araştırmak için ek çalışmaların yapılmasının değerli olabileceği sonucuna varıldı.

Anahtar sözcükler: *BoNT-C, BoNT-D, SNAP-25, Sinaptobrevin, Immunohistokimya*

How to cite this article?

Ateş MB, Terzi F, Çiftçi MK, Ortatatlı M, Çelik Z: Detection of botulinum neurotoxin serotypes C and D, and their effects on expressions of SNAP-25 and synaptobrevin in ruminants: An immunohistochemical study. *Kafkas Univ Vet Fak Derg,* 27 (2): 191-201, 2021.
DOI: 10.9775/kvfd.2020.25060

(*) Corresponding Author

Tel: +90 332 223 2734 Fax: +90 332 241 0063
E-mail: mehmetburakates@selcuk.edu.tr (M.B. Ateş)

Introduction

Botulism is a neuroparalytic disease characterized by muscle laxity and generalized and progressive paralysis arising from the specific effects of the botulinum neurotoxins (BoNT) produced by *Clostridium botulinum (C. botulinum*), which occurs in most mammals, birds, and fish [1,2]. Apart from *C. botulinum*, some BoNT isoforms have been reported to be produced by *C. baratii*, *C. butyricum* and *C. argentinense* [3-5]. Although botulism is seen sporadically throughout the world, it often occurs in herds as outbreaks and causes significant economic losses, especially in farm animals, because the disease has a high mortality rate [1,6]. *C. botulinum* is a gram-positive, anaerobic bacterium with a spore form. Seven different antigenic types of neurotoxins, from A to G, belonging to *C. botulinum* have been described [7]. Then, a new type of BoNT (BoNT-H), responsible for infant botulism, was defined as the eighth isoform [3,8]. BoNT-A, B, E, and F primarily induced botulism in humans, while BoNT-C and D were only harmful to animals [4]. Mosaic recombinant toxins (both type C and D weak chains) defined as BoNT-C+D and BoNT-D+C can also be seen in cases of botulism in ruminants [4].

Notwithstanding the differences in the amino acid sequence and immunological variations, all BoNTs are synthesized in their inactive form and activated later by tissue proteases [2]. BoNTs, absorbed from the intestines, are attached to the presynaptic cholinergic nerve terminals and especially the somatic neuromuscular nerve endings, after arriving via the hematogenous route. The tissue proteases in these areas activate the toxin by breaking down the ligaments of the light (LC) and heavy (HC) chains [9]. The HC bind to target cells, helping LC to enter the cell cytoplasm [10]. LC is released into the cytosol, where it inactivates the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex and thence prevents the clamping and fusion of synaptic vesicles [10,11]. Therefore, neurotransmitter release from vesicles containing acetyl-choline in the neuromuscular junction is prevented [7]. SNARE proteins include vesicle-associated membrane proteins (vamp/synaptobrevin), plasma proteins (synaptosomal-associated protein/SNAP-25), and syntaxin [12]. It has been reported that BoNT-C proteolyzes both SNAP-25 and syntaxin, BoNT-A and E only break down SNAP-25, and BoNT-B, D, F, and G proteolyze synaptobrevin and thereby inhibit acetylcholine synthesis [5]. As a result of this inhibition, clinical findings are observed in animals and death occur due to subsequent paralysis of the diaphragm muscles [13,14].

Botulism is an important intoxication that affects both humans and animals on a global scale and is particularly difficult to diagnose owing to difficulties in toxin isolation. The most important problems today in overlooking the botulism cases are the mouse experiments, which are not sensitive enough, and the inadequate interpretation of clinical and necropsy findings in field conditions. Besides, the selection of the sample to be sent to the laboratory, the lack of knowledge of the transfer conditions of the samples, the shortage of equipped laboratories, and the scarcity of rapid and reliable methods for diagnosis are other important factors [15,16]. However, a rapid decision-making mechanism for herd and animal health should be operated without wasting time on such problems. Currently, the mouse inoculation test is still used as a reference method to determine the presence of toxins necessary for the definitive diagnosis of botulism [15-18]. However, this test is not sensitive enough and useful because of having some disadvantages such as the scarcity of accredited laboratories where mouse experiments are performed, the amount of time needed for the tests, and ethical problems related to working with living organisms [15,18-20]. A study showed that cattle are 12.88 times more susceptible to BoNT-C compared to mice relative to their weight [21]. Another limiting factor in ruminant animals is the selection of the sample to be used for testing is uncertain. These situations cause a serious time lag in the definitive diagnosis of the disease, which sometimes may remain hidden because of the inability to determine the toxin. Therefore, in recent reports, some notifications have been made that immunological tests such as enzyme-linked immunosorbent assay (ELISA) and immuno-PCR for the diagnosis of the disease may be an alternative to mouse experiments [22]. After a detailed review of the literature, no studies have been identified in which BoNT toxins were investigated by immunohistochemical (IHC) method based on a similar immunological basis to these tests.

For all these reasons, firstly we aimed to determine the presence and the distribution of BoNT-C and D using the IHC method for the first time in the tissues of thirty ruminants (twenty cattle, seven sheep, and three goats) had clinical findings and a herd history of botulism. In addition, we secondly intended to investigate the interactions of these neurotoxins with SNAP-25 and synaptobrevin receptors, which are responsible for the development of clinical findings, in the ruminant neuropil tissue. In light of all these assessments, we finally aimed to analyse every detail of the disease from anamnesis information, environmental, feeding and sheltering observations to clinical, necropsy, and microscopic findings to make a preliminary diagnosis of the disease and take necessary protection and control measures immediately.

Material and Methods

Animals and Ethic Statements

The animal material of the study consisted of thirty ruminants (twenty cattle, seven sheep, and three goats), which were brought to the Faculty of Veterinary Medicine of Selçuk University, showing clinical botulism symptoms, and having a herd history of the disease. Each of these animals

represented a separate herd. While some animals were brought dead, others were sent to our laboratory for necropsy with the consent of their owners after necessary examinations in faculty clinics.

It was decided that the study was suitable in terms of research ethics by the Selçuk University Veterinary Faculty Experimental Animal Production and Research Center Ethics Committee (No: 2016/117).

Mouse Experiments

The liver, intestine, kidney, spleen, lung, heart, and brain (cerebrum, cerebellum, and brain stem) samples and rumen contents taken from the animals were directed to the local Veterinary Control and Research Institute authorized by the Ministry of Agriculture and Forestry. But here, mouse inoculation and toxin neutralization experiments were performed from only rumen contents according to the routine official protocol [23]. The rumen content is suspended with physiological saline, centrifuged at 3000 rpm for 15 min, passed through the filter (45 nm) and then administered intraperitoneally (0.5 mL) to the mice. The mice were monitored for 72-120 h in terms of botulism symptoms (respiratory paralysis, hump breathing, death). After the presence of BoNT was confirmed, typing was performed using specific BoNT antitoxins [23].

Histopathological Method

In the systemic necropsy of animals, samples were taken from liver, kidney, spleen, lung, heart, brain, and intestines and fixed in 10% neutral buffered formalin solution. After routine histopathological tissue processing, tissues were embedded in paraffin. Five-µm-thick sections were taken from the paraffin blocks with microtome and were stained with hematoxylin-eosin (HE) before being examined under a binocular light microscope (Olympus BX51, Tokyo, Japan). Photographs were also taken from typical lesions identified in microscopic examinations (Olympus DP12, Tokyo, Japan).

Immunohistochemical Method

For immunohistochemical staining, 5-µm-thick sections from target organs were stained in the immunohistochemistry staining device (Leica, Bondmax) according to the Bond™ polymer refine detection (Leica DS9800) kit procedure. First, all tissues were deparaffinized with heat and dewax solution (Bond™, Leica AR9222) and then rehydrated in graded alcohols (Merck). The sections were washed at least three times with washing solution (Bond™, Leica, AR9590) and deionized water after each step. Then, according to the primary antibody feature used, these sections were applied heat-induced epitope retrieval (epitope 1 antigen retrieval solution, Bond ™, Leica, AR9961, citrate buffer pH: 6.0, 100°C, 20 min). To remove peroxidase activity and prevent nonspecific binding, peroxidase block and protein block were applied at different times. After the reaction with each primary antibody (anti-*C. botulinum* C toxoid antibody [1:400, Abcam, ab27165], anti-*C. botulinum* D toxoid antibody [1:100, Abcam, ab64402], anti-SNAP25 antibody [1:400, Mybiosource, MBS395111], anti-synaptobrevin antibody [1:400, Mybiosource, MBS500033]) at room temperature, post-primary and polymer applications were performed. Afterward, all sections were incubated with 3,3′-Diaminobenzidine (DAB) for 5 min at room temperature. After the sections were washed with distilled water, contrast staining was performed with Mayer's hematoxylin. Sections were examined by binocular light microscope (Olympus BX51, Tokyo, Japan) and photographed (Olympus DP12, Tokyo, Japan).

IHC results for BoNT-C and BoNT-D were evaluated as positive or negative. IHC scoring methods for SNAP-25 and synaptobrevin were adapted from a previous study [12]. Accordingly, AnalySIS LS starter software (Soft Imaging System GmbH, Olympus, Germany) was used to measure and compare the dye intensity from images obtained from IHC stained brain sections. The areas from each section were selected and a grey scale was obtained in which absolute numbers of minimum and maximum staining density were determined for the regions of interest. SNAP-25 staining intensity was found as maximum 70.20 and minimum 171.05. For synaptobrevin 67.20-169.70 (maximum-minimum density values) were determined. Density values for each marker (SNAP-25 and synaptobrevin) were divided into four categories equally. Then, each case was scored as negative (0, no staining); mild (+1), (SNAP-25; 145.85-171.05, synaptobrevin; 144.09-169,70); moderate (+2), (SNAP-25; 120.63-145.84, synaptobrevin; 118.46-144.08); severe (+3), (SNAP-25; 95.42-120.62, synaptobrevin; 92.83-118.45); or very severe (+4), (SNAP-25; 70.20-95.41, synaptobrevin; 67.20-92.82).

In this study, animals of the same species (four cattle, one sheep and one goat) without any neural, toxic, or paralysis symptoms were used as the control group. We determined that there were no macroscopic or microscopic signs of toxicity in these animals. The organs of the cases that were found to be BoNT positive according to the mouse inoculation test results (1; BoNT-D, 1; BoNT-C) were used as positive control for IHC. Furthermore, the cases with suspected botulism in the study but found negative according to the mouse test and IHC staining results (14 of 30 ruminants) were evaluated as negative groups for SNAP-25 and synaptobrevin analyses.

Statistical Analyses

Statistical analysis of IHC findings was performed with the Statistical Package for the Social Sciences (SPSS for Windows® version 25.0) program. Distribution analyses were performed to check the variation of the data obtained using the Kolmogorov-Smirnov test. In addition, the homogeneity of variances was checked by Levene test. Normally distributed, these data were evaluated with one-way

ANOVA test, and then post-hoc Duncan analysis was performed to determine the differences between the groups. Results were given as mean±standard error (X±Sx), and $P<0.05$ was considered significant. In addition, the correlation among these data was examined by Pearson test (Minitab® version 18.1).

Results

Anamnesis, Clinical and Macroscopic Findings

In the history of suspected botulism cases, each of which represents a separate herd, it was recorded that the owners encountered snake, tortoise, or poultry carcasses in bait bales such as straw, clover, silage, and sugar beet pulp. Common clinical findings observed in preliminary reports by local veterinarians and animal caregivers are as follows: no fever, gait disturbances, tremors, paralysis in the hind legs, sternal lying position, head resting on the shoulder, anorexia, dehydration, hypersalivation, pouring of unswallowed feed and liquids. It was stated that in sheep and goats, paralysis and swallowing difficulties were less pronounced or death occurred without any symptoms. The widely observed macroscopic findings after necropsy were: accumulation of fluid in the chest and to a lesser extent in the abdominal cavity; foam in the trachea, pale lungs, and fluid leakage in the airways; endo-epicardial petechiae; multifocal hemorrhage and congestion in the liver; dilatation of the rumen, especially induration of omasum content; coffee grounds-like content in the abomasum; catarrhal enteritis or constipation; presence of skull imprint in the hemispheres; severe hyperaemia, edema, thickened and/or opaque appearance in meninges; and pushing of the cerebellum toward the occipital foramen *(Fig. 1)*.

Mouse Experiments Results

According to the results of mouse experiments performed from rumen contents in the authorized local laboratory, the presences of BoNT-D and BoNT-C in two and one cattle respectively were reported. In other cases, existence of toxin could not be detected.

Histopathological Findings

The main histopathological findings observed in visceral organs were pulmonary edema and emphysema, hydropic degeneration and focal steatosis in hepatocytes, hydropic degeneration in kidney tubules epithelium, and catarrhal enteritis. Meningeal changes consisted primarily of hyperemia, perivascular hemorrhage and edema in the cerebrum and cerebellum. Pronounced hyperemia, edematous dilatation in the perivascular space and hemorrhages were determined in the neuropil tissue of cerebrum, cerebellum, and medulla oblongata. Embolism or thrombosis was not detected. In the grey matter of cerebrum, cerebellum and brain stem, degeneration, chromatolysis and necrosis in neurons,

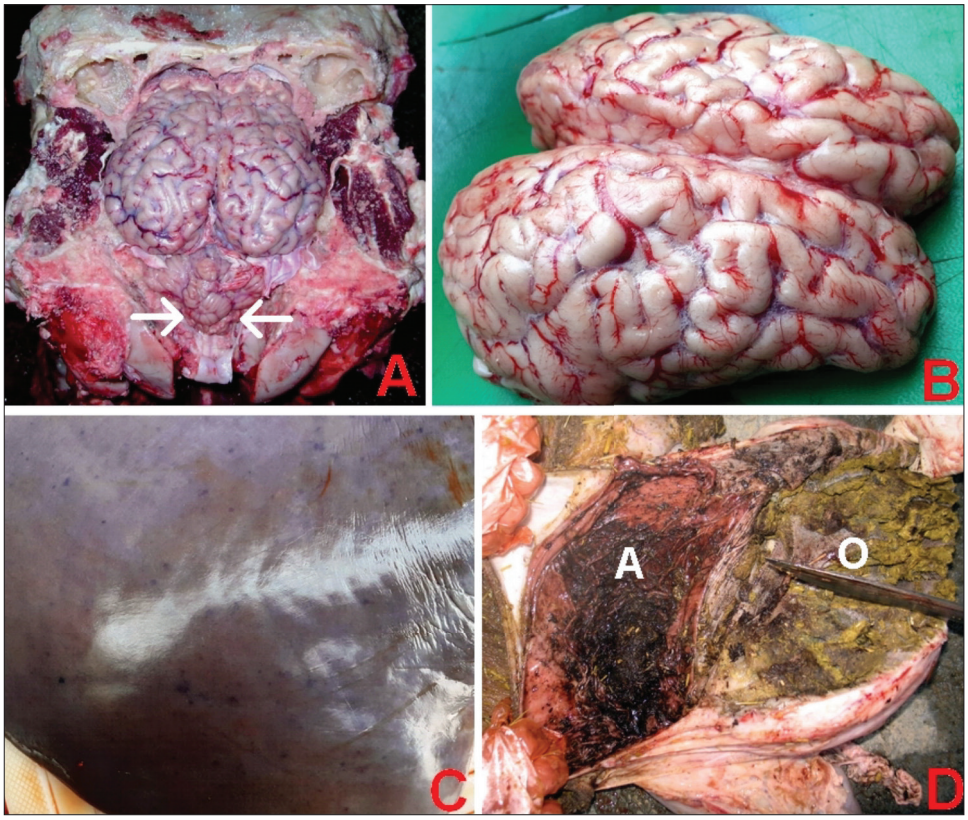


Fig 1. **(A)** Thickened and opaque meninges and hyperaemia in the brain and compression of the cerebellum to the occipital foramen *(arrows),* **(B)** Oedema and hyperaemia in meninges, **(C)** Multifocal hemorrhages in liver, **(D)** Induration and dryness of the omasum content **(O)**, a coffee grounds-like content in the abomasum **(A)**

perineuronal edema and gliosis were observed. Irregularly vacuoles and rarely demyelination were seen in the white matter of the whole central nervous system *(Fig. 2)*. To a lesser extent, hyperemia in the abomasum mucosa and depletion in lymphoid foci in the spleen were determined. The distribution of histopathological findings observed according to the BoNT type determined is presented in *Table 1* in detail. In addition, in the histopathological examination of two animals (one sheep, one goat), a micro-abscess and perivascular cuffing were found in the brain stem, and it was determined that these cases were listeriosis as a result of IHC staining.

Immunohistochemical Findings

The presence of BoNT type C and type D, and the density of SNAP-25 and synaptobrevin, were determined immunohistochemically in the various tissues of research animals. No immunoreactivity was found in animals used as negative control in the study. The positive reaction was obtained in two cases whose mouse test result was found to be positive and was used as positive control in the study. BoNT-C was detected in two of twenty cattle and one of seven sheep. BoNT-C immunoreactivity was not observed in goats. BoNT-D was determined in seven cases of cattle, two of sheep, and two of goats. BoNT-C+D immunoreactivity was observed in two cases of cattle. In summary, BoNT immunopositivity was achieved in a total of sixteen of thirty ruminants *(Table 2)*. Immunoreactivity was found in the cytoplasm and sometimes in the nucleus of the parenchymal cells *(Fig. 3)*. When the distribution of neurotoxins by organs was examined, no positive staining in the heart tissue was observed in any of the animal species. The detailed distribution of BoNT by organs and animal species is presented in *Table 3*.

The IHC scores for SNAP-25 and synaptobrevin are summarized in *Table 4*. SNAP-25 immunoreactivity in BoNT-C- and BoNT-C+D-positive cases were found to be significantly reduced compared to the control group, toxin-undetectable cases (negative animals), and BoNT-D-positive cases ($P<0.05$). In BoNT-D-positive cases, this effect

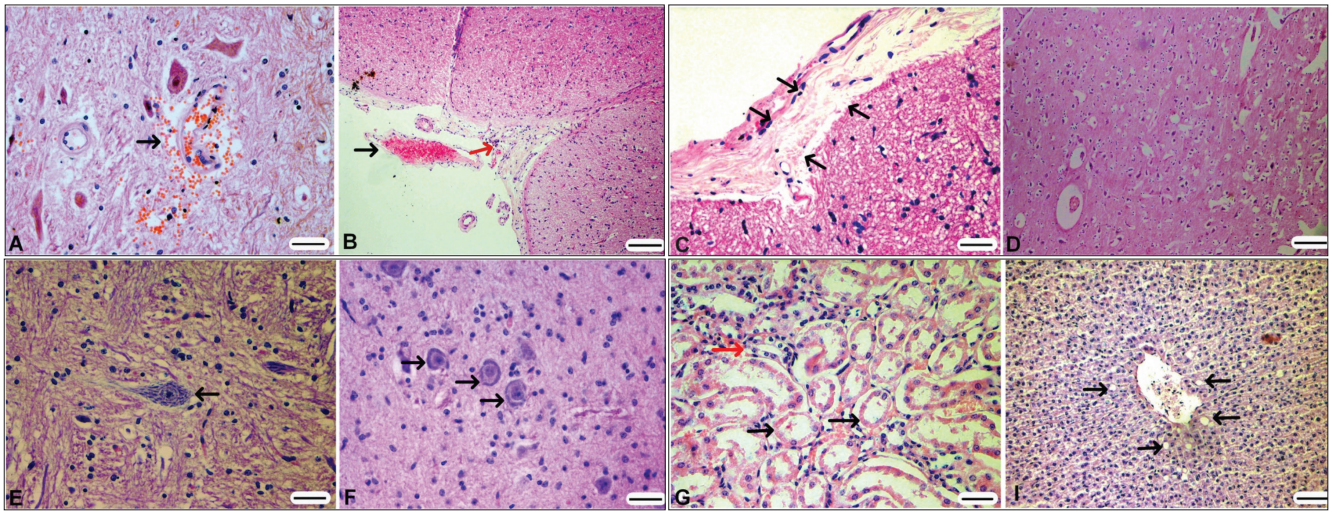


Fig 2. Botulism microscopic images (A) Bleeding in neuropil tissue (arrow), (B) Hyperemia (black arrow) and edema (red arrow) in meninges, (C) Edema and thickening in meninges (arrows), (D) Perineuronal oedema and gliosis, (E) Central chromatolysis in neurons (arrow), (F) Degeneration in neurons (arrows), (G) Degeneration (red arrow) and necrosis (black arrows) in kidney tubular epithelium, (I) Steatosis (arrows) in hepatocytes. HE, Scale bars: 50 µm (A, C, E, F, G), 100 µm (B, I) and 200 µm (D)

Table 1. *Histopathological findings according to the type of toxin*

Histopathological Findings	Toxin Types			
	Negative (n:14)	BoNT-C (n:3)	BoNT-D (n:11)	BoNT C+D (n:2)
Lung oedema and emphysema	5/14	3/3	9/11	2/2
Hydropic degeneration/fatty changes in hepatosit	4/14	2/3	8/11	1/2
Hydropic degeneration in kidney tubules epithelium	3/14	2/3	6/11	2/2
Catarrhal enteritis	4/14	2/3	7/11	1/2
Degeneration/necrosis and gliosis in neurons	6/14	3/3	8/11	2/2
Hyperaemia in brain vessels	6/14	3/3	8/11	2/2
Oedema in neuropil tissue	5/14	2/3	7/11	2/2
Haemorrhage in neuropil tissue	-/14	2/3	5/11	1/2

Table 2. *Distribution of BoNTs detected by IHC method and mouse test according to animal species*

Animal Species	IHC				Mouse Test	
	BoNT-C	BoNT-D	BoNT-C+D	Total	BoNT-D	BoNT-C
Cattle (n:20)	2	7	2	11 (55 %)	2	1
Sheep (n:7)	1	2	0	3 (42.85 %)	0	0
Goat (n:3)	0	2	0	2 (66.66 %)	0	0
Total	3	11	2	16 (53.33 %)	2	1

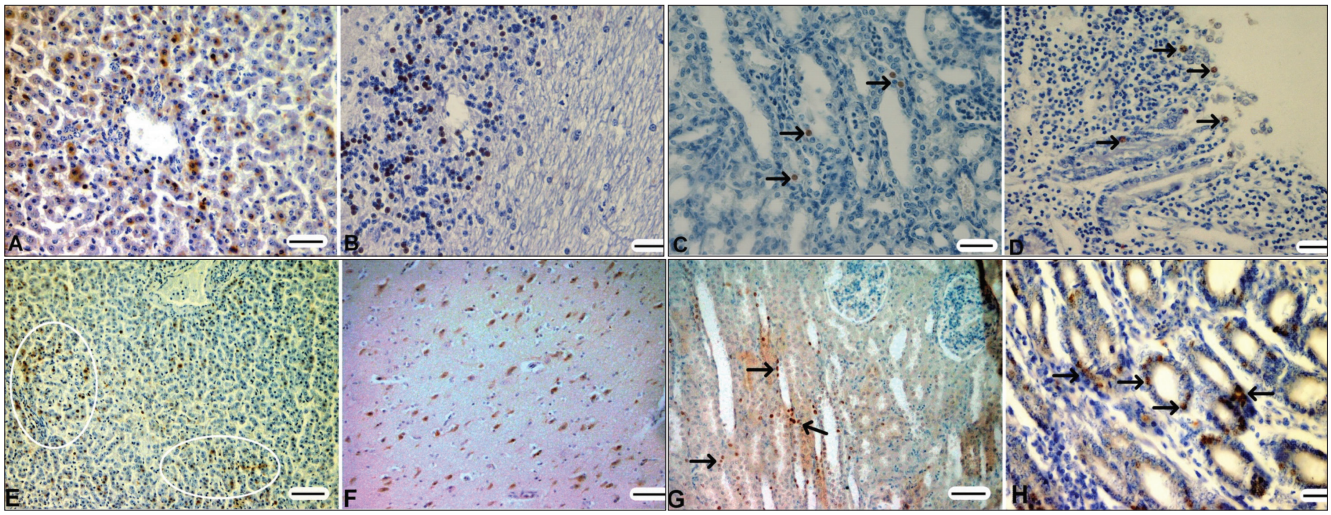


Fig 3. BoNT-C **(A-D)** and BoNT-D **(E-H)** immunoreactivity. Immunoreactivities in hepatocytes **(A)**, stratum granulosum of cerebellum **(B)**, tubular epithelial cells *(arrows)* **(C)**, intestinal epithelial cells *(arrows)* **(D)**, hepatocytes *(rings)* **(E)**, neurons in cerebrum **(F)**, tubular epithelial cells *(arrows)* **(G)**, intestinal gland epithelial cells *(arrows)* **(H)**. IHC staining, DAB chromogen, Mayer haematoxylin, Scale bars: 50 µm (A-D, H) and 100 µm (E-G)

Table 3. *Distribution of BoNT according to organs and animal species*

Tissue/Toxin Type	Cattle		Sheep		Goat		Total	
	BoNT-C	BoNT-D	BoNT-C	BoNT-D	BoNT- C	BoNT-D	BoNT- C	BoNT-D
Liver	1	3		1		2	1	6
Intestines	1	5	1				2	5
Brain	1	2	1	2		1	2	5
Kidney	2	1				1	2	2
Spleen						1		1

Table 4. *IHC staining scores of SNAP-25 and synaptobrevin by BoNT types**

SNARE Protein Type	Toxin Type				
	Control	BoNT-C (+)	BoNT-D (+)	BoNT-C+D (+)	Neg (-)
SNAP-25	3.75±0.25^{a}	1.33±0.33^{c}	2.63±0.24^{b}	1.50±0.50^{c}	3.35±0.17^{ab}
Synaptobrevin	3.50±0.28^{a}	3.33±0.33^{a}	1.18±0.12^{b}	1.00±0.00^{b}	3.28±0.24^{a}

$^{a-c}$ Values in columns with no common superscripts are significantly different according to the post-hoc Duncan test after the one-way ANOVA (P<0.01).
** The numbers represent the average values of IHC staining intensity scores for SNAP-25 and synaptobrevin according to BoNT types. Scores: Negative (0), mild (+1), moderate (+2), severe (+3), very severe (+4)*

was more partial. The immunoreactivity of synaptobrevin was found to be significantly reduced in BoNT-D- and BoNT-C+D-positive cases compared to other groups (P<0.05). There was no statistically significant difference in BoNT-C-positive cases (P>0.05). In addition, a moderately negative correlation (r:-0.50, P<0.005) between BoNT-C and SNAP-25 and a stronger negative correlation (r:-0.75, P<0.000) between BoNT-D and synaptobrevin were

Table 5. *Correlation relationship between BoNT types and SNAP-25 and synaptobrevin**

SNARE Protein Type	Toxin Type			
	BoNT-C (+)	BoNT-D (+)	BoNT-C+D (+)	Neg (-)
SNAP-25	r:-0.50 **P<0.005**	r:-0.104 P<0.585	r:-0.354 P<0.055	r:+0.578 **P< 0.001**
Synaptobrevin	r:+0.269 P<0.150	r:-0.75 **P<0.000**	r:-0.305 P<0.101	r:+0.718 **P<0.000**

* *Correlation value (r) was calculated by Pearson test*

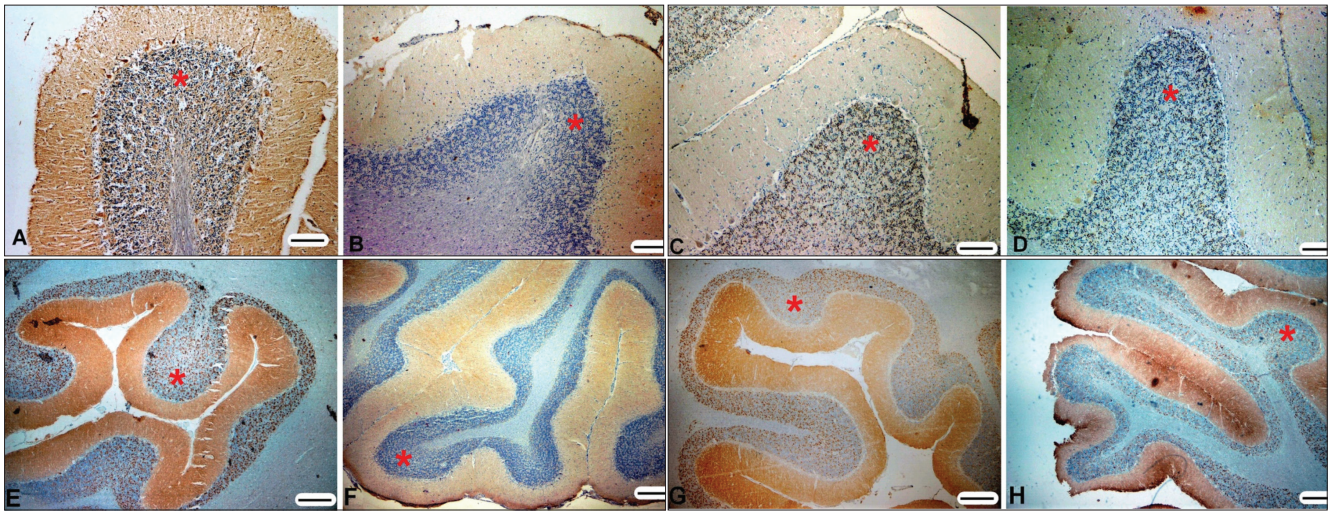


Fig 4. SNAP-25 **(A-D)** and synaptobrevin **(E-H)** immunoreactivity (Imr). **(A)** Very severe Imr in molecular, granular *(star)* and ganglionic layers, cerebellum, control group, **(B)** Mild Imr in granular layer *(star)*, cerebellum, BoNT-C positive group, **(C)** Severe Imr in granular layer (star), cerebellum, BoNT-D positive group, **(D)** Moderate Imr in granular layer *(star)*, cerebellum, BoNT-C+D positive group, **(E)** Very severe Imr in molecular, granular, and ganglionic layers, cerebellum, control group, **(F)** Mild Imr in granular layer, cerebellum, BoNT-D positive group, **(G)** Severe Imr in granular layer, cerebellum, BoNT-C positive group, **(H)** Moderate Imr in granular layer, cerebellum, BoNT-C+D positive group. IHC staining, DAB chromogen, Mayer haematoxylin, Scale bars: 500 µm (A-D), 200 µm (E-H)

observed, both of which were statistically significant. A positive correlation was found between negative cases and both SNAP-25 and synaptobrevin *(Table 5)*. Although SNAP-25 immunoreactivity was observed in neuropil tissue of the cerebrum, it was not found in neurons, glia cells, and endothelial cells. In the substantia grisea of cerebellum, intense immunoreactivity was observed in the granulosa layer and mild immunoreactivity in Purkinje cells. Substantia alba was not stained positive *(Fig. 4, A-D)*. Synaptobrevin IHC staining pattern in the cerebrum and cerebellum was detected to be exactly like the SNAP-25 *(Fig. 4, E-H)*.

Discussion

It has been reported that the most significant cause of widespread botulism outbreaks in farm animals is feed poisoning [24]. *C. botulinum* agents proliferate rapidly in deceased animal carcasses or rotted organic substances (such as improperly stored silage) and secrete neurotoxins [25]. Ingestion of feed and water contaminated with neurotoxins and, in recent years, large silage bales produced without acidification in large plastic airtight bags and the use of tight round bales of straw have been associated with increased cases of botulism in horses and cattle [25]. In the anamnesis of cases with suspected botulism that were brought to our laboratory and constitute the material of the current study, animal owners often stated that some dead rodents or other animals were found in the bales. That said, it is common for cadavers of deceased animals to be thrown into the pasture or around places where animals are housed. However, these cadaveric pieces, especially when eaten by animals that tend to eat foreign matter, are one of the important factors causing the disease. In addition, based on the technological development of agricultural machinery, the reaping of baby birds, snakes, lizards, turtles, and mice by harvesting machines during mowing while closest to the ground, and their mixing into the bales, may be another important reason for the disease. Therefore, it is understood that a good anamnesis knowledge and careful analysis of the shelter and the environment are highly important in the detection of the disease.

The clinical findings in the study are consistent with those observed in cases or outbreaks of botulism previously reported in the literature [9,13,18,20]. Although these clinical findings observed in animals vary according to the course

of the disease, they are not pathognomonic [4]. However, the most characteristic finding we observed was partial or complete paralysis in locomotor muscles. This paralysis usually starts from the back of the body and spreads forward. This condition, which occurs as a result of the inhibition in the release of acetylcholine, progresses rapidly and causes the animal to have difficulty in standing up and eating [18]. In addition, it was stated that most of the animals in our study died without any clinical signs.

The general judgment in botulism is that necropsy findings are not specific to the disease. However, systematic, and careful necropsy can provide important clues to indicate the disease. In this study, we evaluated that dilatation in the anterior stomachs, especially induration of the omasum and the dryness of its content, was caused by paralysis of the synaptic and presynaptic nerves [26]. Although these findings observed in the forestomach are also seen in some febrile diseases, they are thought to be highly important in cases where there is no fever but paralysis and toxicity symptoms are present. Despite this stagnation in the front stomachs, it is also remarkable to find watery content in the intestines. It has been interpreted that the findings such as fluid retention in the body cavities, pulmonary edema, bleeding in the visceral organs, developed because of damage caused by the toxin in the vascular endothelium. Similarly, compression of the cerebellum into the occipital foramen, the presence of skull imprints in the hemispheres, and thickened and/or opaque meninges may also be due to brain edema raised by vascular damage. Although macroscopic findings have occasionally been included in the literature [21,24], it is understood that they have not been analyzed in detail, the authors claiming that they are not specific to the disease. However, in the diagnosis of botulism, which is highly difficult to diagnose even in the most equipped laboratory conditions, the importance of evaluating and analyzing all kinds of information, especially necropsy findings, is undisputed, particularly in field conditions where resources are extremely limited.

In the study, important histopathological findings were observed in the central nervous system. Gliosis, ischemic neuronal changes, neuronal necrosis, and chromatolysis were seen. In addition, perivascular and perineural edema, hyperemia, and bleeding, which are the causes of macroscopically hyperemic and opaque appearance of meninges, were common *(Fig. 2)*. Similar degenerative vascular findings and cell degenerations were found in visceral organs such as the liver and kidney. This strongly suggests that most of these lesions might consist of circulatory disorders caused by toxin damage to the vascular endothelium. It should also be noted that relaxation in the vascular muscles, which may occur because of inhibition of acetylcholine release by neurotoxins, can contribute these circulatory disorders.

It is noteworthy that the histopathological findings belong to botulism in the literature are very limited [21,25]. However, in this study, the presence of significant microscopic lesions in cases with BoNTs suggests that these findings may often be overlooked *(Table 1)*. We predicted that the findings detected histopathologically, especially in the central nervous system, could provide important clues about botulism when evaluated together with other data, although they were not sufficient for the definitive diagnosis of the disease. Up to this point, anamnesis, clinical findings, environmental observation, feed and shelter examination, macroscopic findings during necropsy, and general evaluation of microscopic findings have been highly important for the prediagnosis of the disease. Immediately after the findings are observed, it is vital for herd health to take necessary protection and control measures such as vaccine application, suspicious feed and material disposal, and environmental cleaning until toxin isolation by the authorities.

In the study, well-known and used routinely in many areas, the IHC staining technic was used for the first time for the detection of BoNT-C and BoNT-D through specific monoclonal and polyclonal antibodies, and the results were evaluated. As such, the presence of BoNT was detected with IHC method in eleven of twenty cattle, three of seven sheep, and two of three goats (sixteen of thirty ruminants; 53.33%) with suspected botulism *(Table 2)*.

The rapid course of most cases owing to the high toxicity of BoNT poses a major challenge both in the diagnosis of botulism and in the implementation of appropriate treatment or precautions. The gold standard method for the detection of BoNT is considered to be the mouse inoculation test that can detect BoNT at the level of 10 pg/mL [16]. This test requires observation of mice for two to four days after intraperitoneal injection with samples prepared from serum or gastrointestinal contents [18]. Furthermore, additional neutralization steps are required to identify the antigenically different serotypes of BoNT [14,27]. However, new diagnostic methods need to be developed because this test is highly laborious, time-consuming, and potentially hazardous to personnel during injection; involves ethical problems; and requires a specially equipped laboratory [22]. For this purpose, a number of immunological test formats have recently been reported for the detection of the antigenic nature of the botulism toxin or toxin complexes [27]. Compared with the mouse test, immunological tests are performed and interpreted technically, simply, and quickly [28]. Most early diagnostic tests such as radioimmunoassay [29], ELISA [16], and immuno-PCR [22,27] provided equal sensitivity to mouse experiments thanks to advances in signal amplification [19]. In the past thirty years, ELISA, a test based on antigen-antibody interaction, has been the most widely used technique for serotyping and toxin detection [19]. With the introduction of high-affinity antibodies in ELISA, lower toxin detection limits were achieved than that of possible with mouse inoculation tests (2 pg/mL-2 ng/mL) [16,30]. In a study on

the serum of botulism-suspected animals (bovine and avian), ELISA and mouse inoculation tests were compared and found that the sensitivity and specificity of ELISA were higher [31]. From all these findings, it can be interpreted that immunologically based tests are successful in determining BoNT.

In addition to the above-mentioned disadvantages of mouse tests, there are some extra factors that limit sensitivity in ruminants [4]. Firstly, in order to perform mouse tests with samples prepared from blood serum, internal organs such as liver and stomach-intestines contents, autolysis should not have occurred yet in suspicious cadavers [18]. Because, toxigenic clostridia species rapidly proliferate in decomposed organic materials and can expose BoNT through autolysis [5]. Furthermore, the neurotoxigenic *C. botulinum* found in ruminants as part of the gut microbiota can invade post-mortem cadaver [5,18]. There may also be variations between species in the sensitivity to mouse tests [18]. Cattle have been found to be 12.88 times more susceptible to BoNT-C than mice [21]. It is therefore stated that mouse tests are not sensitive enough in ruminants, especially cattle [5,18,21]. For the mouse tests, only ruminal content samples taken in accordance with the standards from animals, in this study, were accepted by the official local laboratory authorized by the Ministry of Agriculture and Forestry. Here, the presences of BoNT-D and BoNT-C in two and one cattle respectively were able to be detected with the mouse tests *(Table 2)*. As in this study, there are many studies in which insufficient results were obtained in toxin isolation and identification as a result of the mouse tests [32-34]. The consensus in these studies is that the sensitivity of mouse bioassay is insufficient to detect the presence of BoNT in ruminants due to the very low amounts of circulating toxin and the toxin is rapidly degraded. In the Republic of Ireland, it has been reported that 65 dairy cows died or euthanized in botulism outbreak and BoNT could not found in mouse experiments [34]. In France, BoNT isolation could not be obtained by mouse tests in a major botulism outbreak resulting in the death of 80 of 110 cattle, but *C. botulinum* D/C spores were detected by PCR and anaerobic cultivation in silage [32]. In another botulism outbreak that resulted in the death of 427 cattle, toxin was obtained in mouse tests in the decayed cat cadaver, which is thought to cause the epidemic, while could not be detected in the rumen content, milk, and organs of the affected animals. However, it was reported that the presence of BoNT in liver and rumen content was detected with ELISA [24]. When all these are evaluated together, it can be concluded that mouse tests have low sensitivity in ruminants, positive results highly indicate the presence of botulism, but negative results are insufficient to exclude the disease [17,18,33,34].

The formalin-treated form of the toxin is called toxoid [27]. As is known, the tissues used in the application of the IHC method are fixed with formalin. Therefore, the use of antitoxoid primary antibodies, as in our study of toxin screening with IHC, is important to increase the affinity and specificity of staining. Rapid absorption of BoNT, which can be in minuscule amounts but with a strong toxic effect, to neuromuscular junctions also complicates toxin isolation [4,8,16,18]. This may be one of the reasons why most of the blood or fecal samples used in other diagnostic methods for live animals are often not positive. Similarly, methods such as mouse tests may give false negativity because of degradation of the toxin as a result of rapid enzymatic activity and other post-mortem changes, or false positives may arise owing to the toxins produced by the agents in the gut of dead animals [5,18]. However, in this study, thanks to formalin fixation of the tissues before the IHC method, these changes and disadvantages were prevented, and then the toxins in the tissues and organs were made visible using a toxoid form specific antibodies.

Also, visualization of toxin localization on tissues has been evaluated as another important advantage of IHC. Especially after death, there is doubt which tissue or sample to send to the laboratory for the diagnosis of botulism. Likewise, there are some contradictions about which types of samples should be used in diagnostic laboratories. In this regard, with this study we presented the organs determined to have BoNT by IHC method *(Table 3)*. But unfortunately, for the diagnosis of BoNT in Turkey, the mouse tests are performed from only gastrointestinal content, blood serum, or feed samples because of official rules [23]. This may be another reason why immunohistochemically positive cases in the study were negative in the mouse experiments (From 30 animals, a total of 16 in IHC but 3 in the mouse test; *Table 2*).

It has been reported that the H_{CC} subdomains of BoNT-C and BoNT-D's heavy chains interact with protein receptors, gangliosides (GD1b, GT1b) and phosphatidylethanolamine, which are expressed in many cell types such as hepatocytes and crypt epithelial cells in the gut. But the high presence of both gangliosides and protein receptors in neural cells is probably the explanation for why BoNT has a particular affinity for these cells and can cause the disease even at very low concentrations [10,11,35]. Also, it is stated that BoNTs can target many neurons, though not all, and inhibit the release of various compounds by affecting non-neuronal cells at high concentrations [10,36,37]. This situation is seen as a possible cause of the presence of toxin in brain and parenchymal organs in the study. As can be understood from both the prior literature [1,14,19,24,27,31,38,39] and the findings of this study that the samples prepared from the brain and internal organs, especially the liver, which is the first station for the toxins absorbed from the intestine, should be used in the diagnostic tests.

BoNT-C and D were found to be localized in the cytoplasm and sometimes in the nucleus. There are important differences in terms of intracellular localization of BoNT types. Indeed, it has been reported that the LC of BoNT-A

and BoNT-E are localized in the plasma membrane and the cytosol respectively while the LC of BoNT-B is distributed throughout the cell including the nucleus [36,40]. Although there is no data on the intracellular localization of BoNT-C and D in literature, it can be noted from the findings of the study that they distributed throughout cell compartments as similar to BoNT-B.

BoNTs proteolyze the SNARE proteins (SNAP-25 and synaptobrevin), preventing neurotransmitter release from vesicles containing acetylcholine in the neuromuscular junction and thereby causing loose paralysis [7]. BoNT-A, C1, and E proteolyze SNAP-25, while BoNT-B, D, F, and G cause proteolysis of synaptobrevin [41]. Because the proteolysis in SNARE proteins differs according to the toxin type, the researchers have conducted studies on the usability of this feature in determining the toxin type of the disease. In hippocampal culture samples given BoNT-C, it was found that IHC staining of syntaxin and SNAP-25 were decreased [42]. It has been noted, in a study, that BoNT-A, C, and D cause significant reductions in immunoreactivity of SNAP-25, syntaxin I, and synaptobrevin II in the mouse phrenic nerve-hemidiaphragm preparation [43]. Similarly, others have reported that SNAP-25 or synaptobrevin is specifically proteolyzed by BoNTs [44,45]. However, to the best of our knowledge, there are no data in the literature on the interaction between these toxins and SNARE proteins in ruminants that are heavily exposed to BoNTs. In our study, in cases in which BoNT-C and BoNT C+D were determined, SNAP-25 expression decreased significantly compared to the control and negative groups. This situation was partially detected in cases in which BoNT-D was determined. However, only BoNT-C from these groups was found to have a significant negative correlation with SNAP-25; that is, this toxin reduced the SNAP-25. On the other hand, synaptobrevin expression was decreased in BoNT-D and BoNT-C+D groups compared to other groups, but a negative correlation was observed between only BoNT-D and synaptobrevin *(Table 4, Table 5)*. When the immunoreactivity data of SNAP-25 and synaptobrevin obtained in our study were evaluated together, it was found that BoNT-C specifically proteolyzes SNAP-25 while BoNT-D specifically proteolyzes synaptobrevin in ruminants. This indicates that the flaccid paralysis in ruminants because of BoNTs, as in other species [12,41] is a result of the proteolysis of SNAP-25 and synaptobrevin. At the same time, it can be a guide both for confirming a diagnosis of botulism made with IHC and in cases where botulism is suspected but toxins could not be detected. In addition, SNAP-25 and synaptobrevin proteolysis in the central nervous system can disrupt acetylcholine release, resulting in impaired communication between neurons. This situation may contribute to the aggravation of the observed symptoms or the increase of deaths in ruminates.

As a result, in this study, we attempted to conduct a comprehensive analysis of macroscopic and microscopic findings, which take a very limited part in the literature, as well as the diagnostic approach from the anamnesis stage to clinical findings of botulism disease. In this context, we emphasized that anamnesis, clinical symptoms, and macroscopic and microscopic findings should be evaluated together for the preliminary diagnosis of the disease. Also, we have suggested that, if necessary, preventive measures (vaccination, destruction of contaminated feed and materials, etc.) should be taken without waiting for the results of tests to reveal the presence of toxins. In addition, as an alternative to mouse inoculation tests, the effectiveness of IHC staining was questioned, and it was thought that this method might be worthy comparing with other tests. It was also determined that the proteolysis status of SNAP-25 and synaptobrevin, which are responsible for the emergence of clinical findings in patients, may contribute to the diagnosis.

Conflict of Interest

The authors have no conflict of interest.

Authors Contributions

M.B. Ateş and M.K. Çiftci made the experiment and the histological and immunohistochemical interpretation, and wrote the manuscript. F. Terzi and Z. Çelik planned methodology and investigated resources, M. Ortatatlı wrote and review and editing the manuscript. All authors discussed the results and contributed to the final manuscript.

References

1. Quinn PJ, Markey BK, Leonard FC, Hartigan P, Fanning S, Fitzpatrick E: Veterinary microbiology and microbial disease. 2nd ed. 237-239, John Wiley & Sons, Iowa, USA, 2011.

2. Pirazzini M, Rossetto O, Eleopra R, Montecucco C: Botulinum neurotoxins: Biology, pharmacology, and toxicology. *Pharmacol Rev,* 69 (2): 200-235, 2017. DOI: 10.1124/pr.116.012658

3. Poulain B, Popoff MR: Why are botulinum neurotoxin-producing bacteria so diverse and botulinum neurotoxins so toxic? *Toxins,* 11 (1): 34, 2019. DOI: 10.3390/toxins11010034

4. Cope RB: Botulinum neurotoxins. **In,** Gupta RC (Ed): Veterinary Toxicology. 3rd ed., 743-757, Elsevier, London, United Kingdom, 2018.

5. Rossetto O, Pirazzini M, Montecucco C: Botulinum neurotoxins: Genetic, structural and mechanistic insights. *Nat Rev Microbiol,* 12 (8): 535-549, 2014. DOI: 10.1038/nrmicro3295

6. Soares MC, Gaspar AO, Brumatti RC, Gomes DC, Neves DA, Alcântara LOB, Leal PV, Lemos RAA: Economic impact of an outbreak of botulism in a cattle feedlot. *Pesq Vet Bras,* 38 (7): 1365-1370, 2018. DOI: 10.1590/1678-5150-PVB-5643

7. Rossetto O, Pirazzini M, Bolognese P, Rigoni M, Montecucco C: An update on the mechanism of action of tetanus and botulinum neurotoxins. *Acta Chim Slov,* 58 (4): 702-707, 2011.

8. Poulain B, Lemichez E, Popoff MR: Neuronal selectivity of botulinum neurotoxins. *Toxicon,* 178, 20-32, 2020. DOI: 10.1016/j.toxicon.2020.02.006

9. Böhnel H, Gessler F: Botulinum toxins-cause of botulism and systemic diseases? *Vet Res Commun,* 29 (4): 313-345, 2005. DOI: 10.1023/b:verc.0000048489.45634.32

10. Popoff MR, Poulain B: Bacterial toxins and the nervous system: neurotoxins and multipotential toxins interacting with neuronal cells. *Toxins,* 2 (4): 683-737, 2010. DOI: 10.3390/toxins2040683

11. Popoff MR, Bouvet P: Clostridial toxins. *Future Microbiol,* 4 (8): 1021-1064, 2009. DOI: 10.2217/fmb.09.72

12. Raptis A, Torrejon-Escribano B, Gomez de Aranda I, Blasi J: Distribution of synaptobrevin/VAMP 1 and 2 in rat brain. *J Chem Neuroanat,* 30 (4): 201-211, 2005. DOI: 10.1016/j.jchemneu.2005.08.002

13. Mariano V, Nardi A, Gradassi S, De Santis P, Anniballi F, Bilei S, Scholl F, Auricchio B, Bielli C, Culicchi M, Casali De Rosa GL: A severe outbreak of botulism in cattle in Central Italy. *Vet Ital,* 55 (1): 57-62, 2019. DOI: 10.12834/VetIt.768.3714.2

14. Cai S, Singh BR, Sharma S: Botulism diagnostics: From clinical symptoms to *in vitro* assays. *Crit Rev Microbiol,* 33 (2): 109-125, 2007. DOI: 10.1080/10408410701364562

15. Stern D, von Berg L, Skiba M, Dorner MB, Dorner BG: Replacing the mouse bioassay for diagnostics and potency testing of botulinum neurotoxins - progress and challenges. *Berl Munch Tierarztl Wochenschr,* 131 (9-10): 375-394, 2018. DOI: 10.2376/0005-9366-17110

16. Hobbs RJ, Thomas CA, Halliwell J, Gwenin CD: Rapid detection of botulinum neurotoxins-A review. *Toxins,* 11 (7): 418, 2019. DOI: 10.3390/toxins11070418

17. Otter A, Uzal FA: Clostridial diseases in farm animals: 2. Histotoxic and neurotoxic diseases. *In Pract,* 42 (5): 279-288, 2020. DOI: 10.1136/inp.m1984

18. Hogg R, Livesey C, Payne J: Diagnosis and implications of botulism. *In Pract,* 30 (7): 392-397, 2008. DOI: 10.1136/inpract.30.7.392

19. Lindstrom M, Korkeala H: Laboratory diagnostics of botulism. *Clin Microbiol Rev,* 19 (2): 298-314, 2006. DOI: 10.1128/CMR.19.2.298-314.2006

20. Le Marechal C, Hulin O, Mace S, Chuzeville C, Rouxel S, Poezevara T, Mazuet C, Pozet F, Sellal E, Martin L, Viry A, Rubbens C, Chemaly M: A case report of a botulism outbreak in beef cattle due to the contamination of wheat by a roaming cat carcass: From the suspicion to the management of the outbreak. *Animals,* 9 (12): 1025, 2019. DOI: 10.3390/ani9121025

21. Moeller RB, Puschner B, Walker RL, Rocke T, Galey FD, Cullor JS, Ardans AA: Determination of the median toxic dose of type C botulinum toxin in lactating dairy cows. *J Vet Diagn Invest,* 15 (6): 523-526, 2003. DOI: 10.1177/104063870301500603

22. Ryabko AK, Kozyr' AV, Kolesnikov AV, Khlyntseva AE, Zharnikova IV, Shemyakin IG: Strategies for upgrading analyte detection in immuno-PCR studied on identification of type A botulinum neurotoxin. *Appl Biochem Microbiol,* 52 (1): 110-120, 2016. DOI: 10.1134/s0003683816010117

23. Commission: Bakteriyoloji. **In,** Diker KS (Ed): Teşhiste Metot Birliği. 98-100, Ministry of Agriculture and Forestry, Ankara, Turkey, 2014.

24. Galey FD, Terra R, Walker R, Adaska J, Etchebarne MA, Puschner B, Fisher E, Whitlock RH, Rocke T, Willoughby D, Tor E: Type C botulism in dairy cattle from feed contaminated with a dead cat. *J Vet Diagn Invest,* 12 (3): 204-209, 2000. DOI: 10.1177/104063870001200302

25. Myllykoski J, Lindstrom M, Keto-Timonen R, Soderholm H, Jakala J, Kallio H, Sukura A, Korkeala H: Type C bovine botulism outbreak due to carcass contaminated non-acidified silage. *Epidemiol Infect,* 137 (2): 284-293, 2009. DOI: 10.1017/S0950268808000939

26. Pamukcu AM: Hemorrhagic encephalomyelitis due to botulism in cattle in Turkey. *Zentralbl Veterinarmed,* 1 (8): 707-722, 1954. DOI: 10.1111/j.1439-0442.1954.tb00039.x

27. Grate JW, Ozanich Jr RM, Warner MG, Bruckner-Lea CJ, Marks JD: Advances in assays and analytical approaches for botulinum-toxin detection. *Trends Analyt Chem,* 29 (10): 1137-1156, 2010. DOI: 10.1016/j.trac.2010.07.005

28. Ekong T: Immunological detection of botulinum neurotoxins. *Anaerobe,* 6 (2): 125-127, 2000. DOI: 10.1006/anae.1999.0322

29. Ashton AC, Crowther JS, Dolly JO: A sensitive and useful radioimmunoassay for neurotoxin and its haemagglutinin complex from *Clostridium botulinum*. *Toxicon,* 23 (2): 235-246, 1985. DOI: 10.1016/0041-0101(85)90146-1

30. Singh AK, Stanker LH, Sharma SK: Botulinum neurotoxin: Where are we with detection technologies? *Crit Rev Microbiol,* 39 (1): 43-56, 2013. DOI: 10.3109/1040841X.2012.691457

31. Thomas RJ: Detection of *Clostridium botulinum* types C and D toxin by ELISA. *Aust Vet J,* 68 (3): 111-113, 1991. DOI: 10.1111/j.1751-0813.1991.tb00769.x

32. Relun A, Dorso L, Douart A, Chartier C, Guatteo R, Mazuet C, Popoff MR, Assie S: A large outbreak of bovine botulism possibly linked to a massive contamination of grass silage by type D/C *Clostridium botulinum* spores on a farm with dairy and poultry operations. *Epidemiol Infect,* 145 (16): 3477-3485, 2017. DOI: 10.1017/S0950268817002382

33. Dlabola J, Hashish E, Pauly B, Kubisiak B, Behm I, Heseler R, Schliephake A, Wieler LH, Neubauer H, Seyboldt C: *Clostridium botulinum* type D/C intoxication in a dairy cow stock in Saxony-Anhalt (Germany)--report on an innovative diagnostic approach. *Berl Munch Tierarztl Wochenschr,* 129 (3-4): 111-117, 2016.

34. Sharpe AE, Brady CP, Byrne W, Moriarty J, O'Neill P, McLaughlin JG: Major outbreak of suspected botulism in a dairy herd in the Republic of Ireland. *Vet Rec,* 162 (13): 409-412, 2008. DOI: 10.1136/vr.162.13.409

35. Kroken AR, Blum FC, Zuverink M, Barbieri JT: Entry of botulinum neurotoxin subtypes A1 and A2 into neurons. *Infect Immun,* 85 (1): e00795-16, 2017. DOI: 10.1128/IAI.00795-16

36. Beard M, Chaddock JA: *Clostridium botulinum* and associated neurotoxins. **In,** Tang YW, Sussman M, Liu D, Poxton I, Schwartzman J (Eds): Molecular Medical Microbiology. 2nd ed., 1015-1029, Academic Press, Boston, 2015. DOI: 10.1016/B978-0-12-397169-2.00057-3

37. Poulain B, Popoff MR, Molgó J: How do the botulinum neurotoxins block neurotransmitter release: From botulism to the molecular mechanism of action. *Botulinum J,* 1 (1): 14-87, 2008. DOI: 10.1504/TBJ.2008.018951

38. Le Maréchal C, Woudstra C, Fach P: Botulism. **In,** Uzal FA, Prescott JF, Songer JG (Eds): Clostridial Diseases of Animals. 303-330, John Wiley & Sons, Iowa, USA, 2016.

39. Kruger M, Neuhaus J, Herrenthey AG, Gokce MM, Schrodl W, Shehata AA: Chronic botulism in a Saxony dairy farm: Sources, predisposing factors, development of the disease and treatment possibilities. *Anaerobe,* 28, 220-225, 2014. DOI: 10.1016/j.anaerobe.2014.06.010

40. Fernández-Salas E, Ho H, Garay P, Steward LE, Aoki KR: Is the light chain subcellular localization an important factor in botulinum toxin duration of action? *Mov Disord,* 19 (Suppl. 8): S23-S34, 2004. DOI: 10.1002/mds.20006

41. Rodloff AC, Kruger M: Chronic *Clostridium botulinum* infections in farmers. *Anaerobe,* 18 (2): 226-228, 2012. DOI: 10.1016/j.anaerobe.2011.12.011

42. Capogna M, McKinney RA, O'Connor V, Gähwiler BH, Thompson SM: Ca^{2+} or Sr^{2+} partially rescues synaptic transmission in hippocampal cultures treated with botulinum toxin A and C, but not tetanus toxin. *J Neurosci,* 17 (19): 7190-7202, 1997. DOI: 10.1523/jneurosci.17-19-07190.1997

43. Kalandakanond S, Coffield JA: Cleavage of intracellular substrates of botulinum toxins A, C, and D in a mammalian target tissue. *J Pharmacol Exp Ther,* 296 (3): 749-755, 2001.

44. Zhou JY, Wang ZF, Ren XM, Tang MZ, Shi YL: Antagonism of botulinum toxin type A-induced cleavage of SNAP-25 in rat cerebral synaptosome by toosendanin. *FEBS Lett,* 555 (2): 375-379, 2003. DOI: 10.1016/s0014-5793(03)01291-2

45. Whelchel DD, Brehmer TM, Brooks PM, Darragh N, Coffield JA: Molecular targets of botulinum toxin at the mammalian neuromuscular junction. *Mov Disord,* 19 (Suppl. 8): S7-S16, 2004. DOI: 10.1002/mds. 20004

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

Kafkas Univ Vet Fak Derg
27 (2): 203-208, 2021
DOI: 10.9775/kvfd.2020.25067

RESEARCH ARTICLE

Evaluation of the Recombinant *Eg*AgB8/2 Antigen for the Diagnosis of Cystic Echinococcosis in Tibetan Sheep on the Qinghai-Tibetan Plateau, China

Hong DUO [1,a] Yong FU [1,b (*)] Xiuying SHEN [1,c] Zhihong GUO [1,d] Xueyong ZHANG [1,e] Yijuan MA [1,f] Mohammed YOSRI [2,g] Tiancong SUN [1,h] Youlu CHEN [1,i] Gengqiu JIUNAI [3,j]

[1] Qinghai Academy of Animal Science and Veterinary Medicine, Qinghai University, State Key Laboratory of Plateau Ecology and Agriculture, Xining 810016, CHINA
[2] The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo 11787, EGYPT
[3] Animal Husbandry and Veterinary Station, Zhenqin Township, Chindu County 815100, CHINA
ORCIDs: [a] 0000-0002-3013-9818; [b] 0000-0003-3354-9107; [c] 0000-0002-3804-2113; [d] 0000-0003-0049-607X; [e] 0000-0001-5076-9211 [f] 0000-0003-0423-1730; [g] 0000-0001-6197-0690; [h] 0000-0002-1009-4042; [i] 0000-0003-0029-4507; [j] 0000-0002-7926-1611

Article ID: KVFD-2020-25067 Received: 12.10.2020 Accepted: 20.01.2021 Published Online: 21.01.2021

Abstract

The unique geographical and ecological conditions of the Qinghai-Tibetan Plateau (QTP) constitute the environmental conditions of the natural foci of cystic echinococcosis (CE). It is a rare and highly endemic area in the world. The main host of the pathogen, Tibetan sheep, is widely distributed, and the population density is on the rise in recent years, which increases the pressure on the transmission and risk of the natural epidemic source. Currently, the developments of serological diagnosis test for *Echinococcus granulosus* infection on the QTP Tibetan sheep are scanty, which seriously restricts the epidemiological investigation of CE in Tibetan sheep on the QTP. *Eg*AgB8/2 is one of the most important targets of host antibody response in CE and has better diagnostic performance in livestock. This study expressed the recombinant *Eg*AgB8/2 antigen of *E. granulosus* from Tibetan sheep in prokaryotic expression vector, and also preliminarily evaluated its potential value for diagnosing CE in Tibetan sheep using indirect ELISA. Our preliminary results shown that the recombinant *Eg*AgB8/2 antigen had good immunogenicity and exhibited high sensitivity (95%) and high specificity (100%) and no cross-reacted with both *Taenia multiceps* and *Taenia hydatigena*. Further studies are needed to collect more Tibetan sheep sera from *E. granulosus* and other parasitic infections, which may evaluate further sensitivity, specificity and cross-reactive of recombinant *Eg*AgB8/2 antigen in Tibetan sheep positive for CE of the QTP, China.

Keywords: *Cystic echinococcosis, Tibetan sheep, EgAgB8/2 antigen, Serological diagnosis*

Çin'in Qinghai-Tibet Platosu'ndaki Tibet Koyunlarında Kistik Ekinokokkozisin Tanısında Rekombinant *Eg*AgB8/2 Antijeninin Değerlendirilmesi

Öz

Qinghai-Tibet Platosu'nun eşsiz coğrafyası ve ekolojisi, kistik ekinokokkozisin (KE) doğal odaklarının çevresel koşullarını oluşturur. Burası dünyada nadir bulunan ve oldukça endemik bir bölgedir. Patojenin ana konakçısı olan Tibet koyununun yaygınlığı ve popülasyon yoğunluğu son yıllarda artmaktadır ve bu durum doğal salgın kaynağı riskini ve bulaş üzerindeki baskıyı artırmaktadır. Günümüzde, Qinghai-Tibet Platosu'ndaki Tibet koyunlarında *Echinococcus granulosus* enfeksiyonunun serolojik tanı testlerindeki gelişmeler yetersizdir ve bu, bölgede Tibet koyunlarında KE'a yönelik epidemiyolojik araştırmaları ciddi şekilde kısıtlamaktadır. *Eg*AgB8/2, KE'da konakçı antikor yanıtının en önemli hedeflerinden birisidir ve çiftlik hayvanlarında enfeksiyonun teşhisinde daha iyi bir performansa sahiptir. Bu çalışmada, prokaryotik vektör kullanılarak Tibet koyunlarından elde edilen *E. granulosus*'un rekombinant *Eg*AgB8/2 antijeninin ekspresyonu sağlandı ve bu antijenin Tibet koyunlarında KE'un teşhisinde kullanım potansiyeline yönelik ön değerlendirmesi indirekt ELISA ile gerçekleştirildi. Çalışmamıza ait ön bulgular, rekombinant *Eg*AgB8/2 antijeninin iyi bir immünojeniteye sahip olduğunu, yüksek duyarlılık (%95) ve özgüllük (%100) sergilediğini ve hem *Taenia multiceps* hem de *Taenia hydatigena* ile çapraz reaksiyon vermediğini gösterdi. *E. granulosus* ve diğer parazitik etkenlerle enfekte daha fazla Tibet koyunundan toplanacak serumlarla, rekombinant *Eg*AgB8/2 antijeninin duyarlılık, özgüllük ve çapraz reaksiyonlarının değerlendirmesine yönelik daha fazla çalışma yapılmasına ihtiyaç vardır.

Anahtar sözcükler: *Kistik ekinokokkozis, Tibet koyunu, EgAgB8/2 antijeni, Serolojik tanı*

How to cite this article?

Hong Duo H, Fu Y, Shen X, Guo Z, Zhang X, Ma Y, Yosri M, Sun T, Chen Y, Jiunai G: Evaluation of the recombinant *Eg*AgB8/2 antigen for the diagnosis of cystic echinococcosis in Tibetan sheep on the Qinghai-Tibetan Plateau, China. *Kafkas Univ Vet Fak Derg,* 27 (2): 203-208, 2021. DOI: 10.9775/kvfd.2020.25067

(*) Corresponding Author

Tel: +86 0971 5226221
E-mail: qhfuyong@163.com (Y. Fu)

Introduction

Echinococcosis is a zoonosis that can be caused by cestodes of the genus *Echinococcus*, especially cystic echinococcosis (CE) caused by the larvae of *Echinococcus granulosus*, occurs worldwide and is highly endemic on the Qinghai-Tibetan Plateau (QTP), China [1-3]. CE not only seriously endangers the health of both humans and livestock but also is a significant public health issue worldwide [4-7]. The diagnosis of CE is primarily based on imaging and ultrasound techniques which are sometimes inconclusive, but serological tests can be applied as a supplemented test [8]. Especially, the serological test is generally needed in livestock as imaging methods are not practical due to cost factors [9]. Tibetan sheep is the main livestock species on the QTP, which the infection rate of CE in Tibetan sheep is relatively high in this area [10,11]. Currently, epidemiological surveillance of CE in Tibetan sheep on the QTP is based mainly on necropsy procedures. In the absence of serological diagnostic test, infected Tibetan sheep remain in the morbid state, leading to substantial economic losses to the development of animal husbandry. Therefore, the development of an accurate serological diagnostic test of CE in Tibetan sheep is essential for devising effective strategies to control this disease on the QTP.

Over the past years, serological diagnotic test of CE in sheep mainly depends on hydatid cyst fluid (HCF), a crude protoscolex preparation and recombinant proteins as recombinant EG95 oncosphere protein and the AgB hydatid cyst fluid protein [12]. Particularly, *E. granulosus* hydatid cyst fluid has been extensively investigated in serological tests, and its protein components of *Eg*AgB antigens has better diagnostic performance [13]. To date, five different but closely related genes of *Eg*AgB have been identified, corresponding to genes *Eg*AgB1, *Eg*AgB2, *Eg*AgB3, *Eg*AgB4 and *Eg*AgB5 [14]. Several studies have shown that *Eg*AgB8/2 is the most promising subunit of *Eg*AgB for the development of a reliable sensitive and specific test for the serodiagnosis of CE [14-16], which has been widely used in the serological test of CE in inter-mediate host including cattle, sheep, goats, buffaloes, camels, etc. [9,17-19]. Previous studies have shown that the recombinant *Eg*AgB8/2 antigen is very immunogenic [20], and the recombinant *Eg*AgB2 protein has been found to improve the performance of ELISA for serodiagnosis of CE [21], which developing the ELISA test has proved the sensitive and convenient for detecting CE.

Tibetan sheep are predominantly infected with cystic echinococcosis when compared with other animals on the Qinghai-Tibetan Plateau, China. However, there have been currently fewer reports on the development of serological tests for CE in Tibetan sheep. Therefore, in the present study, we expressed the recombinant antigen *Eg*AgB8/2 from cystic echinococcosis in Tibetan sheep. In addition, we preliminarily investigated the recombinant *Eg*AgB8/2 antigen potential value for diagnosing CE in Tibetan sheep using indirect ELISA. These findings are essential for large-scale epidemiological investigations of CE on the QTP endemic regions.

Material and Methods

Parasites and Sera Collection

Hydatid cysts were collected from Tibetan sheep at a local abattoir in Qinghai province, China. Hydatid cysts were identified as *E. granulosus* sensu stricto (G1) [22], and protoscolices were separated from hydatid cyst fluid as previously described [23]. Positive sera (n=20) and negative sera (n=20) for CE infection in Tibetan sheep were provided by the zoonosis laboratory, Qinghai University. Positive sera against *Taenia hydatigena* (n=20) and *Taenia multiceps* (n=20) were obtained from naturally infected Tibetan sheep. All sera were stored at -20°C.

cDNA Synthesis of EgAgB8/2

Total RNA was isolated from the protoscolices using Trizol reagent (Sangon, Shanghai, China) according to the manufacturer's recommendations. The cDNA was synthesized with M-MLV kit (Sangon, Shanghai, China) following standard protocols of cDNA synthesis. The cDNA coding sequence of *Eg*AgB8/2 target protein was PCR amplified using the primers 5′-GCGAATCCTCTGCGTGTGA CATTTGTGGAG-3′ (including an *EcoR* I site, restriction sites underlined) and 5′-GCAAGCTTTGGCAAATCATGTGT CCCGAC-3′ (including a *Hind* III site, restriction sites underlined). The cDNAs coding of *Eg*AgB8/2 protein was cloned in the pET32a (+) plasmid (Invitrogen, Carlsbad, California, USA). after their PCR amplification with primers designed with suitable restriction enzyme sites.

Expression, Purification and Data Analyses of Recombinant Protein

The target fragment and the vector fragment were recovered individually and ligated using T4 DNA ligase (Invitrogen, Carlsbad, California, USA) at 4°C overnight, and subsequently transformed into *Escherichia coli* BL21 (DE3) (Solarbio, Beijing, China). The expression was induced using 0.5 mM/L IPTG at 37°C for 5 h. Then, the bacterial cells were harvested and lysed using ultrasonication. Briefly, the bacteria were resuspended in 25 mL of binding buffer and ultra-sonicated until the solution was clear. The solution was centrifuged, and the supernatant was collected and filtered through a 0.22 μm filter. The recombinant protein was purified to complete homogeneity using Ni-NTA agarose resin (Qiagen, Hilden, Germany). Briefly, the recombinant protein was allowed to bind to Ni-NTA resin at room temperature for 2 h with constant shaking. Affinity column was washed with wash buffer (pH 6.5) supplemented with 12 mM imidazole and recombinant protein eluted with elution buffer at pH 4.2. The expression of the *Eg*AgB8/2

recombinant protein was analyzed by SDS-PAGE, and the protein concentration was determined with UV spectrophotometry.

Western Blotting Analysis

The recombinant protein was run on 10% SDS-PAGE gel and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA). Membranes were incubated using a 1:1000 dilution of a mouse anti-His monoclonal antibody (Sigma, St. Louis, USA), negative serum (1:200 dilution), and sheep anti-*E. granulosus* serum (1:200 dilution) at 37°C for 1 h. After washing for three times, a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma, St. Louis, USA) was used as secondary antibody at 1:1500 dilution and was incubated at 37°C for 2 h. Finally, reactions were visualized using the TMB Chromogenic Reagent kit (Sangon, Shanghai, China) according to manufacturer's instructions.

Establishment of Indirect ELISA

Optimization experiments were used to determine the optimum working conditions of the antigen *Eg*AgB8/2 and sera. Briefly, *Eg*AgB8/2 was diluted to concentrations from 20 µg/mL to 0.625 µg/mL in 1:2 dilution steps, and each dilution was added to the ELISA plate (100 µL/well) and incubated overnight at 4°C. The wells were blocked with 5% skim milk, washed, and incubated with serum samples in dilutions (1:10, 1:50, 1:100, 1:200 and 1:400) at 37°C for 1 h. The plates were then washed three times and incubated with HRP-labeled rabbit anti-goat IgG (Boster Bio-project Co, Wuhan, China) for 1 h. Color reactions were developed with *o*-phenylenediamine solution (OPD) for 15 min in the dark, and the reaction was terminated with 2 M H_2SO_4. The optical density at 450 nm was measured with a microplate reader (Thermo Scientific, Pittsburgh, PA, USA). The cut-off value, sensitivity and specificity were determined as previously described [24,25]. Briefly, the cut-off value was determined from the mean OD_{450} of the 20 negative sheep serum samples plus three standard deviations. The sensitivity of the recombinant *Eg*AgB8/2 antigen ELISA was evaluated using 20 positive sheep serum samples against *E. granulosus*. The specificity was evaluated using 20 negative sheep serum samples. The sensitivity and specificity were calculated as follows:

sensitivity (%) = ELISA positive/true positive ×100%
specificity (%) = ELISA negative/true negative ×100%

The true positive and true negative were determined by the necropsy of Tibetan sheep. The cross reactivity was evaluated using 20 positive serum samples against *T. hydatigena* and 20 positive serum samples against *T. multiceps*.

Statistical Analysis

ELISA data are represented as the mean ± SEM. Statistical analyses were performed using Mann-Whitney test, and the cross-reactivity of the recombinant *Eg*AgB8/2 antigen with sera from Tibetan sheep infected with *T. hydatigena* and *T. multiceps* were plotted using GraphPad Prism software. Values of $P<0.05$ are considered statistically significant.

Results

Expression, Purification and Western Blotting

After IPTG induction, the expression products of *E. coli* BL21 (DE3) containing pET32a(+)-*Eg*AgB8/2 were analyzed by SDS-PAGE electrophoresis. Analysis of the protein solution analysis showed that *Eg*AgB8/2 was successfully expressed in *E. coli* and the recombinant protein was approximately 29 kDa (including a His-tag of 17 kDa), which was consistent with the expected size. The purified recombinant protein *Eg*AgB8/2 was a single band *(Fig. 1)*. Western blotting analysis showed that the mouse anti-His monoclonal antibody could specifically bind to the recombinant protein, and the recombinant protein could react with serum from *E. granulosus*-infected sheep and it formed a single band. The results indicate that the protein is the predicted protein and could be specifically recognized by anti-*Eg* positive serum.

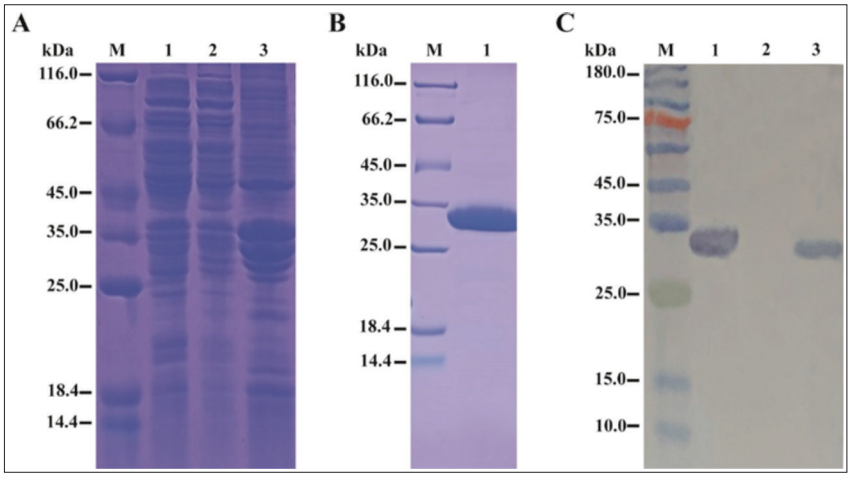


Fig 1. Expression, purification, and western blotting of recombinant *Eg*AgB8/2. **(A)** M, molecular mass marker in kDa; lane 1, the total proteins before induction; lane 2, the precipitated proteins after induction; lane 3, the supernatant proteins after induction, **(B)** M, molecular mass marker in kDa; lane 1, identification of recombinant *Eg*AgB8/2 antigen by SDS-PAGE, **(C)** M, molecular mass marker in kDa; lane 1, purified *Eg*AgB8/2 probed with mouse anti-His monoclonal antibody; lane 2, purified *Eg*AgB8/2 probed with healthy sheep serum; lane 3, purified *Eg*AgB8/2 probed with serum from *E. granulosus* infected sheep

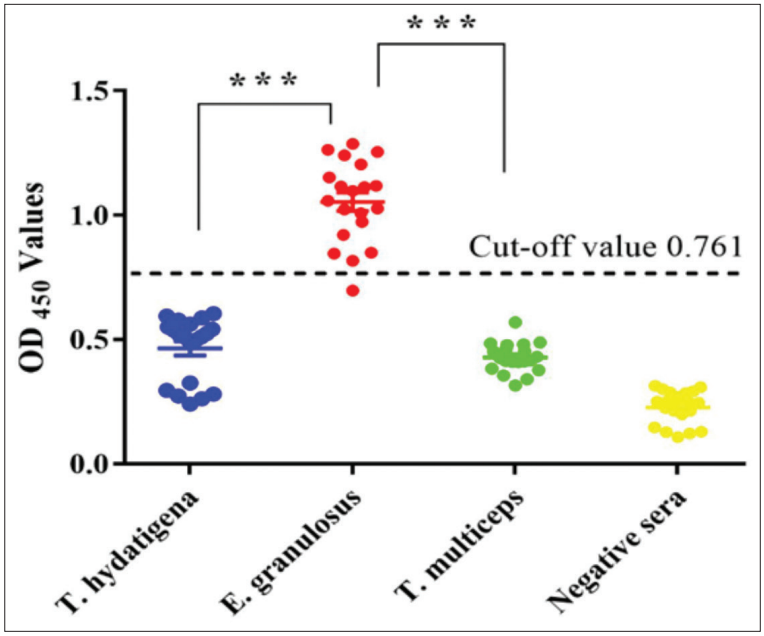


Fig 2. Indirect ELISA of Tibetan sheep sera showing specificity and sensitivity and cross-reactions of recombinant *Eg*AgB8/2 antigen for diagnosis of *E. granulosus*. Data are expressed as mean ± SEM of 2 independent experiments. *** P<0.001 by Student's t test

Evaluation of Recombinant Antigen by Indirect ELISA

The 96-well plate was coated with recombinant *Eg*AgB8/2 antigen (100 µL/well) at the optimum concentration of 5.0 µg/mL carbonate buffer (pH 9.6), and the optimal working concentration of serum was 1:100. The cut-off value was determined as 0.761 from the 20 samples of *E. granulosus*-negative serum. The sensitivity of *Eg*AgB8/2 was 95% (19/20) and the specificity was 100% (20/20). The recombinant *Eg*AgB8/2 antigen showed no cross-react with positive sera against *T. hydatigena* and positive sera against *T. multiceps* with OD_{450} above cut-off value (P<0.001) *(Fig. 2)*. There was a statistically significant difference in the mean OD values of *E. granulosus*-positive sera and *E. granulosus*-negative sera (Mann-Whitney *U*, $z = -5.410$, P<0.001).

Discussion

The serological tests for CE are mainly based on ELISA using hydatid cyst fluid (HCF) [26,27], but different sources of HCF to differing diagnostic effects when testing the sera. *Echinococcus* spp. harbor considerable variability of the strain from different geographical environments and different hosts [28-30], which cause variation in pathogenicity and antigen-antibody reactions between hosts [31]. The research showed that the sensitivity and specificity of CE serological diagnosis largely depends on HCF source, mainly due to the antigenic variability of the HF among different *E. granulosus* s. s. genotypes [32]. For a long time, crude preparations of HCF from *E. granulosus* cysts isolated from sheep was the only antigen available for serological diagnosis of CE in domestic animals [33]. However, HCF is scarce and heterogeneous for the detection of infected animals. Interestingly, *Eg*AgB is the largest portion of the parasite-derived proteins and the most abundant ones inside the HCF [34], which became important reagents for serological diagnosis in cystic echinococcosis. Currently, the recombinant *Eg*AgB antigens from hydatid cyst fluid have been comprehensively investigated in serological diagnosis [9,35]. One of the the recombinant *Eg*AgB antigens is *Eg*AgB2, a secretory protein of the larval stage of *E. granulosus* [36], was a highly antigenic molecule in CE infections [37], and was used as an effective diagnostic antigen for sero-diagnosis of CE [15,38-39]. It has been demonstrated that the recombinant *EgAgB8/2* antigen has better sensitivity and specificity compared to the other recombinant antigens from hydatid cyst fluid in CE [9,40]. In this study, the recombinant *Eg*AgB8/2 antigen for CE in Tibetan sheep showed a sensitivity of 95% and specificity of 100%, which was consistent with the ranges of the different native antigens of HCF were from 64.8% to 100% for sensitivity, and from 40% to 100% for specificity. Therefore, it suggests the different diagnostic performance of CE in animals from different geographical areas.

Tibetan sheep is the main livestock species on the QTP, but relatively high infection rate of CE seriously affects the development of animal husbandry in this area [41-44]. Although the abattoir data of Tibetan sheep are important, particularly in the surveillance of CE, it can be very difficult to identify specifically small lesions in the liver and lungs of young animals without additional histological examination. In addition, DNA technology, particularly the advent of the polymerase chain reaction (PCR), provides an approach for the unambiguous diagnosis of *E. granulosus* but currently this necessitates using metacestode material excised from infected intermediate hosts [45,46]. Therefore, developing a serological diagnostic method would be useful for diagnosis in CE of Tibetan sheep. However, some studies found that there were cross-reactivity between *E. granulosus* and other parasitic infections such as *Echinococcus multilocularis*, *Taenia multiceps*, *Taenia*

hydatigena, *Taenia solium,* and *Fasciola hepatica*, etc, which result in reduced performance for the serological diagnosis of CE in animals [16,32,47]. Therefore, that is why recombinant antigen based diagnostic methods of CE in animals were sought. The recombinant *Eg*AgB8/2 antigen in the hydatid cyst fluid was used to detect cystic echinococcosis, which was very immunogenic [20]. Currently, the serological test based on recombinant *Eg*AgB8/2 antigen is well established in diagnosis of human CE, however little information is available in diagnostic or epidemiological assessment of *E. granulosus* infections in animal intermediate hosts.

In the current study, we expressed the recombinant *Eg*AgB8/2 antigen in the HCF of *E.g* from Tibetan sheep, and preliminarily evaluated the extent of cross-reactivity of the recombinant *Eg*AgB8/2 antigen for other cestode infection commonly found on the QTP such as *Taenia multiceps* and *Taenia hydatigena*. We found the recombinant *Eg*AgB8/2 antigen had good immunogenicity and no cross-reacted with both *Taenia multiceps* and *Taenia hydatigena* which are common parasitic diseases on the QTP. However, more other cestode serums of Tibetan sheep need to be collected for cross-reaction verification.

In conclusion, this study may be a first step toward development of a reliable diagnostic test for CE infection in Tibetan sheep, and can also provide important experimental basis for the future study of CE epidemiological on the QTP. In the meanwhile, a larger panel of sera should be needed in future studies to evaluate the recombinant antigen with better sensitivity and specificity in the detection of CE infection in Tibetan sheep.

Acknowledgements

The authors would like to thank all staff and workers working in Chengduo, Qilian, Xinhai, Guinan veterinary station and the slaughterhouse for their kind help during sample collection.

Financial Support

This study was supported by the Transformation Project of Science and Technology of Qinghai Province (Grant No. 2018-NK-104) and the Natural Science Foundation of China (NSFC) (Grant No. 31960708).

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Standards

Not application.

Author Contributions

H.D., T.S., and Y.C. designed and performed experiments and analyzed results. X.S., Z.G., X.Z., Y.M. and G.J. collected samples. M.Y. provided advice. Y.F supervised the study and wrote the manuscript.

References

1. Deplazes P, Rinaldi L, Rojas CAA, Torgerson PR, Harandi MF, Romig T, Antolova D, Schurer JM, Lahmar S, Cringoli G, Magambo J, Thompson RCA, Jenkins EJ: Global distribution of alveolar and cystic echinococcosis. *Adv Parasitol,* 95, 315-493, 2017. DOI: 10.1016/bs.apar.2016.11.001

2. Zhang W, Zhang Z, Wu W, Shi B, Li J, Zhou X, Wen H, McManus DP: Epidemiology and control of echinococcosis in central Asia, with particular reference to the People's Republic of China. *Acta Trop,* 141, 235-243, 2015. DOI: 10.1016/j.actatropica.2014.03.014

3. Wang Q, Huang Y, Huang L, Yu W, He W, Zhong B, Li W, Zeng X, Vuitton DA, Giraudoux P, Craig PS, Wu W: Review of risk factors for human echinococcosis prevalence on the Qinghai-Tibet Plateau, China: A prospective for control options. *Infect Dis Poverty,* 3:3, 2014. DOI: 10.1186/2049-9957-3-3

4. Qian MB, Abela-Ridder B, Wu WP, Zhou XN: Combating echinococcosis in China: Strengthening the research and development. *Infect Dis Poverty,* 6:161, 2017. DOI: 10.1186/s40249-017-0374-3

5. Han XM, Cai QG, Wang W, Wang H, Zhang Q, Wang YS: Childhood suffering: Hyper endemic echinococcosis in Qinghai-Tibetan primary school students, China. *Infect Dis Poverty,* 7:71, 2018. DOI: 10.1186/s40249-018-0455-y

6. Li K, Zhang L, Zhang H, Lei Z, Luo H, Mehmood K, Shahzad M, Lan Y, Wang M, Li J: Epidemiological investigation and risk factors of *Echinococcus granulosus* in yaks *(Bos grunniens),* Tibetan pigs and Tibetans on Qinghai Tibetan plateau. *Acta Trop,* 173, 147-152, 2017. DOI: 10.1016/j.actatropica.2017.06.019

7. Thompson RCA: Biology and systematics of echinococcus. *Adv Parasitol,* 95, 65-109, 2017. DOI: 10.1016/bs.apar.2016.07.001

8. Hernández-González A, Sánchez-Ovejero C, Manzano-Román R, Sánchez MG, Delgado JM, Pardo-García T, Soriano-Gálvez F, Akhan O, Cretu CM, Vutova K, Tamarozzi F, Mariconti M, Brunetti E, Vola A, Fabiani M, Casulli A, Siles-Lucas M: Evaluation of the recombinant antigens B2t and 2B2t, compared with hydatid fluid, in IgG-ELISA and immunostrips for the diagnosis and follow up of CE patients. *PLoS Negl Trop Dis,* 12 (9): e0006741, 2018. DOI: 10.1371/journal.pntd.0006741

9. Rialch A, Raina OK, Tigga MN, Anandanarayanan A, Ganaie ZA, Aftab A, Lalrinkima H, Singh MN, Varghese A, Samanta S, Banerjee PS, Singh P, Verma MR: Evaluation of *Echinococcus granulosus* recombinant EgAgB8/1, EgAgB8/2 and EPC1 antigens in the diagnosis of cystic echinococcosis in buffaloes. *Vet Parasitol,* 252, 29-34, 2018. DOI: 10.1016/j.vetpar.2018.01.020

10. Yang YR, McManus DP, Huang Y, Heath DD: *Echinococcus granulosus* infection and options for control of cystic echinococcosis in Tibetan communities of Western Sichuan Province, China. *PLoS Negl Trop Dis,* 3 (4): e426, 2009. DOI: 10.1371/journal.pntd.0000426

11. Hu XJ, Yang J, Xie XL, Lv FH, Cao YH, Li WR, Liu MJ, Wang YT, Li JQ, Liu YG, Ren YL, Shen ZQ, Wang F, Hehua E, Han JL, Li MH: The genome landscape of Tibetan sheep reveals adaptive introgression from Argali and the history of early human settlements on the Qinghai-Tibetan Plateau. *Mol Biol Evol,* 36 (2): 283-303, 2019. DOI: 10.1093/molbev/msy208

12. Lightowlers MW, Lawrence SB, Gauci CG, Young J, Ralston MJ, Maas D, Heath DD: Vaccination against hydatidosis using a defined recombinant antigen. *Parasit Immunol,* 18 (9): 457-462, 1996. DOI: 10.1111/j.1365-3024.1996.tb01029.x

13. Mamuti W, Sako Y, Nakao M, Xiao N, Nakaya K, Ishikawa Y, Yamasaki H, Lightowlers MW, Ito A: Recent advances in characterization of *Echinococcus* antigen B. *Parasitol Int,* 55, S57-S62, 2006. DOI: 10.1016/j.parint.2005.11.008

14. Haag KL, Alves-Junior L, Zaha A, Ayala FJ: Contingent, non-neutral evolution in a multicellular parasite: Natural selection and gene conversion in the *Echinococcus granulosus* antigen B gene family. *Gene,* 333, 157-167, 2004. DOI: 10.1016/j.gene.2004.02.027

15. Rott MB, Fernandez V, Farias S, Ceni J, Ferreira HB, Haag KL, Zaha A: Comparative analysis of two different subunits of antigen B from

Echinococcus granulosus: Gene sequences, expression in *Escherichia coli* and serological evaluation. *Acta Trop,* 75 (3): 331-340, 2000. DOI: 10.1016/s0001-706x(00)00069-3

16. Virginio VG, Hernandez A, Rott MB, Monteiro KM, Zandonai AF, Nieto A, Zaha A, Ferreira HB: A set of recombinant antigens from *Echinococcus granulosus* with potential for use in the immunodiagnosis of human cystic hydatid disease. *Clin Exp Immunol,* 132 (2): 309-315, 2003. DOI: 10.1046/j.1365-2249.2003.02123.x

17. Golassa L, Abebe T, Hailu A: Evaluation of crude hydatid cyst fluid antigens for the serological diagnosis of hydatidosis in cattle. *J Helminthol,* 85 (1): 100-108, 2011. DOI: 10.1017/S0022149X10000349

18. Moro P, Verastegui M, Gilman RH, Falcon N, Bernal T, Gavidia C, Gonzalez A, Malqui V, Moro MH, Dueger E: Enzyme-linked immuno-electrotransfer blot assay for diagnosis of hydatidosis *(Echinococcus granulosus)* in sheep. *Vet Rec,* 140 (23): 605-606, 1997.

19. Ibrahem MM, Rafiei A, Dar FK, Azwai SM, Carter SD, Craig PS: Serodiagnosis of cystic echinococcosis in naturally infected camels. *Parasitology,* 125, 245-251, 2002. DOI: 10.1017/s0031182002002044

20. Bashiri S, Nemati Mansoor F, Valadkhani Z: Expansion of a highly sensitive and specific ELISA test for diagnosis of hydatidosis using recombinant EgB8/2 protein. *Iran J Basic Med Sci,* 22 (2): 134-139, 2019. DOI: 10.22038/ijbms.2018.29024.7021

21. Hernández-González A, Santivañez S, García HH, Rodríguez S, Muñoz S, Ramos G, Orduña A, Siles-Lucas M: Improved serodiagnosis of cystic echinococcosis using the new recombinant 2B2t antigen. *PLoS Negl Trop Dis,* 6 (7): e1714, 2012. DOI: 10.1371/journal.pntd.0001714

22. Wang J, Wang N, Hu D, Zhong X, Wang S, Gu X, Peng X, Yang G: Genetic diversity of *Echinococcus granulosus* in Southwest China determined by the mitochondrial NADH dehydrogenase subunit 2 gene. *Sci World J,* 2014:867839, 2014. DOI: 10.1155/2014/867839

23. Song X, Yan M, Hu D, Wang Y, Wang N, Gu X, Peng X, Yang G: Molecular characterization and serodiagnostic potential of a novel dithiol glutaredoxin 1 from *Echinococcus granulosus. Parasit Vectors,* 9:456, 2016. DOI: 10.1186/s13071-016-1741-9

24. Wu M, Yan M, Xu J, Yin X, Dong X, Wang N, Gu X, Xie Y, Lai W, Jing B, Peng X, Yang G: Molecular characterization of triosephosphate isomerase from *Echinococcus granulosus. Parasitol Res,* 117 (10): 3169-3176, 2018. DOI: 10.1007/s00436-018-6015-3

25. Jacobson RH: Validation of serological assays for diagnosis of infectious diseases. *Rev Sci Tech,* 17 (2): 469-526, 1998. DOI: 10.20506/rst.17.2.1119

26. Brunetti E, Kern P, Vuitton DA, Writing Panel for the WHO-IWGE: Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. *Acta Trop,* 114 (1): 1-16, 2010. DOI: 10.1016/j.actatropica.2009.11.001

27. Kordafshari S, Hosseini SH, Jalousian F, Rajabibazl M, Jones M, Etebar F: Evaluation of dot immunogold filtration assay (DIGFA) by recombinant protein EPC1 for anti-*Echinococcus granulosus* IgG antibody. *Iran J Parasitol,* 10 (1): 30-38, 2015.

28. Mcmanus DP: Immunodiagnosis of sheep infections with *Echinococcus granulosus:* In 35 years where have we come? *Parasit Immunol,* 36 (3), 125-130, 2013. DOI: 10.1111/pim.12072

29. Fathi S, Jalousian F, Hosseini SH, Najafi A, Darabi E, Koohsar F: Design and construction of a new recombinant fusion protein (2b2t+EPC 1) and its assessment for serodiagnosis of cystic echinococcosis. *Apmis,* 126 (5): 428-439, 2018. DOI: 10.1111/apm.12838

30. Bart JM, Knapp J, Gottstein B, El-Garch F, Giraudoux P, Glowatzki ML, Berthoud H, Maillard S, Piarroux R: EmsB, a tandem repeated multi-loci microsatellite, new tool to investigate the genetic diversity of *Echinococcus multilocularis. Infect Genet Evol,* 6 (5): 390-400, 2006. DOI: 10.1016/j.meegid.2006.01.006

31. Torgerson PR, Heath DD: Transmission dynamics and control options for *Echinococcus granulosus. Parasitology,* 127, S143-S158, 2003. DOI: 10.1017/s0031182003003810

32. Manzano-Roman R, Sanchez-Ovejero C, Hernandez-Gonzalez A, Casulli A, Siles-Lucas M: Serological diagnosis and follow-up of human cystic echinococcosis: A new hope for the future? *Biomed Res Int,* 2015:428205, 2015. DOI: 10.1155/2015/428205

33. McManus DP: Immunodiagnosis of sheep infections with *Echinococcus granulosus:* In 35 years where have we come? *Parasite Immunol,* 36 (3): 125-130, 2014. DOI: 10.1111/pim.12072

34. Juyi L, Yan J, Xiufang W, Zhaoqing Z, Junliang L, Mingxing Z, Wei Z: Analysis of the chemical components of hydatid fluid from *Echinococcus granulosus. Rev Soc Bras Med Trop,* 46 (5): 605-610, 2013. DOI: 10.1590/0037-8682-0154-2013

35. Lightowlers MW, Gottstein B: Echinococcosis/hydatidosis: Antigens, immunological and molecular diagnosis. *Cab International*, 1995.

36. Maddison SE, Slemenda SB, Schantz PM, Fried JA, Wilson M, Tsang VCW: A specific diagnostic antigen of *Echinococcus granulosus* with an apparent molecular weight of 8 kDa. *Am J Trop Med Hyg,* 40 (4): 377-383, 1989. DOI: 10.4269/ajtmh.1989.40.377

37. Abdi J, Kazemi B, Mohebali M, Bandehpour M, Rahimi MT, Rokni MB: Gene cloning, expression and serological evaluation of the 12-kDa antigen-B subunit from *Echinococcus granulosus. Ann Trop Med Parasitol,* 104 (5): 399-407, 2010. DOI: 10.1179/136485910X12743554760261

38. Chen X, Chen X, Lu X, Feng X, Wen H: The production and comparative evaluation of native and recombinant antigens for the fast serodiagnosis of cystic echinococcosis with dot immunogold filtration assay. *Parasit Immunol,* 37 (1): 10-15, 2015. DOI: 10.1111/pim.12151

39. Khalilpour A, Sadjjadi SM, Moghadam ZK, Yunus MH, Zakaria ND, Osman S, Noordin R: Lateral flow test using *Echinococcus granulosus* native antigen B and comparison of IgG and IgG4 dipsticks for detection of human cystic echinococcosis. *Am J Trop Med Hyg,* 91 (5): 994-999, 2014. DOI: 10.4269/ajtmh.14-0170

40. Golassa L, Abebe T, Hailu A: Evaluation of crude hydatid cyst fluid antigens for the serological diagnosis of hydatidosis in cattle. *J Helminthol,* 85 (1): 100-108, 2011. DOI: 10.1017/S0022149X10000349

41. Zhong X, Wang N, Hu D, Wang J, Liu T, Gu X, Wang S, Peng X, Yang G: Sequence analysis of *cytb* gene in *Echinococcus granulosus* from Western China. *Korean J Parasitol,* 52 (2): 205-209, 2014. DOI: 10.3347/kjp.2014.52.2.205

42. Hu D, Song X, Wang N, Zhong X, Wang J, Liu T, Jiang Z, Dawa T, Gu X, Peng X, Yang G: Molecular identification of *Echinococcus granulosus* on the Tibetan Plateau using mitochondrial DNA markers. *Genet Mol Res,* 14 (4): 13915-13923, 2015. DOI: 10.4238/2015.October.29.12

43. Liu CN, Lou ZZ, Li L, Yan HB, Blair D, Lei MT, Cai JZ, Fan YL, Li JQ, Fu BQ, Yang YR, McManus DP, Jia WZ: Discrimination between *E. granulosus* sensu stricto, *E. multilocularis* and *E. shiquicus* using a multiplex PCR assay. *PLoS Negl Trop Dis,* 9 (9): e0004084, 2015. DOI: 10.1371/journal.pntd.0004084

44. Ma J, Wang H, Lin G, Zhao F, Li C, Zhang T, Ma X, Zhang Y, Hou Z, Cai H, Liu P, Wang Y: Surveillance of *Echinococcus* isolates from Qinghai, China. *Vet Parasitol,* 207 (1-2): 44-48, 2015. DOI: 10.1016/j.vetpar.2014.11.012

45. Yang YR, Rosenzvit MC, Zhang LH, Zhang JZ, McManus DP: Molecular study of *Echinococcus* in west-central China. *Parasitology,* 131, 547-555, 2005. DOI: 10.1017/S0031182005007973

46. Yan N, Nie HM, Jiang ZR, Yang AG, Deng SJ, Guo L, Yu H, Yan YB, Tsering D, Kong WS, Wang N, Wang JH, Xie Y, Fu Y, Yang DY, Wang SX, Gu XB, Peng XR, Yang GY: Genetic variability of *Echinococcus granulosus* from the Tibetan plateau inferred by mitochondrial DNA sequences. *Vet Parasitol,* 196 (1-2): 179-183, 2013. DOI: 10.1016/j.vetpar.2013.02.010

47. Tawfeek GM, Elwakil HS, El-Hoseiny L, Thabet HS, Sarhan RM, Awad NS, Anwar WA: Comparative analysis of the diagnostic performance of crude sheep hydatid cyst fluid, purified antigen B and its subunit (12 Kda), assessed by ELISA, in the diagnosis of human cystic echinococcosis. *Parasitol Res,* 108 (2): 371-376, 2011. DOI: 10.1007/s00436-010-2074-9

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

Kafkas Univ Vet Fak Derg
27 (2): 209-215, 2021
DOI: 10.9775/kvfd.2020.25080

RESEARCH ARTICLE

Effect of Recombinant Transglutaminase on the Quality Characteristics of Cooked Beef Meatballs

Fatma ERSÖZ [1,a] Elif AYKIN DİNÇER [1,b (*)] Aysun Türkanoğlu ÖZÇELIK [2,c] Mehmet İNAN [1,2,d]

[1] Department of Food Engineering, Engineering Faculty, Akdeniz University, TR-07070 Antalya - TURKEY
[2] Food Safety and Agricultural Research Center, Akdeniz University, TR-07070 Antalya - TURKEY
ORCIDs: [a] 0000-0002-9647-1231; [b] 0000-0003-4427-9819; [c] 0000-0003-2537-4220; [d] 0000-0003-1806-7927

Article ID: KVFD-2020-25080 Received: 15.10.2020 Accepted: 25.01.2021 Published Online: 01.02.2021

Abstract

Transglutaminase (TGase) is an enzyme widely used in the food industry. In this study, the effect of transglutaminase enzyme on the chemical and physical characteristics of cooked beef meatballs was evaluated. For this aim, beef meatballs were prepared by using recombinant microbial transglutaminase (MTGase) and commercial TGase enzymes, after that physical and chemical tests were applied to meatball samples. The addition of MTGase enzyme improves the quality parameters of the beef meatballs. The myofibrillar proteins of cooked meatball samples were also analyzed with SDS-PAGE analysis. It was observed that, actin and myosin proteins bind covalently to form a new high molecular weight protein by the help of MTGase addition. These results indicated that recombinant MTGase enzyme can be used to obtain high quality restructured beef meat products.

Keywords: *Beef, Meatball, Recombinant enzyme, Transglutaminase, Pichia pastoris*

Rekombinant Transglutaminazın Pişmiş Sığır Köftelerinin Kalite Özelliklerine Etkisi

Öz

Transglutaminaz (TGase), gıda endüstrisinde yaygın olarak kullanılan bir enzimdir. Bu çalışmada, transglutaminaz enziminin pişmiş sığır köftelerinin kimyasal ve fiziksel özellikleri üzerine etkisi değerlendirilmiştir. Bu amaçla, sığır köfteleri rekombinant mikrobiyal transglutaminaz (MTGase) ve ticari TGase enzimleri kullanılarak hazırlanmış, ardından köfte örneklerine fiziksel ve kimyasal analizler uygulanmıştır. MTGase enziminin ilavesi, sığır köftelerinin kalite parametrelerini iyileştirmiştir. Pişirilmiş köfte örneklerinin miyofibriler proteinleri de SDS-PAGE yöntemi ile analiz edilmiştir. MTGase ilavesi ile, aktin ve miyosin proteinlerinin kovalent bağlanarak yeni bir yüksek moleküler ağırlıklı protein oluşturduğu görülmüştür. Bu sonuçlar, yüksek kaliteli yeniden yapılandırılmış sığır eti ürünleri elde etmek için rekombinant MTGase enziminin kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Sığır eti, Köfte, Rekombinant enzim, Transglutaminaz, Pichia pastoris*

INTRODUCTION

Meatballs, an important ready-to-eat meat product, are the most common among hot pot materials and are well-liked by Turkish consumers. The cooking process contributes a special texture to meatballs due to the gelation of myofibrillar proteins [1,2]. However, high temperature cooking adversely affects the water holding capacity and the textural properties of the meatballs due to the poor gelation capacity of these proteins. The gel properties of heat-induced myofibrillar proteins are enhanced by modification of their structure. For this purpose, the transglutaminase enzyme is widely used in the food industry [3]. Transglutaminase enzyme (TGase, protein-glutamine g-glutamyltransferase, EC 2.3.2.13) is a binder agent that induces protein aggregation in muscle foods through isopeptide covalent cross-linking between glutamine residues (acting as acyl donor) and lysine residues (acting as acyl acceptor) [4,5]. TGase has been employed to improve the gel properties, water holding capacity and emulsion stability of food protein [6,7], and the quality characteristics of meat products [4,8,9], and during recent years, it has been used in the production of

How to cite this article?

Ersöz F, Aykın Dinçer E, Türkanoğlu Özçelik A, İnan M: Effect of recombinant transglutaminase on the quality characteristics of cooked beef meatballs. *Kafkas Univ Vet Fak Derg,* 27 (2): 209-215, 2021.
DOI: 10.9775/kvfd.2020.25080

(*) **Corresponding Author**

Tel: +90 539 584 7958
E-mail: elifaykin@akdeniz.edu.tr (E. Aykın Dinçer)

restructured meat products [3,10,11]. Therefore, it increases the economic value of meat products and decreases waste. Besides, crosslinking proteins catalyzed by TGase containing various essential amino acids improve the nutritional value of meat products [12]. Studies have shown that TGase alone enhanced the functional and textural properties of meat products.

In general, the TGase enzyme is commercially obtained by extracting and purifying from the tissues or body fluids of plants and animals [13-15]. But low yield, time-consuming, high cost and complex purification procedures are the main problems in the extraction of this enzyme. Therefore, researchers have developed effective bacterial TGase expression systems like *Streptomyces lividans*, *Escherichia coli*, and *Corynebacterium glutamicum* to obtain high amount of TGase enzyme [16,17]. Because of rapid growth, bacterial expression systems are frequently used to express MTGase enzyme but there are some restrictions on the use of this system like inclusion body formation, complex refolding processes and incompatible with post-translational modifications. To overcome these problems yeast expression systems have become a good alternative. For recombinant protein production, *Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha, Yarrowia lipolytica,* and *Kluyveromyces lactis* are commonly used. *P. pastoris* is a methylotrophic yeast that is capable of high heterologous extracellular protein production. Additionally, this yeast can grow high cell densities in basic media (containing methanol, ethanol, glucose, and glycerol) and is suitable for genetic manipulations. Yang and Zhang [12] reported that *Streptomyces fradiae* pro-MTGase enzyme was expressed under the control of methanol inducible *AOX1* promoter in *P. pastoris* and the effect of the recombinant MTGase enzyme on the quality of restructured pork meat was evaluated. The results showed that the hardness and chewiness of the restructured meat were increased, and the adhesiveness decreased after the MTGase treatment.

To our current knowledge, there is no data available concerning the effect of recombinant MTGase enzyme on the quality of cooked beef meat in Turkey. Therefore, this study was designed to investigate the effect of the recombinant MTGase on the quality of cooked beef meatballs. For this aim, recombinant *Streptomyces mobaraensis* pro-MTGase enzyme was expressed in *P. pastoris* under the constitutive *GAP* promoter in a 5-L bioreactor [18] and then, beef meatballs were prepared by using the recombinant MTGase enzyme to evaluate chemical and physical characteristics of cooked beef meatballs.

Materials and Methods

Chemical and Reagents

The protein marker used in this study was obtained from Thermo Fisher Scientific (ABD). Cultivation media constituents were purchased from Becton Dickinson and Company (BD) (Franklin Lakes, NJ, USA). Other chemicals and reagents were analytical grade and acquired from Sigma-Aldrich Co. (MO, USA), Merck (Deutschland). The recombinant MTGase enzyme produced in *P. pastoris* was used [18]. The *P. pastoris* X33 strain used in this study was obtained from Life Technologies (Carlsbad, CA. USA). Commercial transglutaminase enzyme was obtained from Ajinomoto Foods Europa SAS (Paris, France). The composition of the commercial enzyme consisted of 99% maltodextrin and 1% transglutaminase and its enzyme activity was reported as 100 Units (U)/g by the manufacturer. Ground beef with approximately 20% beef fat and 1% salt was obtained from a well-known butcher (Veli Cengiz Meat Products Ltd.) in Antalya. The purchased ground beef was a homogeneous mixture of lean beef cuts, beef fat and salt. Considering the tendencies to reduce food salt [4], this salt concentration would be sufficient to ensure the eating salinity (≈2%) of cooked meatballs.

The Production of Recombinant MTGase

The recombinant MTGase enzyme was produced under the control of constitutive *GAP* promoter in *P. pastoris* X33 strain as previously described [18]. In bioreactor level production of the MTGase, fermentation was conducted two-step fed-batch process. For this purpose, a vial of frozen culture was used to inoculate 100 mL BMGY and cultivated for 12 h. 100 mL (10 OD_{600nm}) pre-culture were inoculated to 2 L pH 5 citric acid media (2.0 g/L citric acid monohydrate, 45.6g/L glycerol (86%), 12.6g/L $(NH_4)_2HPO_4$, 0.5 g/L $MgSO_4*7H_2O$, 0.9 g/L KCl, 0.022 g/L $CaCl_2*2H_2O$) at the first step of the fermentation, the batch phase, and continued until sudden rise in dissolved oxygen (DO) level (about 16-20 h) at 28°C, pH: 5 and 900 rpm stirring conditions. After this carbon-exhaustion signal the second step, the fed-batch phase, was started with 50% glucose feed. At the beginning of the fed-batch phase, the pH and temperature values were adjusted to optimum pH and temperature values (pH 7 and 20°C) to obtain maximum enzyme production in the culture supernatants. During the 70 h fed-batch phase the feed rate of glucose solution was exponentially increased; it started with 3 mL/L/h and finished with 18 mL/L/h flow rate. DO level (20% saturation) of this phase was controlled by agitation speed, adding 1.5vvm airflow and supplying pure oxygen as necessary. At the end of enzyme production phase cells were separated by centrifugation and supernatants were collected. The harvested supernatant samples were analyzed with the BCA Protein Assay Kit (Thermo Fisher Scientific [USA]) to determine the amount of total protein. The enzyme was produced in an inactive pro-MTGase form and activated with Dispasel, considering the amount of protein. After activation of the enzyme MTGase activity was calculated as previously described [19] and used in the production of meatballs.

Preparation of the Meatballs

The meatballs used in the research were prepared in

three different compositions: control, commercial and recombinant. Differences in the formulation were sourced from the TGase enzyme. Except for salt, no ingredients such as black pepper, paprika or cumin were used in the formulation of the meatball. The meatballs that included no TGase enzyme were used as a control. Both commercial and recombinant MTGase enzymes were added 400 U to 1 kg ground beef. The enzyme amount and concentration were determined as 0.4 U/g, which is the concentration used by a local company conducting restructured meat products experiment in Antalya. Ground beef was randomly divided into 3 groups for different compositions. All groups consisted of 1 kg ground beef (about 10 meatballs), and a total of 3 kg ground beef were used for one replication. Two replications were carried out for all analyses, so a total of 6 kg ground beef was used.

After the TGase enzyme was added to the ground beef, the mixture was kneaded by hand for five minutes, and homogeneous meatball dough was obtained. After the meatball dough was spread in about 2 cm thickness, meatballs were shaped using petri dishes of 9 cm in diameter. Then, the samples were spread in one layer on cooking paper and kept in an incubator at 40°C for 2 h to catalyze the enzymatic reaction before cooking. A maximum period of 2 h was applied for enzyme activation at 40°C to avoid beef spoilage. The meatballs were cooked in a preheated oven (Siemens HB86K575, South Africa) for about 20 min using the meatball cooking program (the cooking temperature set to 180°C) until the temperature at the geometric centre reached 72°C. At the end of cooking, all samples were allowed to cool at room temperature (25°C), packed in seal plastic bags and stored at refrigerator temperature (4°C) overnight before determining their quality properties.

Chemical and Physical Analysis

The cooking loss of samples was calculated by the difference in the weight of meatballs before and immediately after cooking. The dimension change of the samples was calculated by the difference of the diameter and thickness of the meatballs, measured with a caliper, before and after they were cooked.

The thiobarbituric acid reactive substances (TBARS) were determined according to the method of Lemon [20] and expressed as µmol malondialdehyde (MDA)/kg of the sample.

The water holding capacity (WHC) of meatballs was determined based on the method detailed by Wang et al.[2] Meatballs were cut into cubes (approximately 30×30×20 mm^3). Each cube was placed between the filter papers and then pressed with a 5 kg mass for 2 min. Values of WHC were calculated by the ratio between weight before pressing and weight after pressing.

Color parameters of the samples were measured by using a CR-400 Chromameter (Konica Minolta Inc., Osaka, Japan) and expressed as L^* (lightness), a^* (redness), and b^* (yellowness) values. The color device was calibrated by using its white ceramic plate before actual use. Color values were measured using 3 cubes per group and 3 measurements per cube. Accordingly, the results were reported as the mean value of nine replicates for each group.

The texture profile of the meatball samples was determined using a TA.XTplus Texture Analysis Device (Stable Microsystems, UK). The meatballs removed from the refrigerator were kept at 25°C for 1 h and then cut into cubes to be subjected to texture profile analysis (TPA). The hardness, springiness, cohesiveness and chewiness properties of the meatball cubes were determined. For the analysis, a 100 mm cylinder probe (P/100) and Heavy Duty Platform (HDP/90) accessories were used. Before and after the TPA test, the probe speed was set to 2 mm/sec, the test speed was set to 5 mm/sec, the waiting time was set to 2 sec, the trigger strength was set to 5 g, the load cell was set to be 50 kg and the distance was set to the distance that would provide 40% deformation.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The meatball samples were analyzed with the SDS-polyacrylamide gel electrophoresis method to show the formation of a covalent cross-link between intermolecular protein. The raw minced beef meat and, the commercial and recombinant MTGase enzyme treated cooked beef meatballs were analyzed to observe the changes in protein patterns. Proteins were extracted according to method described by Sorapukdee and Tangwatcharin [21]. In order to solubilize the samples, 27 mL of 5% SDS was added to 3 g samples and homogenized with ultra-turrax (IKA-T18, Staufen, Germany) and incubated at 85°C for 1 h and centrifuged at 3000 g for 20 min. After centrifugation, undissolved debris was removed and supernatant samples were collected. The amount of total protein was determined by using BCA Bradford Assay kit and about 15 µg protein was treated with dithiothreitol (DTT) and incubated 70°C for 10 min. Then samples were loaded to 10% SDS-PAGE gels and subjected to electrophoresis for 1 hour at 100V to determine myofibrillar protein bands. After separation, the gel was stained with Coomassie Blue (G250) and scanned with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). The protein bands aligned to Page Ruler unstained protein ladder (Thermo Fisher Scientific, MA, USA) and actin, myosin and the other protein bands were identified. The relative quantification of the target protein bands was determined with ImageJ program.

Statistical Analysis

The meatball production was made in two replicates, and the analyses of the samples were held in parallel. Variance analysis (ANOVA) was made for the data and a Duncan Multiple Comparison Test was applied to the important

factors. All statistical calculations were done using SAS Statistics Software (v.7.00, SAS Institute Inc., Cary, NC, USA), and the values were given as mean±standard error.

Results

Table 1 shows the cooking loss for the meatballs formulated with different TGase enzymes. The cooking loss values of recombinant and commercial meatballs were slightly lower than that of the control, but not significantly (P>0.05). The increase in thickness (22.73%) and the decrease in diameter (34.75%) of control meatballs were higher than those of other meatballs *(Table 1)*, indicating that both TGase enzymes were useful in retaining moisture in the product during cooking and maintaining the shape of the meatballs. The results of the WHC were also presented in *Table 1*. There is no significant difference in WHC of meatballs after it is treated with TGase (P>0.05).

The TBARS values of meatball samples were significantly affected (*P*<0.01) from the use of the TGase enzyme *(Table 1)*. The highest TBARS value was detected in control meatballs. Both TGase enzymes slowed down lipid oxidation but did not inhibit it. The *L** value of commercial and recombinant meatballs was higher than that of the control *(Table 2)*. The other color parameters *a** and *b** were not significantly affected by the formulations. The textural properties of the meatballs are given in *Table 3*. Except for chewiness (P<0.05), the textural properties were not significantly (P>0.05) affected by the addition of TGase. Hardness, springiness and, cohesiveness showed a mean value of 0.18 kg, 0.91 and 0.74, respectively.

The result of SDS-PAGE analysis of raw minced meat and restructured cooked meatballs was presented in *Fig. 1*. The raw minced meat without MTGase addition was used as a control, and actin and myosin bands were detected on the gel. The densitometric profiles of actin, myosin and newly formed protein band were shown in *Fig. 2*. The relative quantities of actin, myosin and newly formed protein bands were analyzed and the calculated peak areas were shown above the related peaks. When compared to the control group, actin and myosin peak areas were decreased and newly formed protein peak areas were increased in both commercial and recombinant MTGase treatments *(Fig. 2)*.

Discussion

Lower cooking loss value may have been due to the TGase enzyme promoted strong protein interactions, enhancing the water holding capacity and consequently, decreasing the cooking loss. Tseng et al.[4] reported that the cooking yield of low-salt chicken meatballs containing the TGase enzyme was significantly higher than the control group. Monteiro et al.[10] reported that the levels of TGase enzyme from 0% to 0.8% led to a significant increase in cooking yield of restructured tilapia steaks. It was reported that the cooking loss of pork sausages decreased with the addition of a combination of TGase, hydrocolloids, acorn powder, and mung bean powder, due to improved water

Table 1. *The physico-chemical properties of meatballs*

Meatballs	Cooking Loss (%)	Increase in Thickness (%)	Decrease in Diameter (%)	WHC (%)	TBARS (µmol MDA/kg)
Control	51.97±0.39^{a}	22.73±0.19^{a}	34.75±1.37^{a}	99.68±0.05^{a}	43.09±1.62^{a}
Recombinant	46.45±3.17^{a}	17.87±0.06^{b}	27.20±1.55^{ab}	99.72±0.03^{a}	32.73±0.07^{b}
Commercial	45.77±0.92^{a}	16.22±0.11^{c}	26.59±2.09^{b}	99.71±0.02^{a}	32.03±0.36^{b}

^{a,b,c} *Means with different letters within the column indicate differences*

Table 2. *Color values of meatballs*

Meatballs	*L**	*a**	*b**
Control	50.26±0.07^{b}	7.69±0.22^{a}	11.76±0.03^{a}
Recombinant	53.36±0.40^{a}	8.09±0.41^{a}	11.81±0.20^{a}
Commercial	52.92±0.38^{a}	7.36±0.11^{a}	11.92±0.07^{a}

^{a,b} *Means with different letters within the column indicate differences*

Table 3. *Textural properties of meatballs*

Meatballs	Hardness (kg)	Springiness	Cohesiveness	Chewiness (kg)
Control	0.16±0.01^{a}	0.91±0.02^{a}	0.75±0.01^{a}	0.10±0.00^{c}
Recombinant	0.19±0.04^{a}	0.91±0.01^{a}	0.73±0.02^{a}	0.15±0.01^{a}
Commercial	0.19±0.02^{a}	0.92±0.00^{a}	0.75±0.01^{a}	0.13±0.01^{b}

^{a,b,c} *Means with different letters within the column indicate differences*

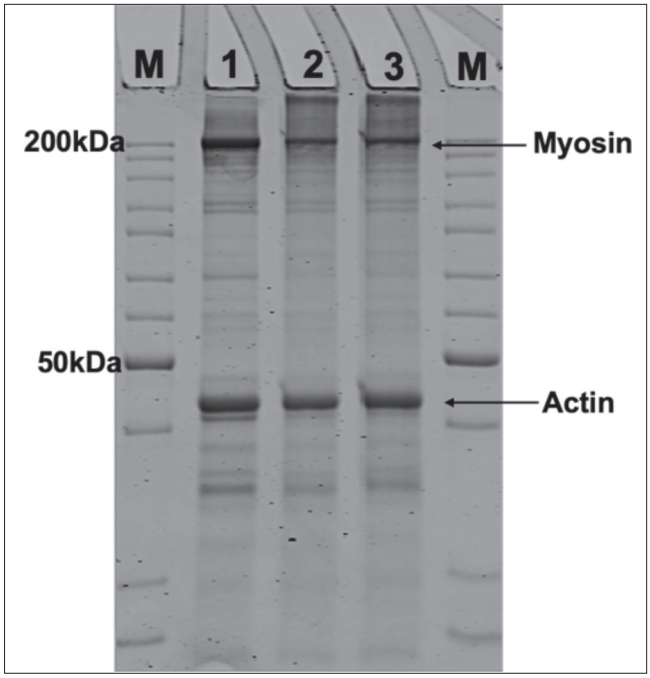


Fig 1. SDS-PAGE analysis of myofibrillar proteins of raw minced meat (Lane 1) and commercial (Lane 2) and recombinant MTGase (Lane 3) treated cooked meatballs, respectively. M, Page ruler unstained protein ladder

binding properties [22]. Similar results were also reported for reduced-salt frankfurters treated with sea mustard [23] and for restructured beef steaks with plant proteins [24].

Shrinking of control meatballs may be sourced from the volume of the diverging water and the mobility of the protein matrix during cooking. TGase enzymes used in meatballs provided the formation of covalent disulfide bonds and aggregation of the exposed hydrophobic amino acids via hydrophobic interactions, in turn leading to the formation of a regular gel network. Tseng et al.[4] also reported that low-salt chicken meatballs made with TGase formed firmer and more regular gel network structures than the control samples which has a looser gel network.

High WHC values indicated the retention of less moveable water and the maintenance of juiciness in meatballs. These results were probably due to the salt content of meatballs (salinity, 1%). It was reported that the use of microbial TGase (without salts) can result in meat products with poor water-binding properties [25]. Tseng et al.[4] reported that low-salt (1%) chicken meatballs with TGase had better emulsion stability and hydration properties. These results suggest that salts are therefore required to improve the protein-water interactions in cooked meat products, along with the TGase enzyme.

Thiobarbituric acid reactive substances results showed that free radicals could be more stable in meatballs with TGase. These results are in line with Gharibzahedi et al.[26], where cross-linked TGase microcapsules with edible oils

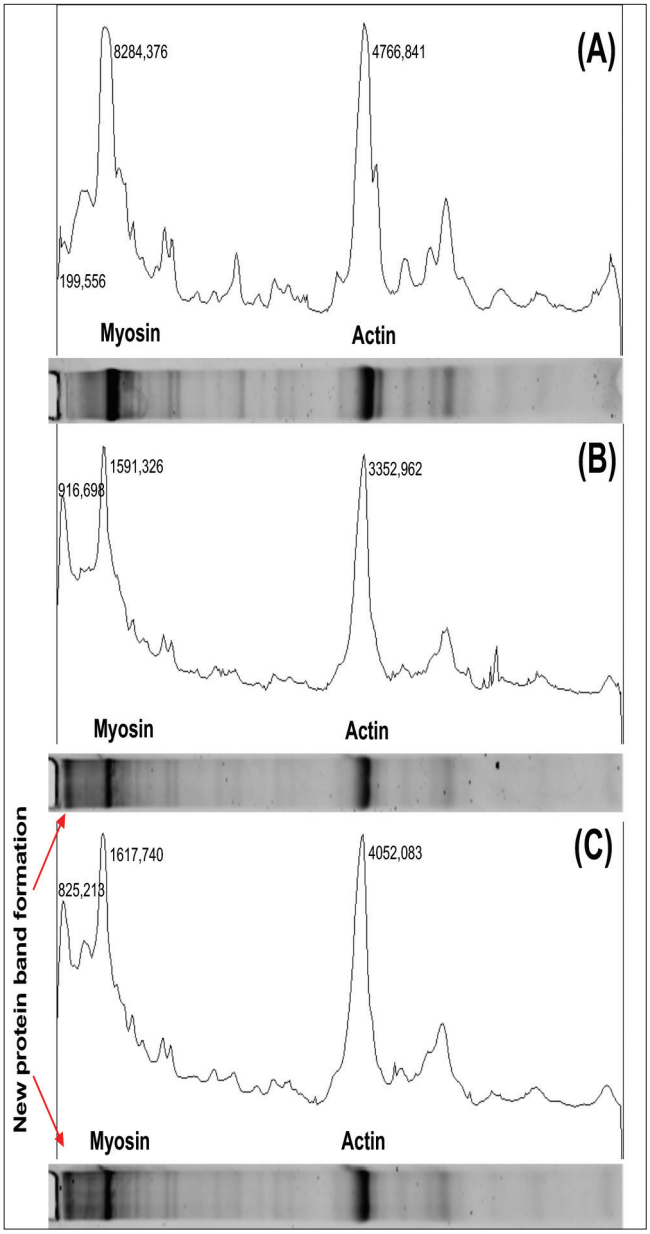


Fig 2. Densitometry plots of SDS-PAGE gels of myofibrillar protein. A: Control group, B: Cooked meatballs with the addition of commercial MTGase, C: Cooked meatballs with the addition of recombinant MTGase

had high effectiveness to delay the lipid oxidation process because the use of TGase could stabilize the polymeric structure of the microcapsule against the diffusion of prooxidants and digestive enzymes. Additionally, it was reported that emulsions stabilized by TGase treated protein isolate showed the inhibiting effects against lipid oxidation due to their larger particle size than the control emulsion [27]. However, it was reported that the addition of different percentages of binder admixture including TGase did not affect lipid oxidation in restructured meat [28]. Baugreet et al.[24] reported that meat alone was affected by lipid oxidation during processing.

Similar to color results, Martínez et al.[29] found that *L**, *a**

and *b** values in beef patties with TGase were 50.60, 9.65 and 16.02, respectively. It was reported that doses of TGase from 0% to 0.5% led to a slight increase in the *L** value of chicken breast patties, from 41.81 to 43.10 [30]. Park et al.[31] reported that *a** and *b** values of cooked meat batters with or without TGase treatment showed no significant difference. Cofrades et al.[32] also reported that the addition of TGase did not affect any of the color parameters of the raw and cooked meat products. Additionally, color is one of the most important visual traits of the beef products perceived by consumers [33]. Therefore, the results indicate the application potential of this recombinant TGase enzyme in beef meatballs.

The hardness, springiness and cohesiveness values of control group were not different from those of recombinant and commercial enzyme added meatballs. However, the chewiness of meat products was related to these properties and reflected the acceptability of food by the consumer. The chewiness of recombinant meatballs was significantly higher than those of the others, possibly from an increase in the formation of cross-linking between glutamine and lysine residues. It was reported that increasing levels of TGase (from 0% to 2%) increased the chewiness of the reduced-salt frankfurters from 0.09 kg to 0.13 kg but did not have a significant effect on the springiness and cohesiveness properties [23]. Yang and Zhang [12] reported that the chewiness of the restructured pork catalyzed by recombinant TGase was higher than that of the control group which had no TGase and suggested that recombinant TGase can improve tenacity and the taste of mixed foods. This study suggests that the addition of recombinant TGase, followed by controlled heating at 40°C, could improve the textural properties of meatballs.

When MTGase was added to restructured meat, there was a decrease in the density of the actin and myosin bands, while a new extra band was formed at the top of the gel (Lane 2 and 3 in *Fig. 1)*. The formation of this new band was observed in both commercial and recombinant MTGase enzyme treatments but was not observed in the control group. Our findings were consistent with the other studies in the literature [21,34]. In addition, *Fig. 2* showed that myosin and actin covalently bond to form a new, cross-linked, high molecular weight protein on the top of the gels. The reduction of actin and myosin bands with the addition of MTGase has been shown in many studies [35,36]. Both commercial and recombinant MTGase enzyme induced cross-linking of polypeptide chains in the cooked beef meatball samples owing to the disulfide bonds. According to these findings, the recombinant MTGase enzyme [18] produced in *P. pastoris* can be used as meat glue like commercial TGases to obtain restructured meat products.

This study showed that the effects of MTGase enzyme on the cooking loss, water holding capacity (WHC), color parameters and texture profile of the meatball samples. The addition of the MTGase enzyme into the meatballs improves the textural properties of the samples. In addition, the TBARS values of the meatballs decreased with MTGase treatment. All of these physical, chemical and SDS-PAGE analysis showed that the recombinant MTGase and the commercial MTGase enzymes had similar effects on the restructured beef meat. As a conclusion, the recombinantly produced MTGase can be a good alternative for cooked beef meatballs in the reconstituted meat industry.

Conflict of Interest

The authors declared no potential conflicts of interest

Statement of Author Contributions

Conception and design: M. İnan. Analysis and interpretation of data: F. Ersöz, E. Aykın-Dinçer. Drafting the article: F. Ersöz, E. Aykın-Dinçer. Revising it for intellectual content: A. Türkanoğlu Özçelik. Final approval of the completed article: F. Ersöz, E. Aykın-Dinçer, A. Türkanoğlu Özçelik, M. İnan.

References

1. Aykın Dinçer E, Büyükkurt ÖK, Candal C, Bilgiç BF, Erbaş M: Investigation of the usability of retrograded flour in meatball production as a structure enhancer. *Korean J Food Sci Anim Resour,* 38 (1): 78-87, 2018. DOI: 10.5851/kosfa.2018.38.1.78

2. Wang KQ, Luo SZ, Zhong XY, Cai KZ, Cai J, Jiang ST, Zheng Z: Effect of modified wheat gluten on boiling resistance capacity of pork meatballs. *J Food Sci,* 81 (2): E430-437, 2016. DOI: 10.1111/1750-3841.13207

3. Sun XD, Arntfield SD: Gelation properties of myofibrillar/pea protein mixtures induced by transglutaminase crosslinking. *Food Hydrocoll,* 27 (2): 394-400, 2012. DOI: 10.1016/j.foodhyd.2011.11.001

4. Tseng TF, Liu DC, Chen MT: Evaluation of transglutaminase on the quality of low-salt chicken meat balls. *Meat Sci,* 55, 427-431, 2000. DOI: 10.1016/S0309-1740(99)00172-2

5. García-Gómez B, Romero-Rodríguez Á, Vázquez-Odériz L, Muñoz-Ferreiro N, Vázquez M: Sensory evaluation of low-fat yoghurt produced with microbial transglutaminase and comparison with physicochemical evaluation. *J Sci Food Agric,* 99 (5): 2088-2095, 2019. DOI: 10.1002/jsfa.9401

6. Li H, Zhang T, Li J, Li H, Xu Y, Yu J: Expression of *Zea mays* transglutaminase in *Pichia pastoris* under different promoters and its impact on properties of acidified milk protein concentrate gel. *J Sci Food Agric,* 99 (10): 4518-4523, 2019. DOI: 10.1002/jsfa.9688

7. Stangierski J, Baranowska HM: The influence of heating and cooling process on the water binding in transglutaminase-modified chicken protein preparation, assessed using Low-Field NMR. *Food Bioprocess Tech,* 8 (12): 2359-2367, 2015. DOI: 10.1007/s11947-015-1618-5

8. Campagnol PCB, Dos Santos BA, Lorenzo JM, Cichoski AJ: A combined approach to decrease the technological and sensory defects caused by fat and sodium reduction in Bologna-type sausages. *Food Sci Technol Int,* 23 (6): 471-479, 2017. DOI: 10.1177/1082013217701859

9. Lee HC, Chin KB: Application of microbial transglutaminase and functional ingredients for the healthier low-fat/salt meat products: A review. *Korean J Food Sci Anim Resour,* 30, 886-895, 2010. DOI: 10.5851/kosfa.2010.30.6.886

10. Monteiro MLG, Márcico ET, Lázaro CA, da Silva ACVC, da Costa Lima BRC, da Cruz AG, Conte-Júnior CA: Effect of transglutaminase on quality characteristics of a value-added product tilapia wastes. *J Food Sci Technol,* 52 (5): 2598-2609, 2015. DOI: 10.1007/s13197-014-1327-5

11. Stangierski J, Rezler R, Lesnierowski G: Analysis of the effect of heating on rheological attributes of washed mechanically recovered chicken meat modified with transglutaminase. *J Food Eng,* 141, 13-19, 2014. DOI: 10.1016/j.jfoodeng.2014.05.005

12. Yang X, Zhang Y: Expression of recombinant transglutaminase gene in *Pichia pastoris* and its uses in restructured meat products. *Food Chem,* 291, 245-252, 2019. DOI: 10.1016/j.foodchem.2019.04.015

13. Beninati S, Piacentini M: The transglutaminase family: An overview: Minireview article. *Amino Acids,* 26, 367-372, 2004. DOI: 10.1007/s00726-004-0091-7

14. Yasueda H, Kumazawa Y, Motoki M: Purification and characterization of a tissue-type transglutaminase from Red Sea Bream *(Pagrus major). Biosci Biotechnol Biochem,* 58 (11): 2041-2045, 1994. DOI: 10.1271/bbb.58.2041

15. Falcone P, Serafini-Fracassini D, Duca D: Comparative studies of transglutaminase activity and substrates in different organs of *Helianthus tuberosus*. *J Plant Physiol,* 142, 265-273, 1993. DOI: 10.1016/S0176-1617(11)80421-9

16. Liu S, Wang M, Du G, Chen J: Improving the active expression of transglutaminase in *Streptomyces lividans* by promoter engineering and codon optimization. *BMC Biotechnol,* 16:75, 2016. DOI: 10.1186/s12896-016-0304-7

17. Salis B, Spinetti G, Scaramuzza S, Bossi M, Jotti GS, Tonon G, Crobu D, Schepfer R: High-level expression of a recombinant active microbial transglutaminase in *Escherichia coli*. *BMC Biotechnol,* 15:84, 2015. DOI: 10.1186/s12896-015-0202-4

18. Türkanoğlu Özçelik A, Ersöz F, İnan M: Extracellular production of the recombinant bacterial transglutaminase in *Pichia pastoris*. *Protein Expr Purif,* 159, 83-90, 2019. DOI: 10.1016/j.pep.2019.03.003

19. Folk JE: Mechanism of action of Guinea pig liver transglutaminase. VI. Order of substrate addition. *J Biol Chem,* 244 (13): 3707-3713, 1969.

20. Lemon DW: An improved TBA test for rancidity. New Series Circular, No: 51. Halifax Laboratory, Halifax: Nova Scotia, 1975.

21. Soparukdee S, Tangwatcharin P: Quality of steak restructured from beef trimmings containing microbial transglutaminase and impacted by freezing and grading by fat level. *Asian-Australas J Anim Sci,* 31 (1): 129-137, 2018. DOI: 10.5713/ajas.17.0170

22. Lee HC, Chin KB: Effect of transglutaminase, acorn, and mungbean powder on quality characteristics of low-fat/salt pork model sausages. *Korean J Food Sci Anim Resour,* 29, 374-381, 2009. DOI: 10.5851/kosfa.2009.29.3.374

23. Choi YS, Jeong TJ, Kim HW, Hwang KE, Sung JM, Seo DH, Kim YB, Kim CJ: Combined effects of sea mustard and transglutaminase on the quality characteristics of reduced-salt Frankfurters. *J Food Process Preserv,* 41 (3): e12945, 2017. DOI: 10.1111/jfpp.12945

24. Baugreet S, Kerry JP, Brodkorb A, Gomez C, Auty M, Allen P, Hamill RM: Optimisation of plant protein and transglutaminase content in novel beef restructured steaks for older adults by central composite design. *Meat Sci,* 142, 65-77, 2018. DOI: 10.1016/j.meatsci.2018.03.024

25. Serrano A, Cofrades S, Colmenero FJ: Transglutaminase as binding agent in fresh restructured beef steak with added walnuts. *Food Chem*, 85 (3): 423-429, 2004. DOI: 10.1016/j.foodchem.2003.07.021

26. Gharibzahedi SMT, George S, Greiner R, Estevinho BN, Frutos Fernández MJ, McClements DJ, Roohinejad S: New trends in the microencapsulation of functional fatty acid-rich oils using transglutaminase catalyzed crosslinking. *Compr Rev Food Sci Food Saf,* 17 (2): 274-289, 2018. DOI: 10.1111/1541-4337.12324

27. Liu C, Damodaran S, Heinonen M: Effects of microbial transglutaminase treatment on physiochemical properties and emulsifying functionality of faba bean protein isolate. *LWT,* 99, 396-403, 2019. DOI: 10.1016/j.lwt.2018.10.003

28. Tseng TF, Tsai CM, Yang JH, Chen MT: Porcine blood plasma transgluataminase combined with thrombin and fibrinogen as a binder in restructured meat. *Asian-Australas J Anim Sci,* 19 (7): 1054-1058, 2006. DOI: 10.5713/ajas.2006.1054

29. Martínez B, Miranda JM, Franco CM, Cepeda A, Vázquez M: Evaluation of transglutaminase and caseinate for a novel formulation of beef patties enriched in healthier lipid and dietary fiber. *LWT-Food Sci Technol,* 44 (4): 949-956, 2011. DOI: 10.1016/j.lwt.2010.11.026

30. Uran H, Aksu F, Yılmaz İ, Durak MZ: Effect of transglutaminase on the quality properties of chicken breast patties. *Kafkas Univ Vet Fak Derg,* 19 (2): 331-335, 2013. DOI: 10.9775/kvfd.2012.7842

31. Park YS, Choi YS, Hwang KE, Kim TK, Lee CW, Shin DM, Han SG: Physicochemical properties of meat batter added with edible silkworm pupae *(Bombyx mori)* and transglutaminase. *Korean J Food Sci Anim Resour,* 37 (3): 351-359, 2017. DOI: 10.5851/kosfa.2017.37.3.351

32. Cofrades S, Lopez-Lopez I, Ruiz-Capillas C, Triki M, Jimenez-Colmenero F: Quality characteristics of low-salt restructured poultry with microbial transglutaminase and seaweed. *Meat Sci,* 87, 373-380, 2011. DOI: 10.1016/j.meatsci.2010.11.014

33. Holman BWB, van de Ven RJ, Mao Y, Coombs CEO, Hopkins DL: Using instrumental (CIE and reflectance) measures to predict consumers' acceptance of beef colour. *Meat Sci,* 127, 57-62, 2017. DOI: 10.1016/j.meatsci.2017.01.005

34. Kilic B: Effect of microbial transglutaminase and sodium caseinate on quality of chicken döner kebab. *Meat Sci,* 63 (3): 417-421, 2003. DOI: 10.1016/s0309-1740(02)00102-x

35. Kumazawa Y, Seguro K, Takamura M, Motoki M: Formation of **ε**-(γ-glutamyl) lysine cross-link in cured horse mackerel meat induced by drying. *J Food Sci,* 58 (5): 1062-1064, 1993. DOI: 10.1111/j.1365-2621.1993.tb06112.x

36. Ramirez-Suarez JC, Xiong YL: Effect of transglutaminase-induced cross-linking on gelation of myofibrillar/soy protein mixtures. *Meat Sci,* 65, 899-907, 2003. DOI: 10.1016/S0309-1740(02)00297-8

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

Kafkas Univ Vet Fak Derg
27 (2): 217-224, 2021
DOI: 10.9775/kvfd.2020.25093

RESEARCH ARTICLE

Determination of MIC Values of Various Antimicrobial Agents and Presence of Resistance Genes in *Pasteurella multocida* Strains Isolated from Bovine [1]

Ozgul GULAYDIN [1,a (*)] Kemal GURTURK [1,b] Ismail Hakki EKIN [1,c] Cihat OZTURK [1,d]

[1] *This study was supported by Research Fund of Van Yuzuncu Yil University with project number of TSA-2019-7924*

[1] Van Yuzuncu Yil University, Faculty of Veterinary Medicine, Department of Microbiology, TR-65090 Tusba, Van - TURKEY
ORCIDs: [a] 0000-0001-8376-2008; [b] 0000-0002-9372-8951; [c] 0000-0001-5029-8130; [d] 0000-0003-2868-2317

Article ID: KVFD-2020-25093 Received: 19.10.2020 Accepted: 15.02.2021 Published Online: 18.02.2021

Abstract

Pasteurella multocida is an important bacterium that can cause respiratory infections in cattle. Due to the usage of antimicrobial agents in the treatment of the disease frequently, it is critical to follow the antimicrobial susceptibility of the isolates. In this study, minimal inhibitory concentrations (MIC) of various antimicrobial agents and presence of genes related to resistance were investigated in 59 *P. multocida* strains isolated from the respiratory tract of cattle. According to MIC values determined by E-test, all of the isolates were susceptible to enrofloxacin, chloramphenicol and gentamicin, but resistant to cefoxitin. In addition, high resistance to ampicillin (88.14%), tilmicosin (64.41%), clindamycin (83.05%) and streptomycin (59.32%) were observed in the isolates. When the resistance genes were examined by PCR, it was determined that bla_{ROB-1}, *tet* H, *sul* II, *str A/aphA* 1 and *erm* 42 genes could play an important role in penicillin, tetracycline, sulfamethoxazole + trimethoprime, aminoglycoside and macrolide resistance, respectively. It was concluded that the usage of ampicillin, tetracycline, sulfamethoxazole + trimethoprime, macrolide and aminoglycosides should be considered for the treatment of respiratory tract infections caused by P. multocida in cattle. Also, it was determined that antimicrobial resistance genes could play an important role in the development of resistance in *P. multocida*.

Keywords: *Pasteurella multocida, Antimicrobial susceptibility, MIC, Resistance gene*

Sığırlardan İzole Edilen *Pasteurella multocida* Suşlarında Çeşitli Antimikrobiyal Maddelerin MİK Değerlerinin ve Antimikrobiyal Direnç Genlerinin Belirlenmesi

Öz

Pasteurella multocida, sığırlarda solunum yolu enfeksiyonlarına neden olan önemli bir bakteriyel etkendir. Hastalığın tedavisinde sıklıkla antimikrobiyal tedavi uygulanması nedeniyle etkene yönelik antimikrobiyal duyarlılık sonuçlarının takip edilmesi kritik öneme sahiptir. Bu çalışmada, sığırların solunum yolundan izole edilen 59 adet *P. multocida* izolatında çeşitli antimikrobiyal maddelerin minimal inhibitör konsantrasyonları (MİK) ve antimikrobiyal direnç ile ilişkili genlerin varlığı araştırıldı. E-test yöntemiyle belirlenen MİK değerlerine göre izolatların tamamı enrofloxacin, chloramphenicol ve gentamicine duyarlı, cefoxitine ise dirençli bulundu. Ayrıca ampicillin (%88.14), tilmicosin (%64.41), clindamycin (%83.05) ve streptomycine (%59.32) yüksek oranda direnç tespit edildi. PCR ile antimikrobiyal direnç genlerinin varlığı incelendiğinde ise penicillin, tetracycline, sulfamethoxazole + trimethoprime, aminoglikozid ve makrolid direncinde sırasıyla bla_{ROB-1}, *tet* H, *sul* II, *str A/aphA* 1 ve *erm* 42 genlerinin önemli rol oynadığı belirlendi. Bu çalışmada, sığırlarda *P. multocida* suşlarının neden olduğu solunum yolu enfeksiyonlarının tedavisinde ampicillin, tetracycline, sulfamethoxazole + trimethoprime ile makrolid ve aminoglikozid antibiyotiklerin kullanımına dikkat edilmesi gerektiği sonucuna varıldı. Ayrıca, antimikrobiyal direnç ile ilişkili genlerin izolatlarda direnç gelişiminde önemli rol oynadığı belirlendi.

Anahtar sözcükler: *Pasteurella multocida, Antimikrobiyal duyarlılık, MİK, Direnç genleri*

INTRODUCTION

Respiratory disease of cattle is one of the infections leading to significant economic losses in cattle breeding. It is known that bacterial and viral factors, as well as stres factors caused by improper transport, weaning, and nutritional conditions are also involved in the etiology of this disease [1]. *Pasteurella multocida* is one of the

How to cite this article?

Gulaydin O, Gurturk K, Ekin IH, Ozturk C: Determination of MIC values of various antimicrobial agents and presence of resistance genes in *Pasteurella multocida* strains isolated from bovine. *Kafkas Univ Vet Fak Derg,* 27 (2): 217-224, 2021.
DOI: 10.9775/kvfd.2020.25093

(*) **Corresponding Author**

Tel: +90 432 225 1128-22529 Mobile: +90 543 263 6648
E-mail: ozgulgulaydin@yyu.edu.tr (O. Gulaydin)

bacterial agent that can cause respiratory disease in cattle [2].

There are a limited number of vaccine types that can achieve a specific immune response in the control of infections caused by *P. multocida*. Due to a wide host spectrum and having different capsular polysaccharides can affect the achievement of the vaccine negatively [3]. Therefore, antimicrobial therapy is often preferred for the treatment and control of pasteurellosis cases.

Prolonged and uncontrolled usage of antimicrobial agents can lead to development of resistance in isolates [4]. Because laboratory tests are time consuming, veterinarians have to use a broad spectrum antimicrobial agents, especially in the treatment of acute infections, which leads to the development of resistance in isolates. For this reason, it is critical that the antimicrobial susceptibility of *P. multocida* isolates should be monitored in national and international aspect, periodically [3].

It is known that the genes which can be located in chromosomal DNA or extra chromosomal structures in bacteria can also cause antimicrobial resistance. Aminoglycoside resistance genes *(str* A, *str* B, *aadA* 14, *aphA* 1, *aad* B and *aadA* 25) [5-7], macrolide resistance genes *(erm* 42, *msr* E, *mph* E, *erm* A and *erm* C) [6,8-10], tetracycline resistance genes (*tet* H, *tet* B, *tet* M, *tet* C, *tet* L and *tet* O) [3,5,8,11], β-lactam resistance gene *(bla_{ROB-1})* [12] and sulfonamide resistance gene *(sul* II) [3] have been reported to be associated with the antimicrobial resistance in *Pasteurellacae* family.

In Turkey, there are various researches [13-15] that were conducted on identification of bacterial agents causing respiratory diseases in cattle and determination of their antimicrobial susceptibilities by disc diffusion method that can be obtained qualitative data about antimicrobial susceptibilities. However, investigation of MIC values of antimicrobial agents and the presence of the genes associated with the antimicrobial resistance can make to be clarified resistant mechanisims in bacterial agents and offers quantitative data.

In this study, MIC values of various antimicrobial agents and the presence of genes related to the antimicrobial resistance in *P. multocida* isolates isolated from the respiratory tract of cattle in Van, Turkey were investigated.

Material and Methods

In this study, 59 *P. multocida* strains isolated from swab samples of upper and lower respiratory tract of the cattle between 2016 and 2019, were used. Nineteen of the isolates were obtained from nasal swab samples of cattle that had pneumonia symptom clinically. Also, 32 and 8 strains were isolated from nasal swabs and trachea-bronchial swabs of slaughtered cattle, respectively. This study was approved by Van Yuzuncu Yil University Animal Researches Local Ethic Committee with the number of 2019/01.

Preliminary identification of the isolates were performed according to hemolitic activity on blood agar, Gram staining, oxidase reaction and growth on MacConkey agar [16]. PCR method reported by Townsend et al.[17] was used for the identification of the isolates at the species level.

Determination of MIC Values

MIC values of penicillin, ampicillin, tetracycline, sulfamethoxazole + trimethoprim, cephalotin, cefotaxime, cefoxitin, enrofloxacin, ciprofloxacine, erythromycin, tilmicosin, clindamycin, chloramphenicol, streptomycin and gentamicin were determined by using E-test stript (Himedia, India and Liofilchem, Italy). The criteria of European Commitee on Antimicrobial Susceptibility Testing [18] and Clinical Laboratory Standards Institute [19,20] were considered in applying and evaluating the tests. For determination of MIC values using E-test method, overnight culture of the isolates on Columbia blood agar (Oxoid, CM 0331, England) supplemented with 5% defibrinated sheep blood were suspended into 2 mL sterile physiological saline (pH:7.0) and the suspension was adjusted to McFarland 0.5 turbidity. Then, 0.1 mL of suspension was inoculated Mueller Hinton agar (Oxoid, CM0337, England) supplemented with 5% defibrinated sheep blood. E-test stript was placed on the agar and incubated at 37°C for 18-24 h. After incubation period, the point where the inhibition ellipse intersected the strip was accepted as the MIC value. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, supplied from culture collection of Van Yuzuncu Yil University, Faculty of Veterinary Medicine, Department of Microbiology, were used as control strains.

Determination of Antimicrobial Resistance Genes

The genes that were related to antimicrobial resistance were investigated by PCR using gene spesific primer *(Table 1)*. Genomic DNA was obtained by boiling method. For this purpose, *P. multocida* colonies were picked from Columbia blood agar and mixed into 200 µL PCR water. Then, suspension was boiled at 100°C in a dry block for 10 min. After chilled on ice, suspension was centrifuged at 10,000 X g for 5 min and supernatant was used as genomic DNA. PCR mixture was consisted of 9.5 µL of mastermix (Abm® 2X PCR Taq Plus Mastermix), 5 µL of genomic DNA and 1 µL of each primer (10 µM) and the total volume was completed to 25 µL with PCR water. Pre-denaturation was performed at 94°C for 5 min and the final extension was performed at 72°C for 10 min. The amplification process that was applied for each gene was shown in *Table 1*. Amplicons were electrophoresed in a 1.5% agarose gel at 80 V for 1.5 h and visualized in a gel imaging system (Spektroline, GL-500).

Results

MIC values of penicillin, ampicillin, tetracycline, sulfamethoxazole + trimethoprim, cephalotin, cefoxitin,

Table 1. *Primers used for the determination of antimicrobial resistance genes by PCR*

Gene	Oligonucleotid (5'-3')	bp	PCR Conditions (denaturation/anneling/elongation)	Reference
β-lactamase				
bla$_{ROB-1}$	**F:** CATTAACGGCTTGTTCGC **R:** CTTGCTTTGCTGCATCTTC	852	94°C-30 sec/50°C-30 sec/72°C-30 sec 25 cycles	[21]
Sulfonamide				
sul II	**F:** ACAGTTTCTCCGATGGAGGCC **R:** CTCGTGTGTGCGGATGAAGTC	704	94°C-60 sec/56°C-60 sec/72°C-60 sec 30 cycles	[22]
Tetracycline				
tet B	**F:** CCTTATCATGCCAGTCTTGC **R:** ACTGCCGTTTTTTTCGCC	774	94°C-30 sec/53°C-30 sec/72°C-90 sec 25 cycles	[23]
tet H	**F:** ATACTGCTGATCACCGT **R:** TCCCAATAAGCGACGCT	1076	94°C -60 sec/47°C-60 sec/72°C-60 sec 30 cycles	[11]
tet M	**F:** GTTAAATAGTGTTCTTGGAG **R:** CTAAGATATGGCTCTAACAA	657	94°C -30 sec/48°C-30 sec/72°C-90 sec 30 cycles	[24]
Macrolide				
erm 42	**F:** TGCACCATCTTACAAGGAGT **R:** CATGCCTGTCTTCAAGGTTT	173	94°C-30 sec/51°C-30 sec/72°C-45 sec 25 cycles	[10]
msr E	**F:** TATAGCGACTTTAGCGCCAA **R:** GCCGTAGAATATGAGCTGAT	395	94°C-30 sec/52°C-30 sec/72°C-30 sec 25 cycles	[10]
mph E	**F:** ATGCCCAGCATATAAATCGC **R:** ATATGGACAAAGATAGCCCG	271	94°C-30 sec/52°C-30 sec/72°C-45 sec 25 cycles	[10]
Aminoglycoside				
str A	**F:** TGACTGGTTGCCTGTCAGAGG **R:** CAGTTGTCTTCGGCGTTAGCA	646	94°C-60 sec/57°C-60 sec/72°C-60 sec 30 cycles	[22]
aph A1	**F:** GCCGTTTCTGTAATGAAGGAG **R:** GGCAATCAGGTGCGACAATCT	642	94°C-30 sec/55°C-30 sec/72°C-30 sec 25 cycles	[25]

cefotaxime, enrofloxacin, ciprofloxacin, erythromycin, tilmicosin, clindamycine, chloramphenicol, streptomycin and gentamicin were determined as 0.125 - >256, 0.125 - >256, 0.25 - 32, 0.004 - 32, 0.016 - 32, 0.064 - >256, 0.002 - 0.094, 0.002 - 0.50, 0.002 - 3, 0.032 - >256, 2 - >32, 1.5 - >256, 0.25 - 8, 2 - >256, ve 0.19 - 2 µg/mL in *P. multocida* isolates, respectively *(Table 2)*. According to these values, all of the isolates were found to be susceptible to enrofloxacin, chloramphenicol and gentamicine, but resistant to cefoxitin. In addition, 4 (6.77%), 52 (88.14%), 21 (35.59%), 23 (38.98%), 1 (1.69%), 2 (3.39%), 14 (23.73%), 18 (30.51%), 38 (64.41%), 49 (83.05%) and 35 (59.32%) of the isolates were resistant to penicilin, ampicillin, tetracycline, sulfamethoxazole + trimethoprim, cephalotin, cefotaxime, ciprofloxacin, erythromycin, tilmicosin, clindamycine and streptomycine, respectively *(Table 3)*.

Distribution of antimicrobial resistance genes in the isolates were shown in *Table 4*.

Bla$_{ROB-1}$ gene was detected in 3 of 4 isolates that were resistant to both penicillin and ampicillin. However 48 isolates, found to be resistant to ampicillin only, did not harbour *bla*$_{ROB-1}$ gene.

Tet H gene were detected in 20 of the 21 tetracycline resistant isolates, but *tet* B gene was found only in 1 of these isolates. *Tet* M could not be found in any of these resistant isolates.

Sul II gene was found in all 23 of the isolates which were determined to be resistant to sulfamethoxazole + trimethoprime.

Whereas eleven of 18 erythromycin resistant isolates harboured *erm* 42 gene only, both *msr* E and *mph* E gene were detected only in 4 of the resistant isolates. Also, 12 and 4 of 38 isolates resistant to tilmicosine were observed to harbour *erm* 42 and *msr* E/*mph* E genes, respectively. *Erm* 42 and *msr* E/*mph* E genes were detected in 12 and 3 of the 49 isolates resistant to clindamycin, respectively but, macrolide resistance genes could not be found in the rest of the isolates. Additionally, 11 of the 17 *P. multocida* isolates resistant to both erythromycin, tilmicosine and clindamycin were determined to harbou *erm* 42 gene, but *msr* E and *mph* E genes were detected only in 3 of macrolide resistant isolates. Any of macrolide resistance genes were not determined in other 3 of 17 macrolide resistant isolates (data not shown).

All of *P. multocida* isolates were susceptible to gentamicin, but 35 isolates were found to be resistant to streptomycin. The *str* A gene was determined in all streptomycin resistant isolates, while the *aphA* 1 gene was detected in 34 isolates.

Table 2. *Distribution of MIC value of antimicrobial agents in P. multocida isolates*

Antimicrobial	MIC (µg/mL)																																		
	0.002	0.003	0.004	0.006	0.008	0.012	0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.50	0.75	1	1.5	2	3	4	6	8	12	16	24	32	>32	48	64	128	>256
P	-	-	-	-	-	-	-	-	-	-	-	-	4	9	17•	22	**3**	-	-•	-	-	-	-	-	-	1	1	-	1	-	-	-	-	1
AMP	-	-	-	-	-	-	-	-	-•	-	-	-	2	5	18•	24	**6**	-	1	-	-	-	-	-	-	-	-	-	2	-	-	-	-	1
TE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	9	8	4	-	-	-•	3	4	7	3•	7	**5**	5	1	-	-	-	-	-
SXT	-	-	1	-	1	2	1	3	2	8	5	1	2	1	9•	4•	5	3	4	**1**	1	1	-	-	-	-	-	-	-	4	-	-	-	-
KF	-	-	-	-	-	-	1	1	-	2	5	7	13	9	8	3	**5**	3	1	-	-	-	-	-	-•	-	-	-	1•	-	-	-	-	-
FOX	-	-	-	-	-	-	-	-	-•	-•	1	-	2	2	14	15	17	**7**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
CTX	20	1	9	11	2	4	**6**	3	1•	1•	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ENR	4	4	10	12	8	1	4	6	**6**	3	-	-	-	-	-•	-	1	-	-	-	-•	-	-	-	-	-	-	-	-	-	-	-	-	-
CIP	2	-	-	2	6	9	10	5	4	4	3•	5•	**4**	-	3	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	7•	15	15	3	-	-	-	-	1•	-	2	1	2	-	3	**3**	-	6
TIL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	5	7	2	4	-	-•	-	-	**38**•	-	-	-	-
CLI	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-•	-	-	1	3	6	7•	6	3	2	1	-	-	-	-	-	-	**30**
CHL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2	13	27	**10**	2	-	2	-	1	1•	-	-	-	-•	-	-	-	-	-
S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2	-	2	2	8	6	-	3•	-	-	-	-•	**35**
GEN	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	-	6	17	18	**13**	1	-	-•	-	-	-	-•	-	-	-	-	-	-	-

P: *Penicilin,* ***AMP:*** *Ampicillin,* ***TE:*** *Tetracycline,* ***SXT:*** *Sulfamethoxazole+trimethoprim,* ***KF:*** *Cephalothin,* ***Fox:*** *Cefoxitin,* ***CTX:*** *Cefotaxime,* ***ENR:*** *Enrofloxacin,* ***CIP:*** *Ciprofloxacin,* ***E:*** *Erythromycine,* ***TIL:*** *Tilmicosin,* ***CLI:*** *Clindamycin,* ***CHL:*** *Chloramfenikol,* ***S:*** *Streptomycin,* ***GEN:*** *Gentamicin*
Underlined Value: MIC_{50}
Bold Written Value: MIC_{90}
•: *Interpretive criteria*

Table 3. *Antimicrobial susceptibilities of P. multocida determined by E-test*

Antimicrobial Agent	S (%)	I (%)	R (%)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
P†	30 (50.84)	25 (42.37)	4 (6.7)	0.25	0.50
AMP†	0	7 (11.86)	52 (88.14)	0.38	0.50
TE†	24 (40.68)	14 (23.73)	21 (35.59)	4	16
SXT††	36 (61.02)	0	23 (38.98)	0.25	1.5
KF†††	58 (98.31)	0	1 (1.69)	0.19	0.50
FOX*,††	0	0	59 (100)	0.38	0.75
CTX††	57 (96.61)	0	2 (3.39)	0.004	0.016
ENR†	58 (98.31)	1 (1.69)	0	0.006	0.032
CIP††	45 (76.27)	0	14 (23.73)	0.023	0.125
E†††	8 (13.56)	33 (55.93)	18 (30.51)	1	64
TIL†	21 (35.59)	0	38 (64.41)	>32	>32
CLI†††	0	10 (16.95)	49 (83.05)	>256	>256
CHL†	59 (100)	0	0	0.75	1
S††††	24 (40.68)	0	35 (59.32)	>256	>256
GEN†††	59 (100)	0	0	1	1.5

P: *Penicilin,* ***AMP:*** *Ampicillin,* ***TE:*** *Tetracycline,* ***SXT:*** *Sulfamethoxazole + trimethoprim,* ***KF:*** *Cephalothin,* ***Fox:*** *Cefoxitin,* ***CTX:*** *Cefotaxime,* ***ENR:*** *Enrofloxacin,* ***CIP:*** *Ciprofloxacin,* ***E:*** *Erythromycine,* ***TIL:*** *Tilmicosin,* ***CLI:*** *Clindamycin,* ***CHL:*** *Chloramphenicol,* ***S:*** *Streptomycin,* ***GEN:*** *Gentamicin*
** Interpretive criteria for Cefotaxime was taken into consideration;† Interpretive criteria reported by CLSI, 2018 [20] was taken into consideration;†† Interpretive criteria reported by EUCAST, 2019 [18] was taken into consideration;††† Interpretive criteria reported by CLSI, 2002 [19] was taken into consideration;†††† Interpretive criteria reported by Benedict et al.[26] was taken into consideration*

Table 4. *Presence of antimicrobial resistance genes in P. multocida isolates*

Antimicrobial Agent		Resistance Genes	Number of Isolates	MIC (µg/mL)
β-lactam	Penicillin	Phenotypic Resistance	4	12 - >256
		bla_{ROB-1}	3	16 - >256
	Ampicillin	Phenotypic Resistance	52	0.25 - >256
		bla_{ROB-1}	3	32 - >256
	Tetracycline	Phenotypic Resistance	21	8 - 32
		tet B	1	24
		tet H	20	3 - 32
		tet M	0	-
Sulfonamide	Sulfamethoxazole + Trimethoprime	Phenotypic Resistance	23	0.38 - >32
		sul II	23	0.38 - >32
Macrolide	Erythromycin	Phenotypic Resistance	18	8 - >256
		erm 42	11	32 - >256
		msr E+ *mph* E	4	8 - 24
	Tilmicosin	Phenotypic Resistance	38	>32
		erm 42	12	>32
		msr E + *mph* E	4	>32
	Clindamycine	Phenotypic Resistance	49	4 - >256
		erm 42	12	>256
		msr E + *mph* E	3	2 - >256
Aminoglycoside	Streptomycin	Phenotypic Resistance	35	>256
		str A	35	>256
		aphA 1	34	>256
	Gentamicin	Phenotypic Resistance	0	0.19 - 2

Discussion

Because the identification and determination of antimicrobial susceptibility of the bacterial agents usually take a long time, the usage of broad-spectrum antimicrobial agents is preferred for the treatment of acute clinical disease and this can lead to the development of antimicrobial resistance in bacteria. Therefore, antimicrobial susceptibility of the bacterial agents and the determination of the MIC values of antimicrobial agents used for the treatment of bacterial infections, have a critical importance in national and international area. In this study, antimicrobial susceptibility of *P. multocida* strains isolated from bovine respiratory tract were evaluated by the determination of MIC values of various antimicrobial agents. Additionaly, genes related to antimicrobial resistance were investigated to identify possible resistance mechanisms developing in the strains.

Yoshimura et al.[27] reported that MIC values of penicillin, dihidro-streptomycin, oxytetracycline and tilmicosin in *P. multocida* strains were 0.05-25 unit/mL, 0.39 - >100, 0.1-25 and 0.1-100 µg/mL, respectively. In another study, MIC values of tetracycline, tilmicosin and sulfamethoxazole + trimethoprime were determined as 0.06-256, 1-128 and 0.015-1 µg/mL, respectively [28]. Anholt et al.[29] found that MIC values of penicillin, ampicillin, tilmicosin, clindamycin and gentamicin were ≤0.12-8, 0.25-8, 4-64, 8-16 and 1-16 µg/mL, respectively. In another study and MIC values of oxytetracyclin and ampicillin were 0.25 - >512 and 0.125-128 µg/mL, respectively. In the research, *tet* H gene was found in 89% of oxytetracyclin resistant isolates, while *tet* B gene was reported to be detected in 4.76% of them. Also, 16 of 22 ampicillin resistant isolates were reported to be harboured bla_{ROB-1} gene [30].

In the presented study, phenotypic and genotypic findings about resistance to penicillin were similar to the findings reported by Dayao et al.[31], whereas the MIC values of penicillin and ampicillin (0.125 - >256 µg/mL) was higher than the values reported by Anholt et al.[29] and Katsuda et al.[30]. Also, that the genes associated with resistance to β-lactam antibiotics are mostly encoded by plasmids, may cause that these genes are found in a low level in chromosomal DNA of ampicillin resistant isolates.

In this study MIC value of tetracycline was found to be lower than those of reported by Garch et al.[28] and Katsuda et al.[30]. However, this value was higher than that of reported for oxytetracycline by Yoshimura et al.[27]. *Tet* H gene was detected in 20 (95.2%) of 21 tetracycline resistant isolates, while *tet* B gene was only found in 1 (4.8%) isolate. Additionally, *tet* M gene could not be detected in the isolates. These findings were similar to the findings reported by Katsuda et al.[30]. In contrast to this study, Dayao et al.[31] reported that *tet* H gene was not detected whereas *tet* B gene was found in 57% of the examined isolates. As in our study, Dayao et al.[31] reported that *tet* M gene could not be detected in tetracycline resistant isolates.

In the presented study, the MIC value of sulfamethoxazole + trimethoprime was observed to be similar to value reported by Garch et al.[28]. However, it was observed that value detected for sulfamethoxazole + trimethoprime was highly lower than MIC value of sulfamethoxazole (≥512 µg/mL) reported by Kehrenberg and Schwarz [22]. It was assumed that the use of sulfamethoxazole without trimethoprime could lead to this difference. However, as in our study, *sul* II gene was reported to be detected in all resistant isolates in both studies.

Generally, MIC value of enrofloxacin was found to be low [27-29] and resistance to this antimicrobial agent was not significant in *P. multocida* isolates [32-33]. As indicated previous studies, MIC value of enrofloxacin was determined as 0.002-0.5 µg/mL and no isolates were found to be resistant to enrofloxacin in this study.

MIC values of streptomycine in *P. multocida* isolates were reported as 0.39 - >100, ≥128 and 1-32 µg/mL by Yoshimura et al.[27], Kehrenberg and Schwarz [22] and Wang et al.[25], respectively. But, in this study, this value was determined to be higher (2 - >256 µg/mL). Also, in the presented research, *str* A ve *aph*A 1 (excepting 1 isolate) genes were found in all streptomycine resistant isolates same as in other studies [22,25]. MIC value (0.19-2 µg/mL) of gentamicin was found to be lower than reported by Wang et al.[28] and Anholt et al.[28].

Kadlec et al.[34] reported that 8 to 32-fold increase were determined in MIC values of erythromycin, tilmicosin and clindamycin when *erm* 42 gene was cloned into *P. multocida* isolates via plasmid vector. It was also reported that the MIC values of erythromycin and tilmicosin increased to 32-128 times when *msr* E+*mph* E genes were cloned. In another study, it was reported that MIC value of tilmicosin ranged from 128 to >128 µg/mL in *erm* 42 positive isolates while that was 32 µg/mL in *msr* E+*mph* E positive isolates. Additionally, in isolates were positive for all three genes, MIC value of tilmicosin was reported to be >128 µg/mL [10]. Similarly, in another study it was revealed that MIC values of tilmicosin and clindamycin in *erm* 42 positive isolates were 128 - >128 and 1024 µg/mL, respectively. It was also reported that MIC values of tilmicosin and clindamycine were 32 and 16 µg/mL in *msr* E and *mph* E genes positive isolates, respectively. In addition, these values were determined as 128 and >1024 µg/mL in the isolates harbouring all those three genes [9].

In this study, MIC value of erythromycin was detected as 32 - >256 µg/mL in *erm* 42 positive isolates. While this gene was determined in 12 of 38 tilmicosin resistant isolates and 11 of 49 clindamycin resistant isolates, MIC values for both antibiotics were found to be >32 µg/mL and >256 µg/mL, respectively. On the other hand, MIC value of erythromycin

varied from 8 to >24 µg/mL in erythromycin resistant isolates that were positive for *msr* E+*mph* E. Whereas both genes were determined in 4 tilmicosin resistant and in 3 clindamycin resistant isolates, MIC values of both antibiotics were detected as >32 µg/mL and 2 - >256 µg/mL, respectively. However, Dayao et al.[31] reported that *msr* E and *mph* E genes could not be detected in *P. multocida* isolates that were resistant to macrolides.

Although in this study macrolide resistance in *P. multocida* isolates were determined to be higher than that of reported by other researcher, the presence of resistance genes were observed in a limited number. It was assumed that other genes or different resistance mechanisms [6,8,9] could play a role in the development of resistance.

In this study, it was determined that *P. multocida* isolates that cause respiratory diseases in cattle was highly susceptible to penicillin, cephalothin, cefotaxime, chloromphenicol, gentamicine and enrofloxacin. Also, it was determined that it should be paid attention to the use of ampicillin, tetracycline, sulfamethoxazole + trimethoprime, macrolide and aminoglycoside antibiotics for the treatment of infections caused by this agent. Although the genes associated with tetracycline, sulfonamide and aminoglycoside resistance have an important role in the development of resistance in *P. multocida* isolates, the presence of resistance genes in extra chromosomal elements as well as other mechanisms that are responsible for macrolide and β-lactam antibiotics should be investigated in further studies.

Conflict of Interest

There is no conflict of interest.

Author Contributions

All authors contributed to laboratory examinations and writing manuscript.

References

1. Horwood PF, Mahony TJ: Multiplex real-time RT-PCR detection of three viruses associated with the bovine respiratory disease complex. *J Virol Methods*, 171 (2): 360-363, 2011. DOI: 10.1016/j.jviromet.2010.11.020

2. Dziva F, Muhairwa AP, Bisgaard M, Christensen H: Diagnostic and typing options for investigating diseases associated with *Pasteurella multocida*. *Vet Microbiol*, 128 (1-2): 1-22, 2008. DOI: 10.1016/j.vetmic.2007.10.018

3. Kehrenberg C, Schulze-Tanzil G, Martel JL, Chaslus-Dancla E, Schwarz S: Antimicrobial resistance in *Pasteurella* and *Mannheimia*: Epidemiology and genetic basis. *Vet Res*, 32 (3-4): 323-339, 2001. DOI: 10.1051/vetres:2001128

4. Sarangi LN, Thomas P, Gupta SK, Priyadarshini A, Kumar S, Nagaleekar VK, Kumar A, Singh VP: Virulence gene profiling and antibiotic resistance pattern of Indian isolates of *Pasteurella multocida* of small ruminant origin. *Comp Immunol Microbiol Infect Dis*, 38, 33-39, 2015. DOI: 10.1016/j.cimid.2014.11.003

5. Kehrenberg C, Catry B, Haesebrouck F, de Kruif A, Schwarz S: Novel spectinomycin/streptomycin resistance gene, *aadA14*, from *Pasteurella multocida*. *Antimicrob Agents Chemother*, 49 (7): 3046-3049, 2005. DOI: 10.1128/AAC.49.7.3046-3049.2005

6. Michael GB, Kadlec K, Sweeney MT, Brzuszkiewicz E, Liesegang H, Daniel R, Murray RW, Watts JL, Schwarz S: ICE Pmu1, an integrative conjugative element (ICE) of *Pasteurella multocida*: Analysis of the regions that comprise 12 antimicrobial resistance genes. *J Antimicrob Chemother*, 67 (1): 84-90, 2012. DOI: 10.1093/jac/dkr406

7. Eidam C, Poehlein A, Leimbach A, Michael GB, Kadlec K, Liesegang H, Daniel R, Sweeney MT, Murray RW, Watts JL, Schwarz S: Analysis and comparative genomics of ICE*Mh1*, a novel integrative and conjugative element (ICE) of *Mannheimia haemolytica*. *J Antimicrob Chemother*, 70 (1): 93-97, 2015. DOI: 10.1093/jac/dku361

8. Wasteson Y, Roe DE, Falk K, Roberts MC: Characterization of tetracycline and erythromycin resistance in *Actinobacillus pleuropneumoniae*. *Vet Microbiol*, 48, 41-50, 1996. DOI: 10.1016/0378-1135(95)00130-1

9. Desmolaize B, Rose S, Wilhelm C, Warrass R, Douthwaite S: Combinations of macrolide resistance determinants in field isolates of *Mannheimia haemolytica* and *Pasteurella multocida*. *Antimicrob Agents Chemother*, 55 (9): 4128-4133, 2011. DOI: 10.1128/AAC.00450-11

10. Rose S, Desmolaize B, Jaju P, Wilhelm C, Warrass R, Douthwaite S: Multiplex PCR to identify macrolide resistance determinants in *Mannheimia haemolytica* and *Pasteurella multocida*. *Antimicrob Agents Chemother*, 56 (7): 3664-3669, 2012. DOI: 10.1128/AAC.00266-12

11. Blanco M, Gutiérrez-Martin CB, Rodríguez-Ferri EF, Roberts MC, Navas J: Distribution of tetracycline resistance genes in *Actinobacillus pleuropneumoniae* isolates from Spain. *Antimicrob Agents Chemother*, 50 (2): 702-708, 2006. DOI: 10.1128/AAC.50.2.702-708.2006

12. Livrelli VO, Darfeuille-Richaud A, Rich CD, Joly BH, Martel JL: Genetic determinant of the ROB-1 beta-lactamase in bovine and porcine *Pasteurella* strains. *Antimicrob Agents Chemother*, 32 (8): 1282-1284, 1988. DOI: 10.1128/AAC.32.8.1282

13. Onat K, Kahya S, Çarli KT: Frequency and antibiotic susceptibility of *Pasteurella multocida* and *Mannheimia haemolytica* isolates from nasal cavities of cattle. *Turk J Vet Anim Sci*, 34 (1): 91-94, 2010. DOI: 10.3906/vet-0901-1

14. Ulker H, Kucuk D, Cantekin Z, Solmaz H: Hatay yöresinde kesimhanede kesilen sığır akciğerlerinden *Pasteurella multocida* ve *Mannheimia haemolytica* izolasyonu ve antibiyotiklere duyarlılığı. *AVKAE Derg*, 2 (2): 10-14, 2012.

15. Guler L, Gunduz K, Sarisahin AS: Capsular typing and antimicrobial susceptibility of *Pasteurella multocida* isolated from different hosts. *Kafkas Univ Vet Fak Derg*, 19 (5): 843-849, 2013. DOI: 10.9775/kvfd.2013.8936

16. Quinn PJ, Markey BK, Leonard FC, Fitzpatrick ES, Fanning S, Hartigan PJ: *Pasteurella* species, *Mannheima haemolytica* and *Bibersteinia trehalosi*. **In,** Veterinary Microbiology and Microbial Disease. 2nd ed., 300-308, John Wiley & Sons Ltd., UK, 2011.

17. Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJS: Development of PCR assays for species and type-specific identification of *Pasteurella multocida* isolates. *J Clin Microbiol*, 36 (4): 1096-1100, 1998.

18. European Committee on Antimicrobial Susceptibility Testing: Clinical Breakpoint Tables v. 9.0, valid from 2019-01-01.

19. Clinical Laboratory Standart Institute: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard. 2nd ed., Wayne, PA: CLSI, 2002.

20. Clinical Laboratory Standart Institute: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 4th ed., Vet 08. Wayne, PA: CLSI, 2018.

21. Matter D, Rossano A, Limat S, Vorlet-Fawer L, Brodard I, Perreten V: Antimicrobial resistance profile of *Actinobacillus pleuropneumoniae* and *Actinobacillus porcitonsillarum*. *Vet Microbiol*, 122 (1-2): 146-156, 2007. DOI: 10.1016/j.vetmic.2007.01.009

22. Kehrenberg C, Schwarz S: Occurrence and linkage of genes coding for resistance to sulfonamides, streptomycin and chloramphenicol in bacteria of the genera *Pasteurella* and *Mannheimia*. *FEMS Microbiol Lett*, 205, 283-290, 2001. DOI: 10.1111/j.1574-6968.2001.tb10962.x

23. Maynard C, Fairbrother JM, Bekal S, Sanschagrin F, Levesque RC, Brousseau R, Maason L, Lariviere S, Harel J: Antimicrobial resistance

genes in enterotoxigenic *Escherichia coli* O149: K91 isolates obtained over a 23 year period from pigs. *Antimicrob Agents Chemother*, 47 (10): 3214-3221, 2003. DOI: 10.1128/aac.47.10.3214-3221.2003

24. Akinbowale OL, Peng H, Barton MD: Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *J Appl Microbiol*, 103 (5): 2016-2025, 2007. DOI: 10.1111/J.1365-2672.2007.03445.X

25. Wang Z, Kong LC, Jia BY, Liu SM, Jiang XY, Ma HX: Aminoglycoside susceptibility of *Pasteurella multocida* isolates from bovine respiratory infections in China and mutations in ribosomal protein S5 associated with high-level induced spectinomycin resistance. *J Vet Med Sci*, 79 (10): 1678-1681, 2017. DOI: 10.1292/jvms.17-0219

26. Benedict KM, Gow SP, Checkley S, Booker CW, McAllister TA, Morley PS: Methodological comparisons for antimicrobial resistance surveillance in feedlot cattle. *BMC Vet Res*, 9:216, 2013. DOI: 10.1186/1746-6148-9-216

27. Yoshimura H, Ishimaru M, Endoh YS, Kojima A: Antimicrobial susceptibility of *Pasteurella multocida* isolated from cattle and pigs. *J Vet Med B*, 48 (7): 555-560, 2001. DOI: 10.1111/j.1439-0450.2001.00468.x

28. Garch F, de Jong A, Simjee S, Moyaert H, Klein U, Ludwig C, Marion H, Haag-Diergarten S, Richard-Mazer A, Thomas V, Siegwart E: Monitoring of antimicrobial susceptibility of respiratory tract pathogens isolated from diseased cattle and pigs across Europe, 2009-2012: VetPath results. *Vet Microbiol*, 194, 11-22, 2016. DOI: 10.1016/j.vetmic.2016.04.009

29. Anholt RM, Klima C, Allan N, Matheson-Bird H, Schatz C, Ajitkumar P, Otto SJG, Peters D, Schmid K, Olson M, McAllister T, Ralston B: Antimicrobial susceptibility of bacteria that cause bovine respiratory disease complex in Alberta, Canada. *Front Vet Sci*, 4:207, 2017. DOI: 10.3389/fvets.2017.00207

30. Katsuda K, Hoshinoo K, Ueno Y, Kohmoto M, Mikami O: Virulence genes and antimicrobial susceptibility in *Pasteurella multocida* isolates from calves. *Vet Microbiol*, 167 (3-4): 737-741, 2013. DOI: 10.1016/j.vetmic.2013.09.029

31. Dayao DAE, Gibson JS, Blackall PJ, Turni C: Antimicrobial resistance genes in *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* and *Pasteurella multocida* isolated from Australian pigs. *Aust Vet J*, 94 (7): 227-231, 2016. DOI: 10.1111/avj.12458

32. Cucco L, Massacci FR, Sebastiani C, Mangili P, Bano L, Cocchi M, Luppi A, Ortenzi R, Pezzotti G, Magistrali CF: Molecular characterization and antimicrobial susceptibility of *Pasteurella multocida* strains isolated from hosts affected by various diseases in Italy. *Vet Ital*, 53 (1): 21-27, 2017. DOI: 10.12834/VetIt.661.3256.2

33. Timsit E, Hallewell J, Booker C, Tison N, Amat S, Alexander TW: Prevalence and antimicrobial susceptibility of *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* isolated from the lower respiratory tract of healthy feedlot cattle and those diagnosed with bovine respiratory disease. *Vet Microbiol*, 208, 118-125, 2017. DOI: 10.1016/j.vetmic.2017.07.013

34. Kadlec K, Michael GB, Sweeney MT, Brzuszkiewicz E, Liesegang H, Daniel R, Watts JL, Schwarz S: Molecular basis of macrolide, triamilide, and lincosamide resistance in *Pasteurella multocida* from bovine respiratory disease. *Antimicrob Agents Chemother*, 55 (5): 2475-2477, 2011. DOI: 10.1128/AAC.00092-11

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

Kafkas Univ Vet Fak Derg
27 (2): 225-233, 2021
DOI: 10.9775/kvfd.2020.25106

RESEARCH ARTICLE

The Usability of Cytological and Immunocytological Methods for Rapid Diagnosis of Encephalitic Listeriosis in Ruminants [1][2]

Özgür ÖZDEMİR [1,a] Mustafa ORTATATLI [1,b] Funda TERZİ [2,c]
Fatih HATİPOĞLU [1,3,d] Mustafa Kemal ÇİFTÇİ [1,e] Mehmet Burak ATEŞ [1,f] (*)

[1] *Presented as an oral presentation in 3rd International Conference on Science, Ecology and Technology 14-16 August 2017, Rome, Italy*
[2] *The study was supported by the Scientific Research Projects Coordination Office of Selcuk University (Project No: 15401088)*

[1] Selcuk University, Faculty of Veterinary Medicine, Department of Pathology, TR-42130 Konya - TURKEY
[2] Kastamonu University, Faculty of Veterinary Medicine, Department of Pathology, TR-37200 Kastamonu - TURKEY
[3] Kyrgyz-Turkish Manas University, Faculty of Veterinary Medicine, Department of Pathology, KG-720044, Bishkek, KYRGYZSTAN
ORCIDs: [a] 0000-0002-1595-0557; [b] 0000-0002-3713-813X; [c] 0000-0002-6184-5408; [d] 0000-0002-0103-5868; [e] 0000-0003-4476-2114
[f] 0000-0003-1297-426X

Article ID: KVFD-2020-25106 Received: 23.10.2020 Accepted: 14.02.2021 Published Online: 15.02.2021

Abstract

Although the clinical and pathological findings are important in the diagnosis of listeriosis, to isolation or to be shown the presence of the bacterium must be required for the definitive diagnosis. This study aims to investigate the availability of imprint cytological (IC) and immunocytochemical (ICC) methods in comparison with histopathological and immunohistochemical (IHC) methods for the rapid diagnosis of encephalitic listeriosis . In the study, the touching and smear preparations taken from the pons and medulla oblongata of 25 ruminants suspected with listeriosis by neurological symptoms were stained with modified giemsa and also with ICC technic for revealing antigens, as a new method. Same tissue sections were stained with Hematoxylin&Eosin and IHC methods too, and examined under light microscope by scoring. In IC examinations, there were intensive neutrophils in 14 cases and few neutrophils in 4 cases, and no neutrophils were observed in 7 cases. In histopathological examinations, 13 of these 14 cases revealed typical microabscesses and listeria positivity in IHC staining. ICC positivity was detected in 12 (92.3%) of the listeria positive 13 cases. A highly positive correlation was observed among cytology (14), ICC (12), histopathology and IHC (13) scores ($r2> 0.8$; $P<0.01$). In conclusion, the cytological examination of the pons and medulla oblongata of listeriosis-suspected ruminants revealed that a rapid pre-diagnosis could be made with the presence of intense neutrophils. Also, with ICC staining of cytological preparations, the diagnosis could be performed with 92.3% accuracy. Since ICC is an easy and fast method, it is concluded that it can be used safely especially in field studies, along with cytological examination.

Keywords: *Cytology, Histopathology, Immunocytology, Immunohistochemistry, Listeriosis*

Ruminantlarda Ensefalitik Listeriozisin Hızlı Tanısı İçin Sitolojik ve İmmunositolojik Yöntemlerin Kullanılabilirliğinin Araştırılması

Öz

Listeriozisin tanısında klinik ve patolojik bulgular önemli ise de, kesin teşhiste etken izolasyonu veya etkenin varlığının gösterilmesi gerekmektedir. Bu çalışmada ruminantlarda ensefalitik listeriozisin hızlı teşhisinde sitolojik ve immunositokimyasal (ICC) yöntemlerin, histopatoloji ve immunohistokimyasal (IHC) yöntemle kıyaslanarak rutinde kullanılabilirliklerinin araştırılması amaçlandı. Çalışmada, sinirsel semptomlarla listeriozisten şüphelenilen 25 adet ruminantın (1 sığır, 1 buzağı, 11 koyun, 9 kuzu, 1 keçi ve 2 oğlak) pons ve medulla oblongatasından alınan sürme ve kazıntı preparatlar modifiye Giemsa ile ve ayrıca ICC yöntemle boyandı. Yine aynı bölümlerden alınan doku örnekleri Hematoksilen-Eozin ve IHC yöntemleriyle de boyandı ve skorlanarak ışık mikroskobunda incelendi. Sitolojik muayenelerde 14 olguda yoğun, 4 olguda ise az sayıda nötrofil görülürken, 7 olguda hiç nötrofil gözlenmedi. Sitolojik olarak listeriozis olduğu değerlendirilen bu 14 vakanın 13'ünde histopatolojik incelemelerde tipik mikroapse ve perivaskuler hücre infiltrasyonu ile IHC boyamalarında listeria pozitifliği bulundu. IHC yöntemi baz alındığında, listeria pozitif 13 vakanın 12'sinde ICC pozitifliği (%92.3) belirlendi. Sitolojide az sayıda nötrofil görülen 4 olgu ile nötrofil görülmeyen 7 olguda ise mikroapseye rastlanmadı, IHC ve ICC de negatif olarak bulundu. Sitoloji (14), ICC (12) ile histopatoloji ve IHC (13) skorları arasında yüksek oranda ($r2>0.8$; $P<0.01$) pozitif korelasyon gözlendi. Sonuç olarak listeriozis şüpheli ruminantların pons ve medulla oblongatasının sitolojik muayenesinde, yoğun nötrofil görülmesiyle hızlı ön tanı konulabileceği, yine sitolojik preparatların ICC yöntemle boyanarak, %92.3 doğrulukla teşhisin yapılabileceği ortaya konuldu. ICC'nin kolay ve hızlı bir yöntem olması sebebiyle özellikle saha çalışmalarında sitolojik muayene ile birlikte güvenle kullanılabileceği sonucuna varıldı.

Anahtar sözcükler: *Sitoloji, Histopatoloji, İmmunositoloji, İmmunohistokimya, Listeriosis*

How to cite this article?

Özdemir Ö, Ortatatlı M, Terzi F, Hatipoğlu F, Çiftçi MK, Ateş MB: The usability of cytological and immunocytological methods for rapid diagnosis of encephalitic listeriosis in ruminants. *Kafkas Univ Vet Fak Derg,* 27 (2): 225-233, 2021.
DOI: 10.9775/kvfd.2020.25106

(*) **Corresponding Author**

Tel: +90 332 223 2734
E-mail: mehmetburakates@selcuk.edu.tr (M.B. Ateş)

Introduction

Listeriosis causes meningoencephalitis and abortus in adult animals and septicemia characterized by milier visceral abscesses in youngs, while rarely can also cause mastitis, purulent conjunctivitis and keratitis in cattle. The causative agent of the disease, *Listeria monocytogenes* is also an important foodborne pathogen and infection source for humans [1-3]. *L. monocytogenes* is easily grown in contaminated water, straw, grass, beet pulp, wet feed, and especially in silages and then taken by animals. The disease sometimes causes important epidemics and economic losses [1].

Encephalitic listeriosis is an endemic problem which is quite common in sheep, cattle and goats worldwide. In encephalitic listeriosis, the causative agent usually enters from the oral mucosal wounds, and comes to the trigeminal ganglion via the sensory axons and then arrives to the medulla oblongata [4,5]. In addition, it sometimes affects the cervical part of the medulla spinalis and thalamus, and causes encephalitis [6]. In encephalitic listeriosis, purulent encephalitis develops and meningitis occurs secondarily [7].

Macroscopically, there is usually no lesion in listeriosis, but sometimes leptomeningeal opacity and gray-white melting foci with a few mm diameter can be seen on the cross-sectional area of medulla oblongata [7,8]. Typical microscopic lesion of the disease is the microabscess where the melting areas are present in the brainstem (in pons and medulla oblongata). These microabscesses may be in the form of intense neutrophil leukocytes and macrophage infiltrations, or may be formed due to microglial reaction. In addition, white matter close to purulent foci has vasculitis and fibrin exudation. Perivascular inflammatory cell infiltration (perivascular cuffing) is severe. These include lymphocytes, histiocytes, plasma cells, and few neutrophils and eosinophil leukocytes [5,9].

Clinical findings, necropsy and histopathological examinations in ruminants cause to suspect encephalitic listeriosis, but there is a need to demonstrate the agent by bacteriological culture, PCR or immunohistochemical methods (IHC) for the definitive diagnosis of the disease [10,11]. These specific methods for the presence of the agent take time and require a specific workforce. Previously, the relationship between neutrophil granulocyte presence in cytology and microabscess formation was investigated for the prediagnosis of listeriosis, and it was reported that 90% of microabscess formation was observed in cases with intense neutrophils in cytology. However, no examination was performed to determine the agent in that study [10].

With this study, it was aimed to perform cytological and immunocytological examination of the touching and smear imprint preparations taken from the pons and medulla oblongata region in ruminants. Then, routine histopathological examination of the same regions was carried out and also the presence of the agent was investigated using IHC method. The findings were compared with cytological and immunocytological results. Thus, it was aimed to determine the usabilty of imprint cytology (IC) and immunocytochemistry (ICC) as a new method in the rapid diagnosis of listeriosis.

Material and Methods

In the study, 25 ruminants (1 cow, 1 calf, 11 sheep, 9 lambs, 1 goat and 2 kids), which have neural symptoms such as depression, leaning or lateral bending of the head, unilateral facial paralysis and suspected of encephalitic listeriosis in clinical examination, then sent for necropsy to our laboratory, were used. Each animal was originated from different herds located in Konya province and its districts. After systemic necropsies were performed routinely, the skulls were opened, firstly imprint cytological specimens were taken from the cut surface of the brainstem (pons and medulla oblongata) using the touching and/or scraping method and then tissue samples were taken for histopathological and IHC studies. For the study, ethics committee approval was obtained with the decision no. 2015/48 of the Selçuk University Veterinary Faculty Experimental Animals Production and Research Center Ethics Committee.

Imprint Cytological Examination

After the touching and smear preparations of pons and medulla oblongata region were air dried, they were stained with a rapid Modified Giemsa Technique [12]. For this purpose, 0.5 mL of Giemsa (Merck) solution was dropped on the imprint cytological preparations fixed in 96% alcohol for one minute. Tap water was added until it completely covered the preparation and it was stirred gently with a pipette and washed in the tap water after being stained for 2 - 3 min. The underside of the slides was dried on blotter paper and the wet top side was covered with coverslip and examined in terms of neutrophil content under a light microscope (Olympus BX 51, Tokyo, Japan). The averages of neutrophil numbers in 10 different areas in each specimen were accounted using 40× objective (×400 magnification) and results were scored as shown in *Table 1*.

Immunocytochemical Examination

After air drying, touching and smear preparations taken from the pons and medulla oblongata region were fixed in 96% alcohol for 10 min and washed in distilled water for 5 min. They were then stained according to the NovoLink™ Max Polymer Detection System (RE7280-K, Leica, Buffalo Grove,United States) kit procedure. Rabbit polyclonal Anti-*Listeria monocytogenes* antibody (ab35132, 1:100, one hour at room temperature, Abcam, Cambridge, United Kingdom) was used as primary antibody and 3,3'-diaminobenzidine (DAB) were used as chromogen. In negative controls, phosphate buffer saline (PBS) was

Table 1. *Scoring methods of cytological, histopathological, IHC and ICC results*

Scores	Cytology (× 400)	Microabscess	Perivascular Cuffing	IHC Staining (× 400)	ICC Staining (× 400)
Mild (+1)	1-2 neutrophil granulocytes	One small microabscess	1-2 layers of cells	Staining in 1-10 cells	Positivity in 1-3 cells or areas
Moderate (+2)	3-5 neutrophil granulocytes	Several small microabscesses	3-4 layers of cells	Staining in 11-20 cells	Positivity in 4-8 cells or areas
Severe (+3)	More than 6 neutrophil granulocyte	Medium-sized and some of them adjoining abscesses	5-6 layers of cells	Staining in more than 20 cells	Positivity in more than 9 cells or areas
Very severe (+4)	No scoring	Numerous and large micro-abscesses in the parenchyma	More than 6 layers of cells	No scoring	No scoring

used instead of primer antibody. All stained sections were examined under a light microscope (Olympus BX 51) and cytoplasmic brown staining was considered positive. Positivity scoring was calculated according to the number of stained cells in the whole slide area at ×40 objective magnification *(Table 1)*.

Histopathological Examination

After the cytological specimens were taken into the slides, tissue samples taken from especially the brain stem (pons and medulla oblongata), cerebral cortex and cerebellum longitudinally and horizontally were fixed in 10% formaldehyde solution for one day and then routine tissue processing procedures were performed. Five-micron thick sections were taken from the paraffin blocks and stained with Hematoxylin-Eosin (H&E). The changes observed in histopathological examinations were scored as shown in *Table 1*, similar to those performed by Oevermann, Di Palma [5].

Immunohistochemical Staining

The samples were cut to five microns thick and taken to polylisine slides. After deparaffinized in xyloles and rehydrated in graded alcohols, slides were stained according to NovoLinkTM Max Polymer Detection System (RE7280-K, Leica, Buffalo Grove, United States) kit procedure. Proteinase K was used in the antigen retrieval process, Rabbit polyclonal Anti-*Listeria monocytogenes* antibody was used as primary antibody (ab35132, 1:100, one hour at room temperature, Abcam, Cambridge, United Kingdom) and DAB was used as chromogen. In negative controls, PBS was used instead of primer antibody. All stained sections were examined in a light microscope (Olympus BX 51) and scored as shown in *Table 1* according to the number of cells stained positively at ×40 objective magnification.

Statistical Analysis

IHC and ICC staining results were compared using t test. IC, ICC, histopathological findings and IHC staining scores were compared using Pearson Correlations test (SPSS 13.0 for Windows/SPSS® Inc., Chicago, USA). Results were interpreted according to $P<0.05$ and r^2 values.

Results

Macroscopic Results

Macroscopically, there was no significant finding other than hyperemia in the meninges. Although a large melting and bleeding area was seen in the brainstem of only one sheep, this case was found to be negative for listeriosis in IHC staining.

Microscopic Results

Histopathological lesions, IC and IHC scores and ICC staining results are summarized in *Table 2*.

Histopathological examinations revealed microabscesses *(Fig. 1-A,C)* at different scores in the brainstem of 13 cases, and perivascular inflammatory cell infiltrations including neutrophil granulocytes were observed in 16 cases *(Fig. 1-C,D)*. In these microabscesses, there were few or more neutrophil granulocyte infiltrations according to the scores and glia cells, and sometimes necrotic neurons in the middle of them. In some areas, the microabscess foci were longitudinally extending along the axons. Besides, coenurosis, purulent meningoencephalitis and malacia were detected in 3 other lambs with perivascular inflammatory cell infiltration but not microabscess. In the lamb with purulent meningoencephalitis, severe purulent meningitis was seen and more widespread neutrophil granulocyte infiltrations, which were not typical micro-abscess-shaped, were noticed in brain tissue.

The definitive diagnosis of listeriosis was performed by IHC method in the study. According to this, of the 25 cases, 13 (52%) were found to be positive for *L. monocytogenes* antigen. Positive staining was observed in microabscesses at the brain stem, usually in the cytoplasm of neutrophils and glia cells *(Fig. 2-A,D)*. In addition, in some perivascular areas near the microabscess, positive staining was observed in the cytoplasm of few neutrophil granulocytes among the mononuclear cells *(Fig. 2-D)*.

In the examination of imprint cytological brainstem preparations prepared during necropsy, intensive neutrophils (+ 2 and + 3 scores = mean 3 - 5 or more) were observed in 14 patients *(Fig. 3-A,C)*, and in 13 of them, purulent

Table 2. *Distribution of cytological, histopathological, immunohistochemical and immunocytochemical findings in animal species*

Findings		Scores	Animals						
			Calf (n:1)	Cow (n:1)	Sheep (n:11)	Lamb (n:9)	Goat (n:1)	Kid (n:2)	TOTAL (n:25)
Histopathological findings	Microabscess scores*	0	1	1	1	8	-	1	12
		+1	-	-	3	-	-	-	13
		+2	-	-	-	-	-	-	
		+3	-	-	2	-	1	1	
		+4	-	-	5	1	-	-	
	Perivascular cuffing scores **	0	1	1	1	5	-	1	9
		+1	-	-	-	2	-	-	16
		+2	-	-	-	-	-	-	
		+3	-	-	2	-	-	-	
		+4	-	-	8	2	1	1	
	IHC staining scores ***	0	1	1	1	8	-	1	12
		+1	-	-	1	-	1	-	13
		+2	-	-	1	-	-	1	
		+3	-	-	8	1	-	-	
Cytological/immuno-cytological findings	Neutrophil scores ****	0	-	-	1	5	-	1	7
		+1	1	1	-	2	-	-	18
		+2	-	-	3	-	-	-	
		+3	-	-	7	2	1	1	
	ICC staining scores *****	0	1	1	2	8	-	1	13
		+1	-	-	1	-	1	1	12
		+2	-	-	2	-	-	-	
		+3	-	-	6	1	-	-	

Scores: ** 0 = no lesions, +1 = one small microabscess, +2 = several small microabscesses, +3 = Medium-sized and some of them adjoining microabscesses, +4 = Numerous and large microabscesses in the parenchyma; ** 0 = no lesions, +1 = Perivascular cell infiltration in 1-2 layers, +2 = Perivascular cell infiltration in 3-4 layers, +3 = Perivascular cell infiltration in 5-6 layers, +4 = Perivascular cell infiltration forming more than 6 layers; *** 0 = No staining, +1 = positive staining in 1-10 cells, +2 = positive staining in 10-20 cells, +3 = positive staining in more than 20 cells; **** 0 = No neutrophil, +1 = 1-2 neutrophils (mild), +2 = 3-5 neutrophils (moderate), +3 = 6 and more neutrophils (severe); ***** 0 = No staining, +1 = positivity in 1-3 cells or areas, +2 = positivity in 4-8 cells or areas, +3 = positivity in more than 9 cells or areas*

encephalitis characterized by microabscess and perivascular cell infiltration were identified in histopathological examinations. In IHC staining of these 14 cases, listeria positivity in accordance with microabscess was found in 13 (92.8%) of them. In one lamb with neutrophils at +3 score in the imprint cytology, there was no IHC or ICC positive staining and typical microabscess formation, but there was severe purulent meningoencephalitis. No microbscess formation and IHC positive staining were observed in none of the cases with 1 - 2 neutrophils (+ 1 score, 4 cases, *Fig. 1-A)* or no neutrophils (7 cases) in IC examination.

In the study, 12 cases (48% of total number of animals) were positive in ICC staining of brainstem tissue imprint preparations. Based on the IHC method in this study (13 cases), a positivity of 92.3% was determined in ICC. ICC scoring was similar to that of IHC. It was noted that positive staining was usually found in neutrophil granulocytes and in the cytoplasm of round or oval nucleated cells, which were considered to be glia cells, and have a diffuse-dense staining pattern that was sprinkled in granular or sometimes filled cytoplasm *(Fig. 4)*. While the cases showing strong positivity (+3 scoring) in the preparation can be evaluated more easily and quickly during the ICC examination, the entire preparation had to be screened laboriously and patiently in order to reach a final decision in negative cases.

On the basis of animal species, listeriosis was found in 10 of the 11 sheep and in only one of the 9 lambs, and only one positive case was found in goats and kids. One cattle and one calf were negative for listeriosis.

In this study, very high (r^2>0.8; P<0.01) positive correlation was determined between microabscess formation (13 cases), perivascular cuffing (16 cases), IHC (13 cases), intense neutrophils in imprint cytology (14 cases) and ICC (12 cases) positivity *(Table 3)*.

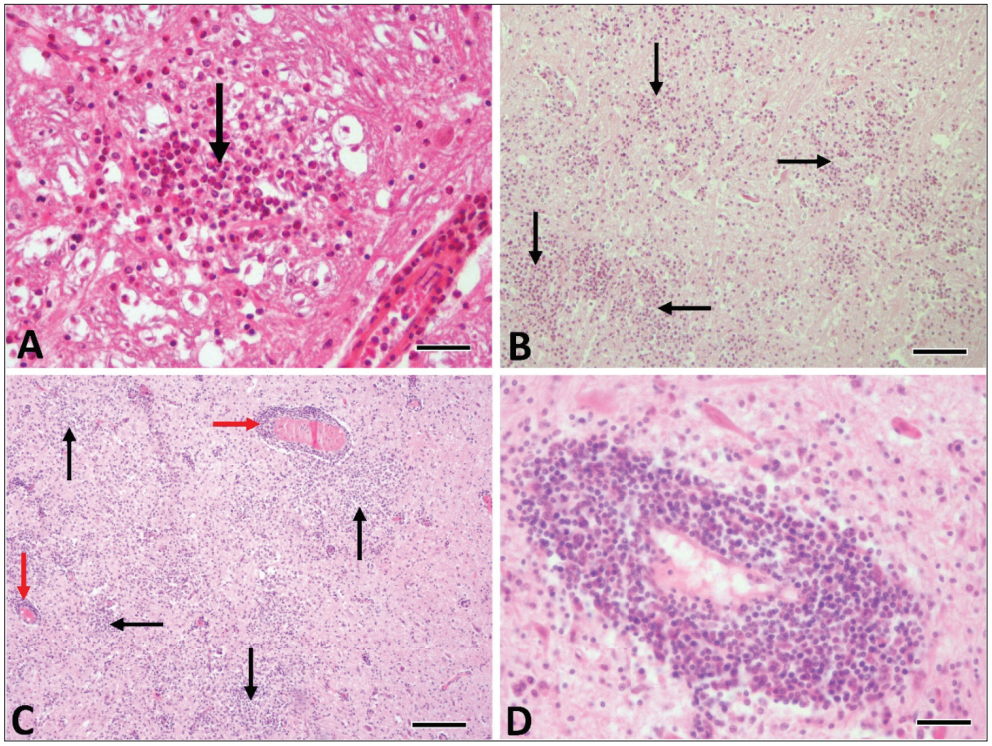


Fig 1. Microabscess and perivascular cell infiltrations in brain stem. **A.** Mild (+1) microabscess *(arrow),* sheep, **B.** Severe (+3) (Large, small and confluent) microabscesses *(arrows),* sheep, **C.** Perivascular cell infiltration (+3) *(red arrows)* and numerous, common microabscesses (+4) *(black arrows),* kid, **D.** Intensive perivascular cell infiltration (+4), sheep, H&E. Scala bar = 50 µm (A,D) and 100 µm (B) and 200 µm (C)

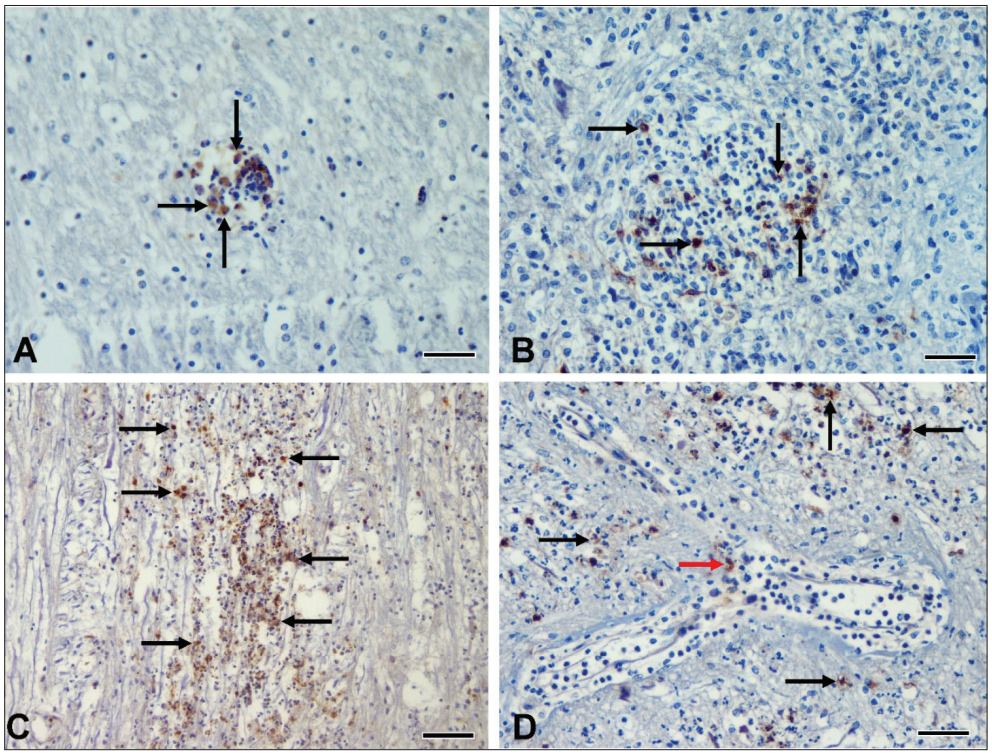


Fig 2. *L. monocytogenes* positive IHC staining in the cytoplasm of neutrophils and glia cells in the brain stem *(black arrows).* **A.** Mild (+1), lamb, **B.** Moderate (+2), kid, **C.** Severe (+3), microabscess along the longitudinal line (parallel to the axon length) in the brain stem, lamb, **D.** Positive staining in parenchyma (Severe (+3) and in perivascular cells in the area close to the microabscess *(red arrow),* sheep, IHC (DAB). Scala bar = 50 µm (A-B,D) and 100 µm (C)

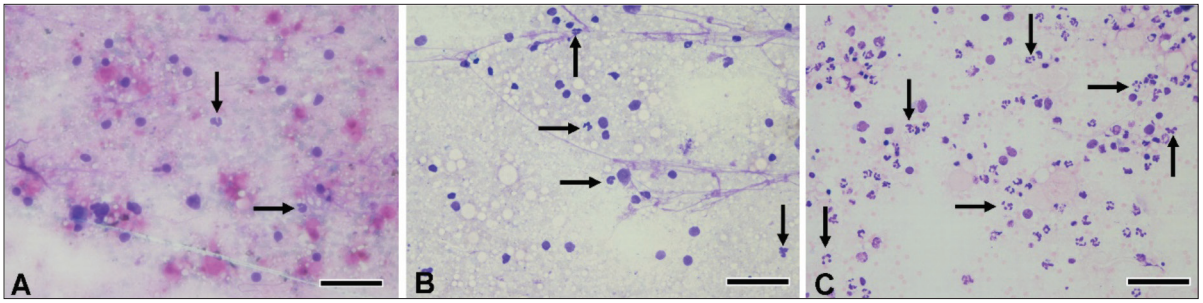


Fig 3. Neutrophil *(arrows)* density in brain stem cytology, sheep, Modified Giemsa, **A.** Mild (+ 1), **B.** Moderate (+ 2), **C.** Severe (+ 3). Scala bar = 20 µm (A-C)

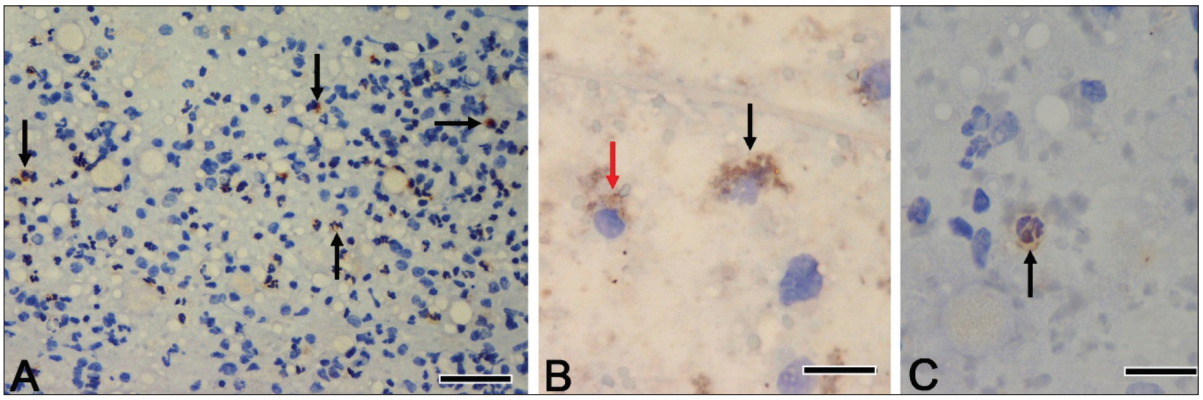


Fig 4. A-C. *L. monocytogenes* positive ICC staining in cytoplasm of neutrophil *(black arrow)* and glia cells *(red arrow),* **A-B:** kid, **C:** lamb, ICC (DAB). Scala bar = 50 µm (A) and 20 µm (B-C)

Table 3. *Degree of correlation between groups. Pearson Correlation Test*

Findings	Perivascular Cuffing	IHC	Neutrophil in Cytology	ICC
Microabscess	$r^2 = +0.90$ P<0.001	$r^2 = +0.97$ P<0.001	$r^2 = +0.90$ P<0.001	$r^2 = +0.92$ P<0.001
Perivascular cuffing		$r^2 = +0.92$ P<0.001	$r^2 = +0.96$ P<0.001	$r^2 = +0.94^*$ P<0.001
IHC			$r^2 = +0.92$ P<0.001	$r^2 = +0.94$ P<0.001
Neutrophil in cytology				$r^2 = +0.91$ P<0.001

Discussion

Listeriosis is not only an important problem in animal husbandry due to the fact that it causes widespread deaths in farm animals and is difficult to diagnose and treat, but also a very important foodborne infection for people. The disease occurs in single cases in cattle and as herd problems in sheep and goats [1,10,13]. It is stated that the disease is less common in goats than sheep and this may be due to the fact that goats are usually grown in mountainous areas and may have different dietary habits due to their free movements [14]. Similarly, it was attracted attention, in this study, that 11 of 13 cases diagnosed as encephalitic listeriosis were sheep while only 2 of them were goat.

The rate of listeriosis in ruminants clinically presenting neurological symptoms and suspected of listeriosis was 52% (13/25 cases) in this study. It is seen that this rate is 55% (11/20) for sheep plus lamb and 90.9% (10/11) for sheep only. Thus, as stated by other researchers [15,16], more than half of the sheep showing neurological symptoms in the winter and spring periods may be related to listeriosis. Therefore, it would not be wrong to evaluate this kind of events as listeriosis from the beginning to save time for treatment.

L. monocytogenes is considered to be the most effective neuroinvasive bacterium and it has been reported that it has the potential to overcome the blood-brain barrier or blood-placental barrier within infected leukocytes or free bacteria in the blood can enter the brain tissue by invading vascular endothelium directly [1,4,17]. Alternatively,

the bacterium reaches the brain through neuronal pathway with centripedal movement via axons [18,19]. In a neuropathogenesis study [5], the researchers reported that in most cases the agents reached the brain via axonal migration through 12th (hypoglossal), 7th (facial) and 5th (trigeminal) cranial nerves, and rarely the 3rd, 6th, 8th and 10th nerves. The same researchers [5] also showed that the infection spread to other regions axially within the brain, and that the microabscess and the active antigens were longitudinally aligned with the axon. Similarly, in this study, it was found that some microabscesses *(Fig. 2-C)* showed longitudinal alignment in parallel direction to axons, and there was significant degeneration in these axons. In addition, because it was determined that there was usually no IHC staining in perivascular areas, it was evaluated that as the researchers reported, the disease reached the brain through the nerves.

Typical neuropathological lesions in listeriosis are the microabscesses that are sometimes accompanied by melting areas in the brainstem. These microabscesses consist of different numbers of neutrophil granulocyte clumps and different amount of macrophage (microglia) infiltrations. Perivascular cell infiltrations containing lymphocytes, histiocytes, plasma cells, and few neutrophil and eosinophil leukocytes in the disease may be severe [1,5,9]. Oevermann, Di Palma [5] scored microabscess and perivascular cuffing in the disease and considered the microabscess in which neutrophils were dense as acute, the microabscess dominated by macrophages as chronic, and the cases where they were both together as subacute. In our study, microabscesses were observed in all of the cases with listeriosis (13 cases). According to the scoring, 3 of them had mild (+ 1; small, single microabscess, *Fig. 1-A),* and 10 of them had many wide-reaching microabscesses (scores + 3 and + 4, *Fig. 1-B,C)*. In all cases, intense presence of neutrophils were observed in microabscesses and were interpreted as acute according to the way the researchers' categorization [5]. As reported in the above literature, microabscesses are the most prominent and specific histopathological findings of encephalitic listeriosis. Severe perivascular inflammatory cell infiltrations were observed in 16 cases *(Fig. 1-C,D),* and 13 of them had listeriosis, and the other three were due to different inflammatory causes (purulent meningoencephalitis, malacia and coenurosis) and had no typical microabscess. Thus, it was seen that perivascular inflammatory cell infiltrations, including neutrophils, were significant in terms of demonstrating a purulent encephalitis but had no specific significance when they were not associated with microcabscesses.

Although the standard bacteriological culture method, which has been used for the definitive diagnosis of encephalitic listeriosis for years, is important in the isolation of the agent, it has negative aspects such as long duration of the method, the need for costly additional applications and the lack of growth in cases where antibiotics are used [5,20].

It is seen that IHC method has been used effectively in brain tissue in the etiological diagnosis of the disease in recent years and bacterial antigens can be detected although antibiotics have been used in animals [5,15,20-22]. In a study [23] listeria antigen was found positive in IHC staining method in 34 (80.9%) of 42 ruminants but determined 28.5% growth in culture. Campero, Odeon [21] reported that bacterial isolation could be performed in only 10 of IHC positive 17 cases with typical histopathological findings. Similarly, in another study the researchers [20] also reported positive results with IHC in patients with negative culture. As a result of these studies, it was emphasized that IHC was a faster and more specific technique in the diagnosis of encephalitic listeriosis in ruminants. In this study, *L. monocytogenes* positivity was found in IHC staining (52%) in 13 of 25 ruminants showing neurological symptoms and the IHC method was used as the base in confirmation of the diagnosis. It was observed that listeriosis was determined in all cases with typical microabscess (13 cases) and the result was fully compatible with IHC. Thus, it was considered that in cases where etiological diagnosis was not possible, a high rate diagnosis of listeriosis could be made by the detection of typical microabsscess histopathologically in the brain stem as observed in the study.

In IHC staining, generally no positive staining of the agent was observed in the perivascular areas and meninges, except few positive staining in some neutrophils in the perivascular areas, only adjacent to the microabscess or in very close *(Fig. 2-D)*. They were thought to be caused by transporting the neutrophils in the microabscess to the adjacent perivascular areas. In this case, it can be said that the agent reaching the brain by neuro-axonal way in accordance with the generally accepted pathogenesis of encephalitic listeriosis creates a lesion in the parenchyma first and then tends to spread towards the periphery and meninges.

A group of our researchers [10] previously researched the relationship between the presence of neutrophils in the brain stem imprint cytology and microabscess formation in suspected cases of listeriosis in sheep and cattle and they reported that neutrophil granulocytes were found in the cytological examinations in 9 of 10 cases with microabscess, and that the presence of neutrophil granulocytes in the cytology could be evaluated as "positive" in the preliminary diagnosis of encephalitic listeriosis. In this study, neutrophil granulocytes were found in 18 cases in the IC preparations prepared by touching and scraping method. While neutrophils were seen in 14 of them with significant and severe score (+2 and +3), few neutrophils were observed in 4 of them. Both IHC and ICC were found negative for listeriosis in cases with few neutrophils and no microabscess was observed in histopathological examination. In these cases, neutrophil granulocytes in the vessels associated with other septicemic diseases could be taken into cytological preparations during scraping/

rubbing and were therefore interpreted as being seen in cytology. IHC positivity was found in 13 out of 14 cases with intense neutrophil granulocytes (+2 and +3 intensity) and the rate of listeriosis compliance was determined as 92.8% with IC examination. In one case, intense (+3) neutrophil granulocytes were detected, but IHC and ICC stainings were negative. Severe purulent meningoencephalitis not associated with listeriosis was detected in this case and this infection was interpreted as the source of neutrophil granulocytes in imprint cytology.

Marco, Ramos [24] and Liu, van Kruiningen [25] each conducted a study on the diagnosis of listeriosis with immunocytochemical method, however these studies had been carried out in the brain tissues with the known IHC method not in cytological preparations, but this method was called "immunocytochemical" examination by the authors. Apart from this, a study truely carried out with ICC method in the cytological preparations is not present previously. Therefore, in this study conducted with ICC staining technic as a novel method, 92.3% (12 of 13 cases) positivity was determined when compared to IHC. ICC staining was not seen in only one case with listeria positive, but it was thought that this could be related to staining error or that possible slight positive staining may be overlooked in the laborious and sensitive evaluation process.

Despite the material number was low in the study, histopathological, IC, IHC and ICC values were not statistically different from each others (t test; $P>0.05$), and IC (92.8%) and ICC (92.3%) methods were considered to be an alternative in the rapid diagnosis of listeriosis. In addition, the presence of neutrophil in imprint cytology, microabscess formation, perivascular cuffing, and a high positive correlation ($r^2 >0.8$; $P<0.01$) between ICC and IHC positivity support this conclusion.

With ICC method, touching and/or scraping preparations taken from the brainstem can be stained after a few minutes of alcohol fixation and evaluated in the same day without any procedure requiring several days, such as tissue fixation, washing, paraffin embedding and sectioning-staining stages. It was seen that the cases showing intense positivity in IC and ICC examinations (+3 scoring cases) were more easily evaluated and the result could be reached easily. On the other hand, it was understood that all the preparation should be scanned and examined thoroughly in order to reach the final decision of negative cases and weakly stained or low scored (+1) cases since there was no tissue integrity in the imprint cytology and no lesion localization in a specific area. In addition, it was noticed that DAB staining precipitates and artifacts, which might be present in ICC preparations, could adversely affect the evaluation process and would cause erroneous results if attention is not paid. Therefore, the advantages of ICC staining were that it was easier and took shorter time than IHC, the disadvantages were that errors could be made frequently, positivity was more subjective than IHC and it required a patient microscopic examination. On the other hand, lesioned tissue fresh cytology may have some risks on the contamination of possibly living organisms, through the bench, microscope, and staining set, even for the alcohol fixed smear slides. Therefore, care should be taken to reduce the risk of contamination to clean areas and the staff while performing fresh tissue cytology during necropsy.

The availability of ICC method, which had a staining procedure in a much shorter time than culture and IHC methods, was investigated in this study, and it was concluded that immunocytology could be recommended as a fast, reliable and effective new method in the diagnosis of encephalitic listeriosis. In addition, it was suggested that the studies about the detection of the agent from CSF taken from live animals showing the clinical findings of encephalitic listeriosis could be performed with ICC method based on the idea that *Listeria monocytogenes* went through CSF due to lesions in the brain.

Conflict of Interest

The authors have no conflict of interest.

Authors Contributions

Ö. Özdemir, M. Ortatalı, F. Terzi, F. Hatipoğlu, M. K. Çiftçi, and M. B. Ateş made the experiment and the histological and immunohistochemical interpretation, and wrote the manuscript. Ö. Özdemir and M. Ortatatlı planned methodology. F. Terzi and M. B. Ateş investigated resources. Ö. Özdemir, F. Hatipoğlu, M. K. Çiftçi, M. Ortatatlı, F. Terzi, and M. B. Ateş wrote and review and editing the manuscript. All authors discussed the results and contributed to the final manuscript.

References

1. Cantile C, Youssef S: Nervous system. **In,** Maxie M (Ed): Jubb, Kennedy, and Palmer's Pathology of Domestic Animals 6th ed., 250-407, W.B. Saunders, Nottingham-UK, 2016.

2. Ren J, Yang M, Wang P, Jiang J, Yan G: A mutant of *Listeria monocytogenes* shows decreased virulence and confers protection against Listeriosis in mice. *Kafkas Univ Vet Fak Derg,* 26 (3): 419-426, 2020. DOI: 10.9775/kvfd.2019.23466

3. Fang X, Hu W, Zhang Y, Wang C, Luo Q, Wu H, Liang X, Gu Y, Fang C, Yang Y: Roles of histidine kinase gene *yycG* in the pathogenicity of *Listeria monocytogenes*. *Kafkas Univ Vet Fak Derg,* 26 (5): 679-686, 2020. DOI: 10.9775/kvfd.2020.24179

4. Castro H, Jaakkonen A, Hakkinen M, Korkeala H, Lindstrom M: Occurrence, persistence, and contamination routes of *Listeria monocytogenes* genotypes on three finnish dairy cattle farms: A longitudinal study. *Appl Environ Microbiol,* 84 (4): e02000-02017, 2018. DOI: 10.1128/AEM.02000-17

5. Oevermann A, Di Palma S, Doherr MG, Abril C, Zurbriggen A, Vandevelde M: Neuropathogenesis of naturally occurring encephalitis caused by *Listeria monocytogenes* in ruminants. *Brain Pathol,* 20 (2): 378-390, 2010. DOI: 10.1111/j.1750-3639.2009.00292.x

6. Zachary J: Nervous system. **In,** McGavin M, Zachary J (Eds): Pathological

Basis of Veterinary Disease. 4th ed., 833-972, Mosby Elsevier, Missouri, USA, 2016.

7. Çiftçi M, Hatipoğlu F: Sinir sistemi. **In,** Çiftçi M, Erer H (Eds): Veteriner Sistemik Patoloji. 4th. ed., 44-46, Nobel Tıp Kitapevi, Konya-Turkey, 2020.

8. Jaguezeski AM, Perin G, Rhoden LA, da Silva TMA, Mendes RE, Bottari NB, Baldissera MD, Morsch VM, Schetinger MRC, Stefani LM, da Silva AS: Changes on the activity of cholinesterase's in an immunomodulatory response of cattle infected by *Listeria monocytogenes*. *Microb Pathog,* 114, 36-40, 2018. DOI: 10.1016/j.micpath.2017.10.038

9. Dhama K, Karthik K, Tiwari R, Shabbir MZ, Barbuddhe S, Malik SV, Singh RK: Listeriosis in animals, its public health significance (food-borne zoonosis) and advances in diagnosis and control: a comprehensive review. *Vet Q,* 35 (4): 211-235, 2015. DOI: 10.1080/01652176.2015.1063023

10. Ozdemir O, Yavuz O, Ciftçi M, Sertkaya N, Hatipoglu F: Can brainstem cytology be used for a pre-diagnostic criter in encephalitic listeriosis? **In**, *VI National Veterinary Pathology Congress (International Participation)*, 19 - 23 September, Aydın, Turkey, 2012.

11. Siregar N, Purwati E, Aritonang S: Identification of listeriosis and potency of antimicrobial probiotics by *in vitro* test on beef cattle. *IOP Conf Ser: Earth Environ Sci,* 497 (1): 012050, 2020. DOI: 10.1088/1755-1315/497/1/012050

12. Ozdemir O, Terzi F, Ortatatli M, Hatipoğlu F, Tuna M, Çiftçi M, Yavuz O, Sayin Z: Cytological pre-diagnosis of anthrax: A trial of rapid modified giemsa method. **In,** *VII. National Veterinary Pathology Congress (International Participation),* 08-10 September, Kars, Turkey, 2014.

13. Dreyer M, Thomann A, Bottcher S, Frey J, Oevermann A: Outbreak investigation identifies a single *Listeria monocytogenes* strain in sheep with different clinical manifestations, soil and water. *Vet Microbiol,* 179 (1-2): 69-75, 2015. DOI: 10.1016/j.vetmic.2015.01.025

14. Kumar H, Singh BB, Bal MS, Kaur K, Singh R, Sidhu PK, Sandhu KS: Pathological and epidemiological investigations into listerial encephalitis in sheep. *Small Ruminant Res,* 71 (1-3): 293-297, 2007. DOI: 10.1016/j.smallrumres.2006.05.010

15. Oevermann A, Botteron C, Seuberlich T, Nicolier A, Friess M, Doherr MG, Heim D, Hilbe M, Zimmer K, Zurbriggen A, Vandevelde M: Neuropathological survey of fallen stock: Active surveillance reveals high prevalence of encephalitic listeriosis in small ruminants. *Vet Microbiol,* 130 (3-4): 320-329, 2008. DOI: 10.1016/j.vetmic.2008.01.015

16. Fairley RA, Pesavento PA, Clark RG: *Listeria monocytogenes* infection of the alimentary tract (enteric listeriosis) of sheep in New Zealand. *J Comp Pathol,* 146 (4): 308-313, 2012. DOI: 10.1016/j.jcpa.2011.08.004

17. Radoshevich L, Cossart P: *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. *Nat Rev Microbiol,* 16 (1): 32-46, 2018. DOI: 10.1038/nrmicro.2017.126

18. Henke D, Rupp S, Gaschen V, Stoffel MH, Frey J, Vandevelde M, Oevermann A: *Listeria monocytogenes* spreads within the brain by actin-based intra-axonal migration. *Infect Immun,* 83 (6): 2409-2419, 2015. DOI: 10.1128/iai.00316-15

19. Antal EA, Loberg EM, Bracht P, Melby KK, Maehlen J: Evidence for intraaxonal spread of *Listeria monocytogenes* from the periphery to the central nervous system. *Brain Pathol,* 11 (4): 432-438, 2001. DOI: 10.1111/j.1750-3639.2001.tb00411.x

20. Johnson GC, Fales WH, Maddox CW, Ramos-Vara JA: Evaluation of laboratory tests for confirming the diagnosis of encephalitic listeriosis in ruminants. *J Vet Diagn Invest,* 7 (2): 223-228, 1995. DOI: 10.1177/104063879500700210

21. Campero CM, Odeon AC, Cipolla AL, Moore DP, Poso MA, Odriozola E: Demonstration of *Listeria monocytogenes* by immunohistochemistry in formalin-fixed brain tissues from natural cases of ovine and bovine encephalitis. *J Vet Med B Infect Dis Vet Public Health,* 49 (8): 379-383, 2002. DOI: 10.1046/j.1439-0450.2002.00586.x

22. Dag S, Akca D, Karaman M, Celebi O, Ozen H, Buyuk F, Sahin M: Use of immunoperoxidase technique in smears prepared from vaginal secretions in early diagnosis of listerial abortions in cattle. *Kafkas Univ Vet Fak Derg,* 19 (1): 1-6, 2013. DOI: 10.9775/kvfd.2012.6358

23. Loeb E: Encephalitic listeriosis in ruminants: Immunohistochemistry as a diagnostic tool. *J Vet Med A,* 51 (9-10): 453-455, 2004. DOI: 10.1111/j.1439-0442.2004.00656.x

24. Marco A, Ramos JA, Dominguez L, Domingo M, Gonzalez L: Immunocytochemical detection of *Listeria monocytogenes* in tissue with the peroxidase-antiperoxidase technique. *Vet Pathol,* 25 (5): 385-387, 1988. DOI: 10.1177/030098588802500508

25. Liu Y, van Kruiningen HJ, West AB, Cartun RW, Cortot A, Colombel JF: Immunocytochemical evidence of *Listeria*, *Escherichia coli*, and *Streptococcus* antigens in Crohn's disease. *Gastroenterology,* 108 (5): 1396-1404, 1995. DOI: 10.1016/0016-5085(95)90687-8

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

Kafkas Univ Vet Fak Derg
27 (2): 235-242, 2021
DOI: 10.9775/kvfd.2020.25159

RESEARCH ARTICLE

Effects of Zinc Oxide Nanoparticles on the Expression of Zinc Transporter 1-4 Genes in the Hippocampus of Male Rats Under Acute Stress [1]

Maede NILECHI [1,a] Akram EIDI [1,b] Hamid GALEHDARI [2,c (*)] Mahnaz KESMATI [3,d]

[1] *This work was supported by genetic laboratory of the faculty of science and biotechnology research center of Shahid Chamran University of Ahvaz and Narges Genetic & PND Laboratory, Ahvaz, Iran*

[1] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, IRAN
[2] Department of Genetics, Faculty of Sciences, Shahid Chamran University of Ahvaz, 6135743337, Ahvaz - IRAN
[3] Department of Biology, Faculty of Sciences, Shahid Chamran University of Ahvaz, 6135743337, Ahvaz - IRAN
ORCIDs: [a] 0000-0002-6134-3248; [b] 0000-0002-6673-5347; [c] 0000-0001-6281-4809; [d] 0000-0002-0168-6441

Article ID: KVFD-2020-25159 Received: 15.11.2020 Accepted: 15.02.2021 Published Online: 16.02.2021

Abstract

Zinc transporters (ZnT) and ZIP proteins maintain Zinc homeostasis in the live organisms. On the other hand, the impacts of zinc oxide nanoparticles (ZnO NPs) on the expression of the *Znt* genes in biological systems were not clear yet. So that in this experimental study we have tried to find the effects of ZnO NPs on *Znt1-4* genes expression in the hippocampus of male rats under acute stress. Adult male rats were divided into groups of control and treated with 5 or 10 mg/kg of ZnO NPs alone and under acute restraint stress for 90 min. The changes in the expression of the selected genes were monitored using real-time qRT-PCR. The ZnT4 protein expression also was measured by Western blotting. Real-time qRT-PCR expression analysis revealed that the *Znt1* gene expression was up-regulated in the stress group, while the expression of the *Znt1* and *Znt4* genes was significantly up-regulated in the group receiving 10 mg/kg of ZnO NPs. Furthermore, in the ZnO NPs 10 mg/kg group under stress, the *Znt2* gene expression was down-regulated, while the *Znt4* gene expression was up-regulated. Moreover, the levels of ZnT4 protein were significantly increased after 10 mg/kg of ZnO NPs injection in the stress and normal groups. According to these results ZnO NPs administration can cause changes in the expression of a number of zinc transporter genes under stress conditions and increases the ZnT4 protein level. Therefore, this is a valuable approach for forecast investigation in biomedicine and pharmacogenetics studies.

Keywords: *Hippocampus, Nanoparticles, Rats, RNA, Zinc*

Akut Stres Altındaki Erkek Ratların Hipokampusundaki Çinko Taşıyıcı Genler 1-4'ün Ekspresyonu Üzerine Çinko Oksit Nanopartiküllerinin Etkileri

Öz

Canlı organizmalarda çinko homeostazını çinko taşıyıcılar (ZnT) ve ZIP proteinleri korur. Öte yandan, çinko oksit nanopartiküllerinin (ZnO NP) biyolojik sistemlerde *Znt* genlerinin ekspresyonu üzerine etkileri henüz tam olarak netlik kazanmamıştır. Bu nedenle, bu deneysel çalışmada, ZnO NP'lerin akut stres altındaki erkek ratların hipokampusundaki *Znt1-4* genlerinin ekspresyonu üzerine etkilerini araştırmaya çalıştık. Yetişkin erkek ratlar, kontrol, yalnızca 5 mg/kg ve 10 mg/kg ZnO NP uygulananlar ve 90 dk'lık kısıtlamaya bağlı oluşan stres süresince 5 mg/kg ve 10 mg/kg ZnO NP uygulananlar olmak üzere gruplara ayrıldı. İlgili genlerin ekspresyonlarındaki değişiklikler gerçek zamanlı qRT-PCR kullanılarak izlendi. ZnT4 protein ekspresyonu ayrıca Western Blot yöntemi ile ölçüldü. Gerçek zamanlı qRT-PCR ekspresyon analizi, stres uygulanan grupta *Znt1* gen ekspresyonunda bir artışın olduğunu, 10 mg/kg ZnO NP uygulanan grupta ise *Znt1* ve *Znt4* genlerinin ekspresyonunda önemli ölçüde bir artış olduğunu ortaya çıkardı. Ayrıca, stres altında 10 mg/kg ZnO NP uygulanan grupta, *Znt2* gen ekspresyonunda azalma saptanırken, *Znt4* gen ekspresyonunda artış belirlendi. Bunun haricinde, 10 mg/kg ZnO NP uygulanan stres ve normal gruplarda uygulamalardan sonra ZnT4 protein seviyeleri önemli ölçüde arttı. Bu sonuçlara göre, ZnO NP uygulaması, stres koşulları altında çinko taşıyıcı bazı genlerin ekspresyonunda değişikliklere neden olabilir ve ZnT4 protein seviyesini artırabilir. Bu nedenle, bu çalışma, biyotıp ve farmakogenetik çalışmaları tasarlamak için değerli bir yaklaşım sunmaktadır.

Anahtar sözcükler: *Hipokampus, Nanopartiküller, Rat, RNA, Çinko*

How to cite this article?

Nilechi M, Eidi A, Galehdari H, Kesmati M: Effects of zinc oxide nanoparticles on the expression of zinc transporter 1-4 genes in the hippocampus of male rats under acute stress. *Kafkas Univ Vet Fak Derg,* 27 (2): 235-242, 2021.
DOI: 10.9775/kvfd.2020.25159

(*) **Corresponding Author**

Tel: +61 33 333 1045
E-mail: galehdari@scu.ac.ir (H. Galehdari)

Introduction

Psychological stress (PS) is considered as the risk-increasing factors for the central nervous system (CNS) diseases. The stress-related problems can have detrimental effects on regulation of inflammatory and immune processes. The latter in turn is claimed to have the potentials to exacerbate depression, autoimmune, coronary artery disease with the possibility of developing some kinds of cancers [1]. Stressful environmental conditions can more often than not, affect zinc homeostasis and alter its spread throughout various body organs, particularly the brain [2].

Zinc (Zn) is considered as a vital micronutrient with several crucial catalytic and regulatory roles especially for efficient brain functioning [3]. This is because of the extracellular and intracellular zinc transport in the hippocampal mossy fibers during neutral activities [4]. Zinc deficiency on the other hand, is instrumental in bringing about irregular glucocorticoid secretion. This, in turn, is seen as a major contributory factor to the emergence of various types of neurobehavioral complications. These include certain cases of Alzheimer and related brain malfunctioning like epilepsy [5]. Endowed with its anti-anxiety, anti-depression, anti-inflammatory, anti-edematous and analgesic functionary, Zinc can be widely employed as a novel therapeutic intervention agent in order to deal with certain behavioral complications [6].

Zinc transport is differently regulated based on dietary zinc levels and physiological conditions through several zinc transporters in different tissues [7]. Results of clinical and experimental observations clearly relate any dysregulation in the zinc transporters expression to the development of cancer, asthma, diabetes, brain malfunctioning and mental illnesses, such as depression [8].

Zinc homeostasis maintenance in mammals is principally achieved by two types of proteins such as the ZIP (SLC39) and Zn transporters (ZnT). The ZIP proteins increase intracellular zinc by transporting extracellular zinc into the cells, while ZnT (SLC30) proteins transfer zinc out of the cytoplasm to the organelles or the extracellular matrix [9].

As the first member of ten proteins in the ZnT family, ZnT1 is located on the plasma membrane of various tissues like brain [10]. It exports cytosolic zinc ions to extracellular space. Under the circumstances of zinc deficiency, the amount of ZnT1 in brain decreases markedly. This is the case, bearing in mind that ZnT1 prevents zinc aggregation in cytoplasm under different physiological conditions [11]. ZnT2 is a component of intercellular acid vehicle. It is often expressed in intestine, kidneys and testis in rats, though not so often in brain [12]. ZnT3 plays a very vital role in several neurotransmitter signaling pathways in the hippocampus [13]. ZnT2 and ZnT3 proteins transport cytoplasmic zinc into vesicular components that in turn facilitate decreasing the intercellular zinc [3]. ZnT4 by nature ensures zinc homeostasis maintenance in various intercellular organelles of tissues like mammalian glands and brain. It facilitates zinc transport into trans-Golgi network. It is also instrumental in transferring Zn^{2+} ion into milk during lactation process [14].

The unique physiochemical nature of nanoparticles renders their universal application in biotechnological and medical science arena [15]. Nanoparticles of oxidized elements like zinc oxide (ZnO NPs) have also been employed in a wide spectrum of technological and medical purposes [16]. Research observation shows that MgO and ZnO nanoparticles treatment grows the glutamate level under stressful conditions. The ZnO NPs also increase zinc and Nr2a expression in rat's hippocampus [17]. The acute restraint stress method decreases zinc level in serum with a simultaneous increment in hippocampus [18].

While several studies have investigated the effects of ZnO NPs in mice and rats, the effects of ZnO NPs on the expression of zinc transporter genes in the hippocampus under restraint stress is an important and new goal. Therefore, in the current study, we evaluated the effects of ZnO NPs on the mRNA expression of the *Znt1*, *Znt2*, *Znt3,* and *Znt4* genes in the hippocampus of male rats under acute restraint stress and normal conditions. In addition, the ZnT4 protein was selected to determination its level in hippocampal tissue.

Material and Methods

Animals and Treatment Groups

This study was performed on thirty male Wistar rats (200±20 g) from the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz. The rats were kept separate with free access to water and food, except during the experiment. Room temperature (22-24°C) and a 12 h light/dark cycle were maintained throughout the study. Animals were randomly classified into six treatment groups, each containing five rats (N = 5) [19]. Animal groups were: control (Saline 0.9%), ZnO NPs (5 and 10 mg/kg), Saline + stress 90 min and ZnO NPs (5 and 10 mg/kg) + stress 90 min.

Experimental rats were raised in an environment that was in strict accordance with the "Guide for the Care and Use of Laboratory Animals". The use of these experimental animals was approved by the "Shahid Chamran University of Ahvaz (Approval No. EE/96.24.3.88369/SCU.AC.IR)".

Preparation of ZnO NPs Compound

The spherical morphology of ZnO NPs (US Nano Co., Texas, USA) was determined by scanning electron microscopic (SEM) images (Hitachi S4160., Co, Japan) and the particle sizes were ranged from 10 to 30 nm. ZnO NPs solution was prepared in an ultrasonic bath by sonication for 16 and shaking for 1 min before each injection. Two doses of

ZnO NPs suspension (5 and 10 mL/kg) were administered through intraperitoneal injection (IP), while the control group received 0.9% saline (1 mL/kg).

Acute Stress Induction

To induce acute stress, rats were placed in semicircular Plexiglas tubes (19.5 × 4.5 × 6 cm), where they were not able to move for 90 min. After stress induction, the rats received 5 or 10 mg/kg of ZnO NPs suspension or saline. Corticosterone hormone levels in serum were assessed to ensure induction of stress in rats.

Tissue Collection and Expression Analysis

Two hours after the ZnO NPs injection or stress induction, all rats were euthanized with ether, and the hippocampal tissue of their brains was excised. Trizol Reagent (Thermo Fisher Scientific, USA) was used to extract total RNA according to the manufacturer's protocol, and the extracted RNA was stored at -80°C. The RNA concentration of each sample was measured using a NanoDrop™ 2000/c spectrophotometer. In addition, RNA integrity was assessed using electrophoresis on a 1% agarose gel containing SafeStain (CinnaGen), and the 28 s, 18 s, and 5 s bands were observed. Extracted RNAs were treated using DNaseI (Takara Bio, Japan). Furthermore, the Primescript™ RT reagent kit (Takara Bio, Japan) was used for the reverse transcription of RNA to cDNA. A total of 1 µL of random hexamers (100 µM) and 1 µL of the oligo(dT) primer (50 µM) were added to 1 µg of DNase-treated RNA, and RNase-free water was added to a final volume of 5 µL, followed by incubation at 65°C for 5 min. Subsequently, 1 µL of the reverse transcriptase enzyme (1 U/µL) and 4 µL of 5X buffer were added, and RNase-free H_2O was added to a final volume of 20 µL, followed by incubation at 37°C for 25 min for cDNA synthesis, and incubation at 80°C for 5 s to deactivate the enzyme. Then, the cDNA was stored at -20°C. For real-time PCR, the following reagents were mixed as noted: 8 µL of SYBR Green I (Takara Bio, Japan), 1 µL each of forward and reverse primers, 2 µL of cDNA, and 3 µL of DNase-free water. Conventional real-time PCR was performed using the ABI 7900HT system. The thermocycling conditions were: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s; and a dissociation stage of 95°C for 15 s, 60°C for 60 s, and 95°C for 1 s. In this study, we used beta actin (act-β) as a housekeeping gene for normalizing the expression analysis. Primers for the act-β, *Znt1*, *Znt2*, *Znt3*, and *Znt4* genes in rats were designed by Gen Script online tool according to the cDNA sequences by Gene Bank *(Table 1)*. To validate the real-time qRT-PCR data, melt curves were plotted, and the accuracy of the curves was confirmed for each analyzed gene and primer dimer strands.

Table 1. *List of primers used in this study*

Gene ID	Gene Bank Accession Number	Sequence for Forward Primer (5'–3') and Reverse Primer (5'–3')
Act-β	[NM_031144.3]	F- TATCGGCAATGAGCGGTTCC
		R- AGCACTGTGTTGGCATAGAGG
Znt1	[NM_022853.2]	F- ACCAGGAGGAGACCAACAC
		R- CTCAACTTCTCTGGCTCTGC
Znt2	[NM_001083122.1]	F- GCACCTTCCTCTTCTCCATC
		R- GTAAGGCTTCCACACCATCC
Znt3	[XM_008764526.2]	F- TCAGCACCTTCCTCTTCTC
		R- GTGGTAAGTAAGCGTCAGC
Znt4	[NM_172066.1]	F-AGTCGTTGATGAAGATAGAAGATG
		R- CGAATGTGTTCAGCAAGAGG

ZnT4 Western Blotting

For western blot analysis, hippocampal tissue isolated from rats, after washing with cold PBS buffer PBS, in a micro-tubes with RIPA lysis buffer containing complete protease inhibitor cocktail (Santa Cruz, USA) homogenized. Then, complete lysis was performed for 1 h on ice bucket. Protein concentrations were determined using a Bradford assay. 20 µg of each tissue lysate was boiled for 10 min in a 2x sodium dodecyl sulfate sample buffer and run via 10% polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis proteins were transferred to Polyvinylidene Fluoride (PVDF) paper. The paper was incubated with first anti-ZnT4 monoclonal antibodies (orb227160) diluted 1:200 in PBS. Secondary antibody, goat anti-mouse IgG-HRP (Bio-Rad, USA) was used and diluted 1:1000 in PBS. Approximately 5% skim milk in Tris Buffered Saline plus 0.1% Tween-20 (TBST) was used as an antibody blocking and dilution buffer. Act-β (Bio-Rad, USA) was used as an endogenous reference to determine the relative density of ZnT4 protein in the sample. Finally, with the addition of chemiluminescence (Najm biotec, Iran) to the PVDF paper ZnT4 protein was detected. Digital image analysis was accomplished by importing the images into the image analysis software Image j.

Data Analysis

The data are expressed as mean ± standard error of the mean (S.E.M.), and the graphs are plotted using MS Excel. Student's t-test was used for the comparison of the unpaired data means using the Instat3 software application in real-time PCR and western blot data analysis. The results were considered as statistically significant when P-values were <0.05. Moreover, Relative Expression Software Tool (REST, version 2009) was used to detect alterations in the expression of the *Znt1*, *Znt2*, *Znt3,* and *Znt4* genes versus the act-β gene in different groups, and relative gene expressions were calculated as $2^{-\Delta\Delta ct}$. Gene expression analysis for the target and act-β genes was repeated three times for each sample. We prepared several dilutions of every cDNA before analysis, and the efficiency of the expression analysis was determined through standard curves.

Results

The Znt1, Znt2, Znt3, and Znt4 Expression in the Hippocampus Tissue

Our data showed a significant up-regulation in the *Znt1* gene expression in the group receiving restraint stress for 90 min and saline (P=0.0021), and the group receiving 10 mg/kg of ZnO NPs compared to the control group (P=0.0008). However, the results of this study did not show any significant difference in terms of the *Znt1* gene expression between the group receiving 5 mg/kg of ZnO NPs and the control group (P=0.6668), and between the group receiving 5 and 10 mg/kg of ZnO NPs with restraint stress compared to the group receiving restraint stress for 90 min and saline (P=0.1355 and P=0.4027, respectively) *(Fig. 1-A)*.

In the current study, there was a significant down-regulation in the *Znt2* gene expression in the group receiving 10 mg/kg of ZnO NPs with restraint stress compared to the group receiving restraint stress for 90 min and saline (P=0.0001). However, the *Znt2* gene expression did not differ significantly between the group receiving restraint stress for 90 min and saline (P=0.1803), the group receiving 5 and 10 mg/kg of ZnO NPs compared to the control group (P=0.2534 and P=0.1643, respectively), and the group receiving 5 mg/kg of ZnO NPs with restraint stress compared to the group receiving restraint stress for 90 min and saline (P=0.1296) *(Fig. 1-B)*.

In this study, there was not any significant change in the *Znt3* gene expression in different groups, including the group receiving restraint stress for 90 min and saline (P=0.1487), the group receiving 5 and 10 mg/kg of ZnO NPs compared to the control group (P=0.1892 and P=0.1106, respectively), and the group receiving 5 and 10 mg/kg of ZnO NPs with restraint stress compared to the group receiving restraint stress for 90 min and saline (P=0.0617 and P=0.0697, respectively) *(Fig. 1-C)*.

As seen in *Fig. 1-D*, there was a significant change in the *Znt4* gene expression in the group receiving 10 mg/kg of ZnO NPs with restraint stress compared to the group receiving restraint stress for 90 min and saline (P=0.0011). Moreover, the *Znt4* gene expression was significantly up-regulated in the group receiving 10 mg/kg of ZnO NPs compared to the control group (P=0.0005), while the *Znt4* gene expression did not differ significantly between the group receiving restraint stress for 90 min and saline (P=0.0773), the group receiving 5 mg/kg of ZnO NPs compared to the control group (P=0.0761), and the group receiving 5 mg/kg of ZnO NPs with restraint stress compared to the group receiving restraint stress for 90 min and saline (P=0.0605).

Western Blot Analysis

Since the *Znt4* gene expression up-regulated in hippocampus due to receiving 10 mg/kg of ZnO NPs with or

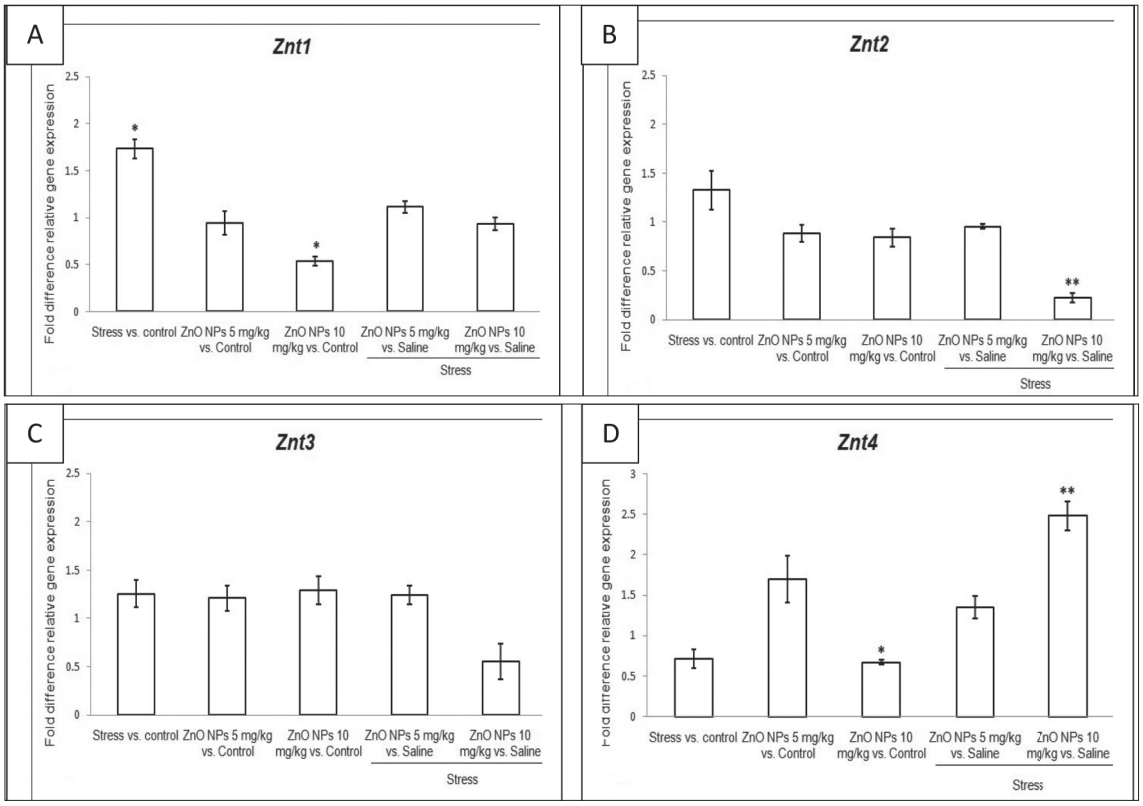


Fig 1. Fold-difference in relative expression of **A-** the *Znt1*, **B-** the *Znt2*, **C-** the *Znt3*, and **D-** the *Znt4* assessed by quantitative Real-Time reverse transcription-polymerase chain reaction, in the hippocampus tissue (* $P<0.05$ vs. the control group, ** $P<0.05$ vs. group receiving acute restraint stress for 90 min and saline). Bars represent mean ± S.E.M. of the genes relative expressions ($2^{-\Delta\Delta ct}$)

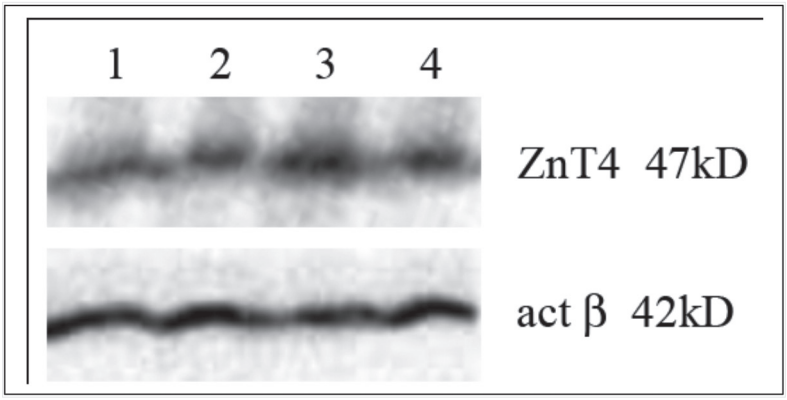


Fig 2. Representing western blot analysis of ZnT4 and act-β (as a control) in the hippocampal tissue of rats (1- The group receiving 10 mg/kg of ZnO NPs, 2- Control group, 3- The group receiving 10 mg/kg of ZnONPs under stress, 4- Stress group). Bands were semi-quantified by image j software and results showed the relative density of ZnT4 protein

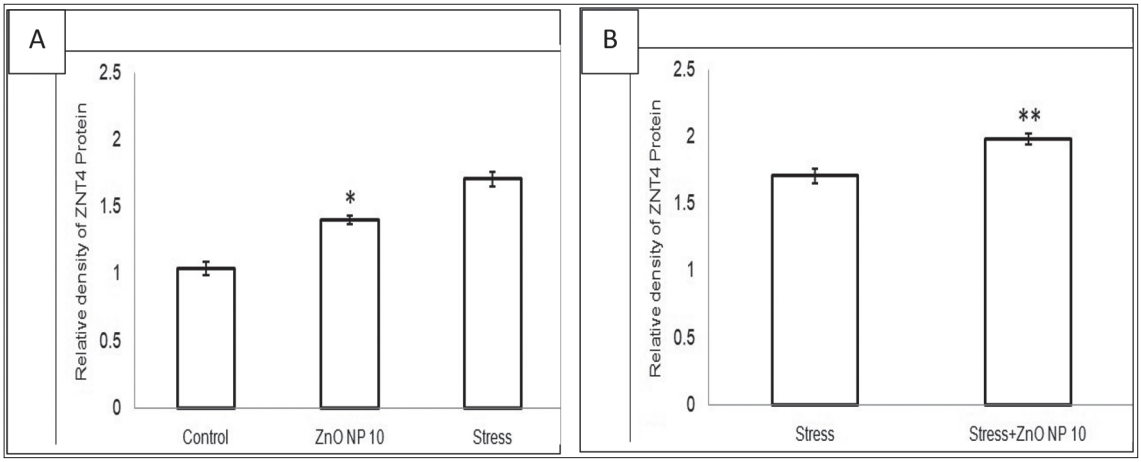


Fig 3. Western blot showing the relative density of ZnT4 protein in hippocampus of **A-** the control group, the group receiving 10 mg/kg of ZnO NPs, and the stress group **B-** the stress group and the group receiving 10 mg/kg of ZnO NPs under stress (* P<0.05 vs. control group, ** P<0.05 vs. group receiving acute restraint stress for 90 min and saline

without stress, the ZnT4 protein level was evaluated using Western blot analysis. The protein level was determined in hippocampal tissue via normalizing with act-β as a control *(Fig. 2)*. Quantitative analysis showed that, in the group receiving 10 mg/kg of ZnO NPs, the level of ZnT4 protein was significantly higher than in the control group (P=0.0004). The ZnT4 protein was significantly differenced in the group receiving 10 mg/kg of ZnO NPs under stress in comparison with the stress group (P=0.0003). Also, no significant change in the level of ZnT4 protein was observed in the stress group compared to the control group (P=0.2207) *(Fig. 3)*.

Discussion

The hippocampus has the highest amount of zinc in the brain and the central nervous system [20]. A great part of the nervous system in hippocampus is affected by stress, bearing in mind that acute stress is instrumental in prompting the zinc level in hippocampus [21,22]. Neurological complaints, depressions and addictions have shown to be stress-related problems [23,24]. The methodology employed in this paper involved deliberations on potential effects of acute restraint stress and ZnO NPs injection on expression of the *Znt1*, *Znt2*, *Znt3*, and *Znt4* genes in hippocampus of various male rats. Each of the latter was subjected to the injection of 5 or 10 mg/kg of ZnO NPs with or without restraint stresses. The effects of 10 mg/kg of ZnO NPs dosages with or without restraint stress on ZnT4 protein expression levels were further assessed.

It was found that the expression of *Znt1* was significantly up-regulated in the observation group subjected to restraint stress. According to the previous studies the acute restraint stress reduces the level of zinc in the serum, while increasing it in the hippocampus [18]. In addition, corticosterone can increase intracellular zinc levels in the hippocampus, causing the production of a type of inactive oxygen in the hippocampal cells [25]. The results are in congruence with the proven homeostasis role of the ZnT1 protein in zinc efflux from the intercellular space. This reportedly acts as an effective protection agent against potential zinc cytotoxicity of nervous system [26].

In this study, there were no significant statistical differences in the expression of studied genes in the different groups subjected to 5 mg/kg of ZnO NPs. However, the expression of *Znt1* and *Znt4* genes was significantly up-regulated in groups subjected to 10 mg/kg of ZnO NPs. Some previous

studies have indicated that ZnO NPs significantly enhances the expression of multiple zinc transporter genes like *Znt1*, *Znt2*, and *Znt4* in mice [17]. Also, it has been reported that zinc deficiency decreases ZnT1 expression in the hippocampus of rats [27]. Considering the role of the *ZnT1* protein in the extraction of cytosolic zinc into the extracellular space and the ZnT4 protein function in the transfer of zinc from the cytoplasm to the Golgi network [11], the increased genes expression in response to ZnO NPs is justified.

In this study, we could indicate that injection of ZnO NPs in the presence of acute restraint stress changed the expression of *Znt2* and *Znt4 in* the rat hippocampus. The ZnT2 protein is responsible for transporting cytosolic zinc to secretory granules and exocytosis [3]. Therefore, by reducing the expression of *Znt2*, the transfer of zinc from the cell is slowed down and zinc is accumulated in the intracellular organelles by the ZnT4 protein [14]. Our results showed that the mutual changes in expression of these two genes could be the cause of zinc homeostasis during stress induction and receiving of ZnO NPs. The close relationship between stress and zinc homeostasis disorder has been previously reported [2]. In conjunction with our results some studies indicated that administration of ZnO NPs under acute stress conditions produce positive effects on behavioral responses [28]. This may indicate the proper accumulation of zinc in the hippocampus by regulation of *Znt2* and *Znt4* genes expression.

In this study, for the first time, we could indicate that acute injection of ZnO NPs in the presence and absence of acute restraint stress could increase the expression of *Znt4* in the rat hippocampus.

Zinc homeostasis in the hippocampus differs from increased zinc levels due to stress or receiving ZnO NPs. During stress, up-regulation in *Znt1* gene expression causes zinc to be exported to the extracellular space. While receiving ZnO NPs, the expression of the *Znt4* gene increases to store the imported zinc in the organelles.

The *Znt3* gene expression showed no significant change among our different groups. Similarly, *ZnT3* mRNA expressions were not affected in the whole brain of rats during zinc deficiency [27]. It has been shown that the expression of the *Znt1*, *Znt2*, and *Znt4* genes was up-regulated, while the levels of *Znt3* mRNAs were unchanged in the cerebral cortex after transient ischemia [29]. More studies are needed to investigate the effects of different doses and treatment times of ZnO NPs supplementation on the *Znt3* gene expression.

In addition, the ZnT4 protein level was analyzed using Western blot because of up-regulation of the *Znt4* gene expression in hippocampus due to receiving 10 mg/kg of ZnO NPs with or without stress. Results have indicated that ZnO NPs significantly increased ZnT4 protein levels in rats with and without stress. Likewise, Zinc-containing imipramine has reportedly been instrumental in increasing ZnT4 protein level in prefrontal context of mice subjected to stress conditions [30]. It seems that the up-regulation of *Znt4* expression followed by an increase in ZnT4 protein levels is an important mechanism of zinc homeostasis in the hippocampus due to receiving zinc-containing drugs.

The advantage of using the form of ZnO NPs is determined by the fact that other types of zinc, such as the usual ZnO or zinc methionine, have not previously altered the expression of *Znt1*, *Znt2* and *Znt5* genes [31]. ZnT3 protein has been shown to play a protective role against oxidative stress and ER stress (Endoplasmic reticulumstr) in the body [32]. Studies show that mice in which the Znt3 gene has been knocked out are less able to adapt to chronic stress and exhibit anxious and depressive behaviors [33,34]. Sudden changes in zinc signaling, or in other words, sudden changes in zinc levels, induce a certain stress to the cell, which is called zinc stress (zinc stress) and can lead to dysfunction of cells, especially in the nervous system [35].

One of the causes of this problem (zinc stress) is psychological and behavioral stimuli such as psychological stress [36]. Regulating the expression of zinc transporter genes by receiving the appropriate dose of a drug containing zinc nanooxide after induction of stress can prevent dysfunction of the central nervous system. Because changes in the expression of zinc-transferring family genes vary during different psychological stimuli such as psychological stress, physical stress, anxiety, and depression; The study of these genes in various diseases and changes in their expression due to receiving different forms of the element zinc has been considered. On the other hand, the expression of the studied genes is different in different parts of the body due to the difference in the amount of zinc absorption in tissues, cells and intracellular organs [36]. Stress-induced centralization, as well as zinc-containing drugs, does not necessarily lead to a single result of comparing the expression changes of the studied genes with other tissues and cells. Our results in this study showed that, as in depression in rats, stress increased the expression of the *Znt1* gene and did not alter the expression of the *Znt2*, *Znt3* and *Znt4* genes, resulting in a loss of intracellular zinc storage. This can be the cause of stress-induced nervous system disorders due to lack of zinc [36] As a result of receiving zinc oxide nanoparticles under stress conditions, zinc homeostasis is in order to maintain intracellular zinc storage, which is done by altering the expression of *Znt2* and *Znt4* genes. *Znt4* gene expression is mediated by the uptake of zinc oxide nanoparticles under conditions with and without stress to increase cellular storage, which is confirmed by the presence of this protein [37].

Finally, we investigated for the first time the effects of zinc oxide nanoparticles on changes in the expression of four zinc transporter genes in the hippocampus of rats under stress and non-stress conditions, which revealed part of

the mechanism of zinc regulation. These findings can be valuable in the field of pharmacy and medicine. Evaluation results showed the application of ZnO NPs to be valuable in forecasting the necessary investigation in the fields of biomedicine and pharmacogenetics as substantiated by comparable recent researches. The present research showed ZnO NPs to be an effective catalyst to transform the expression of zinc transporter genes in hippocampus of rats subjected to stress and non-stress experimental contributions. Moreover, the zinc homeostasis in rat's hippocampus was shown to vary on its own under restraint stress experimental conditions.

Acknowledgments

The authors hereby express thank to the genetic laboratory of the faculty of science and biotechnology research center of Shahid Chamran University of Ahvaz and Narges Genetic & PND Laboratory, Ahvaz, Iran for technical assistance.

Author Contributions

MN, AD, planned and designed the research. HG and MK provided help in the experiment. All authors discussed the results and contributed to the final manuscript.

Ethical Principles and Publication Policy

All authors state that the paper presented contains the main results of the research and that the study data have been properly analyzed and prepared for publication using sufficient and appropriate sources.

Declaration of Conflict of Interest

None.

References

1. Chasapis CT, Ntoupa PSA, Spiliopoulou CA, Stefanidou ME: Recent aspects of the efects of zinc on human health. *Arch Toxicol*, 94 (5): 1443-1460, 2020. DOI: 10.1007/s00204-020-02702-9

2. Herman JP, Cullinan WE: Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci*, 20, 78-84, 1997. DOI: 10.1016/s0166-2236(96)10069-2

3. Gower-Winter SD, Levenson CW: Zinc in the central nervous system: From molecules to behavior. *Biofactors*, 38 (3): 186-193, 2012. DOI: 10.1002/biof.1012

4. Takeda A, Tamano H, Ogawa T, Takada S, Ando M, Oku N, Watanabe M: Significance of serum glucocorticoid and chelatable zinc in depression and cognition in zinc deficiency. *Behav Brain Res*, 226 (1): 259-264, 2012. DOI: 10.1016/j.bbr.2011.09.026

5. Dou X, Tian X, Zheng Y, Huang J, Shen Z, Li H, Wang X, Mo F, Wang W, Wang S, Shen H: Psychological stress induced hippocampus zinc dyshomeostasis and depression-like behavior in rats. *Behav Brain Res*, 273, 133-138, 2014. DOI: 10.1016/j.bbr.2014.07.040

6. Prasad AS: Impact of the discovery of human zinc deficiency on health. *J Am Coll Nutr*, 28, 257-265, 2009. DOI: 10.1080/07315724.2009.10719780

7. Song Y, Elias V, Wong CP, Scrimgeour AG, Ho E: Zinc transporter expression profiles in the rat prostate following alterations in dietary zinc. *Biometals*, 23 (1): 51-58, 2010. DOI: 10.1007/s10534-009-9266-8

8. Pfaender S, Föhr K, Lutz AK, Putz S, Achberger K, Linta L, Liebau S, Boeckers TM, Grabrucker AM: Cellular zinc homeostasis contributes to neuronal differentiation in human induced pluripotent stem Cells. *Neural Plast*, 2016:3760702, 2016. DOI: 10.1155/2016/3760702

9. Bin BH, Seo J, Kim ST: Function, structure, and transport aspects of ZIP and ZnT zinc transporters in immune cells. *J Immunol Res*, 2018:9365747, 2018. DOI: 10.1155/2018/9365747

10. Downey AM, Hales BF, Robaire B: Zinc transport differs in rat spermatogenic cell types and is affected by treatment with cyclophosphamide. *Biol Reprod*, 95 (1): 22, 2016. DOI: 10.1095/biolreprod.116.140558

11. Jobarteh ML, Mcardle HJ, Holtrop G, Sise EA, Prentice AM, Moor SE: mRNA levels of placental iron and zinc transporter genes are upregulated in gambian women with low iron and zinc status. *J Nutr*, 147, 1401-1409, 2017. DOI: 10.3945/jn.116.244780

12. Cousins RJ, Liuzzi JP, Lichten LA: Mammalian zinc transport, trafficking and signals. *J Biol Chem*, 281 (34): 24085-24089, 2006. DOI: 10.1074/jbc.R600011200

13. Lee JY, Kim JS, Byun HR, Palmiter RD, Koh JY. Dependence of the histofluorescently reactive zinc pool on zinc transporter-3 in the normal brain. *Brain Res*, 1418, 12-22, 2011. DOI: 10.1016/j.brainres.2011.08.055

14. McCormick NH, Kelleher SL: ZnT4 provides zinc to zinc-dependent proteins in the trans-Golgi network critical for cell function and Zn export in mammary epithelial cells. *Am J Physiol Cell Physiol*, 303 (3): C291-C297, 2012. DOI: 10.1152/ajpcell.00443.2011

15. Bahmani M: A new method for promoting biologic synthesis and reducing the size of titanium dioxide nanoparticles (Tio2 NPs) synthesized by *Origanum vulgare*. *Plant Biotechnol Persa*, 1 (1): 10-12, 2019. DOI: 10.29252/pbp.1.1.10

16. Scherzad A, Meyer T, Kleinsasser N, Hackenberg S: Molecular mechanisms of zinc oxide nanoparticle-induced genotoxicity. *Materials*, 10 (12): 1427, 2017. DOI: 10.3390/ma10121427

17. Wang C, Lu J, Zhou L, Li J, Xu J, Li W, Zhang L, Zhong X, Wang T: Effects of long-term exposure to zinc oxide nanoparticles on development, zinc metabolism and biodistribution of minerals (Zn, Fe, Cu, Mn) in mice. *PLoS One*, 11 (10): e0164434, 2016. DOI: 10.1371/journal.pone.0164434

18. Kozlowska K: A stress-system model for functional neurological symptoms. *J Neurol Sci*, 383, 151-152, 2017. DOI: 10.1016/j.jns.2017.10.044

19. Torabi M, Kesmati M, Galehdari H, Varzi HN, Pourreza N: MgO and ZnO nanoparticles anti-nociceptive effect modulated by glutamate level and NMDA receptor expression in the hippocampus of stressed and non-stressed rats. *Physiol Behav*, 214:112727, 2020. DOI: 10.1016/j.physbeh.2019.112727

20. Portbury SD, Adlard PA: Zinc signal in brain diseases. *Int J Mol Sci*, 18 (12): 2506, 2017. DOI: 10.3390/ijms18122506

21. Bafaro E, Liu Y, Xu Y, Dempski RE: The emerging role of zinc transporters in cellular homeostasis and cancer. *Signal Transduct Target Ther*, 2:17029, 2017. DOI: 10.1038/sigtrans.2017.29

22. Nuttall JR, Oteiza PI: Zinc and the aging brain. *Genes Nutr*, 9 (1): 379, 2014. DOI: 10.1007/s12263-013-0379-x

23. Olesen RH, Hyde TM, Kleinman JE, Smidt K, Rungby J, Larsen A: Obesity and age-related alterations in the gene expression of zinc-transporter proteins in the human brain. *Transl Psychiatry*, 6:e838, 2016. DOI: 10.1038/tp.2016.83

24. Cieślik K, Sowa-Kucma M, Ossowska G, Legutko B, Wolak M, Opoka W, Nowak G: Chronic unpredictable stress-induced reduction in the hippocampal brain-derived neurotrophic factor (BDNF) gene expression is antagonized by zinc treatment. *Pharmacol Rep*, 63 (2): 537-543, 2011. DOI: 10.1016/s1734-1140(11)70520-5

25. Zheng Y, Huang J, Tao L, Shen Z, Li H, Mo F, Wang X, Wang S, Shen H: Corticosterone increases intracellular Zn^{2+} release in hippocampal HT-22 cells. *Neurosci Lett*, 588, 172-177, 2015. DOI: 10.1016/j.neulet.2015.01.016

26. Lehvy AI, Horev G, Golan Y, Glaser F, Shammai Y, Assaraf YG: Alterations in ZnT1 expression and function lead to impaired intracellular zinc homeostasis in cancer. *Cell Death Discov*, 5:144, 2019. DOI: 10.1038/s41420-019-0224-0

27. Chowanadisai W, Kelleher SL, Lönnerdal B: Zinc deficiency is

associated with increased brain zinc import and LIV-1 expression and decreased ZnT-1 expression in neonatal rats. *J Nutr*, 135 (5): 1002-1007, 2005. DOI: 10.1093/jn/135.5.1002

28. Torabi M, Kesmati M, Pourreza N, Varzi HN, Galehdari H: Neurobehavioral and biochemical modulation following administration of MgO and ZnO nanoparticles in the presence and absence of acute stress. *Life Sci*, 203, 72-82, 2018. DOI: 10.1016/j.lfs.2018.04.023

29. Aguilar-Alonso P, Martinez-Fong D, Pazos-Salazar NG, Brambila E, Gonzalez-Barrios JA, Mejorada A, Flores G, Millan-Perezpeña L, Rubio H, Leon-Chavez BA: The increase in zinc levels and upregulation of zinc transporters are mediated by nitric oxide in the cerebral cortex after transient ischemia in the rat. *Brain Res*, 1200, 89-98, 2008. DOI: 10.1016/j.brainres.2007.11.077

30. Rafało-Ulinska A, Poleszak E, Szopa A, Serefko A, Rogowska M, Sowa I, Wojciak M, Muszynska B, Krakowska A, Gdula-Argasinska J, Kala K, Jasiewicz B, Opoka W, Szewczyk B, Nowak G: Imipramine influences body distribution of supplemental zinc which may enhance antidepressant action. *Nutrients*, 12 (9): 2529, 2020. DOI: 10.3390/nu12092529

31. Ma F, Wo Y, Li H, Chang M, Wei J, Zhao S, Sun P: Effect of the source of zinc on the tissue accumulation of zinc and jejunal mucosal zinc transporter expression in holstein dairy calves. *Animals*, 10 (8): 1246, 2020. DOI: 10.3390/ani10081246

32. Turan B: A brief overview from the physiological and detrimental roles of zinc homeostasis via zinc transporters in the heart. *Biol Trace Elem Res*, 188 (1): 160-176, 2019. DOI: 10.1007/s12011-018-1464-1

33. McAllister BB, Dyck RH: Zinc transporter 3 (ZnT3) and vesicular zinc in central nervous system function. *Neurosci Biobehav Rev*, 80, 329-350, 2017. DOI: 10.1016/j.neubiorev.2017.06.006

34. McAllister BB, Pochakom A, Fu S, Dyck RH: Effects of social defeat stress and fluoxetine treatment on neurogenesis and behavior in mice that lack zinc transporter 3 (ZnT3) and vesicular zinc. *Hippocampus*, 30 (6): 623-637, 2020. DOI: 10.1002/hipo.23185

35. Fukada T, Bin BH, Hara T, Takagishi T, Yoshigai E, Lian X: Zinc transporters in physiology and pathophysiology. **In,** Brewer GJ, Prasad AS (Eds): Essential and Toxic Trace Elements and Vitamins in Human Health, 55-67, Academic Press, 2020.

36. Rafalo A, Zadrozna M, Nowak B, Kotarska K, Wiatrowska K, Pochwat B, Sowa-Kucma M, Misztak P, Nowak G, Szewczyk B: The level of the zinc homeostasis regulating proteins in the brain of rats subjected to olfactory bulbectomy model of depression. *Prog Neuropsychopharmacol Biol Psychiatry,*72, 36-48, 2017. DOI: 10.1016/j.pnpbp.2016.08.009

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

Kafkas Univ Vet Fak Derg
27 (2): 243-251, 2021
DOI: 10.9775/kvfd.2020.25033

PRELIMINARY REPORT

High-throughput Sequencing Analysis of miRNA Expression in Embryonic Chicken Breast Muscle

Lei ZHANG [1,a] Song REN [1,b] Herong LIAO [1,c] Jie SUN [1,d (*)]

[1] College of Animal Science and Technology, Shihezi University, Shihezi, 832000, CHINA
ORCIDs: [a] 0000-0001-5378-1179; [b] 0000-0003-0914-0141; [c] 0000-0001-8174-3989; [d] 0000-0003-2607-4991

Article ID: KVFD-2020-25033 Received: 03.10.2020 Accepted: 22.02.2021 Published Online: 23.02.2021

Abstract

The embryonic period is a critical period for the development of muscle and adipose tissue in chickens. MicroRNAs (miRNAs) have been shown to play important roles in various biological processes, but little is known about miRNAs in chicken embryos. To investigate functional miRNAs regulating the meat quality of adult chickens, embryonic breast muscle tissues of Cobb broilers (CB) and rose-crowned chickens (RC) were collected and analyzed with high-throughput small RNA deep sequencing. The results showed that 842 known miRNAs and 598 novel miRNAs were identified from the four sequencing libraries, of which 592 were differentially expressed (DE) miRNAs ($P<0.05$). Taking Cobb broilers as a reference, 321 miRNAs were upregulated, and 271 miRNAs were downregulated. Real-time PCR confirmed that the trends in differentially expressed miRNAs were completely consistent with the sequencing results. We identified 120,666 target genes of the differentially expressed miRNAs, and functional enrichment analysis revealed that these genes were mainly involved in the Wnt signaling pathway, Adherens junction, Butirosin and neomycin biosynthesis and the Hedgehog signaling pathway. Furthermore, regulatory networks of interactions among miRNAs and their targets were constructed, and fatty acid binding protein 5 *(FABP5)* was confirmed as a target of miR-3532-5p by dual-luciferase assay. The results of this study enriched the relevant data on chicken muscle miRNA expression, and they laid a foundation for further analysis of the miRNA-mediated regulation of genes and the link between miRNA and chicken meat quality traits.

Keywords: *Cobb broilers, Rose-crowned chicken, miRNA, Meat quality, Embryo, High-throughput sequencing*

Embriyonik Tavuk Göğüs Kasında miRNA Ekspresyonunun Yüksek Verimli Dizi Analizi

Öz

Embriyonik dönem, tavuklarda kas ve yağ dokusunun gelişimi için kritik bir dönemdir. MikroRNA (miRNAs)'ların çeşitli biyolojik süreçlerde önemli roller üstlendikleri gösterilmiştir, fakat tavuk embriyolarında miRNA'lar hakkında çok az şey bilinmektedir. Yetişkin tavuklarda et kalitesini düzenleyen işlevsel miRNA'ları araştırmak için Cobb broylerlerinin (CB) ve gül taçlı tavukların (RC) embriyonik göğüs kas dokuları toplandı ve yüksek verimli küçük RNA dizi analizleri gerçekleştirildi. Sonuçlar, 592'si farklı olarak eksprese olan (DE) miRNA'lar ($P<0.05$) olmak üzere 842 bilinen miRNA ve 598 yeni miRNA'nın dört sekans kütüphanesinden tanımlandığını gösterdi. Cobb broylerleri referans alındığında, 321 miRNA'nın ekspresyonu artmış ve 271 miRNA'nın ekspresyonu azalmıştır. Real-time PCR analizi, farklı olarak eksprese olan miRNA'lardaki eğilimlerin dizileme sonuçlarıyla tamamen tutarlı olduğunu doğruladı. Farklı olarak eksprese olan miRNA'ların 120,666 hedef genini belirledik ve fonksiyonel zenginleştirme analizi, bu genlerin esas olarak Wnt sinyal yolağı, Adherens bağlantısı, Butirosin biyosentezi, neomisin biyosentezi ve Hedgehog sinyal yolağında yer aldığını ortaya çıkardı. Ayrıca, miRNA'lar ve hedefleri arasında düzenleyici etkileşim ağları oluşturuldu ve ikili lusiferaz analizi ile yağ asidi bağlayıcı protein 5 (FABP5), miR-3532-5p'nin bir hedefi olarak doğrulandı. Bu çalışmaya ait bulgular, tavuk kası miRNA ekspresyonu ile ilgili verileri zenginleştirdi ve genlerin miRNA aracılı regülasyonunun ve miRNA ile tavuk eti kalite özellikleri arasındaki bağlantının daha fazla analizi için bir temel oluşturdu.

Anahtar sözcükler: *Cobb broyleri, Gül taçlı tavuk, miRNA, Et kalitesi, Embriyo, Yüksek verimli dizi analizi*

INTRODUCTION

Poultry meat is one of the edible meat proteins accepted by consumers all over the world. With the continuous improvement of people's living standards, the demand for poultry products has gradually changed from an increase in quantity to an increase in quality. Cobb broilers (CB) is a special-purpose commercial matching variety introduced,

How to cite this article?

Zhang L, Ren S, Liao HR, Sun J: High-throughput sequencing analysis of miRNA expression in embryonic chicken breast muscle. *Kafkas Univ Vet Fak Derg,* 27 (2): 243-251, 2021.
DOI: 10.9775/kvfd.2020.25033

(*) Corresponding Author

Tel: +86 135 79742370
E-mail: sunjie_shzu@126.com (J. Sun)

which has the advantages of fast growth rate, high feed conversion rate and high slaughter rate. The Rose-crowned chicken (RC) is a Chinese native chicken breed with good meat quality and unique flavor. Previous studies in our lab found that there are significant differences in the growth rate and muscle quality between the two chicken breeds, and the content of inosinic acid and amino acid in breast muscle and leg muscle of RC are higher than that of CB [1]. Therefore, these two breeds are ideal animal models for studying the genetic mechanism of chicken quality. Meat quality is influenced by many factors, among which the content of intramuscular fat (IMF) affects the juiciness, tenderness and flavor of meat [2-4]. Therefore, an in-depth understanding of the differentially expressed genes and their molecular regulatory mechanisms that affect intramuscular fat would be beneficial for controlling intramuscular fat content and improving muscle quality.

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs that regulate post-transcriptional regulation of gene expression through base pairing with complementary sequences in the 3' untranslated region of mRNA [5]. An increasing number of studies have indicated that miRNAs have important regulatory roles in various biological and metabolic processes, such as cell proliferation, differentiation, apoptosis and lipid metabolism [6-8]. Several miRNAs have been identified to be responsible for adipogenesis or muscle development [9,10]. Gga-miR-140-5p was found to promote intramuscular adipocyte differentiation in chicken muscle via targeting retinoid X receptor gamma [11]. Furthermore, miR-130a [12], miR-223 [13], miR-125b [14], miR-15a [15], miR-143a-3p [16], etc. were found to be associated with adipocyte differentiation and lipid metabolism. Therefore, miRNAs may play an important role in affecting meat quality characteristics. Studies showed that the development of muscle and intramuscular fat in the embryonic stage plays a decisive role in the meat production and meat quality of poultry after hatching [17,18]. In our previous study, a remarkable difference in the meat traits and flavor between Rose-crowned chicken and Cobb broilers were found during the embryonic period. We speculated that miRNAs in embryonic stage might serve as important regulatory factor of chicken meat quality.

Fatty acid-binding proteins (FABPs) are approximately 14-15 kDa, mainly cytoplasmic proteins, which can reversibly bind saturated and unsaturated long-chain fatty acids with high affinity [19]. FABP5 is a class of intracellular lipid carrier that can transport fatty acids to PPARγ [20], and plays an important regulatory in lipid metabolism. FABP5 was reported to be a molecular regulator of fat synthesis [21], deposition [22], and obesity [23]. At the same time, FABP5 is also regulated by miRNAs, such as miRNA-122 regulates liver metabolism in the chicken by targeting to inhibition expression of FABP5 [24].

The aim of this study was to identify the miRNAs affecting the differences in meat quality between Rose-crowned chicken and Cobb broilers using small RNA deep sequencing. Differentially expressed miRNAs and their target genes were identified to elucidate the regulatory patterns of miRNAs and their network. Subsequently, based on combined analysis of miRNAs and potential target mRNAs, the candidate miRNAs involved in intramuscular adipocyte differentiation were further characterized. Our results may provide a theoretical basis for the subsequent study of the molecular mechanisms of muscle development and fat formation in chicken embryos.

Material and Methods

Ethics Statement

This study was approved by the Medical Ethics Committee, First Affiliated Hospital, Medical College, Shihezi University (A2016-095, 9 March 2016). All samples were collected in strict accordance with the committee's guidelines.

Sample Collection and RNA Isolation

Embryonic Cobb broilers (CB) and rose-crowned chicken (RC) were used for high-throughput sequencing. Thirty fertilized RC and CB eggs were incubated at 37°C and 60% humidity, then ED 8 breast muscle tissues were collected by surgery at a clean bench. Meanwhile, the embryonic brain was collected for gender identification, using a method reported by Milos Vucicevic [25]. Total RNA was extracted from each breast muscle using TRIzol (Invitrogen, Carlsbad, CA, USA). The quality and concentration of all RNA samples were determined by 1.5% agarose gel electrophoresis and by analyzing the ratio of absorbance at A260/280, and then the samples were divided into four mixed RNA pools: CBM (male cobb broilers) and RCM (male rose-crowned chicken), CBF (female cobb broilers) and RCF (female rose-crowned chicken), each with 3 biological replicates.

Small RNA Library Construction and Deep Sequencing

Total RNA (3 μg per sample) was used as input material for generating each small RNA library. Four small RNA libraries were constructed using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) following the manufacturer's recommendations. The quality and quantity of the cDNA library were assessed using Qubit2.0 (Life Technologies) and Agilent 2100 (Agilent Technologies) systems. Finally, the assessed cDNA libraries were sequenced using an Illumina HiSeq2500 sequencing platform at Novogene (Beijing, China).

Sequence Analysis and Identification of miRNAs

Raw sequencing reads were processed by evaluating the sequencing quality, removing low-quality reads, adaptor sequences and reads smaller than 18 nt or longer than 35 nt. To analyze the distribution of small RNAs based on the reference sequence, all of the clean sequencing reads were mapped to the chicken genome using Bowtie2

(https://sourceforge.net/projects/bowtie-bio/files/). The reads mapped to the chicken genome were BLASTed against the non-coding RNA sequences in GenBank *(http://www.ncbi.nlm.nih.gov/)* and the RNA families in Rfam *(http://rfam.sanger. ac.uk/)* to identify miRNA types and numbers and sRNA fragments generated from mRNA degradation. Finally, the remaining sequences were then searched against the mature chicken miRNAs in miRBase (Release 21.0), and miREvo [26] and mirdeep2 [27] were used to identify known and unknown miRNAs.

Analysis of Differently Expressed miRNAs

The expression levels of miRNAs in the different libraries constructed were estimated based on the Illumina sequencing data according to the transcripts per million clean reads (TPM). The normalized expression values were calculated using the following formula: (read count x 1.000.000)/total miRNA read counts in the library. The DESeq2 program was used to analyse the DE miRNAs. miRNAs with |log2(fold change)| <5 and P<0.05 were identified as DE miRNAs.

Quantitative Real-Time PCR (qPCR)

The relative expression levels of eight randomly selected DE miRNAs were validated using quantitative real-time reverse transcription PCR (qRT-PCR). The *U6* gene was chosen as the reference gene for miRNA expression. The primers used are described in *Table 1*. Total RNA was extracted using TRIzol reagent, and then it was reverse transcribed using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) following the manufacturer's instructions. A miScript SYBR Green PCR kit was used to perform qPCR and determine expression levels of miRNAs. All of the reactions were repeated in triplicate, and the relative expression levels were calculated using the $2^{-\Delta\Delta ct}$ method. P<0.05 were considered to indicate significant differences.

Target Gene Prediction, Pathway and Network Analysis

Target genes were predicted using miRanda *(http://www.microrna.org/microrn-a/home.do)* and RNAhybrid *(http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/)*. GO enrichment and KEGG pathway analysis were performed using GOseq *(http://www.geneontology.org/)* and Kobas 2.0 *(http://kobas.cbi.pku.edu.cn/help.do)*, respectively.

Vector Construction

The 3′ untranslated region (UTR) of the *FABP5* gene, containing the gga-miR-3532-5p binding site, was amplified from chicken genomic DNA by PCR and subcloned into the XhoI-NotI site of a psiCHECK-2 vector (FABP5-3′-UTR-WT). Mutant FABP5-3′-UTR plasmids were generated by changing the gga-miR-3532-5p binding site from AGTGCAA to GTACGAA (FABP5-3′-UTR-Mut). The primers used were described in *Table 2*. Plasmid DNA was sequenced by Sangon Biotech (Shanghai, China) and extracted using an EndoFree Maxi Plasmid kit (TIANGEN, Beijing, China).

Luciferase Reporter Assay

Luciferase reporter experiments were performed in DF-1 cells. Cells were seeded in 12-well plates and cultured under routine conditions with 10% foetal bovine serum for 24 h. Then, the cells were cotransfected with 1000 ng of FABP5-3′UTR-WT or FABP5-3′UTR-Mut and 50 nM gga-miR-3532-5p mimic or negative control using Lipofectamine 2000, and the medium was replaced 6 h later. Forty-eight hours after transfection, the activities of firefly and Renilla luciferase were analyzed using a dual-luciferase reporter assay system (Promega) following the manufacturer's instructions.

Cell Transfection

The miR-3532-5p mimics and mimic NC were purchased from Ribobio (Guangzhou, China). Chicken preadipocyte line was cultured at 37°C in high-glucose medium supplemented with 10% fetal bovine serum and 100 μg/mL penicillin/streptomycin. Upon reaching 60-70%

Table 1. *Primer sequences for differential miRNA of qPCR verification*

miRNA	Primer Sequences (5′-3′)
gga-miR-140-5p	cgcgAGTGGTTTTACCCTATGGTAG
gga-miR-133a-3p	ttGGtCCCCttCAACCAGCtG
gga-miR-3532-5p	GTTGCACTGCAGCTGCTCTTGG
gga-miR-218-5p	cgcgTTGTGCTTGATCTAACCATGT
gga-miR-128-1-5p	CGGGGCCGTAACACTGTCT
gga-miR-1677-5p	TCCTGCACCGCTGAAGTCAAT
gga-miR-1306-3p	TGGACGTTGGCTCTGGTGG
novel_4	cgcTGAGATGAAGCACTGTAGCTC
U6-F	CAAATTGGCTAAGCGGGCCT
U6-R	CTAACAGCGTCGAGACTGCG

Note: *Lowercase letters in the primer sequences are protective bases*

Table 2. *Primer sequences of candidate target genes*

Gene	Primer Sequences	Product Length
gga-FABP5-F-W	*CCG*CTCGAGGAGTAGCTGAGTGCAATC	
gga-FABP5-F-M	*CCG*CTCGAGGAGTAGCTG**GTACG**AATC	308 bp
gga-FABP5-R-W	*ATTT*GCGGCCGCAGAGCATGAACTTTGAAT	

Note: *Italics are protective bases of restriction enzymes. Underlined letters are recognition sequences that introduce Not I and Xho I restriction enzymes. The points in bold are the base mutation sites. W stands for wild type and M stands for mutant*

confluence, the cells were transfected with a gga-miR-3532-5p mimic (50 nM) or a negative control (50 nM) using 5 μL of Lipofectamine 2000, and the medium was replaced 6 h later. After 24 h, the cells were used for RNA extraction.

Statistical Analyses

All experiments were carried out at least three times. Data are presented as the mean±standard deviation of the mean based on at least three replicates of each treatment. Data were analysed using SPSS 22.0 software (IBM, Chicago, IL, USA), and differences between groups were evaluated by one-way ANOVA; $P<0.05$ was considered significant.

Result

Overview of Small RNA Deep Sequencing Data

Four small RNA libraries were constructed as follows: CBM, RCM, CBF and RCF, with three replicates for each treatment. After quality control steps and adaptor removal were performed, a total of 11,540,611, 13,512,883, 11,660,807, and 12,108,093 clean reads were obtained from the four libraries *(Table 3)*. The size distribution of clean reads was assessed for all four groups. The small RNA sequence length was mainly concentrated at 21-24 nt, and the length of 22 nt was the most common size *(Fig. 1)*.

Identification of Differentially Expressed miRNAs

A Pearson correlation analysis showed that the correlation between samples was high, greater than 0.902 *(Fig. 2-a)*, indicating that the sample expression patterns were highly similar. The expression of miRNA was normalized by TPM calculation. The results showed that the expression level of 15 miRNAs in all samples was greater than 10000 TPM. These highly expressed miRNAs may be closely related to the proliferation and differentiation of embryonic cells. In total, 592 differentially expressed miRNAs were identified, including 518 known miRNAs and 74 novel miRNAs. Of these, 161 were upregulated, and 151 were downregulated in comparing RCM vs CBM; 160 were upregulated, and 120 were downregulated miRNAs in comparing RCF vs CBF *(Fig. 2-b)*. Among the identified miRNAs, 217 were found in all four libraries *(Fig. 2-c)*. Clustering analysis indicated that high similarity was shown within three replicates for each group and among the significant differences in miRNA expression between the two breeds of chicken *(Fig. 2-d)*.

qRT-PCR Validation of the Sequencing Data

To verify the RNA-Seq data, 8 miRNAs identified as differentially expressed between the two groups were validated by qRT-PCR *(Fig. 3)*. Our results showed that the expression patterns of these 8 miRNAs were consistent with the RNA-Seq results, indicating that the deep sequencing results were reliable and appropriate for further analysis.

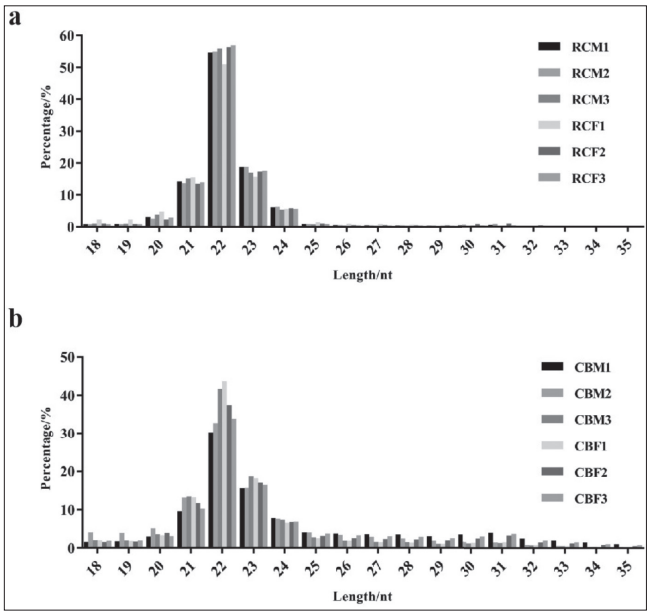


Fig 1. Length distribution of miRNA sequences in RC (**a**) and CB (**b**)

Table 3. *The information of raw data filtering*

Sample Name	Total Reads	Clean Reads	Q20/%	Q30/%	GC Content/%
RCM1	11168561	11050590 (98.94%)	97.50	94.11	49.29
RCM2	11194256	11055720 (98.76%)	97.51	94.15	49.22
RCM3	12647857	12515524 (98.95%)	97.52	94.15	49.35
RCF1	15928743	15725381 (98.72%)	98.84	97.74	49.63
RCF2	13499911	13337495 (98.80%)	98.90	97.88	49.07
RCF3	11594373	11475773 (98.98%)	97.59	94.31	49.19
CBM1	12532506	12318734 (98.29%)	96.89	93.34	51.80
CBM2	12317713	12100224 (98.23%)	96.49	92.51	52.10
CBM3	12450399	12272280 (98.57%)	98.52	96.79	51.42%
CBF1	12880091	12686254 (98.50%)	98.11	95.68	51.05%
CBF2	12151906	11977217 (98.56%)	96.91	93.40	51.44%
CBF3	11857817	11660807 (98.34%)	96.95	93.47	51.68%

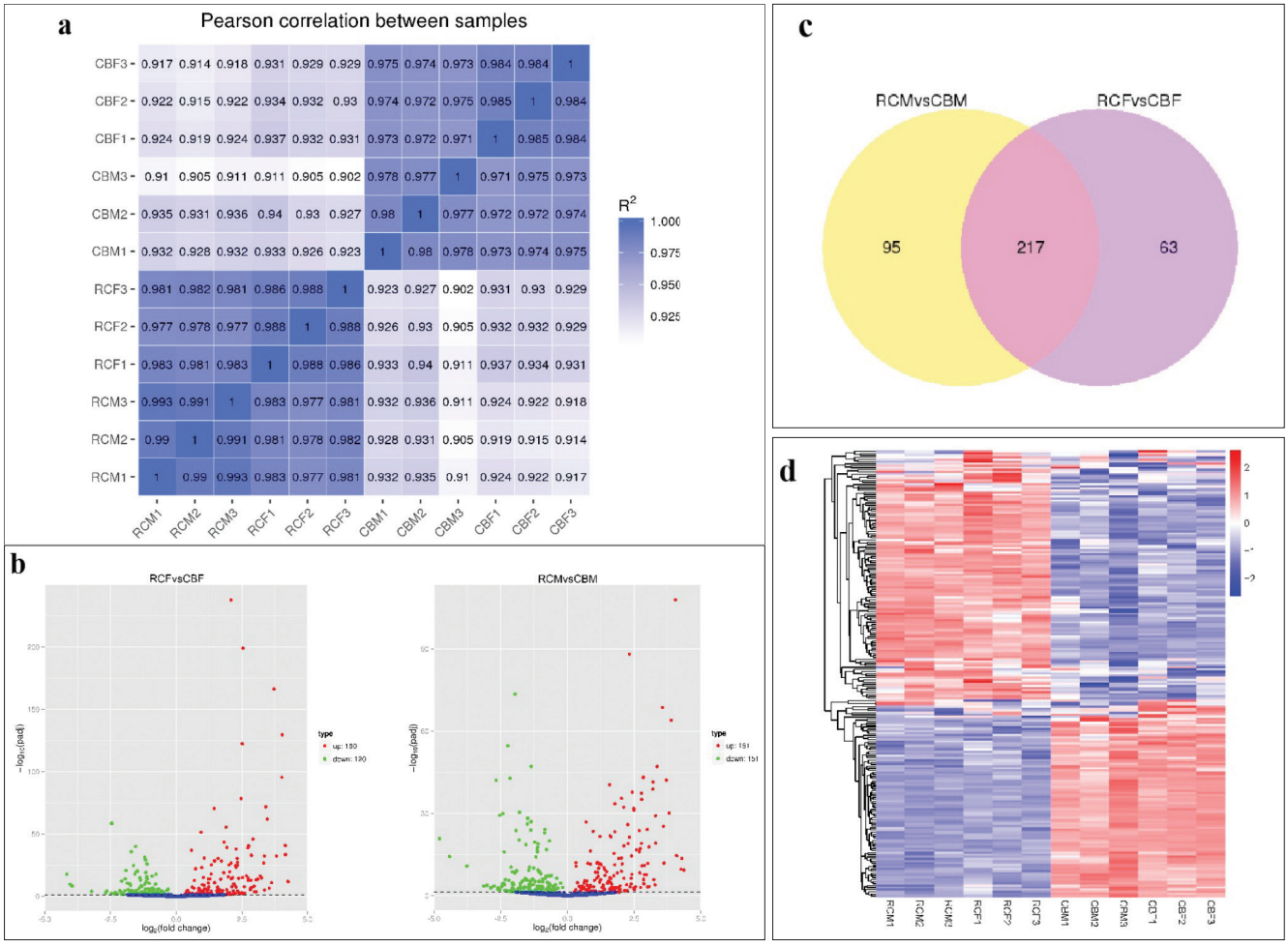


Fig 2. Overview of small RNA Deep Sequencing Data. **a-** Correlation analysis between samples, **b-** Volcano plot of DE miRNAs between two comparisons, **c-** Venn plot of DE miRNAs between two comparisons, **d-** Heat map of coexpressed miRNAs between the two groups

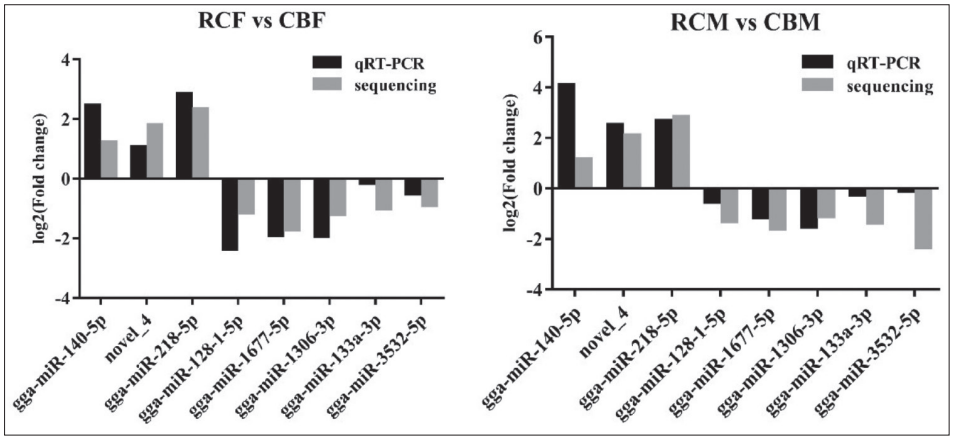


Fig 3. qRT-PCR validation of differentially expressed miRNAs. All experiments were carried out at least three times. log2 (Fold change) was the multiple of the difference in expression between samples to calculate the log2 value

Target Gene Prediction and Functional Enrichment Analysis

Using miRanda and RNAhybrid, a total of 120,666 consensus potential miRNA targets were identified for all differentially expressed miRNAs. For all potential targets, GO annotation and KEGG pathway analysis were performed to identify functional modules. All of the target genes were mainly enriched in biological metabolic processes, biosynthesis of cellular components, cell proliferation and differentiation, and embryonic development. Pathway analysis of all targets revealed that 9 KEGG pathways were significantly enriched ($P<0.05$), including 4 in RCM vs CBM and 5 in RCF vs CBF. The significantly enriched pathways included Ribosome, Wnt signalling pathway, Adherens junction, Butirosin and neomycin biosynthesis and Hedgehog signalling pathway

Table 4. *Top 5 KEGG signaling pathways of two comparisons*

Sample	KEGG	Rich Factor	P-Value	Gene Number
RCM vs CBM	Ribosome	0.4	0.02	48
	Wnt signaling pathway	0.370	0.011	44
	Adherens junction Butirosin and neomycin biosynthesis Hedgehog signaling pathway	0.397 1 0.4	0.022 0.029 0.05	27 5 18
RCF vs CBF	Ribosome	0.375	0.003	45
	Wnt signaling pathway	0.361	0.007	43
	Adherens junction Hedgehog signaling pathway Butirosin and neomycin biosynthesis	0.382 0.422 1	0.018 0.019 0.023	26 19 5

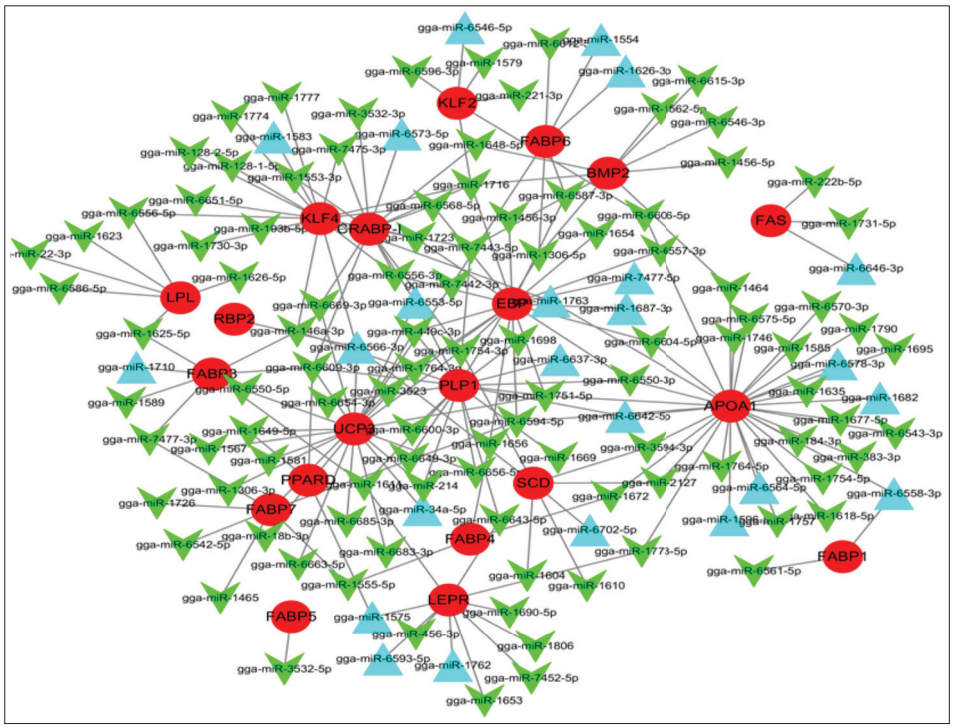


Fig 4. Interaction network of highly differentially expressed miRNAs and their potential targets. In this network, the target genes are displayed as red circles, the upregulated miRNAs are displayed as blue triangles, and the down regulated miRNAs are displayed as green arrows

(Table 4). According to the results of GO and KEGG analysis, we have identified key molecular players in the development of chicken breast muscle. In order to further understand the function of miRNA and their target genes, we focused on some of the differentially expressed miRNA and mRNA related with lipid metabolism, and a regulatory network for miRNA-mRNA interaction was constructed *(Fig. 4)*. In total, 33 up-regulated miRNA-mRNA pairs, and 160 down-regulated miRNA-mRNA pairs were identified. Among them, FABP5 as a potential target may be down-regulated by miR-3532-5p, this is the first discovery. EBP was the network core gene, a total of 19 miRNA-mRNA pairs were found, including gga-miR-1716, gga-miR-1751-5p and gga-miR-6604-5p, etc. gga-miR-1716 was also targets BMP2, CRABP-I, KLF2 and KLF4. APOA1 was targeted by 32 miRNAs, including 7 up-regulated and 25 down-regulated miRNAs. LPL was targeted by 7 down-regulated miRNAs, FAS was targeted by down-regulated gga-miR-222b-5p and gga-miR-1731-5p, and up-regulated gga-miR-6646-3p.

Validation of the miR-3532-5p Targeted FABP5 Gene

To verify the direct binding site between miR-3532-5p and *FABP5*, a 3'UTR fragment containing a seed region binding site was inserted into a psiCHECK-2 vector *(Fig. 5-a,b)*. Luciferase assays revealed that the luciferase reporter activity of FABP5-3'UTR-WT was significantly repressed by the miR-3532-5p mimic, while the luciferase activity of FABP5-3'UTR-MUT was not changed *(Fig. 5-c)*. Chicken preadipocytes were transfected with miR-3532-5p mimics, and the results show that overexpression of miR-3532-5p extremely significant downregulated *FABP5* mRNA expression *(Fig. 5-d)*. These results indicated that the predicted site of *FABP5* is a target of miR-3532-5p.

Discussion

Small RNA sequencing technology based on the Illumina high-throughput sequencing platform can directly assess

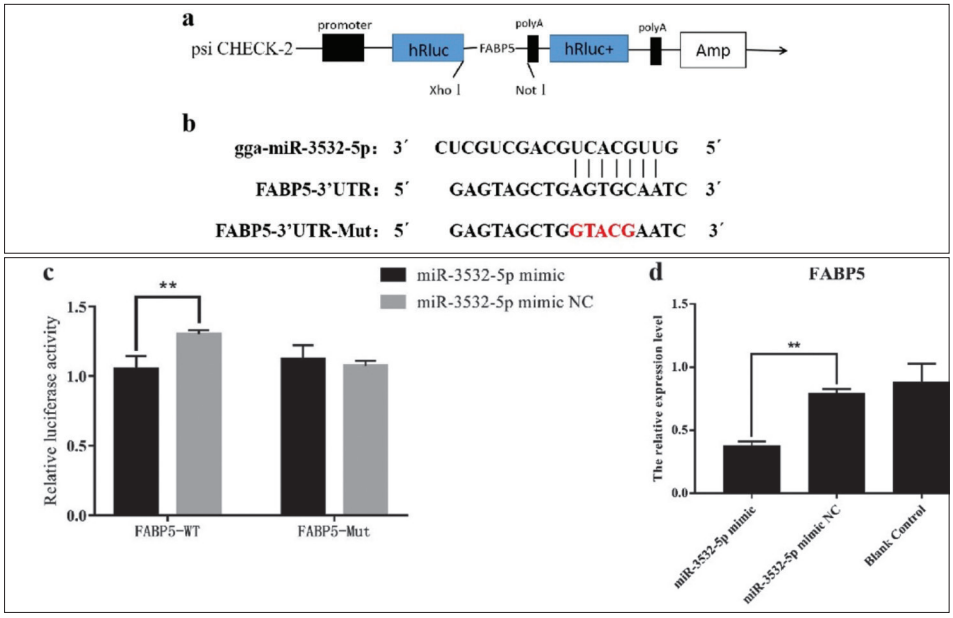


Fig 5. Validation of *FABP5* as a direct target of gga-miR-3532-5p. **a-** Target site of gga-miR-3532-5p within chicken *FABP5* mRNA 3'UTR and the mutation design of its 3'UTR, **b-** The sequence with the miR-3532-5p and *FABP5* binding sites was inserted into the psiCHECK-2 vector, **c-** miR-3532-5p mimics was transfected into DF1 cells along with FABP5-3'UTR-WT or FABP5-3'UTR-Mut (**P<0.01), **d-** The mRNA expression of *FABP5* is significantly reduced after overexpression miR-3532-5p

miRNAs in samples and has been used to identify known and predicted possible miRNAs [28]. The emergence of this method has greatly increased the discovery of new miRNAs. Many miRNAs have been identified as being associated with animal intramuscular fat deposition performance by high-throughput sequencing [11,29], but little is known about miRNAs in chicken embryos associated with adipogenesis.

In this study, the breast muscles on ED 8 from RC and CB were analyzed by high-throughput sequencing. To identify differentially expressed miRNAs that may play important regulatory roles in chicken meat quality, we compared the expression levels of miRNAs in the embryonic muscles of rose-crowned and Cobb broilers. A total of 842 known miRNAs were identified, and 598 new miRNAs were predicted. A total of 592 DE miRNAs were identified, of which 321 in the CB group were upregulated, and 271 were downregulated. MiRNAs are important regulatory factors in the process of fat deposition. Many of the miRNAs we have screened have been shown to regulate the proliferation and differentiation of adipocytes [30,31]. Previous studies showed that miR-128-3p inhibits the differentiation and generation of 3T3-L1 adipocytes by targeting *PPARG* and *Sertad2* [32]. Overexpression of miR-199a-5p promoted the proliferation of preadipocytes, and it was found that miR-199a-5p plays a role in the proliferation and differentiation of preadipocytes by downregulating the expression of Cav-1 [33]. The role of miRNA is mainly achieved by regulating the expression of target genes. In this study, miRanda and RNAhybrid were used to obtain 120,666 potential target genes for the differentially expressed miRNAs. MiRNAs affect cell signaling pathway transmission by regulating key genes in the signal transduction pathway to indirectly regulate adipocyte differentiation. The GO results show that the target genes are mainly concentrated in biological processes such as biological metabolism, cellular component biosynthesis, cell proliferation and differentiation, and embryonic development. Based on the GO annotation classification, KEGG pathway enrichment analysis was performed on the predicted target genes, and the results showed that a total of 5 signaling pathways were significantly enriched, of which the Wnt signaling pathway and the Hedgehog signaling pathway have been confirmed to play an important role in fat metabolism. According to the gene functions and their signaling pathway, we identified some candidates that affect fat formation and differentiation, including the *FABP* gene family, *FAS*, *APOA1*, and *LPL*.

In understanding the function of miRNA and its target genes and constructing a regulatory network of miRNA-mRNA interactions, miR-3532-5p may be an important regulator of intramuscular fat formation. Liu et al. showed that miR-3532 is closely related to the chicken reproductive process and ovarian steroidogenesis [34]. Wang et al.[35] mentioned that miR-3532 is a miRNA that is differentially expressed by chicken lung and trachea, but no further study on its target genes or its function was performed. To our knowledge, no previous studies have associated gga-miR-3532-5p with chicken IMF deposition. We used miRanda and RNAhybrid software to predict target genes, and we found that *FABP5* may be a target gene of miR-3532-5p. As a lipid carrier, *FABP5* is specific for the binding of fatty acids, preferentially binding long-chain unsaturated fatty acids and long-chain saturated fatty acids. It plays a vital role in triglyceride synthesis, fatty acid transport, and fat metabolism [36,37]. *FABP5* is also a key gene in the PPAR signaling pathway, which promotes adipocyte growth and differentiation by regulating PPARδ signaling [38]. Ma et al.[39] found that the activity of preadipocytes in the induction of differentiation into adipocytes decreased when the *FABP5* gene was knocked out, and the expression of *PPARγ* and *C/EBPα* genes associated with adipocyte differentiation was downregulated. This result indicates

that *FABP5* is an important factor influencing the activity and differentiation of preadipocytes. Previous studies have shown that the *FABP5* gene is closely related to fat deposition in pigs, and its polymorphic locus has an important effect on intramuscular fat content in chickens [40]. In our study, dual-luciferase assays and qRT-PCR confirmed the targeting relationship between miR-3532-5p and *FABP5*. It is speculated that gga-miR-3532-5p participates in the process of intramuscular fat formation in chicken embryos by inhibiting the expression of the *FABP5* gene, which may be an important regulatory factor affecting the differences in meat quality and flavor between RC and CB.

In conclusion, our study successfully constructed a miRNA library of embryonic breast muscles from RC and CB chickens and identified miRNAs that are associated with chicken quality. GO and KEGG analyses were performed to investigate the functional roles of these miRNAs. We constructed an interaction network of these miRNAs and their putative targets that may affect chicken breast meat quality. Furthermore, our study presents evidence that miR-3532-5p target *FABP5*, thereby participating in the process of intramuscular fat formation in chicken embryos. Our results lay the foundation for future analysis of the regulatory mechanism of miR-3532-5p in chicken intramuscular fat deposition. The results of this analysis provide new information that may be applied to further studies of the molecular regulatory mechanisms involved in the different meat quality traits exhibited by chicken embryos.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 31660654 & 31860641).

Conflict of Interest

No conflict of interest between all authors.

Author Contributions

In the process of writing the article, L. Zhang and S. Ren are responsible for the provision, integration and writing of the article data, H. Liao is responsible for the provision of research animals, J. Sun is the article reviewer.

References

1. Yang XJ, Han YX, Wang HL, Ren S, Zhang L, Qin YM, Qi MF, Zhang WZ, Liao HR, Sun J: Study on nutritional value and flavor of muscle from different breed of chicken. *China Poultry*, 40, 9-14, 2018. DOI: 10.16372/j.issn.1004-6364.2018.02.003

2. Wang Y, Hui X, Wang H, Kurban T, Hang C, Chen Y, Xing J, Wang J: Association of H-FABP gene polymorphisms with intramuscular fat content in Three-yellow chickens and Hetian-black chickens. *J Anim Sci Biotechnol*, 7:9, 2016. DOI: 10.1186/s40104-016-0067-y

3. Wu G, Shi X, Zhou J, Wang L, Li Y, Song C, Xiang A, Dong P, Sun S: Differential expression of meat quality and intramuscular fat deposition related genes in Hanjiang black pigs. *Acta Biochim Biophys Sin (Shanghai)*, 47, 145, 2015. DOI: 10.1093/abbs/gmu127

4. Bonny SPF, Hocquette JF, Pethick DW, Farmer LJ, Legrand I, Wierzbicki J, Allen P, Polkinghorne RJ, Gardner GE: The variation in the eating quality of beef from different sexes and breed classes cannot be completely explained by carcass measurements. *Animal*, 10, 987-995, 2016. DOI: 10.1017/S175173111500292X

5. Ambros V: The functions of animal microRNAs. *Nature*, 431, 350-355, 2004. DOI: 10.1038/nature02871

6. Mens MMJ, Ghanbari M: Cell cycle regulation of stem cells by MicroRNAs. *Stem Cell Rev Rep*, 14, 309-322, 2018. DOI: 10.1007/s12015-018-9808-y

7. Ge Y, Sun Y, Chen J: IGF-II is regulated by microRNA-125b in skeletal myogenesis. *J Cell Biol*, 192, 69-81, 2011. DOI: 10.1083/jcb.201007165

8. Lee EK, Lee MJ, Abdelmohsen K, Kim W, Kim MM, Srikantan S, Martindale JL, Hutchison ER, Kim HH, Marasa BS, Selimyan R, Egan JM, Smith SR, Fried SK, Gorospe M: miR-130 suppresses adipogenesis by inhibiting peroxisome proliferator-activated receptor gamma expression. *Mol Cell Biol*, 31, 626-638, 2010. DOI: 10.1128/MCB.00894-10

9. Guan L, Hu X, Liu L, Xing Y, Zhou Z, Liang X, Yang Q, Jin S, Bao J, Gao H, Du M, Li J, Zhang L: bta-miR-23a involves in adipogenesis of progenitor cells derived from fetal bovine skeletal muscle. *Sci Rep*, 7:43716, 2017. DOI: 10.1038/srep43716

10. Jebessa E, Ouyang H, Abdalla BA, Li Z, Abdullahi AY, Liu Q, Nie Q, Zhang X: Characterization of miRNA and their target gene during chicken embryo skeletal muscle development. *Oncotarget*, 9, 17309-17324, 2018. DOI: 10.18632/oncotarget.22457

11. Zhang M, Li DH, Li F, Sun JW, Jiang RR, Li ZJ, Han RL, Li GX, Liu XJ, Kang XT, Sun GR: Integrated analysis of MiRNA and genes associated with meat quality reveals that Gga-MiR-140-5p affects intramuscular fat deposition in chickens. *Cell Physiol Biochem*, 46, 2421-2433, 2018. DOI: 10.1159/000489649

12. Ma X, Wei D, Cheng G, Li S, Wang L, Wang Y, Wang X, Zhang S, Wang H, Zan L: Bta-miR-130a/b regulates preadipocyte differentiation by targeting PPARG and CYP2U1 in beef cattle. *Mol Cell Probes*, 42, 10-17, 2018. DOI: 10.1016/j.mcp.2018.10.002

13. Li F, Li D, Zhang M, Sun J, Li W, Jiang R, Han R, Wang Y, Tian Y, Kang X, Sun G: miRNA-223 targets the GPAM gene and regulates the differentiation of intramuscular adipocytes. *Gene*, 685, 106-113, 2019. DOI: 10.1016/j.gene.2018.10.054

14. Cheng X, Chen X, Wang P, Chen T, Sun J, Xi Q, Zhang Y: Exploration of targets regulated by miR-125b in porcine adipocytes. *In Vitro Cell Dev Biol Anim*, 56, 103-111, 2020. DOI: 10.1007/s11626-019-00427-3

15. Li G, Fu S, Chen Y, Jin W, Zhai B, Li Y, Sun G, Han R, Wang Y, Tian Y, Li H, Kang X: MicroRNA-15a regulates the differentiation of intramuscular preadipocytes by targeting ACAA1, ACOX1 and SCP2 in chickens. *Int J Mol Sci*, 20:4063, 2019. DOI: 10.3390/ijms20164063

16. Zhang P, Du J, Wang L, Niu L, Zhao Y, Tang G, Jiang Y, Shuai S, Bai L, Li X, Wang J, Zhang S, Zhu L: MicroRNA-143a-3p modulates preadipocyte proliferation and differentiation by targeting MAPK7. *Biomed Pharmacother*, 108, 531-539, 2018. DOI: 10.1016/j.biopha.2018.09.080

17. Velleman SG: Muscle development in the embryo and hatchling. *Poult Sci*, 86, 1050-1054, 2007. DOI: 10.1093/ps/86.5.1050

18. Chartrin P, Bernadet MD, Guy G, Mourot J, Hocquette JF, Rideau N, Duclos MJ, Baéza E: Do age and feeding levels have comparable effects on fat deposition in breast muscle of mule ducks? *Animal*, 1, 113-123, 2007. DOI: 10.1017/S1751731107658029

19. Furuhashi M, Hotamisligil GS: Fatty acid-binding proteins: Role in metabolic diseases and potential as drug targets. *Nat Rev Drug Discov*, 7, 489-503, 2008. DOI: 10.1038/nrd2589

20. O'Sullivan SE, Kaczocha M: FABP5 as a novel molecular target in prostate cancer. *Drug Discov Today*, 25, 2056-2061, 2020. DOI: 10.1016/j.drudis.2020.09.018

21. Li P, Yu M, Zhou C, Qi H, Wen X, Hou X, Li M, Gao X: FABP5 is a critical regulator of methionine- and estrogen-induced SREBP-1c gene expression in bovine mammary epithelial cells. *J Cell Physiol*, 234, 537-

549, 2019. DOI: 10.1002/jcp.26762

22. Li B, Qiao L, An L, Wang W, Liu J, Ren Y, Pan Y, Jing J, Liu W: Transcriptome analysis of adipose tissues from two fat-tailed sheep breeds reveals key genes involved in fat deposition. *BMC Genomics*, 19:338, 2018. DOI: 10.1186/s12864-018-4747-1

23. Shibue K, Yamane S, Harada N, Hamasaki A, Suzuki K, Joo E, Iwasaki K, Nasteska D, Harada T, Hayashi Y, Adachi Y, Owada Y, Takayanagi R, Inagaki N: Fatty acid-binding protein 5 regulates diet-induced obesity via GIP secretion from enteroendocrine K cells in response to fat ingestion. *Am J Physiol Endocrinol Metab*, 308, E583-591, 2015. DOI: 10.1152/ajpendo.00543.2014

24. Wang X, Shao F, Yu J, Jiang H, Gong D, Gu Z: MicroRNA-122 targets genes related to liver metabolism in chickens. *Comp Biochem Physiol B Biochem Mol Biol*, 184, 29-35, 2015. DOI: 10.1016/j.cbpb.2015.02.002

25. Vucicevic M, Stevanov-Pavlovic M, Stevanovic J, Bosnjak J, Gajic B, Aleksic N, Stanimirovic Z: Sex determination in 58 bird species and evaluation of CHD gene as a universal molecular marker in bird sexing. *Zoo Biol*, 32, 269-276, 2013. DOI: 10.1002/zoo.21010

26. Friedländer MR, Mackowiak SD, Li N, Chen W, Rajewsky N: miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res*, 40, 37-52, 2012. DOI: 10.1093/nar/gkr688

27. Wen M, Shen Y, Shi S, Tang T: miREvo: An integrative microRNA evolutionary analysis platform for next-generation sequencing experiments. *BMC Bioinformatics*, 13:140, 2012. DOI: 10.1186/1471-2105-13-140

28. Ouyang H, He X, Li G, Xu H, Jia X, Nie Q, Zhang X: Deep sequencing analysis of miRNA expression in breast muscle of fast-growing and slow-growing broilers. *Int J Mol Sci*, 16, 16242-16262, 2015. DOI: 10.3390/ijms160716242

29. Fu S, Zhao Y, Li Y, Li G, Chen Y, Li Z, Sun G, Li H, Kang X, Yan F: Characterization of miRNA transcriptome profiles related to breast muscle development and intramuscular fat deposition in chickens. *J Cell Biochem*, 119, 7063-7079, 2018. DOI: 10.1002/jcb.27024

30. Kajimoto K, Naraba H, Iwai N: MicroRNA and 3T3-L1 pre-adipocyte differentiation. *RNA*, 12, 1626-1632, 2006. DOI: 10.1261/rna.7228806

31. Wang Q, Li YC, Wang J, Kong J, Qi Y, Quigg RJ, Li X: miR-17-92 cluster accelerates adipocyte differentiation by negatively regulating tumor-suppressor Rb2/p130. *Proc Natl Acad Sci U S A*, 105, 2889-2894, 2008. DOI: 10.1073/pnas.0800178105

32. Chen C, Deng Y, Hu X, Ren H, Zhu J, Fu S, Xie J, Peng Y: miR-128-3p regulates 3T3-L1 adipogenesis and lipolysis by targeting Pparg and Sertad2. *J Physiol Biochem*, 74, 381-393, 2018. DOI: 10.1007/s13105-018-0625-1

33. Shi XE, Li YF, Jia L, Ji HL, Song ZY, Cheng J, Wu GF, Song CC, Zhang QL, Zhu JY, Yang GS: MicroRNA-199a-5p affects porcine preadipocyte proliferation and differentiation. *Int J Mol Sci*, 15, 8526-8538, 2014. DOI: 10.3390/ijms15058526

34. Liu L, Xiao Q, Gilbert ER, Cui Z, Zhao X, Wang Y, Yin H, Li D, Zhang H, Zhu Q: Whole-transcriptome analysis of atrophic ovaries in broody chickens reveals regulatory pathways associated with proliferation and apoptosis. *Sci Rep*, 8:7231, 2018. DOI: 10.1038/s41598-018-25103-6

35. Wang Y, Brahmakshatriya V, Zhu H, Lupiani B, Reddy SM, Yoon BJ, Gunaratne PH, Kim JH, Chen R, Wang J, Zhou H: Identification of differentially expressed miRNAs in chicken lung and trachea with avian influenza virus infection by a deep sequencing approach. *BMC Genomics*, 10:512, 2009. DOI: 10.1186/1471-2164-10-512

36. Dallaglio K, Marconi A, Truzzi F, Lotti R, Palazzo E, Petrachi T, Saltari A, Coppini M, Pincelli C: E-FABP induces differentiation in normal human keratinocytes and modulates the differentiation process in psoriatic keratinocytes in vitro. *Exp Dermatol*, 22, 255-261, 2013. DOI: 10.1111/exd.12111

37. Descalzi Cancedda F, Dozin B, Zerega B, Cermelli S, Cancedda R: Ex-FABP: A fatty acid binding lipocalin developmentally regulated in chicken endochondral bone formation and myogenesis. *Biochim Biophys Acta*, 1482, 127-135, 2000. DOI: 10.1016/s0167-4838(00)00159-x

38. Yu CW, Liang X, Lipsky S, Karaaslan C, Kozakewich H, Hotamisligil GS, Bischoff J, Cataltepe S: Dual role of fatty acid-binding protein 5 on endothelial cell fate: a potential link between lipid metabolism and angiogenic responses. *Angiogenesis*, 19, 95-106, 2016. DOI: 10.1007/s10456-015-9491-4

39. Ma X, Ren X, Han P, Hu S, Wang J, Yin J: SiRNA against Fabp5 induces 3T3-L1 cells apoptosis during adipocytic induction. *Mol Biol Rep*, 37, 4003-4011, 2010. DOI: 10.1007/s11033-010-0059-5

40. Estellé J, Pérez-Enciso M, Mercadé A, Varona L, Alves E, Sánchez A, Folch JM: Characterization of the porcine FABP5 gene and its association with the FAT1 QTL in an Iberian by Landrace cross. *Anim Genet*, 37, 589-591, 2006. DOI: 10.1111/j.1365-2052.2006.01535.x

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

Kafkas Univ Vet Fak Derg
27 (2): 253-258, 2021
DOI: 10.9775/kvfd.2020.25021

SHORT COMMUNICATION

Development of Humoral Immune Response to Thermostable Newcastle Disease Vaccine Strain I-2 in Ring-Necked Pheasant *(Phasianus colchicus)*

Faisal SIDDIQUE [1,a] Rao Zahid ABBAS [2,b] Asif IQBAL [3,c] Masood RABBANI [4,d] Azhar RAFIQUE [5,e] Irshad HUSSAIN [4,f] Rais AHMED [1,g] M. Shahid MAHMOOD [6,h (*)] Alireza LOTFI [7,i]

[1] Department of Microbiology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, PAKISTAN
[2] Department of Parasitology, University of Agriculture, Faisalabad, PAKISTAN
[3] Department of Parasitology, Riphah International University, Lahore, PAKISTAN
[4] Department of Microbiology, Quality Operation Laboratory, University of Veterinary and Animal Sciences, Lahore, PAKISTAN
[5] Department of Zoology, GC University Faisalabad, PAKISTAN
[6] Institute of Microbiology, University of Agriculture Faisalabad, PAKISTAN
[7] Ilkhchi Branch, Islamic Azad University, Ilkhchi, IRAN

ORCIDs: [a] 0000-0002-5237-5256; [b] 0000-0001-7344-6212; [c] 0000-0003-1127-1941; [d] 0000-0001-6777-1879; [e] 0000-0001-8147-8820 [f] 0000-0002-1471-363X; [g] 0000-0002-8843-0616; [h] 0000-0001-8709-1029; i 0000-0002-2879-9841

Article ID: KVFD-2020-25021 Received: 27.09.2020 Accepted: 11.01.2021 Published Online: 12.01.2021

Abstract

Newcastle Disease (ND) was ranked as a List A and trade limited disease by the World Health Organization. It is one of the deadly diseases of domestic and zoo birds especially pheasants that are highly susceptible species to Newcastle disease virus (NDV). This study was designed to determine the humoral immune response (HIR) of thermostable NDV vaccine strain I-2 in same age pheasants. For this purpose, forty-five pheasants of the same age were separated and placed in cages. Thermostable NDV I-2 vaccine was mixed with feed and administered through oral route to the same age pheasants. HIR was detected using haemagglutination inhibition test (HI) and enzyme linked immunosorbent assay (ELISA) on 0, 7th, 14th, 21th and 28th days post-vaccination (DPV). Optimum geometric mean anti-NDV-ELISA (2380) and anti-NDV-HI (Log27.5) antibodies titers were identified on 14th DPV. It was concluded that the oral administration of NDV I-2 strain is able to elicit a protective immune response in pheasants. Moreover, the use of this novel vaccine technique at the same age of pheasants overwhelms the attempt to catch these birds for single vaccination.

Keywords: *Thermostable, Pheasant, Newcastle, ELISA, Vaccine*

Halka Boyunlu Sülün'de *(Phasianus colchicus)* Termostabil Newcastle Hastalığı Aşı Suşu I-2'ye Karşı Humoral İmmun Yanıtın Gelişimi

Öz

Newcastle Hastalığı, Dünya Sağlık Örgütü'nün A listesinde yer alan ve ticareti sınırlayan bir hastalıktır. Evcil ve hayvanat bahçesi kuşlarının, özellikle de Newcastle Hastalığı Virüsüne (NDV) oldukça duyarlı türler olan sülünlerin ölümcül hastalıklarından birisidir. Bu çalışma, aynı yaştaki sülünlerde termostabil NDV aşı suşu I-2'ye karşı şekillenen humoral immün yanıtı belirlemek için tasarlanmıştır. Bu amaç doğrultusunda kırk beş sülün ayrılarak kafeslere yerleştirildi. Yemle karıştırılan termostabil NDV I-2 aşısı, sülünlere oral yolla verildi. Humoral immun yanıt, aşılamadan sonraki 0., 7., 14., 21. ve 28. günlerde (DPV) Hemaglütinasyon İnhibisyon (HI) testi ve Enzim İşaretli İmmünosorbent Testi (ELISA) kullanılarak tespit edildi. Optimum geometrik ortalama anti-NDV-ELISA (2380) ve anti-NDV-HI (Log27.5) antikor titreleri aşı uygulamasını takiben 14. günde saptandı. NDV I-2 suşunun oral yolla uygulamasının sülünlerde koruyucu bir immün yanıt oluşturabileceği kanaatine varıldı. Ayrıca, aynı yaştaki sülünlerde kullanılan bu yeni aşı uygulama tekniği, aşılama sırasında bu kuşların tek tek yakalanma girişimlerinin üstesinden de gelmektedir.

Anahtar sözcükler: *Termostabil suş, Sülün, Newcastle Hastalığı, ELISA, Aşı*

How to cite this article?

Siddique F, Abbas RZ, Iqbal A, Rabbani M, Rafique A, Hussain I, Ahmed R, Mahmood MS, Lotfi A: Development of humoral immune response to thermostable Newcastle disease vaccine strain I-2 in ring-necked pheasant *(Phasianus colchicus)*. *Kafkas Univ Vet Fak Derg,* 27 (2): 253-258, 2021. DOI: 10.9775/kvfd.2020.25021

(*) **Corresponding Author**

Tel: +92 333 6629016
E-mail: shahiduaf@gmail.com (M.S. Mahmood)

Introduction

Small population of ring-necked pheasant *(Phasianus colchicus)* is bred as a game bird in Asia. They are primarily bred in various parts of Europe, America as a game bird. It belongs to family *Phasianidae* genus order *Galliformes* and subfamily *Phasianinae* [1]. Males are dominant, long tails and extremely decorated with bright colors as compared to females. Ring-necked pheasant may serve as biological indicator for the study of wildlife species and ecosystems fitness [2]. Fifty two species are present worldwide and six of these species are found in Pakistan. Most of the species are known to be endangered due to hunting and susceptibility to various diseases such as Newcastle disease (ND). Hunters choose this bird because of its higher quality of meat (high essential amino acid, fatty acids and low fat profile) as compared to ducks, broilers and geese [3-6]. Newcastle disease has been identified as one of the most pathogenic diseases of birds worldwide [7,8]. This disease affects approximately two hundred fifty species of birds and results in high morbidity and mortality that may pose significant economic losses and limit trade and embargoes [9-11]. Ring-necked pheasants are more susceptible to ND virus and the virus often causes death [12]. The main causative agent of ND in pheasants is avian paramyxovirus serotype 1 (APMV-1), which belongs to the Paramyxoviridae family and the Avula virus genus [13]. It is a negative sense, enveloped, non-segmented and filamentous RNA virus with a genome size of 15.2 kb [14]. Contamination of feed and water with NDV containing bird's droppings is the main reason for the spread of this disease to healthy pheasant [15]. Flying birds, such as doves and pigeons, are also capable of spreading this virus to nearby pheasants and are primarily responsible for many recent epidemics [16,17]. Pheasants of all ages are susceptible to NDV and the average incubation period of NDV is 5-6 days [18]. The clinical signs and symptoms of pheasants infected with ND depend on many factors, including age, host immune status, virus strain and environmental conditions [19]. Many clinical symptoms of pheasants have been observed, such as white green diarrhea, head shaking, lack of appetite, reduced egg production, sneezing, coughing and trouble in breathing. In pheasants, mortality rate can easily reach to 100% since pheasants are known to have higher morbidity and mortality during the ND epidemic [12,20]. The control of Newcastle disease in pheasant reduced the risk of spread to commercial poultry [21]. In addition to biosecurity steps, vaccination is the most effective method to control the disease. There are two important concerns related to the vaccination of flying birds. First, if the cold chain system is not adequately managed when delivered to end users in warehouses and drug stores, the consistency of commercially available NDV vaccines would typically degrade rapidly. Secondly, administration of vaccines is very difficult in wild birds [22]. To counter these issues, Thermostable NDV vaccine has been prepared, and can be administered by mixing with feed, so there is no need to capture birds for vaccination. Thermostable vaccine is a promising approach to control ND in pheasants as this vaccine maintains its efficacy at 28°C for 6-8 weeks and at 4-8°C for 1 year.

Material and Methods

Ethical Statement

The experiment was conducted under the regulations stipulated by the Independent Ethics Committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan (Number of bio-ethical committee letter is ORIC 1749, 1.6.2020).

Study Area

The experiment was performed at Safari Zoo Lahore. The Park was established in 1982 and spreads over 242 acres. The park contains the country's largest walkthrough aviary.

Source of NDV Vaccine Strain

The thermostable NDV I-2 strain was obtained from the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore.

Preparation of Vaccines

- In Ovo Propagation of Thermostable NDV

Five hundred (nine-day old) chicken embryos were procured from well reputed hatchery and transferred to an incubation facility available at Vaccinology Laboratory of the Department. Candling of embryos were done for evaluating livability of the embryos followed by proper labeling and documentation. After thorough scrubbing of egg shells using 70% ethanol, air sac was marked using lead pencil. A bore was made in few centimeters above air sac of egg shell of each embryo for inoculation of 0.1-0.2 mL of thermostable NDV strain (0.1 mL) under sterile environment. The bore was sealed using molten wax and re-incubated at 37°C. Candling process was repeated at 10^{th} day after incubation and dead embryos were discarded. While on day 11, remaining embryos were transferred in refrigerator at 4°C for 6 h. The amniotic-allantoic fluid was collected and stored at -20°C for further processing [22].

- Titration of I-2 NDV Virus

Titration of I-2 NDV was calculated by Egg infective dose 50 (EID_{50}). Briefly, tenfold serial dilution of virus was made from 10^{2}-10^{10}. The virus (0.1 mL) from each dilution was injected into nine days old chicken embryonated eggs. These eggs were incubated at 37°C for 24-72 h. The eggs were chilled at 4°C overnight. Allantoic fluid was collected from each egg and haemagglutination test was performed to examine the presence of the virus. The live thermostable ND virus was subjected to lyophilization for further use as oral vaccine/mixing with feeds.

- Sterility Test

Vaccine sterility was confirmed by culturing it on bacterial and fungal media for the presence of any contamination. For this purpose, 3 mL of vaccine fluid was centrifuged (500 x g) for 10 min and the residue was streaked on nutrient agar, MacConkey's agar, blood agar, and Sabouraud's dextrose agar plates. Loop full sediment was also inoculated in Frey's modified medium for Mycoplasma. These plates were incubated at 37°C for 48 h except Sabouraud's agar and Frey's medium. The Sabouraud's agar plates were incubated at 25°C in a humid chamber and Frey's medium plates were incubated for 7-10 days at a 37°C. All the plates were observed for microbial contamination [23].

- Safety Testing

After the vaccine was proved to be sterile, each batch of vaccine was inoculated in 4-5 day-old 20 chickens through drinking water. The inoculated chickens were monitored for any pyrogenic effect, vaccine shock and/or vaccinal reaction.

- Experimental Design

Forty-five ring-necked pheasants of same age were separated from other birds at Safari Zoo Lahore. The birds were examined for antibodies against NDV and those which were found to be seronegative were included in the study. These birds were kept under standard management conditions. The forty-five pheasants were separated into three groups (G1, G2 and G3), fifteen pheasants in each. 5 mL of fresh, sterilized and non-chlorinated water has been added to the lyophilized NDV I-2 vaccine bottle (UVAS-CASTLE VAC) containing 10^6 Egg Infective Dose 50 per chicken. This viral suspension was further diluted in 50 mL water. The vaccine suspension was sprayed on feed (15 mL per 150 g of feed) and mixed thoroughly. Then, this vaccine treated feed (150 g) was spread on a clean cloth and fifteen pheasants from G1 were allowed to eat until the feed finished. Therefore, each pheasant ate approximately 10 g of vaccine coated feed. G2 was vaccinated with commercially available thermolabile NDV LaSota vaccine (Intervac Pvt. Ltd) as a positive control. The G3 was kept as a negative control (phosphate buffer saline) in the complete experimental design. Pheasants of each group were caged separately. The pheasants were checked daily two times (morning and evening) throughout the whole experiment to observe for any abnormal behavior [23,24].

- Collection of Samples

Blood samples (2-3 mL) form each group were collected directly from brachial vein in sterile blood collection tubes. Samples were transported to Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore and stored at -80°C for further use in serological tests.

- Serological Test to Determine Humoral Immune Response

The HIR in terms of anti-NDV antibodies were determined through Haemagglutination inhibition (HI) test and Enzyme-linked immunosorbent assay (NDV ELISA Kit, IDEXX Laboratories, Westbrook, ME) on days 0, 7, 14, 21 and 28 post-vaccination (DPV).

Statistical Analysis

The data for HI and ELISA tests were analyzed by calculating geometric mean titers (GMTs), mean±SD and through one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS software (version 20.0).

Results

In the present study, single feed mixing NDV I-2 vaccine developed protective antibody response in same age pheasants after seven days of post vaccination. Two main serological tests, HI and ELISA, were used to evaluate antibody titers. The outcomes of anti-NDV-ELISA antibody titers at 0, 7^{th}, 14^{th}, 21^{th}, and 28^{th} days of post-vaccination have been depicted in *Table 1*. Two means of UVAS-CASTLE VAC and LaSota NDV are significantly different from each other even at day 0. In G1, maximum ELISA geometric mean antibody titers Log_2 was accomplished at day 14, e.g., 2380 as followed in G2, i.e., 1867, respectively. The geometric mean ELISA antibody titers Log_2 were significantly higher ($P<0.05$) in G1 as compared to G2. The results of geometric anti-NDV-HI antibody titers at 0, 7^{th}, 14^{th}, 21^{th}, and 28^{th} days of post-vaccination have been presented in *Table 2*. Optimum geometric anti-NDV-HI antibody titers were observed in G1 at day 14, e.g. 7.5 as compared to G2 i.e. 6, respectively. These findings in contrast with the outcomes from the earlier studies, pheasants were vaccinated with commercially available NDV LaSota vaccine and humoral immune response was evaluated on the basis of agglutinin titer. Anti-NDV antibodies were found up to 75% of birds. Booster dose was given at 21^{th} day of post vaccination, and then antibodies were depicted in all experimental birds [33]. Similar contrast findings were reported when ring-necked pheasants were vaccinated with different strains of Newcastle disease virus such as Ulster, B1 and LaSota. No maternally derived antibodies were detected at seven days of age. First protective antibodies response was observed at 10^{th} day of post vaccination. Booster dose was given to maintain antibody titer against NDV up to 94 days of age.

The results of this project have shown that the use of UVAS-CASTLE VAC (Thermostable NDV I-2 strain) vaccine in pheasants is effective, immunogenic, economical and feasible, and leads to a protective immunogenic response. This may prevent the transmission of NDV into other birds especially poultry birds. Furthermore, administration of oral vaccine in feed to the same age free range pheasants overcomes the individual catching hurdles of these birds during vaccination.

Table 1. *Comparative geometric mean anti-NDV-ELISA titer of G1 UVAS-CASTLE VAC (I-2 NDV strain), G2 LaSota NDV vaccine and G3 Negative control (PBS) in ring-necked pheasant*

Group No.	Vaccination	ELISA Mean Antibody Titer (Days)				
		0	7	14	21	28
G1	I-2 NDV	1235±0.141	1796±0.346	2280±0.454	1960±0.379	1144±0.126
G2	LaSota NDV	1231±0.141	1523±0.237	1767±0.354	1680±0.335	1144±0.126
G3	Negative Control (PBS)	1231±0.141	1120±0.134	1061±0.097	1064±0.099	1079±0.111

The anti-NDV-ELISA titer pheasant vaccinated with NDV 1-2 vaccine in G1 was significantly higher as compared to that pheasant G2 vaccinated with LaSota vaccine during 7 to 28 days post vaccination (P>0.05); NDV (Newcastle disease virus); NC (Negative control); PBS (Phosphate buffer saline); D/W (Drinking water); G (Group); ELISA (Enzyme-linked immunosorbent assay)

Table 2. *Comparative geometric mean anti-NDV-HI titer of G1 UVAS-CASTLE VAC (I-2 NDV strain), G2 LaSota NDV vaccine and G3 Negative control (PBS) in ring-necked Pheasant*

Group No.	Vaccination	Route	HI Mean Antibody Titer (Days)				
			0	7	14	21	28
G1	I-2 NDV	D/W	3.8±0.62	6.6±0.64	7.6±0.75	6.2±0.61	4.4±0.59
G2	LaSota NDV	D/W	3.8±0.52	4.8±0.56	5±0.61	5.5±0.60	3.6±0.51
G3	NC (PBS)	D/W	3.8±0.52	2.8±0.48	2.2±0.41	1.8±0.34	1.2±0.30

The anti-NDV-HI titer pheasant vaccinated with NDV 1-2 vaccine in G1 was significantly higher as compared to that pheasant G2 vaccinated with LaSota vaccine during 7 to 28 days post vaccination (P>0.05); NDV (Newcastle disease virus); NC (Negative control); PBS (Phosphate buffer saline); D/W (Drinking water); G (Group); HI (Haemagglutination Inhibition test)

Discussion

ND is one of the most pathogenic diseases which hampered the development of pheasant farming, if not successfully controlled. This includes the development of effective methods to combat this deadly disease. It was difficult to immunize the free-range or uncaged pheasants since conventional or old techniques were developed for the use in commercial and domestic birds. ND spread through contamination of feed and water with NDV infected droppings. Moreover, flying birds have the capability to transfer this virus among various species of birds and mainly responsible for many epidemics in the recent past [21]. Pheasants are carriers of virulent Newcastle disease virus strains. Hence, control of NDV in pheasant by vaccination is necessary to control or minimize the chances of the spread of ND in commercial birds [25,26]. It was therefore the objective of this project to prepare a thermostable NDV I-2 vaccine and evaluation of its efficacy in flying birds like pheasants. For this purpose, 8-9 days old chicken embryos were used to obtain maximum growth of NDV I-2 strain. Similarly, thermostable NDV I-2 virus has been grown in 8-day-old embryonic eggs in central laboratories in the developing countries. These embryonic eggs are not strictly pathogen-free, but are harvested from a healthy poultry flock that is routinely evaluated for main viral diseases such as ND [27]. Biological method i.e. egg infective dose fifty (EID_{50}) for titration of NDV I-2 strain presented optimum titers 10^9 EID_{50}/mL in the next 24 h of incubation, when grown in nine days old chicken embryos. Similar findings were reported when thermostable NDV I-2 strain produced optimum titer 10^9 tissue culture infective dose 50 mL ($TCID_{50}$/mL), after growing in Vero cell line [23]. In other recent studies, when NDV grown in 8-9 days embryonated eggs, 10^8 EID_{50}/mL titer has been detected [28].

The protective antibody titer is an indication of the good quality of any vaccine [29]. The HI and ELISA tests were used to evaluate the antibody titer in pheasant birds following oral administration of the Thermostable NDV I-2 vaccine in this study. The results of both tests showed that optimum antibody titer was reached on day 14 after vaccination. Statistically, the highest mean protective antibody titers Log 2 was observed in G1 ($P<0.05$) as contrasted to G2. In zoo birds, the antibody titer above $\geq 2^3$ or 3 $\log_2$ is intended to be protective against virulent NDV in experimental or field trials of chickens [30]. This protective antibody titer may also be applied to wild birds such as pigeons and pheasants [31]. Similar consequences have been documented in feral pigeons [32] and guinea fowls when NDV I-2 strain vaccine was administered orally and mixed with feed. Vaccine administration route has significant effect on the production of protective antibodies titer. Eye drop administration produced maximum protective titer of antibodies as compared to other routes [33,34]. Ulster 2C NDV strain produced low antibody titer as compared to LaSota and B1 NDV strain [22]. Pheasants did not show any adverse effect after oral vaccination in the present study. It was concluded that the use of thermostable NDV vaccination techniques in wild birds, particularly pheasants, led to a reduction in the risk of transmitting of this horrible disease from these birds to poultry birds.

The present research concluded that the wild birds particularly pheasants have highlighted the spread of ND in commercial as well as rural poultry birds, therefore, they are significant risk factors for ND epidemics. Vaccination is the only way of preventing infectious diseases in the world. Commercially available NDV vaccines are usually thermolabile in nature and required a cold storage system to maintain their shelf life. In developing countries like Pakistan, where electric supply is a burning issue, it provides a viable and effective alternative to combat ND. In the future, we hope that the thermostable NDV I-2 vaccine will be much more widely used and implemented and help to manage Newcastle disease more effectively in the region.

Acknowledgements

Authors are immensely obliged to Higher Education Commission, Pakistan for their financial support in terms of IPFP start-up grant. Moreover, many thanks to workers and staff of Lahore, Safari Zoo supporting to conduct this study.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contributions

F. Siddique, M. Rabbani, I. Hussain, have planned and designed the research. R. Z. Abbas, A. Iqbal, A. Rafique has contributed to the experimental method. R. Ahmad, M.S. Mahmood, A. Lotfi is helping to give the final form of the manuscript. All contributors discussed the findings and contributed to the final manuscript.

References

1. Ashoori A, Kafash H, Varasteh Moradi H, Yousefi M, Kamyab H, Behdarvand N, Mohammadi S: Habitat modeling of the common pheasant *Phasianus colchicus* (Galliformes: Phasianidae) in a highly modified landscape: Application of species distribution models in the study of a poorly documented bird in Iran. *Eur Zool J,* 85, 372-380, 2018.

2. Ashraf S, Javid A, Ashraf M, Akram M, Malik S, Altaf M: Influence of egg weight on egg quality parameters and growth traits in ring necked pheasants *(Phasianus colchicus)* in captivity. *J Anim Plant Sci*, 26, 331-338, 2016.

3. Strakova E, Suchy P, Vitula F, Vecerek V: Differences in the amino acid composition of muscles from pheasant and broiler chickens. *Arch Tier*, 49, 508-514, 2006. DOI: 10.5194/aab-49-508-2006

4. Zhu H, Zhang H, Wang Y, Ciren D, Dong H, Wu Q, Rehman MU, Nabi F, Mehmood K, Xu J, Li J: Genetic characterization and evolutionary analysis of emerging newcastle disease virus isolated from tibetan chickens. *Kafkas Univ Vet Fak Derg*, 23, 385-389, 2017. DOI: 10.9775/kvfd.2016.16753

5. Abd-Elsamee MO, Fathy Abbas Motawe H, Maher Selim M, Ramadan Elsherif HM: Effect of different dietary crude protein levels and citric acid on broiler chickens' performance, carcass characteristics, intestinal morphology, and blood components. *World Vet J,* 10 (3): 362-374, 2020.

6. Mieczkowska A, Kokoszyński D, Wasilewski R, Bernacki Z: Carcass composition and meat quality of common pheasants *(Phasianus colchicus)* depending on sex of birds. *Food Sci Technol Qual*, 3, 95-106, 2015.

7. Rahman AU, Momena H, Muhammad ZS: Adaptation of Newcastle disease virus (NDV) in feral birds and their potential role in inter-species transmission. *Open Virol J,* 12, 52-68, 2018. DOI: 10.2174/1874357901812010052

8. Xiang B, Chen R, Liang J, Chen L, Lin Q, Sun M, Kang Y, Ding C, Liao M, Xu C, Ren T: Phylogeny, pathogenicity, and transmissibility of a genotype XII Newcastle disease virus in chicken and goose. *Transbound Emerg Dis*, 67, 159-170, 2020. DOI: 10.1111/tbed.13335

9. Igwe AO: Comparative study of the pathogenicity and pathogenesis of a local Nigerian velogenic Newcastle disease virus in guinea fowls and chickens. MSc Dissert University Nigeria, Nsukka, pp.112-116, 2009.

10. Diel DG, Susta L, Garcia SC, Killian ML, Brown CC, Miller PJ, Afonso CL: Complete genome and clinicopathological characterization of a virulent Newcastle disease virus isolate from South America. *J Clin Microbiol*, 50, 378-387, 2012. DOI: 10.1128/JCM.06018-11

11. Ibrahim DS, Elsanousi AA, ElDeeb AH, Arafa A, Selim AA: Effect of avian influenza (subtype H9N2) on the pathogenesis and virulence of velogenic Newcastle disease virus in chicken under experimental co-infection. *Int J Vet Sci,* 9, 136-140, 2020.

12. Aldous EW, Manvell RJ, Cox WJ, Ceeraz V, Harwood D, Shell W, Alexander DJ, Brown IH: An outbreak of Newcastle disease in pheasants (*Phasianus colchicus*) in South East England in 2005. *Vet Rec*, 160, 482-484, 2007. DOI: 10.1136/vr.160.14.482

13. Brown VR, Bevins SN: A review of virulent Newcastle disease viruses in the United States and the role of wild birds in viral persistence and spread. *Vet Res*, 48:68, 2017. DOI: 10.1186/s13567-017-0475-9

14. Wajid A, Dimitrov KM, Wasim M, Rehmani SF, Basharat A, Bibi T, Arif, S, Yaqub T, Tayyab, M, Ababneh M, Sharma P, Miller PJ, Alfonso CL: Repeated isolation of virulent newcastle disease viruses in poultry and captive non-poultry avian species in Pakistan from 2011 to 2016. *Prev Vet Med,* 142, 1-6, 2017. DOI: 10.1016/j.prevetmed.2017.04.010

15. Zhang S, Wang X, Zhao C, Liu D, Hu Y, Zhao J, Zhang G: Phylogenetic and pathotypical analysis of two virulent Newcastle disease viruses isolated from domestic ducks in China. *PLoS One*, 6 (9): e25000, 2011. DOI: 10.1371/journal.pone.0025000

16. Rehan M, Aslam A, Khan M, Abid M, Hussain S, Umber J, Anjum A, Hussain A: Potential economic impact of Newcastle disease virus isolated from wild birds on commercial poultry industry of Pakistan: A review. *Hosts Virus*, 6, 1-15, 2019. DOI: 10.17582/journal.hv/2019/6.1.1.15

17. Shabbir MZ, Goraya MU, Abbas M, Yaqub T, Shabbir MAB, Ahmad A, Anees M, Munir M: Complete genome sequencing of a velogenic viscerotropic avian paramyxovirus 1 isolated from pheasants *(Pucrasia macrolopha)* in lahore Pakistan. *J Virol,* 86, 13828-13829, 2012.

18. Mousa MR, Mohammed FF, Khalefah HS, El-Deeb AH, Ahmed KA: Comparative serological, histopathological and immunohistochemical evaluation of immune status of broiler chickens experimentally infected with velogenic Newcastle disease virus in different ages. *Int J Vet Sci*, 8 (3): 143-150, 2019.

19. Shabbir MZ, Malik AN, Wajid A, Rehmani SF, Munir M: Newcastle disease virus: Disease appraisal with global and Pakistan perspectives. *J Infect Mol Biol*, 1, 52-57, 2013.

20. Mahmood MS, Siddique F, Hussain I, Ahmad SI, Rafique A: Thermostable vaccines for Newcastle disease: A review. *World's Poult Sci J*, 70, 829-838, 2014. DOI: 10.1017/S0043933914000889

21. Chowdhary M, Nashiruddullah N, Abrol R, Sood S, Rahman S, Ahmed JA, Maqbool R: Newcastle disease and their pathology in fowls affected with genotype XIII and pigeons with genotype II. *Int J Curr Microbiol App Sci,* 9, 3800-3810, 2020. DOI: 10.20546/ijcmas.2020.910.437

22. Faisal S, Mahmood MS, Hussain I, Deeba F: Antibody response of broilers to cell culture adapted thermostable newcastle disease i-2 strain vaccine. *J Anim Plant Sci,* 25:1562-1565, 2015.

23. Siddique F, Mahmood MS, Hussain I, Deeba F: Evaluation of efficacy of Vero cell-adapted, thermostable Newcastle disease vaccine in broilers. *J Appl Poult Res*, 26, 145-153, 2017. DOI: 10.3382/japr/pfw055

24. Mahmood MS, Sabir R: Preparation and evaluation of avian influenza

(H9) and Newcastle disease (thermostable I-2 strain) bivalent vaccine for commercial poultry. *Agrobiol Rec,* 3, 17-23, 2021. DOI: 10.47278/journal.abr/2020.017

25. Carrasco AOT, Seki MC, Raso TF, Paulillo AC and Pinto AA: Experimental infection of Newcastle disease virus in pigeons *(Columba livia)*: Humoral antibody response, contact transmission and viral genome shedding. *Vet Microbiol*, 129, 89-96, 2008. DOI: 10.1016/j.vetmic.2007.11.012

26. Manar AAK, El-Kady MF, Shaheed IB: Pathogenesis of Newcastle disease virus genotype vii in chickens vaccinated with LaSota and inactivated Newcastle disease vaccines. *Int J Vet Sci*, 9 (2):196-202, 2020. DOI: 10.37422/IJVS/20.011

27. Adwar T, Lukešová D: Evaluation of thermostable vaccines against Newcastle disease in village chicken used in tropics and subtropics. *Agric Trop Subtrop,* 41, 74-79, 2008.

28. Echeonwu GON, Iroegbu CU, Echeonwu BC, Ngene A, Nwosu CI, Joannis TM, Ndako J: Immune response and protection of free range chickens vaccinated orally with feeding of Newcastle vaccine-coated cassava granules. *Afr J Microbiol Res*, 2, 120-125, 2008.

29. Zahid M, Irshad A, Shakoor S, Samiullah TR, Shahid N, Iqbal A, Tanveer S, Akram SJ, Ali MA, Ahmed S, Husnain T, Shahid AA, Rao AQ: Engineered recombinant NDV-Fusion protein and its polyclonal antibodies production. *Pak Vet J,* 40, 499-503, 2020. DOI: 10.29261/pakvetj/2020.034

30. Bensink Z, Spradbrow P: Newcastle disease virus strain I_2-a prospective thermostable vaccine for use in developing countries. *Vet Microbiol*, 68, 131-139, 1999. DOI: 10.1016/S0378-1135(99)00069-3

31. Musa U, Abdu PA, Mera UM, Emmenna PE, Ahmed MS: Vaccination with newcastle disease vaccines strain I_2 and LaSota in commercial and local chickens in Plateau State Nigeria. *Nigerian Vet J*, 31, 46-55, 2010. DOI: 10.4314/nvj.v31i1.68940

32. Wambura PN, Kataga S: Putative protective antibody response following oral vaccination of multi-age free ranging helmeted guinea fowls *(Numida meleagris)* with Newcastle disease virus strain I-2 coated on oiled rice. *Trop Anim Health Prod*, 43, 99-102, 2011. DOI: 10.1007/s11250-010-9659-2

33. Graczyk S, Wieliczko A, Pliszczak-Król A, Janaczyk B: Humoral and cellular response of pheasants vaccinated against Newcastle disease and haemorrhagic enteritis. *Acta Vet Brno*, 75, 379-386, 2006**.** DOI: 10.2754/avb200675030379

34. Schmidt EMS, Paulillo AC, Locatelli-Dittrich R, Beltrame O, Denadai J: Serum protein profiles of juvenile ring-necked pheasants vaccinated or not against Newcastle disease. *Int J Poult Sci*, 8, 359-362, 2009. DOI: 10.3923/ijps.2009.359.362

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

Kafkas Univ Vet Fak Derg
27 (2): 259-264, 2021
DOI: 10.9775/kvfd.2020.24929

CASE REPORT

Neuroendocrine Tumor of the Large Intestine in a Dog

Sebastian SLODKI [1,2,a (*)] Joanna BOGUCKA [1,b]

[1] UTP University of Science and Technology, Faculty of Animal Breeding and Biology, Department of Animal Physiology, Physiotherapy and Nutrition, Mazowiecka 28, 85-084 Bydgoszcz, POLAND
[2] Sebastian Słodki 'SweetVet' Bydgoszcz, Wyzwolenia 60,85-790 Bydgoszcz, POLAND
ORCIDs: [a] 0000-0002-3433-7512; [b] ORCID: 0000-0001-5298-1144

Article ID: KVFD-2020-24929 Received: 06.09.2020 Accepted: 31.01.2021 Published Online: 01.02.2021

Abstract

This paper describes the diagnosis and treatment of a 5-year-old Standard Schnauzer dog admitted to the clinic due to a liver failure and chronic bloody diarrhea. Based on the initial examination, no diagnosis could be made, therefore an endoscopic examination of the gastrointestinal tract was performed. The endoscopy revealed a tumor within the ostium *ileocaecocolicum*. Unfortunately, shortly after this procedure, a massive hemorrhage occurred, which led to the animal's death. The necropsy confirmed the presence of the tumor. The histopathological and immunohistochemical results of the collected specimens showed a neuroendocrine tumor (NET G1) of the large intestine. In conclusion, gastrointestinal carcinoids in dogs are relatively rare and often misdiagnosed due to complex or non-specific symptomatology.

Keywords: *Dog, Immunohistochemistry, Large intestine, Neuroendocrine carcinoma*

Bir Köpekte Kalın Bağırsak Nöroendokrin Tümörü

Öz

Bu makale, karaciğer yetmezliği ve kronik kanlı ishal nedeniyle kliniğe kabul edilen 5 yaşındaki bir Standart Schnauzer köpeğinde nöroendokrin tümör teşhisini açıklamaktadır. İlk muayeneye bağlı tanı konulamadığı için köpeğin gastrointestinal sisteminin endoskopik muayenesi yapıldı. Endoskopik muayenede *ostium ileocaecolicum* içerisinde bir tümöre rastlandı. Ne yazık ki, bu işlemden kısa bir süre sonra, hayvanın ölümüne yol açan büyük bir kanama meydana geldi. Otopside tümörün varlığı doğrulandı. Alınan örneklerin histopatolojik ve immünohistokimyasal analizi sonucu, kalın bağırsakta bir nöroendokrin tümör (NET G1) belirlendi. Sonuç olarak, köpeklerde gastrointestinal karsinoidler nispeten nadirdir ve karmaşık veya spesifik olmayan semptomatolojisi nedeniyle sıklıkla yanlış teşhis edilirler.

Anahtar sözcükler: *İmmunohistokimya, Kalın bağırsak, Köpek, Nöroendokrin karsinom*

Introduction

Neuroendocrine tumors (NETs) derived from the diffuse endocrine system cells are rare cancers in both humans and animals, including dogs [1]. In most cases, they are localized in the digestive tract, and over 50% of them are carcinoids. Diagnosis of neuroendocrine tumors, including carcinoid carcinoma, is challenging because of the atypical set of disease symptoms and often the small size of the active tumor, and the difficulty in detecting in medical imaging [2]. In our case, we describe a detailed diagnostic procedure that leads to the diagnosis of the disease, i.e. case history, and clinical examination.

Case History

A miniature Schnauzer male five years old dog was admitted to the Veterinary Office, in June 2017. The dog (weight 11.2 kg) was diagnosed with liver insufficiency and hypothyroidism. Despite the treatment with medicines containing phospholipids, ornithine, silymarin, and the hepatic diet, the results of laboratory tests were outside the reference ranges. The blood tests presented by the owner performed in another veterinary clinic showed the following results: aminotransferase - alanine (ALT 236.3 U/L), aspartate aminotransferase (AST 94 U/L), total protein (TP 76.5 g/L), total bilirubin (BIL) 3.9 µmol/L, urea (UREA

How to cite this article?

Slodki S, Bogucka J: Neuroendocrine tumor of the large intestine in a dogs. *Kafkas Univ Vet Fak Derg,* 27 (2): 259-264, 2021.
DOI: 10.9775/kvfd.2020.24929

(*) Corresponding Author

Tel: +48 503 770 268
E-mail: sebastian.slodki@utp.edu.pl (S. Slodki)

8.62 mmol/L), free T4 (free thyroxine-fT4) 1.07 ng/dL, cholesterol 7.39 mmol/L. The other biochemical blood parameters did not differ from the reference values. The general condition of the dog was good. The palpation of the liver revealed an enlargement and slight pain. The ultrasound examination (US) showed moderately enlarged liver with mild remodeling features; liver edges rounded without focal changes, gallbladder size around 1 cm, a smooth wall without visible stones and deposits, bile ducts not dilated. The ultrasound image of the rest of the internal organs was normal. The following treatment was introduced: timonacic (Heparegen 100 mg, Jelfa Ltd., Jelenia Gora, Poland) 2 x 0.5 tablet, ursodeoxycholic acid (Ursocam 250 mg, Polfarmex Ltd., Kutno, Poland) 1 x 0.25 tablet, silybin 30% (Hepaxan Dog tablets, Vebiot, Debica, Poland) 1 x 2 tablets, levothyroxine (Euthyrox100 µg, Merck KGaA, Darmstadt, Germany) 1 tablet twice a day and a blood test in 3 weeks.

At the end of April 2018, blood and mucus appeared in the stool. During the clinical examination, the general condition was good, the abdomen slightly taut, in the rectum the feces with an admixture of mucus and traces of blood. Symptomatic treatment, fluid therapy, anti-hemorrhagic treatment, and antibiotic therapy were applied. The day after the examination, the dog's clinical condition improved; after another five days, bloody diarrhea, single vomiting, aversion to water, and food appeared. Additional blood tests were performed, including lipase (reference values) as well as x-ray and control abdominal ultrasound. The radiological examination did not show foreign bodies in the gastrointestinal tract or features indicative of intestinal obstruction. The abdominal US revealed large amounts of gases, with a well-preserved wall layer, gallbladder size about 2.5 cm with the presence of numerous deposits. The wall of the intestine in the area of the *ostium ileo-caecocolicum* showed pathologic thickening of the wall and disturbance of the layered structure on the section of about 15 mm, which suggested proliferative changes; the liver normoechogenic, without focal changes, other organs within the normal range. In the morphological examination of the blood, apart from the increase in the level of leukocytes 24 G/L and thrombocytes 690 G/L, the remaining parameters were within normal limits. The hepatic parameters of ALT and AST were respectively: 324.72 U/L and 92.736 U/L. The drugs used included drotaverine (No-spa 20 mg/mL, Sanofi Aventis Ltd., Warsaw, Poland) - 1 mL subcutaneously, ranitidine (Solvertyl 25 mg/mL, ICN Polfa Ltd., Rzeszow, Poland) - 1 mL subcutaneously, tylosin (Biotyl 50 mg/mL, Biowet Drwalew Ltd., Drwalew, Poland) 1.3 mL subcutaneously seven days, and maropitant citrate (Cerenia10 mg/mL, Zoetis Poland Ltd., Warsaw, Poland) - 1 mL subcutaneously. The pharmacological treatment was continued, additional metronidazole (Metronidazol Polpharma 250 mg, Polpharma, Warsaw, Poland) 2 x 0.5 tablet for seven days was administrated and a stool test for the presence of parasites was performed. The flotation result and *Giardia* spp-antigen (ELISA) were negative. Unfortunately, despite the treatment admixtures of mucus and a small amount of blood were present in the stool; therefore, the endoscopic examination of the gastro-intestinal tract was recommended, and phytomenadione was orally prescribed (Vitacon10 mg, Polfa Warszawa, Warsaw, Poland), 2 x tablet daily.

At the end of June 2018, the morphology showed a decrease in erythrocytes 5.25 T/L (5.5-8.5), a decrease in hemoglobin of 11.4 g/dL, and an increase in thrombocytes of 595 G/L. Over the next days, there was a further decrease in erythrocytes (4.93 T/L), hematocrit (30%), hemoglobin (8.3 g/dL). After ten days, the level of erythrocytes dropped to 3.86 T/L, hematocrit 22.8%, hemoglobin 7 g/dL. Despite the deteriorating blood test results, the dog's clinical condition was better and the occurrence of bleeding from the gastrointestinal tract was much less. Oral preparations with iron and folic acid were introduced, and blood was collected on reticulocytes, B12, iron, folic acid, and blood group determination. The level of reticulocytes was 4.75%, iron 3.30 µg/mL, folic acid 22.4 ng/mL, and vitamin B12 804.0 pg/mL. During the following days, the patient's condition did not change significantly.

At the end of August, an infusion colonoscopy was performed under general anesthesia. In the endoscopic examination, the device was introduced into the ileocecal valve area. At this point, the area of the highly congested and slightly bleeding mucous membrane was found *(Fig. 1)*. Also, tissue samples for histopathological examination were collected from this area. Other sections of the mucous membrane were normal. The image of the examination raised the suspicion of intestinal cancer. The day after the colonoscopy, the dog was in clinically stable condition.

On the fourth day after the procedure, the patient's condition deteriorated suddenly. According to the information

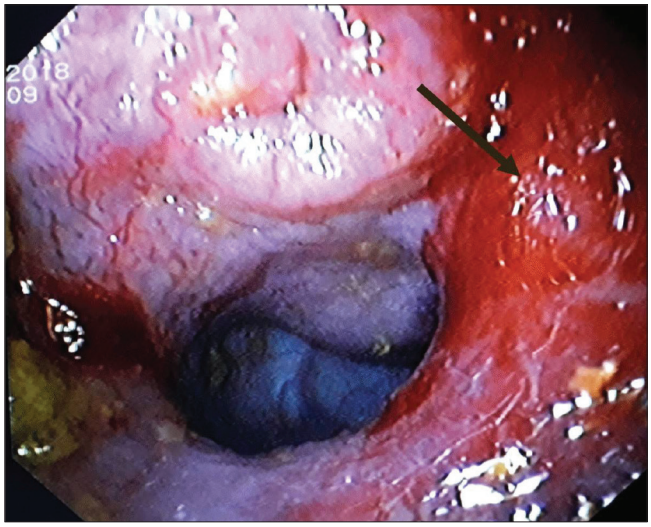


Fig 1. Colonoscopy- inflammation of the mucosa with bleeding in the area of the ostium ileocaecocolicum

obtained from the owner, the dog was very weak, breathed heavily, did not eat or drink, and there was much blood in the feces. In the clinical examination, a very bad general condition was found, conjunctiva and mucous membranes were porcelain white. The stomach was painful palpable, and the spleen strongly enlarged. The dog was administrated 1 mL of dexamethasone (Dexasone 2 mg/mL, ScanVet Poland, Warsaw, Poland) intravenously, and blood was collected for morphology. The blood glucose level was above 600 mg/dL and 5 iu of insulin subcutaneously (Caninsulin 40 iu/mL, MSD Animal Health Poland, Warsaw, Poland) was given. Approximately 30 min after the administration of the drugs, there was an acute cardiorespiratory failure followed by cardiac arrest. Despite the attempted resuscitation, it was not possible to regain vital functions.

We obtained permission from the owners to conduct the necropsy. The examination showed properly developed subcutaneous tissue and muscles, pale red, minor blood extravasation in muscle tissue. A small amount of bloody, clear fluid in the abdomen was present. The digestive tract was gassed, filled with a small amount of mushy food, light brown. In the large intestine, tarry digestive content with visible blood clots was found**.** The necropsy also revealed an enlarged, swollen, and congestive spleen without focal lesions and a thickened, hard fragment of the intestine in the ostium ileocaecocolicum area *(Fig. 2)*. After an incision of the intestinal wall, an extremely altered mucosa with visible ulceration and submucosal hemorrhages, enlarged local lymph nodes, and a structure resembling a neoplastic

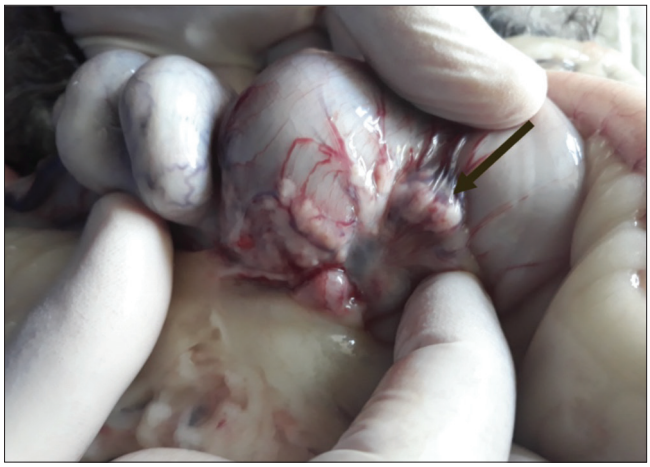


Fig 2. Necropsy - the affected area of the intestine

infiltration were found, the intestinal lumen was filled with blood in this part. The liver was enlarged, firm in consistency, bright red, with no visible nodular changes.

The kidneys were of normal size and shape. The nerve capsule was easily removable, the ratio of the cortex to the medullary layer was maintained. In the chest, a small amount of bloody fluid was found. Lungs were of normal size and shape, aerated, light red. The heart was of normal size and position, with a small amount of clotted blood in the left ventricle. Apart from the primary neoplastic tumor, no macroscopic metastatic changes in the abdominal and thoracic cavity organs were found.

The samples were fixed in a buffered formalin solution and sent to the accredited Laboratory of Oncological Prevention and Diagnostics "Patolog" J&J Głowaccy. The above-mentioned laboratory prepared the preparations using the paraffin technique and performed H&E and mucicarmine staining. The immunohistochemical tests were conducted using Roche's antibodies against synaptophysin, cd 56, ki 67, and chromogranin A, according to the procedures developed by this company *(Table 1)*.

Postmortem samples for histopathological and immunohistochemical examinations were collected.

The result of the histopathological examination of the intestinal wall sections taken in vivo during the endoscopic examination did not give the final diagnosis. In the tissue collected during colonoscopy, the presence of dilated, thrombotic capillaries in the vessels of the lamina propria, and the resulting circulatory disturbances, as well as the accompanying slight lymphatic infiltration were found. The above-described histopathological changes may be the cause of lamina propria bleeding even in minor injuries. The histopathological examination of the specimen taken during the section indicated the presence of carcinoid (neuroendocrine tumor-NET G1) of the large intestine. In this case, the routine H&E staining and immunohistochemistry were performed. The H&E staining revealed the presence of numerous tumor cells in small clusters in the submucosa of the intestinal wall. The microscopic examination revealed small tumor cells, concentrated in clusters, round nucleoli, few figures of the mitotic division *(Fig. 3, Fig. 4)*. There were also single clusters of tumor cells in lymph and capillary vessels *(Fig. 5)*. Staining of synaptophysin *(Fig. 6)* and cd 56 *(Fig. 7)* gave a positive result which confirmed the neuroendocrine activity of tumor cells. Chromogranin A

Table 1. *Panel of primary antibodies used in immunohistochemical techniques*

Antibody	Source	Clone	Dilution	Result
Synaptophysin	Roche	Rabbit Monoclonal Antibody (MRQ-40)	00.04 µg/mL	(+)
Chromogranin A	Roche	Mouse Monoclonal (LK2H10)	1 µg/mL	(-)
CD56	Roche	Rabbit Monoclonal Antibody (MRQ- 42)	0.21 µg/mL	(+)
Ki67	Roche	Rabbit Monoclonal Antibody (30-9)	2 µg/mL	(+)

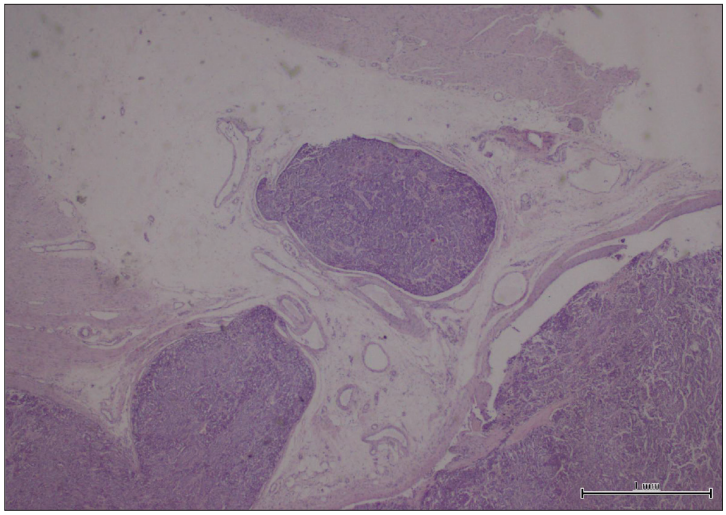


Fig 3. Histological picture of small tumor cells (neuroendocrine tumor - NET G1) of the large intestine. H & E staining. Bar=1 mm

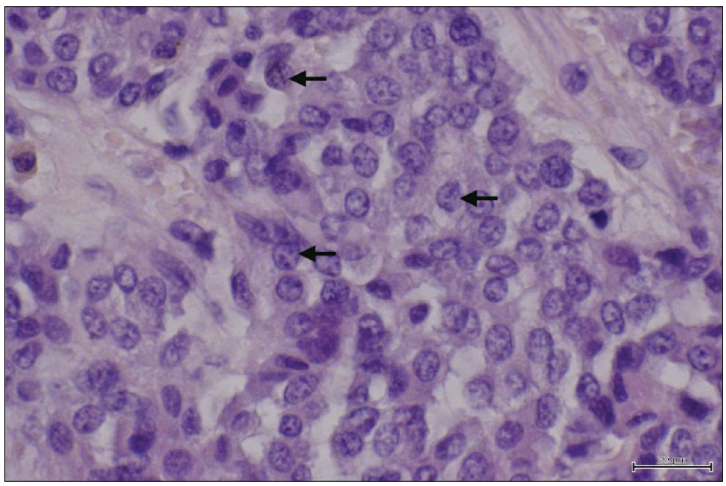


Fig 4. Carcinoma histological image. Small tumor cells with round nucleoli and few figures of mitotic division . Bar=20 µm

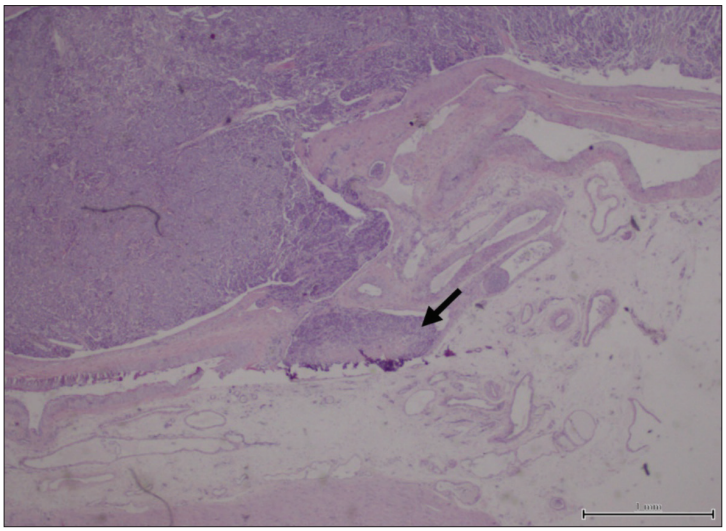


Fig 5. Cancer cells infiltration in lymphatic vessels. H&E staining. Bar=1 mm

staining did not give positive results in both the control sample and tumor cells, which indicates the unsuitability of selected antibodies to staining on dog tissues. Other antibodies should be chosen in this case, or the staining methodology should be modified. However, the positive synaptophysin staining results, and cd 56 were enough to make the diagnosis. In contrast, Ki 67 showed a very low sub-cell index based on which the tumor was classified as NET G1 *(Fig. 8)*. Also, mucicarmine staining was performed to demonstrate the tumor's exocrine capacity and the precise secretion of mucus. This time the staining was negative, which means that the tumor has only endocrine activity and does not produce mucus.

Discussion

Carcinoids pose a diagnostic challenge because they are often innocuous at the time of presentation, requiring a multidisciplinary diagnostic approach with detailed biochemical analysis, cross-sectional and nuclear medicine imaging [3]. Neuroendocrine tumors have rarely been reported in dogs mainly in the intestine, liver, bile duct, lungs, gallbladder, esophagus, skin, and nasal cavity [4]. The most common laboratory techniques used to diagnose the GEP-NEN include the following tests: fasting blood glucose, insulin, and C-peptide concentrations, serum gastrin concentration and gastric pH, serum glucagon concentration, serum VIP concentration, serum chromogranin A concentration, concentration of 5-hydroxyindoleacetic acid in a 24-h urine sample. Tests for chromogranin A and 5-HIAA can give false-positive results for various reasons [5].

A tumor location is crucial for diagnosis and treatment; however, no one technique is sensitive enough; hence, CT (Computed Tomography) or MRI (Magnetic Resonance Imaging) scans should be combined with nuclear medicine imaging [6]. Detection of the tumor and finding its location also created significant problems. It was only at the end of the disease when the tumor was detected in the ultrasound examination, which was then confirmed in the endoscopic examination. Performing CT or MRI scans would have probably helped to diagnose the patient earlier.

However, the final diagnosis was made based on histopathological examinations, in particular, immunohistochemistry performed from collected ex-vivo or post-mortem tissue of the tumor. In addition to the classic H&E staining, to assess the tumor texture and cell structure, immunohistochemical staining (for example, Ki67) allows determining the division capacity of the tumor cells, the so-called

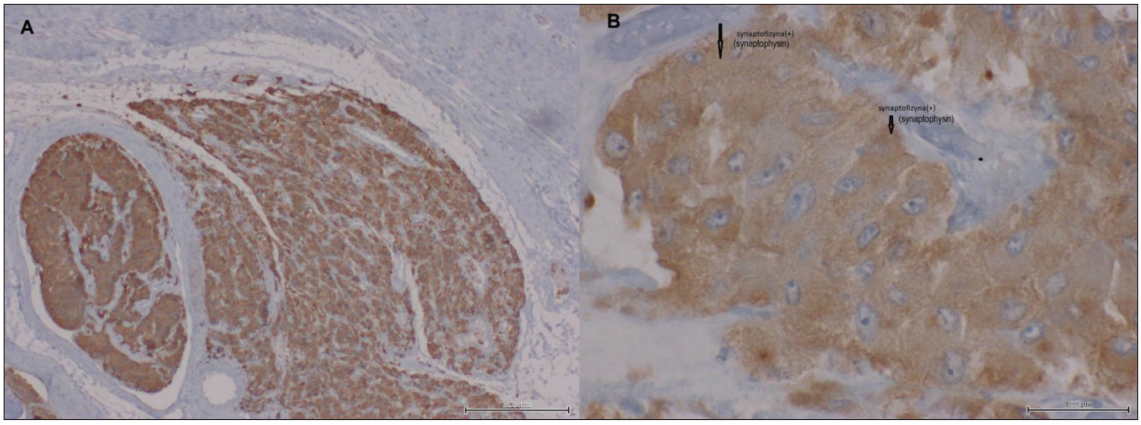


Fig 6. Immunohistological picture of neuroendocrine tumor NET-G1. Positive intracytoplasmic synaptophysin staining. A -Bar=200 µm; B-Bar-=100 µm

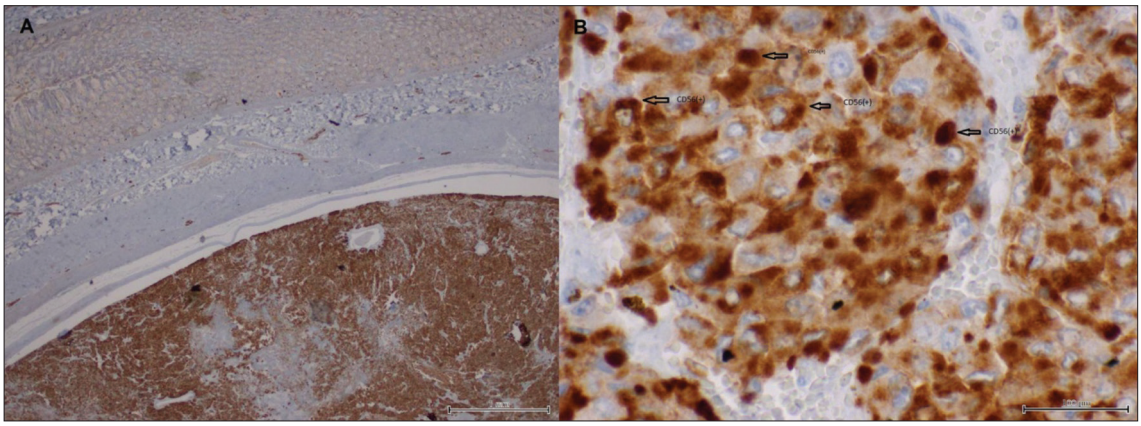


Fig 7. Positive intracytoplasmic CD 56 staining. Single cells within the mucosa. A-Bar=1 mm; B=100 µm

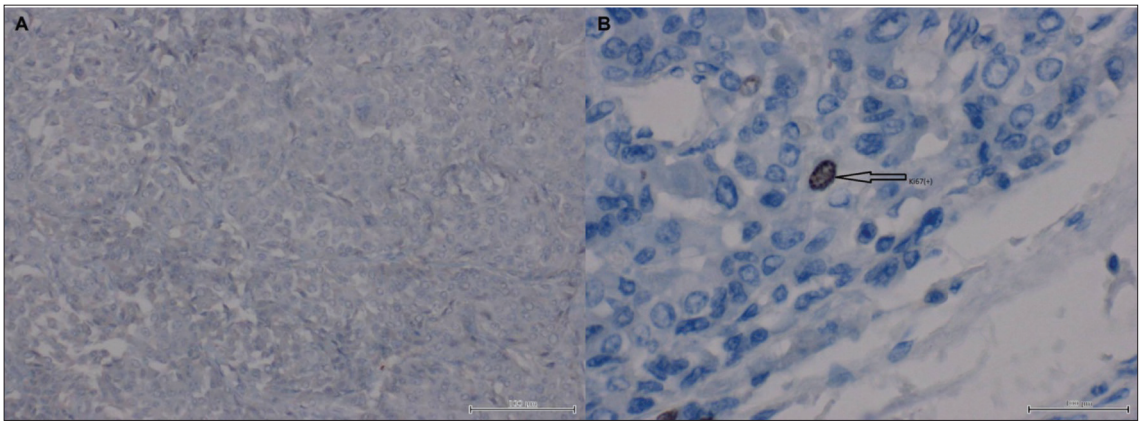


Fig 8. Positive intranuclear Ki-67 staining proliferaion index 3.A-Bar=200 µm ; B=100 µm

mitotic index, which is important in the classification of cell malignancy and their tendency to metastasis. In our case, there was no metastasis observed in other organs. Additional staining, such as synaptophysin, chromogranin A, cd 56, allows demonstrating the neuroendocrine activity of the tumor, which is characteristic for carcinoid cells. The mucicarmine staining for the presence of mucus assesses whether the tumor also has exocrine features. Clinical symptoms depend on the functionality of the tumor. The hormonal activity of tumor cells determines the specific set of symptoms and endocrinological disorders, e.g., insulinoma, glucagonoma, gastrinoma, VIPoma [7]. In the case of our patient, we suspect that chronic liver failure may have been caused by endocrine and biochemical disorders associated with the presence of the tumor. Although non-functioning NETs may secrete hormones [8],

they do not cause any symptoms. Therefore, they are often diagnosed later in the course of the disease with symptoms of metastasis [7].

In conclusion, in our report, we present a neuroendocrine tumor NET, G1 (based on the 2019 WHO Classification) [9] of the gastrointestinal tract in a dog. The extreme difficulty in diagnosing the carcinoid was due to non-specific symptoms, the early onset of the disease, and the limited availability of specialized imaging techniques, including MRI and CT.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contribution

J. Bogucka and S. Slodki conceived and designed the study. S. Slodki collected the data. J. Bogucka analyzed the data. All authors interpreted the data, draft the manuscript, critically revised the manuscript for important intellectual contents, and approved the final version. All authors are in agreement with the content of the manuscript.

Acknowledgements

The pathological part of the study was conducted by the Laboratory of Oncological Prevention and Diagnostics "Patolog" J&J Głowaccy.

References

1. Modlin IM, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP, Moss SF, Nilsson O, Rindi G, Salazar R, Ruszniewski P, Sundin A: Gastroenteropancreatic neuroendocrine tumors. *Lancet Oncol,* 9, 61-72, 2008. DOI: 10.1016/S1470-2045(07)70410-2

2. Kuwata K, Shibutani M, Kemmochi Y, Taniai E, Morita R, Ogawa B, Mitsumori K: A neuroendocrine carcinoma of undetermined origin in a dog. *J Toxicol Pathol,* 23 (3): 151-155, 2010. DOI: 10.1293/tox.23.151

3. Dasari A, Shen C, Halperin D, Zhao B, Zhou S, Xu Y, Shih T, Yao JC: Trends in the incidence, prevalence, and survival outcomes in patients with neuroendocrine tumors in the United States. *JAMA Oncol,* 3 (10): 1335-1342,2017. DOI: 10.1001/jamaoncol.2017.0589

4. Head KW, Else RW, Dubielzig RR: Tumors of the alimentary tract. **In,** Meuten DJ (Ed): Tumors in Domestic Animals. 4th ed., 401-481, Iowa State Press, Ames, IA, 2002.

5. Schott M, Klöppel G, Raffel A, Saleh A, Knoefel WT, Scherbaum WA: Neuroendocrine neoplasms of the gastrointestinal tract. *Dtsch Arztebl Int,* 108 (18): 305-312, 2011. DOI: 10.3238/arztebl.2011.0305

6. Plöckinger U, Rindi G, Arnold R, Eriksson B, Krenning EP, de Herder WW, Goede A, Caplin M, Oberg K, Reubi JC, Nilsson O, Delle Fave G, Ruszniewski P, Ahlman H, Wiedenmann B: Guidelines for the diagnosis and treatment of neuroendocrine gastrointestinal tumours. *Neuroendocrinology,* 80, 394-424, 2004. DOI: 10.1159/000085237

7. Leoncini E, Carioli G, La Vecchia C, Boccia S, Rindi G: Risk factors for neuroendocrine neoplasms: A systematic review and meta-analysis. *Ann Oncol,* 27, 68-81, 2016. DOI: 10.1093/annonc/mdv505

8. Garcia-Carbonero R, Capdevila J, Crespo-Herrero G, Díaz-Pérez JA, Martínez Del Prado MP, Alonso Orduña V, Sevilla-García I, Villabona-Artero C, Beguiristain-Gómez A, Llanos-Muñoz M, Marazuela M, Alvarez-Escola C, Castellano D, Vilar E, Jiménez-Fonseca P, Teulé A, Sastre-Valera J, Benavent-Viñuelas M, Monleon A, Salazar R: Incidence, patterns of care and prognostic factors for outcome of gastroenteropancreatic neuroendocrine tumours (GEP-NETs): Results from the National Cancer Registry of Spain (RGETNE). *Ann Oncol,* 21 (9): 1794-1803, 2010. DOI: 10.1093/annonc/mdq022

9. Nagtegaal ID, Odze RD, Klimstra D, Paradis V, Rugge M, Schirmacher P, Washington KM, Carneiro F, Cree IA: The 2019 WHO classification of tumours of the digestive system. *Histopathology,* 76 (2): 182-188, 2020. DOI: 10.1111/his.13975

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

Kafkas Univ Vet Fak Derg
27 (2): 265-269, 2021
DOI: 10.9775/kvfd.2020.25113

CASE REPORT

A Case of Ventral Abdominal Hernia Associated with an Ectopic Egg in an Albino Budgerigar and Evaluation by Infrared Thermography

Buse ERSENAL [1,a (*)] Serhat ÖZSOY [2,b]

[1] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa, TR-34320 Avcılar, Istanbul - TURKEY
[2] Department of Wild Animal Diseases and Ecology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa, TR-34320 Avcılar, Istanbul - TURKEY
ORCIDs: [a] 0000-0003-2404-2988; [b] 0000-0003-0988-7691

Article ID: KVFD-2020-25113 Received: 28.10.2020 Accepted: 02.02.2021 Published Online: 03.02.2021

Abstract

A four-year-old albino budgerigar was presented to Department of Wild Animal Diseases and Ecology clinic, Faculty of Veterinary Medicine, İstanbul University-Cerrahpaşa, with complaints of abdominal swelling. An ectopic egg and abdominal hernia were diagnosed as a result of radiographic evaluation, and then the hairless, swollen abdominal surface area was evaluated by infrared thermography. In a budgerigar, an abdominal hernia case with ectopic egg was presented for the first time and evaluated for the first time by thermography. In thermographic imaging, the maximum temperature was obtained from the skin surface area on the right side of the hernia sac corresponding to the area where the egg was found on the radiograph. It is thought that heat increase has occurred due to inflammatory fibrin formation process around the ectopic egg. Clinical parameters of the patient were in normal range so the surgical operation was successfully performed under general anesthesia, and the ectopic egg was removed and the abdominal hernia was repaired.

Keywords: *Thermography, Budgerigar, Ectopic egg, Abdominal hernia*

Bir Albino Muhabbet Kuşunda Ektopik Yumurta İle İlişkili Ventral Abdominal Fıtık Olgusu ve Kızılötesi Termografi İle Değerlendirilmesi

Öz

Dört yaşındaki albino muhabbet kuşu, İstanbul Üniversitesi-Cerrahpaşa, Veteriner Fakültesi Yabani Hayvan Hastalıkları ve Ekoloji Anabilim Dalı kliniğine karın şişliği şikayeti ile getirildi. Radyografik değerlendirme sonucu ektopik yumurta ve ventral abdominal fıtık varlığı teşhis edildi ve daha sonra kızılötesi termografi ile tüysüz, şişmiş karın yüzeyi değerlendirildi. Muhabbet kuşunda, ektopik yumurta ile birlikte görülen abdominal fıtık olgusu ilk kez sunuldu ve termografi ile ilk kez değerlendirildi. Termografik görüntülemede, yumurtanın röntgende bulunduğu alana karşılık gelen, fıtık kesesinin sağ tarafındaki deri bölgesi yüzeyinden maksimum sıcaklık elde edildi. Ektopik yumurta çevresindeki enflamatuar fibrin oluşum süreci nedeniyle ısı artışının meydana geldiği düşünülmektedir. Hastanın klinik parametreleri normaldi, bu nedenle cerrahi operasyon genel anestezi altında başarıyla uygulandı, ektopik yumurta uzaklaştırıldı ve karın fıtığı onarıldı.

Anahtar sözcükler: *Ektopik yumurta, Karın fıtığı, Muhabbet kuşu, Termografi*

INTRODUCTION

Egg production, together with the complex structure of the female sex organs, causes reproductive diseases [1]. Eggs called "ectopic eggs" or "internal laying", they become extraoviductal due to reverse peristalsis in the infundibulum or rupture of the oviduct and fall into the abdominal cavity [2,3]. Therefore, egg binding and ectopic egg reproductive diseases may show similar clinical signs in birds such as abdominal enlargement, soiled feathers at the vent and base of the tail, retention of droppings and tail pumping [2,4]. An abdominal hernia appears in relation with egg laying and weak abdominal muscles in Psittacine species especially budgerigars with clinical signs of abdominal swelling and skin surface changes [5,6].

How to cite this article?

Ersenal B, Özsoy S: A case of ventral abdominal hernia associated with an ectopic egg in an albino budgerigar and evaluation by infrared thermography. *Kafkas Univ Vet Fak Derg,* 27 (2): 265-269, 2021.
DOI: 10.9775/kvfd.2020.25113

(*) Corresponding Author

Tel: +90 507 338 9119
E-mail: buse_ersenal@hotmail.com (B. Ersenal)

Egg-binding, ectopic egg, ectopic egg yolk and peritonitis cases require application of female reproductive system surgery for urgent intervention; and also hernia also requires the herniorapy, after radiological diagnosis [1,6-10]. Radiological diagnosis shows where the egg is located in the abdominal cavity and what the hernial content is [3,11]. Infrared thermography is a complementary diagnostic method that can evaluate temperature changes on the body surface and in many animal species it is becoming more and more important in the diagnosis of various types of diseases [12-15]. Also, in avian research, there have been many studies involving the welfare and physiological thermal images of birds [16-20]. In this study, the presence of a ventral abdominal hernia simultaneously with an ectopic egg in a budgerigar and its evaluation by thermography is presented for the first time.

Case History

A four-year-old albino budgerigar was referred with one month history of abdominal swelling. On physical examination; pulse rate (272 beats/min), respiratory rate (65 breaths/min) and general state of the bird were in normal ranges but spherical swelling was located in the ventral abdomen and pericloacal region *(Fig. 1)*. Her diet consisted of a commercially available food rich for seeds, and herappetite and defecation were normal and she was not laying for two years according to anamnesis. After physical examinations, radiographic and then termographic evaluation by FLIR Series E50 (FLIR Systems AB) were obtained. According to the radiograph findings, intestines and an egg were detected in the hernia sac *(Fig. 2)*. Thermography detects the points that give the maximum and minimum temperature in the scanned area. In thermograms, the warmest areas appear as white, the coldest areas as blue and black [13]. In this case, when the entire hernia sac was scanned by thermography *(Fig. 3)*, it was found that the area was wider and white in color (warmer) at the right side, and also red spot showed the maximum temperature (41.2°C), which corresponded to the area where the egg was radiographly determined. The thermal image was taken at a distance of 40 cm from the un-feathered swollen area and the emission rate was taken from 0.96. Based on the clinical and radiographic examinations, it was decided that surgical application should be performed. Antibiotic (Amoklavin Ped. 100 mg/kg PO, for 2 days, Deva, Turkey) was provided before surgery. A bird was restrained in dorsal recumbency under the 2% isoflurane anesthesia and oxygen 2L through a breathing mask. Serum supplementation was applied during the operation (%5 Dekstroz Laktatlı Ringer 1 mL SC, PF, Turkey). The skin was prepared for an aseptic operation

Fig 1. An albino budgerigar with abdominal swelling

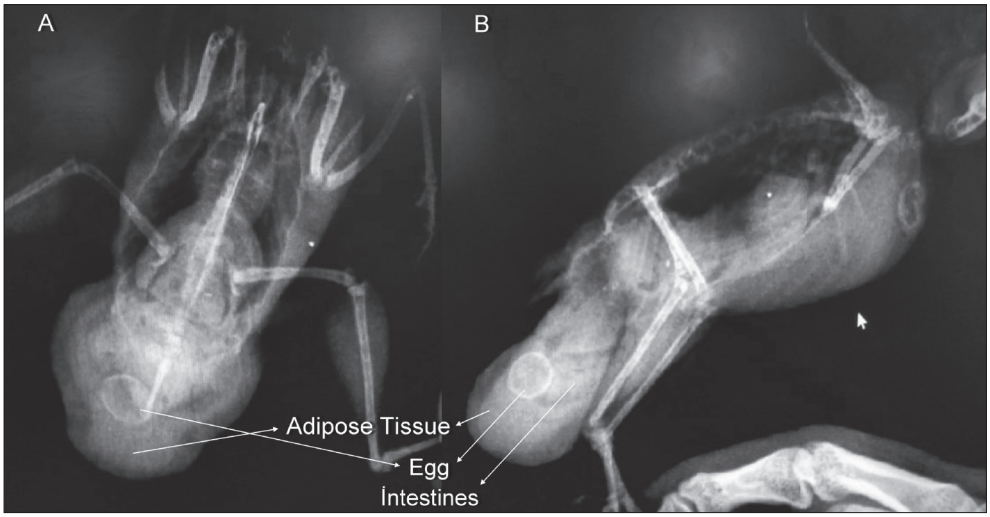


Fig 2. On the ventrodorsal (A) and right laterolateral radiographs (B); intestines and egg are visible in the hernia sac

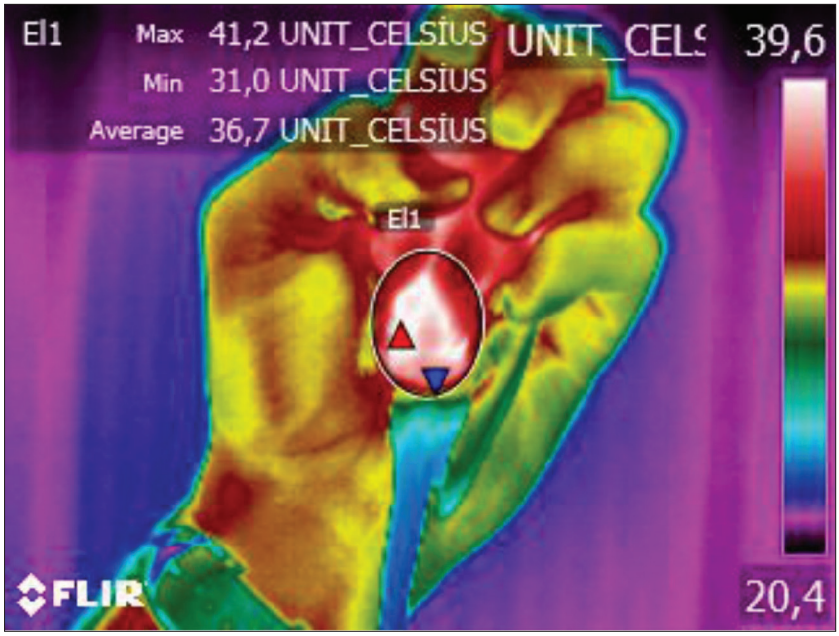


Fig 3. Thermographic evaluation of abdominal hernia. Red spot shows the maximum temperature for egg region.*Color should be used for this figure in print

Fig 4. Ectopic egg removed from the coelomic cavity

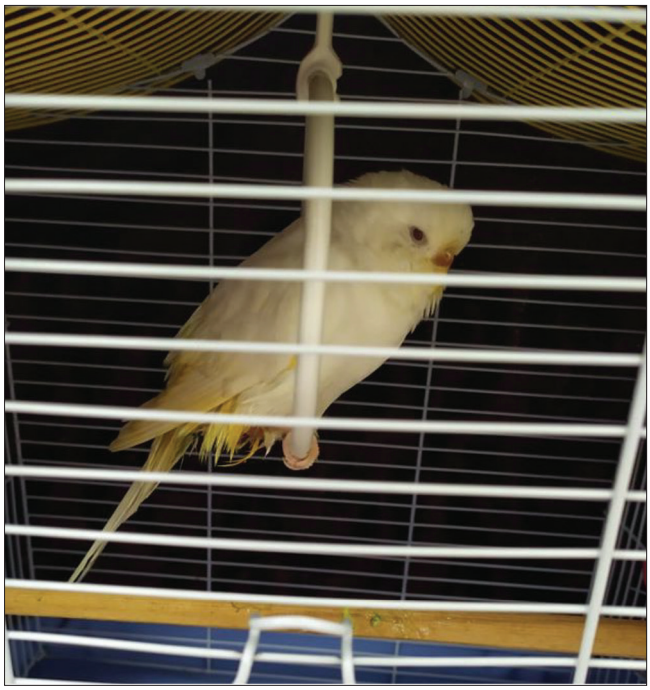


Fig 5. Bird stood up half an hour after surgery

and surgical intervention with transabdominal incision through the skin and abdominal muscles ensured. After the incision, the ectopic egg surrounded by the fibrin mesh first appeared in the hernia sac, and the intestines were behind it. The egg *(Fig. 4)* and fibrin mesh were first removed, then all the contents were returned to the abdominal cavity, the size of the swollen hernia sac reduced by incisions for normal size and the abdominal wall closure was successfully performed. Both the abdominal muscles and the incision line were sutured by a simple continuous suture pattern using No. 4/0 monocryl. Pain reliever meloxicam (Meloxicam 0.5 mg/kg SC, Bavet, Turkey) was given and the bird stood up half an hour after surgery *(Fig. 5)*. Amoxicillin clavulanic acid (Amoklavin Ped. 100 mg/kg PO, for 5 days, Deva, Turkey), multivitamin and calcium supplements were prescribed. Vegetables were added to the diet and movement restriction recommended. Five days after the operation, her general condition improved and she regained her health.

Discussion

The presence of an ectopic egg is vital in small birds [4]. Ectopic eggs may easily move around the abdomen or the egg yolk may enter the abdominal cavity and cause yolk peritonitis [3,9]. Chronic laying, oviductal trauma, or oviductal infection are common causes for ectopic eggs, egg-binding and egg-related coelomitis [2]. Poor breeding conditions and stress, especially hypocalcemia, systemic diseases, oviduct muscle weakness, genetic predisposition may also predispose factors [3,21]. Clinical signs include broody or egg-laying behavior without egg production, abdominal distention, soiled feathers at the vent and base of the tail, tail wagging, imbalance, sit on the floor, obturator paresis, oxygen deficiency and at the end death [2,22]. Good prognosis depends on the correct treatment done in time [22]. Abdominal hernias in birds can be congenital or acquired and often associated with reproduction, egg binding or straining, endocrine imbalances, hormonal effects, other causes such as hepatic lipidosis, malnutrition, coelomic masses,and urate concretion [23,24]. In this case, the ectopic egg, which was perceived as a mass in the coelomic cavity,

was thought to trigger hernia development. Abdominal hernias in birds are not true hernias because there is no opening in the aponeurosis of abdominal muscles so skin was the only hernia sac structure holding the content of abdominal organs [24]. Radiographic and ultrasonographic examinations are used to visualize the hernia content and egg to confirm the diagnosis [11,25]. Infrared thermography, which is a developing diagnostic method of assessing surface emitted temperature, is in the field of obstetrics and gynecology, in different races, in the evaluation of different pathological and physiological conditions such as mastitis, mammary tumors, detection of oestrus, pregnancy and omphalitis [13-15,26-30]. However, according to our research thermography has not been studied in avian reproductive diseases. Because the well feathered areas are closer to the ambient temperature, the region that gives the most accurate surface temperature in avian species is the un-feathered regions [16]. In this case, the image was easily taken, as the enlarged sac was un-feathered. Infrared thermography is able to discover changes that have not yet caused clinical signs in apparently healthy subjects and provides significant advantages thanks to its ability to measure surface temperature remotely, non-invasively and quickly in crowded bird populations [16,31]. For normal tissue repair due to peritoneal injury, an inflammatory reaction is initiated that produces fibrous exudates and causes fibrin formation [10,32]. In the formed fibrin, erythrocytes, leukocytes and platelets make a network that accumulates, thereby forming a blood clot [33]. In this case, the ectopic egg appears to be perceived as a foreign body causing irritation in the coelom and initiated the inflammatory fibrin formation process as a result of immune response. It is thought that due to these active reactions in the region, there is an increase in temperature at that point compared to other hernia sac skin areas and is reflected as maximum temperature in the thermographic image obtained. The reason why the detected temperature is not higher; It may be because it is in the process of chronic inflammation, not acute. It is concluded that by reporting this case in birds, ectopic egg can trigger hernia development and can be successfully treated with surgical intervention. Infrared thermography, may have the potential to detect pathological changes in the abdominal cavity, such as ectopic eggs, as a complementary diagnostic tool.But this study is the first study in the field of avian obstetrics and gynecology to evaluate a pathological condition by infrared thermography so more studies on this issue are needed to speak more clearly.

Conflict of Interest

The authors report no conflicts of interest.

Author Contributions

The authors alone are responsible for the content and writing of this case report.

References

1. Hasholt J: Diseases of the female reproductive organs of pet birds. *J Small Anim Pract*, 7 (4): 313-320, 1966. DOI: 10.1111/j.1748-5827.1966.tb04448.x

2. Jenkins JR: Surgery of the avian reproductive and gastrointestinal systems. *Vet Clin North Am Exot Anim Pract*, 3 (3): 673-692, 2000. DOI: 10.1016/s1094-9194(17)30069-5

3. McMillan MC: Imaging of avian urogenital disorders. *AAV Today*, 2 (2): 74-82, 1988. DOI: 10.2307/30134405

4. Gorham SL, Akins M, Carter B: Ectopic egg yolk in the abdominal cavity of a cockatiel. *Avian Dis*, 36 (3): 816-817, 1992. DOI: 10.2307/1591792

5. Harrison GJ, Harrison LR: Clinical Avian Medicine and Surgery. 225, WB Saunders, Philadelphia, 1998.

6. Parrah JD, Dar KH, Athar H, Qureshi B: Surgical management of abdominal hernia in a duck *(Anas Platyrhynchos)*. *Mathews J Vet Sci*, 1 (1): 002, 2016.

7. Gunduz MC, Toydemir S, Enginler SÖA, Yılmaz ÖT, Yigit F, Ucmak M: Hysterectomy operation for the treatment of the egg binding in the Lovebird *(Agapornis comprise)*: Case report. *J Fac Vet Med Istanbul Univ*, 35 (1): 43-48, 2009.

8. Mans C, Sladky KK: Clinical management of an ectopic egg in a Timneh African grey parrot *(Psittacus erithacus timneh)*. *J Am Vet Med Assoc*, 242 (7): 963-968, 2013. DOI: 10.2460/javma.242.7.963

9. Harrison GJ: Reproductive medicine. **In,** Harrison GJ and Harrison LR (Eds): Clinical Avian Medicine and Surgery. 620-634, WB Saunders, Philadelphia, 1986.

10. Smolec O, Kos J, Vnuk D, Babic T, Bottegaro NB: Abdominal ventral hernia in a pigeon *(Columba livia)*: A case report. *Vet Med*, 54 (6): 291-294, 2009. DOI: 10.17221/89/2009-vetmed

11. Amer MS, Hassan EA, Torad FA: Radiographic and ultrasonographic characteristics of ventral abdominal hernia in pigeons *(Columba livia)*. *J Vet Med Sci*, 80 (2): 292-296, 2018.

12. Rekant SI, Lyons MA, Pacheco JM, Artz J, Rodriguez LL: Veterinary applications of infrared thermography. *Am J Vet Res*, 77 (1): 98-107, 2016. DOI: 10.2460/ajvr.77.1.98

13. Durrant BS, Ravida N, Spady T, Cheng A: New technologies for the study of carnivore reproduction. *Theriogenology*, 66 (6-7): 1729-1736, 2006. DOI: 10.1016/j.theriogenology.2006.02.046

14. Colak A, Polat B, Okumus Z, Kaya M, Yanmaz LE, Hayirli A: Short communication: Early detection of mastitis using infrared thermography in dairy cows. *J Dairy Sci*, 91 (11): 4244-4248, 2008. DOI: 10.3168/jds.2008-1258

15. Cebulj-Kadunc N, Frangež R, Kruljc P: Infrared thermography in equine practice. *Vet Stanica*, 51 (2): 109-116, 2020. DOI: 10.46419/vs.51.2.1

16. McCafferty DJ: Applications of thermal imaging in avian science. *Ibis*, 155 (1): 4-15, 2013. DOI: 10.1111/ibi.12010

17. Edgar JL, Lowe JC, Paul ES, Nicol CJ: Avian maternal response to chick distress. *Proc R Soc B*, 278, 3129-3134, 2011.

18. Soroko M, Zaborski D: Investigation of the effects of probiotic, *Bacillus subtilis* on stress reactions in laying hens using infrared thermography. *Plos One*, 15(6): e0234117, 2020. DOI: 10.1371/journal.pone.0234117

19. Giloh M, Shinder D, Yahav S: Skin surface temperature of broiler chickens is correlated to body core temperature and is indicative of their thermoregulatory status. *Poult Sci,* 91, 175-188, 2012. DOI: 10.3382/ps.2011-01497

20. Wilcox CS, Patterson J, Cheng HW: Use of thermography to screen for subclinical bumblefoot in poultry. *Poult Sci,* 88, 1176-1180, 2009. DOI: 10.3382/ps.2008-00446

21. Yaqoob AB, Rashid DR, Omer D, Kipjen SL, Ajaz A, Abhishek K, Kishan PJ, Kumar GS: Egg binding in female Budgerigar *(Melopsittacus undulatus)*: Case report. *SKUAST J Res*, 21 (1): 112-113, 2019.

22. Reddy BS, Sivajothi S: Egg binding in Budgerigar *(Melopsittacus undulatus)*-an emergency condition. *Int J Avian Wildl Biol*, 3 (5): 352-

353, 2018. DOI: 10.15406/ijawb.2018.03.00119

23. Bennet R.A: Soft tissue surgery. **In,** Ritchie BW, Harrison GJ, Harisson LR (Eds): Avian Medicine: Principles and Application. 1097-1136, Lake Worth FL, Wingers Publishing; 1994.

24. Jahromi AR, Nazhvani SD, Haddadi S: Ventral abdominal hernia in a common Myna *(Acridotheres tristis)* - A case report. *Vet Arhiv*, 79 (6): 621-625, 2009.

25. Rosen LB: Avian reproductive disorders. *J Exot Pet Med*, 21 (2): 124-131, 2012. DOI: 10.1053/j.jepm.2012.02.013

26. Topalidou A, Ali N, Sekulic S, Downe S: Thermal imaging applications in neonatal care: A scoping review. *BMC Pregnancy Childbirth*, 19:381, 2019. DOI: 10.1186/s12884-019-2533-y

27. Shecaira CL, Seino CH, Bombardelli JA, Reis GA, Fusada EJ, Azedo MR, Benesi FJ: Using thermography as a diagnostic tool for omphalitis on newborn calves. *J Therm Biol*, 71, 209-211, 2018.

28. Pavelski M, Silva DM, Leite NC, Junior DA, De Sousa RS, Guérios SD, Dornbusch PT: Infrared thermography in dogs with mammary tumors and healthy dogs. *J Vet Intern Med*, 29 (6):1578-1583, 2015. DOI: 10.1111/jvim.13597

29. Talukder S, Kerrisk KL, Ingenhoff L, Thomson PC, Garcia SC, Celi P: Infrared technology for estrus detection and as a predictor of time of ovulation in dairy cows in a pasture-based system. *Theriogenology*, 81 (7): 925-935, 2014.

30. Bowers S, Gandy S, Anderson B, Ryan P, Willard S: Assessment of pregnancy in the late-gestation mare using digital infrared thermography. *Theriogenology,* 72 (3): 372-377, 2009. DOI: 10.1016/j.theriogenology. 2009.03.005

31. Pampariene I, Veikutis V, Oberauskas V, Zymantiene J, Zelvyte R, Stankevicius A, Marciulionyte D, Palevicius P: Thermography based inflammation monitoring of udder state in dairy cows: Sensitivity and diagnostic priorities comparing with routine California mastitis test. *J. Vibroengineering*, 18 (1): 511-521, 2016.

32. Holmdahl L: The role of fibrinolysis in adhesion formation. *Eur J Surg Suppl*, (577), 24-31, 1997.

33. Lasne D, Jude B, Susen S: From normal to pathological hemostasis. *Can J Anaesth*,53, S2-S11, 2006. DOI: 10.1007/BF03022247

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 on 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 includes all aspects of veterinary medicine and animal science.

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.

The official language of our journal is **English**. Additionally, all the manuscripts must also have Turkish title, keywords, and abstract (translation will be provided by our journal office for foreign authors).

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. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and supplementary files (figure etc.) should be submitted by using online manuscript submission system at the address of *http://vetdergi.kafkas.edu.tr/*

During the submission process, 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 sent to the editorial office.

3- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

4- Authors should know and take into account the issues listed in the "**Ethical Principles and Publication Policy**" section regarding scientific research and authors.

5- 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 does 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 the abstract 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 does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an 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 be followed by the Introduction, Case History, Discussion and References. The length of the text should be no longer than 4 pages in total. The page limit does 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 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a 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 be followed by introduction, text (with appropriate titles), conclusion, and references. The length of the text should be no longer than 15 pages in total.

6- 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 the title.

7- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

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. DOI: 10.9775/kvfd.2017.17726

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 a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply 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

8- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

9- The editorial board has the right to perform necessary modifications and a reduction in 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 the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

10- All responsibilities from published articles merely belong to the authors. According to the 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 a broad database of academic publications.

11- There is no copyright fee for the authors.

12- The authors are charged a fee on acceptance of the manuscript to cover printing costs and other expenses. This payment information can be found at *http://vetdergi.kafkas.edu.tr/*

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

• 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.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere.

- Title page

• Title, running title (should be a brief version of the title of your paper, no exceed 50 characters)

• The author's name, institutional affiliation, Open Researcher and Contributor ID **(ORCID)**

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• 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

Further considerations

• Journal policies detailed in this guide have been reviewed

• The manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided