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Kafkas Üniversitesi Veteriner Fakültesi Dergisi Editörlüğü
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Research Article

Enzymological Properties and Nematode-Degrading Activity of Recombinant Chitinase AO-379 of *Arthrobotrys oligospora*

Wenqiang ZHONG [1,†] Ying CHEN [2,†] Shasha GONG [1,†] Jun QIAO [1,3] Qingling MENG [1]
Xingxing ZHANG [3] Xifeng WANG [1] Yunfu HUANG [1] Lulu TIAN [1] Yanbing NIU [2]

[†] *These authors contribute equally*

[1] Key Laboratory of Control and Prevention of Animal Disease of Xinjiang Production & Construction Corps, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang, 832003, CHINA

[2] College of Animal Science and Technology, Xinjiang Agricultural Vocational and Technical College, Changji, Xinjiang 31100, CHINA

[3] State Key Laboratory for Sheep Genetic Improvement and Healthy Production, Xinjiang Academy of Agricultural and Reclamation Science, Shihezi, Xinjiang, 832000, CHINA

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Abstract

Chitinase is an important virulence factor produced by nematode trapping fungi in the process of infection, and plays an important role in the cleavage of nematodes and their eggshells. In this study, the cDNA sequence of *Arthrobotrys oligospora* chitinase AO-379 was amplified by RT-PCR and inserted into the vector pPIC9K to induce the expression of AO-379 in *Pichia pastoris* GS115. The recombinant AO-379 (reAO-379) was purified by nickel ion affinity chromatography, and enzymological properties and nematode-degrading activity of reAO-379 was analyzed. SDS-PAGE and Western blot analysis showed that the reAO-379 with molecular weight of about 44 kDa was successfully obtained. The reAO-379 showed strong chitinase activity at pH 5.5 and 30°C. Using reAO-379 to treat *Strongylus equinus, Caenorhabditis elegans* and *Haemonchus contortus* for 12, 24, and 36 h , the killing rates of reAO-379 in *S. equinus* were 42%, 89% and 100%; in *C. elegans* were 50%, 90% and 97%; in and H. contortus were 53%, 62% and 84%, respectively. Using reA-379 to treat *Fasciola hepatica* and *Dicrocoelium chinensis* eggs for 24, 48 and 72 h, the degradation rates of reAO-379 were 12%, 43% and 65% in F. hepatica eggs, and were 15%, 33% and 55% in *D. chinensis* eggs, respectively. Our study suggests that the reAO-379 is potentially valuable for development of biological control agent against digestive tract nematodes in livestocks.

Keywords: *Chitinase AO-379, Molecular characteristics, Expression, Nematode-degrading activity, Arthrobotrys oligospora*

Arthrobotrys oligospora Rekombinant Çitinaz AO-379'un Enzim Özellikleri ve Nematod İndirgeyici Aktivitesi

Öz

Çitinaz, enfeksiyon sürecinde nematod tutucu mantar tarafından üretilen önemli bir virulans faktörüdür ve nematodların yumurta kabuklarından ayrılmalarında önemli rol oynar. Bu çalışmada, *Arthrobotrys oligospora* çitinaz AO-379 cDNA sekansı RT-PCR ile amplifiye edildi ve Pichia pastoris GS115'de AO-379 ekspresyonunu oluşturmak amacıyla pPIC9K vektörüne yerleştirildi. Rekombinant AO-379 (reAO-379) nikel iyon affinite kromotografi ile saflaştırıldı, enzim özellikleri ve nematod indirgeme aktivitesi araştırıldı. SDS-PAGE ve Western blot analizleri yaklaşık 44 kDa moleküler ağırlığında olan reAO-379'ın başarıyla elde edildiğini gösterdi. reAO-379 pH 5.5 ve 30°C'de güçlü çitinaz aktivitesi gösterdi. reAO-379 ile *Strongylus equinus, Caenorhabditis elegans* ve *Haemonchus contortus* 12, 24 ve 36 saat muamele edildiklerinde sırasıyla *S. equinus* için %42, %89 ve %100, *C. elegans* için %50, %90 ve %97 ve *H. contortus* için %53, %62 ve %84 öldürme oranlarına sahip olduğu gözlemlendi. *Fasciola hepatica* ve *Dicrocoelium chinensis* yumurtaları reA-379 ile 24, 48 ve 72 saat muamele edildiğinde, indirgenme oranları *F. hepatica* yumurtalarında sırasıyla %12, %43 ve %65 iken *D. chinensis* yumurtalarında sırasıyla %15, %33 ve %55 olarak belirlendi. Bu çalışma, reAO-379'un yetiştiricilikte sindirim sistemi nematodlarına karşı potansiyel bir biyolojik kontrol ajan olarak kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Çitinaz AO-379, Moleküler özellikler, Ekspresyon, Nematod-indirgeyici aktivite, Arthrobotrys oligospora*

INTRODUCTION

Animal digestive tract parasitic nematodes are a class of parasites that cause serious damage to livestock husbandry, leading to huge economic losses to the world's livestock industry each year [1]. In recent decades, the use

İletişim (Correspondence)
+90 86-993-2055036 Fax: 86-993-2055038
xjmqlqj@163.com

of chemical anthelmintic drugs, to a large extent, has relieved nematode caused economic losses in livestock husbandry. However, long-term, high-dose chemical drugs also cause serious problems such as drug resistance of nematodes, drug residues in food products of animal origin, destruction of ecosystems and biodiversity, and pollution to soil and water sources [2,3]. Therefore, there is an urgent need to develop effective animal and environment-friendly biological agents to control digestive tract nematodes in livestocks [4].

Nematode trapping fungi are one of the natural predators of nematodes, which can capture nematodes by producing complex predatory structures (such as trapping hyphae, adhesive net, constriction loop, or non-constricting adhesive network) [5-7]. Numerous studies have shown that nematode trapping fungi have potential value for the development of biological control agents [8-12]. It has been demonstrated that chitin is an important component of the nematode body wall and the eggshell [13]. Recently, more and more studies have shown that nematode trapping fungi can secrete a variety of extracellular hydrolases, such as serine protease, chitinase and collagenase, to digest and degrade nematode body wall and eggshell, thus penetrating and infecting nematodes or eggs [14,15].

As a typical representative strain of nematode trapping fungi, *Arthrobotrys oligospora* can capture and kill the parasitic nematodes of livestock, and is one of the important fungi for the study of fungi-nematode interaction [16-20]. So far, many scholars have identified and isolated different chitinases from *A. oligospora* [15]. However, the role and mechanism of chitinase in the infection and degradation of nematodes by *A. oligospora* is still unclear.

The aim of this study is to explore the enzymological properties and nematode-degrading activity of recombinant chitinase AO-379 (reAO-379) of *Arthrobotrys oligospora*. Chitinase AO-379 gene of *A. oligospora* was cloned and it's molecular characterization was analyzed. Then reAO-379 was prepared using *Pichia pastoris* expression system, and enzymological properties and nematode-degrading activity of reAO-379 were determined.

MATERIAL and METHODS

Cultivation of the Fungus and Induction of Predatory Hyphae

A. oligospora strain XJ-A1 that was isolated in Xinjiang, and stored in Shihezi University was inoculated in corn meal agar (CMA) medium and incubated at 25°C for 6 days. Appropriate amount of nematode was added to the culture to induce the formation of predatory hyphae (hyphae ring). When the three-dimensional hyphae network was induced by the nematode, the hyphae was collected and stored at -80°C for further study.

Amplification and Cloning of A. oligospora AO-379 Gene

The total RNA was extracted by Trizol reagent (Takara, Japan). The cDNA was reverse transcribed with the reverse transcription kit (Qiagen, Germany). The primer sequences that were used for the amplification of AO-379 cDNA (MG279142) were as follows: forward primer: 5'-GATACGTAATGCATCATCATCATCATCATTTGTTCAAATATAT-3' (containing the *Sna*B1 restriction site); reverse primer: 5'-CGAGCGGCCGCTTAGTTGGACAAGAAGCCC-3' (containing the *Not*1 restriction site). The PCR products were recovered using Agarose Gel DNA Extraction Kit (Takara, Japan) ligated with pMD18-T vector (Takara, Japan) at 16°C overnight, and transformed into *E. coli* DH5α competent cells. The positive transformants were confirmed by PCR and double-enzyme digestion. The screened positive clones were sent to The Beijing Genomics Institute (BGI) for sequencing. The sequencing results were aligned and at least two identical cloning sequences were selected to analyze the molecular characteristics of the encoded protein using the online biology software *(http://www.expasy.org/tools/)*.

Construction and Screening of Recombinant Pichia pastoris

Briefly, the RT-PCR product and the pPIC9K (Invitrogen, USA) were digested with *Sna*B1 and *Not*1 (Takara, Japan), respectively. The vector and the target gene fragments were recovered individually and ligated with T4 ligase (Promega, USA) at 16°C. Then, the ligation product was transformed into *E. coli* DH5α competent cells, and the recombinant vector pPIC9K-AO-379 was screened by PCR and double-enzyme digestion. The pPIC9K-AO-379 was linearized by *Sal* I (Takara, Japan) digestion and transformed into *Pichia pastoris* GS115 (Invitrogen, USA) by electroporation under the conditions of 1.5kV, 20 μF and 200 Ω.

Expression and Purification of Recombinant Protein AO-379 of A. oligospora

The recombinant yeast colony was inoculated into 20 mL BMGY medium (Invitrogen, USA), cultured at 28°C with 250 r/min rotation until $OD_{600\,nm}$ = 2-4. The yeast cells were collected by centrifugation and induced with methanol in 20 mL 0.5% BMMY medium (Invitrogen, USA). The culture medium was collected at 24, 48, 72 and 96 h following induction. The supernatant was collected by centrifugation and analyzed by SDS-PAGE. Take out the purified protein that was expressed in prokaryotic expression system, fully mixed with Freund's complete adjuvant (1:1 dilution), and subcutaneously inoculated in 12 mice (Each mouse was inoculated with 200 μm). Two weeks later, the purified protein was fully mixed with Freund's incomplete adjuvant (1:1 dilution) and the mice were inoculated again. At the third week, take blood through the heart of mice. After

centrifugation (3 000 r/min 10 min), the positive mouse anti-reAO-379 serum was used as the primary antibody and HRP-labeled goat anti-mouse antibody (Abcam, USA) was used as the secondary antibody for Western blot analysis. The recombinant chitinase AO-379 (reAO-379) was purified according to the Ni-NTA Purification System (Invitrogen, USA) and lyophilized.

Analysis of Chitinase Activity of A. oligospora reAO-379

Chitinase hydrolyzes chitin to produce N-acetylglucosamine and produces red compound with p-dimethylaminobenzaldehyde. The chitinase activity of reAO-379 was determined using $OD_{585\ nm}$ absorbance according to the instructions of the chitinase detection kit (Takara, Japan) in culture supernatants that were collected at different induction timepoints. At 37°C, 1 mg chitinase decomposition of chitin in 1 hour could produce 1 mg N-acetylglucosamine, the amount of enzyme was an enzyme activity unit (U). Each experiment was repeated three times.

Determination of Optimum Temperature and pH for A. oligospora reAO-379

According to the methods reported by Wang et al.[13] and Zhao et al.[21] the optimum temperature and optimum pH of reAO-379 chitinase were determined using a protein/nucleic acid analyzer ($OD_{585\ nm}$). Each experiment was repeated three times. Briefly, 40 µL reAO-379 solution was added into 400 µL nematodes and eggs-containing Tris-HCl (0.01 M, pH 7.0). The reaction mixture was incubated at different temperatures between 10°C to 60°C to determine the optimum temperature. The recombinant chitinase AO-379 was added into pH 3.0-8.0 Tris-HCl buffer (0.01 M) to determine the optimum pH of the enzyme.

Analysis of the Nematode-degrading Activity of A. oligospora reAO-379

Twenty-five mg reAO-379 was dissolved in 500 µL Tris-HCl buffer (optimum pH) to prepare 50 mg/mL enzyme solution. Subsequently, about 1.500 *S. equinus*, *C. elegans* and *H. contortus* were added separately into 150 µL reAO-379 enzyme solution and incubated at optimum temperature for 12, 24, and 36 h. The changes in nematode body wall were observed by using inverted microscope (Leica, Germany). Normal saline (NS) and commercialized chitinase (Chitinase from *Streptomyces griseus*, Sigma, USA) solution were used as the negative and positive control, respectively. The number of dead bodies in different solutions that were incubated for different time were observed and calculated. Each experiment was repeated three times.

Analysis of the Nematode Eggshell-degrading Activity of A. oligospora reAO-379

Twenty-five mg reAO-379 was dissolved in 500 µL Tris-HCl buffer (optimum pH) to prepare 50 mg/mL enzyme solution. Then, about 2.000 *F. hepatica* and *D. chinensis* eggs were added individually into 150 µL reAO-379 enzyme solution and incubated at optimum temperature for 24, 48 and 72 h. The changes in eggs were observed by using inverted microscope (Leica, Germany). Normal saline (NS) and commercialized chitinase (Sigma, USA) solution were used as the negative and positive control, respectively. Each experiment was repeated three times.

Statistical Analysis

Statistical analysis was conducted using SAS software (version9.1, SAS Institute, Inc., Cary, NC). A comparison of the killing or degradation rates among different nematodes and eggs was performed using the $\chi 2$-test. The value with $P<0.05$ was considered statistically significant.

RESULTS

The conidia of *A. oligospora* were pointed at the base *(Fig. 1G)*. After 15 days of culture, chlamydospores were

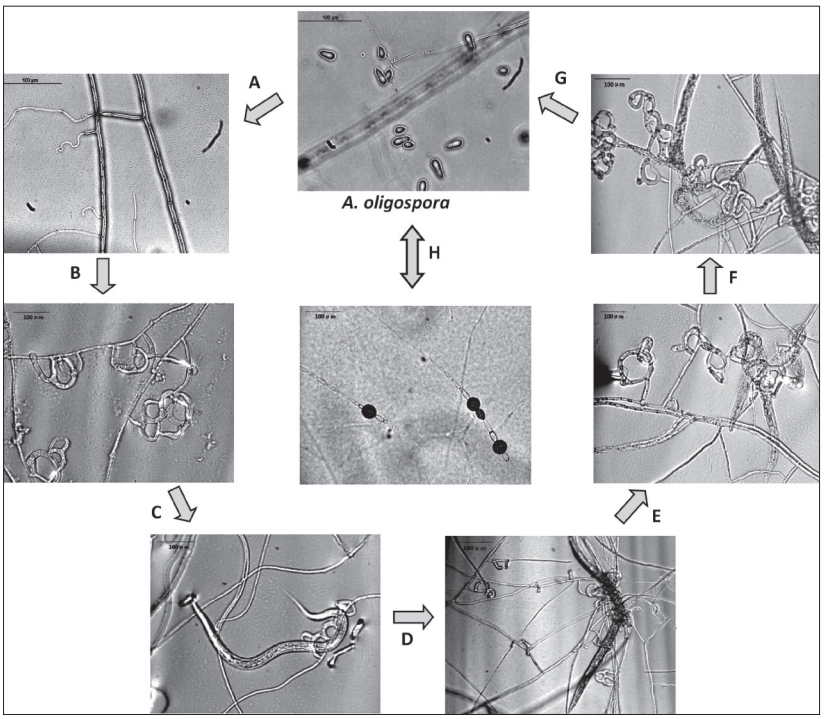


Fig 1. Saprophytic and parasitic stages of the nematode-trapping fungus *A. oligospora.* The life cycle of *A. oligospora* includes three stages: Dormancy stage, saprophytic stage, and parasitic stage. The all stage can be divided into the following eight steps: **A-** The formation of nutrient mycelium; **B-** The formation of the predation ring; **C-** Adhesion of nematodes; **D-** Nematode trapping; **E-** Penetration and immobilization; **F-** Digestion and assimilation; **G-** The saprophytic stage and H. Dormancy stage

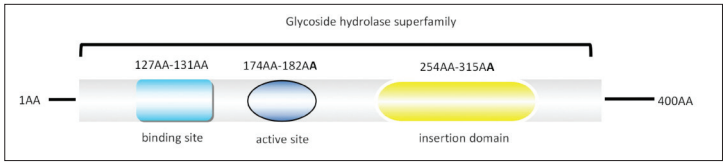
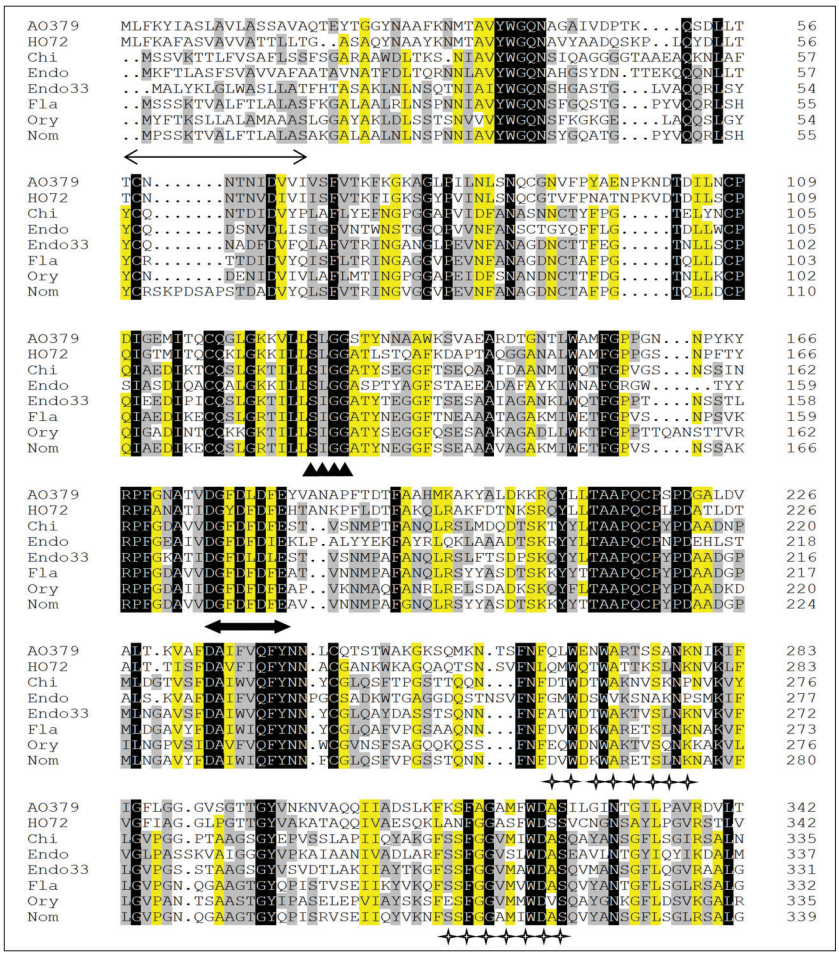


Fig 2. Domain pattern of chitinase AO-379 gene encoding protein of *Arthrobotrys oligospora*

Fig 3. Comparison of amino acid sequence of major catalytic active regions of chitinase among different fungi. The GenBank accession numbers of AO-379, HO72, CHI, Endo, Endo33, Fla, Ory and Nom chitinase from different fungi are MG279142, XP_011110546.1, XP_013323183.1, CCX31601.1, GAQ05774.1, KOC09654.1, XP_001822714.2 and XP_015410999.1, respectively. The black portion is highly homogeneous (100%) of the area; The middle part of the gap between the chitin enzyme variable region; (SXGG, DGXDXDWE) and the binding region of Chitinase (ENGVMDYKA, GLCCGMWWETSG) of the glycosyl hydrolase 18 family of conserved hydrolysates; *Double arrows* denotes a signal peptide; *triple asterisk* represents a substrate binding site; *Left* and *right arrows* represents the active site; *Cross star* represents the chitinase binding domain



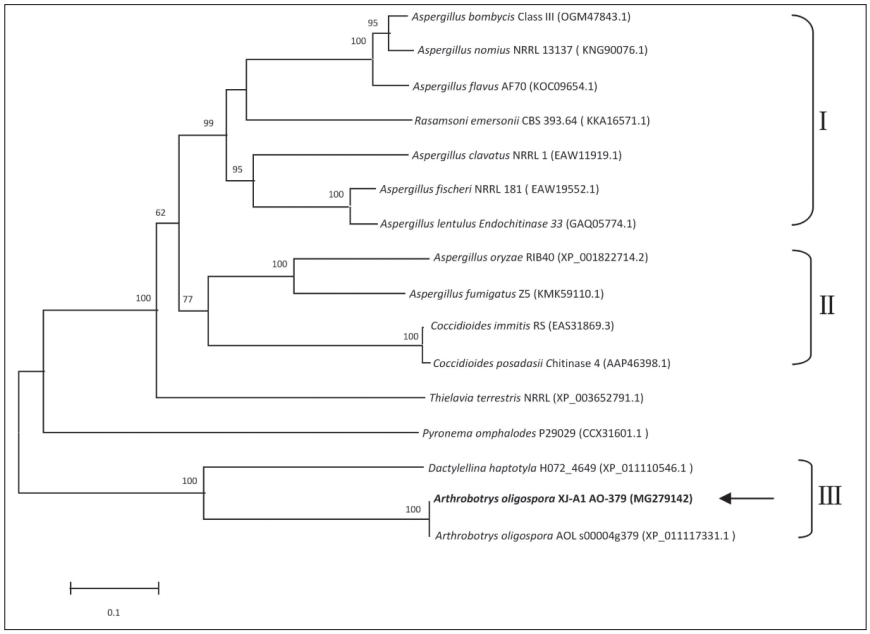


Fig 4. Phylogenetic analysis of chitinases based on amino acid sequences from different fungi. Phylogenetic tree were constructed using MEGA 6.0 software. The data were subjected to neighbor-joining (NJ) method of phylogenetic analysis, and bootstrap probabilities for each node are calculated from 1000 repetitions

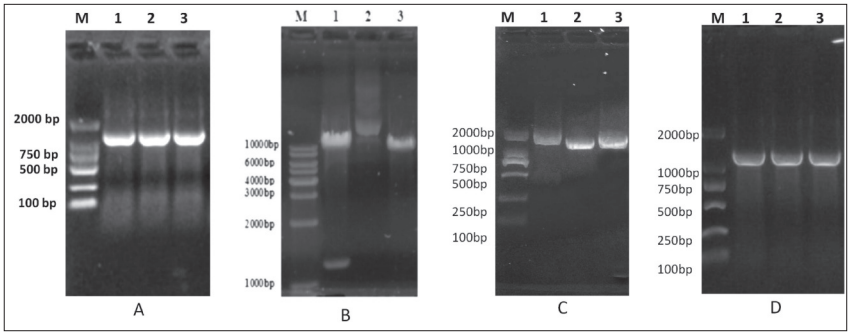


Fig 5. Construction and identification of recombinant plasmid pPIC9K-AO-379 and positive transformants. **A-** PCR amplification of cDNA gene with primers AO-379-F-SnaBI and AO-379-R-NotI using fungal gene/pPIC9K-AO-379 as a template. Lanes 1, 2, and 3 were the PCR products. M was the DNA marker, following lane M is the same; **B-** pPIC9K-AO-379 digested by SnaBI and NotI. Lane 1, empty vector; lane 2, recombinant vector; lane 3, product after enzyme digestion; **C-** PCR amplification of genes (wild-type AOX1 and vector AOX1 with the gene of interest) from Pichia pastoris GS115/pPIC9K-AO-379 using primers 5'AOX1 and 3'AOX1. Lanes 1 and 2 were the PCR products; **D-** PCR amplification of cDNA gene from P. pastoris GS115/pPIC9K-AO-379 using primers AO-379-F-SnaBI and AO379-R-NotI. Lanes 1 and 2 were the PCR products

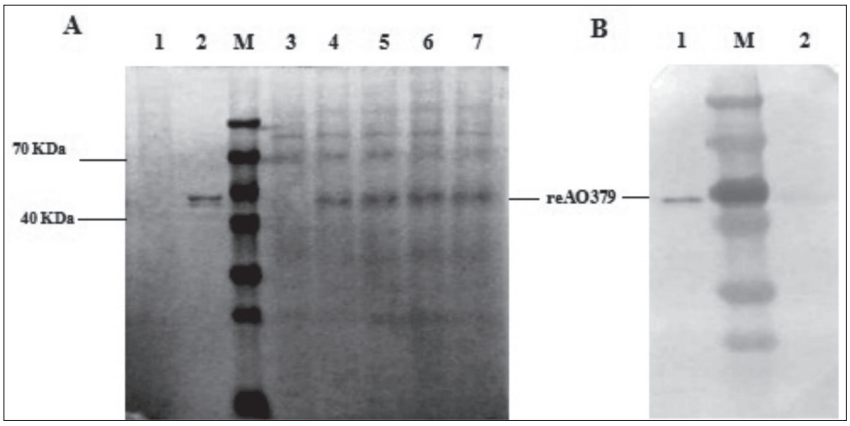


Fig 6. SDS-PAGE and Western blot analysis of expression of reAO-379. **A-** SDS-PAGE analysis of reAO-379. Lane M, protein molecular weight marker (broad). Lane 1: untransformed GS115; lane 2: The purified reAO-379; lane 3: GS115/pPIC9K; lane 4, 5, 6 and 7: GS115/pPIC9K-AO-379 after induction of 24 h, 48 h, 72 h and 96 h, respectively; M: molecular mass standard; **B-** Western blot analysis of reAO-379. Lane 1: purified reAO-379. Lane 2: supernatant from the negative stain transformed with pPIC9K (negative control). Lane M: protein molecular weight marker (broad)

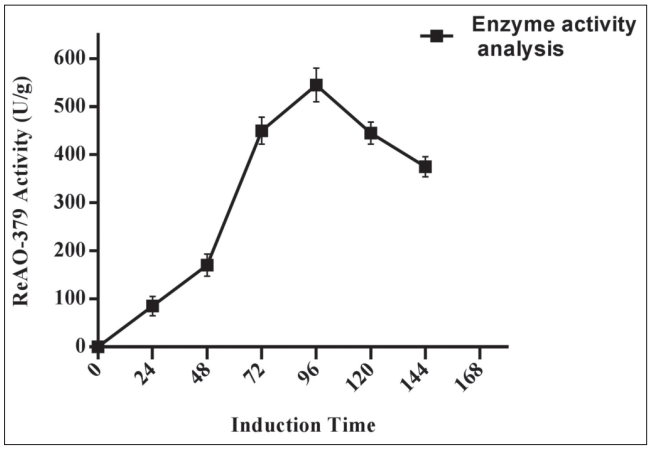


Fig 7. Analysis of chitinase activity of reAO-379 expressed in the cultures of *Pichia pastoris;* The activity of reAO-379 was determined by the reaction of N-acetylglucosamine with p-dimethylaminobenzaldehyde to produce a red compound resulting in a change in absorbance. Results are expressed as A_{585}/min×10^{-3} (unit)

produced in *A. oligospora* *(Fig. 1H)*. After being cultured at 25°C for 24 h, the conidia germinated to form ordinary hyphae *(Fig. 1A,B)*. After 10 h of induction with nematodes, *A. oligospora* began to catch the nematodes *(Fig. 1C)*; The three-dimensional network structure emerged 12 h following induction, the nematodes were trapped by the hyphae ring *(Fig. 1D)*. Fourteen h later, some trapped nematode body shrinked and some hyphae penetrated the nematode body wall *(Fig. 1E)*. Sixteen h later, the contents of the nematodes that were captured were gradually reduced. Finally, the nematodes only contained an empty shell which was ablated eventually *(Fig. 1F)*.

The sequencing result showed that the full length *A. oligospora* AO-379 gene was 1.203bp, encoding 400 amino acids. The signal peptide was located at positions 1-21. The sequence of chitinase AO-379 shared 97.08% homology with the sequence of the *A. oligospora* standard strain (ATCC 24927). Scanprosite software analysis showed that the encoded protein AO-379 belonged to the family of glycoside hydrolase 18, which is characterized by the sequence of VDGFDLDFE at 174-182 amino acids. AO-379 contained two conserved regions: SLGGS was located at positions 127-131 and was the substrate binding site; VDGFDLDFE was located at positions 174-182 and was the hydrolase catalytic activity site *(Fig. 2, Fig. 3)*. Phylogenetic analysis showed that the fungal chitinase formed three major branches. *Aspergillus*-produced seven chitinases formed the first branch; AO-379 belonged to the third branch, which was closest to *Dactylellina haptotyla* chitinase. Endophytic fungi and insect pathogenic fungi produced chitinases (RIB40, Z5, RS and Chitinase 4) formed the second branch *(Fig. 4)*.

The pPIC9K-AO-379 recombinant vector was successfully constructed *(Fig. 5)*. The recombinant *Pichia pastoris* was inoculated in the medium and the recombinant enzyme was induced to express by methanol. After 72 h induction, SDS-PAGE analysis showed that the expressed recombinant protein had a molecular weight of 44 kDa, which was consistent with the expected molecular weight of reAO-379 *(Fig. 6A)*. Western blot showed that the recombinant protein could specifically react with the positive mouse anti-AO-379 serum, confirming that the expressed recombinant protein was reAO-379 *(Fig. 6B)*.

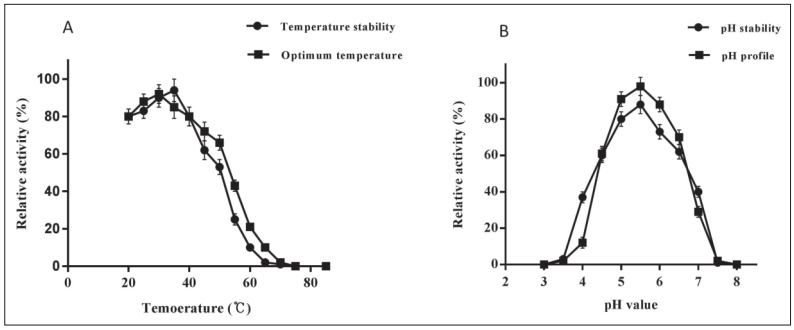
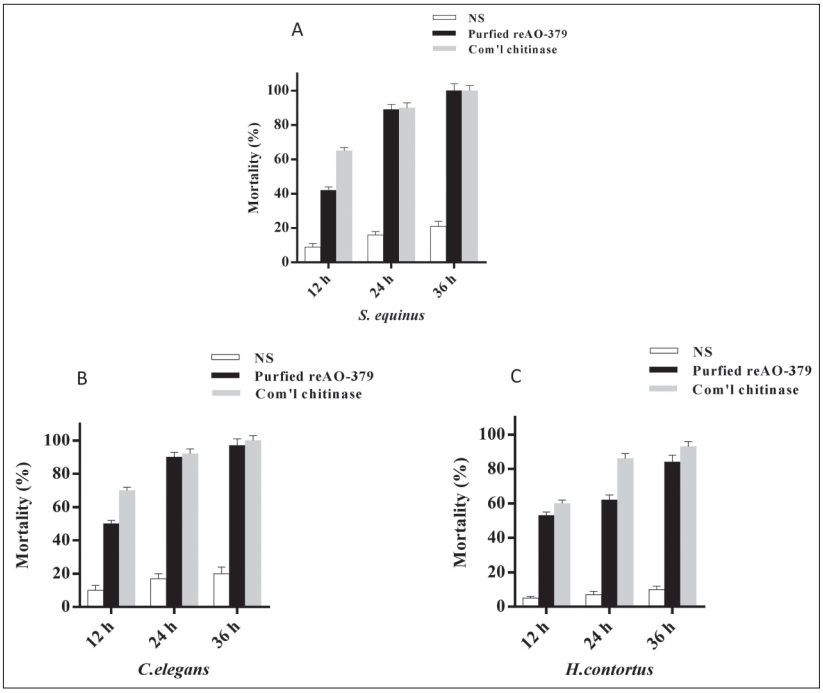


Fig 8. Effects of temperature and pH on activity of purified reAO-379. **A**- Different temperature; **B**- Different pH. Relative activity is expressed as a percentage of the maximum activity under standard assay conditions. The activity of reAO-379 (50 mg/mL) before incubation was defined as 100%. The value is the average of three replications

Fig 9. Analysis of nematicidal activity of the purified reAO-379. **A**- *S. Equinus*; **B**- *C. elegans*; **C**- *H. contortus*. The nematodes in the different time periods were digested by purified reAO-379. The normal saline (NS) commercial chitinase (Com'l chitinase) were used as negative and positive control, respectively. Data represent the average values of three independent assays



The chininase activity of reAO-379 in *Pichia pastoris* culture medium was low during 0-48 h methanol induction, and was increased rapidly after 48 h induction. The chitinase activity reached the maximum (545 U/g) after 96 h induction *(Fig. 7)*, and the chitinase concentration detected in the supernatant of the culture medium was 6.6 mg/mL.

The enzymatic activity of reAO-379 increased gradually with increasing temperature in the range of 10-30°C, and reached the maximum at 30°C. The enzymatic activity of reAO-379 was relatively stable at 20-40°C, while the enzyme was almost inactivated when being incubated at 70°C for 30 min *(Fig. 8A)*. The reAO-379 was active at pH 3.5-7.5, and reached its maximum activity at pH 5.5 *(Fig. 8B)*.

Using reAO-379 to treat *S. Equinus*, *C. elegans* and *H. contortus* separately for 12, 24, and 36 h, we found that the killing rates of reAO-379 in *S. equinus* were 42%, 89% and 100%, respectively *(Fig. 9A)*, in *C. elegans* were 50%, 90% and 97% *(Fig. 9B)*, and in *H. contortus* were 53%, 62% and 84%, respectively *(Fig. 9C)*. The nematode killing rate in AO-379 treated groups was significantly different when compared with the negative control group ($P<0.05$); while it was not significantly different when compared with the commercialized chitinase ($P>0.05$). After 12 h reAO-379 treatment, the nematode cuticle was partially degraded *(Fig. 10A-a4, Fig. 10B-b4, Fig. 10C-c4)*. After 24 h treatment, the body wall of the nematodes was largely degraded *(Fig. 10A-a5, Fig. 10B-b5, 10C-c5)*. After 36 h treatment, the body wall of the nematodes was completely degraded and the internal tissue of the nematodes was destroyed *(Fig. 10A-a6, Fig. 10B-b6, Fig. 10C-c6)*, whereas the body wall of the nematodes without reAO-379 treatment was relatively smooth *(Fig. 10A-a1, a2, a3; Fig. 10B-b1, b2, b3; Fig. 10C-c1, c2, c3)*.

Using reAO-379 to treat *F. hepatica* and *D. chinensis* eggs individually for 24, 48, and 72 h, we found that the killing rates of reAO-379 in *F. hepatica* eggs were 12%, 43% and 65%, respectively *(Fig. 11A)*, and in *D. chinensis* eggs were 15%, 33% and 55%, respectively *(Fig. 11B)*. The nematode eggshells in the negative control group were not degraded at all. After 24 h treatment with reAO-379, the nematode cuticle was slightly degraded *(Fig. 12A-a4, Fig.*

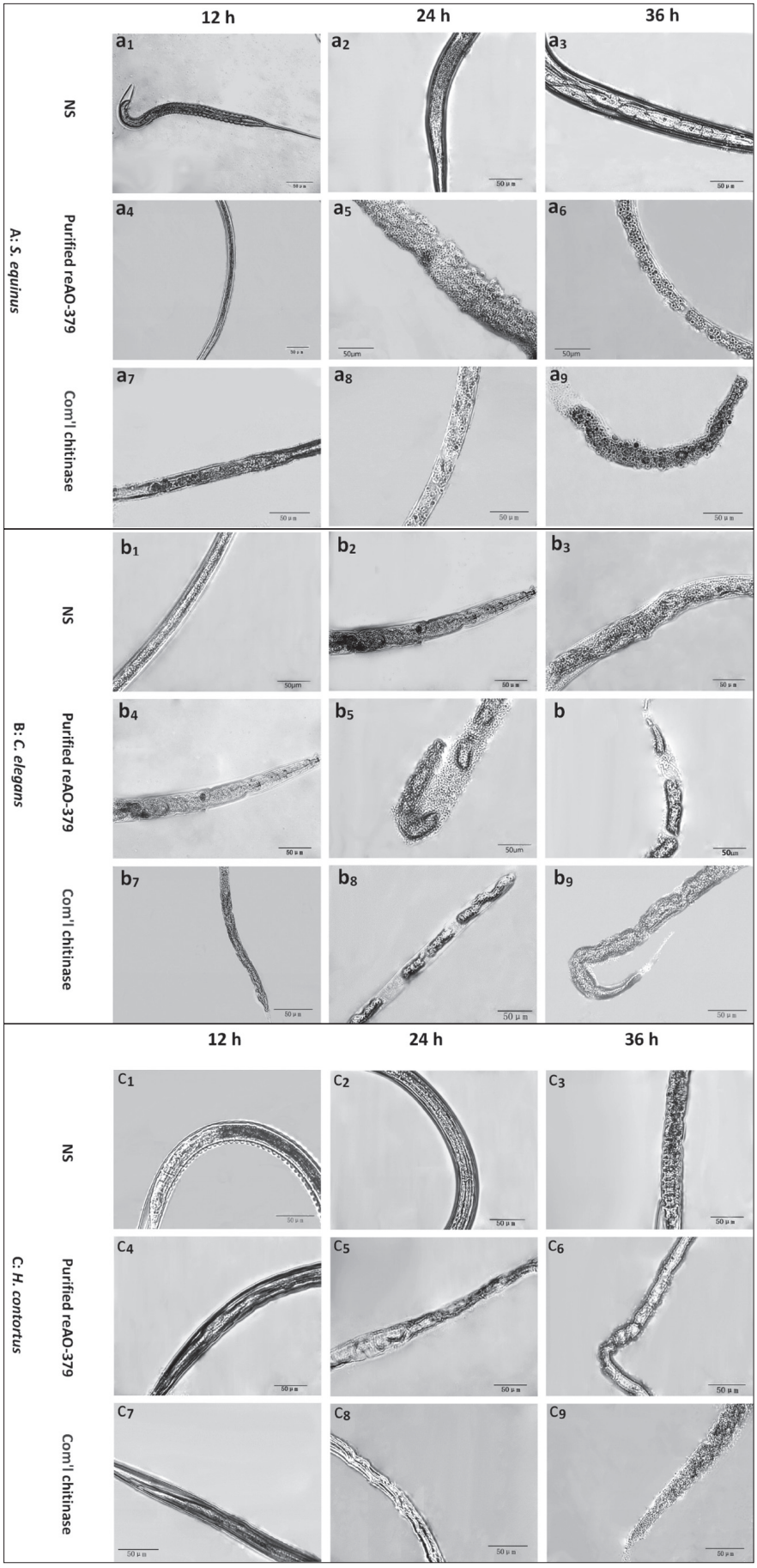


Fig 10. Immobilization and degradation of nematodes treated by purified reAO-379. **A-** *S. equinus* treated by the purified reAO-379 and commercial chitinase were degraded after 12 h, 24 h and 36 h of incubation (a_4, a_5 and a_6), respectively; *S. equinus* treated with the commercial chitinase was degraded after 12 h, 24 h and 36 h of incubation (a_7, a_8 and a_9), respectively; **B-** *C.elegans* treated with the purified reAO-379 and commercial chitinase were degraded after 12 h, 24 h and 36 h of incubation (b_4, b_5 and b_6), respectively; *C. elegans* treated with the commercial chitinase was degraded after 12 h, 24 h and 36 h of incubation (b_7, b_8 and b_9), respectively; **C-** *H.contortus* treated with the purified reAO-379 and commercial chitinase were degraded after 12 h, 24 h and 36 h of incubation (c_4, c_5 and c_6), respectively; *H.contortus* treated with the commercial chitinase was degraded after 12 h, 24 h and 36 h of incubation (c_7, c_8 and c_9), respectively

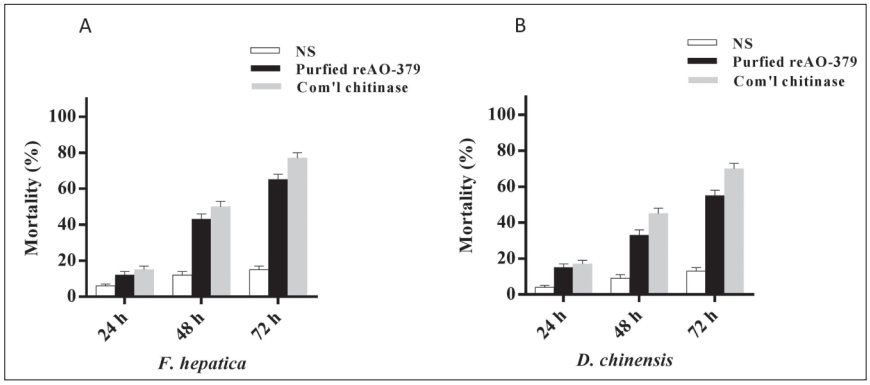


Fig 11. Analysis of degrading activity of the purified reAO-379 in *F. hepatica* and *D. chinensis* eggs. **A-** *F. hepatica*; **B-** *D. Chinensis*. The eggshells were digested in the different time periods. The normal saline (NS) commercial chitinase (Com'l chitinase) were used as negative and positive control, respectively. Data represent the average values of three independent assays

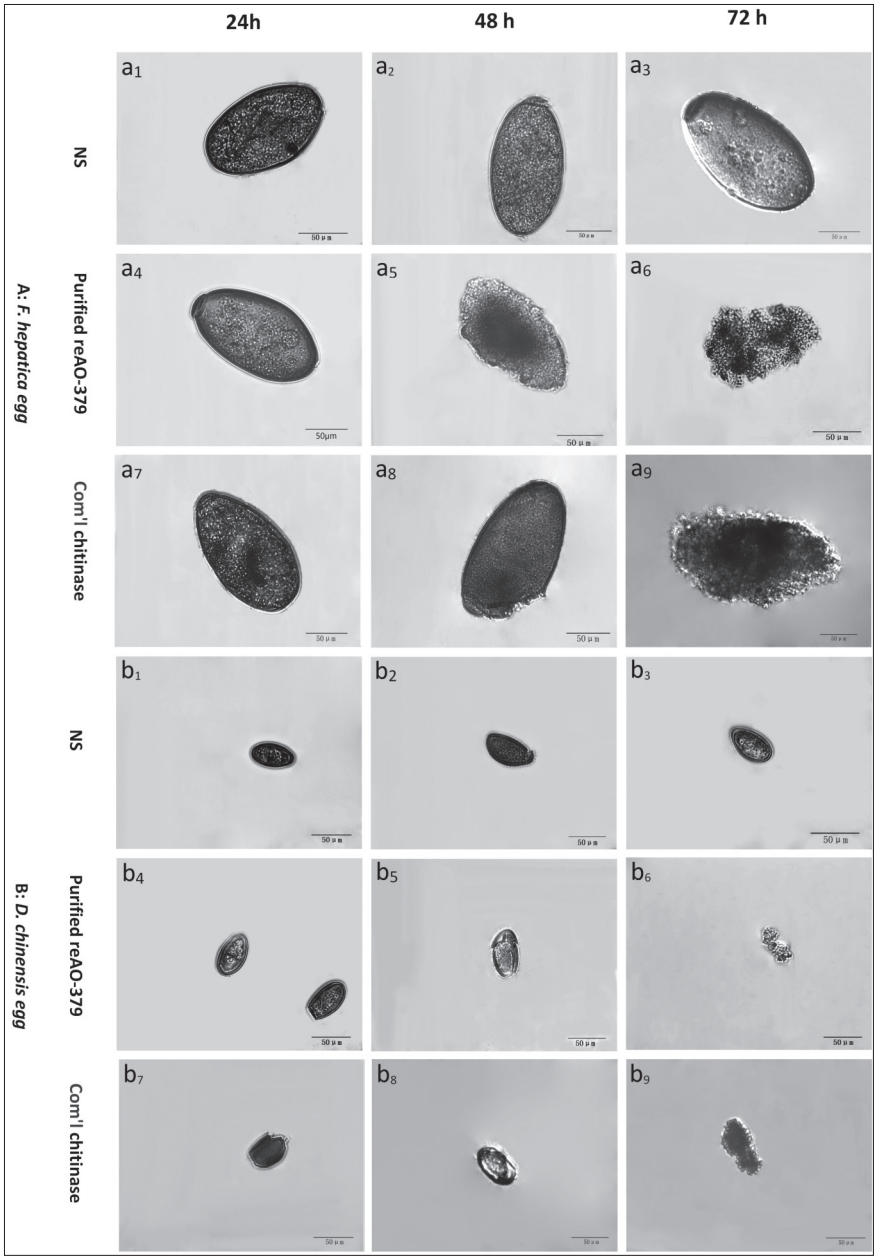


Fig 12. Immobilization and degradation of *F. hepatica* and *D. chinensis* eggs by purified reAO-379. **A-** *F. hepatica* eggs treated with purified reAO-379 was degraded after 24 h, 48 h and 72 h of incubation (a_4, a_5 and a_6), respectively; *F. hepatica* eggs treated with the commercial chitinase (Com'l chitinase) was degraded after 24 h, 48 h and 72 h of incubation (a_7, a_8 and a_9) (positive control), respectively; *F. hepatica* eggs treated with NS was as negative control (a_1, a_2 and a_3); **B-** *D. chinensis* eggs treated with the purified reAO-379 was degraded after 24 h, 48 h and 72 h of incubation (b_4, b_5 and b_6), respectively; *D. chinensis* eggs treated with the commercial chitinase (Com'l chitinase) was degraded after 24 h, 48 h and 72 h of incubation (b_7, b_8 and b_9) (positive control), respectively. *D. chinensis* eggs treated with NS was as negative control (b_1, b_2 and b_3)

12B-b4). After 48 h treatment, the eggshells were almost completely degraded *(Fig. 12A-a5, Fig. 12B-b5)*. After 72 h treatment, the eggshells were degraded and some tissues were destroyed *(Fig. 12A-a6, Fig. 12B-b6)*, but the nematode body without reAO-379 treatment was smoother *(Fig. 12A-a1, a2, a3; Fig. 12B-b1, b2, b3)*.

DISCUSSION

Chitinase is a protease that is widely expressed in bacteria, prokaryotes and eukaryotes and is capable of hydrolyzing beta-1,4-bond in chitin [22]. The chitin degradation activity of chitinase is carried out by a chitinolytic system with synergistic and continuous action, which hydrolyzes chitin into N-acetyl-β-D-glucosamine (GlcNAc) [23,24]. *A. oligospora* is a representative fungus of nematode trapping fungi, which can produce a variety of extracellular proteases to infect nematodes [15]. Here, through analysis of the sequence of *A. oligospora* chitinase AO-379 gene, we found that the gene encoded protein AO-379 contains the characteristic sequence of VDGFDLDFE and belongs to the family of glycoside hydrolase 18 (GH18) [25]. Simltaneously, it also has a substrate binding site and a hydrolase catalytic activity site. Phylogenetic analysis showed that AO-379 is closely related to *Dactylellina haptotyla* chitinase, but is far from the chitinases of endophytic fungi and insect pathogenic fungi.

Tunlid et al.[26] purified serine protease PII from *A. oligospora* and confirmed that it can degrade the cuticle of the nematode body wall. Subsequently, chitinase was found in various fungi [27-29]. At present, 15, 18, 21 and 30 chitinases have been identified in the genomes of pathogenic fungi (such as *M. oryzae*, *T. reesei*, *M. acridus* and *M. anisopliae*); ten chitinases have been identified individually in the genome of non-pathogenic *A. nisulans* and *N. crassa* [27-30]. Yang et al.[15] for the first time, identified 16 chitinase-encoding ORFs from the genome of *A. oligospora*. Transcriptome analysis results show that at carbon/nitrogen deficiencies or in the presence of chitin, the expression level of chitinase AO-379 was increased by 1.64 times, suggesting that AO-379 may play an important role in nematode infection in *A. oligospora* [15].

As an important biocontrol resource for livestock parasitic nematodes, fully understanding *A. oligospora* infection mode and its virulence factor is the key to reveal its infection mechanism and to develop efficient and stable biocontrol agents against nematodes [31]. Current studies have shown that nematode trapping fungi can secrete extracellular proteases that degrade the cuticle of nematode body wall or the eggshells [13], which may play a very important role in nematode infection [32]. In order to elucidate the biological function of *A. oligospora* chitinase AO-379, we expressed the chitinase AO-379 gene in *Pichia pastoris*. The recombinant AO-379 (reAO-379) was purified by nickel ion affinity chromatography. SDS-PAGE analysis showed that the reAO-379 with molecular weight of about 44 kDa was successfully obtained. Western blot showed that the recombinant protein could specifically react with the positive mouse anti-AO-379 serum, confirming that the expressed recombinant protein was reAO-379 Enzymatic activity analysis results showed that the activity of purified reAO-379 was high and stable at 30°C and pH 5.0-6.0, with the maximum activity at pH 5.5. When treated with reAO-379, the body wall of *S. equinus*, *C. elegans* and *H. contortus* was almost completely degraded and some of the internal tissue was also destroyed. The eggshells of the nematodes *F. hepatica* and *D. chinensis* were completely degraded after being treated with reAO-379 for 72 h.

In conclusion, we analyzed the molecular characteristics of chitinase AO-379 of *A. oligospora*, heterologously expressed AO-379 in *Pichia pastoris* and demonstrated that reAO-379 had strong biological activity to degrade the nematode body wall and eggshell, which provides the theoretical basis for the further development of nematode biological control agents using reAO-379.

Supplementary Date

Supplementary data are available in supplementary files.

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Ethical Statement

Ethical approval for this study was given by the Research and Ethical Committee of Shihezi University.

Declaration of Conflicting Interests

This manuscript has not been simultaneously submitted for publication in another journal and been approved by all co-authors. The authors declare that they do not have any conflict of interest.

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Research Article

Development of a Monoclonal Antibody Against Bovine α-Casein to Evaluate Functional Status of Mammary Epithelial Cells During Mastitis

Wentao MA [1,a] Yi WANG [1] Fei GAO [1] Mengxia NING [1] Ahua LIU [1]
Yanyan LI [1] Yang GAO [1] Ping LU [2] Dekun CHEN [1]

[1] Veterinary Immunology Laboratory, College of Veterinary Medicine, Northwest Agriculture and Forestry University, Yangling 712100, Shaanxi Province, CHINA
[2] China Animal Health and Epidemiology Center, Qingdao 266032, Shandong Province, CHINA
[a] ORCID: 0000-0002-4747-1489

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Abstract

Mastitis is a widespread disease in dairy animals and causes huge economic losses around the globe. Mastitis affects the health of animals and also reduces the protein content in the milk. In lactating animals, the surface of mammary gland is coated and lined with mammary epithelial cells (MECs). The functional status of MECs can be evaluated by determining the expression of caseins. The total content of caseins can largely reflect the quality of milk. In the previous literature, there is no general information about the milk quality and expressions of casein in mastitis. The current study was designed to determine if there is any correlations between mastitis and α-casein expression in MECs. We prepared a hybridoma cell line that produces antibody against bovine α-casein to evaluate the casein expression status *in vitro* on the MECs during lipopolysaccharide (LPS)-induced mastitis. The results showed that the expression of α-casein has not changed significantly under the stimulation of LPS. Our study established a useful tool to determine the expression profile of α-casein in bovine MECs. Furthermore, the result indicated that the expression level of α-casein remained stable during mastitis.

Keywords: *α-casein, Monoclonal antibody, Mammary epithelial cells, Mastitis*

Mastitis Süresince Meme Epitel Hücrelerinin Fonksiyonel Durumunu Değerlendirmek Amacıyla Sığır α-Kazein Proteinine Karşı Monoklonal Antikor Geliştirilmesi

Öz

Mastitis sütçü hayvanlarda yaygın bir hastalıktır ve dünya çapında büyük ekonomik kayıplara neden olmaktadır. Mastitis hayvanların sağlığını etkilemekte ve sütteki protein miktarını düşürmektedir. Laktasyondaki hayvanlarda, meme bezlerinin yüzeyleri meme epitel hücreleri ile döşelidir. Meme epitelyum hücrelerinin fonksiyonel durumu kazein ekspresyonu belirlenerek değerlendirilebilir. Total kazein miktarı büyük ölçüde sütün kalitesini yansıtmaktadır. Literatürde süt kalitesi ve mastitiste kazein ekspresyonu hakkında bir bilgi bulunmamaktadır. Bu çalışma, mastitis ile meme epitelyum hücrelerinde α-kazein ekspresyonu arasında bir ilişki bulunup bulunmadığını belirlemek amacıyla yapılmıştır. Lipopolisakkarit (LPS) ile oluşturulmuş mastitis süresince meme epitelyum hücrelerinde *in vitro* kazein ekspresyonunu değerlendirebilmek amacıyla sığır α-kazeinine karşı antikor üreten hibridoma hücre hattı hazırlanmıştır. Elde edilen sonuçlar, α-kazein ekspresyonunun LPS stimulasyonu altında anlamlı derecede değişmediğini göstermiştir. Bu çalışma sığır meme epitel hücrelerinde α-kazein ekspresyonunu belirlemek amacıyla yararlı olmuştur. Aynı zamanda, sonuçlar mastitis süresince α-kazein ekspresyon seviyesinin stabil olduğunu göstermiştir.

Anahtar sözcükler: *α-kazein, Monoklonal antikor, Meme epitel hücreleri, Mastitis*

INTRODUCTION

Casein is one of the most important components of bovine milk and constitute for more than 80% of total milk protein [1]. It is mainly composed of four components: α-casein (αs1- and αs2-), β-casein, and κ-casein. These four components respectively account for 38%, 10%, 35%, and 13% of the total caseins in the milk [2]. The α-casein is consisted of (αs1- and αs2)-parts that are the most important part of caseins. The contents of caseins can largely reflect the quality

İletişim (Correspondence)
+86-29-87092134
mawentao@nwafu.edu.cn (W. Ma); cdk@nwafu.edu.cn (D. Chen)

of milk. Several methods have been reported in order to detect certain types of casein. An immunomagnetic bead-based enzyme-linked immunosorbent assay with monoclonal antibodies (mAbs) against β-casein has been established to determine the content of β-casein in bovine milk [3,4]. In addition, mass spectrometry method has been used to determine the content of α-casein in milk by using an mAb against αs1-casein [5]. The level of caseins in the milk is not always the same under different feeding conditions, environmental stress and lactation cycles.

Mammary epithelial cells (MECs) are the functional unit for lactation in animals and produce caseins [6,7]. The amount of casein secretion is closely related to the functional state of the MECs; therefore, casein content is a useful indicator of the functional state of MECs [6]. However, there are no previous reports which analyzed the change of caseins during mastitis. Therefore, it is reasonable to study the level of casein expression in order to determine the functional status of MECs, especially during mastitis.

The amount of α-casein is the highest among the four caseins secreted by MECs [8]. Some studies have shown that the hydrolysis-derived peptide Met-Lys-Pro of α-casein has strong angiotensin-converting enzyme-inhibitory activity and can significantly reduce the systolic blood pressure of hypertensive rats [9]. Anastasia et al.[10] found that α-casein of different species can inhibit the proliferation of human breast cancer cell line T47D. Therefore, the preparation of mAbs against α-casein can not only measure its expression level in MECs but also evaluate the functional status of MECs. In this study, mice were immunized with α-casein to prepare mAbs against bovine α-casein, and the expression of α-casein in MECs was measured under different conditions.

MATERIAL and METHODS

Cells and Antigen

The SP2/0 cells were preserved at the laboratory and cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ExCell Bio, Shanghai, China) and 100 units/mL penicillin-streptomycin. Bovine mammary epithelial cells (BMECs) were isolated from fresh raw milk and cultured in Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12) (Thermo Fisher Scientific) supplemented with 10% FBS (ExCell Bio), 100 units mL^{-1} penicillin-streptomycin, 10 μg mL^{-1} bovine insulin (Sigma Aldrich, St. Louis, Missouri, USA), 10 ng mL^{-1} of human epidermal growth factor (hEGF Sigma Aldrich), and 5 μg mL^{-1} of hydrocortisone. Bovine alpha-casein was also utilized in this study (Sigma Aldrich).

Ethical Approval

The animal-related protocols in this study were reviewed and approved by the Research Ethics Committee of Northwest A&F University according to the guidelines of the Ministry of Health in China for the care and use of laboratory animals.

Animals

Female 6- to 7-week-old BALB/c mice were purchased from the Laboratory Animal Center of the Fourth Military Medical University.

Animal Immunization

Six-week-old mice were immunized subcutaneously with 100 μg α-casein (Sigma Aldrich) emulsified in complete Freund's adjuvant (Sigma Aldrich) for the first immunization. Three weeks later, the second immunization was injected subcutaneously and was comprised of 100 ug α-casein (Sigma Aldrich) emulsified in incomplete Freund's adjuvant (Sigma Aldrich). Three weeks later, the third immunization was conducted according to the dose and method of the second immunization. Two weeks following this, the fourth immunization was conducted intraperitoneally with 100 μg α-casein (Sigma Aldrich). After one week, the fifth immunization was conducted intraperitoneally with 100 μg α-casein (Sigma Aldrich). To test the antibody titers, an indirect-enzyme-linked immunosorbent assay (ELISA) was conducted by using the peripheral blood of the immunized mice. Mice with the highest antibody titers were selected as the donors of splenocytes.

Hybridoma Generation

Splenocytes of the mice with the highest antibody titers were collected and fused with SP2/0 cells in the presence of PEG 1500 (Roche, Basel, Schweizer). Hybridoma cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific) with hypoxanthine-aminopterin-thymidine (HAT) selection medium (Sigma Aldrich), supplied with 10% FBS (ExCell Bio) and 100 units mL^{-1} of penicillin and streptomycin. Culture supernatants were screened by ELISA. The specific mAbs were selected using western blot and immunofluorescence techniques.

Indirect Enzyme-Linked Immunosorbent Assay

Polystyrene microtiter 96-well plates (Corning Costar, Corning, New York, USA) were coated with 1 ug/well of bovine α-casein (at the concentration of 1 mg mL^{-1} in 0.1 M sodium carbonate buffer with a pH of 9.6), fresh raw cow milk, or fresh raw goat milk overnight (14 h at 4°C). After saturating the residual sites with 2% cold fish gelatin in PBS at 37°C for 1 h, the plates were washed three times with PBS containing 0.05% Tween-20 (PBST). The cell culture supernatant of the mAbs was incubated with the plates at 37°C for 1 h and then washed with PBST. Furthermore, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Bioss, Beijing, China) was diluted to 1:5000 in PBST and incubated at 37°C for 1 h. The substrate (0.3% H_2O_2, 0.1% TMB in 0.1M citric acid, and

pH=5) (TIANGEN, Beijing, China) was incubated for 18 min at 37°C, and the reaction was stopped with 0.2 M H_2SO_4 [3]. The absorbance at 450nm was determined (BIO-RAD, Model 680).

Isotype Identification of mAbs

Two clones (1-C3 and 2-F1) of strong response were further cloned and propagated. Thye were given additional isotype identification using the Mouse Monoclonal Antibody Isotyping Kit (Roche) according to the manufacturer's instructions.

Western Blot Analysis

Samples of fresh raw cow milk (50 μg), goat milk (50 μg), and bovine α-casein (2 μg) were boiled in sodium dodecyl sulfate (SDS) (Sigma Aldrich) sample buffer supplied with β-mercaptoethanol for 15 min, respectively. The proteins were electrophoresed on 6%-12% polyacrylamide gels and were transferred onto a polyvinylidene difluoride (PVDF) membrane under 85 V for 50 min. After blocking with 3.5% fish gelatin in PBST at room temperature for 2 h, the PVDF membrane was incubated with the supernatant of anti-bovine α-casein (1-C3 and 2-F1 mAbs) diluted by 20 times in 1% blocking buffer at 4°C overnight. Bound antibodies were recognized with an HRP-conjugated goat anti-mouse IgG secondary antibody (Sigma Aldrich) at a 1:6000 dilution in blocking buffer. The signal was visualized with enhanced chemiluminescence reagent (ECL) and ChemiDoc™MP Imaging System (BIO-RAD).

BMECs Isolated From Cow Milk

Bovine milk was analyzed with a mastitis diagnosis kit developed by our laboratory. Milk from healthy teat was sterilely collected and diluted 1:1 with phosphate buffered saline (with 1000 units mL^{-1} of penicillin-streptomycin) and centrifuged at 1500 rpm for 20 min at room temperature. Layer of fat and whey in the milk were discarded and the cell pellet was washed twice with PBS by centrifugation at 1000 rpm for 10 min. Subsequently, the pellets were resuspended in proliferation medium consisting of DMEM/F12 (Thermo Fisher Scientific) supplemented with 10% FBS, 10 μg mL^{-1} bovine insulin (Sigma Aldrich), 10 ng mL^{-1} human epidermal growth factor (hEGF) (Sigma Aldrich) [11], 5 μg mL^{-1} of hydrocortisone, and 100 units mL^{-1} of penicillin-streptomycin. Cells were cultured at 37°C in 5% CO_2 and the culture medium was refreshed after every 24 h. Cells were passaged at a ratio of 1:2 until they had a 70%-90% confluence.

α-Casein Secretion by BMECs After Stimulation of LPS In Vitro

Bovine mammary epithelial cells were seeded into 6-well plates at 3 generations. The cells were preincubated with medium containing 0.5% FBS for 6 h prior to lipopolysaccharide (LPS) treatment (Sigma Aldrich) at 10 ng μL^{-1} (n=5) or 100 ng μL^{-1} (n=5) for 3 h after they grew up to 70%. The control group was treated with medium containing 0.5% FBS and simultaneously refreshed with new medium (n=5). Goat MECs were also used as a control (n=5). All cells were cultured for an additional 12 h. Immunofluorescence assays were carried out to analyze the secretion of bovine α-casein by BMECs with the 1-C3 mAb. All the experimental and control groups were repeated three times.

Immunofluorescence Analysis of Bovine α-casein Secretion by BMECs In Vitro

Immunofluorescence assays were performed to analyze the secretion of bovine α-casein from BMECs with the 1-C3 mAb. BMECs were passaged to 3 generations and cultured for an additional 12 h. After that they were harvested for immunofluorescence analysis. Cells were fixed with 4% polyoxymethylene at room temperature for 30 min and briefly washed with filtered PBS for three times. After the cells were infused with 0.5% triton-X 100 for 15 min, the cells were blocked with 3.5% fish gelatin (Sigma Aldrich) at room temperature for 30 min. The primary antibody of mAb 1-C3 (diluted in 1% gelatin) was reacted with BMECs overnight at 4°C and the bound antibody was detected with FITC-conjugated goat anti-mouse IgG (San Ying, Wuhan, China). Finally, 5 μg mL^{-1} DAPI (Sigma Aldrich) was added for 5 min. Images were observed under a microscope (Leica, Model DMI3000B, Germany) and analyzed by an image pro 6.0 software [12].

RESULTS

In order to obtain mAbs that specifically react against bovine α-casein, we screened out the splenocytes and SP2/0 myeloma cell hybridoma supernatant by indirect ELISA. Plates were coated with bovine α-casein, raw cow milk, and raw goat milk separately. Four positive hybridoma clones were obtained which were named as 1-C3, 2-H1, 3-H4, and 4-G6 *(Fig. 1)*. The results showed that all of these antibodies could react with cow milk and bovine α-casein. In addition, 2-F1, 1-H4, and 3-G4 reacted with goat milk, while 1-C3 showed no reactivity *(Fig. 1)*.

Subsequently, we selected 1-C3 and 2-F1 for further identification via western blot analysis. We tested the subtype of 1-C3 and 2-F1 in order to obtain a broad-spectrum IgG subtype antibody. The results showed that both 1-C3 and 2-F1 mAbs were IgG1 subtype and had κ-type light chains *(Fig. 2A)*.

To examine the availability of the mAbs, we performed a western blot analysis using bovine α-casein, cow milk, and goat milk. The results showed that the 2-F1 mAb reacted with α-casein from both goat milk and cow milk *(Fig 2B)*. In comparison, 1-C3 did not exhibit reactivity to goat milk, cow milk, or purified bovine α-casein. These results may because 2-F1 mAb recognized a linear epitope which existed in both bovine and goat caseins, even though

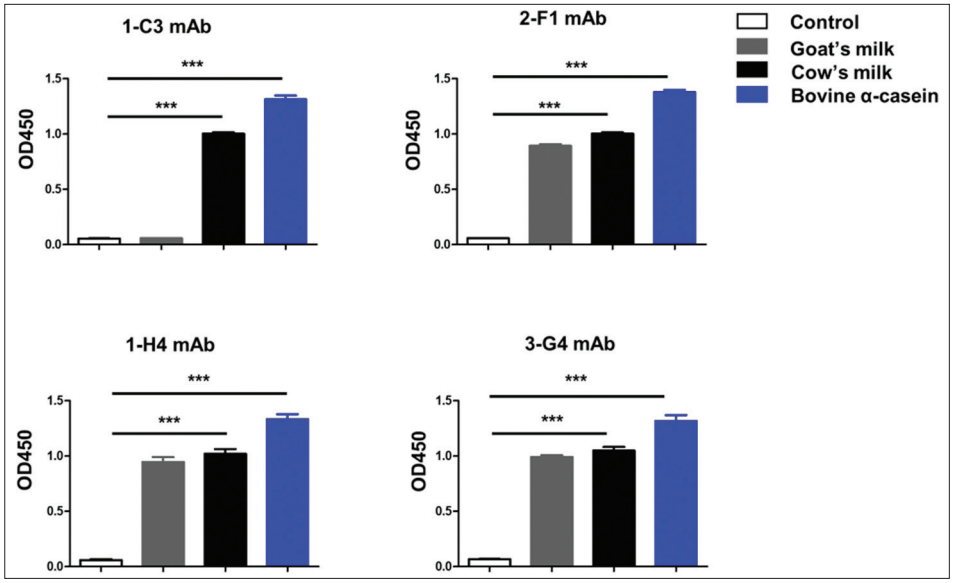

Fig 1. Specificity identification of four α-casein mAbs (1-C3, 2-F1, 1-H4, and 3-G4) with indirect ELISA (n = 3, P<0.05)

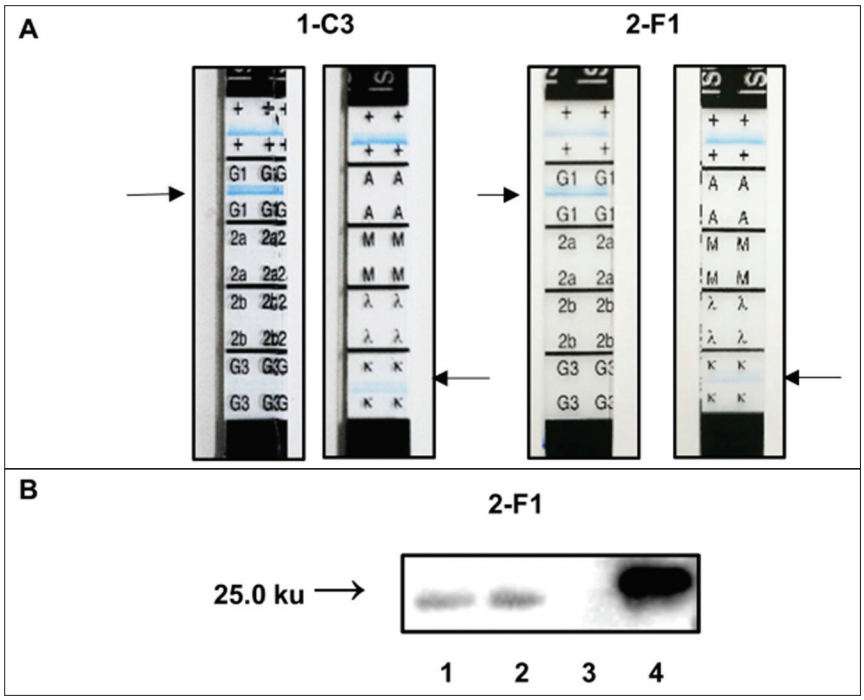

Fig 2. Subtype identification of 1-C3 and 2-F1. (A) The band on the top of the strip was the positive control. Subtype of anti-bovine α-casein mAb was IgG1 (the left strip), the light-chain was κ-type (the right strip). The blue bands on the top of each band indicate a positive result. (B) Western blot analysis of 2-H1 supernatants against α-casein was carried out. In lane 1, the loaded sample was 50 μg of goat milk; in lane 2, the loaded sample was 50 μg of cow milk; In lane 3, the loaded sample was buffer only; and in lane 4, the loaded sample was 2 μg of bovine α-casein. The molecular weight of α-casein is about 25 kDa

these epitopes were denatured. However, the lack of the reactivity with the mAb1C3 in western blot analysis suggested that the mAb was most likely directed against a conformational epitope in bovine α casein.

In this study, we used a described *in vitro* method to establish a mastitis model of MECs *in vitro* via stimulation with 10 ng μL^{-1} or 100 ng μL^{-1} of LPS [13,14]. Both the experimental and control group were analyzed by an immunofluorescence assay after culture of an additional 12 h. The results showed that α-casein synthesized and secreted by BMECs could be detected by the 1-C3 mAb at a steady level *(Fig. 3A)*. In addition, LPS (10 ng/μL or 100 ng/μL) stimulation did not affect the expression of α-casein *(Fig. 3B,C)*. Compared with the bovine MECs, the goat MECs could not be detected by any bovine α-casein antibodies *(Fig. 3D)*. Our results are consistent with previous studies and showed that the expression of caseins were not significantly changed or even decreased when an animal is in mastitis [15,16].

DISCUSSION

Mastitis is a worldwide problem in dairy animals that can result in huge economic losses around the globe. During mastitis, MECs play a critical role in initiating the inflammatory responses in the mammary gland and sequentially the function of MECs may also be affected by the immune response [17,18]. However, there have not been any relevant studies reporting the expressions of

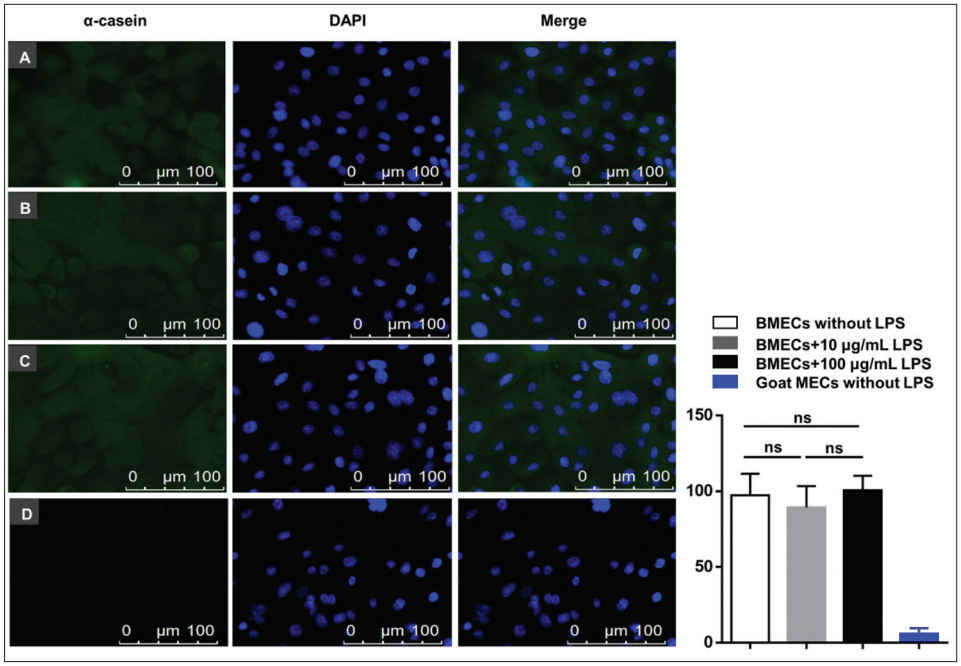

Fig 3. Immunofluorescence analysis of the developed mAbs. (A) Unstimulated primary BMECs (B) BMECs stimulated with 10 μg mL^{-1} of LPS (C) BMECs stimulated with 100 μg mL^{-1} of LPS (D) Unstimulated primary goat MECs. Scale bar = 100 μm

caseins by MECs during mastitis. In this study, we aimed at determining the expression of caseins for the evaluation of MEC function. Using the antibody specifically directed against bovine α-casein, we found that the level of α-casein did not change significantly in the *in vitro* mastitis model. Our results may provide an efficient tool for the determination of α-casein expression.

Caseins are the most import nutritional components in bovine milk. Generally, there are four types of bovine caseins and classified as αs1-casein, αs2-casein, β-casein and κ-caseins [2]. Among these four types of caseins, α-caseins (including αs1-casein and αs2-casein) are the most abundant form of casein in bovine milk [1]. Importantly, α-caseins have been reported to be involved in a variety of physical or pathological conditions, with αs1- and αs2-subtypes playing redundant but usually distinct roles [19,20]. However, in the present study, although we developed mAbs directed against bovineα-caseins, the antibody cannot distinguish αs1- from αs2-caseins, because we cannot separate these two proteins during the development of the mAbs. Thus, the mAbs developed by this study need further optimization in the future.

As an important part of the mammary gland, MECs not only have important lactation function, but also cooperate with immune cells to defend against microbial infection. In most of the studies, MECs serve as a key element in the study of the physiological functions of mammary gland. However, it is difficult to obtain primary MECs. Alternatively, immortalized mammary epithelial cell lines have been generally used in many studies [21-23]. Accumulating evidence shows that the level of casein transcriptome changes greatly during the *in vitro* culture of MECs, while primary MECs with high casein synthesis and secretion ability yield results closer to the real condition of mammary gland tissues [6,7]. Thus, the primary MECs used in the present study are more appropriate in studying casein expression profile to reflect a real *in vivo* condition.

At present, there have been several technical methods developed to access expression profiles of casein. Reverse transcription polymerase chain reaction (RT-PCR) analysis has been used by a few studies to determine the mRNA expression level of casein in MECs. However, drawbacks of this method are that mRNA expression level may not always truly reflect the level of the protein, and this method requires high-quality templates, and the process is complicated and time-consuming [24]. In addition, liquid chromatography tandem-mass spectrometry (LC-MS) has been used to determine the casein contents at different times after *E. coli* infection [25]. However, this is also time-consuming and labor-intensive. In the present study, a regular immunofluorescence assay using mAbs against bovine α-casein were used to determine casein levels in MECs. Compared with the above mentioned methods, our method is easier to conduct and the yield results are more accurate.

Plenty of evidence exists that confirms the ability of MECs to synthesize and secrete casein changes under different conditions [15,26], and it is very important to measure these changes for the evaluation of cellular function. The total casein content showed a decreasing trend in an *in vitro* mastitis model. However, the changes of different caseins are different. Using an ELISA method, it was found that β-casein decreased significantly during mastitis, while the content of α-casein did not change significantly [15], which was in compliance with the results of several other studies [25,27]. In this study, we found that the content of α-casein of primary MECs did not change significantly under the stimulation of various concentrations of LPS, indicating that

the expression level of α-casein does not alter significantly during mastitis. Thus, α-casein alone is not suitable for the evaluation of the function and state of MECs. However, as discussed above, the mAbs developed in the present study cannot distinguish αs1- from αs2-casein. It is possible that if the expression levels of these two casein subtypes show an opposite trend, the stable expression profile of α-casein during mastitis will also be generated. Therefore, further investigations into developing mAbs with better specificity to αs1- and αs2-caseins are needed. In addition, a more appropriate biomarker for MEC function evaluation is also needed in the future.

Conflict of Interest

The authors declare that no conflict of interest exists.

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Research Article

Antimicrobial Susceptibility, Serotypes, and Genetic Diversity of Streptococcus suis in Diseased and Healthy Pigs in Southern China

Ling PENG [1,2,a] Chongbo XU [1] Xufu YANG [1,2] Boting LIU [1]

[1] Yingdong College of Life Science, Shaoguan University, Shaoguan 512005, CHINA
[2] Joint Laboratory of Animal Infectious Diseases Diagnostic Center-Harbin Veterinary research Institute of Chinese Academy of Agriculture Science, Shaoguan University, Shaoguan 512005, CHINA
[a] ORCID: 0000-0002-7798-703X

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Abstract

In the study, the characteristics of 81 *Streptococcus* suis isolates recovered from healthy and diseased pigs in Southern China were analyzed. These isolates showed differential resistance rates of over 40% to 22 and over 80% to 11 antibiotics out of 42 tested drugs. These isolates belonged to 6 serotypes (2, 3, 7, 9, 19, and 28), 9.9% of which were characterised as non-typable (NT), and were classified into 15 sequence types (STs), 8 of which were new (ST812-ST815, ST821-823, and ST832). Additionally, 6 virulence-associated gene patterns (VAGPs) and 10 random amplified polymorphic DNA (RAPD) patterns were found among the 81 strains. Among the 4 genotyping methods used in this study, multilocus sequence typing (MLST) provided the highest resolution for identification of S. suis strains. Significant differences in STs, RAPD patterns, and VAGPs were observed between strains isolated from healthy pigs and those isolated from diseased pigs. This study did not find any statistically significant correlations between antimicrobial resistance and serotypes, STs, RAPD patterns, VAGPs, or strain sources. Serotype 2 strains were predominant (44.4%) in the study and all of these strains showed the molecular characteristics of highly pathogenic strains in VAGP, GDH sequence types, and MLST. Our results showed that *S. suis* strains in Southern China are continually evolving, and therefore increased surveillance of *S. suis* in piggeries must be considered.

Keywords: *Streptococcus suis, Antimicrobial susceptibility, Multilocus sequence typing, Virulence genes, RAPD, Glutamate dehydrogenase*

Güney Çin'de Hastalıklı ve Sağlıklı Domuzlarda *Streptococcus suis*'in Antimikrobiyal Direnci, Serotipleri ve Genetik Çeşitliliği

Öz

Bu çalışmada Güney Çin'de sağlıklı ve hasta domuzlardan elde edilen 81 *Streptococcus suis* izolatının özellikleri incelendi. İzolatlar, test edilen 42 antibiyotikten 22'sine %40'ın üzerinde ve 11'ine %80'in üzerinde değişen direnç gösterdi. Bu izolatlar 6 serotipe (2, 3, 7, 9, 19 ve 28) ait olup %9.9'u tiplendirilemeyen olarak karakterize edildi ve 8'i yeni (ST812-ST815, ST821-823 ve ST832) olmak üzere 15 sekans tipine (ST) sınıflandırıldı. Ayrıca, 81 suşta 6 adet virulans ile ilişkili gen şeması (VAGP) ve 10 adet rastgele amplifiye polimorfik DNA (RAPD) şeması bulundu. Çalışmada kullanılan 4 genotiplendirme metodu arasında multilokus sekans tiplendirme (MLST) S. suis suşlarının tespitinde en yüksek rezolusyonu sağladı. Sağlıklı ve hasta domuzlardan izole edilen suşlar arasında sekans tiplerinde, RAPD şemasında ve VAGP'lerde anlamlı farklılıklar gözlemlendi. Çalışmada antimikrobiyal direnç ile serotipler, ST, RAPD şeması, VAGP'ler veya suş kaynakları arasında istatistiksel olarak bir ilişki tespit edilmedi. Serotip 2 suşu çalışmadaki predominant olandı ve tüm bu suşlar VAGP, GDH sekans tipi ve MIST'de oldukça patojenik suşların moleküler özelliklerini göstermekteydi. Elde edilen sonuçlar Güney Çin'de *S. suis* suşlarının sürekli olarak değiştiğini ve bu yüzden domuzlarda *S.suis* takibinin gerekli olduğunu göstermiştir.

Anahtar sözcükler: *Streptococcus suis, Antimikrobiyal direnç, Multilokus sekans tiplendirmesi, Virulans genler, RAPD, Glutamat dehidrogenaz*

INTRODUCTION

Streptococcus suis (S. suis) is an opportunistic zoonotic pathogen. *S. suis* can infect humans and various animals, causing many serious diseases such as meningitis, septicemia, endocarditis, pneumonia, and pyogenic arthritis [1]. At least 33 serotypes (1-31, 1/2, and 33) of *S. suis* have been described according to the differences in capsule antigen [2-4]. In recent years, novel variant serotypes of *S. suis* have been reported [5].

İletişim (Correspondence)
+86-13826319175
penglingfx@126.com

Serotyping of *S. suis* is one of the most useful methods to understand the pathogen epidemiology. Although the disease largely should be mostly caused by a small number of serotypes, it is recognized that the capsular serotype is a poor marker of virulence. Not all isolates allocated to the same serotype cause the same type of disease, and virulence can vary substantially both within and among serotypes [6]. Differences in virulence among *S. suis* isolates are related to virulence-associated genes. At present, more than 70 virulence-associated genes have been found in *S. suis*, most of which are bacterial surface components, surface proteins, extracellular proteins, enzymes, and regulatory factors. These virulence genes are directly or indirectly involved in adhesion to host cells, survival *in vivo,* and immune escape [7]. By analyzing the main virulence genes of *S. suis* strains, it would be easier to understand the molecular characteristics and pathogenicity of the strains [8-10].

Antimicrobial agents play an important role in the treatment and control of *S. suis* infection. However, irrational use of antibiotics leads to a continual increase in drug resistance of *S. suis* worldwide [11-15]. Therefore, antimicrobial susceptibility testing is a crucial step preceding the prescription of antibiotic treatment.

S. suis genotyping and associated research techniques can be used to compare the epidemic strains from different regions, better understand *S. suis* epidemiology, predict the origins, causes, and epidemic trends of the disease. *S. suis* genotyping has been carried out using a variety of methods [16-18].

In this study, the antimicrobial susceptibility of 81 isolates from healthy and diseased pigs in Southern China were tested against 42 antimicrobial agents. These isolates were characterized by serotyping, multilocus sequence typing (MLST), random amplified polymorphic DNA (RAPD), virulence-associated gene pattern (VAGP), and sequence analysis of the gene encoding glutamate dehydrogenase (GDH). The potential relationship between antimicrobial resistance and these characteristics was also analyzed.

MATERIAL and METHODS

Strains and Serotyping

A total of 81 *S. suis* strains were isolated from healthy and diseased pigs in Southern China between 2013 and 2016. Fourteen were from the nasal cavity of healthy pigs and 67 were from the blood, joint fluid, and lungs of diseased pigs. The isolates were serotyped using the method described in Okural et al.[19].

Antimicrobial Susceptibility

The antimicrobial susceptibility of the *S. suis* isolates was assessed using the disk diffusion method described by Soares et al.[20]. Antimicrobial disks (Hangzhou Tianhe Microbiological Co., Hangzhou, China) and associated concentration are described in *Table 1*. Growth inhibition was assessed against NCCLS standards and the isolates were classified as susceptible, intermediate or resistant. Strains ATCC 49619 *(Streptococcus pneumoniae)* was included as experimental control.

Detection of Virulence-Associated Genes

The virulence-associated genes extracellular protein factor *(epf),* murimidase-released protein *(mrp),* suilysin *(sly),* arginine deiminase *(arcA)*, 38-kDa protective antigen *(bay046),* hyaluronidase *(hyl),* and virulence-associated sequences *orf2* were detected by PCR, as previously described [21-25]. Details of all oligonucleotide primers used are listed in *Table 2*.

Nucleotide Sequence Determination of Glutamate Dehydrogenase

In this study, the *gdh* gene was PCR amplified using the primers GDHF (5'AACA TTCGGATTTTGCAATAAAAA3') and GDHR (5'ATTAGCACGTCAATTTTGGGG3'). The primers were designed using a previously reported *gdh* nucleotide sequence in GenBank (GenBank accession number AF229683) [26]. Subsequently, the PCR product obtained from each isolate was sub-cloned into the pUCm-T vector and sequenced using the dideoxy chain-termination method (Sangon Biotech Ltd., Shanghai, China). GDH sequence types of the *S. suis* isolates were analyzed using the method described by Kutz et al.[27].

DNA Fingerprinting by Random Amplified Polymorphic DNA

RAPD of the *S. suis* isolates was performed based on the method previously described [28-30]. The RAPD primer S298 (5'-GTGGAGTCAG-3') was used to analyze *S. suis* isolates. PCR reaction mixtures contained 10 mmol/L Tris-HCl (pH8.3), 10 mmol/L KCl, 2.0 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 1.5 U Taq DNA polymerase, 0.5 µmol/L of each primer, and 1.0 uL of genomic DNA. The PCR conditions were as follows: 1 cycle at 94°C for 2 min; 40 cycles at 94°C for 30 sec, 36°C for 30 sec, and 72°C for 2 min; and a cycle at 72°C for 6 min. The amplified products were analyzed using 1.4% agarose gel electrophoresis.

Multilocus Sequence Typing

A total of seven housekeeping genes were used in the MLST analysis of the *S. suis* isolates: *aroA*, *cpn60*, *dpr*, *gki*, *mutS*, *recA*, and *thrA*. These genes were amplified by PCR described in King et al.[31]. The PCR products were sequenced from both directions by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The housekeeping gene sequences, obtained for each isolate, were edited, assembled, and aligned by the software MEGA version 5.0 [32]. Then, the sequences were submitted to the MLST

database *(https://pubmlst.org/ssuis/)* for the analysis of allele number and sequence type (ST) according to the methodology described by Jolley et al.[33]. The population structure and clonal complexes of the *S. suis* isolates were determined using the eBURST software [34].

RESULTS

In this study, the antibiotic resistance of the 81 *S. suis* isolates against 42 different antibiotics were tested. The resistance rates to 11 antibiotics were high (over 80%), to 11 antibiotics were intermediate (40-80%) and to 15 antibiotics were low (beow 30%) *(Table 1)*. Furthermore, all isolates showed 0% resistance to cefradine, cefazolin, teicoplanin, vancomycin and rifampicin *(Table 1)*.

Six distinct serotypes within the 81 serotyped *S. suis* isolates were identified. Serotype 2 (44.4%) was the most prevalent, followed by serotypes 7 (22.2%), 3 (8.6%), 9 (7.4%), 19 (4.9%), and 28 (2.5%). A total of 9.9% of the isolates tested were non-typable (NT). All isolates from diseased pigs were classified as serotypes 2, 3, 7, and 9. All of the isolates from healthy pigs were classified as serotypes 19, 28, and NT *(Table 3)*.

There were 6 kinds of VAGPs in all 81 strains tested. All *S. suis* serotype 2 strains were positive for 7 virulence genes and were classified as VAGP1. Serotype 3, 7, and 9 strains were positive for 5 virulence genes and were classified as VAGP2 (they lacked *epf* and *siy)*. There were 4 kinds of VAGPs in 14 strains from healthy pigs. All strains were positive for virulence genes *acrA* and *bay046*. Seventy-nine strains (97.5%) were positive for virulence genes *mrp*, while only 2 strains from healthy pigs were negative. Seventy-six strains (93.8%) were positive for virulence genes *orf2* and only 5 strains from healthy pigs were negative. All of the strains from diseased pigs were positive for virulence genes *hyl* and all of the strains from healthy pigs were negative for virulence genes *hyl*. Virulence genes *epf* and *siy* were only distributed in serotype 2 strains *(Table 3)*.

In the study, RAPD patterns of 3 to 8 bands with sizes ranging between 300 and 2.000 bp were obtained using RAPD primer S298. The 81 *S. suis* strains showed 10 RAPD patterns (RAPD-A to RAPD-J) according to the differences in numbers and band sizes *(Table 3)*.

In this study, we identified 2 new alleles *(cpn60* 311 and *cpn60* 312). A total of 15 STs were found and 8 STs (ST812-ST815, ST821-823, and ST832) were new MLST STs. ST1, ST86, ST242, ST253, ST812, and ST813 belonged to the clonal complex 1 (CC1), ST117 and ST29 belonged to CC2, ST243 and ST814 belonged to CC55, ST815 belonged to CC67, ST821 and ST822 belonged to CC71, ST823 belonged to CC53, and ST833 were singletons *(Table 3; Fig. 1)*.

Highly virulent strains of *S. suis* serotype 2 can be distinguished from moderately virulent and nonvirulent strains on the basis of their GDH [26,27]. The isolates of *S. suis* serotype 2 were divided into 3 groups according to deduced GDH amino acid sequence. Group I consisted of highly virulent isolates, containing Ala299-to-Ser, Glu305-to-Lys, and Glu330-to-Lys amino acid substitutions compared

Table 1. *Antimicrobial resistance profiles of S. suis strains from southern China*

Antibiotic Tested (μg)	Level of Susceptibility		
	Resistant (%)	Intermediate (%)	Sensitive (%)
Clarithromycin (15)	70.4	3.7	25.9
Azithromycin (15)	81.5	0	18.5
Erythromycin (15)	70.4	3.7	25.9
Roxithromycin(15)	74.1	3.7	22.2
Amoxicillin (10)	22.2	0	77.8
Ampicillin (10)	29.6	0	70.4
Carbenicillin (100)	40.7	0	59.3
Penicillin (10IU)	14.8	0	85.2
Cefradine (30)	0	0	100
Cefazolin (30)	0	0	100
Cefalexin (30)	3.7	3.7	92.6
Cefixime (30)	81.5	11.1	7.4
Cefotaxime (30)	22.2	0	77.8
Cefatriaxone (30)	11.1	0	88.9
Cefuroxime (30)	7.4	25.9	66.7
Gentamicin (10)	77.8	11.1	11.1
Kanamycin (30)	66.7	29.6	3.7
Amikacin (30)	88.9	3.7	7.4
Spectinomycin (100)	3.7	0	96.3
Streptomycin (10)	48.1	18.5	33.3
Tetracycline (30)	85.2	3.7	11.1
Deoxytetracycline (30)	22.2	40.7	37.1
Lomefloxacin (10)	85.2	11.1	3.7
Enrofloxacin (5)	3.7	0	96.3
Ciprofloxacin (5)	11.1	63	25.9
Norfloxacin (10)	44.4	29.6	25.9
Fleroxacin (10)	48.1	33.3	18.5
Levofloxacin (5)	3.7	3.7	92.6
Ofloxacin (5)	3.7	0	96.3
Enoxacin (10)	81.5	14.8	3.7
Nalidixan (30)	100	0	0
Trimethoprim (1.25) + Sulfamethoxazole (23.75)	44.4	7.4	48.1
Sulfisoxazole (300)	85.2	7.4	7.4
Trimethoprim	70.4	3.7	25.9
Clindamycin (30)	81.5	0	18.5
Cillimycin (2)	96.3	0	3.7
Chloramphenicol (30)	7.4	25.9	66.7
Teicoplanin (30)	0	3.7	96.3
Vancomycin (30)	0	0	100
Foroxone (300)	92.6	0	7.4
Furadantin (300)	22.2	29.6	48.1
Rifampicin (5)	0	7.4	92.6

Table 2. *Oligonucleotide primer sequences used in this study*

Genes	Primer Sequence (5'-3')	PCR Product Size (bp)	Reference
gdh	AACATTCGGATTTTGCAATAAAAA ATTAGCACGTCAATTTTGGGG	1500	This study
epf	CGCAGACAACGAAAGATTGA AAGAATGTCTTTGGCGATGG	744	[21]
sly	ACTCTATCACCTCATCCGC ATGAGAAAAAGTTCGCACTTG	1400	[22]
mrp	ATTGCTCCACAAGAGGATGG TGAGCTTTACCTCATCCGC	188	[21]
hyl	CTCAGATGAAAGCCTTTCTA TTTGTCCTTGGTCGTTGTC	1290	[23]
arcA	TGATATGGTTGCTGCTGGTC GGACTCGAGGATAGCATTGG	118	[21]
bay046	ATGCCACGGATTACCTTCCC CCGTCTCCTTAATGATCCGC	253	[24]
orf2	CAAGTGTATGTGGATGGG ATCCAGTTGACACGTGCA	858	[25]

Table 3. *Source of isolation and characteristics of the 81 S. suis isolates in this study*

ST/ CC	Presence of Virulence-Associated Genes							VAGP	Serotype	RAPD Pattern	Number of Strains	Source
	epf	sly	mrp	hyl	orf2	acrA	bay046					
1/1	+	+	+	+	+	+	+	1	2	A	19	The blood, joint fluid and lung of diseased pigs
86/1	+	+	+	+	+	+	+	1	2	A	2	
812	+	+	+	+	+	+	+	1	2	A	2	
813	+	+	+	+	+	+	+	1	2	A	2	
242	+	+	+	+	+	+	+	1	2	B	9	
253	+	+	+	+	+	+	+	1	2	C	2	
117/2	-	-	+	+	+	+	+	2	3	D	7	
29/2	-	-	+	+	+	+	+	2	7	E	18	
243/55	-	-	+	+	+	+	+	2	9	F	4	
814/55	-	-	+	+	+	+	+	2	9	F	2	
815/67	-	-	+	-	+	+	+	3	19	G	4	The nose swabs of healthy pigs
833/-	-	-	+	-	+	+	+	3	28	H	2	
821/71	-	-	-	-	+	+	+	4	NT	I	1	
821/71	-	-	+	-	-	+	+	5	NT	I	2	
821/71	-	-	-	-	-	+	+	6	NT	I	1	
822/71	-	-	+	-	+	+	+	3	NT	I	2	
823/53	-	-	+	-	-	+	+	5	NT	J	2	

with groups II and III. Groups II and III consisted of moderately virulent and nonvirulent strains, which are separated from each other by Tyr72-to-Asp and Thr296-to-Ala substitutions [27]. In this study, the GDH gene of 36 strains of *S. suis* serotype 2 obtained a 1.500-bp fragment containing an open reading frame of 1.344 nucleotides by PCR and nucleotide sequence determination. According to the deduced amino acid sequence, these strains were all classified as GDH Group I.

The resistance to 42 antimicrobial agents among each characteristic of all these isolates as a whole was counted and compared it with the distribution of serotypes, STs, RAPD patterns, VAGP, and strain sources to the distribution of antimicrobial resistance. The isolation frequency of each serotype was very similar to the incidence of antimicrobial resistance in the corresponding serotype, the isolation frequency of each ST was very similar to the incidence of antimicrobial resistance in the corresponding ST, the isolation frequency of each RAPD pattern was very similar to the incidence of antimicrobial resistance in the corresponding RAPD pattern, the isolation frequency of each VAGP was very similar to the incidence of antimicrobial resistance in the corresponding VAGP, and the isolation frequency of diseased pigs or healthy pigs was also very similar to the incidence of antimicrobial resistance in the corresponding source of strains. The statistical results showed that there was no significant correlation between antimicrobial resistance and serotypes, STS, RAPD patterns, or VAGPs *(Fig. 2-Fig. 6)*.

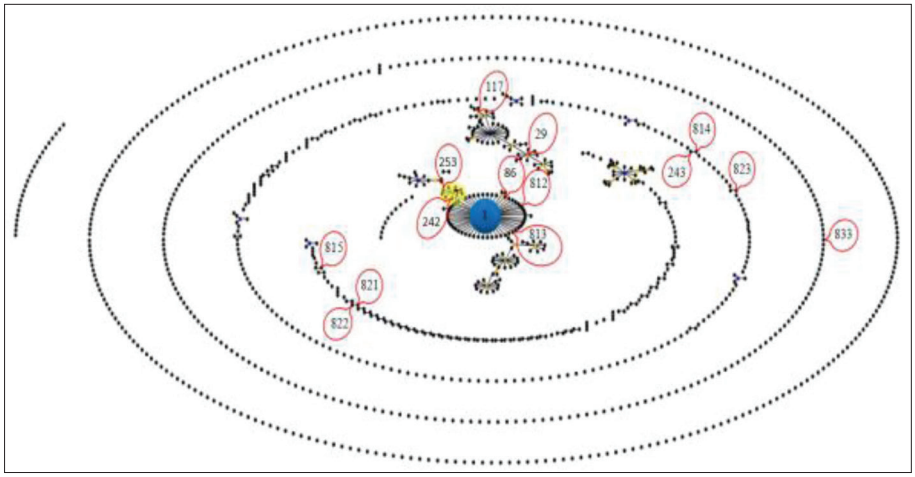
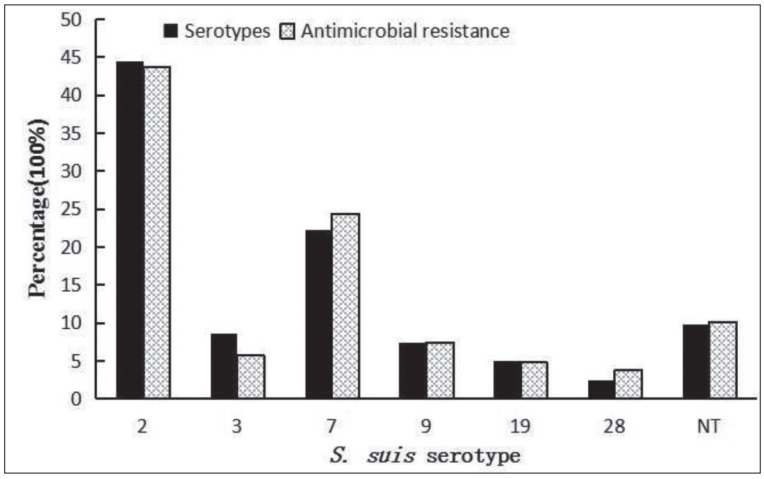


Fig 1. The population structure based 1096 STs from the MLST database of *Streptococcus suis*

Fig 2. Distribution of *S. suis* serotypes and antimicrobial resistance among each of these serotypes. Serotypes: frequency (%) = number of strains belonging to a certain serotyp/total number of strains ×100%; Antimicrobial resistance: incidence (%) = number of resistances to all tested antimicrobial agents among strains of a certain serotype/ total number of resistances to all tested antimicrobial agents across all strains×100%



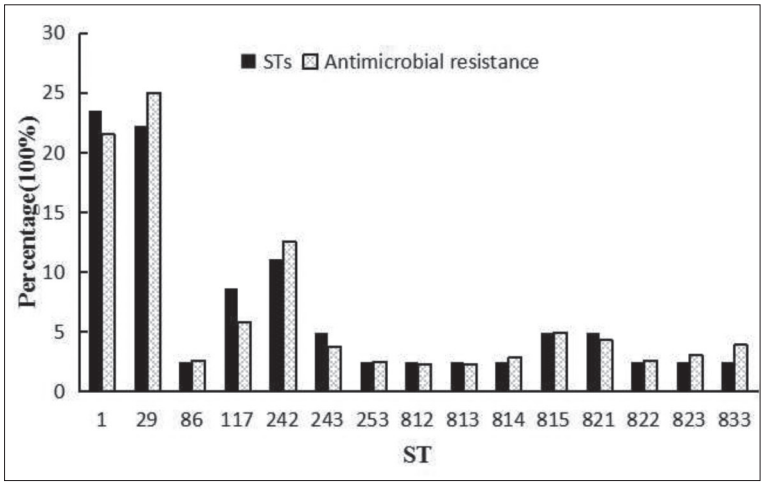


Fig 3. Distribution of *S. suis* STs and antimicrobial resistance among each of these STs. STs: frequency (%) = number of strains belonging to a certain ST/total number of strains ×100%; Antimicrobial resistance: incidence (%) = number of resistances to all tested antimicrobial agents among strains of a certain ST/total number of resistances to all tested antimicrobial agents across all strains×100%

DISCUSSION

In this study, the antimicrobial susceptibility of 81 isolates of *S. suis* from Southern China was tested against 42 antimicrobial agents. Testing of 42 antibiotics indicated that these isolates were differentially resistant to 22 (over 40%) and 11 (over 80%) antibiotics.

Previous reports suggested that *S. suis* strains had a high resistance to macrolides, lincosamides, tetracycline, and sulfonamides [11-15]. The *S. suis* isolates in this study showed a high resistance to macrolides, lincosamides, tetracycline, and sulfonamides, as well as a high resistance to quinolones and aminoglycosides.

Interestingly, a high resistance to foroxone (92.6%) and lomefloxacin (85.2%) was found in this study. The use of foroxone has been banned in veterinary medicine in China

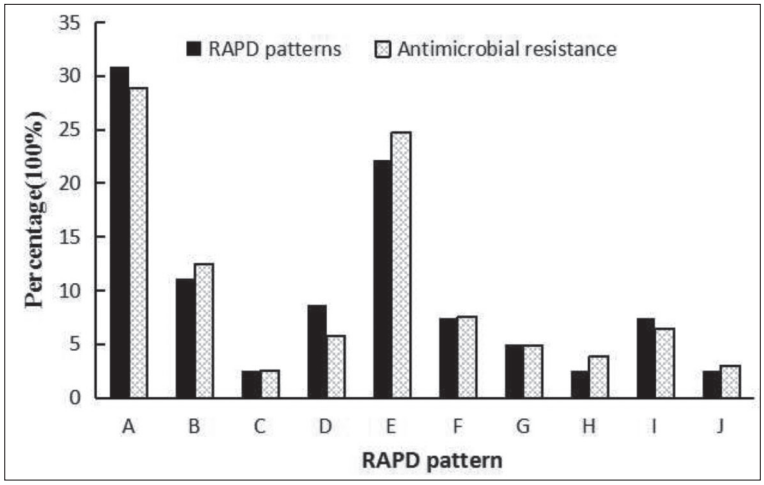
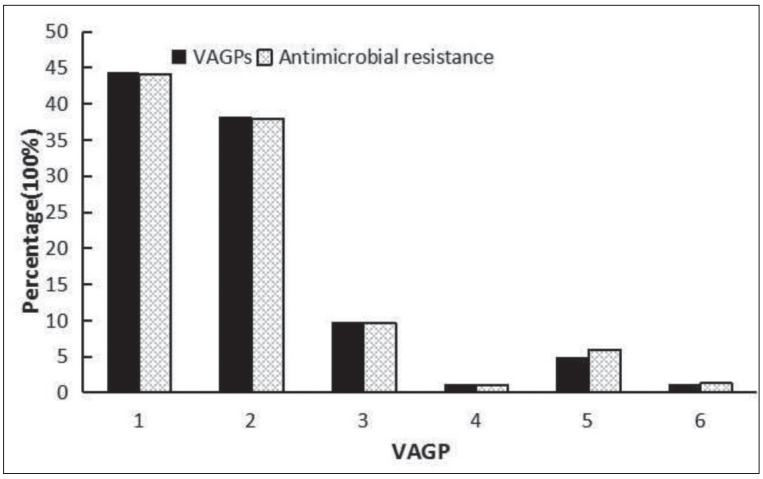


Fig 4. Distribution of *S. suis* RAPD patterns and antimicrobial resistance among each of these RAPD patterns. RAPD patterns: frequency (%) = number of strains belonging to a certain RAPD pattern/total number of strains ×100%; Antimicrobial resistance: incidence (%) = number of resistances to all tested antimicrobial agents among strains of a certain RAPD pattern/total number of resistances to all tested antimicrobial agents across all strains×100%

Fig 5. Distribution of *S. suis* VAGPs and antimicrobial resistance among each of these VAGPs. VAGPs: frequency (%) = number of strains belonging to a certain VAGP/total number of strains ×100%; Antimicrobial resistance: incidence (%) = number of resistances to all tested antimicrobial agents among strains of a certain VAGP/total number of resistances to all tested antimicrobial agents across all strains×100%



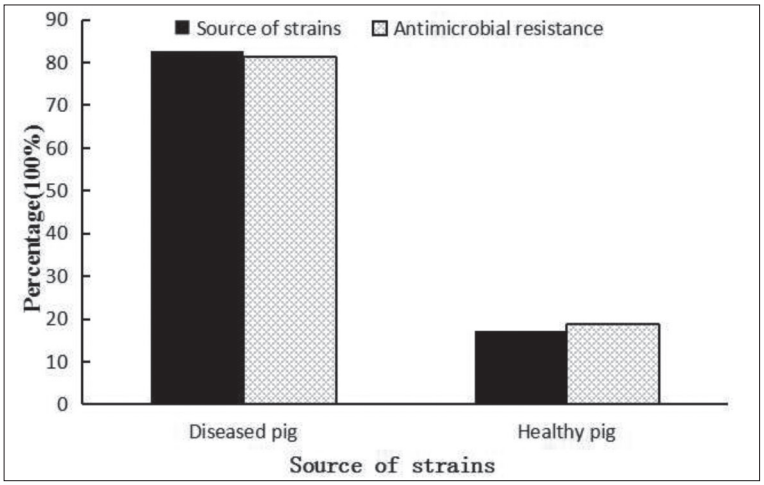


Fig 6. Distribution of source of strains and antimicrobial resistance in corresponding source of strains. Source of strains: frequency (%) = number of strains isolated from diseased pigs or healthy pigs/total number of strains ×100%; Antimicrobial resistance: incidence (%) = number of resistances to all tested antimicrobial agents among strains isolated from diseased pigs or healthy pigs /total number of resistances to all tested antimicrobial agents across all strains×100%

since 2002, while lomefloxacin was banned in 2015. Bischoff et al.[35] reported that antimicrobial resistance can be sustained when antimicrobial selective pressure is removed. The reason for this may be that resistance selection of bacteria can occur through a variety of mechanisms and is not always related to the use of specific antibiotics.

Previous reports suggested the resistance rate for β-lactams in *S. suis* was low [11,14,36]. In this study, the resistance rate of other β-lactams was relatively low except for cefixime, which indicates that β-lactams may be the primary drugs to treat the infection of *S. suis*.

Previous studies indicated that tetracycline-resistance was an important cofactor in the selection of resistance to macrolides/lincosamides [37,38]. In this study, 85.2% of the 81 isolates were resistant to tetracycline, 82.6% of which

were coresistant to macrolides and lincosamides. This indicated more frequent coresistance to tetracyclines and macrolides/lincosamides in *S. suis* isolates from Southern China.

The potential correlation between the antimicrobial resistance and serotypes, STS, RAPD patterns, VAGPs, or strain sources were examined,no statistically significant correlations between them were found (x^2-test, $P>0.05$).

In this study, 14 *S. suis* strains were isolated from healthy pigs, while 67 were isolated from diseased pigs. Seventy-three strains belonged to 6 distinct serotypes (2, 3, 7, 9, 19, and 28). The 8 remaining strains were NT. Six VAGPs, 10 RAPD patterns, and 15 STs were found among the 81 strains. The strains classified as same ST had the same serotype, VAGP, and RAPD patterns. All serotype 2 strains in the study belonged to VAGP1, but they were divided into 6 STs and 3 RAPD patterns. Serotype 2 strains with VAGP1/RAPD-A profiles were classified as ST1, ST86, ST812, and ST813. Serotype 9 strains with VAGP3/RAPD-F profiles were classified as ST243 and ST814. The 6 NT strains with an RAPD-I pattern were classified as ST821 and ST822; the 2 NT strains with an RAPD-J pattern were classified as ST823, but the 8 NT strains possessed 4 VAGPs. Accordingly, the MLST provided the highest resolution for *S. suis* strains among the 4 typing methods used in the study. There was a certain correlation among serotypes, STs, VAGPs, and RAPD patterns.

Serotype 2 strains were predominant in this study. These results are in line with previous reports [39,40]. All of these serotype 2 strains belonged to the ST1 clonal complex, indicating one route of clonal dissemination of these serotype 2 isolates. The ST1 clonal complex is the most widely distributed, possessing the highest pathogenicity and the largest number of isolates reported worldwide. In China, ST7 strains have been often isolated. ST7 is a SLV of ST1 (allelic profile 1, 1, 1, 1, 1, 1, 1) with increased virulence [41], but ST7 strains were not found in this study. However, 19 ST1 strains, 9 ST242 strains, and 2 ST253 strains were found. ST242 and ST253 are SLVs of ST7 (allelic profile 1, 1, 1, 1, 1, 1, 3). 4 serotype 2 strains classified as ST812 and ST813 were found; ST812 and ST813 are SLVs of ST1. The results show that *S. suis* strains in Southern China are continually evolving.

Serotype 2 strains possessed all 7 virulence genes, while serotypes 3, 7, and 9 possessed 5 virulence genes (lacking *epf* and *sly)*. The strains isolated from healthy pigs possessed 2-4 virulence genes (lacking *epf*, *sly,* etc.).

In this study, the 36 strains of *S. suis* serotype 2 were all classified as GDH Group I by the analysis of the *gdh* gene. As mentioned earlier, Group I consisted of highly virulent isolates. Therefore, these serotype 2 strains showed the molecular characteristics of highly pathogenic strains in VAGP, GDH sequence types, and MLST.

For the first time, this study provided an overall and systemic description of the correlation between the anti-microbial resistance and serotypes, STS, RAPD patterns, VAGPs, or strain sources in *S. suis* from Southern China,but, no significant correlation was observed. Significant differences in STs, RAPD patterns, and VAGPs were observed between strains isolated from healthy pigs and diseased pigs. β-lactams are still the most effective drugs to be used for the treatment of *S. suis* infections in Chinese veterinary clinics. eBURST analysis of *S. suis* isolates showed that the *S. suis* isolates in China are continually evolving. Therefore, increased surveillance of *S. suis* in piggeries must be considered. Detection of virulence-associated genes and determination of GDH and MLST can help predict the virulence of *S. suis* serotype 2 isolates.

Acknowledgment

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Research Article

Inducible Nitric Oxide Synthase (iNOS) Enzyme Activity and Transcription Level as Well as Ultrastructural Changes in Different Tissues of Grass Carp in Response to *Ichthyophthirius multifiliis*

Yingjie LU [1] Shuwen TAN [1] Bingqing O [1] Xiwen ZHANG [2]
Ying YANG [1] Shujian HUANG [1] Saeed EL-ASHRAM [1,3,a]

[1] College of Life Science and Engineering, Foshan University, 18 Jiangwan Street, Foshan TR-528231 Guangdong Province, CHINA
[2] The University of Georgia, Athens, USA
[3] Kafrelsheikh University, Kafr el-Sheikh, 33516, EGYPT
[a] ORCID: 0000-0003-0389-1980

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Abstract

This study aims to contribute to this growing area of research by exploring the gene expression and enzyme activity of iNOS, and provide new insights into the ultrastructural changes in the kidneys, spleen, skin and gills infected with *Ichthyophthirius* on grass carp. Furthermore, electron microscopy (EM) findings illustrated the uneven distribution of the macrophages and lymphocytes, and the concentrated and fragmented and/or disappeared cell nuclei in the kidneys, spleen, gills and skin. The extreme ultrastructural changes resulted in the reduction in the number of immune cells and subsequent downregulation of iNOS enzyme activity, especially in the gills and skin of *Ichthyophthirius*-infected grass carp. Our results showed that enzyme activity and gene expression of iNOS were significantly down-regulated in the kidneys and spleen ($P>0.05$), gills and skin ($P<0.05$) compared with the negative control group. The data showed the different levels of damages against *I. multifiliis* infection in the kidneys, spleen, gills and skin. Taken together, expression and activity of inducible nitric oxide synthase could serve as an important indicator to determine the pathological status of the *I. multifiliis*-grass carp.

Keywords: *Ichthyophthirius multifiliis, Gene expression, Enzyme activity Ctenopharyngodon idella iNOS, Ultrastructural changes*

Ichthyophthirius multifiliis'e Karşı Çim Sazanının Farklı Dokularında İndüklenebilir Nitrik Oksit Sentaz (iNOS) Enzim Aktivitesi, Transkripsiyon Seviyesi ve Ultrastrüktürel Değişiklikler

Öz

Bu çalışma *Ichthyophthirius* ile enfekte edilmiş Çim sazanının böbrek, dalak, deri ve kuluçkasında meydana gelen ultrastrüktürel değişiklikler ile iNOS enzim aktivitesi ve gen ekspresyonunu araştırmak suretiyle katkıda bulunmayı amaçlamaktadır. Böbrek, dalak, solungaç ve deride elektron mikroskopi bulguları makrofaj ve lenfositlerin dengesiz dağılımı ile konsantre ve parçalanmış ve/veya gözden kaybolmuş çekirdeğin olduğunu göstermiştir. *Ichthyophthirius* ile enfekte Çim sazanının özellikle solungaç ve derisinde aşırı ultrastrüktürel değişiklikler, immun hücrelerin sayısında azalma ve sonrasında iNOS enzim aktivitesinde aşağı yönde ayarlanma ile sonuçlandı. Elde edilen sonuçlar, iNOS enzim aktivitesi ve gen ekspresyonunun negatif kontrol grubu ile karşılaştırıldığında böbrek ve dalakta anlamlı derecede ($P>0.05$), solungaçlarda ve deride ($P<0.05$) aşağı yönde ayarlandığını gösterdi. Elde edilen veriler *I. multifiliis* enfeksiyonuna karşı böbrek, dalak, solungaç ve deride farklı derecelerde hasarların oluştuğunu göstermiştir. *I. multifiliis* ile enfekte Çim sazanında patolojik durumun belirlenmesinde indüklenebilir nitrik oksit sentaz gen ekspresyonu ve enzim aktivitesi bir belirteç olarak kullanılabilir.

Anahtar sözcükler: *Ichthyophthirius multifiliis, Gen ekspresyonu, Ctenopharyngodon idella iNOS Enzim aktivitesi, Ultrastrüktürel değişiklikler*

İletişim (Correspondence)
+86-18620403131; Fax: +86-757-85505013
huangshujian@fosu.edu.cn (S. Huang); saeed_elashram@yahoo.com (S. El-Ashram)

INTRODUCTION

Ichthyophthirius multifiliis, ciliated protozoan that causes "Ich" or "white spot disease" is a major burden for fish farmers and aquarists world-wide. The parasite has been detected globally in both cultured and wild fish, and all species of freshwater fish are considered susceptible. Ich has both host life stage, such as trophont and environmental life stages, such as tomont, tomite and theront. *I. multifiliis* causes remarkable economic losses in all freshwater fish, resulting in huge mortalities [1,2]. The grass carp *(Ctenopharyngodon idellus)* is one of the major kinds of freshwater fish cultured in China, belonging to family *Cyprinidae*. Interest in grass carp as a food has emerged throughout the world because of their delicious and unique flavor. Grass carp has become an attractive food source, with good economic potential and high commercial interest in China. In recent years, the production of the grass carp has decreased due to infection of *I. multifiliis*. Our research group has reported the up-regulation of *MH II-DAB* gene in spleen, gills and skin, and cell degeneration and necrosis are observed immune and other related tissues of *Ichthyophthirius*-infected grass carp [3]. Subsequent studies have recorded the marked upregulation of superoxide dismutase (SOD) in the spleen, liver and muscle tissue of *Ichthyophthirius*-infected grass carp with mechanical damages of different tissues as well as mitochondrial damages [4]. Nitric oxide (NO) is a significant biologically active molecule that plays a key part in host defense against microbial infections. NO is formed by the nitric oxide synthase (NOS), which is one of the important immune regulatory factors [5-7]. There are three NOS isoforms, endothelial constitutive NOS (ecNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [8,9]. iNOS is expressed in many cell types, including macrophages, neutrophils and fibroblasts in response to a range of immunological stimuli, such as cytokines and lipopolysaccharide (LPS). It has dual roles as a critical agent of host defense in infection and as a central mediator of pathogenesis [10,11]. Very little is known about iNOS enzyme activity and gene expression level as well as pathologic and ultrastructural changes in different tissues of the *Ichthyophthirius*-infected grass carp. Therefore, the present study fills a gap in the literature by studying the gene expression and enzyme activity of iNOS, and providing new insights into the ultrastructural changes in the kidneys, spleen, skin and gills of the *Ichthyophthirius*-infected grass carp.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Animal Ethics Committee of the College of life science and engineering, Foshan University, Guangdong, China. The College did not issue a number or ID to this animal study, because the studied fish are not an endangered or protected species. Specimen collection was carried out based upon the protocol issued by the Animal Ethics Committee of the College of life science and engineering. Furthermore, all methods were performed in accordance with the relevant guidelines and regulations

Parasite

Laboratory cultures of *I. multifiliis* obtained from a fish farm (Guangzhou, Guangdong province, China) which was maintained at 20°C by serial passage in grass carp *(Ctenopharyngodon idella)* [12].

Fish and Exposure to Ichthyophthirius Infection

Grass carp *(C. idellus)*, weighing 40±2 g, was obtained from the Holdone Aquaculture Breeding Limited Company. The fish were acclimatized for one week before infection experiments initiated. A total of 100 fish were randomly divided into four groups at a density of 25 fish per each group. Fish were fed once at a level of 1% average fish weight per meal daily with a commercial fish pellet feed, manufactured by the Institute of Hydrobiology, Chinese Academy of Sciences. Three groups were infected by *I. multifiliis*, and the 4th group is negative control (uninfected grass carp). Fish were infected with a high dose of live trophont (immersion) in the dark for 8 h. At 4 to 6 days after infection (after some trial runs, we decided to set up the experiments at 4 to 6 days to avoid grass carp death) at 21±2ºC, pH 7.0, with dissolved oxygen 6.0-7.8 mg/L, ammonia content (total nitrogen) 0.5-2.0 mg/L and total hardness ($CaCO_3$) 85.0-104.5 mg/L, six fish were gently transferred to a small glass aquarium containing a mild anesthetic (MS 222, 20 mg/L). In the laboratory, the fish were killed quickly with an overdose of MS222 (200 mg/L), then the spleen, kidneys, skin and gills of the control and infection groups were collected and divided into small pieces and stored in liquid nitrogen (LN2) and fixed in 2.5% glutaraldehyde in the phosphate buffer for total RNA extraction and transmission electron microscope (TEM), respectively.

Ultrastructural Changes of The Kidneys, Spleen, Skin and Gills

Transmission electron microscopy preparations were obtained by fixing the kidneys, spleen, skin and gills in 2.5% glutaraldehyde in the phosphate buffer, and 2% osmium tetroxide is used for postfixation. After rinsing with phosphate buffer, the specimens were dehydrated in graded ethanol and then embedded in Epon 812 (Epikote resin). Ultrathin sections were sliced with glass knives on a UCT ultramicrotome (Leica Ltd, Germany), stained with uranyl acetate and lead citrate, and examined under a Tecnai 12 electron microscope (FEI, Acht, Netherlands) [12].

Detection of iNOS Enzyme Activity

The iNOS enzyme activity in the kidneys, spleen, skin and gills of the infected and uninfected control groups was

measured employing the NOS Activity Assay Kit according to manufacturer's instructions (Jiancheng, Nanjin, China) [4].

Identification of Inducible Nitric Oxide Synthase Gene

Primers for *iNOS* gene were designed within the highly conserved regions of grass carp *iNOS* gene (GenBank accession number HQ589354.1): *iNOS* F (5'-CAC CTT CAA TCC GAC CTT A-3') and *iNOS* R (5'-AAT CAC GAC AGC CGA ACA C-3') [3]. Total RNA was extracted from grass carp kidneys, spleen, skin and gills using the RNAiso Plus reagent (Takara, Dalian, China) according to the manufacture's instruction. cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Polymerase chain reaction (PCR) amplification was performed using a 2× EasyTaq PCR SuperMix (TransGen Biotech, Beijing, China). PCR was carried out as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C *(iNOS)* for 30 s , and extension at 72°C for 1 min; followed by 10 min at 72°C for the final extension. The amplified PCR products were processed by electrophoresis in 1% agarose gel. The PCR products were purified and ligated into a pMD19-T vector (Takara, Dalian, China), transformed into competent *E. coli* DH5α, and plated on the Amp-LB-agar petri dish, respectively. Positive colonies containing expected size inserts were screened by colony PCR, and sequenced by Sangon (Shanghai, China) [3].

Quantitative Real Time PCR

Quantitative real-time PCR was performed on ABI PRISM 7500 Fast Real-time PCR System with SYBR-Green as fluorescent dye referring to manufacturer's protocol (Takara, Dalian, China). Grass carp β2m gene (GenBank accession number AB190816) was used as an internal reference ($\beta_2 m$F: 5'-GGCTGGCAGTTTCACCTCAC-3', $\beta_2 m$R: 5'-CCACCCTTT GTCTGGCTTTG-3'). Quantitative primers for *iNOS* gene (*iNOS* F: 5'-CAC CTT CAA TCC GAC CTT A-3'; *iNOS* R: 5'-AAT CAC GAC AGC CGA ACA C-3') was designed according to the sequencing results above [3]. All reactions were performed in triplicates. The qPCR conditions were carried out as follows: 60 s at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C, and 40 s at 72°C. The reaction specificity was confirmed by observing a single peak at the expected Tm on the melting curve. Gene expression in different tissues was determined as a ratio of target gene *vs* reference gene and calculated according to the following equation: the relative expression ratio of a target gene (R) = $(E_{\text{reference}})^{\text{Ct reference}}/(E_{\text{targer}})^{\text{Ct target}}$ where *E* is the amplification efficiency, and Ct is the number of PCR cycles needed for the signal to exceed a predetermined threshold value. R = $(E_{\text{target}})^{\text{Ct target (control – sample)}}/(E_{\text{target}})^{\text{Ct reference (control – sample)}}$ [3].

Statistical Analysis

iNOS gene expression data were analyzed by one-way analysis of variance (one-way ANOVA), and $P<0.05$ was considered significant. All the statistical analyses were performed using SPSS® Version 17.0 software (SPSS Inc., Chicago IL, USA). All the measurements were made in triplicate.

RESULTS

The trophonts were seen on the fish body surface within 3~5 days of infection with *I. multifiliis.* White spots were observed on the mucus of head and scale under the microscope of affected fish *(Fig.1A)*. The scale showed the yellowish-white lesions, and the gills appeared necrotic with encysted organisms "white spots" *(Fig. 1B)*.

Kidneys *(Fig. 2A)*, spleen *(Fig. 2B)*, gills *(Fig. 2C)* and skin *(Fig. 2D)* of the control group showed normal morphology. Cells of the control group had evident nuclei, nucleopores, and exhibited normal morphology (arrowheads). The reticular fibers in the kidneys and skin were very visible; lymphocytes and their intercellular space in the spleen were observed clearly; and blood and dermal cells in the skin were found, with obvious dermal cell nuclei. However, pathologic and ultrastructural changes of *I. multifiliis* infection in kidneys, spleen, gills and skin showed that the distribution of the macrophages and lymphocytes was uneven, and cell nuclei were concentrated and fragmented and/or disappeared in kidneys, spleen, gills and skin. The reticular fibers were loosely organized and damaged, leaving large spaces in between. The intercellular spaces were loose and irregular with large holes. Many cellular structures had already disappeared *(Fig. 3A,B,C,D)*.

Furthermore, iNOS enzyme activity in the kidneys *(Fig. 4A)* and spleen *(Fig. 4B)* ($P<0.05$), and in the gills *(Fig. 4C)* and

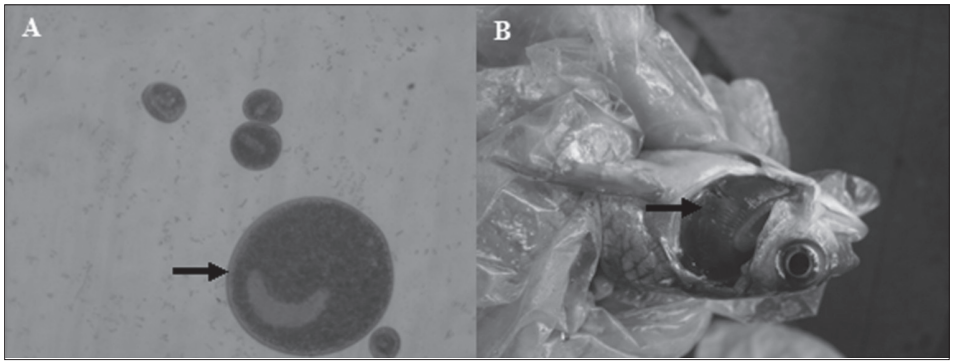

Fig 1. Microscopic examination of the body surface and gross pathology of *Ichthyophthirius* -infected grass carp. Trophonts of *I. multifiliis* were observed on the fish body surface of grass carp, some trophonts grew up into theronts (A) (400×); gills of infected grass carp showed necrotic spots and with white spots on their gill filaments (B) (400×)

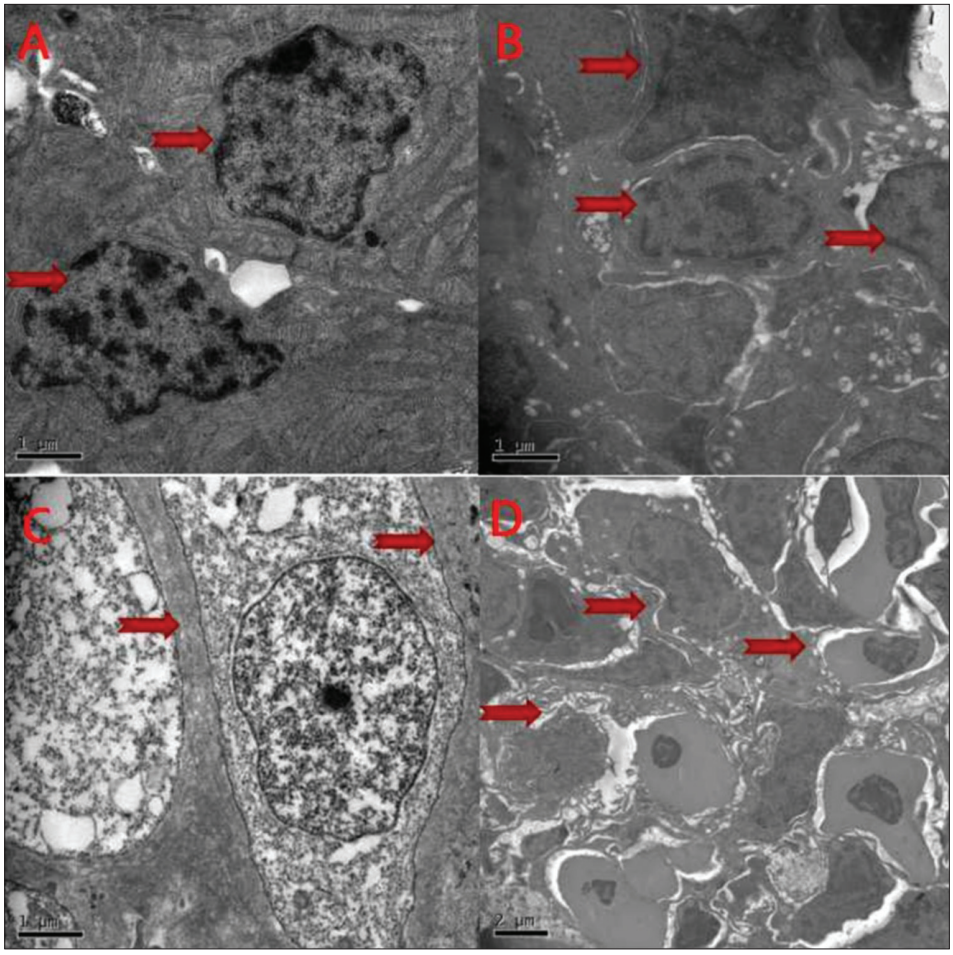

Fig 2. Ultrathin sections in kidneys (A) (3000×), spleen (B) (3000×), gills (C) (3000×) and skin(D) (1900×) of the control group

skin *(Fig. 4D)* (P<0.01) was significantly down-regulated in the *Ichthyophthirius*-infected groups compared to the uninfected control group.

iNOS enzyme activity in the infected group was significantly down-regulated compared to the uninfected control group *(Fig. 4)*. Furthermore, iNOS enzyme activity in the gills and skin were substantially different after *Ichthyophthirius*-infection (P<0.01). Similarly, iNOS enzyme activity in the kidneys and spleen were signifcant different (P<0.05). The expression of *iNOS* gene in the kidneys *(Fig. 5A)* and spleen *(Fig. 5B)* (P<0.05) and in the gills *(Fig. 5C)* and skin *(Fig. 5D)* (P<0.01) was markedly down-regulated after *Ichthyophthirius* infection *(Table 1)*.

iNOS gene expression in the infected tissues and organs were substantially down-regulated compared to the uninfected control group. In the kidneys and spleen, *iNOS* gene expression were significantly different (P<0.05). Similarly, *iNOS* gene expression were highly significantly different in the gills and skin (P<0.01). The results denoted that damages were more obvious in the gills and skin after *Ichthyophthirius* infection in comparison with those in kidneys and spleen.

The expression of *iNOS* gene in the gills, skin, kidneys, and spleen of *Ichthyophthirius*- infected grass carp was significantly down-regulated compared with the control groups as shown in *Table 1*. *iNOS* gene expression ration were highly significantly different in the gills and skin (P<0.01), and significantly different in kidneys and spleen after infection (P<0.05).

DISCUSSION

Nitric oxide is a biological messenger and immune-regulation factor. Its immune action was found in human, rats and pigs earlier than in freshwater fish [13,14]. Nitric oxide is generated by nitric oxide synthases and plays a major role in immune-mediated protection against parasitic diseases [15]. The NOS enzyme, the primary source of NO, has three isoforms, including inducible NO synthesis (iNOS), which plays an important role in the parasitic infections [8]. iNOS, an important effector molecule, restricts pathogen growth in infected hosts [16,17]. Our results showed that the enzyme activity and gene expression of iNOS were substantially down-regulated in the kidneys and spleen (P>0.05) and in the gills and skin (P<0.05) compared to that in the negative controls. Similarly, recent evidence shows that the downregulation of iNOS, SOD and H_2O_2 expressions in *Clonorchis*-infected freshwater [18]. However, Gonzalez et al.[19] found that *iNOS* gene expression was significantly up-regulated in the

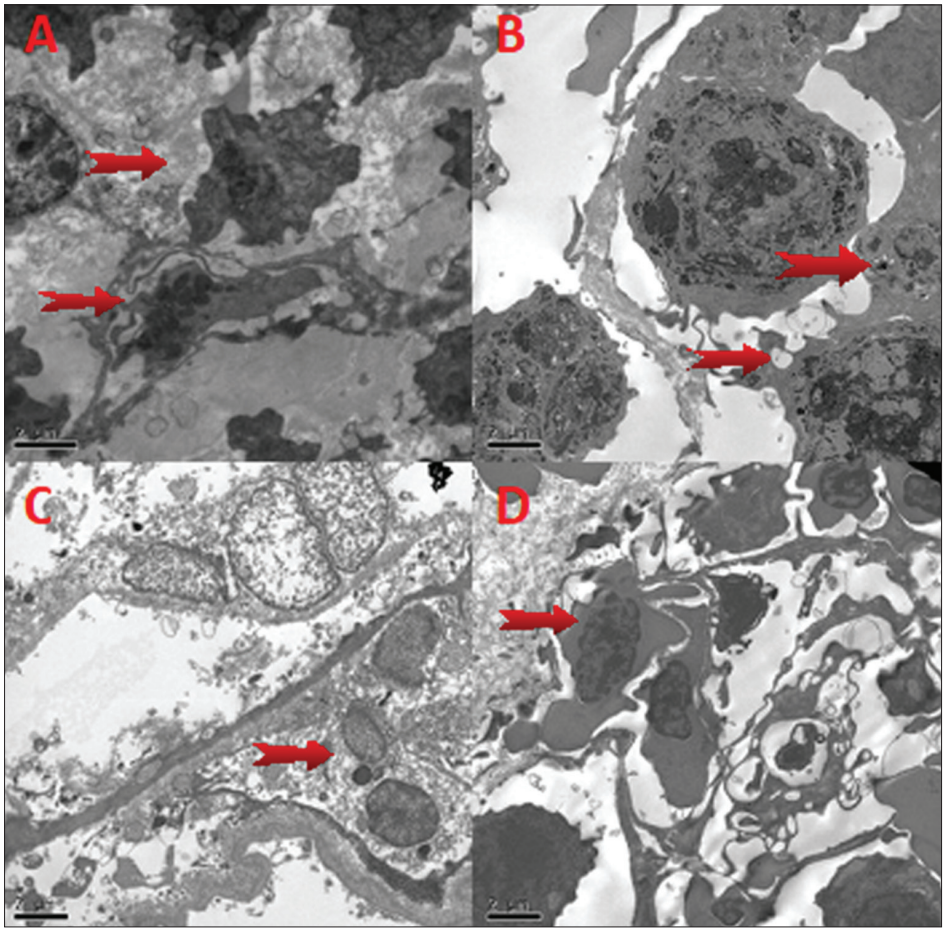

Fig 3. Pathologic and ultrastructural changes of *Ichthyophthirius* infected tissues. The necrosis and disappearance of lymphocyte and epithelial cell nuclei of the kidneys (A); the uneven distribution the macrophages and lymphocytes, and fragmented and degenerative lymphocyte cell nuclei in spleen (B); the necrotic and degenerative epithelial cells of the gills (C) and skin (D); the uneven distribution of the immune or immune-related cell nuclei and its concentration and disappearance (arrow, 1900×)

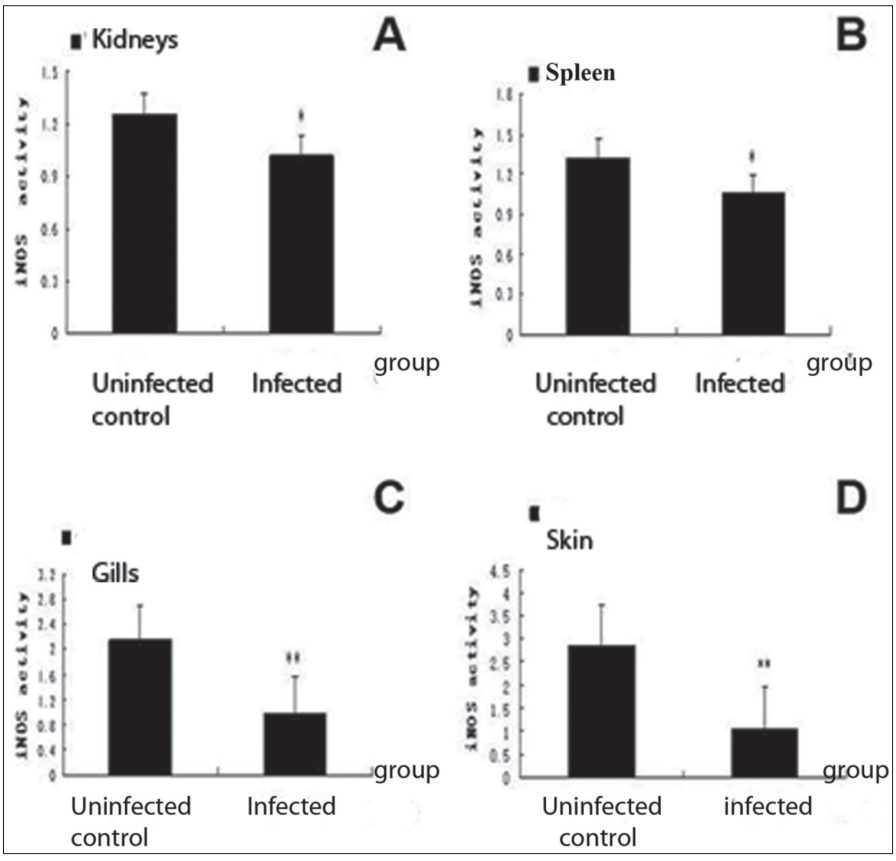

Fig 4. iNOS enzyme activity. iNOS enzyme activity in the kidneys (A) and spleen (B), and in the gills (C) and skin (D) are significantly down-regulated in the *Ichthyophthirius*-infected groups compared to the uninfected control group, * P<0.05, ** P<0.01

Table 1. *iNOS gene expression in different tissues*				
Relative Expression Ratio of *iNOS* Gene	**Kidneys**	**Spleen**	**Gills**	**Skin**
Control group	1	1	1	1
Infection group	0.828±0.756*	0.854±0.663*	0.12±0.09**	0.0082±0.0018**
*Values are means±standard errors. * P<0.05, ** P<0.01*				

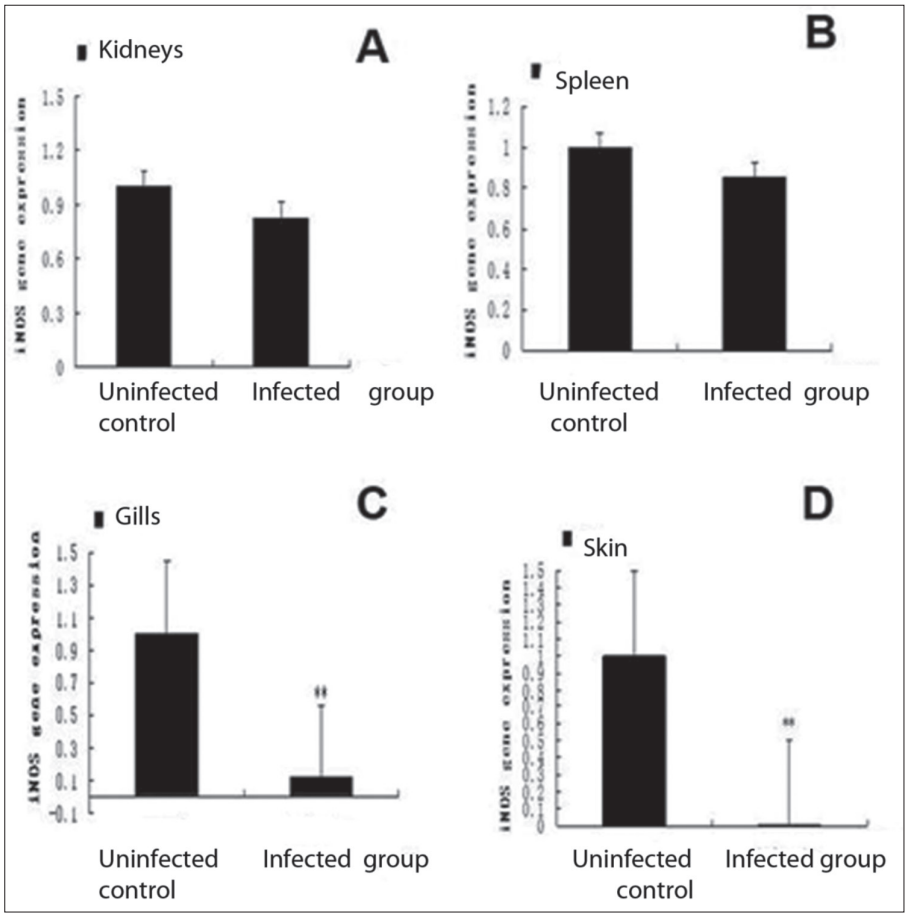

Fig 5. *iNOS* genes expression. The expression of *iNOS* gene in the kidneys (A) and spleen (B) and in the gills (C) and skin (D) was markedly down-regulated after infection, * P<0.05, ** P<0.01

blood of infected carp. Similarly, *iNOS* gene expression was substantially up-regulated in the skin of rainbow trout by immune radiovaccine against *Ichthyophthirius multifiliis*, and in the gills of *Ichthyophthirius*-infected rainbow trout larvae [20,21]. These findings may help us to understand the iNOS expression profile in the naïve and primed fish. Additionally, the low level of iNOS enzyme activity and gene expression in the gills and skin were significant compared with that in the kidneys and spleen of grass carp infected with *I. multifiliis* indicating the gills and skin are the main protective barrier with different immune regulators. Meanwhile, the results of iNOS enzyme activity and gene expression in the skin and gills were related with the active immune cells. Interestingly, the immune cells in the skin and gills were more degenerated and necrotic than in the kidneys and spleen of the *Ichthyophthirius* -infected grass carp. Similarly, previous studies showed that iNOS can be limited to certain organs and stages of infection in mice [22,23]. While Losada et al.[24] reported the obvious increase of iNOS positive cells in the intestine more than in the spleen and kidneys of the *Enteromyxum* -infected turbot, indicating that the iNOS level delayed the response, and made tissue lesion worse. Therefore, different parasites invaded the different target organs and the gills and skin are the main sites of parasitic invasion [22,25,26]. Low levels of iNOS enzyme activity and gene expression in the spleen and kidneys of the *Ichthyophthirius*-infected grass carp owing to its immune cell damage, which are connected with the iNOS production [27,28]. Gills and skin are the mucosal tissues associated with the immune system of fish. Gills are involved in the immune defense through the mucosa associated lymphoid tissues as a respiratory organ [29-31]. *I. multifiliis* infected grass carp by invading the skin and gill barrier, destroyed the lymphocyte structure and capillary circulation. We also showed that prerequisites for intact gills as sources of gas exchange and nutrition for all the fish body, including the spleen and kidneys. The reduction of immune cells in the absence of nutrients and oxygen

is a reliable conclusion to be drawn [32,33]. On the other hand, our previous studies showed that the disappearance and destruction mitochondria in the spleen and kidneys of the *Ichthyophthirius*-infected grass carp, which may attribute to the loss of nutrients and oxygen caused by *I. multifiliis* without the oxidation injury [4]. Furthermore, NO is also a kind of vasodilator activity, gills and skin are rich in the blood capillary. From our studies, the red cells were destroyed in the gills and skin of the fish [34]. Therefore, this study proved that iNOS enzyme activity and gene expression in the gills and skin were more down-regulated than in the spleen and kidney of the infected group due to the reduction in the number of immune cells via oxygen deficiency. These results are in line with those of previous studies [27,28,35,36]. Expression and activity of inducible nitric oxide synthase could serve as an important indicator to determine the pathological status of the *I. multifiliis*-grass carp. This could perhaps also be useful for better management of the aquaculture conditions.

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Competing Financial Interests

The authors declare no competing financial and non-financial interests.

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Research Article

Establishment and Evaluation of a Suckling Mouse Integrin $\alpha_v\beta_8$ Transgenic CHO-677 Cell Line with Increased Susceptibility to Type O/BY/CHA/2010 Foot-and-Mouth Disease Virus

Kaiqi LIAN [1,2] Fan YANG [2] Lingling ZHOU [1] Mingliang ZHANG [1] Yuwei SONG [1]

[1] Academician Workstation of Animal Disease Control and Nutrition Immunity in Henan Province, Henan Joint International Research Laboratory of Veterinary Biologics Research and Application, School of Biotechnology and Food Science, Anyang Institute of Technology, Anyang 455000, CHINA

[2] Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, CHINA

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Abstract

Integrin $\alpha_v\beta_8$ may play an important role in the initial stage of FMDV infection and influence the host specificity and tissue tropism. To study the role of integrin $\alpha_v\beta_8$ in FMDV infection, a cell line, which stably expressed the suckling mouse αvβ8 heterodimer, was established and designated as CHO-677-m$\alpha_v\beta_8$. Polymerase chain reaction (PCR) and indirect immunofluorescent assay (IFA) were used to determine the presence of integrin subunits α_v and β_8 in the cell line at the 20th passage, respectively. The results showed that these genes were successfully transduced and expressed in the cell line. Biological characteristics of FMDV were determined in the cell line. After the cell line was infected with FMDV type O/BY/CHA/2010, there was an obvious increase for viral RNA and proteins, compared with those in the parental cells. Our data showed that a stable cell line was successfully established. Additionally, the results of antibody blockade assay show that the cells treated with anti-β_8 serum had a lower virus titre, compared to the mock-treated cells, implying that the β_8 was important for maintaining $\alpha_v\beta_8$ in receptor function required for productive infection by FMDV. The cell line may be used to isolate the FMDV, study the function of integrin $\alpha_v\beta_8$ in infection and entry of FMDV.

Keywords: *$\alpha_v\beta_8$, Cell line, Foot-and-Mouth Disease Virus, Integrin receptor, Mouse*

Tip O/BY/CHA/2010 Şap Hastalığı Virusuna Karşı Artırılmış Duyarlılıkta Süt Emen Fare $\alpha_v\beta_8$ Transgenik CHO-677 Hücre Hattının Oluşturulması ve Değerlendirilmesi

Öz

İntegrin $\alpha_v\beta_8$ Şap hastalığının ilk aşamasında önemli bir rol oynayabilir ve konak spesifikliği ile doku tropizmini etkileyebilir. Şap hastalığında integrin $\alpha_v\beta_8$'ınrolünü araştırmak amacıyla stabil olarak süt emen fare αvβ8 heterodimer eksprese eden bir hücre hattı geliştirildi ve CHO-677-m$\alpha_v\beta_8$ olarak adlandırıldı. Yirminci pasajda hücre hattında α_v ve β_8 integrin subünitlerinin varlığını belirlemek amacıyla sırasıyla polimeraz zincir reaksiyonu ve immunofloresan teknikleri kullanıldı. Sonuçlar bu genlerin hücre hattına başarıyla aktarıldığını ve eksprese edildiğini gösterdi. Şap hastalığı virusunun biyolojik karakterleri hücre hattında belirlendi. Hücre hattı Şap hastalığı virusu tip O/BY/CHA/2010 ile enfekte edildikten sonra hücre hattında viral RNA ve proteinleri parental hücreler ile karşılaştırıldığında belirgin olarak artmıştı. Elde edilen veriler stabil bir hücre hattının başarıyla oluştuğunu gösterdi. Ayrıca, antikor blokajı yönteminin sonuçları, anti-β_8 serumu uygulanan hücrelerin plesibo uygulanan hücrelerle karşılaştırıldığında daha düşük virus titresine sahip olduğunu ve böylece Şap hastalığı virusu ile hastalık oluşturmak için gerekli reseptör fonksiyonunda $\alpha_v\beta_8$'nin oluşturulmasında β_8'in önemli olduğunu gösterdi. Bu hücre hattı Şap hastalığı virusunun izolasyonunda, enfeksiyonda integrin $\alpha_v\beta_8$'in fonksiyonunun ve virusun hücreye girişinin çalışılmasında kullanılabilir.

Anahtar sözcükler: *$\alpha_v\beta_8$, Hücre hattı, Şap hastalığı Virusu, İntegrin reseptörü, Fare*

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly infectious disease of domestic and wild cloven-hoofed animals, including cattle, swine, sheep, goats, and over 70 species of wild animals. The causative agent, foot-and-mouth virus (FMDV), belongs to

İletişim (Correspondence)
+86 372 2909876
liankaiqi616@163.com

a member of the family *Picornaviridae*, genus *Aphthovirus*, and has at least seven serological types, A, C, O, Asia1, South Africa serotype (SAT)1, 2, and 3, and multiple subtypes [1]. The virus is a non-enveloped particle of icosahedral symmetry containing a single-stranded, positive-sense RNA genome approximately 8.5 kb in length. There is a single open reading frame (ORF) in the viral genome, which is translated into ten nonstructural proteins (NSP) and four structural proteins [2,3].

The recognition of specific receptors on the cell plasma membrane by proteins on the virus surface is necessary for virus attachment and subsequent infection [4,5]. Thus far, four integrins ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$) have been demonstrated by several laboratories to be FMDV receptors [6-9]. Integrin $\alpha_v\beta_8$ is the fourth integrin receptor identified for FMDV and may play an important role in the initial stage of FMDV infection and influence the host specificity and tissue tropism. While the natural hosts of FMDV include domestic and wild cloven-hoofed animals, the suckling-mouse is one of the most important experimental animals in the laboratory [1]. Thus, in the present study, suckling-mouse integrin α_v and β_8 subunits were cloned, and the Chinese hamster ovary 677 (CHO-677) cell line was transduced to stably express suckling-mouse integrin $\alpha_v\beta_8$ using a highly efficient lentivirus-based gene transfer technology [10]. The CHO-677 cell line expresses neither four integrin receptors nor heparin sulfate (HS) receptor for FMDV and is usually used in the studies of receptor pathway for FMDV [11-13]. IBRS-2 cell doesn't only expresses single integrin $\alpha_v\beta_8$ of four integrin receptors, but also HS receptor. Therefore, it is necessary for establishing the CHO-677-m$\alpha_v\beta_8$ cell line.

MATERIAL and METHODS

Cells, Virus and Antibodies

Human embryonic kidney (HEK) 293T cells and HS-deficient Chinese hamster ovary (CHO-677 or pgsD-677, ATCC, CRL-2244) cells were cultured in Ham'S/F-12 (SH30026.01, HyClone, USA) medium supplemented with 10% fetal bovine serum (FBS, SH30070.03, HyClone, USA), 1% streptomycin (0.2 mg/mL) and penicillin (200 U/mL). Baby hamster kidney (BHK-21, ATCC, CCL-10) cells were maintained in Eagle's Minimal essential Medium (EMEM, Invitrogen, USA) containing 10% FBS. All the cells were incubated at 37°C with 5% CO_2.

Foot-and-mouth virus type O/BY/CHA/2010 (GenBank accession No. JN998085.1) was isolated from a naturally infected pig in the city of Baiyun in Guangdong Province, China, during the 2010 outbreak and was propagated on BHK-21cells.

Guinea pig anti-FMDV serum and rabbit polyclonal anti-serum directed against mouse integrin subunit α_v (anti-α_v serum) and β_8 (anti-β_8 serum) were obtained from the Lanzhou Veterinary Research Institute. Fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea-pig IgG antibody (F6261) and FITC-conjugated goat anti-rabbit IgG antibody (F0382) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cloning and Sequencing The Integrin Subunit α_v and β_8Genes

Genomic RNA was extracted from the tongue or lung tissues of suckling mice using an RNeasy Mini kit (74104, Qiagen, Hilden, Germany), according to the manufacturer's specifications. The use of all animals in this study was approved by the Review Board of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. cDNA was synthesized from the extracted RNA with AMV reverse transcriptase (12328-019, Invitrogen, USA) using random primers (20 pmol/mL), and used as the template for the amplification of the α_v and β_8 transcripts with PCR. The PCR primer pairs, α_vF/α_vR and β_8R/β_8F are shown in *Table 1*. The PCR reaction system was: 25 μL Premix LA Taq(RR903A, Takara Bio, Japan), 1 μL primer (10 pmol/L), 2 μL DNA, 21 μL dH_2O. The cycling parameters were: 40 cycles of denaturation at 95°C for 3 min, annealing at 58°C for 30 sec, and the elongation at 72°C for 4 min, followed by a final elongation step at 72°C for 10 min before the reaction was cooled to 16°C for further processing. The amplicon was purified with the MiniBEST Agarose Gel DNA Extraction KitVer.4.0 (9762, Takara Bio, Japan) and cloned separately into the pGEM-T Easy vector (A1360, Promega, Shanghai, China). The positive plasmids were sequenced in both directions by GeneWizInc (Suzhou, China).

Construction of the Recombinant Lentiviral Plasmid

The recombinant plasmid was constructed as previously described [14]. Briefly, pOK_{12} and the internal ribosome entry site (IRES) fragment were amplified from pOK_{12} and the pIRES2-EGFP plasmid (stored in our laboratory), respectively, with the corresponding primer pairs *(Table 1)*, and the IRES fragment was then cloned into pOK_{12} to generate pOK-IRES. The α_v PCR product was digested by the restriction enzymes *Not*(1623) and *Nhe*(1622) (Takara Bio, Japan), and cloned into the pOK-IRES plasmid to generate the recombinant plasmid pOK-α_v-IRES, which was digested to obtain the α_v-IRES fragment by the restriction enzymes *Not*(1623) and *Xba*(1634) (Takara Bio, Japan). This fragment was then cloned into the pLVX-Tight-Puro vector (Clontech, USA) to generate the recombinant plasmid pLVX-α_v-IRES. The amplified β_8 fragment was digested by the restriction enzymes *Xba*(1634) and *Mlu*(1619) (Takara Bio, Japan), and cloned into pLVX-α_v-IRES to generate the recombinant lentiviral plasmid pLVX-α_v-IRES-β_8. All the products were sequenced by GeneWiz Inc.

Establishment of The CHO-677-m$\alpha_v\beta_8$Cell Line

Using the Lenti-X™ Tet-Off® Lentiviral Advanced Induced

Table 1. *Oligonucleotides used in this study*

Name	Nucleotide sequence (5′-3′) [a, b]	Genome Position
α_vF	ATGGCTGCTCCCGGGCGC	1-18
α_vR	TCAGGTTTCAGAGTTTCCT	3117-3135
β_8F	ATGTGCGGCTCGGCCCTGGCT	1-21
β_8R	TTAGAAGTTGCACCTGAAG	2286-2304
IRESF IRESR pL-α_vF	CTAGCTAGCGCCCCTCTCCCTCCCCCCCCCCTAA CGGTCTAGATGTGGCCATATTATCATCGTGT TTTGCGGCCGCGCCACCATGGCTGCTCCCGGGCGC	1-25 562-584 1-18
pL-α_vR	CTAGCTAGCTCAGGTTTCAGAGTTTCCTTCGCCATT	3117-3143
pL-β_8F	CGGTCTAGAGCCACCATGTGCGGCTCGGCCCTGGCT	1-20
pL-β_8R	TCGACGCGTTTAGAAGTTGCACCTGAAG	2343-2364

[a] *Red letters represent restriction enzyme site;* [b] *Blue letters represent Kozak sequence*

Expression System (TakaraBio, Japan), we transduced 293T packaging cells with the lentiviral vector pLVX-α_v-IRES-β_8 to yield the disguised lentivirus, which was then used to infect the CHO-677 target cells according to the Lentiviral Expression System User Manual. To establish a stable CHO-677-m$\alpha_v\beta_8$ cell line, a single clone was cultured under selection with 500 mg/mL G418(E859, Amresco, US) and 2 mg/mL puromycin (P9620, Sigma-Aldrich, St. Louis, MO, USA). After approximately 2 weeks, cell cloning islands were observed. After 20 rounds of continuous cloning, a stable $\alpha_v\beta_8$-transgenic CHO-677 cell line was obtained and designated as "CHO-677-m$\alpha_v\beta_8$". The presence of α_v and β_8 genes in the CHO-677-m$\alpha_v\beta_8$ cells at the twentieth passages were analyzed by PCR using the primers (α_vF, α_vR and β_8F, β_8R) *(Table 1)*. The PCR reaction system was: 12.5 μL Premix LA Taq, 0.5 μL primer (10 pmol/L), 0.5 μL template, 11 μL dH_2O. The cycling parameters were: 30 cycles of denaturation at 95°C for 3 min, annealing at 58°C for 30 sec, and the elongation at 72°C for 4 min, followed by a final elongation step at 72°C for 10 min. Furthermore, the expression of integrin subunits α_v and β_8 in CHO-677-m$\alpha_v\beta_8$ cells were confirmed by an indirect immunofluorescence assay (IFA).

Growth Characterization of FMDV Type O/BY/CHA/2010 in CHO-677-m$\alpha_v\beta_8$Cell Line

In order to examine the functional activity of $\alpha_v\beta_8$ in CHO-677-m$\alpha_v\beta_8$ cell line, FMDV was used to infect the cell line. Plaque assay, growth kinetic, IFA and quantitative real-time PCR (qRT-PCR) of FMDV type O/BY/CHA/2010 were analyzed in the CHO-677-m$\alpha_v\beta_8$ cell line.

Plaque Assay

CHO-677-m$\alpha_v\beta_8$ and the parental CHO-677 cells were seeded in six-well cell culture plates 48 h before infection. Dilutions (10-fold) of the virus were prepared in Ham's F-12 medium. The inoculum volume was 200 mL per well. After One hour adsorption, the culture medium was removed, and then the cells were overlain with 50% gum tragacanth(G1128, Sigma-Aldrich, St. Louis, MO, USA) and 50% 2′ minimal essential medium supplemented with 2% fetal bovine serum. The plates were incubated for 48 h, fixed with acetone and methanol (1:1), and stained with crystal violet (Histochoice, Amresco, Solon, Ohio).

Growth Kinetics of the Virus

Growth kinetic of the virus was analyzed in BHK-21 cells. The parental and m$\alpha_v\beta_8$ expressing cells were infected with FMDV type O/BY/CHA/2010 at the same multiplicity of infection (MOI=1), and the virus was allowed to adsorb for 1 h at 37°C. The medium was removed and then the cells were washed. Fresh medium was added. At 12, 24, 36, 48, 60, and 72 h post-infection (hpi), the plates were freeze/thawed three times and the viral titers in cell culture media were determined by $TCID_{50}$ on BHK-21 cells using the Reed-Muench method [15]. Three independent experiments were performed and the mean value was used to determine the viral growth curve.

qRT-PCR

To determine the level of viral replication, the parental and m$\alpha_v\beta_8$ expressing cells were prepared in triplicate as described above and the viral supernatants were harvested at the indicated times after infection. Total RNA was extracted from the cells with the QIAxtractor kit (Qiagen), according to the manufacturer's instructions. qRT-PCR was performed with the StrataGene Mx3000P® Real-time PCR System (Agilent, Santa Clara, USA) using the SYBR Premix Ex Taq Kit (TaKaRa), as described previously [16].

IFA

Viral proteins were detected with IFA. CHO-677-m$\alpha_v\beta_8$ and CHO-677 cells were seeded on 35 mm diameter plates, grown to approximately 80% confluence, and then

infected with FMDV type O/BY/CHA/2010 at MOI=1. At 24 hpi, the cells were fixed, permeabilized, and blocked as described previously [17]. The cells were incubated with guinea pig anti-FMDV serum (1:500 dilution) at 37°C for 2 h, and then reacted with FITC-conjugated goat anti-guinea-pig IgG antibody (1:500 dilution) for 1.5 h in a dark room. The cell nuclei were stained by the 4′, 6-diamidino-2-phenylindole (DAPI, Invitrogen).The treated cells were viewed by the microscope.

The Effect of Antibody Blockade for β_8 Subunit in FMDV Infection

Antibody blockade assay was performed to examine the role of β_8 subunit in the FMDV infection. CHO-677-m$\alpha_v\beta_8$ cells were seeded in 35-mm-diameter dishes and incubated for 48 h at 37°C with 5% CO_2. Confluent cell monolayers were rinsed with PBS (pH 7.5, 2 mM $CaCl_2$, 1 mM $MgCl_2$). The cells were divided into two groups and there were three dishes in each group. One group was incubated with PBS as control; while the other was incubated with the anti-β_8 serum (1:500 dilution). After 1.5 h, the supernatants were removed, and then both groups were infected with FMDV type O/BY/CHA/2010 (MOI=2) at 37°C. At 24 hpi, the titer of virus in the cell culture media was determined by $TCID_{50}$ on BHK-21 cells.

RESULTS

Cloning and Sequencing The Integrin Subunit α_v and β_8 Genes

To study the integrin $\alpha_v\beta_8$ of suckling mouse, we amplified the integrin subunit α_v and β_8 genes from the tongue or lung tissues, respectively *(Fig. 1)*. The results of DNA sequencing confirmed that the sequences of both genes were consistent with the corresponding one from the GenBank.

Construction of Lentivirus Recombinant Plasmid

In order to confirm the lentiviral recombinant plasmid pLVX-α_v-IRES-β_8 was built successfully, double enzyme digestion experiments were performed. The pLVX-α_v-IRES-β_8 plasmid was digested by *Not*I and *Nhe*I to identify the α_v fragment, and digested by *Xba*I and *Mlu*I to identify the β_8 fragment. As shown in *Fig. 2*, the results of double enzyme digestion were verified by electrophoresis.

Construction of the CHO-677-m$\alpha_v\beta_8$ Cell Line

To detect the presence of the suckling mouse integrin subunits α_v and β_8 at the gene in CHO-677-m$\alpha_v\beta_8$ cell line, α_v and β_8 genes at the twentieth passage were amplified by PCR *(Fig. 3A)*. The sequencing results proved the identity of the PCR product. Furthermore, the expression of integrin $\alpha_v\beta_8$ protein in CHO-677-m$\alpha_v\beta_8$ cell line was confirmed by IFA *(Fig. 3B)*, demonstrating that CHO-677-m$\alpha_v\beta_8$ cell line was successfully established.

Growth Characterization of FMDV Type O/BY/CHA/2010 in CHO-677-m$\alpha_v\beta_8$ Cell Line

To study the functional features of $\alpha_v\beta_8$, the cells were infected with FMDV type O/BY/CHA/2010 to analyze viral

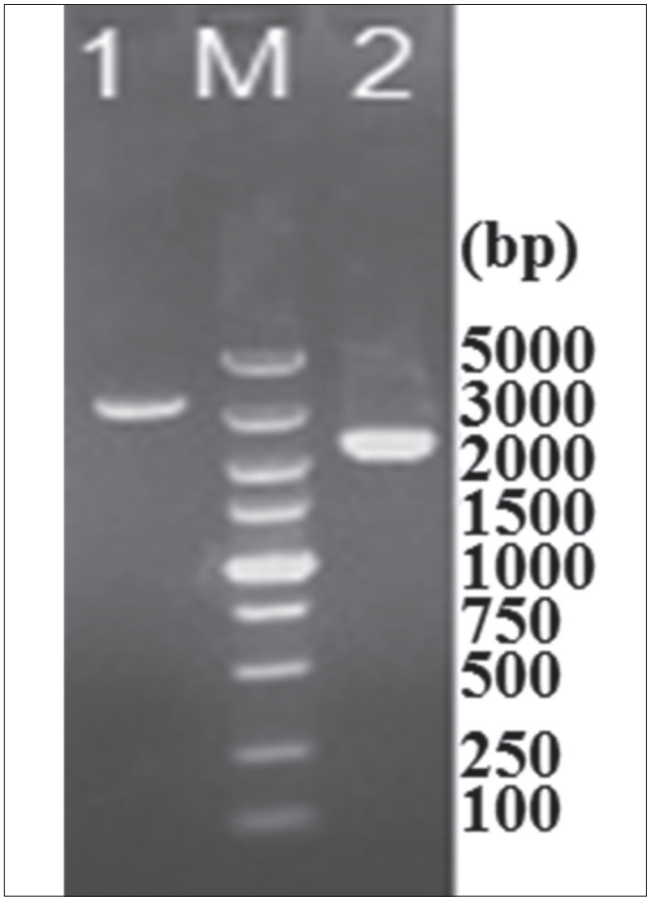

Fig 1. PCR products of both subunit genes of suckling mouse integrin $\alpha_v\beta_8$.Lane1: α_v gene fragment (3135bp.); Lane2: β_8 gene fragment (2304bp.); Lane M: 5000 bp. DNA Ladder

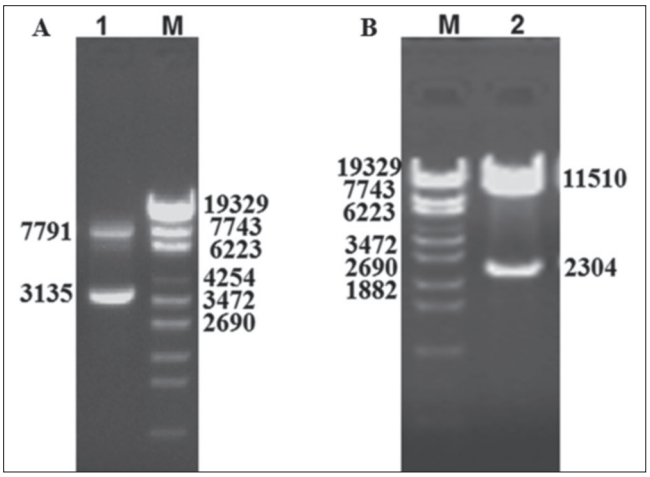

Fig 2. Identification of the recombinant plasmid pLVX-α_v-IRES-β_8 by double enzyme digestion. Lane1: Double enzyme digestion identification about the α_v fragment. Lane 2: Double enzyme digestion identification about the β_8 fragment. Lane M: DNA molecular weight marker λ-EcoT14 I

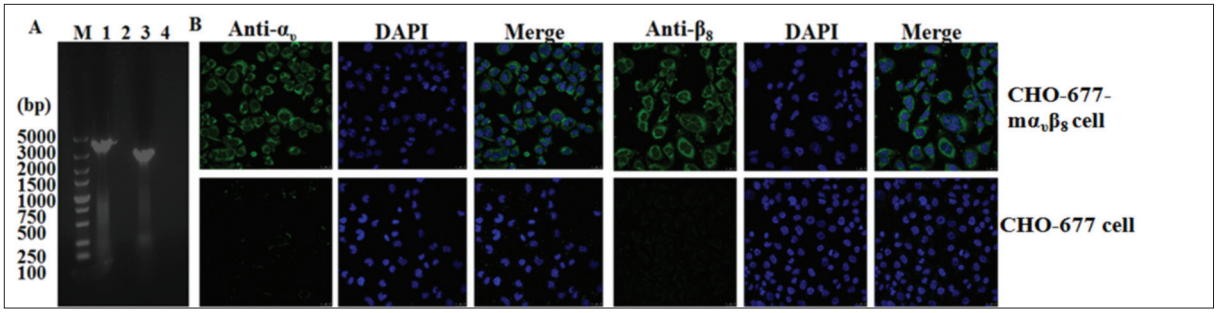

Fig 3. Identification of CHO-677-mα$_v$β$_8$ cell line. A. Detection of integrin subunit α$_v$ and β$_8$ genes in CHO-677-mα$_v$β$_8$ cell line at the twentieth passage. The PCR products of α$_v$ and β$_8$genes (lane 1 and 3, respectively) are shown, relative to the negative controls for the α$_v$ and β$_8$ genes (lanes 2 and 4, respectively). Molecular weight marker in lane M. B. Expression of integrin subunits α$_v$ and β$_8$ were detected in CHO-677-mα$_v$β$_8$ cells at the twentieth passage by IFA. Photographs were taken at a magnification of 40 fold. One representative experiment is shown

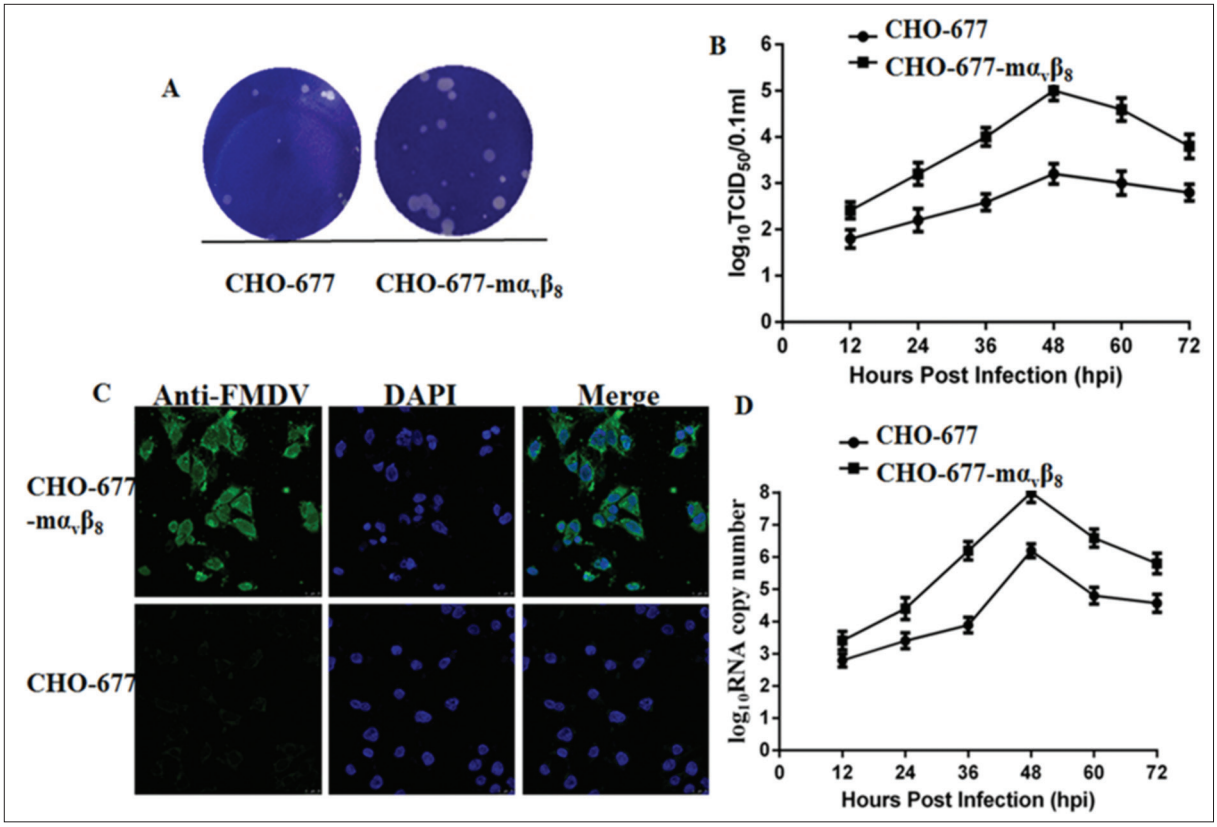

Fig 4. Growth characteristics of FMDV type O/BY/CHA/2010 in CHO-677-mα$_v$β$_8$ and CHO-677 cells. A. Plaque morphology of CHO-677-mα$_v$β$_8$ and CHO-677 cells after inoculation with FMDV type O/BY/CHA/2010. B. Growth kinetics of FMDV type O/BY/CHA/2010 on CHO-677-mα$_v$β$_8$ and CHO-677 cells. C. Immunofluorescence analysis of FMDV type O/BY/CHA/2010 on CHO-677-mα$_v$β$_8$ and CHO-677 cells. The virus proteins were probed with guinea pig anti-FMDV serum and then reacted with FITC-conjugated goat anti-guinea pig IgG antibody. D. The levels of type O/BY/CHA/2010 FMDV RNA copy numbers were determined in infected CHO-677-mα$_v$β$_8$ and CHO-677 cells at different time points by real-time PCR

growth characteristics. Plaque assays were performed to compare the plaque size and quantity produced in CHO-677-mα$_v$β$_8$ and CHO-677 cells, and the plaque phenotypes and FMDV yields were characterized in both cells. As shown in *Fig. 4A*, the virus produced more plaques in the CHO-677-mα$_v$β$_8$ cells than in the CHO-677 cells.

In order to compare the *in vitro* growth characteristics of FMDV type O/BY/CHA/2010 in the CHO-677-mα$_v$β$_8$ cell line and the parental cells, samples were collected at 12h intervals, up to 72 hpi and $TCID_{50}$ was titrated on BHK-21 cells. The peak titers of FMDV type O/BY/CHA/2010 were $10^{5.0}$ $TCID_{50}$/0.1 mL in CHO-677-mα$_v$β$_8$ cells at 48 hpi and $10^{3.2}$ $TCID_{50}$/0.1 mL in the parental cells at 48 hpi *(Fig. 4B)*. We then determined the expression of viral proteins with IFA. Intracellular cytoplasmic fluorescence was detected in the infected CHO-677-mα$_v$β$_8$ cell line, while weak fluorescence was detected in the CHO-677 cells *(Fig. 4C)*, indicating the

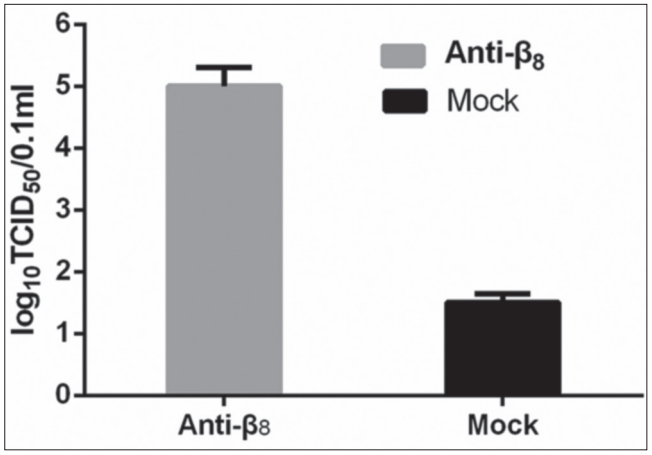

Fig 5. The result of antibody blockade assay. The virus titres were detected by $TCID_{50}$ on BHK cells (mean ± SEM of three independent experiments)

CHO-677- mα$_v$β$_8$ cell line increased the susceptibility to FMDV type O/BY/CHA/2010.

To analyze the replication capacity of the virus, we determined the copy numbers of FMDV type O/BY/CHA/2010 RNAs in both cells with qRT-PCR. As shown in *Fig. 4D*, the parental cells showed clearly lower levels of RNA replication than the CHO-677-mα$_v$β$_8$ cell line. These results indicated that the CHO-677-mα$_v$β$_8$ cells are susceptible to FMDV.

Antibody Blockade Assay

In order to determine whether the integrin subunit β$_8$ plays an important role in FMDV-mediated infection, the antibody of β$_8$ ligand binding domain (LBD) was used to block the integrin subunit β$_8$. Cells treated with anti-β$_8$ serum had a lower virus titer, compared to the mock-treated cells *(Fig. 5)*, indicating that integrin α$_v$β$_8$ increased the susceptibility of CHO-677-mα$_v$β$_8$ cell line to FMDV and that integrin subunit β$_8$ played an important role in FMDV-mediated infection.

DISCUSSION

Many host cells express more than one integrin receptor [18], which may hinder the study of viral infection and invasion mediated by single receptor. Therefore, cells expressing single integrin receptor are necessary for researching individual receptor pathways. PerhapsIBRS-2 cells, which only express integrin α$_v$β$_8$, are commonly used to study FMDV infection mediated by integrin α$_v$β$_8$ [12,19]. However, there is no representative cell from other hosts, which only express integrin α$_v$β$_8$. Suckling mice are widely used as experimental animals for FMDV. For example, serial passages and determining the LD_{50} of FMDV both require suckling mouse as experimental animals. Moreover, the suckling mouse integrin receptor genes have high sequence homology to swine, bovine and camel counterparts. Therefore, we cloned the full-length cDNAs of the integrin subunits α$_v$ and β$_8$ from the suckling mouse, and established a CHO-677-mα$_v$β$_8$ cell line stably expressing suckling mouse integrin α$_v$β$_8$.

Integrin is a family of allosteric, heterodimeric, transmembrane glycoproteins that regulate cell-cell, cell-extracellular matrix, and sometimes cell-pathogen interactions [20,21]. Miller et al.[22] studied the role of the β$_6$ cytoplasmic domain in infection, showing that α$_v$β$_8$ not only contributed to passing the virus onto a next receptor for internalization but also played an important role in the whole infection process. Zhang et al.[23] showed that integrin β$_6$-1 subunit could induce partial protection against FMDV in guinea pigs. Integrin α$_v$β$_8$ was identified as a receptor for FMDV. Zhang et al.[14] established a CHO-677-mα$_v$β$_8$ cell line stably expressing suckling mouse integrin α$_v$β$_8$. Although integrin α$_v$β$_8$ and α$_v$β$_8$ had been studied widely, the study of integrin α$_v$β$_8$ in infection was not well defined. Several reseachers studied the function of integrin by a transient expression system [9,24]. Wang et al. [25]. determined the role of α$_v$β$_8$ and α$_v$β$_8$ in FMDV replication. Although the transient expression system is easy and conveniently manipulated, its repeatability is low. Here, a highly efficient lentivirus-based inducible expression system was used to establish the CHO-677-mα$_v$β$_8$ cell line. As a member of integrin family, α$_v$β$_8$ has many functions, which include the receptor function for FMDV [6]. Therefore, in order to examine the CHO-677-mα$_v$β$_8$ cell line, FMDV was selected to infect the cell line. Our results suggested that the suckling mouse integrin α$_v$β$_8$ increased the susceptibility of CHO-677-mα$_v$β$_8$ cell line to FMDV. In this paper, we used antibody blockade assay to study the role of the β$_8$ subunit in mediated infection, showing that the β$_8$ subunit played a critical role in integrin α$_v$β$_8$-mediated infection. Of course, the CHO-677-mα$_v$β$_8$ cell line should be further determined and evaluated.

In this study, a CHO-677-mα$_v$β$_8$ cell line was established as a cell model for studying the interaction between FMDV and single integrin-expressed cell line. Our results demonstrated that integrin α$_v$β$_8$ expression increased the susceptibility of CHO-677-mα$_v$β$_8$ cell line to FMDV and that the β$_8$ was important for maintaining α$_v$β$_8$ in receptor function required for productive infection by FMDV. The cell line may be used to isolate the FMDV, study the function of integrin α$_v$β$_8$ in infection and entry of FMDV, and so on.

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Competing Interests

The authors declare they have no competing interests.

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Research Article

Effects of *Thymus vulgaris* L. in Acute and Chronic Epilepsy Models in Rats Induced by Pentylenetetrazole

Hülya ÖZDEMİR [1,a] Vedat SAĞMANLIGİL [2,b] Özlem Ergül ERKEÇ [3,c]
Gökhan OTO [1,d] Yıldıray BAŞBUĞAN [4,e] Hasan UYAR [1,f]

[1] Van Yuzuncu Yil University, Medical School, Department of Pharmacology, TR-65100 Van - TURKEY
[2] Near East University, Faculty of Veterinary Medicine, Department of Physiology, Nicosia/TRNC- Mersin 10, TURKEY
[3] Van Yuzuncu Yil University, Medical School, Department of Physiology, TR-65100 Van - TURKEY
[4] Van Yuzuncu Yil University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-65100 Van - TURKEY
[a] ORCID:0000-0002-6045-8342; [b] ORCID : 0000-0001-9335-7348; [c] ORCID :0000-0001-5275-6254; [d] ORCID:0001-7310-7800
[e] ORCID: 0000-0001-5124-7853; [f] ORCID:0000-0002-5982-5467

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Abstract

The aim of this study was to investigate the effects of *Thymus vulgaris* L. (TVL) on oxidative stress, motor coordination and learning/memory in acute and chronic epilepsy models in rats induced by Pentylenetetrazole (PTZ). To this end, 64 male Wistar-albino rats were randomly divided into eight groups with 8 rats each: (1) acute control (AC), (2) acute PTZ (APTZ), (3) acute PTZ + sodium valproate (APTZ+VPA), (4) acute PTZ + TVL (APTZ+TVL), (5) chronic control (CC), (6) PTZ kindling (PTZk), (7) PTZ kindling + VPA (PTZk+VPA) and (8) PTZ kindling + TVL (PTZk+TVL) . Seizures were observed for 30 min after each PTZ injection and were scored. Acute PTZ-induced seizures were created by injecting a single convulsive dose of PTZ (60 mg/kg, ip) in acute groups. PTZ kindling was produced by injecting a subconvulsant dose of PTZ (35 mg/kg, ip) every other day, with 14 injections in total. No significant difference was found among the PTZk + VPA, PTZk, and PTZk + TVL groups with regard to seizure scores. No significant difference was found among all the 8 groups in the learning/memory tests conducted using the Morris Water Maze (MWM) test and the motor activity tests conducted using the rotarod test (P>0.05). The analysis of total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) indicated that the administration of PTZ decreased the antioxidant capacity and increased the oxidant capacity. Moreover, the TVL administration established the oxidant/antioxidant balance, particularly in the chronic groups. Further studies are needed to investigate whether high doses of TVL have an effect on PTZ-induced seizure scores.

Keywords: *Behavior, Epilepsy, Kindling, Seizure, Pentylenetetrazole, Thymus vulgaris* L.

Sıçanlarda Pentilentetrazol İle İndüklenen Akut ve Kronik Epilepsi Modellerinde *Thymus vulgaris* L.'nin Etkileri

Öz

Bu çalışmanın amacı pentilentetrazol ile indüklenen akut ve kronik epilepsi modellerinde *Thymus vulgaris* L. (TVL)'in oksidatif stres, motor koordinasyon ve öğrenme/bellek üzerine etkilerini araştırmaktır. Bu amaçla 64 adet Wistar albino sıçan randomize olarak sekiz gruba ayrıldı. (1) akut kontrol (AC), (2) akut pentilentetrazol (APTZ) (3) akut PTZ + sodyum valproat (APTZ+VPA), (4) akut PTZ + TVL (APTZ+TVL), (5) kronik kontrol (CC), (6) PTZ tutuşma (PTZk), (7) PTZ tutuşma + VPA (PTZk+VPA) ve (8) PTZ tutuşma + TVL (PTZk + TVL). PTZ enjeksiyonundan sonra 30 dk boyunca nöbetler gözlenerek skorlandı. Çalışmada tek doz PTZ (60 mg/kg, ip) enjeksiyonu ile akut PTZ indüklü nöbet modeli oluşturuldu. Tutuşma ise gün aşırı subkonvulsan dozda PTZ (35 mg/kg, ip) uygulanarak toplam 14 enjeksiyonla oluşturuldu. PTZk+VPA, PTZk ve PTZk+TVL grupları arasında nöbet skorları bakımından anlamlı bir fark bulunamadı. Kronik çalışma gruplarında Morris water maze cihazı ile yapılan öğrenme ve bellek testleri yanında rota rod cihazı ile yapılan motor aktivite testlerinde gruplar arasında farklılık görülmedi (P>0.05). Total antioksidan statüsü (TAS), total oksidan statüsü (TOS) ve oksidatif stres indeksi (OSİ) sonuçları, PTZ uygulaması neticesinde antioksidan kapasitenin azaldığı, oksidan kapasitenin ise arttığı belirlendi. *T. vulgaris*'in özellikle uzun süre uygulanması ile total oksidan-antioksidan dengenin sağlandığı görüldü. Sonuç olarak gelecekte, daha yüksek dozlarda *T. vulgaris* L.'nin PTZ indüklü nöbet skorlarını değiştirip değiştirmeyeceğini belirlenmesi için ek çalışmaların yapılabileceği düşünülmektedir.

Anahtar sözcükler: *Davranış, Epilepsi, Tutuşma, Nöbet, Pentilentetrazol, Thymus vulgaris* L.

İletişim (Correspondence)
+90 532 2455888
hulyaozdemir39@gmail.com

INTRODUCTION

Epilepsy is one of the most common and serious brain disorders in the world (WHO, 2004). About 1/3 of the patients undergoing antiepileptic therapy continue to experience seizures and in some of these patients, progressive conditions such as increasing seizure frequency and cognitive decline can also be seen [1]. Although the underlying cause of epilepsy remains unclear, a long-standing hypothesis posits that the immune system plays a role in the pathogenesis of epilepsy. Additionally, this hypothesis has also been supported by the recent reports that implicate the use of steroids or immunoglobulins in the treatment of certain types of epilepsy and those documenting the role of inflammation and antibodies in the clinical course of certain types of epilepsy [2]. Pentylenetetrazole (PTZ) is a widely used behavioral approach in antiepileptic drug discovery studies [3]. Development of novel anticonvulsant drugs and disease-modifying antiepileptogenic agents constitute the next generation of therapeutic approaches for epilepsy. Moreover, accumulating evidence suggests that inflammatory events play a role in the etiopathogenesis of various types of epilepsy [4,5].

Experimental and clinical studies in the past decade have revealed that some specific inflammatory mediators and their cognate receptors are upregulated in epileptic brain tissue. Some of these drugs have already been shown to have a therapeutic effect on chronic peripheral inflammation and to have the potential to facilitate the hyperexcitability processes by reducing inflammation in epileptic brain tissue. Moreover, development of drugs that interfere with the mechanisms involved in the etiopathogenesis of seizures could lead to disease-modifying and curative effects in addition to symptomatic effects [6]. Therefore, drugs with anti-inflammatory or antioxidant properties are commonly studied in experimental epilepsy models for their potential therapeutic effects.

Thyme belongs to the Lamiaceae family and is a well-known therapeutic agent and is commonly used as folk medicine. In particular, the composition of the essential oils from *Thymus vulgaris* L. (TVL) are grown in the Mediterranean parts of Turkey [7,8]. This composition consists of thirty main elements of essential oil that constitute 100% of this composition. Some of these elements include thymol (46.2%), carvacrol (2.44%), linalool (4.0%), gamma-terpinene (14.1%), p-cymene (9.9%), myrcene (3.5%), α-pinene (3.0%), and flavonoids [8,9]. Of these, carvacrol has been shown to have antifungal, antibacterial, anthelmintic, analgesic, insecticidal, cholesterol-reducer, liver protective, antioxidant and anti-cancerogenic effects [10-13]. Its been shown to have anti-diabetic effects and to improve the learning functions of the brain [14].

Epilepsy, mediated by oxidative stress, results in abnormal structural changes in cellular proteins, membrane lipids, DNA, and RNA. Oxidative stress in brain has been implicated as the widespread cause of numerous acute neurological disorders including Parkinson's disease and Alzheimer's disease [15]. Moreover, oxidative stress has been reported to play a key role in various epilepsy models and PTZ is known to cause oxidative stress [16]. The biochemical changes induced by oxidative stress implicate the role of free radicals during seizures. Since antioxidants have a potential to reduce the seizures, though partially, they may be used as additional agents to antiepileptic drugs. Therefore, in the present study, we aimed to investigate the antiepileptic activity of TVL in acute and chronic epilepsy models to evaluate the protective and antioxidant effects of TVL on memory.

MATERIAL and METHODS

First of all, fresh leaves of thyme were collected in May 2017 from the area between the Aydın and İzmir provinces in the Aegean Region of Turkey. Following identification, a voucher specimen was prepared with a code number VAN YYU VANF-164096.

The animal care protocol and the experimental study were approved by the Animal Care and Use Committee of the Experimental Animal Unit and Ethics Committee (2018/05).

Preparation of Extract

The ground leaves were dried in shade, sheltered from direct sunlight, milled to a homogeneous powder, and then sifted through a 1-cm mesh. The powdered plant material (20 g) was extracted in 150 mL of distilled water with decantation method for 5 min. The extract was concentrated to dryness using a rotary evaporator followed by a lyophilizer and then the yield percentage of the extract was calculated. Water extraction of TVL was performed using a modified version of the decoction method used by Eddouks et al.[17].

Experimental Design

Sixty-four male Wistar Albino rats were randomly divided into eight groups with 8 rats each: (1) acute control (AC), (2) acute PTZ (APTZ), (3) acute PTZ+sodium valproate (APTZ+VPA), (4) acute PTZ+TVL (APTZ+TVL), (5) chronic control (CC), (6) PTZ kindling (PTZk), (7) PTZ kindling+VPA (PTZk+VPA) and (8) PTZ kindling + TVL (PTZk+TVL). PTZ (Sigma) was dissolved in isotonic saline (0.9% NaCl) and was administrated by intraperitoneal injection. VPA and TVL extract were administered orally by intragastric gavage daily.

Induction of Acute Seizures and PTZ Kindling

Tonic-clonic seizures were induced by injecting a single convulsive dose of PTZ (60 mg/kg, 0.1 mL/100 g) [18] in the acute groups (APTZ, APTZ+VPA, APTZ+TVL). The rats in the APTZ+VPA group received VPA (100 mg/kg by gavage, 0.1 mL/100 g) [19] and the rats in the APTZ+TVL group received TVL (200 mg/kg by gavage, 0.1 mL/100 g) [14] 2 h before

the acute PTZ injection. The rats in the AC group received saline solution only (0.1 mL/100 g by gavage).

PTZ kindling was produced by injecting a subconvulsant dose of PTZ (35 mg/kg) every other day (three times a week; every Monday, Wednesday and Friday; 14 treatments in total).

The rats in the chronic control group received isotonic saline (0.9% NaCl) TVL by gavage every day. In the PTZk+VPA and PTZk+TVL groups all PTZ injections were administrated 2 h after oral treatments (VPA or TVL). TVL extract was prepared freshly (in isotonic saline, 0.9% NaCl) and administrated daily to the rats (by gavage at dose 0.1 mL/100 g) in the PTZk+TVL group. Blood and tissue samples were collected under ketamine (90 mg/kg ip), and xylazine (10 mg/kg s.c) anesthesia.

Seizures were observed for 30 min after each PTZ injection. Seizure scores were evaluated using the following scoring system: stage 0: no response; stage 1: ear and facial twitching; stage 2: myoclonic jerks without upright position; stage 3: myoclonic jerks, upright position with bilateral forelimb clonus; stage 4; tonic-clonic seizures; stage 5: generalized tonic-clonic seizures, loss of postural control [20,21]. The rats demonstrating at least three consecutive stage 4 or 5 seizures were considered to be kindled.

Statistical Analysis

Statistical Analysis for Seizures, Spatial Learning-Spatial Memory and Motor Activity Tests: Data were analyzed using SPSS for Windows version 13.0 (SPSS Inc. Co., Chicago, IL, USA). Descriptive statistics were expressed as median, mean, standard deviation (SD), minimum, and maximum. The groups were compared using Kruskal-Wallis test. Friedman tests was used to compare days for Morris Water Maze (MWM) data. A P value of $P<0.05$ was considered significant.

Statistical Analysis for TAS-TOS: Results are expressed as Mean±Standart Deviation (SD). Analysis of variance (ANOVA) was performed, and the statistical comparisons among the groups were carried out with post hoc Tukey's test for normally distributed variables, or with nonparametric Bonferroni test for non-normally distributed data using a statistical package program (SPSS 23.0 for Windows).

RESULTS

No significant difference $P>0.05$ was found among the seizure scores in the acute groups *(Table 1)*.

In the PTZk group, the experiment was finalized when kindling was achieved after the 14th injection *(Table 2)*. In the same group, no rat died throughout the experiment.

Table 1. *Comparison of seizure scores in the acute groups*

Group		Median	Mean	SD	Min.	Max.	P
Score	APTZ	5.00	5.00	.00	5.00	5.00	0.329
	APTZ+TVL	5.00	4.71	.76	3.00	5.00	
	APTZ+VPA	5.00	4.43	.98	3.00	5.00	

Table 2. *Effects of VPA and TVL treatment on the development of PTZ-kindled seizures*

Injection Number	PTZk + VPA					PTZk					PTZk + TVL					*P Values for Groups
	Med.	M	SD	Min.	Max.	Med.	M	SD	Min.	Max.	Med.	M	SD	Min.	Max.	
#1	1.0 c	1.3	.5	1.0	2.0	1.0 d	1.3	.5	1.0	2.0	1.0 d	1.4	.7	1.0	3.0	.981
#2	1.0 c	1.9	1.1	1.0	3.0	1.5 cd	1.8	1.0	1.0	3.0	1.0 d	1.4	.7	1.0	3.0	.549
#3	3.0 b	2.3	1.3	1.0	4.0	3.0 c	2.3	1.0	1.0	3.0	1.0 d	1.6	1.2	1.0	4.0	.432
#4	3.0 b	3.0	1.6	1.0	5.0	3.0 c	3.0	1.3	1.0	5.0	2.0 c	2.1	1.2	1.0	4.0	.433
#5	3.0 b	2.7	1.4	1.0	5.0	3.0 c	3.3	.8	3.0	5.0	3.0 b	2.3	1.0	1.0	3.0	.213
#6	3.0 b	2.7	1.4	1.0	5.0	3.0 c	3.0	.9	2.0	4.0	3.0 b	2.4	1.2	1.0	4.0	.649
#7	4.0 a	3.9	.9	3.0	5.0	3.5 bc	3.7	.8	3.0	5.0	3.0 b	3.2	.4	3.0	4.0	.266
#8	3.0 b	3.1	.9	2.0	5.0	4.0 b	3.7	1.0	2.0	5.0	3.0 b	3.0	1.1	2.0	5.0	.379
#9	3.0 b	3.1	1.5	1.0	5.0	4.0 b	3.7	.5	3.0	4.0	3.0 b	2.6	1.3	.0	4.0	.248
#10	4.0 a	3.7	1.0	2.0	5.0	4.0 b	3.8	.4	3.0	4.0	3.0 b	3.4	.9	2.0	5.0	.437
#11	3.0 b	3.3	.8	2.0	4.0	4.0 b	3.8	.4	3.0	4.0	3.0 b	3.1	.8	2.0	4.0	.184
#12	3.0 b	3.3	.8	2.0	4.0	4.0 b	4.0	.0	4.0	4.0	3.0 b	3.4	1.2	2.0	5.0	.210
#13	4.0 a	3.9	1.2	2.0	5.0	5.0 a	4.7	.5	4.0	5.0	3.5 ab	3.8	.9	3.0	5.0	.178
#14	4.0 a	4.1	.9	3.0	5.0	5.0 a	4.7	.5	4.0	5.0	4.0 a	3.6	.9	2.0	5.0	.091
** *P values for injection*	.001					.001					.001					

** Kruskal-Wallis Test→; ** Friedman test ↓; Variables with more than one letter indicate statistical significance between injection numbers (the column)*

On the other hand, no significant difference was found among the PTZk, PTZk+VPA, and PTZk+TVL groups in terms of seizure scores. However, seizure frequency increased progressively in all the three groups (P<0.001, *Table 2*).

Spatial learning/memory was tested in the rats in the chronic groups using the Morris Water Maze (MWM) [22,23]. MWM was performed using the spatial version of the MWM test used by Tuzcu and Baydaş [24]. For pretraining orientation, the rats were made to swim in the platform-free maze for 2 min. Before the testing, environmental cues that aid the rats in spatial learning including high-contrast geometric patterns were placed on the walls visible to the animal from the water and platform throughout the duration of the experiment. Using a computer equipped with Noldus EthoVision Tracking System, the maze was divided into 4 equal quadrants. Care was taken to position the platform in the same quadrant throughout the test. Each rat swam for 4 times over a period of four consecutive days. The time spent for locating the hidden platform (reaction time) and the time spent in the target quadrant were recorded for each rat.

After the completion of these tests, total anti-oxidant status (TAS) and total oxidant status (TOS) were measured using a commercially available kit [25]. Oxidative stress index (OSI) was defined as the ratio of TOS to TAS level [25]. For the calculation of OSI, the resulting unit of TAS (mmol Trolox equivalent/L) was converted to micromole equivalent/L. The OSI value was defined as 'arbitrary unit' (AU) and was calculated using the following formula:

$$OSI = \frac{(TOS,\ \mu mol\ H_2O_2\ Eq./lt)}{(TAS,\ mmol\ Trolox\ Eq./lt\ X\ 10)}$$

The rotarod test consists of a circular rod turning at a constant or increasing speed and is used for estimating the duration a rat can remain on the rod rather than fall onto a platform below. Prior to the test, the rats were trained to remain on the rod rotating at a speed of 6 rpm for a total duration of 3 min. For testing, each rat was placed on the rod turning at 16 rpm for three sessions of 60 sec each. The integral of time spent on the rod over three sessions (maximum, 180 sec) was accepted as the rotarod performance for each rat [26].

Spatial Learning Test

The spatial learning test on MWM indicated that the PTZk+TVL group had a greater decrease in the time spent for locating the hidden platform compared to other chronic groups, although no significant difference was established among the groups (P>0.05) *(Fig. 1)*.

Spatial Memory Test

The spatial memory test on MWM indicated that the time spent in the target quadrant was longer in the PTZk + TVL group compared to other chronic groups, although no

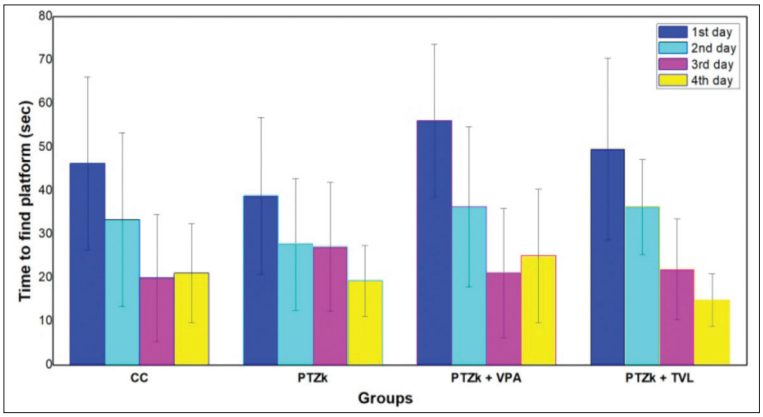

Fig 1. Time spent for locating the hidden platform on the MWM test in the chronic groups *(Friedman* Test was applied there was no significant difference among the groups, P>0.05)

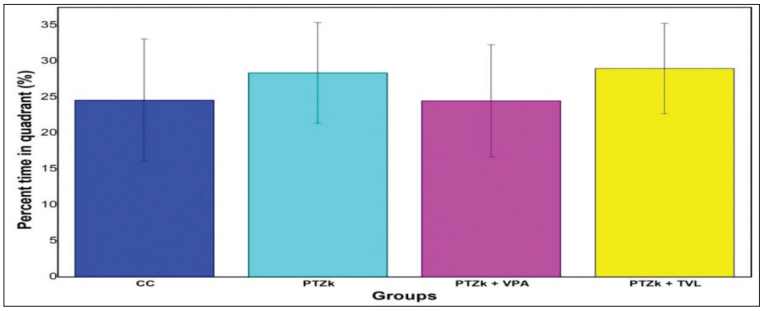

Fig 2. Comparison of the time spent in the target quadrant among the chronic groups *(Friedman* Test was applied there was no significant difference among the groups, P>0.05)

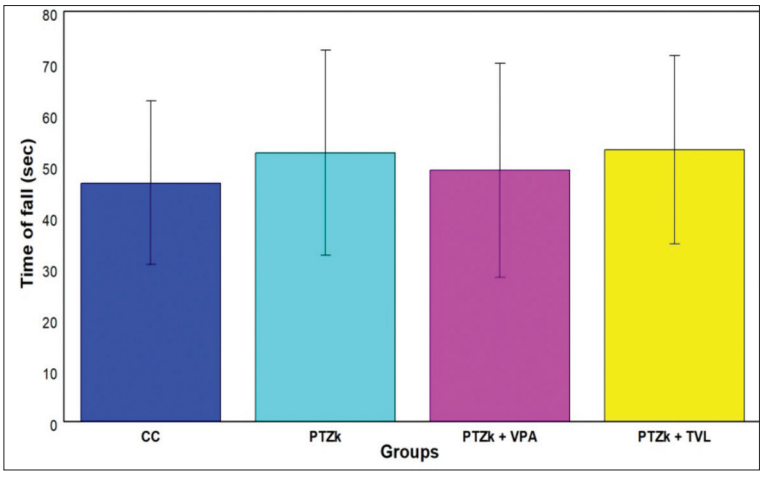

Fig 3. Comparison of the time spent on the rotating rod among the chronic groups *(Friedman* Test was applied there was no significant difference among the groups, *P>0.05)*

Table 3. *Serum TAS, TOS, and OSI levels*

Group	TAS Mean±SD	TOS Mean±SD	OSI Mean±SD
AC	1109±47.65[a]	8.3±0.46[a,c]	0.76±0.05[a,a1]
APTZ	909.2±87.51[c]	11.76±0.50[a1,b,b1,c,c1]	1.32±0.07[a2]
APTZ+VPA	710.2±39.07[b]	7.71±0.49[a2,c1]	1.10±0.09[a3,c]
APTZ+TVL	996.9±104.8[b1]	34.91±1.51[a,a1,a2,a3,a4,a5,a6]	3.74±0.49[a,a2,a3,a4,a5,a6,a7]
CC	1223±146.6[a1,b,c2]	7.67±0.65[a3,b,]	0.69±0.11[a4,a8]
PTZk	434.7±28.36[a,a1,b1,b2, c]	9.36±0.49[a4]	2.17±0.07[a1,a5,a8,a9,c]
PTZk+VPA	781.6±101.1[c2]	10.29±0.83[a5]	1.42±0.20[a6]
PTZk+TVL	1004±90.76[b2]	7.02±0.43[a6,b1]	0.75±0.10[a7,a9]

[a,a1-a9] P<0.001, [b,b1, b2] P<0.01, [c, c1, c2] P<0.05 (Groups with the same letter indicate the presence of a significant difference)

significant difference P>0.05 was established among the groups *(Fig. 2)*.

Motor Activity Test

The Rotarod performance test indicated no significant difference P>0.05 among the chronic groups with regard to motor activity *(Fig. 3)*.

TAS and TOS Measurement

The analysis of serum samples indicated that the TAS levels decreased in the APTZ group compared to the control group and increased, though insignificantly, in the APTZ+TVL group compared to the APTZ group (P>0.05). Moreover, TAS levels decreased significantly (P<0.001) in the PTZk group compared to the control group and increased significantly (P<0.001) in the PTZk+TVL group compared to the PTZk group *(Table 3)*.

In acute groups, the TOS levels were significantly higher in the APTZ and APTZ + TVL groups compared to the control group (P<0.05, P<0.001, respectively). However, no significant different was found among the chronic groups with regard to TOS levels, a significant (P<0.001) difference was found between the APTZ+TVL and PTZk+TVL groups *(Table 3)*.

In acute groups, OSI was increased significantly in the APTZ+TVL group compared to the other groups (P<0.001). In chronic groups, OSI increased significantly (P<0.001) in the PTZk group compared to control group and decreased significantly (P<0.001) in the PTZk+TVL group compared to the PTZk group *(Table 3)*.

DISCUSSION

The present study evaluated the antiepileptic effects of a variant of thyme and its effects on spatial learning/memory, and motor activity and also examined the oxidant and antioxidant capacity levels of TVL in experimental epilepsy models induced by acute or chronic PTZ administrations.

Epilepsy affects more than 65 million people worldwide [27]. Epilepsy is a highly prevalent, serious brain disorder and oxidative stress is regarded as a possible mechanism involved in epileptogenesis. Inflammation and oxidative stress are known as critical factors in the pathophysiology of epilepsy [28,29]. However, it has been suggested that there may also be an association between seizure generation and the disruption of the balance between oxidants and antioxidants [30].

Experimental studies suggest that oxidative stress is a factor contributing to the onset and evolution of epilepsy. Oxidative stress is regarded as a result of the disruption of the balance between the formation and the extermination of reactive oxygen species (ROS) [28]. In addition, excessive ROS formation may alter DNA and thereby may lead to lipid modification and formation of pro- and anti-inflammatory cytokines [31]. Increasing data suggest that there is an association between the immune system and the pathophysiology of epilepsy [32]. The inflammatory responses are also considered to contribute to epileptogenesis [32]. Moreover, it has been shown that there is an association among IL-1β, IL-6, TNFα and epilepsy and it has also been reported that seizures increase both the level of IL-1β and its mRNA and this increase is associated with oxidative stress [32].

Oxidative stress is an important factor in numerous epilepsy models [33]. Moreover, researchers have recently focused on a possible association between oxidative stress and epilepsy, contending that the abnormal Ca^{+2} signaling resulting from oxidative stress may lead to excessive free radical formation, mitochondrial dysfunction, cell injury, and ultimately to epilepsy [34]. Oxidative stress and mitochondrial dysfunction are involved in neuronal death and seizures. However, oxidative damage occurred in proteins, lipids, and mitochondrial DNA after seizure activity [35]. These findings implicate that RNA oxidation is a significant factor contributing to seizure-induced neuronal degeneration and to epileptogenesis [35,36].

Accumulating evidence suggests that antioxidant therapy may reduce lesions induced by oxidative free radicals in

some animal seizure models. Recent studies have also shown that the association between mitochondrial dysfunction and chronic oxidative stress may play an important role in epileptogenesis [28]. Therefore, there is a growing interest into antioxidants that decrease oxidative stress in the treatment of epilepsy [30]. Induced seizures may be partially prevented with the treatment methods that are based on antioxidants such as superoxide dismutase (SOD) mimetics, vitamin E, melatonin, spin traps, vitamin C and coenzyme Q10 [37,38].

Literature indicates that agents with anti-inflammatory or antioxidant properties are commonly studied in experimental epilepsy models for their potential therapeutic effects. Oxidative stress is considered to play a role in epileptogenesis and in PTZ-induced acute seizures. Moreover, herbs with antioxidant properties have also been shown to have therapeutic effects in experimental epilepsy models [39,40]. In the present study, we found that TVL, particularly its chronic administration, strengthened the antioxidant system. Also, it was reported that chronic administration of *Aloe vera* leaf (extract) powder prevented the progression of kindling in PTZ-kindled mice and also reduced brain levels of malondialdehyde (MDA) and increased the glutathione (GSH) levels compared to the PTZ-kindled non-treated group. Depending on these findings, the authors concluded that *Aloe vera* leaf (extract) powder has significant anticonvulsant and antioxidant activity [41]. On the other hand, the phytochemical results presented by the epilepsy studies conducted with plant extracts have indicated the presence of three flavonoids and four additional compounds belonging to the steroid, terpenoid, phenol, alkaloids, saponins, tannins or sugar classes of compounds in jasmine flowers and *P. daemia* roots [42,43]. Flavonoids (luteolin) have been shown to provide protection against some biological epilepsy models such as the seizures induced by PTZ [44].

Skalicka-Wozniak et al.[45] performed efficient purification of single constituents from *Thymus vulgaris* essential oil (EO) (borneol, thymol and carvacrol) and investigated anticonvulsant activities of single constituents from *Thymus vulgaris* EO. The authors indicated that *Thymus vulgaris* EO provided protection against maximal electroshock (MES)-induced seizures. Accordingly, preclinical research on *Thymus vulgaris* EO, as well as on isolated terpenoids, provides evidence suggesting that the essential oil has partial protective activity against seizures.

Sodium valproate (VPA), an antiepileptic drug used in the treatment of epilepsy due to its therapeutic effect, shows gamma-aminobutyric acid (GABA)-mimetic activity and elevates brain GABA levels by inhibiting some enzymes that are responsible for the synthesis and destruction of GABA. Moreover, VPA also enhances the glutamic acid decarboxylase activity that plays a key role in GABA synthesis and weakly inhibits the GABA-aminotransferase that plays a part in GABA metabolism [46]. Accordingly, it is reported that VPA, when used with ethanolic extract of TVL, has no effect on MES- and PTZ-induced convulsions. However, combination treatments such as Thyme+VPA have been shown to provide full protection (100% and 33%, respectively) against convulsions induced by PTZ and MES [47]. In our study, however, we did not use TVL and VPA in combination and we found that the sole application of TVL extract seems to have no effective constituents that can battle these mechanisms. Therefore, it is tempting to consider that TVL extract could not enhance GABA-ergic neurotransmission.

Hippocampus is known to have a crucial role in learning and memory in mammals. Accordingly, any neuronal damage or dysfunction in the hippocampus leads to cognitive impairment [48]. Previous studies indicated that the PTZ-induced seizures resulted in hippocampal neuronal damage, subsequently leading to spatial learning and memory impairment [48,49]. Additionally, it has also been reported that antiepileptic drugs (AEDs), which are known to have limited effectivity even in drug-controlled seizures, have little or no effect on the cognitive deficits in epilepsy [50].

Most AEDs interfere with cognitive functions and thus there is an urgent need for AEDs that are effective but do not show this side effect. Of note, some of the plants that have been investigated in the search for alternative drugs against the neuronal damage caused by epilepsy have been shown to have favorable effects on epilepsy and cognitive functions [48].

For instance, some researchers have shown anti-inflammatory and neuroprotective effects of Sinomenine (SN) in nervous system diseases. Moreover, the researchers also determined the effect of SN on epilepsy in PTZ-induced chronic epilepsy models by assessing spatial learning and memory using MWM and reported that different doses of SN blocked the hippocampal neuronal damage, minimized the impairment of spatial learning and memory in PTZ-kindled rats, and also provided neuroprotection both *in vitro* and *in vivo* [48].

Morteza-Semnani et al.[51] investigated the effect of methanol extracts and essential oils from Thymus species *(Thymus fallax, T.kotschyanus* Boiss. and *T. pubescen* Boiss.) on swimming performance in mice. This finding implicates that the extracts and oils shortened the immobility period during the forced swimming test in the experimental group compared with the control group and exhibited a dose-dependent antidepressant activity.

On the other hand, Zhen et al.[44] investigated the anticonvulsant effects of a natural flavonoid, luteolin (LU), on PTZ-induced cognitive impairment in rats. The researchers revealed that pretreatment with LU suppressed the seizure induction, duration, and severity following PTZ injection, reversed cognitive impairment, reduced neuronal and oxidative stress damage.

Similarly, given that TVL has favorable effects on spatial learning and memory, the present study investigated the protective effects of TVL on neuronal damage. However, since the cognitive functions of the rats were not affected in our experimental epilepsy model, no evaluation could be made regarding the effect of TVL on cognitive functions.

Our results indicated that although there was a decrease in the scores of the PTZk+TVL group compared to the PTZk group, a satisfactory decrease was not obtained probably due to the inadequate dose (200 mg/kg) of TVL. From these findings then, it can be thought that TVL does not involve an effective substance to enhance inhibitory GABA-ergic neurotransmission in the central nervous system. However, favorable effects of TVL on cognitive functions were demonstrated in one of our previous studies. Our results showed that thyme extract improves the cognitive learning functions that are impaired by diabetes [52]. Depending on these successful outcomes, in the present study, we administered TVL at dose of 200 mg/kg.

The performance of a rat on the rotarod test is a valuable measure that can be used for evaluating the aspects of motor function such as balance and coordination [53]. In the present study, we used the accelerating rotarod test to investigate whether epilepsy and the administration of TVL extract have any effect on the motor functions of the rats. The test indicated no significant difference among the chronic groups with regard to motor activity. This finding implicates that the motor activity is not affected in PTZ-kindling epilepsy model and the administration of TVL 200 mg/kg does not affect the motor coordination center in the rat brain. Meaningfully, future studies to be conducted with higher doses of TVL may elucidate whether motor functions are affected at higher doses. Similarly, Addae et al.[42] examined the neuropsychiatric effects of Jasmine leaf extract that is extensively used in folk medicine. The researchers concluded that Jasmine leaf extract provided favorable outcomes in an animal model of acute partial complex epilepsy and also had a significant anxiolytic effect at a dose that does not affect motor coordination. Additionally, it has also been shown that essential oil and any single compound of *Thymus vulgaris* has no significant effect on motor performance of the experimental animals assessed by the chimney test [45].

In our study, the evaluation of TAS, TOS, and OSI levels indicated that the PTZ administration decreased the anti-oxidant capacity and increased the oxidant capacity while the TVL administration established the oxidant/antioxidant balance in the chronic groups. These findings implicate that TVL may protect the organism against oxidative stress in chronic PTZ-induced epilepsy model rather than in acute PTZ-induced seizure model and also suggest that a better understanding of these effects could be obtained by further studies to be conducted dose-dependent. Based on the other studies, it can be considered that these biological and neuroprotective effects are associated with the components of the essential oil such as borneol, thymol, eugenol, carvacrol, flavonoids, and terpenoids. The compounds of *Thymus vulgaris* can be further investigated in acute and chronic PTZ-induced epilepsy models.

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Conflicts of Interest

There are no conflicts of interest.

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Research Article

Preliminary Study of High Efficiency Vaccine of *Rhipicephalus (Boophilus) microplus* in South Xinjiang, China

Yong-Hong LIU [1,a] Kai-Rui LI [1] Bo HE [1] Fei LI [1,2,b] Lu-Yao ZHANG [1,3,c]
Jiao-Jiao PAN [1] Qiang-Rong WANG [1] Li ZHAO [1,d]

[1] College of Animal Science, Tarim University, Aral 843300, P.R. CHINA
[2] Animal Loimia Controlling and Diagnostic Center of Aksu Region, Aksu, Xinjiang 843300, P.R. CHINA
[3] Animal husbandry and veterinary workstations of Barkol kazak autonomous county, Kumul 839200, P.R. CHINA
[a] ORCID: 0000-0003-4432-9433; [b] ORCID: 0000-0001-09739-0663; [c] ORCID: 0000-0003-0689-8811; [d] ORCID: 0000-0001-8538-5605

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Abstract

The direct damage and spread of pathogens of *Rhipicephalus (Boophilus) microplus* to cattle -based livestock is extremely serious, the traditional chemical acaricides control have many disadvantages, and vaccine prevention and control is a potential alternative. The commercially available vaccine, based on *Rhipicephalus (Boophilus) microplus* Bm86, has been favored by farmers and veterinarians in some areas, but it also has drawbacks such as reduced effectiveness due to genetic variation. Based on the fact that Bm91 and Bm86 sharing can enhance the immune effect of Bm86, as well as the characteristics of DNA vaccines, the combination of DNA vaccine and protein vaccine enhanced the immune effect. In this study, adopts the local *Rhipicephalus (Boophilus) microplus* strains, using prokaryotic expression system preparation Bm86 and Bm91 proteins, and using the eukaryotic expression vector pVAXI containing the CpG sequences constructed Bm86 and Bm91 double gene eukaryotic expression system. This research provides materials for the next step of Co-immunizing animals with Bm86 and Bm91 mixed proteins with Bm86 and Bm91 dual-gene carriers, it also provides a way for co-immunization with multi-antigen protein and multi-gene carriers to control ticks.

Keywords: *Rhipicephalus (Boophilus) microplus, Bm86, Bm91, Co-expression*

Çin'in Güney Xinjiang Bölgesinde *Rhipicephalus (Boophilus) microplus*'a Karşı Yüksek Verimli Aşı İçin Ön Çalışma

Öz

Sığır besiciliğinde *Rhipicephalus (Boophilus) microplus*'un doğrudan oluşturduğu hasar ve yayılımı oldukça ciddi bir problem olup, geleneksel kimyasal akarisitlerin kontrolde pek çok dezavantajları vardır ve aşı ile koruma ve kontrol alternatif olabilir. *Rhipicephalus (Boophilus) microplus* Bm86 temelli hazırlanan ve ticari olarak mevcut olan aşı bazı bölgelerde yetiştiriciler ve veteriner hekimler tarafından tercih edilmektedir, ancak genetik varyasyondan dolayı azalmış etkisi gibi negatif tarafları bulunmaktadır. Bm91 ve Bm86'nin birlikte bulunması Bm86'nın bağışıklık oluşturma etkisini artırabileceğinden aynı zamanda DNA aşıların özelliklerinden dolayı DNA aşısının protein aşı ile kombinasyonu bağışıklık etkisini artırabilir. Bu çalışmada yerel *Rhipicephalus (Boophilus) microplus* türü ile Bm86 ve Bm91 proteinlerinin prokaryotik ekspresyon sistemi ve CpG sekansı içeren pVAXI prokaryotik ekspresyon vektörü aracılı Bm86 ve Bm91 çift gen ökaryotik ekspresyon sistemi kullanıldı. Bu çalışma, Bm86 ve Bm91 proteinleri karışımı ile Bm86 ve Bm91 çift gen taşıyan aşı materyali oluşturmuş, böylece kene kontrolünde çoklu antijen protein ve çoklu gen taşıyan aşı ile koimmunizasyon için bir araç oluşturmuştur.

Anahtar sözcükler: *Rhipicephalus (Boophilus) microplus, Bm86, Bm91, Koekspresyon*

INTRODUCTION

The cattle tick *Rhipicephalus (Boophilus) microplus* is parasitic on cattle hematophagous ectoparasite, which spread of various pathogens [1-8]. This parasite was found in West Africa, East Africa, South Africa, the Middle East, Latin America and Asia [6,8-12]. In China, *R. microplus* tick is the most widespread tick species and widely distributed in 23 provinces [12]. The traditional method to control *R. microplus* tick is to use chemical insecticides, but there are many disadvantages,

İletişim (Correspondence)
+86-1829-0612711
zhaolidky@126.com

such as food and environmental pollution, chemical residues and increase of antidrug ticks [13-17]. The vaccine that protects cattle against ticks is a promising alternative way to control ticks, which has additional advantages compared with chemical control agents [14,17-21]. At present, the only commercial anti-tick vaccine in the world is from Cuba, named GavacTM, which is based on the recombinant Bm86 antigen of the midgut membrane protein of *R. microplus* tick [16,17,22-24]. The main protective mechanism of this vaccine is the production of antibodies against Bm86 protein. This humoral immune response directly affects the intestinal tract of ticks, reduces the amount of blood sucking and reduces the weight and fecundity of female ticks [23,25-28]. This antigen does not grant enough protection to several *R. microplus* tick populations [27,29,30]. There is evidence that improving the efficacy of this vaccine against *R. microplus* tick has been achieved satisfactorily by adding more than one antigen into the vaccine [31]. Bm 91 is an antigen isolated from the salivary glands and midgut of *R. microplus* tick. When inoculated to cattle, this antigen will significantly reduce the ability of sucking and laying eggs of ticks infesting the cattle [32]. The anti-tick effect of Bm91 on the immune response against ticks is not as obvious as Bm86 [33]. But, Bm91 induced long-term immune response and showed an increased efficacy of Bm86 vaccine for *R. microplus* tick when co-administered [33,34]. In addition, the sequence variation of antigen sites between *R. microplus* ticks isolated from different geographical areas have been proved to affect vaccine efficacy [35]. Therefore, it is necessary to select the predominant strains in a local region for preparation of effective vaccines [15]. The DNA vaccine has a potential advantage over other types of vaccines, they can induce strong cellular immune responses in addition to the humoral immune response, and they have no risks associated with the use of traditional attenuated vaccines, and the purification of plasmid DNA is easier and cheaper than recombinant protein. Furthermore, the DNA vaccine is stable at room temperature [28,36,37]. At the same time, some studies have shown that the co-immunisation of DNA and protein vaccines boosts the immune effect [38,39].

From the above, this study investigated the preparation of Bm86 and Bm91 recombinant mixed proteins and dual gene plasmid DNA adopts the local *Rhipicephalus (Boophilus) microplus* strain, and laid the foundation for the joint immunological study to enhance the immunological effect of anti-tick vaccine.

MATERIAL and METHODS

Materials

Research Areas and R. microplus Collection

In 2013, *R. microplus* were collected from cows in Makit of Kashgar Prefecture, Xinjiang (1179 m above sea level; 38°54'N, E77°39'E). These tick specimens were placed in sampling vials with sufficient air and transported immediately to the laboratory for cryopreservation.

Methods

RNA Extraction, RT-PCR and Sequence Analysis

The cryopreserved *R. microplus* ticks specimens were washed twice with 0.1% DEPC, then frozen in liquid nitrogen and ground using a mortar. Total RNA was prepared from *R. microplus* ticks using TRIzol® reagent (BBI, Shanghai, China, Code No. B610409) in accordance with the manufacturers' protocols. Total RNA was used for the synthesis of cDNA using the TaKaRa RNA PCR Kit (AMV) Ver.3.0 (Takara, Dalian, China, Code No. RR019A) in accordance with the manufacturer's manual. The cDNA was used for *R. microplus* Bm86 (1953 bp) gene and Bm91 (1833 bp) gene amplification with gene-specific primers. The primers sequence used for Bm86 and Bm91 gene amplification were 5'-ATGCGTGGCATCGCTTTGTTCG-3', 5'-TTACAACGATGCTGCGGTGACTG-3 and 5'-ATG GGCGT TGCCTTTATAGAAGGCT-3', 5'-TCATAACGAGATGTTTTTCC AGC-3', the annealing temperature was 57ºC and 58ºC, respectively. All PCR amplicons were bi-directionally sequenced using ABI PRISMTM 3730 XL DNA Analyzer. The sequencing results were analyzed in online BLAST *(https://blast.ncbi.nlm.nih.gov/)*. The obtained sequences in this study were submitted to GenBank under the accession numbers "GenBank accession NO.: MH165269 and MH165270".

Vector Constructs for Expression Recombinant Protein

The DNA fragments of Bm86 and Bm91 were amplified using the cryopreserved Bm86 and Bm91 gene amplification products using the Premix Taq™ Version 2.0 Kit (Takara, Dalian, China, Code No. R004A). The primers sequence used for Bm86 gene amplification were P1: 5'-CG*G GATCC* TGCGTGGCATCGCTTTGTTC G-3' with the restriction enzyme sites *Bam*H I) and P2: 5'-TCC*CCG GAATCC*TTACAA CGATGCTGCGGTGACTG-3' with the restriction enzyme sites *Eco*R I); The primers sequence used for Bm91 gene amplification were P3: 5'-TCC*CCGGAATCC*ATGGGCGTT GCCT TTATAGAAGGCT-3' with the restriction enzyme sites *Eco*RI) and P4: 5'-TT *GCGGCCGC* TCATAACGAGATGTTTTTCCAGC-3' with the restriction enzyme sites *Not* I). Using Bm86 and Bm91 gene amplification products as the template, the dual DNA fragments Bm were amplified with the P1 and P4 primers. Collect and purify the target fragments of Bm86, Bm91 and Bm using the OMEGA™ Gel Extraction Kit (OMEGA, China, Code No. D2500) in accordance with the manufacturer's manual. The DNA fragments of Bm86, Bm91 and Bm were ligated into pEASY-Blunt vector using the pEASY-Blunt Simple Cloning Vector (TransGen, China) in accordance with the manufacturer's manual. The plasmids were introduced into *E. coli* DH5α, the positive clone strains were detected by the technique of colonies PCR, using the following primers: P1 and P2 for Bm86, P3

and P4 for Bm91, P1 and P4 for Bm. The positive colony were cultured and extracted plasmids using the OMEGA™ Plasmid Mini Kit (OMEGA, China, Code No. D6943) in accordance with the manufacturer's manual. The extracted plasmids were double-enzyme cut to identify, using the following restriction enzyme sites: *Bam*H I and *Eco*R I for Bm86; *Eco*R I and *Not* I for Bm91; *Bam*H I and *Not* I for Bm. Moreover, the vectors were verified by DNA sequencing. The DNA fragments of Bm86 and Bm91 were ligated into pET28a via the restriction enzyme sites *Bam*H I/*Eco*R I and *Eco*R I/*Not* I using T4 DNA ligase. The plasmids were introduced into *E. coli* DH5α, the positive clone strains were detected by the technique of colony PCR, using the following primers: P1 and P2 for Bm86, P3 and P4 for Bm91. The positive colony were cultured and extracted plasmids using the OMEGA™ Plasmid Mini Kit (OMEGA, China, Code No. D6943) in accordance with the manufacturer's manual. The extracted plasmids were double-enzyme cut to identify, using the following restriction enzyme sites: *Bam*H I and *Eco*R I for Bm86; *Eco*R I and *Not* I for Bm91. The correct strain and plasmid DNA were kept for use.

Expression and Purification of the Recombinant Protein

The recombinant plasmid was confirmed by sequencing and introduced into *E. coli* expression strain BL21, the positive clone strains were detected by the technique of colony PCR, using the following primers: P1 and P2 for Bm86, P3 and P4 for Bm91. The positive colony were cultured and purified Bm86 and Bm91 protein by IPTG induction. *E. coli* cells harboring recombinant plasmids, pET28a-Bm86 and Bm91, were respectively grown under continuous shaking at 37°C in LB broth containing kanamycin. The cells were induced at OD600=0.5 with 0.6 mM IPTG, and grown for an additional 20 h at 18°C, and then harvested by centrifugation (5000 g, 15 min) and the pellets were frozen at −80°C until used. All purification steps were carried out at 4°C. Bacterial pellets were thawed in 100 mL lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM DTT, 10% glycerine, 0.5% TritonX-100, 2 mM EDTA, pH 8.0), supplemented with the appropriate protease inhibitor cocktail (Roche, Switzerland). Bacterial cells were lysed using an ultrasonic processor to generate the crude bacterial extract and centrifuged for 30 min at 12.000 × g to collect the supernatant. The supernatants were passed over a Ni column (GE, USA) pre-equilibrated with binding buffer ($NaH_2PO_4{\cdot}2H_2O$ 50 mM, NaCl 300 mM, Imidazole 10 mM, pH 8.0) and washed with 20 column volumes of binding buffer. Then the column was washed with 10 column volumes of washing buffer ($NaH_2PO_4{\cdot}2H_2O$ 50 mM, NaCl 300 mM, Imidazole 20 mM, pH 8.0) and eluted with elution buffer ($NaH_2PO_4{\cdot}2H_2O$ 50 mM, NaCl 300 mM, Imidazole 200 mM, pH 8.0). The proteins were quantified using BCA Protein Assay Kits in accordance with the manufacturer's manual (GenStar, USA). Protein extracts (20 µg per lane) were resolved on 12% sodium dodecyl sulphate-polyacrylamide gels and electro blotted onto Bio-Rad Immun-Blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA). After transfer, PVDF membranes were blocked in Tris-buffered saline-Tween 20 (TBST; containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% bovine serum albumin (Sigma-Aldrich) for 1 h at room temperature and incubated with the primary antibody of mouse His-TAG (Sigma-Aldrich, dilution 1:1000) overnight at 4°C. Membranes were washed three times (10 min) in TBST and incubated with the secondary antibody of anti-mouse immunoglobulin G (Sigma-Aldrich, dilution 1:2000) for 30 min. Subsequently, the membranes were washed three times (10 min) in TBST. Horseradish peroxidase activity was examined by Chemiluminescent Substrate (Roche) in accordance with the manufacturer's protocol. The proteins were collected for the following experiment.

Constructs for Eukaryotic Expression

The full-length DNA fragment of Bm was inserted into pVAX1-CpG vector between *Bam*H I and *Not* I sites for cell transfection and subsequent injection into cattle -based livestock. Cell transfection was performed using HEK293 cells (CRL-1573, American Type Culture Collection). Briefly, HEK293 cells were cultured in petri dish with DMEM medium (Hyclone, Code No.SH30243.01) added 10% FBS (Gemini, Code No.100-106) and 1% Penicillin-Streptomycin (Gibco, Code No.15140), put it in the incubator with 5% CO_2, 37°C. On the day before transfection, the cells are digested by trypsin, enriched by centrifugation or diluted with the medium according to their density, then replacing the medium using OPTI-MEM medium (Gibco, Code No.31985) with 10% FBS. Extracted 200 µL OPTI-MEM medium put into two 1.5 mL EP tubes, one EP tube added 4 µg plasmid DNA to be transfected, and another EP tube added 8 µL PEI (DNA: PEI=1 µg: 2 µL) gently mixed. The culture medium with PEI was added to the medium with the plasmid DNA, incubate at room temperature for 20 min. Add the HEK293 cells slowly to the mixture and gently mixed. The six orifice plates were cultured in the cell incubator, and the old medium was discarded after 4 h, and the new OPTI-MEM medium containing 10% FBS was added, then continued cultivation for 24 h before test.

Total RNA was prepared from transfection cells using TRIzol® reagent (Life Technologies, USA) and purified using a PureLink® RNA Mini Kit (Invitrogen, USA) combined with a PureLink® DNase Kit (Invitrogen), in accordance with the manufacturers' protocols. RNA concentration and quality were measured using a NanoVue spectrophotometer (GE Healthcare, USA). Approximately 2 µg total RNA was used for the synthesis of cDNA using the TransScript One-Step gDNA Removal and cDNA Synthesis Kit in accordance with the manufacturer's manual (TransGen, China). The cDNA was used for PCR reaction with gene-specific primers. The Bm fragment was amplified with P1 and P2 primers, and the pVAX1-Bm-CpG is preserved to provide nucleic acid to immune animal.

RESULTS

To obtain the recombinant protein in *E. coli*, we separately developed the Bm86 and Bm91 prokaryotic expression vector pET28a *(Fig. 1)*.

The field collected *R. microplus* ticks specimens were used for total RNA prepared. Total RNA was used for the synthesis of cDNA, and the cDNA was used for *R. microplus* Bm86 gene and Bm91 gene amplification with gene-specific primers. The gene fragments that were consistent with the desired fragment size were obtained *(Fig. 2)*. The PCR amplicons were bi-directionally sequenced, the sequencing results confirmed the Bm 86 gene with 1953 bp full open reading frame, encoding 650 aa and the Bm 91 gene with 1833 bp full open reading frame, encoding 610 aa, were obtained. The obtained Bm86 gene sequences results were similar with *B. microplus* cell surface glycoprotein Bm86 (GenBank accession NO.: TCKBM86A), and the similarity was 99% (1926/1953). The obtained Bm91 gene sequences results were similar with *Boophilus microplus* angiotensin-converting enzyme-like protein (Bm91) (GenBank accession NO: BMU62809), and the similarity was 98% (1801/1833).

Primers with internal *Bam*H I and *Eco*R I restriction sites were designed to amplify the cDNA of Bm86, and Primers with internal *Bam*H I and *Not* I restriction sites were designed to amplify the cDNA of Bm91. The PCR product was subcloned into the BamHI-*Eco*R I sites of pET28a to produce a fusion protein with a His tag at the N terminus, which was named as pET28a-Bm86 *(Fig. 3)*. The PCR product was subcloned into the *Bam*H I-*Not* I sites of pET28a to produce a fusion protein with a His tag at the N terminus, which was named as pET28a-Bm91 *(Fig. 3)*.

E. coli cells harboring recombinant plasmids were used to express and purify The *R. microplus* Bm86 and Bm91 proteins. Bm86 contained a 650 amino acid, which was predicted as a 71.5 KDa polypeptide and Bm91 contained a 610 amino acid, which was predicted as a 67.1 KDa polypeptide were obtained *(Fig. 4)*.

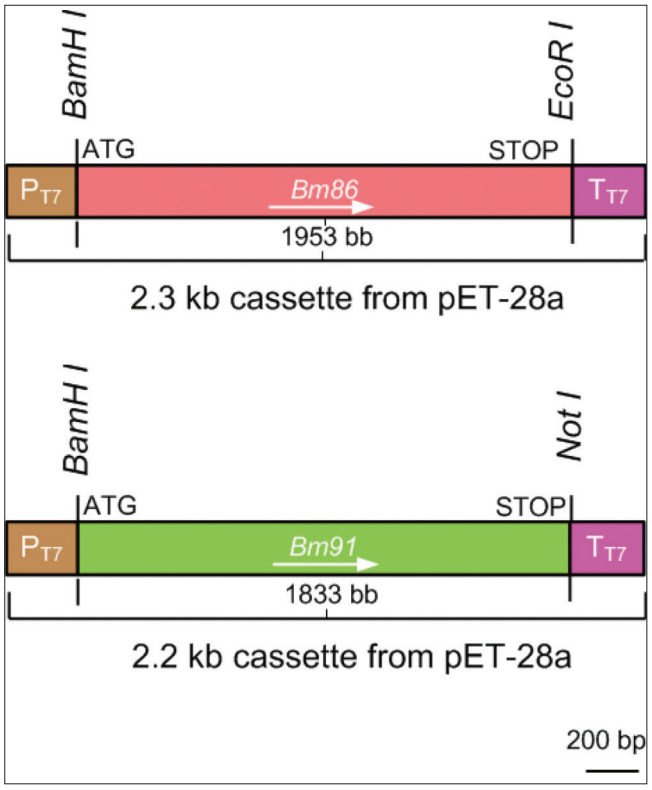

Fig 1. Construct maps of *pET28a-Bm86* and *pET28a-Bm91*. Construct maps of *pET28a-Bm86 (top)* and *pET28a-Bm91 (bottom)*. Arrows indicate the direction of transcription; P_{T7}, T7 promoter; T_{T7}, T7 terminator. The restriction enzymes used for cloning are indicated

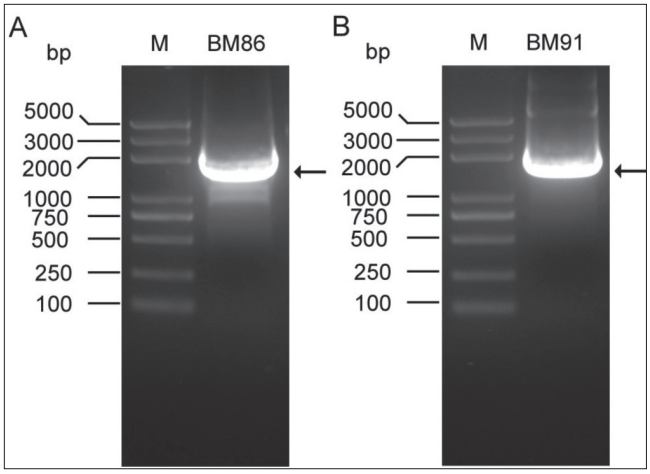

Fig 2. *R. microplus* Bm86 gene and Bm91 gene PCR amplification. **A-** The *R. microplus* Bm86 (1953 bp) gene PCR amplification with gene-specific primers, **B-** The *R. microplus* and Bm91 (1833 bp) gene PCR amplification with gene-specific primers

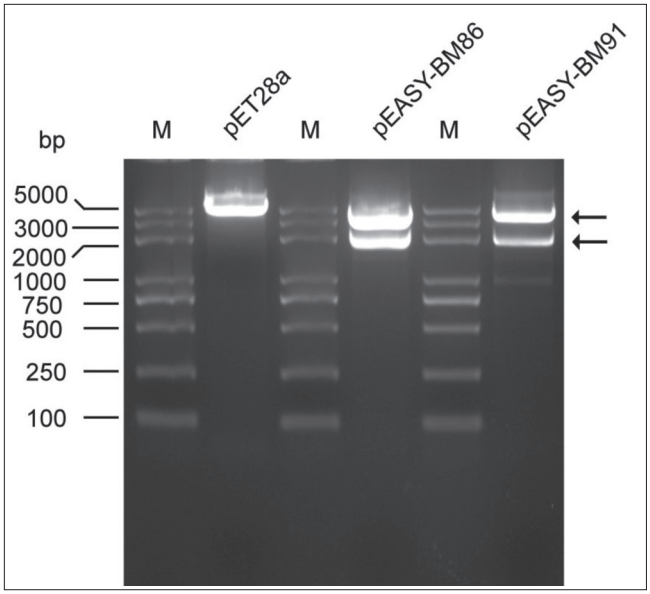

Fig 3. *pET28a,* Bm86 gene and Bm91 gene double enzyme cutting results. The *R. microplus* Bm86 (1953 bp) gene double enzyme cutting use *Bam*H I and *Eco*R I restriction sites and Bm91 (1833 bp) gene PCR amplification double enzyme cutting use *Bam*H I and *Not* I restriction sites

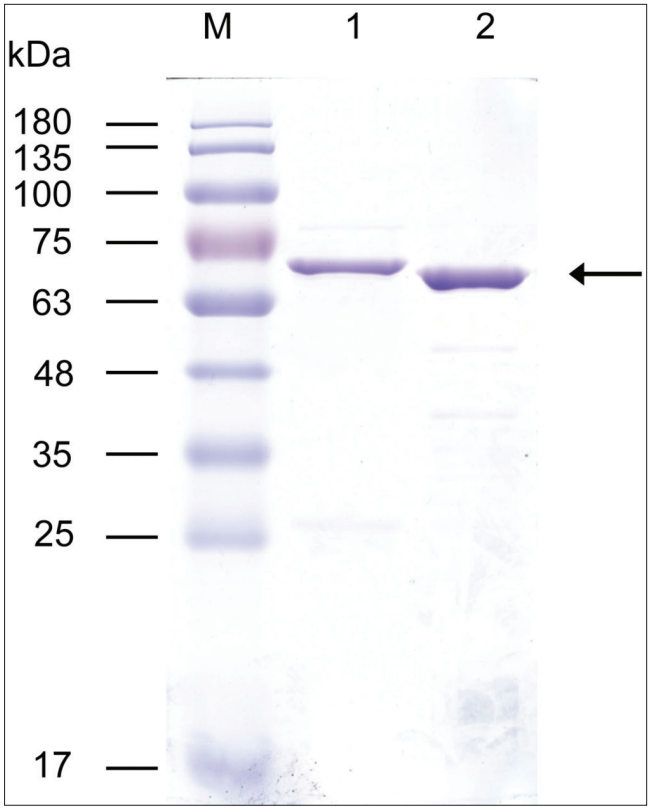

Fig 4. Bm86 and Bm91 recombinant protein SDS-PAGE electrophoresis detection. The *R. microplus* Bm86 protein (lane 1) and Bm91 protein (lane 2) SDS-PAGE electrophoresis detection. The arrow indicate the destination strip location. 5 μL protein was taken for electrophoresis, and the protein concentration of Bm86 and Bm91 were 0.98 mg/mL and 1.68 mg/mL, respectively

To further confirm the obtained proteins were the target proteins, the proteins were detected in the protein level with Western blot analyses *(Fig. 5)*.

Co-immunization DNA and protein vaccines boosts the immune effect, so we investigated the preparation of Bm86 and Bm91 recombinant mixed proteins and dual gene plasmid DNA Bm. To obtain the dual gene plasmid DNA Bm, we developed the double gene expression vector pVAX1-Bm-CpG *(Fig. 6)*.

Primers with internal *Bam*H I and *Not* I restriction sites were designed to amplify the cDNA of Bm. The PCR product was subcloned into the *Bam*HI-*Not* I sites of pVAX1-CpG to produce a double gene expression vector, which was named as *pVAX1-Bm-CpG (Fig. 7)*.

To further study whether the double gene Bm could express in mammalian cells. The double gene expression vector of Bm was performed cell transfection using HEK293 cells. Total RNA was prepared from transfection cells and used for the synthesis of cDNA. The cDNA was used for PCR reaction with gene-specific primers P1 and P2. As shown in the *Fig. 8*, the target band can be detected, and the pVAX1-Bm-CpG is preserved to provide nucleic acid to immune animal.

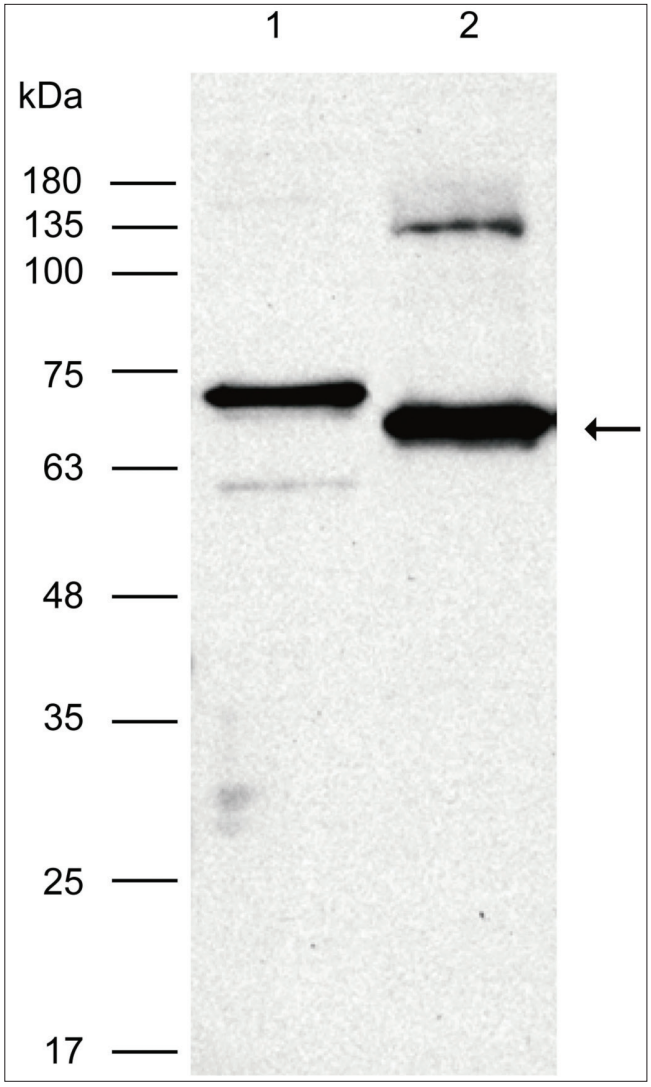

Fig 5. Bm86 and Bm91 recombinant protein SDS-PAGE Western blot detection. The *R. microplus* Bm86 protein (lane 1) and Bm91 protein (lane 2) SDS-PAGE electrophoresis detection. The arrow indicate the destination strip location. 5 μL protein was taken for Western blot, and the protein concentration of Bm86 and Bm91 were 0.98 mg/mL and 1.68 mg/mL, respectively

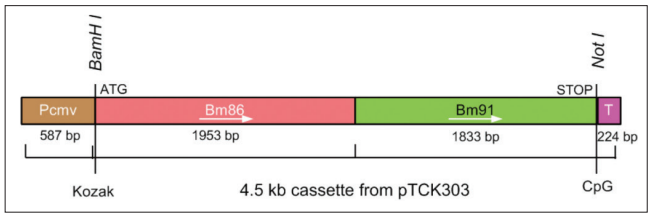

Fig 6. Construct maps of *pVAX1-Bm-CpG*. Construct maps of *pVAX1-Bm-CpG*. Arrows indicate the direction of transcription; P_{CMV}, CMV promoter; T_{BGH}, BGH terminator. The restriction enzymes used for cloning are indicated. CpG means CpG sequence and Kozak sequence GCCACC

DISCUSSION

Ticks rank second to mosquitoes, which are the pathogen carriers of human, livestock and wildlife diseases [2,40]. *Rhipicephalus (Boophilus) microplus* is the most influential

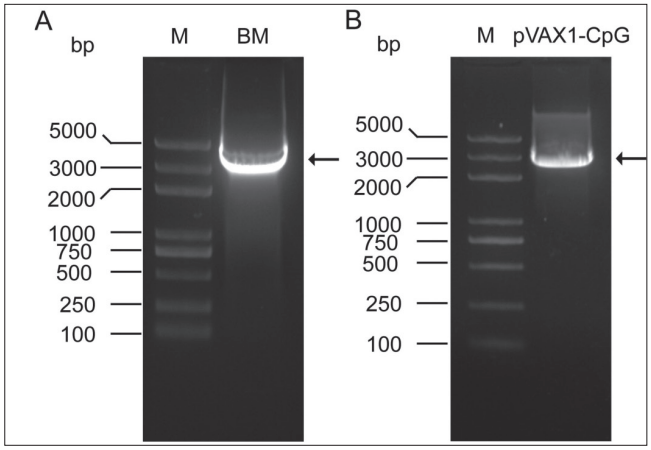

Fig 7. Bm gene and *pVAX1-CpG* vector double enzyme cutting results. The double gene Bm (3800 bp) gene double enzyme cutting use *Bam*H I and *Not* I restriction sites and *pVAX1-CpG* vector used the same double enzyme cutting to developed the double gene expression vector

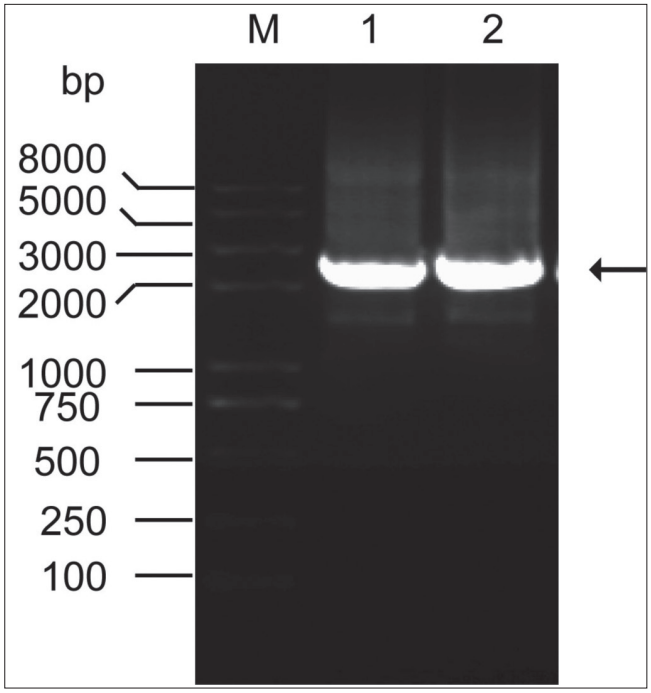

Fig 8. Expressions of *Bm* gene were identified in transfected mammalian cells. The expressions of *R. microplus Bm* gene were detected in the RNA level with reverse transcription polymerase chain reaction. lane 1 and 2 represent two repeats. The arrow indicate the destination strip location

tick species affecting cattle in the world [41,42]. It is known for its aggressiveness and rapid replacement of other species of the same subgenus [8]. *R. (B.) microplus* tick is a harmful species for animal husbandry resulting in huge economic losses to farmers from tropical to subtropical regions [43]. At present, acaricides are no longer effective enough to control ticks alone on farms. The vaccine may provide a complementary treatment in a comprehensive pest management program [17]. The glycoprotein Bm86 is located in *B. microplus* tick midgut cells, and Bm91 located in the salivary gland and B midgut of *B. microplus* [25,44].

At present, the vaccines based on bm86 are used to immunize cattle in order to induce immunoglobulin. When ticks swallow blood, these antibodies, together with other components of the immune system, such as complement, can cause the cleavage of intestinal epithelial cells, causing ticks to die or damage [22,45]. If Bm86 and Bm91 antigens used in combination, the effects of their antibodies will act on different parts of *R. (B.) microplus* ticks, and cause more serious damage, further reduce the fertility rate of ticks and the frequency of acaricide and achieve the result of control ticks and tick-borne diseases. Facts have been proved that the addition of the Bm91 antigen indeed improves the efficacy of Bm86 vaccine alone [33]. In addition, the efficacy of the vaccination with Bm86 and the amino acid sequence variations in the Bm86 protein in challenges with *R. microplus* was negatively correlated [17,46]. Therefore, using local *R. microplus* ticks to prepare multi antigen vaccines may have an ideal effect on prevention and control of local ticks.

Gene vaccine or DNA vaccine, which is encoded antigen DNA, has been evaluated as prophylactic vaccines and therapeutic treatments for the treatment of infectious diseases, allergies or cancer [28]. Studies have shown that pBMC2 DNA immunization potentially induces humoral and cellular immune responses against *B. microplus* [28]. But, using Bm86 antigen vaccines did not achieve an ideal effect on prevention and control of *B. microplus* in sheep, due to Bm86 antigen induces a protective immune response against *B. microplus,* and DNA vaccination did not result in sustained antibody production [47]. The effect of the *B. microplus* tick DNA vaccine and the double DNA vaccine of *B. microplus* tick immune bovine requires further verification. The combined use of different nucleic acid vaccines and recombinant protein vaccines immunization can enhance the humoral and cellular immune responses induced by DNA vaccine, such as raising antibody level, cell proliferation reaction, CTL activity and cytokine secretion, etc., so as to effectively improve the effect of the vaccine [48-50].

In addition, the expression vector of DNA vaccine is also important. The promoter strength of the expression vector is an important factor in determining the transcription efficiency, and the enhancer can promote the transcription ability of the promoter. PVAXl vector contains pCMV strong promoter and enhancer, which is an efficient new eukaryotic expression vector. Antigen gene expression unit and the CpG base motif adjuvant unit are two essential functional units of DNA vaccine. The CpG motif can induce the body to produce Thl immune response and increase the expression of costimulatory molecules [51]. In addition, in order to enhance the translation efficiency of eukaryotic genes, the kozak sequence (GCCACC) was increased at the front end of the antigen gene, which greatly improved the effect of DNA vaccine.

This study successfully expressed the *R. microplus* Bm86

and Bm91 protein using the prokaryotic expression system, combined with the advantages of pVAXl and CpG, successfully constructed the Bm86 and Bm91 dual gene carrier pVAX1-Bm-CpG, which provided the material for the next step of Co-immunizing animals with Bm86 and Bm91 mixed proteins and Bm86 and Bm91 dual-gene carriers, and laid the foundation for the development of the new vaccine. At present, the research progress is proceeding smoothly with the design of this study. It is expected that the double gene nucleic acid vaccine and the mixed protein vaccine have a good effect or provide the research ideas for the future development of the anti-tick vaccine.

Consent for Publication

Not applicable.

Availability of Data and Materials

All the sequences obtained in our laboratory have been uploaded to the GenBank database *(Bm86 and Bm91*: MH165269 and MH165270).

Competing Interests

The authors declare that they have no competing interests.

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Ethical Approval

Ethical treatment of animals was practiced in this study; however, the relevant document number is not available at Tarim University. Permission was obtained from the farm owners before collection of the specimens.

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Research Article

Growth Performance and Meat Quality in Tibetan Sheep Fed Diets Differing in Type of Forage

Ao REN [1,2,#] Bin LI [3,5,#] Hong-dong JIE [1] Liang CHEN [1,2] Bin ZHANG [1] Si-man AO [3]
Zhi-liang TAN [2,4] Chuan-she ZHOU [1,4] Ba-sang ZHUZHA [3] Xiao-ying CHEN [3] Sheng-zhen HOU [6]

[#] *These authors contributed equally to this study*
[1] College of Animal Science and Technology, Hunan Agricultural University, Changsha, 410128, CHINA
[2] Key Laboratory for Agri-Ecological Processes in Subtropical Region, and Hunan Research Center of Livestock & Poultry Sciences, and South Central Experimental Station of Animal Nutrition and Feed Science in Ministry of Agriculture, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, 410125, CHINA
[3] Department of Animal Science and Veterinary Medicine, Tibetan Autonomous Prefecture Academy of Agricultural and Animal Husbandry Science, Lhasa, 850009, CHINA
[4] Hunan Co-Innovation Center of Animal Production Safety, CICAPS, Changsha, 410128, CHINA
[5] State key laboratory of hulless and yak germplasm resources and genetic improvement, Lhasa, 850009, CHINA
[6] Animal Science Department of Agriculture and Animal Husbandry College, Qinghai University, Xining, 810016, CHINA

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Abstract

The Qinghai-Tibetan Plateau is a large and elevated plateau (mean elevation 4500 m) in northwestern China, where with a harsh environment for animal survival. Tibetan sheep is an important dominant livestock that inhabit this harsh environment. The objective of this trial was to preliminarily explore and compare the effects of indigenous barley straw (BS) and artificial cultivated oat hay (OH) on growth performance, visceral organ mass and meat quality of Tibetan sheep. Twenty-four Tibetan sheep with initial weights of 15.9±1.92 kg were randomly assigned to two mixed diets containing the same concentrate mixed with BS or OH, and the experiment was carried out in a completely randomized design. The weight of lungs and thymus for OH was greater (P≤0.05) than that of BS, while the weight of other viscera (heart, liver, kidney, epinephros and spleen) had no significant difference (P>0.05) between the two. The weight of the rumen, reticulum and abomasum for OH was 10.31%, 31.22% and 33.44% (P≤0.01) higher than that of BS. The length of the colon in sheep fed OH diet was longer (P≤0.01) than the sheep fed BS diet. The content of Histidine in longissimus dorsi muscle of OH was greater (P≤0.05) than that of BS, while there were no significant (P>0.05) differences between the two for the other individual amino acids (AA), umami AA, essential AA and total AA. It is concluded that the two types of roughage did not affect the growth and slaughter performance, meat quality, but OH is beneficial for the gastrointestinal development for Tibetan sheep.

Keywords: *Tibetan sheep, Growth performance, Meat quality, Forage*

Farklı Yemlerle Beslenen Tibet Koyunlarında Büyüme Performansı ve Et Kalitesi

Öz

Qinghai-Tibet Platosu Çin'in kuzeybatısında yer alan geniş ve yüksek (ortalama rakım 4500 m) bir plato olup hayvancılık için çetin çevre şartlarına sahiptir. Tibet koyunu bu çetin coğrafyada yaşayan en önemli besi hayvanıdır. Bu çalışmanın amacı, yerli arpa samanı (AS) ve yapay ekili yulaf samanının (YS) Tibet koyununda büyüme performansı, viseral organ ağırlığı ve et kalitesi üzerine etkilerinin ön değerlendirmesini yapmaktır. Başlangıç ağırlıkları 15.9±1.92 kg olan yirmi dört Tibet koyunu iki gruba ayrılarak AS veya YS içeren iki karışık diyetle tamamen rastgele dizayn düzeneğinde beslendi. YS ile beslenenlerde akciğerlerin ve timusun ağırlığı AS ile beslenenlere oranla daha fazlayken (P≤0.05) diğer viseral organlarda (kalp, karaciğer, böbrek, adren ve dalak) iki besleme arasında anlamlı fark gözlemlenmedi (P>0.05). YS ile beslenenlerde rumen, retikulum ve abomazum ağırlıkları AS ile beslenenlere oranla sırasıyla %10.31, %31.22 ve %33.44 daha fazlaydı (P≤0.01). YS ile beslenen koyunlarda kolon, AS ile beslenenlere oranla daha uzundu (P≤0.01). YS ile beslenen koyunların longissimus dorsi kasında histidin miktarı AS ile beslenenlere oranla daha fazla iken (P≤0.05) diğer bireysel amino asitler (AA), umami AA, esansiyel AA ve total AA her iki uygulama arasında fark göstermedi (P>0.05). Tibet koyununda yem tipinin büyüme ve kesim performansı ile et kalitesini etkilemediği fakat YS uygulamanın gastrointestinal gelişim için daha faydalı olduğu sonucuna varıldı.

Anahtar sözcükler: *Tibet koyunu, Büyüme performansı, Et kalitesi, Yem*

İletişim (Correspondence)
+86-18711326324 (A. Ren), +86-13989012147 (B. Li), +86-0731-84615236 (C. Zhou), Fax: +86-0731-84612685
18711326324@163.com (A. Ren), xukesuolibin@163.com (B. Li), zcs@isa.ac.cn (C. Zhou)

INTRODUCTION

The Qinghai-Tibetan Plateau is known as the "third pole" of the world because it is characterized by high altitude, severe cold, low oxygen level, strong UV radiation, a short growing season, and deficiencies in feed supply [1,2]. Tibetan sheep is the major indigenous ruminant that inhabit this harsh environment living at an altitude of 3.000 to 5.000 m, it is the main resource of meat and milk in those areas, and the major source of income for most nomadic and seminomadic people in these regions. The traditional feeding system for Tibetan sheep is free range husbandry, however the harsh environment cause shortage of feedstuffs and nutritional impairment [3]. In order to improve the production efficiency for Tibetan sheep, it is particularly important to conduct scientific supplementary feeding scheme using local resources.

Highland barley is the main crop in Tibet, and the total cultivated area was about 117.900 ha that makes up more than 69.7% of the total area of grain in Tibet. As an important source of roughage for local animals, highland barley straw (BS) can offset the shortage of feedstuffs to some extent, especially during the cold season [4]. Oat is also widely planted in Tibet, although it is not as extensive as highland barley, it is a cereal grain of the family *Gramineae (Poaceae)*. Along with green mass, oat hay (OH) is a valuable feed for agricultural animals [5].

Some studies about OH and BS feed on sheep have been published. The dry matter (DM) and crude protein (CP) intakes were significantly increased with and the average daily gain was higher when OH was replaced by rosemary distillation residues in lamb diets, but carcass composition and gut weights were similar among groups [6]. When feeding with OH, DM, organic matter (OM) and neutral detergent fiber (NDF) digestibilities were higher in Tibetan than fine-wool sheep [7]. Replacement of OH by BS promotes PUFA deposition in the muscle and long-chain FAs in the adipose fat of Tibetan sheep [8]. It is possible to fatten light lambs on a TMR pellet including ground BS by increasing average daily gain and reducing the fattening period, without any negative impact on carcass and meat characteristics [9]. Previously study had shown the difference of nutritional composition between OH and BS, the OM and nitrogen content of OH seemed to higher than that of BS, and the acid detergent fiber content of BS was higher than OH [2]. But there few research comparing the OH and BS on growth performance and meat quality of Tibetan sheep, and how to make full use of the resource in local is meaningful for improving production efficiency. The goal of this study was to evaluate the effect of forage type on growth performance and meat quality of Tibetan sheep.

MATERIAL and METHODS

Animal and Management: The experiment was conducted according to the animal care and use guidelines of the Animal Care Committee, Institute of Animal Husbandry and Veterinary, Tibet Academy of Agriculture and Animal Husbandry. The experimental was conducted at the research farm of Institute of Animal Husbandry and Veterinary, Tibet Academy of Agriculture and Animal Husbandry, Lhasa City, China. The formal experiment commenced on 25 June and ended on 25 July 2018. The average daily temperatures ranged from a minimum of 11°C to a maximum of 26°C. Twenty-four Tibetan sheep with an average body weight (BW) of 15.9±1.92 kg were used in this experiment, all the sheep have the same age and equal number of ram and ewe. The sheep were randomly divided into six pens (four sheep/pen), the animal represented the experimental unit. All the sheep were provided with free access to fresh water.

Experimental Diets and Design: The experiment was carried out in a completely randomized design. Sheep were randomly and equally assigned to two experimental treatments, the sources of roughage for the two dietary treatments consisted of BS or OH, BS (Zangqing 311, a popular local barley breed) was assigned to three pens and OH (Tianyan 1, a popular oat breed large-scale cultivated in Tibet) was assigned to the other three pens. Sheep in both treatments received the same concentrate, and the ingredients of the concentrate were (DM basis): 45 g/kg soybean meal, 470 g/kg corn, 424 g/kg wheat bran, 7 g/kg calcium carbonate, 9 g/kg palm oil, 9 g/kg sodium chloride, 36 g/kg minerals, and vitamins premix. The ratio of concentrate to roughage was 50:50. Diets were prepared to provide 1.3 times the level required for the maintenance of metabolizable energy (ME) according to the Zhang and Zhang [10]. Sheep were fed two equal meals at 09:00 and 18:00 h. Concentrate and roughage were offered at the same time and the refusals were collected and weighed daily for 7 d of the formal experiment to measure the voluntary feed intake, and feed intake was measured by pen, and water was available *ad libitum*. The Chemical composition of the diets and DM intake were shown in *Table 1*.

Sample Collection and Handling: Sheep weight and feed intake were measured weekly during the formal experimental periods. The diets and refusals for both groups were equally sampled, oven dried at 65°C, air equilibrated, ground to pass a 1-mm sieve, and stored pending laboratory analysis. At the end of the experiment (after 20 days of the formal experiment), all the sheep were selected for slaughter after being fasted overnight. All sheep were electrically stunned and slaughtered at the same day, by exsanguination under commercial procedures, according to the animal ethics committee of the Institute of Subtropical Agriculture, the Chinese Academy of Sciences. The sheep were hung to remove the skin, head (at the occipito-atlantal joint), forefeet (at the carpal-metacarpal joint), and hind feet (at the tarsal-metatarsal joint). The viscera, such as heart, liver, kidney, epinephros, lungs, spleen, and thymus, as well as

the gastrointestinal tissues (forestomach, such as rumen, reticulum, omasum and abomasums; the small intestine, such as duodenum, jejunum, and ileum; the large intestine, such as cecum, colon, and rectum) were removed and the weight was recorded, respectively. Additionally gastrointestinal tissues length was recorded. Afterward, carcasses were weighed and subsequently chilled under commercial conditions at 4ºC for 12 h in total darkness. Thereafter, the left side of carcass was used for meat quality variable measurements. Carcass was deboned at 24 h postmortem. About 200 g of left longissimus dorsi muscle (LM) were sampled. Approximately 50 g fresh LM was weighed and placed in a Whirl-Pak bag, suspended in a 4°C for the determination of drip loss. After that, 100 g subsample of LM and GM were taken and stored at 4ºC for 24 h for the determination of cooking loss, the other subsamples was immediately stored at -20ºC for subsequently chemical composition measurements.

Chemical and Physical Analysis: The cooking loss of muscle at 24 h postmortem was measured according to the methods reported by Ramírez et al.[11]. Briefly, the muscle samples were weighed (F), vacuum-packed in plastic bag, and cooked at 80ºC for 1 h by immersion in a water bath. After that, cooked samples were cooled under running water for 30 min, removed from the bags, blotted, and weighed (C). Cooking loss was calculated as (F-C) × 100/F [12]. Dropping loss was estimated without freezing on the day of slaughter according to the following procedures [13]. Approximately 50 g fresh LM was weighed (W1) and placed in a Whirl-Pak bag, suspended in a 4°C cooler for 24 h, reweighed (W2), and dropping loss was calculated. To measure the loin eye area of the longissimus dorsi muscle, each carcass was bisected and a transverse cut was made in the left half of the carcass at the level of the 12th and 13th ribs, an outline of the cranial section was made using a transparency sheet and a pen, which was then used to calculate the area of the section [14].

The frozen meat samples were thawed overnight (approximately 14 h) in a chill at 4ºC. DM contents of the thawed samples were determined by drying at 105ºC in an oven (DHG-9023A, Jing Hong, shanghai) for 48 h to a constant weight, then the samples were ground through a 0.5 mm with a laboratory mill (DF-2, Changsha Instrument Factory, Changsha city, Hunan province, China). Total nitrogen (N) was analyzed according to the methods of AOAC [15]. Fat content was determined according to the method described by Hara and Radin [16]. ADF content and NDF content was determined according to the methods of AOAC [17] and Van Soest et al.[18].

The amino acid (AA) contents were determined according to Mason et al.[19]. To isolate the AA fraction (AAF), 0.5 g of dried tissue was hydrolyzed with 15 mL 6 N HCl under N_2 for 20 h. The hydrolysate was filtered through glass fibers to eliminate particulate matter. Filtered hydrolysate was diluted to 50 mL with pure water. AAs were isolated from 10 mL of the diluted hydrolysate using cation exchange chromatography according to the methods of Nissen and Haymond [20]. The fraction containing AAs was dried under a stream of N_2 at 60ºC.

Calculations: The calculations were calculated as the formula below:

Yields (%) of the hot body(Y):

Y = carcass weight/body weight x 100

The weight of the viscera (the heart, liver, kidney, epinephros, lungs, spleen, thymus, stomach, and intestines) were summed to determine the yield of the non-carcass components relative to body weight at slaughter [14].

Dry matter intake (DMI)

DMI = offered weight (DM) - refusals weight (DM)

Cooking loss:

Cooking loss (%) = (F-C) × 100/F

Crude protein (CP):

CP = total nitrogen x 6.25

Dropping loss:

Dropping loss = (W1-W2)/W1 × 100

Statistical Analyses: Statistical analyses of data were evaluated through a one-way ANOVA procedure, and animal were used as experimental unit. Statistical significance was set at $P<0.05$ and tendencies at $0.05\leq P\leq 0.10$. All statistical analyses were conducted with JMPR 12.1.0. (SAS Institute Inc.).

RESULTS

All the sheep were healthy and consumed their feed allowance throughout the experiment. The average DMI was 582 and 609 g/d for sheep fed BS and OH, respectively *(Table 1)*.

The ADG and slaughter performance (initial BW, final BW and dressing percentage) did not affected between the two treatments *(Table 2)*. The weight of lungs for OH treatment was greater ($P\leq 0.05$) than that of BS treatment, while the weight of other viscera (heart, liver, kidney, epinephros and spleen) had no significant differences ($P>0.05$) between the two treatments *(Table 2)*.

The weight and length of the gastrointestinal tissues in sheep fed BS and OH diets were shown in *Table 3*. The weight of the rumen, reticulum and abomasum for OH treatment was 10.31%, 31.22% and 33.44% ($P\leq 0.01$) higher than that of BS treatment, while there was no significant ($P>0.05$) differences in the weight of the abomasum, the small intestine and the large intestine tissue between BS and OH treatments. The length of the colon in sheep fed

Table 1. *Chemical composition of the diets and dry matter intake of Tibetan sheep*

Items[1]	Forage[2]		Diet[3]		SEM[4]	P
	BS	OH	BS	OH		
DM (%)	95.5	94.9	96.7	95.5	-	-
OM (g/kg•DM)	923	952	918	932	-	-
CP (g/kg•DM)	14.2	43.4	59.4	73.9	-	-
NDF (g/kg•DM)	709	597	539	483	-	-
ADF (g/kg•DM)	458	387	300	264	-	-
Starch (g/kg•DM)	61	75	179	186	-	-
EE (g/kg•DM)	61	62	65.7	69.4	-	-
NFC (g/kg•DM)	139	250	254	306	-	-
ME[5] (MJ/kg•DM)	1.11	1.41	7.13	7.76	-	-
DMI[6] (g/d)	-	-	582	609	13.3	0.32

[1] DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract; NFC = non-fibrous carbohydrates; calculated by the equation of OM-CP-EE-CF; [2] BS = barley straw; OH = oat hey; [3] Forage plus concentrate diet (1:1), the concentrate contained (g/kg): soybean meal (45), wheat bran (424), calcium carbonate (7), palm oil (9), sodium chloride (9), and premix (36); [4] Pooled standard error of means; [5] Metabolizable energy (ME) was estimated according to Zhang and Zhang (1998); [6] Dry matter intake

Table 2. *Effects of dietary fiber sources on the daily gain, dressing percentage and viscera weight in sheep*

Items[1]		Treatments[3]		SEM[4]	P
		BS	OH		
Slaughter performance	Initial BW[1] (kg)	15.83	16.42	0.51	0.43
	Final BW (kg)	20.69	20.78	0.50	0.90
	Carcass weight (kg)	7.20	7.38	0.25	0.62
	Dressing percentage (%)	35.49	34.79	0.69	0.48
	ADG[2] ($g \cdot d^{-1}$)	0.17	0.14	0.01	0.28
Viscera weight (g)	Heart (g)	85.13	86.02	2.65	0.82
	Liver (g)	321.9	339.9	10.46	0.24
	Kindey (g)	24.35	26.54	0.88	0.09
	Epinephros (g)	0.98	1.11	0.05	0.11
	Lungs (g)	198.6^{b}	224.7^{a}	6.08	0.006
	Spleen (g)	40.74	43.48	2.23	0.39

[1] BW = Body Weight; [2] ADG = average daily gain; [3] BS = barley straw, OH = oat hay; [4] Pooled standard error of means; [a,b] Mean values within a row with unlike superscript letters were significantly different (P<0.05)

OH diet was longer (P≤0.01) than the sheep fed BS diet, whereas the length of the small intestine tissues, cecum and rectum had no significant (P>0.05) difference between the two treatments *(Table 3)*.

As shown in *Table 4*, no differences were found in dropping and cooking loss, loin eye area and chemical composition of LM (P>0.05). For AA composition, the content of His in longissimus dorsi muscle of OH treatment was greater (P≤0.05) than that of BS treatment, while there were no significant (P>0.05) differences between the two treatments for the other individual AA, umami AA, essential AA and total AA *(Table 4)*.

DISCUSSION

The average daily gain of sheep was not influenced by the type of forage offered *(Table 2)*, and it was around 150 g/d for both diets. This value was similar to those reported by Costa et al.[21] and Lima et al.[22] for the same genotype consuming diets, because barley and oat both belonged to C3 photosynthetic pathway plants [23], and both forages presented a similar anatomical arrangement. Replacement with an alternative food did not affect the weight gain of the sheep evaluated by Atti and Mahouachi [24]. We obtained dressing percentage similar to those reported by Ekiz et al.[25], Cloete et al.[26], and Andrade et al.[27] for

Table 3. *Effects of dietary fiber sources on weight and length of gastrointestinal tissues in sheep*

Items		Treatments[1]		SEM[2]	P
		BS	OH		
Weight (g)	Rumen	403.5[b]	445.1[a]	10.24	0.009
	Reticulum	74.21[b]	97.38[a]	5.57	0.008
	Omasum	61.86	72.77	5.42	0.17
	Abomasum	76.59[b]	102.2[a]	4.60	0.0007
	Duodenum	11.57	13.64	0.80	0.08
	Jejunum	272.7	290.7	13.51	0.36
	Ileum	144.5	167.3	10.51	0.14
	Cecum	89.89	97.91	3.86	0.16
	Colon	144.8	154.4	6.86	0.33
	Rectum	36.23	35.56	1.79	0.80
Length	Duodenum (cm)	24.08	27.79	1.28	0.06
	Jejunum (m)	13.25	13.50	0.48	0.72
	Ileum (m)	6.34	7.16	0.37	0.13
	Cecum (cm)	78.17	87.08	3.17	0.06
	Colon (m)	3.94[b]	4.62[a]	0.15	0.004
	Rectum (cm)	31.0	31.77	2.05	0.79

[1] BS = barley straw, OH = oat hay; [2] Pooled standard error of means; [a,b] Mean values within a row with unlike superscript letters were significantly different (P<0.05)

indigenous breeds. This indicates that the native breeds of sheep have potential for meat production.

The variations in nutrition level, diet quality and feed intake induced variation in the mass of splanchnic tissues [28]. Although splanchnic tissues only represent 6-10% of body weight, they can account for up to 50% of total energy expenditure [29]. As a consequence, variations in the weight of splanchnic tissues contribute to the adaptations in maintenance requirements of the animal according to its feeding level. The changes in visceral organ mass may in response to dietary CP and ME levels on sheep [30,31] and beef cattle [32]. In the present study, we did not find any changes in visceral organ mass such as heart, liver, kidney epinephros, spleen and thymus, the lack of difference may be related to the insufficient experimental period. However, we found that the lungs mass decreased for sheep fed forage from BS in the present study, the lower organ weight of lung indicated that the lung was more powerful to resist inflammation especially in the special plateau climate environment.

The gastrointestinal tract (GIT) represents only 5 to 7% of body mass, yet consumes roughly 15 to 20% of whole-body oxygen [33]. Therefore, development of GIT is very sensitive to dietary nutrition. Differences have been reported in visceral organ mass and in individual mass of metabolically important organs due to DMI and physical form and energy density of the diet [32,34]. Forestomach mass appears to respond to physical form of the diet and fiber content [32,34], and the intestinal mass appears to be dependent on both [32]. Previous works in lamb [35,36] and steers [32,34] has shown that reticulo-ruminal mass is responsive to physical form of the diet. In the current study, we found that the weight of the rumen, reticulum and abomasums for sheep fed forage from BS decreased than that fed OH, it resulted from the difference of physical form of forage between treatments, especially for the difference of physical form between BS and OH. Even though dietary ME content was not different between treatments, it seems to be similar to previous results, an increased rate of passage and greater ruminal outflow rates for native forage (BS) have contributed to the response [37]. In ruminants, digestive tract tissues are affected by changes in ME intake [30], protein intake [35,38], and nutrient restriction [32,39], as well as dietary density [30,32]. The length of small intestine, cecum and rectum did not affect for both treatments, it might result from the similar dietary composition and actual intake of important ingredients such as protein and ME for both treatments. The greater length of colon when feeding OH compared with BS might be due to the greater supply of energy to the colonic epithelium when feeding OH as demonstrated by the greater absorption of short-chain fatty acids (SCFA) to the portal vein [40].

Previous research reported that there was no effect of crambe meal level on chemical composition and quality of the longissimus dorsi (LD) muscle and cooking loss [41]. Fasae et al.[42] also reported that proximate composition of the meat from the LD muscle indicated that the DM, CP, fat and ash contents were not influenced by the dietary treatment (varying levels of maize and cassava hay).

Table 4. *Effects of dietary fiber sources on meat quality, chemical composition and amino acids contents of the longissimus dorsi muscle in sheep (DM basis)*

Items[1]		Treatments[2]		SEM[3]	P
		BS	OH		
Meat quality	Dropping loss (%)	5.81	4.21	0.83	0.17
	Cooking loss (%)	32.56	28.33	1.77	0.19
	Loin eye area (cm^2)	7.32	5.53	0.52	0.06
Chemical composition	DM (%)	27.45	28.16	1.96	0.26
	CP (%)	21.03	21.88	3.52	0.14
	CF (%)	4.82	4.87	0.65	0.27
AA composition (mg/g)	Asp	20.24	21.09	0.80	0.46
	Thr	11.04	11.50	0.46	0.48
	Ser	9.26	9.59	0.39	0.55
	Glu	44.59	45.94	1.76	0.59
	Gly	8.41	9.04	0.29	0.14
	Ala	12.63	13.37	0.37	0.17
	Cys	1.95	2.15	0.09	0.14
	Val	10.14	10.45	0.26	0.39
	Met	4.81	4.29	0.48	0.45
	Ile	10.05	10.66	0.36	0.23
	Leu	18.12	19.14	0.64	0.27
	Tyr	8.61	9.03	0.34	0.39
	Phe	8.12	8.52	0.27	0.31
	Lys	20.07	21.11	0.82	0.38
	His	6.73^b	7.56^a	0.28	0.04
	Arg	14.29	14.94	0.55	0.41
	Pro	8.12	8.89	0.43	0.22
	Umami AA (%)	36.87	36.63	0.22	0.45
	Essential AA (%)	37.86	37.75	0.15	0.61
	Total AA	217.0	227.5	7.86	0.35

[1] DM = dry matter, CP = crude protein, CF = crude fat; [2] BS = barley straw, OH = oat hay; [3] Pooled standard error of means; [a,b] Mean values within a row with unlike superscript letters were significantly (P<0.05)

Bonanno and Di Miceli et al.[43] determined the effects of sulla forage on meat quality of lambs compared with annual ryegrass, reported that the physical, chemical, and sensory properties of the lamb meat were not influenced. It was similar to the present study, that the dropping loss, cooking loss, loin eye area and the chemical composition (DM, CP and CF) were not affected by the dietary treatment. However, Byong et al.[44] reported that CP of the LD muscle was significantly lower when feeding with mulberry silage supplementation, and as reported by Abdullah et al.[45] that cooking loss, as one measure of water-binding capacity of meat, had a strong genetic determination and was also influenced by handing of the animal and the meat.

There were a large amount of glutamic acid (Glu), lysine (Lys), aspartic acid (Asp), leucine (Leu) and arginine (Arg) founded in the LD muscle of Tibetan sheep, the AA content the LD muscle not only could reflect the character of Tibetan sheep mutton, but also allow for a selection of improving meat quality in the breed analyzed with more attractive sensory attributes. The umani AA, essential AA, total AA and individual AA except His was not different between treatments in the present study. Histidine (His) has been identified as a limiting AA for growing cattle [46], and previous studies showed that different protein concentration in the diet might have affected the histidase activity in cattle and rats [47,48]. The CP concentration in OH was numerically 24.42% higher than that of BS, and CP intake was numerically 30.18% higher than BS, which may improve histidase activity and histidine content in LD muscle.

Our results demonstrate that the growth performance, slaughter performance and meat quality was not affected when fed on indigenous BS compared to artificial cultivated OH for Tibetan sheep, but OH was beneficial for the gastro-intestinal development of Tibetan sheep.

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Research Article

Determining the Change in Retail Prices of Veal in Turkey by GARCH Method Between 2014-2017 [1]

Mehmet Saltuk ARIKAN [1,a] Mustafa Bahadır ÇEVRİMLİ [2,b]
Ahmet Cumhur AKIN [3,c] Mustafa Agah TEKİNDAL [4,d]

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[1] Department of Animal Health Economics and Management, Fırat University Faculty of Veterinary Medicine, TR-23119 Elazıg - TURKEY
[2] Department of Livestock Economics and Management, Selcuk University Faculty of Veterinary Medicine, TR-42003 Konya - TURKEY
[3] Department of Animal Health Economics and Management, Mehmet Akif Ersoy University Faculty of Veterinary Medicine, TR-15030 Burdur - TURKEY
[4] Department of Biostatistic, Selcuk University Faculty of Veterinary Medicine, TR-42003 Konya - TURKEY
[a] ORCID: 0000-0003-4862-1706; [b] ORCID: 0000-0001-5888-242X; [c] ORCID: 0000-0003-3732-0529; [d] ORCID: 0000-0002-4060-7048

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Abstract

In Turkey, the demand for veal in the red meat market is steadily increasing. In the formation of this demand and price; additional factors such as consumer preferences, government interventions in the red meat market, import decisions, also the implemented policies and subsidies are effective besides the main factors. However, the fact that such practices do not affect the veal prices in the market makes it impossible to ensure price stability and a sustainable market. The analysis of the change in retail prices is important for both producers and consumers so that the validity of this view can be tested. The aim of this study was to analyze the retail prices of minced meat and the veal cubes from the first week of January 2014 to the last week of December 2017 via using the volatility estimation modeling method in the context of the effectiveness of the policies and market interventions applied in the red meat sector implemented in Turkey. Non-stationary series were made stationary by differencing. By developing a Generalised Autoregressive Conditional Heteroskedasticity (GARCH 1,1) model using the series subject to a unit root test, it was found to be the optimal model that best explains the fluctuation of the prices. The results of the estimation show that the retail prices of veal cubes and minced meats fluctuated conspicuously in the said period and that the implemented policies and the market interventions were not adequate to eliminate the instability and uncertainty of the prices.

Keywords: *GARCH, Price volatility, Time series, Turkey, Veal*

Türkiye'de Perakende Dana Eti Fiyatlarındaki Değişimin Garch Yöntemiyle Belirlenmesi, 2014-2017

Öz

Türkiye'de kırmızı et tüketiminde büyükbaş hayvan etine olan talep her geçen gün artmaktadır. Bu talebin ve fiyatın oluşmasında tüketici tercihleri, kırmızı et piyasasına yapılan devlet müdahaleleri, alınan ithalat kararları, uygulanan politikalar ve desteklemeler de etkili olmaktadır. Ancak bu uygulamaların etkisinin reel piyasada dana eti fiyatlarına olumlu yansımaması sebebiyle, fiyat istikrarının sağlanması ve sürdürülebilir yapıya kavuşması imkânsızlaşmaktadır. Bu çalışmada; Ocak 2014 ilk hafta ile Aralık 2017 son haftası arasındaki döneme ait verilere göre Türkiye'de kırmızı et sektöründe uygulanan politikalar ve devlet müdahaleleri kapsamında perakende dana kıyma ve kuşbaşı ortalama fiyatları volatilite tahmin modelleme yöntemi ile analiz edilmesi amaçlanmıştır. Durağan olmayan serilerin farkları alınarak durağan hale getirilmiştir. Birim kök testi uygulanan serilere daha sonra Genelleştirilmiş Otoregresif Koşullu Değişen Varyans (GARCH 1,1) modeli kurularak, fiyatlardaki dalgalanmayı açıklayan en uygun model olduğu tespit edilmiştir. Tahmin sonuçları incelenen dönemde perakende dana kuşbaşı ve kıyma fiyatlarında belirgin dalgalanmaların yaşandığını göstermekle birlikte bu dönemde uygulanan politikaların ve piyasaya yapılan müdahalelerin fiyatlardaki istikrarsızlık ve belirsizliği gidermede yeterli olmadığını açıklamaktadır.

Anahtar sözcükler: *GARCH, Fiyat oynaklığı, Zaman serisi, Türkiye, Dana eti*

İletişim (Correspondence)
+90 424 237 00 00-3995 Mobile: +90 505 3254651
msarikan@firat.edu.tr

INTRODUCTION

In Turkey, the demand for red meat is met by sheep, goat, water buffalo and cattle producers, with beef constituting 87.67% of the total red meat production [1]. However, retail beef prices have substantially increased in recent years.

While retail veal prices rose by 130% between 2005-2013, retail mutton prices increased by 154% and the feed prices by 115%. The increases in the prices of red meat were higher than the increases in the CPI (Consumer Price Index), which was 118% in the same period [2]. This drastic increase in the retail prices resulted in approximately 30% decrease in the per capita consumption of red meat [3]. The increase in prices continued in the period from 2014 to 2017 as well, and retail prices of veal rose by 19.54% [4]. The increasing prices of red meat have been a subject of debate both in the media and among the policy-makers in Turkey [2].

The increasing prices both mean a higher level of uncertainty regarding the prices in the next years and poses a significant price risk, which adversely affects producer welfare where there are without risk protection mechanisms in place [5]. The increasing demand for red meat and global pressures on climate change have pushed up wholesale and retail prices of meat products in many parts of the world [6]. Abrupt changes in retail prices usually affect the food security for a part of the population, resulting in changes to the dietary habits [7,8].

Price volatility indicates the range in which the prices may vary in the future [9]. Knowledge of the degree of price volatility is very important in the sense that it would allow policy-makers to formulate appropriate strategies to protect against risks. In primary commodity markets such as the red meat market, production decisions are based on expected prices. When such expectations are not met, producers face the risk of decreasing revenues [10].

Price fluctuations affect all actors in the food supply chain. Volatility decreases the inputs of production and investments, causing a risk for producers who can react [11-13]. Unexpected price increases for consumers who spend a large portion of their income on food have a negative impact on sustainable food consumption [14].

It's a common fact that the volatilities in meat prices can be successfully modelled with the ARCH (Autoregressive Conditional Heteroscedasticity)/GARCH (Generalized Autoregressive Conditional Heteroscedasticity) time series models. GARCH is a model that can be used to analyse both mean and variance effects of policy and market structure changes, and in a changing environment such as the livestock market there is a need for such a model that combines the desirable aspects of dynamics, static equilibrium and price volatility [15,16]. It has been successfully used to analyse and explain the price changes of red meat in various countries [10,15,17,18].

Changes in prices of red meat products are a significant economic risk that affects the level of production. Thus, the research on the changes in retail prices of meat in Turkey is important to understand how to equilibrate the market.

This study is intended to analyse the average retail prices of minced meats and veal cubes under the government policies and interventions in the red meat sector of Turkey between 2014 and 2017 using the volatility estimation modelling method. The main purpose of this study is contributing the literature on the effectiveness of the policies and market interventions applied in the red meat sector. In the long run, this study also aims to provide significant information to the decision and policy makers about the econometric method that they can need.

MATERIAL and METHODS

Data Set

In order to explore the changes in meat prices, the weekly retail prices of minced and veal cubes in Turkey from the first week of 2014 to the last week of 2017 were used as the data for the analysis. The short-term asymmetric effect was determined by considering the time interval to measure the response of price changes. The data sets of the minced meats and veal cubes prices used in the analysis were obtained from the weekly bulletin of the Meat and Milk Board [4].

Analysis Method

The analyses conducted using ARIMA model and ARCH/GARCH models are intended to measure the index uncertainty. In the present study, due to the absence of future predictions, volatility of the price change series has been measured over the current asymmetric effect GARCH model instead of hybrid models. In order to measure the index uncertainty, the ARCH model suggested by Engle [19] for the first time and the GARCH model, which had been developed by Bollerslev [20] were used in the study.

The functioning of the ARCH model can be addressed as follows [21]:

Let's assume a regression model with k explanatory variables.

$$Y_t = \beta_1 + \beta_2 X_{2t} + ...+ \beta_k X_{kt} + \varepsilon_t \quad [1]$$

In equation (1), under the assumption that conditional information can be obtained at time (t-1), the error term ε_t, is normally distributed with zero means and variance $(\alpha_0 + \alpha_1 + \varepsilon^2_{t-1})$, which can be expressed as $\varepsilon_t \sim N[0,(\alpha_0 + \alpha_1 + \varepsilon^2_{t-1})]$. While the error term with zero mean is one of the assumptions of the method of least squares, the addressing of the error term's variance at time t as a function of the square of the error term at t-1 was introduced by the ARCH model. Additionally, the expression of the error term's variance as $(\alpha_0 + \alpha_1 + \varepsilon^2_{t-1})$ is called the ARCH(1) process.

Since relatively long lags are used and a fixed lag structure is suggested in applying the ARCH model, some constraints are imposed on the parameters in the conditional variance equation. In an attempt to avoid problems with negative variance parameter estimates, Bollerslev [20] extended the ARCH model and developed a new one that allowed for both more past information and a more flexible lag structure. As the said model was developed not as an alternative to the ARCH model, but as a model intended to improve it, it is called generalised ARCH, or shortly GARCH [22].

Using the conditional variance equation, standard GARCH(p, q) process can be expressed as follows:

$$\sigma^2_t = \omega + \alpha\, \varepsilon^2_{t-1} + \beta\, \sigma^2_{t-1} \qquad [2]$$

The equation (2) indicating the conditional variance is a function of the mean (ω), ARCH term (ε^2_{t-1}) and GARCH term (σ^2_{t-1}). Therefore, p and q in GARCH(p, q) denote the ARCH term and GARCH term, respectively. In the ARCH(q) process, conditional variance is a function of only the past sample variances, whereas the GARCH(p, q) process allows lagged conditional variances to enter as well.

When working with time series, one should always pay attention to the stationarity of the data. Time series data of economic and financial variables usually involve trend and seasonality, which may violate the principle of stationary time series [23]. A stationary time series can be defined as mean and variance are independent over time. Where time series are not stationary, the estimated econometric models may produce misleading results. Thus, in the econometric analyses conducted using time series, unit root test (stationarity test) is performed on the time series. Therefore, Augmented Dickey-Fuller (ADF) and Phillips-Perron (PP) tests were conducted to test whether the time series had a unit root (stationarity). Results of the unit root test are given in *Fig. 1* and *Fig. 2* as well as in *Table 1* and *Table 2*.

Fig. 1 and *Fig. 2* present the change in the prices of veal cubes and minced meats over time. It is clear that the variables do not follow a stationary course during the periods under consideration. The veal cubes and minced meats series were rendered stationary after the ADF and PP tests.

RESULTS

Decreases and increases in the retail prices of minced meats and veal cubes indicate a significant level of price fluctuation. Following the increases in October 2014 and May 2015, excessive increases are observed in June 2017 *(Fig. 1, Fig. 2)*.

The series used in the model was compiled using the weekly data from January 2014/1 to December 2017/52 and the ARCH and GARCH methods. Firstly, the non-stationary series was made stationary by taking its logarithm and first difference *(Fig. 1, Fig. 2)*. Secondly, it was found that inflation followed the AR(1) and MA(1) process by looking at the correlogram of ACF and PACF *(Table 3)*. The modelling with AR(1) and MA(1) showed that both were significant. AIC and BIC values of the results obtained by running AR(1) and MA(1) individually were compared. As AIC and BIC values of AR(1) were lower than those of MA(1), AR(1) model was selected *(Table 4, Table 5)*. Finally, the estimated ARCH/GARCH model was solved using the maximum likelihood (ML) technique, as shown in *Table 4* and *Table 5*, and it was found that the model had the effect of GARCH (1, 1) and no effect of a higher degree of GARCH. The presence of the GARCH(1, 1) effect in the index series indicates that the GARCH variance series obtained as a result of the solution can be used as a measure of uncertainty in the veal cubes and minced meats series from January 2014/1 to December 2017/52.

The first difference of the data was taken to make the veal cubes series in *Fig. 1* stationary. The series became stationary after taking its first difference.

The first difference of the data was taken to make the minced meats series in *Fig. 2* stationary. The series became stationary after taking its first difference.

ADF and PP unit root tests indicate that the veal cubes series from January 2014/1 to December 2017/52 became stationary after taking its first difference.

ADF and PP test values of the veal cubes series from January 2014/1 to December 2017/52 after taking its first difference are significant at a significance level of 1%, 5%

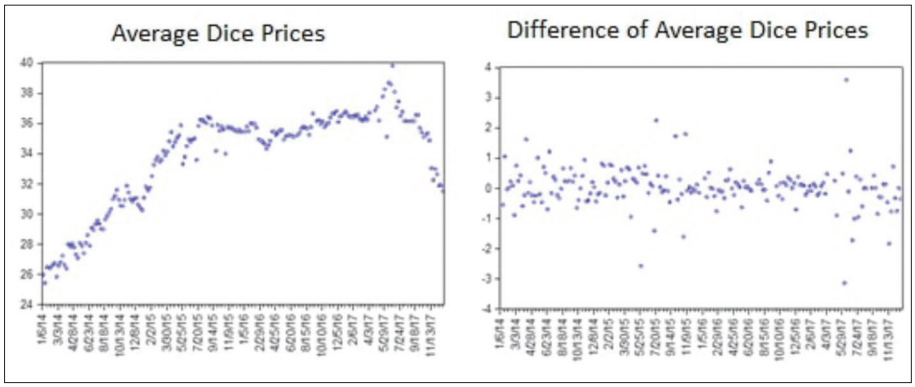

Fig 1. Testing of stationarity of dice series from January 2014/1 to December 2017/52 using ADF test (Avarage exchange rate for 2017: 1 USD = 3.65 Turkish Lira)

Fig 2. Testing of stationarity of mince series from January 2014/1 to December 2017/52 using ADF test (Avarage exchange rate for 2017: 1 USD = 3.65 Turkish Lira)

Table 1. Unit root tests coefficients for the dice series

Variable	Level Value	First Difference	Level Value	First Difference
Veal cubes	ADF		PP	
	-12.01921 **(12)**	-3.467851 **(12)**	-3.471454 **(12)**	-2.879494 **(12)**

Table 2. *Unit root tests coefficients for the mince series*

Variable	Level Value	First Difference	Level Value	First Difference
Minced meats	ADF		PP	
	18.09752 **(12)**	-3.466176 **(12)**	-0.145169 **(12)**	-3.470934 **(12)**

and 10%, respectively. The values in the parentheses are the optimal lag lengths.

ADF and PP unit root tests indicate that the mince series from January 2014/1 to December 2017/52 became stationary after taking its first difference.

ADF and PP test values of the mince series from January 2014/1 to December 2017/52 after taking its first difference are significant at a significance level of 1%, 5% and 10%, respectively. The values in the parentheses are the optimal lag lengths.

In this framework, the model developed below for the mince series from January 2014/1 to December 2017/52 is significant.

$$\sigma_t^2 = 0.087583 + 0.154908\sigma_{t-1}^2 - 0.647365\varepsilon_{t-1}^2 \quad [3]$$

The model indicates that the mince price is affected by the price in the previous week, with 1 unit of increase in the mince price being followed by a 0.15 unit of increase in the mince price in the next week.

$$\sigma_t^2 = 0.140914 + 0.787804\sigma_{t-1}^2 - 0.133606\varepsilon_{t-1}^2 \quad [4]$$

The model indicates that the veal cubes price is affected by the price in the previous week, with 1 unit of increase in the veal cubes price being followed by a 0.78 unit of increase in the veal cubes price in the next week.

As an econometric result, it was found that the minced meats and veal cubes series from January 2014/1 to December 2017/52 between 06.01.2014 and 25.12.2017 had a quite varying variance.

In the models set on the prices of minced meats and veal cubes, RESID (-1) parameter shows the effect of shocks on price changes and GARCH (-1) parameter shows the effect of the previous period volatility on the current period volatility. The fact that the sum of both parameters is close to one means that it will have a long time effect on price volatility if a possible equilibrium situation deteriorates.

According to the results of the analysis, the veal cubes are more sensitive to the price changes compared to minced meats.

DISCUSSION

This study analysed the volatility of the retail prices of minced meats and veal cubes using volatility estimation modelling for the period between 2014 and 2017, and the results of the GARCH(1, 1) model show that it is the optimal model that best explains the change in the prices.

Analysis results indicate that the retail prices of minced meats and veal cubes saw excessive volatility in the period under consideration. Various policies and government interventions were implemented in the red meat sector in an effort to decrease the rising meat prices. In order to prevent price increases in October and November 2014, the General Directorate of Meat and Milk Board was authorised to import 15 thousand tons of beef with zero custom duty [24], and the custom duty for livestock imports

Table 3. *Correlogram results of the mince and dice series from January 2014/1 to December 2017/52*

Type of Meat	Months	Q Statistic of Error Terms				Type of Meat	Q Statistic of Error Terms			
		AC	PAC	Q-Stat	Prob		AC	PAC	Q-Stat	Prob
Minced meats	1	-0.264	-0.264	14.059	0.000	Veal cubes	-0.283	-0.283	16.148	0.000
	2	-0.094	-0.176	15.839	0.000		-0.066	-0.159	17.027	0.000
	3	-0.039	-0.127	16.150	0.001		-0.012	-0.087	17.054	0.001
	4	0.027	-0.045	16.304	0.003		-0.020	-0.069	17.135	0.002
	5	0.142	0.132	20.429	0.001		0.216	0.204	26.746	0.000
	6	-0.076	0.009	21.612	0.001		-0.106	0.023	29.069	0.000
	7	0.035	0.066	21.870	0.003		0.077	0.117	30.313	0.000
	8	-0.110	-0.084	24.412	0.002		-0.201	-0.168	38.709	0.000
	9	0.185	0.146	31.612	0.000		0.159	0.078	44.020	0.000
	10	-0.063	-0.009	32.447	0.000		-0.015	-0.045	44.070	0.000
	11	-0.010	0.017	32.466	0.001		0.044	0.093	44.489	0.000
	12	0.007	0.007	32.477	0.001		0.009	0.015	44.508	0.000
	13	0.031	0.061	32.682	0.002		-0.059	0.062	45.248	0.000
	14	0.023	0.002	32.800	0.003		0.073	0.002	46.392	0.000
	15	-0.085	-0.047	34.380	0.003		-0.059	-0.001	47.142	0.000
	16	0.153	0.120	39.494	0.001		0.113	0.032	49.903	0.000
	17	-0.054	0.036	40.126	0.001		-0.056	0.027	50.583	0.000
	18	0.034	0.028	40.379	0.002		0.041	0.039	50.945	0.000
	19	-0.120	-0.103	43.591	0.001		-0.002	0.036	50.946	0.000
	20	0.057	0.010	44.310	0.001		-0.044	-0.032	51.386	0.000
	21	0.102	0.066	46.649	0.001		0.076	0.023	52.690	0.000
	22	-0.082	-0.039	48.164	0.001		-0.013	0.024	52.728	0.000
	23	0.089	0.081	49.950	0.001		0.066	0.061	53.722	0.000
	24	-0.091	0.013	51.852	0.001		-0.055	0.015	54.410	0.000
	25	0.039	-0.030	52.204	0.001		0.005	0.001	54.415	0.001
	26	0.004	-0.011	52.208	0.002		0.025	0.011	54.563	0.001
	27	0.008	-0.003	52.222	0.002		-0.049	-0.069	55.118	0.001
	28	-0.001	0.019	52.223	0.004		0.034	-0.038	55.394	0.002
	29	-0.008	-0.001	52.239	0.005		0.006	0.025	55.402	0.002
	30	0.014	-0.030	52.288	0.007		0.019	0.028	55.491	0.003
	31	-0.048	-0.015	52.840	0.009		-0.071	-0.047	56.681	0.003
	32	0.065	0.009	53.859	0.009		0.002	-0.043	56.682	0.005
	33	0.033	0.070	54.127	0.012		0.089	0.062	58.574	0.004
	34	0.008	0.040	54.143	0.016		-0.018	0.013	58.654	0.005
	35	-0.026	0.046	54.312	0.020		-0.041	-0.055	59.056	0.007
	36	-0.047	-0.052	54.843	0.023		-0.041	-0.039	59.471	0.008

AC: *Auto correlation,* ***PAC:*** *Partial auto correlation,* ***Q-stat:*** *Q statistic, Prob: Probability*

by the private sector was reduced to 15%, provided that imported livestock would be used for stockbreeding [25]. Although the decisions taken were effective in reducing the retail prices of minced meats and veal cubes until the end of 2014, the prices started to elevate again as from early 2015 until May. The rising prices adversely affected the prices of foods and food services in this period [26]. In an attempt to prevent the price increases, first the custom duty for imports was reduced to 0%, and then the Meat and Milk Board was authorised to import 30,000 tons of fresh or frozen bovine meat [27,28].

To prevent the increase in red meat prices which were rising due to the lack of sufficient cattle for slaughter in Turkey, a market intervention was implemented through the Meat and Milk Board in March 2017, supplying butchers with fresh beef carcass and food and meat industrialists with frozen beef carcass through importation [29]. The upward movement in red meat prices observed since February 2017 continued in June, and the increase in prices was reported to be basically arising from the shortage of domestic supply [30]. Pursuant to the decision taken in response to the drastic price increase in June, the custom

Table 4. *The result of GARCH analysis of the mince series from January 2014/1 to December 2017/52*

Variable	Coefficient	Std. Error	z-Statistic	P
	Variance Equation			
C	0.087583	0.027537	3.180620	0.0015
RESID(-1)^2	0.154908	0.058710	2.638520	0.0083
GARCH(-1)	0.647365	0.094256	6.868192	0.0001
GARCH (1, 1): Conditional Variance Equation; P=0.001				

Table 5. *The result of GARCH analysis of the dice series from January 2014/1 to December 2017/52*

Variable	Coefficient	Std. Error	z-Statistic	p
	Variance Equation			
C	0.140914	0.019868	7.092685	0.0001
RESID(-1)^2	0.787804	0.152150	5.177816	0.0001
GARCH(-1)	0.133606	0.052468	2.546417	0.0109
GARCH (1, 1): Conditional Variance Equation; P=0.001				

duty for bovine animals was reduced from 135% to 26%, and the custom duty for bovine meat ranging between 100-225% was reduced to 40% [31]. This decision granted the authorisation to import livestock and carcass, which had been exclusively held by the Meat and Milk Board, to the private sector as well. One month after the issuance of this decree, the Meat and Milk Board was granted authorisation to import 90 thousand tons of red meat and 975 thousand head of livestock, 500 thousand head being bovine and 475 thousand head being ovine [32].

Price uncertainty seems to be a very significant restrictive factor for meat production where risk protection mechanisms do not exist. As it affects the production decisions, it restricts the efforts of businesses to build new capacity, invest in advanced technologies and enter new markets [10]. The Turkish livestock sector is subject to excessive price fluctuations, low profitability and unforeseeable intervention policies [33].

Another factor leading to the price increases is the role of market concentration and market power, particularly at the level of retail prices [2]. While the four-firm concentration (CR_4) in the red meat market of Turkey was 41% in 2007, it rose to 50% in 2012 [34,35]. The long marketing chain in the red meat market diminishes the effectiveness of government subsidies granted to producers and causes the producer share in consumer prices of many products to gradually decrease. This indicates that the subsidies granted are actually not transferred to producers, but rather to the processes after the production [36]. Due to the asymmetrical price transfer in the red meat market, there are no large-scale enterprises producer cooperatives that have the potential to become suppliers [2]. As producers are small-scale enterprises, it is highly essential that they organise against large retailers and wholesalers so that they can have sufficient bargaining power under the current market conditions [3].

The models developed for the time series data of the minced meats and veal cubes prices in the research show that increases in prices affect the next week's prices. The studies conducted on the same subject found that volatility of beef prices reacted heavily to the market movements [10], that a negative shock in the prices caused a higher volatility than a positive shock of the same degree [37], and that the fluctuations in beef prices resulted in fluctuations in the price of mutton and chicken [18].

Veal meat prices in Turkey were determined to be strictly affected from the economic and political instability, food crisis, natural events etc., and the prices of veal meat could return to normal values after a long time compared to the average price [38]. On the other hand, the dependence on beef and veal red meat has increased over the years and it reflected to the importation as well [39]. Red meat consumption has been sensitive to the price changes and always responded negatively to the beef meat pricing crisis [40]. Red meat imports, unfortunately, couldn't help the price increases and the market price of red meat was increased later because of a lower local supply [41]. On the other hand, due to the high level intermediary margins of beef meat marketing, red meat prices is increasing in Turkey [42].

In conclusion, the results of the estimation prove that the retail prices of veal cubes and minced meats fluctuated conspicuously in the period under consideration and that the implemented policies and the market interventions were not adequate to eliminate the instability and uncertainty of the prices. The presence of oligopsonistic market conditions, the long marketing chain in the sector and the import dependency to meet the demand for red meat attenuate the market power. Hence, it is essential that the price uncertainty is reduced from the perspective of producers and concrete steps other than importation

be taken to prevent increases in retail prices from the perspective of consumers so that the sector can grow and reach a level where it can compete globally.

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Research Article

Effect of Dietary Fatty Acid Pattern on Growth Performance, Carcass Characteristics, Fatty Acid Profile, and Serum Biochemistry Parameters in Broiler Chickens

Branko MILANKOVIĆ [1] Jelena ĆIRIĆ [2,a] Milena KRSTIĆ [1] Marija STARČEVIĆ [3]
Branislav BALTIĆ [2] Dragan ŠEFER [1] Vesna ĐORĐEVIĆ [2] Milka POPOVIĆ [4] Radmila MARKOVIĆ [1,b]

[1] Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, University of Belgrade, 11000 Belgrade, REPUBLIC OF SERBIA
[2] Institute of Meat Hygiene and Technology, 11000 Belgrade, REPUBLIC OF SERBIA
[3] Serbian Armed Forces, 11000 Belgrade, REPUBLIC OF SERBIA
[4] Faculty of Medicine Novi Sad, University of Novi Sad, Serbia and Institute of Public Health of Vojvodina, 21000 Novi Sad, REPUBLIC OF SERBIA
[a] ORCID: 0000-0002-8118-7676; b ORCID: 0000-0002-6178-1758

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Abstract

This study was undertaken to investigate the effect of dietary fat supplementation with linseed oil and/or pig lard on performance, carcass characteristics, fatty acid profile and serum biochemistry parameters in broiler chickens. In the study, 240 one-day-old Cobb 500 broiler chickens were randomly distributed to one control and three experimental dietary groups: diet supplemented with pig lard; diet supplemented with linseed oil, and; diet supplemented with a 1:1 ratio of lard to linseed oil. The fat sources influenced serum biochemistry parameters and fatty acid profiles of drumsticks with thighs, but had no influence on growth performance or carcass characteristics. With dietary linseed oil, eicosapentaenoic acid and docosahexaenoic acid increased ($P<0.05$) in meat from drumsticks with thighs. Supplementation with pig lard significantly ($P<0.05$) increased the saturated and monounsaturated fatty acids in drumsticks with thighs. In conclusion, dietary incorporation of linseed oil and pig lard during starter, grower and finisher phases can enrich broiler chickens meat with n-3 PUFA. This study has clearly shown that linseed oil in broiler nutrition provided the best n-6/n-3 ratio.

Keywords: *Broiler chickens, Carcass characteristics, n-3 PUFA rich oils, Lard*

Etlik Piliçlerde Diyetteki Yağ Asitlerinin Büyüme Performansı, Karkas Özellikleri, Yağ Asidi Profili ve Serum Biyokimyasal Parametreleri Üzerine Etkisi

Öz

Bu çalışma broiler tavuklarda diyette keten tohumu yağı ve/ya domuz yağı ilavesinin performans, karkas özellikleri, yağ asidi profili ve serum biyokimyasal parametreleri üzerine etkisini araştırmak amacıyla yapılmıştır. Çalışmada 240 adet bir günlük Cobb 500 broiler tavuk rastgele olarak kontrol ile domuz yağı katkılı diyet, keten tohumu yağı katkılı diyet ve 1:1 oranında domuz yağı ve keten tohumu katkılı diyet olmak üzere üç deneysel diyet grubuna ayrıştırılmıştır. Yağ katkıları kalçalı butta yağ asidi profilini ve serum biyokimyasal parametrelerini etkilerken büyüme performansı ve karkas özellikleri üzerine etki etmedi. Keten tohumu yağı kalçalı but etinde eikosapentaenoik asit ve dokosahekzaenoik asit miktarını artırırdı ($P<0.05$). Domuz yağı katkısı kalçalı but etinde anlamlı derecede doymuş ve tekli doymamış yağ asidi miktarını artırdı ($P<0.05$). Sonuç olarak, başlangıç, büyüme ve bitirme evrelerinde diyete keten tohumu yağı ve domuz yağı ilavesi broiler tavuklarda etteki çoklu doymamış yağ asidi miktarını artırır. Bu çalışma, broiler yeminde keten tohumu yağının en iyi n-6/n-3 oranını sağladığını göstermiştir.

Anahtar sözcükler: *Etlik piliç, Karkas özellikleri, n-3 çoklu doymamış yağ asidinden zengin yağ, Domuz yağı*

İletişim (Correspondence)
+381 60 6696861
1310jecko@gmail.com

INTRODUCTION

The success of the poultry industry depends on enhancing growth performance and carcass characteristics, reducing fat deposition of growing broiler chickens and improving the products offered to consumers. Nutrition plays a strong role in growing broiler chickens [1]. Poultry meat is considered healthier, with a relatively lower fat content compared with other animal meat [2,3]. Fats from animal (beef tallow, pig lard, fish oil etc.) or plant origins (sunflower oil, linseed oil, corn oil, coconut oil etc.) are added to commercial broiler chicken feeds as a source of fatty acids and a source of energy. Supplementation and manipulation of the fatty acid composition is implemented for nutritional purposes and human health. Previous studies in broiler chickens have shown different relationships between the fatty acid contents of diets and tissues, especially for breast and thigh meat [1,4-7]. The concentration of n-3 PUFA in broiler chicken tissues depends mainly on the fatty acid composition of the diet [1]. The n-3 PUFA fatty acids decrease proinflammatory eicosanoids and inflammatory biomarkers in broiler chickens [8]. Plant oils (e.g., linseed oil) are rich in a-linolenic acid (ALA), which is the metabolic precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [9]. There is a potential to enrich the human diet with n-3 PUFA by modifying poultry feeding practices to satisfy human health, as the ratio of dietary oils and fats in the animals' diets affects the deposition of fatty acids in broiler meat. Recent studies have shown that dietary imbalance of the n-6/n-3 PUFA ratio can affect human health, especially with high n-6/n-3 PUFA ratios [1,10]. Supplementation of linseed oil at up to 4.5% in broiler diet increased the conversion of ALA to EPA and DHA in breast meat. Similarly, the total n-3 PUFA, including EPA and DHA in meat, were significantly increased as a result of decreases in the n-6/n-3 PUFA ratio or the addition of animal fats [11,12].

The positive effect on the nutritional value of chicken meat, achieved by replacing or supplementing fat with n-3 rich linseed oil and other plant oils, has been documented [13-15]. Little information is, however, available on the effects of replacing the saturated fatty acid (SFA) in rendered animal fat with pig lard. Thus, this study was undertaken to investigate the effect of dietary supplementation of linseed oil (n-3 PUFA rich) and/or pig lard (moderate SFA and MUFA) on broiler chicken growth performance, carcass characteristics, and fatty acid profile of drumsticks with thighs, and serum biochemistry parameters.

MATERIAL and METHODS

Animal Ethics

The experimental protocol was approved by the Veterinary Directorate of the Serbian Ministry of Agriculture, Forestry and Water Management and the Ethics Committee of the Faculty of Veterinary Medicine, University of Belgrade.

Housing and Trial Duration

A total of 240 one-day-old broiler chickens of both sexes and the same origin (Cobb 500) were used in this study during a 42-day period. Birds were randomly assigned to one of four dietary treatments (control and three experimental groups), each having 6 replicates (10 birds in each replicate). Birds were placed in an environmentally controlled room (stocking density 6 birds/m^2) with 5 cm thick sawdust. The temperature in the room was 32°C from days 1 to 5, and then gradually lowered to 22°C on day 21. This temperature was maintained until the end of the study. Humidity was 45-50% RH. The lighting of the rooms was 24 h. Water and feed were supplied *ad libitum* throughout the study.

Experimental Diets

From the start of the trial, each group of broilers was fed with one of four different diets, which comprised the same basal diet, but differed only in additive supplementation (lard and linseed oil) *(Table 1)*.

Basal diet was formulated to meet the maintenance and growth requirements of the animals used in the study. Broiler chickens were fed from day 1 to day 42 in three phases with three nutritionally different concentrated feed mixtures, namely, starter (up to 10 days), grower (11-21 days), and finisher (22-42 days) mixtures. The broilers in the control group were given a diet without lard and linseed oil. The other three treatment groups were given the same diet as fed to the control group (C group) but were supplemented with lard and/or linseed oil. Commercially prepared linseed oil (Granum^R, Serbia) and lard were added to the feed for the experimental groups *(Table 1)*.

Feedstuff Composition

The ingredients and chemical composition (calculated analyses) of the basal diets are listed in *Table 2*. All components of the diet were analyzed for moisture [16], crude protein [17], total lipids [18], ash [19], crude fiber [20], calcium [21], and phosphorus [22]. The content of nitrogen-free matter was determined by the formula:

BEM = 100 - (% moisture + % ash + % cellulose + % protein + % fat).

Performance and Carcass Characteristics of Broilers

Growth performance of broiler chickens was evaluated by recording body weight, weight gain, and feed conversion ratio during the 42-day experimental period. Weight gains of broiler chickens were recorded on a pen basis, the uneaten feed was discarded, and fresh feed replaced in feeders at the end of each day. Feed conversion ratio (FCR) was calculated as the amount of feed consumed per unit of body weight gain.

At the end of the study, animals were transported to the slaughterhouse and then individually weighed, electrically

Table 1. *Three broiler chicken diets supplemented with different sources of fat (%)*

Fat	Diets											
	Starter				Grower				Finisher			
	C	L	LO	L+LO	C	L	LO	L+LO	C	L	LO	L+LO
Lard	-	1		0.50	-	2.50	-	1.25	-	5		2.50
Linseed oil	-	-	1	0.50	-	-	2.50	1.25	-		5	2.50
Total	-	1	1	1	-	2.50	2.50	2.50	-	5	5	5

C- *Control diet without supplemented fat;* ***L-*** *Experimental diet supplemented with lard;* ***LO-*** *experimental diet supplemented with linseed oil;* ***L+LO-*** *experimental diet supplemented with 1:1 ratio of lard to linseed oil*

Table 2. *Formulation and calculated contents of the basal diets for broilers*

Ingredients (%)	Starter (up to 10 days)		Grower (days 11-21)		Finisher (days 22-42)	
	C	E	C	E	C	E
Maize	50.85	49.85	44.15	41.65	44.95	39.95
Wheat	-	-	10.00	10.00	15.00	15.00
Soy grits	15.00	15.00	17.00	17.00	20.00	20.00
Soybean meal	12.40	12.40	1.00	1.00	1.00	1.00
Soybean cake	17.00	17.00	23.30	23.30	14.70	14.70
Monocalcium phosphate	1.20	1.20	1.00	1.00	0.90	0.90
Chalk	1.60	1.60	1.60	1.60	1.60	1.60
Salt	0.35	0.35	0.35	0.35	0.35	0.35
Premix**	1.00	1.00	1.00	1.00	1.00	1.00
Lysine	0.20	0.20	0.20	0.20	0.10	0.10
Methionine	0.20	0.20	0.20	0.20	0.20	0.20
Adsorbent	0.20	0.20	0.20	0.20	0.20	0.20
Parameter	**Calculated Values**					
Metabolic energy MJ/kg	12.69	12.71	13.01	13.03	13.11	13.13
Total ash	6.77	6.77	6.66	6.66	6.16	6.15
Total lipids	6.61	6.76	7.39	7.51	7.20	7.29
Crude fiber	3.89	3.89	3.97	3.97	3.44	3.44
Crude protein	22.24	22.22	21.14	21.13	19.62	19.62
Moisture	10.41	10.39	10.20	10.18	10.54	10.53
Lysine	1.50	1.49	1.42	1.42	1.17	1.17
Methionine+ cysteine	0.81	0.81	0.80	0.80	0.76	0.76
Tryptophan	0.31	0.31	0.29	0.29	0.27	0.27
Calcium	1.01	1.01	0.94	0.94	0.90	0.90
Phosphorus	0.59	0.59	0.56	0.56	0.54	0.54
NFE	50.08	49.96	50.63	50.55	53.04	52.97

*** Mineral- vitamin premix provided per kg of diet: Vit. A 12.999 IJ, Vit. D_3 4 950 IJ, Vit. E 75 mg, Vit. K_3 3 mg, Vit. B_1 3 mg, Vit. B_2 7.95 mg, Vit. B_6 4.05 mg, Vit. B_{12} 0.0195 mg, Vit. C 19.95 mg, Biotin 0.15 mg, Niacin 60 mg, Calcium pantothenate 15 mg, Folic acid 1.95 mg, Iodine 1.0005 mg, Selenium 0.3 mg, Choline chloride 399.9 mg, Iron 39.99 mg, Copper 15 mg, Manganese 99.9 mg, Zinc 99.9 mg, Methionine 2100 mg, Lysine 1200 mg*
C- *Control group;* ***E-*** *Experimental group (L, LO, L+LO)*

stunned and immediately slaughtered by severance of the jugular veins. Subsequently, animals were processed following standard industrial techniques, and hot carcass weight was recorded. During the first 24 h post-mortem, carcasses were stored in a ventilated cold room at 2°C, after which cold carcass weight was measured. After chilling, carcasses of broiler chickens from each group were separated into breast, drumsticks with thighs, wings, neck, and back with pelvis. The different carcass parts were weighed.

Meat Quality

At 24 h post-mortem, 6 drumsticks with thigh meat (i.e., muscle) samples from each experimental group were packed in polyethylene bags until analysis of moisture [23], lipid [24], protein [25], and ash [26]. The pH of drumsticks with thighs was determined using a digital pH meter (TESTO 205, Germany).

Fatty Acid Analysis

The fatty acid profiles of the diets are shown in *Table 3*. Total lipids for fatty acid determination were extracted from homogenized samples (diets and drumsticks with thighs) with hexane/isopropanol mixture by accelerated solvent extraction (ASE 200, Dionex, GmbH, Idstein, Germany). After evaporation of solvent until dryness under a stream of nitrogen, total lipids were converted to fatty acid methyl esters (FAME) by trimethylsulfonium hydroxide. FAMEs were determined using a Shimadzu 2010 gas chromatograph equipped with flame ionization detector (FID) and cyanopropyl HP-88 capillary column (100 m x 0.25 mm x 0.20 mm) [27]. Temperature of the injector and detector were 250°C and 280°C, respectively. FAMEs were identified on the basis of relative retention time, compared with the relative retention times of the individual compounds in a standard mixture of fatty acid methyl esters, Supelco Component 37 FAME mix (Supelco, Bellefonte, USA). Quantification of fatty acids was determined relative to an internal standard, heneicosanoic acid, C21:0. The level of fatty acids (diets and drumsticks with thighs) is expressed as a percentage (%) of the total identified fatty acids. According to Fuchs et al.[28], indexes of lipid quality (atherogenic index (AI), index of thrombogenicity (TI), and hypocholesterolemic/hypercholesterolemic fatty acid ratio (HH) were calculated using the following equations:

Table 3. *The fatty acid composition (%) of broiler chicken diets*

Fatty Acid	Diets											
	Control			L			LO			L+LO		
	I	II	III	I	II	III	I	II	III	I	II	III
C14:0	0.72	0.59	0.03	0.94	0.85	1.26	0.63	0.51	0.57	0.67	0.64	0.94
C16:0	11.05	11.72	10.62	14.13	15.00	15.65	10.30	9.27	8.29	10.74	12.16	10.18
C16:1	0.09	0.10	0.09	0.62	0.80	1.08	0.12	0.09	0.11	0.20	0.39	0.62
C17:0	0.08	0.09	0.08	0.14	0.13	0.20	0.09	0.07	0.06	0.09	0.10	0.14
C18:0	4.25	4.69	4.15	6.32	6.15	8.01	4.24	3.77	3.60	4.28	4.58	6.32
C18:1cis-9	24.63	24.72	26.74	32.87	31.58	32.60	24.84	24.79	26.93	25.36	27.60	27.22
C18:1cis-11	1.02	1.01	0.93	1.28	1.56	1.80	0.98	0.81	0.71	1.06	1.13	1.28
C18:2n-6	52.80	51.63	51.08	32.90	33.02	30.96	44.48	46.03	46.44	47.08	44.81	43.45
C20:0	0.42	0.46	0.44	0.35	0.39	0.31	0.40	0.34	0.33	0.39	0.37	0.35
C18:3n-6	0.04	0.04	0.05	0.15	0.09	1.09	0.11	0.13	0.31	0.30	0.12	0.15
C18:3n-3	3.71	3.79	3.51	7.04	7.26	5.92	11.06	11.85	10.14	7.51	5.90	6.09
C20:1	0.15	0.19	0.14	0.46	0.63	0.36	0.16	0.15	0.18	0.20	0.27	0.46
C20:2n-6	0.03	0.05	0.04	0.24	0.18	0.30	0.05	0.09	0.10	0.09	0.11	0.24
C22:0	0.69	0.63	0.50	0.38	0.33	-	0.50	0.49	0.51	0.50	0.04	0.38
C20:3n-6	0	0.00	1.31	1.74	1.57	-	1.34	1.33	1.30	1.09	1.48	1.74
C20:3n-3	0.07	0.03	0.04	0.08	0.06	0.06	0.03	0.03	0.02	0.04	0.06	0.08
C20:5n-3	0.09	0.07	0.08	0.07	0.06	0.10	0.19	0.08	0.11	0.08	0.09	0.07
C24:0	0.16	0.19	0.17	0.12	0.11	0.10	0.24	0.17	0.17	0.15	0.15	0.12
C22:6n-3	-	-	-	0.17	0.23	0.20	0.24	0.00	0.12	0.17	0.00	0.17
SFA	17.37	18.37	15.99	22.38	22.96	25.53	16.40	14.62	13.53	16.82	18.04	18.43
MUFA	25.89	26.02	27.90	35.23	34.57	35.84	26.10	25.84	27.93	26.82	29.39	29.58
PUFA	56.74	55.61	56.11	42.39	42.47	38.63	57.50	59.54	58.54	56.36	52.57	51.99
n-6	52.87	51.72	52.48	35.03	34.86	32.35	45.98	47.58	48.15	48.56	46.52	45.58
n-3	3.87	3.89	3.63	7.36	7.61	6.28	11.52	11.96	10.39	7.80	6.05	6.41
n-6/n-3*	13.66	13.30	14.46	4.76	4.58	5.15	3.99	3.98	4.63	6.23	7.69	7.11

C- *Control diet without supplemented fat;* ***L-*** *Experimental diet supplemented with lard;* ***LO-*** *Experimental diet supplemented with linseed oil;* ***L+LO-*** *Experimental diet supplemented with 1:1 ratio of lard to linseed oil;* ***I-*** *Starter diets;* ***II-*** *Grower diets;* ***III-*** *Finisher diets;* ******* *ratio*

AI = (C12:0 + 4xC14:0 + C16:0)/(Σn-3 PUFA + Σn-6 PUFA + ΣMUFA)

TI = (C14:0 + C16:0 + C18:0)/(0.5xΣMUFA + 0.5xΣn-6PUFA + 3xΣn-3PUFA + Σn-3PUFA/Σn-6PUFA)

HH = (C18:1n-9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3 + C22:6n-3)/(C14:0+C16:0)

Serum Biochemistry Analyses

Blood samples were collected from the slaughtered birds in non heparinized tubes. The samples were centrifuged at 3000 rpm for 15 min, and the serum obtained was stored at -20°C until analysis. HDL-cholesterol and triglyceride were determined by the auto analyzer (CentroLIA LB 961, Berthold Technologies, Germany) using commercially available kits purchased from Accurex biomedical company.

Statistical Analyses

Statistical analysis of the results was conducted using software GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). All parameters were described by means and standard error of means (SEM). One-way ANOVA with Tukey's post hoc test was performed to assess the significance of differences ($P<0.05$) among control and experimental groups.

RESULTS

In the current study, the effects of differing broiler chickens' dietary fatty acid sources on meat from drumsticks with thighs, growth performance, carcass characteristics, fatty acid profile and serum biochemistry parameters (cholesterol and triglyceride levels) in broiler chickens were studied.

Table 4 shows body weight, weight gain, feed-conversion ratio and feed consumption of broilers receiving diets containing lard and/or linseed oil. The body weight did not differ across the treatment groups. However, numerically higher final body weight was observed in the group with added linseed oil than in the other groups. The weight gain for two periods (days 1-21 and days 1-42) was higher in the group with added linseed oil. Broiler chickens fed only with basal diet showed a higher feed-conversion ratio (from day 1 to day 42) than did those supplemented with linseed oil or/and pig lard.

The chemical composition (protein, lipids, moisture and ash), pH, and temperatures of the meat are shown in *Table 4*. Protein in meat from the control group was 17.79%, and it was 17.66% in meat from the group supplemented with lard, 17.11% in meat from the group supplemented with linseed oil and 17.84% in meat from the group supplemented with a 1:1 ratio of lard to linseed oil. The lipid content was the lowest in the lard group (4.24%), while in the control, linseed oil and lard plus linseed oil groups it was 4.63%, 4.74% and 4.76%, respectively. The moisture content in meat from the four groups was approximately the same and ranged from 77.21% (control) to 77.83% (lard).

Table 4. *Growth performance and carcass characteristics of broiler chickens in different diet groups*

Parameters		Groups				SEM	P value
		C	L	LO	L+LO		
BW		2452	2433	2551	2405	44.94	0.020
Weight gain (g)	Day 1-10	254.24	262.16	260.36	265.95	4.72	0.161
	Day 1-21	1034.60	1050.44	1061.24	1048.95	19.71	0.100
	Day 1-42	2354.60	2354.52	2486.57	2363.80	42.65	0.580
FCR	Day 1-10	1.35	1.27	1.30	1.33	0.01	0.0004
	Day 1-21	1.51	1.44	1.47	1.49	0.009	0.0002
	Day 1-42	1.86	1.77	1.79	1.81	0.01	0.0001
Feed consumption (g)	Day 1-10	343.22	332.94	338.46	353.71	3.60	0.14
	Day 1-21	1562.24	1512.63	1560.02	1562.93	8.21	0.0010
	Day 1-42	4379.55	4167.50	4450.96	4278.48	21.38	0.63
Meat characteristics	pH_{45min}	6.32	6.18	6.23	6.24	0.056	0.082
	pH_{24h}	6.07	6.02	6.03	5.95	0.032	0.170
	T_{45min} (°C)	34.27	33.01	33.23[c]	30.42	0.657	<0.0001
	Moisture (%)	77.21	77.83	77.78	77.75	0.189	0.438
	Lipid (%)	4.63	4.24	4.74	4.76	0.124	0.239
	Protein (%)	17.79	17.66	17.11	17.84	0.175	0.251
	Ash (%)	0.82[a]	0.84[a]	0.85	0.90[b]	0.009	0.012

Data are means and SEM (n=60 per group). Within a row, means with different letters significantly differ; [a,b] P<0.05; ***C****- Control diet without supplemented fat;* ***L****- Experimental diet supplemented with lard;* ***LO****- Experimental diet supplemented with linseed oil;* ***L+LO****- experimental diet supplemented with 1:1 ratio of lard to linseed oil;* ***BW****- The average body weight, g/live bird;* ***FCR****- Feed conversion ratio;* ***SEM:*** *Standard error of means*

Table 5. *Serum biochemistry parameters of broiler chickens fed different diets*

Parameters (mmol/L)	Day	Groups				SEM	P value
		C	L	LO	L+LO		
HDL-Cholesterol	Day 20	5.09[a]	2.80[b]	2.60[b]	2.90[b]	0.026	<0.0001
	Day 42	2.40	2.30	2.10	2.21	0.019	<0.0001
Triglyceride	Day 20	0.88	1.03	0.94	1.23	0.003	<0.0001
	Day 42	0.41	0.28	0.44	0.40	0.002	<0.0001

Data are means and SEM. Within a row, means with different letters significantly differ, [a,b] P<0.05; ***C-*** *Control diet without supplemented fat;* ***L-*** *Experimental diet supplemented with lard;* ***LO-*** *Experimental diet supplemented with linseed oil;* ***L+LO-*** *Experimental diet supplemented with 1:1 ratio of lard to linseed oil;* ***SEM:*** *Standard error of means*

Table 6. *Fatty acid profile (%) of meat from drumsticks with thighs of broilers fed different diets for 42 days*

Fatty Acid	Groups				SEM	P value
	C	L	LO	L+LO		
C14:0	0.82	1.03	0.72	0.75	0.020	<0.0001
C15:0	0.08[a]	0.09[a]	0.07[b]	0.08[a]	0.002	0.002
C16:0	17.46	21.11	18.13	20.32	0.201	<0.0001
C16:1	2.04	2.98	3.30	3.13	0.112	0.0003
C17:0	0.16[a]	0.19[a]	0.12[b]	0.15[a]	0.004	<0.0001
C18:0	6.70	7.47	5.96	6.98	0.183	0.011
C18:1cis-9	28.44	37.44	32.25	34.41	0.262	<0.0001
C18:1cis-11	1.27	2.10	1.43	1.66	0.026	<0.0001
C18:2n-6	38.36[a]	23.90[b]	26.64[b]	25.12[b]	0.362	<0.0001
C20:0	0.15	0.13	0.15	0.13	0.005	0.368
C18:3n-6	0.21[a]	0.18[a]	0.14[b]	0.13[b]	0.006	<0.0001
C18:3n-3	2.47	1.21	8.40	4.71	0.098	<0.0001
C20:1	0.30[a]	0.50[b]	0.23[a]	0.35[a]	0.011	<0.0001
C20:2n-6	0.45[a]	0.35[b]	0.28[b]	0.32[b]	0.014	0.0002
C22:0	0.08[a]	0.07[a]	0.11[b]	0.07[a]	0.006	0.014
C20:3n-6	0.30[a]	0.23[b]	0.25[b]	0.31[a]	0.011	0.016
C20:3n-3	0.05	0.05	0.19	0.12	0.006	<0.0001
C20:5n-3	0.10	0.58	0.60	0.50	0.055	0.033
C22:5n-3	0.31[a]	0.25[a]	0.64[b]	0.50[b]	0.019	<0.0001
C22:6n-3	0.18	0.25	0.34	0.34	0.030	0.009
SFA	25.44	30.09	25.25	28.48	0.318	<0.0001
MUFA	32.05[a]	43.02[b]	37.20[a]	39.54[a]	0.330	<0.0001
PUFA	42.51	26.89	37.55	31.98	0.442	<0.0001
n-6	39.27[a]	24.66[b]	27.31[b]	25.87[b]	0.347	<0.0001
n-3	3.24	2.23	10.25	6.11	0.123	<0.0001
n-6/n-3*	12.18	11.24	2.67	4.25	0.255	<0.0001

Data are means and SEM. Within a row, means with the different letter significantly differ; [a,b] P<0.05; ***C-*** *Control diet without supplemented fat;* ***L-*** *Experimental diet supplemented with lard;* ***LO-*** *Experimental diet supplemented with linseed oil;* ***L+LO-*** *experimental diet supplemented with 1:1 ratio of lard to linseed oil* ***SEM:*** *Standard error of means;* * ratio

Ultimate meat pH measured 24 h post-mortem did not change markedly when lard or linseed oil were supplemented in the diet, although the pH tended to decrease when diet was supplemented with lard plus linseed oil (L+LO) *(Table 4)*. However, the pH of meat from drumsticks with thighs was not significantly different (P>0.05) from meat from the other dietary groups. Temperatures after 45 min were significantly decreased (P<0.05) in the lard plus linseed oil group compared to those in the control, lard, and the linseed oil groups.

Table 7. *Effect of different diets on atherogenic index (AI), index of thrombogenicity (TI), and hypocholesterolemic/hypercholesterolemic fatty acid ratio (HH) of broiler chicken meat (42 d)*

Parameter	Groups				SEM	P value
	C	L	LO	L+LO		
AI	0.28[a]	0.36[b]	0.27[a]	0.32[b]	0.031	<0.0001
TI	0.13[a]	0.12[a]	0.10[b]	0.12[a]	0.009	0.034
HH	3.80[a]	1.40[b]	3.61[a]	2.87[b]	0.190	<0.0001

Data are means and SEM. Within a row, means with the different letter significantly differ, [a,b] P<0.05, C- Control diet without supplemented fat; ***L****- experimental diet supplemented with lard;* ***LO****- Experimental diet supplemented with linseed oil;* ***L+LO****- Experimental diet supplemented with 1:1 ratio of lard to linseed oil;* ***SEM:*** *Standard error of means*

The mean values of serum biochemistry parameters (HDL-cholesterol and triglyceride) of broiler chickens are shown in *Table 5*. Supplementation of lard or supplementation of lard plus linseed oil produced significant differences (P<0.05) in the mean concentration of HDL-cholesterol in the broilers' sera. At 42-days-old, cholesterol levels were lower in broilers fed diets with lard (L) or linseed oil (LO). There was a decrease in the triglyceride levels in the broilers fed diet supplemented with lard compared with the control group and other experimental groups (day 42).

Increasing the level of dietary linseed oil and lard in broiler chickens serum reduced the animals' blood cholesterol concentrations *(Table 5)*.

The fatty acid composition of meat (i.e., muscle) from drumsticks with thighs in relation to dietary oil and fat is illustrated in *Table 6*. It was observed that the fatty acid composition of meat from drumsticks with thighs reflected the fatty acid profile of the experimental diet. The major fatty acids detected in the meat from drumsticks with thighs of the lard (L) group and affected by the dietary fat were C14:0, C15:0, C18:0, C18:1n-9, C18:1n-11. The concentration of oleic acid (C18:1 n-9) in the meat from drumsticks with thighs of broilers fed the lard diet was significantly higher than those groups fed linseed oil (LO) or lard plus linseed oil (L+LO) diets. Broilers fed linseed oil (LO) had a significantly higher concentration of C18: 3n-3 (8.40%) compared with L (1.21%), C (2.47%) and L+LO (4.71%) groups. The concentration of C20:0 in meat from drumsticks with thighs did not differ significantly among the dietary treatments (P>0.05). Significantly higher concentrations of EPA, docosapentaenoic acid (DPA) and DHA were found in broiler chickens fed linseed oil than in the control group (P<0.05). The different dietary fat sources influenced (P<0.05) the mono unsaturated fatty acid (MUFA) deposition in the meat from drumsticks with thighs. Similarly, dietary replacement of lard had influence on SFA levels in the meat (drumsticks with thighs). A significant increase in the concentration of total SFA and MUFA was found in broiler chickens fed the lard diet compared with other groups. The PUFA were increased in meat from drumsticks with thighs of the control group (without any supplementation). In the meat from drumsticks with thighs, total n-6 PUFA content was lowered with lard supplementation compared with other groups. Dietary supplementation with linseed oil, a rich n-3 PUFA source, improved the total n-3 PUFA in the meat (LO<C<L+LO<L). The highest total n-3 PUFA deposition in the meat from drumsticks with thighs was recorded with linseed oil supplementation.

The n-6/n-3 ratio differed among sources of fat. Broiler chickens fed linseed oil had lower n-6 /n-3 (2.67) compared with animals fed control (12.18), lard (11.24) and lard plus linseed oil (4.25) diets.

There were significant treatment differences 5 observed in atherogenic index (AI), index of thrombogenicity (TI) and hypocholesterolemic/hypercholesterolemic fatty acid ratio (HH) of meat from drumsticks with thighs in the present study *(Table 7)*. A lower thrombogenic index of meat from linseed oil-supplemented animals with the higher n-3 level has been reported [28]. In the current study, a significantly lower hypocholesterolemic/hypercholesterolemic fatty acid ratio (HH) of meat from drumsticks with thighs was found in the lard group (P<0.05). Thus, a significantly higher 5 atherogenic index was found in in the lard group compared to the control group. Lower values of atherogenic index and index of thrombogenicity indicate a healthier ratio regarding the higher content of fatty acids that inhibit the aggregation of platelets and reduce the potential for coronary diseases. In contrast, a higher hypocholesterolemic/hypercholesterolemic fatty acid ratio indicates a more nutritionally suitable fatty acid profile [29].

DISCUSSION

Many studies indicated no difference in broilers' growth performance parameters when the animals were fed different fat sources [30-32]. Also, Andreotti et al.[33] demonstrated no effects on performance when broiler chickens were fed from days 21 to 49 with diets containing lard. The growth performance of broilers fed on n-3 PUFA-enriched diets (linseed oil) was not different from those fed on a control diet. These results are in agreement with several previous studies [34-36]. The weights of basic cuts of broiler carcasses (breast, drumsticks with thighs, wings, neck, and back with pelvis) did not significantly differ among the compared groups *(Table 4)*. Supplementation of linseed oil improved

the yield of carcass cuts, where the linseed oil group had higher weight of breast, drumsticks with thighs, wings, neck, and back with pelvis compared to other groups, but differences were not significant.

The chemical composition (protein, moisture, and fat) of meat from different groups of broiler chickens was not significantly different ($P>0.05$), but the ash content was significantly different ($P<0.05$). Zelenka et al.[37] noticed no variation in chemical composition of meat from drumsticks with thighs due to dietary incorporation of linseed oil in broiler chicken diet.

The concentration of triglyceride was decreased in the broiler chicken group fed diet supplemented with lard (L) compared with the other groups. These results are in accordance with Ibrahim et al.[1], who reported that increasing dietary n-3 fatty acids in broiler chicken diet reduced cholesterol. El-Katcha et al.[6] stated that feeding broilers on a diet with a 1:5 ratio of n-3/n-6 PUFA reduced the cholesterol content of broiler chicken meat. The effect of dietary fatty acid profile on cholesterol levels of broiler chickens was also reported by Maraschiello et al.[38]. These authors found higher levels of cholesterol for broiler chickens fed lard than for those fed plant oils. Peebles et al.[39] concluded that added lard fed to broiler chickens in starter diets produced responses in serum low density lipoprotein cholesterol concentrations.

After breast meat, drumsticks with thighs is a very important cut in broiler chickens. As a result, many studies focused on the nutritional aspects of broiler chicken meat by assessment of drumsticks with thighs. Earlier studies showed clear correlations between dietary fatty acid composition and the fatty acid composition of chicken meat [1,4,13,40]. Previous studies reported that fatty acid composition of drumsticks with thighs reflected the differences in dietary fatty acid (from plants and animals) intake more than breast meat of broiler chickens [1,4,13,40]. This is related to the higher total lipid content in drumsticks with thighs. The fatty acid composition of meat from drumsticks with thighs is shown in *Table 6*. Broiler chickens fed lard presented higher values of SFA, mainly myristic, palmitic, and stearic acids (C14:0, C16:0, and C18:0), than those fed linseed oil. Also, broiler chickens fed with linseed oil had a substitution of these SFA (mainly stearic acid) with linoleic acid (18:2n-6). The deposition of linoleic (C18:2n-6) and a-linolenic (C18:3n-3) acids in drumsticks with thighs was more correlated with their content in the feed. Thus, broiler chickens fed linseed oil presented the lowest values of stearic acid in drumsticks with thighs, in accordance with Scaife et al.[40]. Rosa [41] used three types of plant and animal oil (linseed, soybean, and a mixture of linseed and fish) at inclusion levels of 1, 2 and 3% in broiler diets and observed that the composition of fatty acids in the rations influenced the fatty acid profile of drumsticks with thighs. In the present study, the proportion of EPA and DHA found in drumsticks with thighs were higher in broiler chickens fed linseed oil. The concept of synergism between animal fats and vegetable oils has been recognized for many years [42-44]. Animal fats such as lard are rich in long-chain saturated fatty acids. Most vegetable oil sources have a high content of unsaturated fatty acids. Sanz et al.[45] observed the effect of sunflower oil and a mixture of bovine tallow and swine fat on fatty acid profiles of broiler chicken meat. These results [44] were similar to ours in the current study. Comparison of fatty acid profiles in drumsticks with thighs evidences changes in the preferential localization of SFA, MUFA and PUFA according to the dietary fat and oil sources. Furthermore, it was well documented that oleic acid is the major fatty acid of drumsticks with thighs [40,46,47]. Nevertheless, the maximal MUFA contents were observed in drumsticks with thighs of lard-fed groups. Crespo and Esteve-Garcia [45] indicated the muscle from drumsticks with thighs of chickens fed with lard had the highest concentrations of linoleic acid. In the current study, broiler chickens fed with supplemented fat and oil (L+LO) showed similar high PUFA contents in muscle from drumsticks with thighs.

Broiler chickens fed lard ingested the highest amount of SFA, so the highest percentages of SFA were found in broilers fed lard and in broiler chickens fed lard plus linseed oil ($P<0.01$). Similar results were found in the study of Crespo and Esteve-Garcia [46]. In the current study, broiler chickens fed linseed oil had higher values of linoleic acid (C18:2 n-6) and other n-6 derivatives than those in the other treatments. These fatty acids replaced SFA and MUFA compared to the broilers fed lard. The same effect was observed with n-3 fatty acids in broilers fed linseed oil [48]. Despite the higher content of linoleic acid in the diet with linseed oil compared with the lard diet, n-6 derivatives were found in higher amounts in broilers in our control group. Supplementation with lard in broiler chicken diets caused increases in the n-6/n-3 fatty acid ratio in drumsticks with thighs compared with other groups. However, the most favorable n-6/n-3 fatty acid ratio in drumsticks with thighs was found in broiler chickens fed linseed oil (2.67). The proportion of total n-6/n-3, fulfilling the demands of health-conscious consumers, should be from 1 to 5, so the best oil supplement in broiler chickens diets in other studies were linseed oil, followed by the mixture of linseed oil and lard [1,4,13,39]. The dietary incorporation of linseed oil and pig lard during starter, grower and finisher phases can enrich broiler chickens meat with n-3 PUFA. This study has clearly shown that linseed oil in broiler nutrition provided the best n-6/n-3 ratio.

Conflict of Interest

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

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Research Article

Efficacy of Topical *Curcuma longa* and *Nigella sativa* Combination for Feline Head and Neck Dermatitis: An Open Pilot Study

Kerem URAL [1,a] Mehmet GÜLTEKİN [1,b] Hasan ERDOĞAN [1,c]

[1] Adnan Menderes University, Veterinary Faculty, Internal Medicine, TR-09016, Aydın - TURKEY
[a] ORCID: 0000-0003-1867-7143; [b] ORCID: 0000-0002-5197-2403; [c] ORCID: 0000-0001-5141-5108

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Abstract

Two medicinal plants, *Curcuma longa* (turmeric) and *Nigella sativa* (black cumin), have generated growing interest for their therapeutic anti-inflammatory action. We hypothesize that commercially available topical *C. longa* and *N. sativa* cream (Dermacumin cream®) will significantly decrease both the Feline Dermatitis Extent (FDE) and Severity Index (SI) and the pruritus scores after 3 weeks of twice-daily treatment of feline head and neck dermatitis. Ten client-owned cats with feline head and neck dermatitis which had received no previous treatment. FDE and SI scores gradually decreased on days 7 (P<0.001) and 28 (P<0.0001) compared to day 0. Similarly, decreased Visual Analog Scale (VAS) scores were detected on days 7 and 28 (P<0.0001). Cats receiving the herbal cream for 3 weeks all had significantly decreased FDE and SI scores, from 171.8 to 0 (P<0.0001), and VAS scores from 6.3 to 0 (P<0.0001). A positively elevated correlation (r=0.62, P<0.001) between FDE and SI and VAS scores for each case through evaluation period was revealed. The present study supports a potential benefit of topical herbal medicine against feline head and neck dermatitis.

Keywords: *Black cumin, Curcumin, Dermatitis, Feline, Pruritus, Treatment*

Kedi Baş ve Boyun Dermatitisi İçin Topikal *Curcuma longa* ve *Nigella sativa* Kombinasyonunun Etkinliği: Açık Pilot Çalışma

Öz

İki şifalı bitki, *Curcuma longa* (zerdeçal) ve *Nigella sativa* (çörek otu), terapötik anti-enflamatuar etkileri yüzünden giderek artan ölçüde ilgi çekmektedir. Sunulan çalışmada piyasada bulunan topikal *C. longa* ve *N. sativa* içeren kremin (Dermacumin krem®), baş ve boyun dermatitli kedilerde günde iki kez tedavi edilmesinden 3 hafta sonra Feline Dermatitis Extent (FDE), Severity Index (SI) ve kaşıntı skorlarını önemli ölçüde azaltacağını varsayıyoruz. Bu amaçla daha önce tedavi görmemiş olan baş ve boyun dermatiti olan on sahipli kedi kullanıldı. FDE ve SI skorlarının 0. güne kıyasla 7. (P<0.001) ve 28. günde (P<0.0001) azaldığı belirlendi. Benzer şekilde, Görsel Analog Skala (GAS) skorlarındaki azalma 7 ve 28. günlerde (P<0.0001) saptandı. Bitkisel kremi 3 hafta boyunca alan kedilerin hepsinde FDE ve SI skorları anlamlı olarak azalmış, 171.8'den 0'a (P<0.0001) ve GAS skorları 6.3'den 0'a (P<0.0001) düşmüştür. Değerlendirme süresi boyunca her bir olgu için FDE/SI ve GAS skorları arasında pozitif olarak yüksek bir korelasyon (r=0.62, P<0.001) ortaya çıkarılmıştır. Bu çalışma, topikal bitkisel ilaçların kedilerin baş ve boyun dermatitine karşı potansiyel faydasını desteklemektedir.

Anahtar sözcükler: *Çörek otu, Zerdeçal, Dermatitis, Feline, Kaşıntı, Tedavi*

INTRODUCTION

Head/neck excoriations and pruritus in feline cases is due to papular and erythematous alterations existing on the face/neck related to alopecia, crusts, miliary dermatitis, seborrhea, and self-induced trauma [1]. Given the limited treatment options reported previously for feline allergic disease, it should not be unwise to state for the known facts, long-term risks of immunosuppressive treatment. There is an unfulfilled demand for a reliable and effective anti-inflammatory and probably non-immunosuppressive treatment option for feline head and neck dermatitis (Fhnd). The purpose of the present open-label, non-controlled study was to elucidate the probable efficacy of a herbal cream containing *Curcuma longa (C. longa*; turmeric) and *Nigella sativa (N. sativa*; black cumin) in Fhnd. It was hypothesized that turmeric and black cumin might effectively mitigate inflammation in cats suffering

İletişim (Correspondence)
+90 256 2470700/6247 Fax: + 90 256 2470720
kural@adu.edu.tr

from Fhnd. It was further suggested that the combined anti-inflammatory effects of the latter compounds might account for the observed pruritus alleviation and relevant clinical signs.

MATERIAL and METHODS

Animals and Samples

The open-label, non-controlled study enrolled with written owner consent due to ethical guidelines of Adnan Menderes University HADYEK Committee (no: 64583101/2017/053) and compared to FEDESI and Vas prurititis scores with values on two subsequent re-evaluations at weeks 1 and 4.

Research Period/Inclusion Criteria

The present study was conducted at the Aydin Adnan Menderes University, Faculty of Veterinary, Department of Internal Medicine (n=6) and to private practices in İzmir (n=2) and Muğla (n=2) municipalities. A total of 10 cats with pruritic dermatoses (Fhnd) were initially analyzed for preliminary allergy background based on owner compliancies, clinical findings, cytological and necessary dermatological examination involving hair plucking, deep skin scraping and acetate tape impression, and beside fungal cultures. Regular flea control by the referring veterinarians was evident in all cats. Prior to the current study, all cats were subjected to a food trial with hydrolyzed proteins (protection for food allergy) (n=7) or novel home cooked (n=3) for at least 8 weeks. No prior drug administration was evident, which was one of the inclusion criteria. Cats were excluded if bacterial or fungal infections (based on laboratory diagnostic); ectoparasites (based on, for example, a flea comb, cytology, dermatoscopy, or skin scraping); flea allergy; or metabolic or other non allergic disorders were diagnosed [2]. Flea control was previously available in all of the cats but was not continued throughout the study as none of the cats had flea allergic dermatitis. Rapid diagnostic ELISA test kits for antigens were used for excluding viral diseases. Complete blood count, serum biochemistry, and endocrine panels were available on days 0 and 28 for all cats involved.

Trial Protocol

The referring veterinary surgeons applied herbal cream (Dermacumin®, Veterinary Herbal Medicine; V.H.M., Antalya, TURKEY) twice daily (at least 2 pumps, similar to what has been described elsewhere) [3] for 3 weeks onto the affected lesions located on head and neck regions. The cream was apportioned in commercial bottles with a pump nozzle able to be used as a spray. The herbal cream included an equal combination of 90% pure *C. longa* and 85% pure *N.sativa*. Entire clinical evaluation were performed on days 0, 7, and 28 at the referring veterinary clinic. The Feline Dermatitis Extent and Severity Index (FeDESI) was used to score the clinical findings involving excoriations/erosions, erythema and self-induced alopecia (presented as self-induced depilation resulting from excessive grooming and licking) [4]. The owners assessed pruritus (defined as scratching) via a 10 cm visual analog scale (VAS) involving signifier [5] similar to another study [3]. The owners were asked to complete a questionnaire for the number of treatment applications, side effects, or other necessary information.

Data Interpretation

Feline Dermatitis Extent and Severity Index and VAS pruritus scores on days 7 and 28 were compared to day 0 for the assessment of therapeutic efficacy. The distribution of data was analyzed with the D'Agostino and Pearson normality test. Friedman's test with Dunn's post-hoc tests were the choice to analyze assess the differences among FeDESI scores. One-way repeated-measures ANOVA tests with Tukey post-hoc tests were performed to make interpretation of VAS pruritus scores. The correlation between FeDESI and VAS pruritus scores for each feline case during study period was assessed via Pearson's correlation test. Statistical software was used to analyze the data and present the results (Prism 6; Graphpad Inc., San Diego, CA, USA).

RESULTS

A total of 10 Fhnd case were involved. To those of cats enrolled 8 were domestic long hairs, 1 each a domestic short hair and a Scottish short hair. Regarding sex 3 were neutered males and the rest 7 were spayed females. At initial referral, the mean age was 3.1 years (SD 1.6 years), and the mean weight was 4.5 kg (SD 1.2 kg). None of the Fhnd cases were removed from the study or received antibiotic therapy.

Complete blood count and serum biochemistry (ALT, AST, urea, creatinine) were available on days 0 and 28 (completion of the study) for all cats involved in which abnormal values were not detected apart from leukocytosis evident in 2 cats. Endocrine panels involving plasma cortisol and total and free thyroxine values were checked for all cases, which determined reference ranges. Rapid diagnostic ELISA test kits gave negative results for feline immunodeficiency virus/feline leukemia and calicivirus antigens.

Post-hoc tests denoted that initial day (0) values were significantly elevated in contrast to scores at the end of weeks 1 and 4 *(Fig. 1* and *Fig. 2)*. Average FeDESI scores decreased from 171.8 to 0 ($P<0.0001$) with a significant difference in average VAS pruritus scores from 6.3 to 0 ($P<0.0001$) on days 0 to 28 respectively. As a brief explanation, two cats with Fhnd also had lesions in other body parts with a total score of 442 and 251 surprisingly, out of maximum FeDESI score of 165 for Fhnd *(Fig. 1* and *Fig. 2)*.

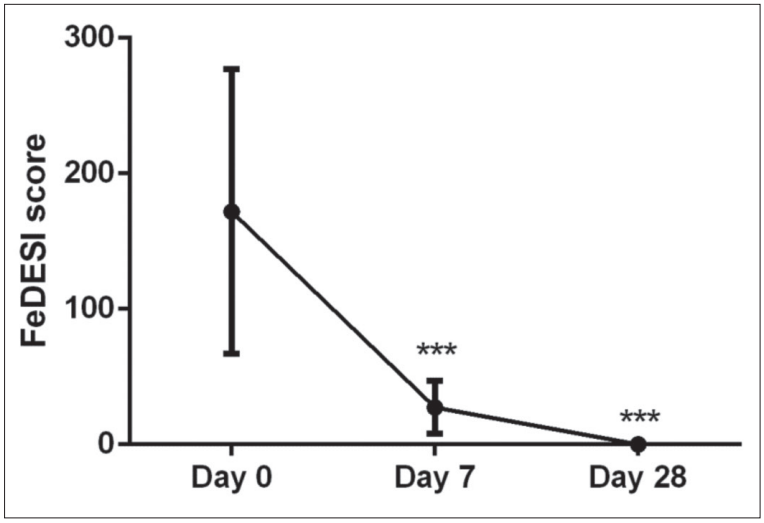
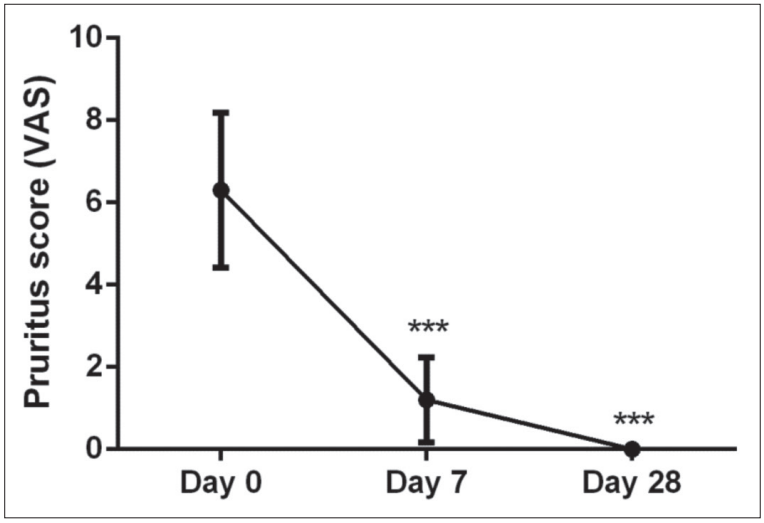


Fig 1. Changes in FeDESI score in cats treated with *Curcuma longa* and *Nigella sativa* (means ± SD)

Fig 2. Changes in VAS pruritus score in cats treated with *Curcuma longa* and *Nigella sativa* (means ± SD)



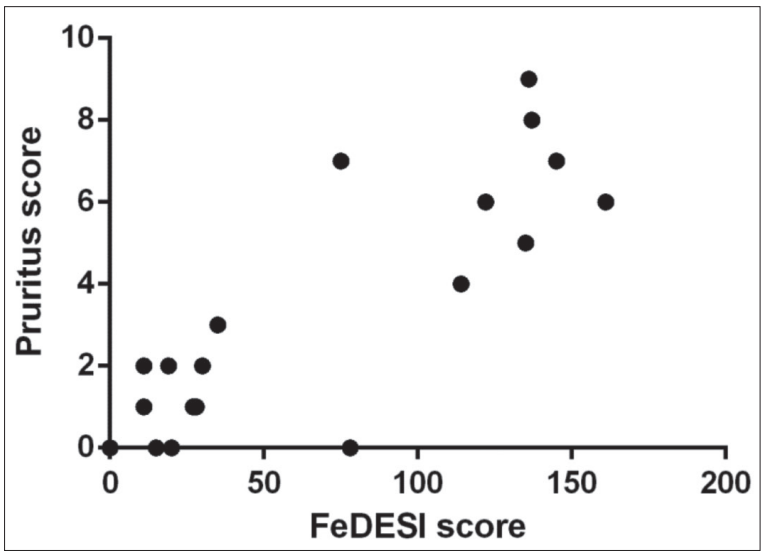


Fig 3. Correlation graph between the FeDESI score and VAS pruritus score for each cat at each time point

In addition, significant differences from days 7 to 28 were also evident, and all scores declined. Apart from lesional scores of each cat, it was evidenced that 9 out of 10 cats were effectively in clinical recovery by day 7, and all were cured without side effects on day 28. It is surprising and interesting to observe that the cats' scores went down to 0.

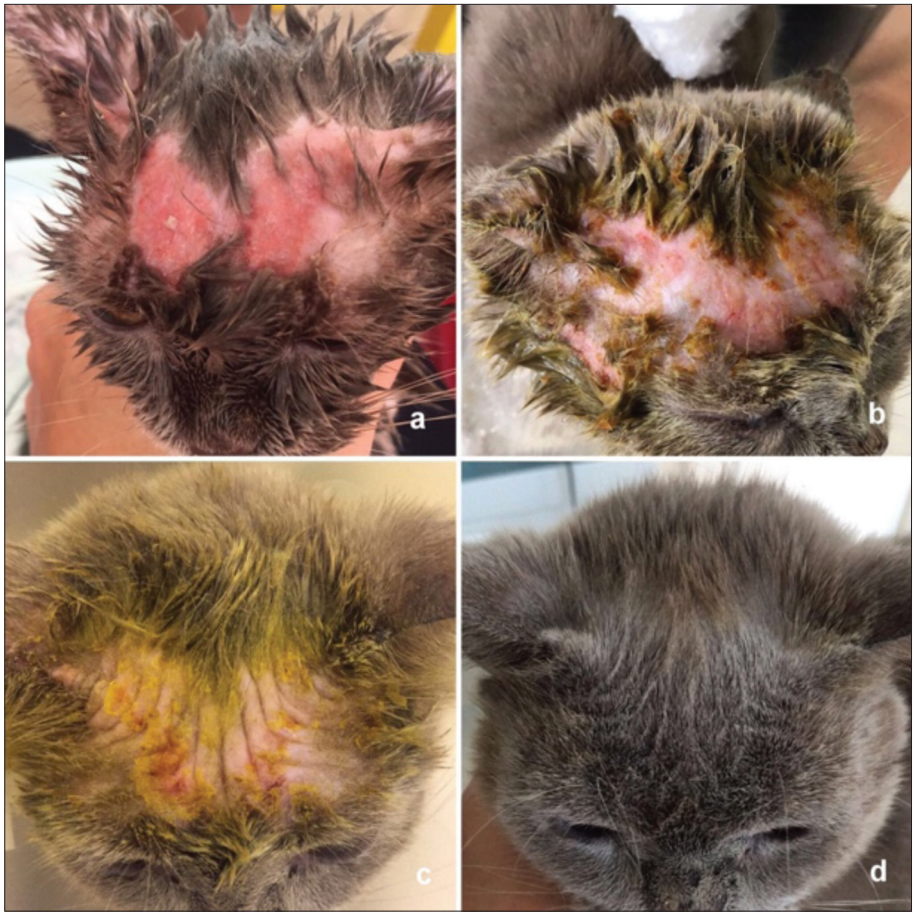
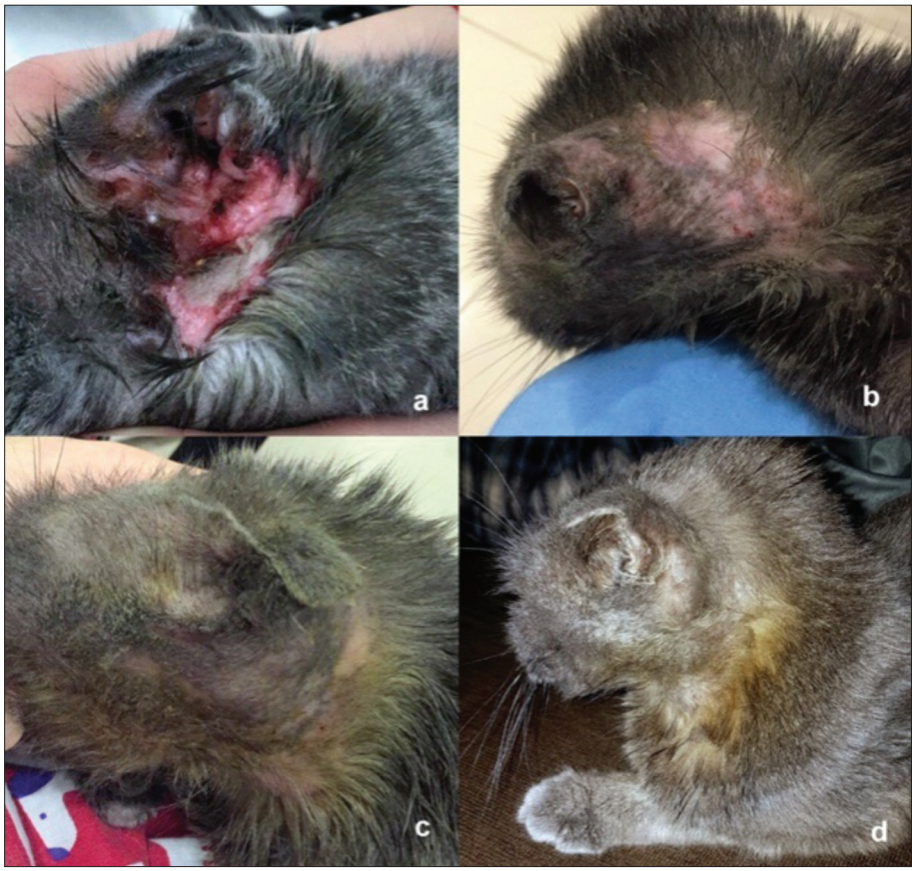


Fig 4. Feline head and neck dermatitis with erythema and alopecia respond to turmeric and black cumin cream treatment. a- Day 0, b- Day 3, c- Day 7, d- Day 28

Fig 5. Feline head and neck dermatitis with erosion, ulceration and partial alopecia respond to turmeric and black cumin cream treatment. a- Day 0, b- Day 3, c- Day 5, d- Day 6



The correlation between FeDESI and VAS pruritus scores for every Fhnd cases at study duration were assessed via Pearson's correlation test, which revealed a high positive correlation (r=0.62, P<0.001; *Fig. 3*).

There was no side effects dedicated to the curcumin and black cumin treatment in any of the cases. The bitter taste of black cumin was over whelmed by vanilla extract included in the present compound, and food binders allowed the patients to lick the cream without adverse effect. Clinical photographs of a selected case were shown in *Fig. 4* and *Fig. 5* describing before and after treatment records.

DISCUSSION

The present study denoted that a topical administration of *C. longa* (turmeric) and *N. sativa* (black cumin) emerged to be a highly effective therapeutical option for Fhnd. There were significant alterations in both pruritus and clinical lesions, as detected by FeDESI. All Fhnd cases (n=10) presented decreased both FeDESI [on days 7 (P<0.001) and 28 (P<0.0001) compared to day 0; from 171.8 to 0 (P<0.0001)] and VAS pruritus scores [on days 7 and 28 (P<0.0001); from 6.3 to 0 (P<0.0001)] at the time point dedicated to a remarkable clinical recovery in this study. The FeDESI and pruritus scores were strikingly have correlation, indicating both that the herbal cream caused alterations among relevant clinical signs and that the augmentation was to the authors' knowledge, relevant to the efficacy, which might be due to turmeric's anti-allergic [6] and anti-inflammatory [7] effects and is probably due to suppression of IgE-mediated allergic reaction and activated mast cells, as reported previously in different models [8]. For a better understanding, the efficacy of the present study's scoring systems should be discussed briefly in depth. In a prior investigation, 32 cats with allergic dermatitis and treated with cyclosporine were examined with both Scoring Feline Allergic Dermatitis (SCORFAD) and FeDESI prior to and after treatment. In that study, FeDESI and SCORFAD scores correlated well, whereas SCORFAD was less correlated with pruritus. According to the latter authors, FeDESI might be more suitable for use. Regarding allergic dermatitis in cats, SCORFAD might be used for assessing the severity of the disease [2]. On the other hand, FeDESI, another scoring system for evaluating hypersensitivity, was found easier to complete than SCORFAD [2] and was preferred as a global and intuitive scoring method in the present study. It may be briefly discussed that the assessment of pruritus in cats could not be easy. As was also reported and used in a prior study and adapted in the present study, the pruritus scale has been approved in dogs [5] but not for cats.

Data regarding turmeric evidenced that this compound is highly pleiotropic, able to interact with different molecular targets participating in inflammation [7,8]. Turmeric attenuates the inflammatory response by down regulating the activities of nitric oxide synthase, lipoxygenase, and cyclooxygenase-2 [8]. On the other hand, black cumin might possess immune-stimulatory, anti-inflammatory [9], and antiallergic [10] effects. Turmeric and black cumin caused complete recovery and totally decreased pruritus scores in this study. This effect might be comparable with cyclosporine, resulted in well clinical achievement ½ portion to all cats involved in that researches [11,12], or with hydrocortisone aceponate spray against allergic dermatitis in cats, where scores of initial day were momentously superior to the scores at weeks 2, 4,6 and 8 (P<0.0001) [3].

In the present study, the response to herbal cream therapy was accelerated, with the majority of the clinical recovery as evidenced by week 1. Clinical scores showed tendency to gradually boost there after in almost all of the cats. Available evidence suggested that this response was similar to that in feline allergic dermatitis, where 8 out of 10 cats achieved clinical remission by 28 days by use of a hydrocortisone aceponate spray [3].

In addition to the important limitation of a lacking control group (non-controlled study due to ethical concerns and the design of the research, as this was a pilot study), a major flaw is the small sample size of the present study. As previous studies were not available to confirm the researchers' hypothesis that assessed the clinical response in Fhnd over a 3-week period of following local turmeric and black cumin treatment, the study was designed to confirm or reject this hypothesis before embarking on a larger, more expensive study. There was no adverse event detected in the present study. Longer studies are promptly needed to see the full effects and/or adverse effects of turmeric and black cumin.

In conclusion, this herbal cream bona fides well efficacy and toleration in 10 cats with head and neck dermatitis. This herbal product has been licensed in Turkey for usage in cats and dogs. Further studies are warranted to support the findings of this small-scale open-label pilot study.

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Research Article

Effects of Exogenous Amylase in Transition Dairy Cows Fed Low-Starch Diets: 1. Lactation Performance

Hıdır GENÇOĞLU [1,a] Çağdaş KARA [1,b] Mukaddes Merve EFİL [1,c] Abdülkadir ORMAN [2,d] Yavuz MERAL [3,e] Elif KOVANLIKAYA [4,f] İsmail ÇETİN [5,g] Randy Duncan SHAVER [6,h] Erkan ŞEN [1,i] Tolga ALTAŞ [1,j]

[1] Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Bursa Uludag University, TR-16059 Bursa - TURKEY; [2] Department of Zootechnics, Faculty of Veterinary Medicine, Bursa Uludag University,TR-16059 Bursa - TURKEY; [3] DSM Animal Nutrition & Health Turkey, TR- 34810 Istanbul - TURKEY; [4] Department of Animal Science, Faculty of Agriculture, Bursa Uludag University, TR-16059 Bursa - TURKEY; [5] Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Tekirdag Namık Kemal University, TR-16059 Tekirdağ - TURKEY; [6] Department of Dairy Science, University of Wisconsin - Madison, 1675 Observatory Dr., Madison 53706, USA

[a] ORCID: 0000-0003-1067-2874; [b] ORCID: 0000-0003-2515-1211; [c] ORCID: 0000-0003-0646-9777; [d] ORCID: 0000-0001-9138-4422
[e] ORCID: 0000-0002-5592-3668; [f] ORCID: 0000-0002-1104-3693; [g] ORCID: 0000-0001-7589-4852; [h] ORCID: 0000-0002-0992-2728
[i] ORCID: 0000-0001-6267-2118; [j] ORCID: 0000-0002-7490-6177

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Abstract

The objective of this trial was to determine the effect of exogenous amylase during the transition period in dairy cows on dry matter intake and lactation performance. The effect of exogenous amylase supplementation on lactation diets with low starch concentration (19.5% of dry matter) and dry period diets with moderate starch concentration (15.5% of dry matter) was evaluated. A total of 30 multiparus Holstein cows were randomly assigned to two groups fed diets with (n=15) or without amylase (n=15). Treatments were granular amylase (0.5 g of Ronozyme RumiStar per kg of total mixed ration dry matter) or control. The research was conducted starting at 21 d prepartum until 84 d postpartum. Starch and neutral detergent fiber concentrations averaged 15.5±0.5% and 15.7±0.9%, 42.6±1.1% and 43.4±1.2% in close up diets and 19.8±2.9% and 19.4±0.5%, 33.6±0.8% and 34.2±0.6% in lactation diets for control and amylase, respectively. Dry matter intake, milk yield and composition were evaluated for differences between treatments. Postpartum intakes of dry matter (DMI) and organic matter (OM), neutral detergent fiber (NDF), crude protein (CP), and starch intake were unaffected by treatment. Milk yield was not influenced by treatment, but numerically greater by 2.0 kg/d for cows fed amylase compared with control diet. The percentages of milk fat, protein and lactose were not impressed by treatment, however fat-, solid-, and energy-corrected milk were 2 kg/d greater for cows fed amylase diet than for cows fed control diet. Fat-, solid-, and energy-corrected milk feed conversions (kg/kg DMI) were 5 to 6% greater for cows fed amylase diet than for cows fed control diet (P<0.01). It was concluded that inclusion of amylase improved the feed efficiency of lactating cows fed a low starch diet, may offer for potential to increase milk yield; but the enzyme did not affect DMI.

Keywords: *Amylase, Dry matter intake, Milk production, Feed efficiency, Dairy cows*

Düşük Nişastalı Rasyonlarla Beslenen Geçiş Dönemindeki İneklerde Amilaz Enziminin Etkisi: 1. Laktasyon Performansı

Öz

Bu araştırmanın amacı geçiş dönemindeki ineklerin rasyonlarına amilaz enzimi ilavesinin kuru madde tüketimi ve laktasyon performansı üzerine etkisini incelemektir. Rasyonların nişasta düzeyi kuru madde esasına göre kuru dönemdeki hayvanlar için %15.5, laktasyon dönemindekiler için ise %19.5 olarak tespit edildi. Araştırmada birden fazla doğum yapmış 30 baş siyah alaca ırkı inekler rastgele amilaz (n=15) ve kontrol (n=15) gruplarına dağıtıldı. Araştırma kontrol ve amilaz (0.5 g Ronozyme RumiStar/kg toplam karma rasyon kuru madde) grupları şeklinde oluşturuldu. Deneme doğumdan önceki 21 gün ile doğumdan sonraki 84. günler arasında yürütüldü. Kuruda bulunan kontrol ve amilaz grubundaki rasyonların nişasta ve nötral deterjant fiber (NDF) içerikleri sırasıyla %15.5±0.5 ve %15.7±0.9 ile %42.6±1.1 ve %43.4±1.2 iken laktasyondakilerde ise %19.8±2.9 ve %19.4±0.5 ile %33.6±0.8 and %34.2±0.6 arasında tespit edildi. Doğum sonrası kuru madde tüketimi, organic madde, NDF, ham protein, nişasta düzeyleri bakımından gruplar arasında bir farklılık bulunmamıştır. Süt verimi uygulamadan etkilenmedi ancak sayısal olarak amilaz ile beslenen ineklerde 2 kg/gün daha fazla süt elde edildi. Süt yağı, proteini ve laktoz düzeyleri denemeden etkilenmedi, ancak yağa, katı maddeye ve enerjiye gore düzeltilmiş süt verimleri amilaz ile beslenen ineklerde 2 kg/gün daha fazla süt üretildi. Yağa, katı maddeye ve enerjiye gore düzeltilmiş süt verimlerinin yeme dönüşüm oranları (kg/kg kuru madde tüketimi) amilaz ile beslenen ineklerde kontrol grubuna gore %5-6 daha yüksek bulundu. Sonuç olarak düşük nişasta içeriğine sahip rasyonlarla beslenen geçiş dönemindeki ineklerde amilaz enzimi ilavesinin yemden yararlanmayı iyileştirdiği, süt veriminde potansiyal bir artış sağlayabileceği; ancak kuru madde tüketimini etkilemediği tespiti yapılmıştır.

Anahtar sözcükler: *Amilaz, Kuru madde tüketimi, Süt verimi, Yemden yararlanma, Süt ineği*

İletişim (Correspondence)
+90 224 2941363 Mobile: +90 532 2969034
gencoglu@uludag.edu.tr

INTRODUCTION

Carbohydrates are the main source of energy, typically providing over half of the energy in ruminant diets [1]. Moreover, intensive production systems for lactating dairy cows are based on starch as the primary source of energy from carbohydrates. Starch contributes approximately 50 and 75% of the energy value of corn silage and grain, respectively [2]. High grain prices have enhanced the interest in feeding reduced-starch diets. There has been interest in feeding low-starch diets with improved starch digestibility because of high corn prices [3]. Therefore, improving starch utilization can reduce feed costs by reducing starch in diets or increase income by increasing milk production. In addition, there are some feed additives [4] and treatments [5] applied for providing improvements in performance of transition dairy cows.

The starch level of diets for lactating dairy cows is not well defined in dairy herds in Turkey. However, we estimated that dietary starch concentrations ranging from 19 to 26% in total mixed ration (DM basis) in Turkey. Total-tract digestibility of starch is variable ranging from 70 to 100% in dairy cows [6] and it was positively related to ruminal and postruminal starch digestibilities [7]. Various feed-related factors influence the digestibility of corn starch by dairy cows including: particle size, processing method, harvest/storage method and maturity, and endosperm type for grain, chop length, and kernel processing for corn silage [7,8]. However, increased ruminal starch digestion can undesirably induce ruminal acidosis, thereby leading to reduction in ruminal microbial synthesis, milk yield and composition.

Some exogenous amylases are resistant to ruminal degradation, and thus can increase diet digestibility in ruminants [9,10]. Several studies have reported that exogenous amylase resistant to ruminal degradation increased organic matter (OM) digestibility in dairy [3,10] and feedlot cattle [11]. The addition of exogenous amylase to the diets of lactating dairy cows has the potential to improve animal productivity. Exogenous amylase addition to TMR of dairy cows has increased milk yield by up to 3.9 kg/cow/d, and positive *in vitro* and *in vivo* digestibility responses to exogenous amylase were also reported [10]. Gencoglu et al.[3] found that adding exogenous amylase to low-starch diets (21% DM) increased feed efficiency when compared with a low-starch diet without amylase (1.77 vs 1.98 kg milk/kg DMI).

The effects of amylase supplementation on lactation performance, DMI, and feed efficiency of lactating cows have been inconsistent. The supplementation of amylase to reduced starch diet (21%) increased milk production, had no effect on DMI, and tended to increase feed efficiency [12]. Gencoglu et al.[3] observed a decreasing in DMI, no effect on milk production, an increase in feed efficiency when cows fed 21% starch diet was supplemented with amylase. Weiss et al.[13] indicated that supplemented amylase to cows fed a 26% starch diet did not detect changes in milk yield, DMI, and feed efficiency.

Low starch diets may be an economic alternative when grain prices are high. The effect of amylase addition to diets with a much lower starch concentration (19.5%) has not been evaluated. Therefore, the objective of the trial was to determine the effect of exogenous amylase during the transition period on dry matter intake (DMI), and lactation performance in dairy cows fed low starch diet. The fact that changing of rumen fermentation with adding of exogenous amylase would improve feed efficiency and lactating performance of transition dairy cows was hypothesized.

MATERIAL and METHODS

The experiment was conducted from January 2011 through August 2011 at Omer Matli Research Center (Karacabey, Bursa Turkey). All the procedures were approved by the Bursa Uludag University, Animal Experiments Local Ethics Committee (Committee Number and Date: 2010-07/02 and 02.11.2010). Thirty (30) multiparous Holstein cows were randomly assigned to with or without (control) exogenous amylase groups in a completely randomized design. Current lactation numbers for control and amylase cows were 2±0.3 and 2±0.4, respectively. Previous lactation 305-d milk yields for control and amylase cows were 8289±1322 and 8332±1779 kg, respectively. Disease incidences for control and amylase cows, respectively, were retained placenta (1 vs. 0), milk fever (2 vs. 1), ketosis (4 vs. 5), dystocia (0 vs. 2), and mastitis (2 vs. 3).

The research was conducted starting at 21 d prepartum until 84 d postpartum. Cows were housed in a free-stall barn and fed diets as a total mixed ration (TMR) with an automatic feeding door system. At 35 d prior to the expected calving date, cows were assigned to their respective diets and housed for adaptation to the automatic feeding door system 2 week before initiation of the experimental period. Cows were housed in individual maternity pens from parturition until 4 days in milk and then cows were moved to free-stall housing equipped with an automatic feeding door system. Cows were fed individually the TMR once daily (0800 h) to allow for *ad libitum* consumption and animals were allowed access to feed at all times, except during milking times. The orts samples were collected to determine dry matter intake (DMI) during the trial period (from d 21 prepartum to d 84 postpartum). Ingredient composition of the experimental diets is in *Table 1*. The all diets were formulated to comply with recommendations from NRC 2001 [2].

The control diet did not contain exogenous amylase. The amylase diet was fed with exogenous amylase addition to the concentrate mixtures. A granular amylase formulation, Ronozyme RumiStar (Lot number: 600 (CT) AU360001) with an amylase activity of 600 Kilo Novo Units (KNU) per g

Table 1. *Ingredient composition of the diets (As dry matter basis)*

Ingredients	-21 to 0 DIM	1 to 84 DIM
Corn silage	27.7	28.3
Alfalfa Hay	7.2	19.1
Wheat Straw	31.7	2.6
Corn	5.3	10.5
Soybean meal-47%	7.4	11.3
Corn gluten meal-60%	0.0	1.8
Sunflower meal-30%	0.5	0.0
Full fat soybean	3.3	4.5
Wheat bran	9.5	11.2
Rice bran	3.5	5.3
By pass fat[1]	1.0	1.5
Molasses	1.7	2.5
Calcium carbonate	0.00	1.05
Salt	0.00	0.17
Vitamin-Mineral Premix[2]	0.36	0.18

[1] Minimum 99.5% total fat content (Ecolex, Malaysia)
[2] Supplied per kilogram of vitamin-mineral premix : Vit. A 12.000.000 IU, Vit. D_3 3.000.000 IU, Vit. E (dl-α-Tokoferol Asetat) 35 g, Mn 50 g, Fe 50 g, Zn 50 g, Cu 10 g, I 0.8 g, Co 0.15 g, Se 0.3 g, Antioxidant 10 g

provided by DSM Nutritional Products (Basel, Switzerland) was used for this study. The targeted dosage of 300 KNU/kg of the total mixed ration (TMR) dry matter (DM) in amylase diet was achieved by adding 1 g of Ronozyme RumiStar per kg of concentrate mixture (as-fed basis). The control and amylase concentrate mixtures were prepared as pelleted feed (pelleting temperature 65°C) by Matli Feed Co. (Karacabey, Turkey). The pelleted concentrates of control and amylase were sampled every 4 week, stored at −20°C, and then sent to DSM Nutritional Products Analytical Services Center (Basel, Switzerland) for analysis of amylase activity [14]. Determined amylase activities for control and amylase pelleted concentrates mixtures were 0±0, and 606.9±53.4 KNU/kg (as-fed basis), respectively. The treatment TMR for lactating cows averaged 303.4±27 KNU/kg of DM, which was similar to the targeted dosage of 300 KNU/kg of DM recommended by DSM Nutritional Products and the dosage used in the trials of Gencoglu et al.[3], and Klingerman et al.[10], Ferraretto et al.[12].

Cows were milked 3 times daily at 0600, 1400, and 2200 h, and daily milk production was recorded at each milking. Milk samples were obtained from all cows weekly from all milkings on the same 2 consecutive days throughout the 12-wk lactation period of the trial and analyzed for fat, protein, and lactose concentrations by infrared analysis (Omer Matli Research Center, Karacabey, Turkey) using a Bentley 150 (near infrared spectroscopy; Bentley 150 Infrared Milk Analyzer, Bentley Instruments, Chaska, MN) with average daily yields of fat, protein, and lactose calculated from these data for each week. Yields of 3.5% fat-corrected milk (FCM), solid-corrected milk (SCM), and energy-corrected milk (ECM) were calculated according to NRC 2001 [2] equations. Actual-milk, FCM, SCM, and ECM feed conversions were calculated by week using average daily yield and DMI data.

The TMR, concentrate mixtures, corn silage, and alfalfa hay were sampled weekly and composited by 3-week periods and analyzed for dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF), starch, sugar, ether extract, ash, and energy values were calculated from the analytical data using NRC 2001 [2] equations. The composited samples were dried in a forced-air oven at 60°C for 48 h for measurement of DM content and then ground through a 1 mm diameter screen using a laboratory 3303 Mill (Hundenge, Sweden). Crude protein was determined by the Kjeldahl method [15]. Ash was determined by combustion at 550°C for 6 h. The NDF, acid detergent fiber (ADF), and lignin contents were determined using the methods described by Van Soest et al.[16] with heat-stable amylase (Sigma No: A-3306, Sigma Chemical Co., St Louis, MO, USA) and sodium sulfite used in the NDF procedure. Starch was measured on composited samples as described by Bal et al.[17].

Data were analyzed as a completely randomized design using the Linear Mixed Model of SPSS (SPSS 13.0, 2004). The model included treatment, time, and treatment × time interaction as Fixed effects and cow within treatment as a Random effect. The REML (Restricted Maximum Likelihood) was the chosen estimation method. Means were determined using the least squares means statement. Statistical significance and trends were considered at $P \leq 0.05$ and $P > 0.05$ to $P < 0.10$, respectively.

RESULTS

The nutrient composition of corn silage, alfalfa hay, wheat straw and concentrates mixtures during the study and the particle size of the corn silage are in *Table 2*. Corn silage and alfalfa hay were of good quality with 36.6% DM and 30.8% starch (DM basis) in corn silage and 16.4% CP and 50.1% NDF (DM basis) in alfalfa hay. Concentrate mixtures fed to control and amylase cows showed similar nutrient concentrations. The nutrient composition and particle size of diets (close up and lactating) are in *Table 3*. The nutrient concentrations and particle size of close up and lactating diets were similar for control and amylase.

Treatment effects on least squares means for DM and nutrient intakes of lactation cows are presented in *Table 4*. Intakes of DM, OM, NDF, and CP were unaffected treatment ($P > 0.10$). Least squares means by week on treatment for DMI are presented in *Fig. 1*; week ($P > 0.10$) and week × treatment ($P > 0.10$) interaction effects did not differ.

Treatment effects on lactation performance are in *Table 5*. Milk yield was unaffected by treatment ($P > 0.10$), but

Table 2. *Nutrient composition and particle size of corn silage, alfalfa hay, and concentrate mixtures*					
Item	**Corn Silage**	**Alfalfa Hay**	**Wheat Straw**	**Concentrate Without Amylase**	**Concentrate With Amylase**
DM, % as fed	36.6±1.4	91.8±0.6	92.0±0.2	90.6±0.4	90.3±0.3
% DM					
OM %	93.9±2.4	89.6±0.3	93.4±0.5	91.7±0.2	91.4±0.2
CP %	8.6±3.6	16.4±1.2	3.7±0.4	25.0±0.2	25.1±0.2
NDF %	46.3±2.0	50.1±0.9	83.7±6.6	21.9±0.4	22.0±0.3
Starch %	30.8±2.2	2.4±0.6	0.6±0.8	21.7±0.3	21.5±0.5
Particle Size[1]	**% As Fed Retained**				
19 mm	3.2±1.0	-------	-------	-------	-------
8 mm	49.2±1.4	-------	-------	-------	-------
1.18 mm	44.7±1.4	-------	-------	-------	-------
Pan	2.9±0.5	-------	-------	-------	-------

[1] Determined as described by Kononoff et al.[18]

Table 3. *Diet nutrient composition and particle size[1]*				
Item	**Control**	**Amylase**	**Control**	**Amylase**
Nutrients	**Close Up**		**Lactating**	
DM, % as fed	71.2±1.1	71.6±0.9	62.7±1.5	62.5±1.7
% DM				
CP %	14±0.4	13.9±0.8	17.2±0.9	17.3±0.9
Ether Extract %	4.5±0.3	4.6±0.4	6.6±0.6	6.5±0.6
NDF %	42.6±1.1	43.4±1.2	33.6±0.8	34.2±0.6
NFC %	30.3±0.8	30.1±0.9	34.5±1.8	34.1±2.2
Starch %	15.5±0.5	15.7±0.9	19.8±2.9	19.4±0.5
TDN_{1X}[3]	67.4±0.8	67.7±0.5	----------	----------
NEL_{3x}, Mcal/kg[3]	----------	----------	1.72±0.4	1.72±0.2
Penn State Separator Sieves[4]	**% As Fed Retained**			
19 mm	41.8±3.2	43.0±4.2	10.9±2.5	9.5±4.7
8 mm	28.1±6.3	27.6±4.1	46.4±5.8	48.0±8.7
1.18 mm	24.1±3.2	24.2±2.5	31.0±5.0	31.6±3.9
Pan	6.0±0.3	5.3±0.3	11.7±0.9	10.9±2.1

[1] Treatments were pelleted concentrate mixture without amylase (Control) and with amylase (Amylase), [2] NFC: Nonfiber carbohydrate, %; calculated as: 100 - (NDF, % + CP, % + EE, % + ash, %), [3] Calculated using NRC (2001) summative energy equation, [4] Determined as described by Kononoff et al.[18]

Table 4. *Effect of treatment on least squares means for DM and nutrient intakes in early lactation cows[1]*				
Item	**Control**	**Amylase**	**SEM[2]**	**P**
DMI, kg/d	17.7	17.9	0.2	NS[3]
OM Intake, kg/d	14.9	14.8	0.2	NS
NDF Intake, kg/d	5.5	5.5	0.1	NS
CP Intake, kg/d	2.8	2.8	0.03	NS
Starch Intake, kg/d	3.2	3.2	0.04	NS

[1] Treatments were pelleted concentrate mixture without amylase (Control) and with amylase (Amylase), [2] Standard error of the mean. [3] Non significant

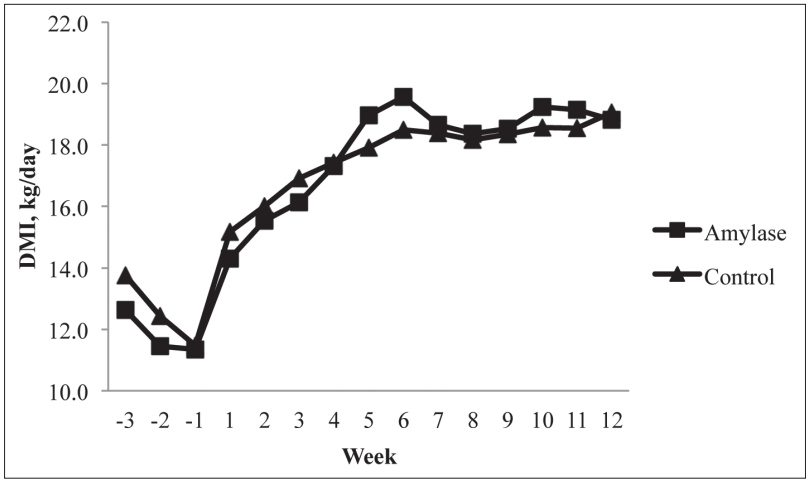


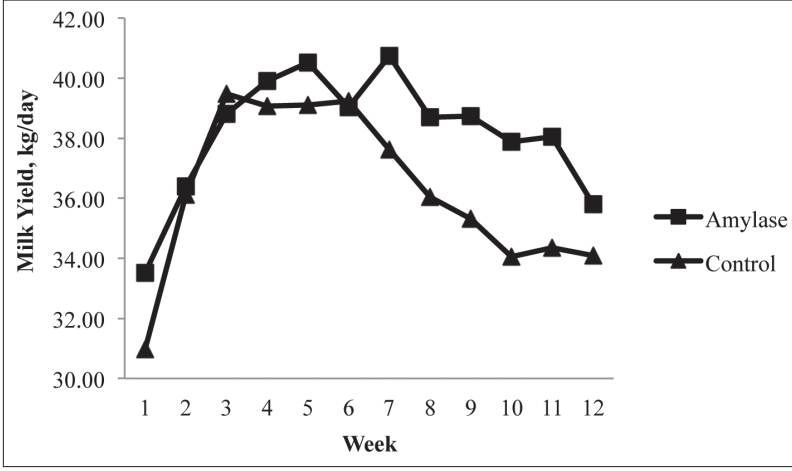
Fig 1. Effect of treatment on DMI (kg/d) least squares means by week on treatment. Treatments were pelleted concentrate mixture without amylase (Control) and with amylase (Amylase)

Table 5. *Effect of treatment on least squares means for DMI of prepartum and early lactation cows and early-lactation lactation performance*[1]

Item		Control	Amylase	SEM[2]	P
DMI Prepartum (kg/day)		12.5	11.5	0.4	NS[3]
DMI Postpartum (kg/day)		17.7	17.9	0.2	NS
Milk Yield (kg/d)		36.3	38.2	0.7	NS
3.5% FCM, kg/d[4]		32.7	34.7	0.4	0.001
SCM, kg/d[5]		32.7	34.9	0.3	0.001
ECM, kg/d[6]		35.4	37.7	0.3	0.001
Fat,	%	3.31	3.42	0.06	NS
	kg/d	1.20	1.30	0.03	0.01
Protein	%	3.08	3.07	0.03	NS
	kg/d	1.12	1.17	0.01	0.01
Lactose	%	4.93	4.97	0.02	NS
	kg/d	1.79	1.90	0.02	0.01
kg Milk/kg DMI		2.05	2.15	0.03	0.01
kg 3.5% FCM/kg DMI		1.84	1.97	0.04	0.005
kg SCM/kg DMI		1.85	1.98	0.03	0.004
kg ECM/kg DMI		2.01	2.14	0.04	0.004

[1] Treatments were pelleted concentrate without amylase (Control) and with amylase (Amylase); [2] Standard error of the mean; [3] Non significant; [4] Fat-corrected milk calculated according to NRC (2001) equations; [5] Solids-corrected milk calculated according to NRC (2001) equations; [6] Energy-corrected milk calculated according to NRC (2001) equations

Fig 2. Effect of treatment on milk yield (kg/d) least squares means by week on treatment. Treatments were pelleted concentrate mixture without amylase (Control) and with amylase (Amylase)



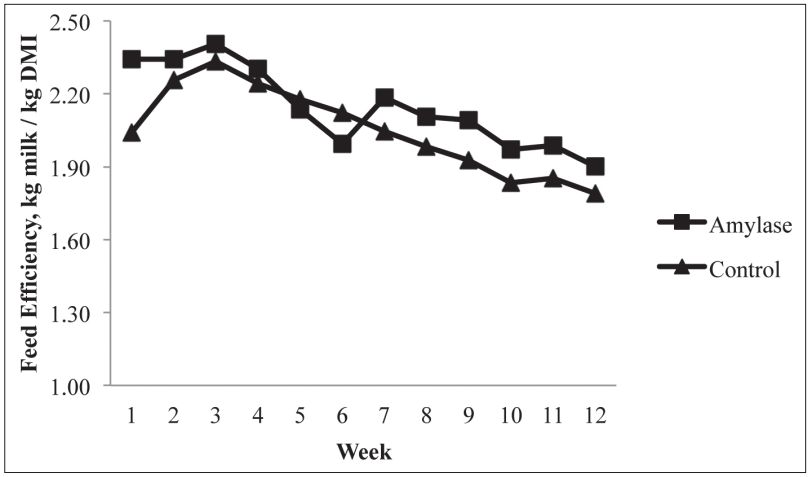
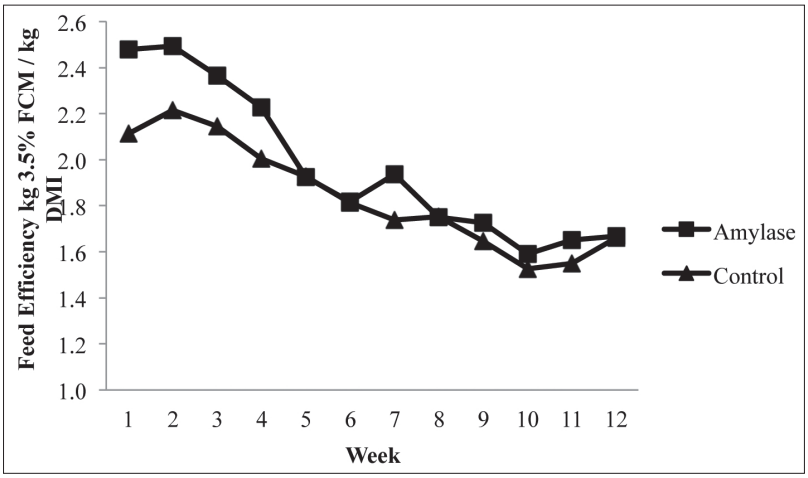


Fig 3. Effect of treatment on feed efficiency (kg of milk yield/kg of DMI) least squares means by week on treatment. Treatments were pelleted concentrate mixture without amylase (Control) and with amylase (Amylase)

Fig 4. Effect of treatment on feed efficiency (kg of 3.5% FCM milk yield/kg of DMI) least squares means by week on treatment. Treatments were pelleted concentrate mixture without amylase (Control) and with amylase (Amylase)



was numerically greater by 2.0 kg/d for cows fed amylase compared to control.

Effect of treatment on milk yield (kg/d) least squares means by week are in *Fig. 2*. Cows fed amylase diet produced more milk than cows fed control in first week of lactation (31.0 vs 33.5 kg/d) but cows fed control and amylase produced a similar amount of milk from 2 to 6 week of lactation.

The FCM, SCM and ECM yields for cows fed amylase were 2.0 ($P<0.001$), 2.2 ($P<0.001$), and 2.3 ($P<0.001$) kg/d greater than for cows fed control. Feed efficiency of the FCM, SCM and ECM was 6.5% greater for amylase than control in the current study. Least squares means by week on treatment for feed efficiency (kg of milk yield/kg of DMI, $P<0.01$) and feed efficiency (kg of 3.5% FCM milk yield/kg of DMI, $P<0.005$) are presented in *Fig. 3* and *Fig. 4*, respectively.

The percentages of milk fat, protein and lactose were unaffected by treatment. However, yields of milk fat, protein and lactose for cows fed amylase were greater ($P<0.001$) than for cows fed control.

DISCUSSION

The DMI was unaffected by treatment in the present study. Average prepartum DMI was not affected by treatment and averaged 12.0 kg/d. Prepartum decrease in DMI was greater for cows fed amylase relative to control cows *(Fig. 1)*. On the other hand, postpartum DMI was not affected by the addition of amylase, averaging 17.8 kg/d during the 84 days in milk. A similar treatment effect was observed for DMI by [19,20] study, they observed no difference in short-term DMI in response to exogenous amylase. Although that is not significant, DMI tended to be lower by about 5% for reduced starch diet with amylase compared to reduced starch diet without amylase [12]. Weiss et al.[13] and McCarthy et al.[21] reported no differences in DMI between reduced starch diets with or without amylase. Exogenous amylase addition to normal starch diets (approximately 26% to 27% DM) has resulted in either similar [13,22] or increased [10] DMI. Similar DMI between control and amylase was consistent throughout the experimental period.

The supplementation of amylase to reduced-starch diets (21% on DM basis) has either not affected [12] or reduced [3,23] DMI. Gencoglu et al.[3] reported that DMI for cows fed by the reduced-starch (21% on DM basis) diet without amylase was 3.2 kg/d greater than for cows fed the reduced starch diet with amylase and thus both of NDF and starch intake was greater for cows fed without amylase reduced starch

diet. In addition, the expected increase in DMI might not be observed as the heat stress actualized in the last term of trial as shown *Fig. 1*.

Milk yield was unaffected by treatment (P>0.10), but was numerically greater by 2.0 kg/d for cows fed amylase compared to control. This observation is in agreement with the reports of Gencoglu et al.[3], Ferraretto et al.[12], Weiss et al.[13], and DeFrain et al.[20] where exogenous amylase addition did not affect milk yields. McCarthy et al.[21] reported a tendency for 1.6 kg/d decreased milk production by cows supplemented with amylase (activity of 351 KNU/kg). On the other hand, exogenous amylase supplementation increased milk yield in experiments of Klingerman et al.[10], Tricarico et al.[22], Harrison and Tricarico [24]. In the current trial, the numerically increased milk yield for cows fed amylase versus control may be related to the greater ruminal total volatile fatty acid (VFA) concentrations. Seymour et al.[25] reported that milk yield was most highly related to ruminal concentrations of butyrate (r^2=0.47) and propionate (r^2=0.23).

Cows fed amylase diet produced more milk than cows fed control in first week of lactation (31.0 vs 33.5 kg/d, *Fig. 2)* but cows fed control and amylase produced a similar amount of milk from 2 to 6 week of lactation. Milk production decreased in all cows during 6 to 12 week of lactation. Decrease in milk production may attributed to the occurrence of heat stress in dairy cows [26]. In the current study, four cows in each group entered the trial the end of April, and heat stress could have affected these cows during the July and August. Orman and Ogan [27] observed that the seasons affect milk production in the Northwest of Turkey. As shown *Fig. 2*, milk production more sharply decreased for cows fed control than for cows fed amylase.

The FCM, SCM and ECM yields for cows fed amylase were 2.3, 2.2, and 2.3 kg/d greater than for cows fed control. The gain in feed efficiency has been driven by increased milk yield at same DMI. Klingerman et al.[10] and Tricarico et al.[22] and reported greater FCM yields with exogenous amylase addition to normal-starch diets (26% on dry matter basis). However, Gencoglu et al.[3], Ferraretto et al.[12], and Vargas-Rodriguez et al.[28] reported similar FCM, SCM and ECM yields for reduced-starch diets with and without exogenous amylase addition. However, McCarthy et al.[21], reported less FCM for reduced starch diet with amylase than reduced starch diet without amylase.

Feed efficiency of the FCM, SCM and ECM was 6.5% greater for amylase than control in the current study. Klingerman et al.[10] reported greater feed efficiency with exogenous amylase supplementation in normal-starch diets, and other researchers [3,12,19] reported greater feed efficiency with exogenous amylase supplementation in low-starch diets. Weiss et al.[13] reported that addition of the exogenous amylase to a low-starch diet had no effect on feed efficiency.

The percentages of milk fat, protein and lactose were unaffected by treatment. However, yields of milk fat, protein and lactose for cows fed amylase were greater than for cows fed control. Addition of exogenous amylase did not affect milk fat percentage for normal-starch [10,22,24] or reduced-starch diets [3,13]. A tendency was found for milk protein percentage to be greater for cows fed reduced starch diet with amylase (3.06 versus 2.99%; P<0.10) compared to increased milk protein percentage (P=0.006, respectively) cows fed reduced starch diet without amylase [3]. McCarthy et al.[21] reported greater milk protein percentage (P=0.006) for cows fed reduced starch with amylase (22.9% as dry matter basis) compared to reduced starch without amylase (23.7% as dry matter basis). Addition of exogenous amylases to normal-starch diets did not affect milk protein percentage in the trials of Klingerman et al.[10], Tricarico et al.[22], Harrison and Tricarico [24]. Ferraretto et al.[12] reported that exogenous amylase addition to reduced-starch diets did not affect (P>0.10) milk protein percentage. Milk lactose content was unaffected by treatment in other trials with exogenous amylase addition [3,12,21].

Cows fed low starch diet with addition of exogenous amylase to either diet, resulted in similar intakes of DM, OM, NDF, and CP but greater FCM, SCM, and ECM yields. It was concluded that inclusion of amylase improved the feed efficiency of lactating cows fed low starch diet; but the enzyme did not affect DMI.

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Research Article

Optimization of Entrapment Substances for Microencapsulation of *Lactobacillus plantarum* and *Lactobacillus casei* Shirota Against Gastric Conditions

Emel UNAL TURHAN [1,a]

[1] Osmaniye Korkut Ata University, Kadirli Applied Sciences School, Department of Food Technology, TR-80760 Osmaniye - TURKEY

[a] ORCID ID: 0000-0002-0284-574X

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Abstract

Microencapsulation is a promising method that has considerable effects on protection of probiotic viability. A variety of coating materials have been utilized to enhance the stability of probiotic microorganisms during the transition through gastrointestinal tract. The aim of this research was to determine optimum coating material combinations for probiotic microencapsulation against gastric conditions. Fructooligosaccharides, peptide, sodium alginate, gelatin and gellan gum were used as entrapment substances to microencapsulate *Lactobacillus plantarum* and *Lactobacillus casei* Shirota with extrusion technique. The response surface technique was applied to detect the optimum proportion of encapsulation substances against gastric condition. Microencapsulation protected probiotic cultures against stress factors such as simulated gastric juice and bile-salt solution. Optimum rate of encapsulation substances varied according to the type of probiotic bacteria. Test results showed that *L. plantarum* should be coated with 1.5% alginate, 0.92% gellan gum, 0.18% gelatin, 0.36% peptide and 1.31% fructooligosaccharides for highest protection. *L. casei* Shirota should also be coated with 2% alginate, 0.98% gellan gum, 0.51% gelatin, 0.86% peptide and 1.98% fructooligosaccharides for highest protection. This research concluded that microencapsulation with encapsulation materials at optimum concentration provided improved protection for the probiotics.

Keywords: *Lactobacillus casei Shirota, Lactobacillus plantarum, Microencapsulation, Response surface method, Extrusion*

Lactobacillus plantarum ve *Lactobacillus casei* Shirota'nın Gastrik Koşullara Karşı Mikroenkapsülasyonu İçin Kaplama Materyallerinin Optimizasyonu

Öz

Mikroenkapsülasyon, probiyotik canlılığının korunması üzerinde önemli etkileri olan umut verici bir yöntemdir. Probiyotik mikroorganizmaların gastrik koşullara karşı dayanımını arttırmak için çeşitli kaplama materyallerinden yararlanılmıştır. Bu çalışmanın amacı gastrik koşullara karşı probiyotik mikroenkapsülasyonu için ideal kaplama materyali kombinasyonunu belirlemektir. Fruktooligosakkarit, peptit, sodyum aljinat, jelatin ve gellan gam ekstrüzyon tekniği ile *Lactobacillus plantarum* ve *Lactobacillus casei* Shirota'yı mikroenkapsüle etmek için tutuklayıcı maddeler olarak kullanılmıştır. Gastrik koşullara karşı enkapsülasyon materyallerinin ideal oranları cevap yüzey tekniği ile elde edilmiştir. Mikroenkapsülasyon işlemi yapay gastrik su ve safra tuzu çözeltisi gibi stres faktörlerine karşı probiyotik kültürleri korumuştur. Kaplama materyallerinin ideal oranları probiyotik bakteri türüne göre değişmiştir. Test sonuçları yüksek düzeyde koruma için *L. plantarum*'un %1.5 aljinat, %0.92 gellan gam, %0.18 jelatin, %0.36 peptit ve %1.31 FOS ile kaplanması gerektiğini göstermiştir. Yüksek düzeyde koruma için *L. casei* Shirota ise %2 aljinat, %0.98 gellan gam, %0.51 jelatin, %0.86 peptit ve %1.98 FOS ile kaplanmalıdır. Bu araştırma, en uygun konsantrasyonda kaplama materyalleri ile mikroenkapsülasyonun, probiyotiklerin canlılığını iyileştirdiği sonucunu çıkarmıştır.

Anahtar sözcükler: *Lactobacillus casei Shirota, Lactobacillus plantarum, Mikroenkapsülasyon, Cevap yüzey tekniği, Ekstrüzyon*

INTRODUCTION

Probiotics have numerous useful properties on human health. Because of their beneficial effects, probiotic cultures often used in several functional food products [1]. As a matter of fact, there is a recent trend towards consumption

İletişim (Correspondence)
+90 328 8880090 Fax: +90 328 8880091
emelunalturhan@gmail.com

of functional foods worldwide [2-4]. World Health Organization (WHO) reported that probiotics are live microorganisms that, "when administered in sufficient amounts, confer a health benefit on the host" [5]. *Lactobacillus* and *Bifidobacterium* species have been the most commonly known probiotics and play an important role in the function and integrity of the intestinal ecosystem and the immune system [6-8]. Especially, *L. plantarum* and *L. casei* Shirota are the most popular and often performed probiotics in food production.

Probiotic bacteria have to survive during gastric transit and have positive effects on health of the host. Maintenance of probiotic viability at the time of consumption and ensuring of sufficient probiotic amounts are challenges on probiotic manufacturers [9,10]. Especially stress factors such as low pH, enzymes and bile salts in gastrointestinal system lead to a negative impact on probiotic robustness and performance. Inhibitory activity of stomach acid and bile salt should be overcome to retain probiotic viability and functionality [9,11]. For the therapeutic effects of probiotics, viable cell counts should be higher than or equal to10^7 CFU/g or mL of product and probiotic bacteria should be able to survive under gastrointestinal conditions [12,13]. In this case, microencapsulation technique is an alternative and effective strategy to protect survival of probiotics against hard conditions. The promised health benefits of probiotics were achieved with microencapsulation [14,15]. Two methods often used for microencapsulation are emulsion and extrusion. Extrusion method has many advantages that it is simple and inexpensive method with gentle operations, does not involve deleterious solvents, does not cause probiotic cell injuries and can be done under aerobic and anaerobic conditions [15,16]. In extrusion technique, probiotic bacteria are added into the hydrocolloid solution (mostly alginate) for entrapping in the gel matrix and then the cell suspension is passed through the syringe needle to form droplets, which free-fall into the solidification solution [8].

There are differences in the characteristics of probiotic strains and the right encapsulation method should be selected for each probiotic [13]. The physicochemical properties of the capsules have a significant impact on the viability of microencapsulated probiotic bacteria. Efficiency of microencapsulation can show differences depending on the kind and the concentration of the encapsulation substances, particle dimensions, initial viable cell counts and microbial strains. As a matter of fact, choose of capsule materials plays an important role in the bacterial cell protection against environmental stresses and affect release of probiotic cells as available and metabolically active state in gastrointestinal system. The appropriate encapsulation substances act as protective agent and may offer the highest robustness of the probiotics in microcapsules during transport from digestive tract of host and/or during exposure to adverse conditions from food matrices [17]. Alginate, gelatin and gellan gum are the most often used polymers for microencapsulation of probiotic bacteria due to their simplicity, non-toxicity, biocompatibility, excellent membrane-forming ability and low cost [18-20].

The other approach utilized to achieve sufficiently high numbers of probiotics in intestinal systems is the use of "prebiotics" [21]. Prebiotics were used to refer non digestible food substances that induce the growth or activity of beneficial microorganisms in the gastrointestinal tract of host [12,22,23]. As a matter of fact, food industry and researchers showed a major concern in the use of prebiotics because of synergistic effects between probiotics and prebiotics [2]. Fructooligosaccharides (FOS) are the most commonly used prebiotics and nowadays peptides are used as growth promoter [21]. However, research on the use of prebiotic in microcapsules is scarce and more work is needed to measure the stability of these capsules system in gastric conditions.

In the present study, determination of optimum entrapment substances combinations for probiotic microencapsulation against gastric conditions and enhancement of probiotic survival were aimed.

MATERIAL and METHODS

Bacterial Strains and Culture Conditions

Probiotic strains used in this research are *L. plantarum* (Blessing-Biotech GmbH-Stuttgart/Germany) and *L. casei* Shirota (Yakult-RIUM/The Netherlands).

Probiotic cultures were grown in de Man, Rogosa Sharpe (MRS) broth (Merck, Germany) at 37°C for 24 h. After incubation, cells were removed by centrifugation (3000×g, 10 min at 4°C), washed and resuspended twice in saline solution. The final cell concentrations of probiotic cultures were adjusted to 10^{10} CFU/mL for microencapsulation.

Optimization of Entrapment Substances for Probiotic Cultures

The kind and proportion of the entrapment substances have effect on the stability of probiotic strains. The detection of entrapment substances in optimum compositions is crucial for highest protection [24]. For this reason, response surface technique was performed for optimization of entrapment substances [25]. Modelling of this experiment was based on variables (coating materials) and responses (probiotic cell viability). Modelling results from the response surface technique was detected with Design expert 6.02 software *(Table 1)*. Alginate, gelatin, gellan gum, FOS and peptide were selected as entrapment substances. Also, responses in this experiment were based on viable cell counts of probiotics in simulated gastric fluid (SGF) and bile-salt solution (BSS) were evaluated.

Table 1. *Variables of experiment*

Combination	Alginate (%)	Gellan Gum (%)	Gelatin (%)	Peptide (%)	FOS (%)
1	2.00	1.00	0.00	1.00	0.00
2	2.00	0.00	0.00	1.00	2.00
3	0.50	0.00	0.00	0.00	0.00
4	0.50	0.00	1.00	1.00	2.00
5	0.50	1.00	1.00	0.00	2.00
6	1.25	0.50	0.50	0.50	1.00
7	1.25	0.50	0.50	0.50	2.00
8	0.50	1.00	0.00	1.00	2.00
9	1.25	0.50	0.50	1.00	1.00
10	1.25	1.00	0.50	0.50	1.00
11	2.00	1.00	1.00	0.00	0.00
12	1.25	0.50	0.00	0.50	1.00
13	1.25	0.50	0.50	0.50	1.00
14	2.00	0.00	1.00	1.00	0.00
15	1.25	0.50	0.50	0.00	1.00
16	0.50	1.00	1.00	1.00	0.00
17	1.25	0.00	0.50	0.50	1.00
18	1.25	0.50	0.50	0.50	0.00
19	2.00	0.00	1.00	0.00	2.00
20	1.25	0.50	1.00	0.50	1.00
21	2.00	1.00	0.00	0.00	2.00
22	0.50	0.50	0.50	0.50	1.00
23	2.00	0.50	0.50	0.50	1.00
24	1.25	0.50	0.50	0.50	1.00
25	1.25	0.50	0.50	0.50	1.00
26	1.25	0.50	0.50	0.50	1.00

Microencapsulation of Probiotic Cultures

Fructooligosaccharides and peptide are prebiotics-promoting probiotic growth and often used for synbiotic effect from synergy between probiotics and prebiotics. Previous researchers were mainly applied calcium alginate, gelatin, and gellan gum as coating materials because these entrapment substances provide better protection for probiotics in food and in the intestinal tract. The proper selection of probiotic strains, prebiotics and coating materials is crucial in obtaining a therapeutic effect [2,19,25,26]. On this sense, microencapsulation in this study were performed with entrapment substances supporting probiotic growth and protection. Probiotic cultures *(L. plantarum* and *L. casei* Shirota) were microencapsulated with entrapment substances consisting of 26 different combinations *(Table 1)* according to extrusion technique. As a preliminary, 26 different solutions containing sodium alginate (0.5-2%), gelatin (0-1%), gellan gum (0-1%), FOS (0-2%) and peptide (0-1%) were sterilized by autoclaving (121°C for 15 min) and cooled to 40°C. For microencapsulation by extrusion technique, probiotic cell suspension including *L. plantarum* or *L. casei* Shirota (10^{10} CFU/mL) was added into 50 mL of this sterile coating material solution to yield a final concentration of 1% (V/V). This mixture was placed in a syringe with 0.11 mm needle and injected into sterilized gelling solution (0.1 M $CaCl_2$). The capsules, 0.5 mm in diameter were retained for 1 h for solidification and then aseptically transferred into a sterile petri dishes [19,24]. Probiotic microcapsules obtained in this study were showed in *Fig. 1*.

Resistance of Entrapped Probiotic Strains to SGF and BSS

A solution consisted of 0.5% sodium chloride and 0.3% pepsin was adjusted to pH 2 with 1 N HCl and was used for the determination of resistance to SGF. The microencapsulated probiotic bacteria (1 g) were added into SGF solution (10 mL) in flask and incubated in shaking water bath (100 rpm) at 25°C for 1 h. To determine the resistance to BSS, microencapsulated probiotics (1 g) were inoculated into solution of 2% ox gall powder (Sigma, USA) and incubated in shaking water bath (100 rpm) at 25°C for 1 h [24,26].

Fig 1. Microencapsulated probiotic cells

Enumeration of Probiotic Strains in Microcapsules

One gram of microencapsulated probiotic bacteria samples were diluted with 9 mL of sterile phosphate buffer solution (0.1 M, pH 7.0) and allowed to homogenize for 15 min. Probiotic bacteria (CFU/g) were plated on de Man, Rogosa Sharpe Agar (Merck, Germany) and incubated at anaerobic conditions (Anaerocult A, Merck) for 48 h at 30°C [19,27].

RESULTS

Viable cell counts in microcapsules containing probiotic strains (*L. plantarum* or *L. casei* Shirota) were measured before and after treatment to SGF and BSS conditions and this measurement results were given in *Table 2*. Additionally, reduction in viable cell counts of probiotic strains after treatment of SGF and BSS for each combination of coating materials (from 1 to 26) was calculated from results in

Table 2. *Responses of experiment (log CFU/g)*

LP Counts Before SGF/BSS	LP Counts After SGF	LP Reduction After SGF	LP Counts After BSS	LP Reduction in BSS	LC Counts Before SGF/ BSS	LC Counts After SGF	LC Reduction in SGF	LC Counts After BSS	LC Reduction BSS
9.04	7.54	1.50	7.53	1.51	9.20	7.00	2.20	7.79	1.41
9.87	8.02	1.85	7.82	2.05	9.14	7.73	1.41	7.04	2.10
9.00	6.84	2.16	7.86	1.14	9.04	7.00	2.04	8.41	0.63
9.23	6.47	2.76	7.69	1.54	9.63	7.04	2.59	8.00	1.63
9.79	6.60	3.19	8.60	1.19	9.11	7.36	1.75	8.14	0.97
9.11	7.00	2.11	8.34	0.77	9.61	7.17	2.44	7.56	2.05
9.85	6.60	3.25	8.20	1.51	9.95	7.04	2.91	8.34	1.61
9.90	8.07	1.83	7.80	2.10	9.07	7.20	1.87	7.47	1.60
9.97	7.90	2.07	8.43	1.54	9.04	7.93	1.11	7.69	1.35
9.88	8.07	1.81	7.85	2.03	9.14	7.73	1.41	7.03	2.11
9.96	7.73	2.23	7.20	2.76	9.95	5.60	4.35	8.27	1.68
9.96	7.07	2.89	8.34	1.62	9.07	6.60	2.47	8.14	0.93
9.49	7.60	1.89	7.60	1.89	9.04	7.17	1.87	7.30	1.74
9.97	7.91	2.06	8.23	1.74	9.14	7.93	1.21	7.65	1.49
9.36	8.04	1.32	8.32	1.04	9.07	7.82	1.25	8.00	1.07
9.07	6.47	2.60	7.77	1.30	9.69	7.32	2.37	8.00	1.69
9.00	5.00	3.00	6.69	2.31	9.07	5.30	3.77	6.11	2.96
9.04	7.77	1.27	8.00	1.04	9.04	7.11	1.93	8.00	1.04
9.67	7.11	2.56	8.04	1.93	9.17	7.43	1.74	8.20	0.97
9.88	8.03	1.85	7.83	2.05	9.11	7.71	1.40	7.00	2.11
9.96	8.14	1.82	8.17	1.79	9.00	7.32	1.68	8.00	1.00
9.85	8.02	1.83	7.84	2.01	9.14	7.72	1.42	7.02	2.12
9.88	8.04	1.84	7.83	2.05	9.14	7.72	1.42	7.00	2.14
9.30	8.36	0.94	7.60	1.70	9.50	7.74	1.76	7.66	1.84
9.84	9.07	0.77	8.60	1.24	9.62	5.60	4.02	8.20	1.42
9.88	8.04	1.84	7.84	2.04	9.14	7.72	1.42	7.04	2.10

LP: *L. plantarum,* ***LC:*** *L. casei Shirota,* ***SGF:*** *simulated gastric fluid,* ***BSS:*** *bile-salt solution,* ***Reduction:*** *Difference between probiotic viable cell counts before SGF or BSS and after SGF or BSS*

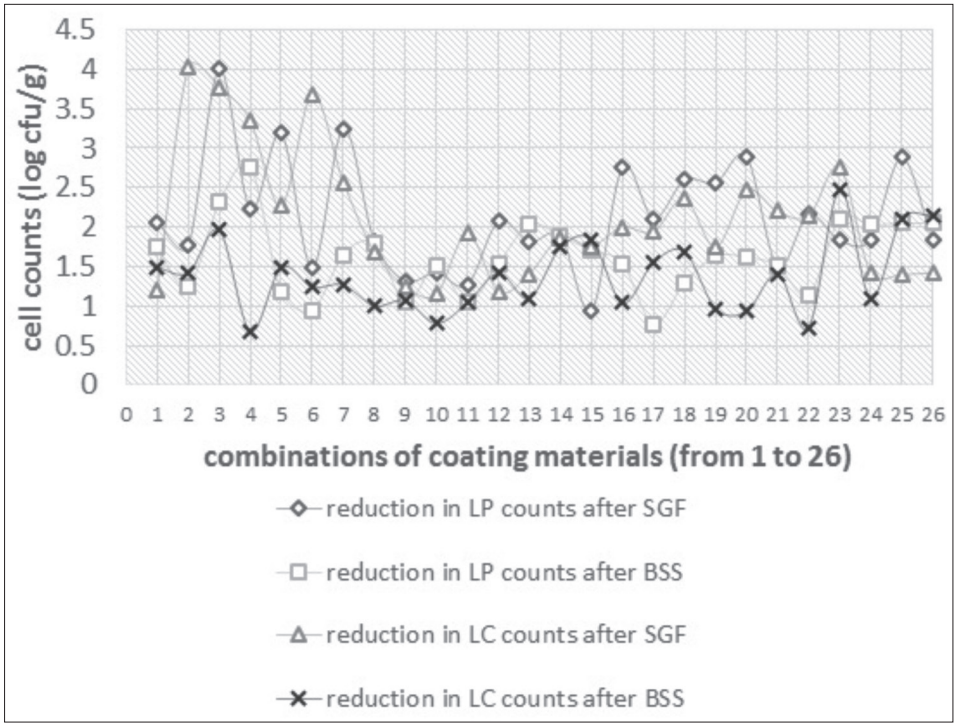

Fig 2. Reduction in viable cell counts of probiotic strains after treatment of SGF and BSS

Table 2 and this reduction was shown in *Fig. 2*.

As observed from results of this research, probiotic viable cell counts in microcapsules changed between 9.04 and 9.97 log CFU/g before exposure to SGF and BSS, while probiotic viable cell counts in microcapsules ranged from 5.0 to 8.60 log CFU/g after exposure to SGF and BSS.

Simulated gastric fluid conditions caused a drop from 0.77 to 3.25 log CFU/g in *L. plantarum* counts and from 1.11 to 4.35 log CFU/g in *L. casei* Shirota counts, respectively. After BSS condition, a reduction in *L. plantarum* and *L. casei* Shirota counts varied from 0.77 to 2.76 log CFU/g and from 0.63 to 2.96 log CFU/g, respectively.

Optimum concentrations of 5 different entrapment substances were predicted through the model established with response surface methodology. As seen in *Table 1*, concentrations of entrapment substances tested in this study were adjusted between 0.5-2% for alginate, 0-1% for gellan gum, 0-1% for gelatin, 0-1% for peptide and 0-2% for FOS. The encapsulation material composition and concentration providing the highest probiotic cell viability were calculated by using prediction model according to results obtained in *Table 1*. The optimum values for the obtainment of microcapsules with highest probiotic robustness were found as the mix of 1.5% alginate, 0.92% gellan gum, 0.18% gelatin, 0.36% peptide and 1.31% FOS for *L. plantarum* and as the mix of 2% alginate, 0.98% gellan gum, 0.51% gelatin, 0.86% peptide and 1.98% FOS for *L. casei* Shirota.

DISCUSSION

It is known from literature works that free cells of probiotic strains are more susceptible than microencapsulated cells under gastrointestinal conditions. As a matter of fact, several researchers reported that microencapsulation provided additional protection to probiotic cells with a physical barrier against stress factors in intestinal system and exhibited more robustness during gastric transit than their free cell [28-31]. Based on previous studies, there was no need to test the viability of free probiotic strains after exposure to SGF and BSS because encapsulation enhances the viability of probiotic strains. Microencapsulation technique is required to ensure survival or stability of probiotics bacteria during the passage to digestive tract of host. However, coating materials used in microencapsulation had differently effect on protection of probiotic against adverse factors [32]. In accordance with this, the present results showed that resistance in probiotic viability changed according to coating material combinations (26 different microcapsule) after SGF and BSS. The use of prebiotic materials (peptide and FOS) in addition to gelling agents such as sodium alginate and gelatin for microencapsulation provides a better protection to probiotic bacteria. These prebiotic agents may act as a supporter of probiotic viability. As a matter of fact, various coating material combinations with regard to their compositions and concentrations have caused different levels of probiotic resistance against gastric conditions according to earlier studies [18,19,26,33]. Similarly, the present study confirmed this different effect of coating material combinations on probiotic resistance. Additionally, statistical analysis showed that microencapsulation with different coating material combinations had effect on resistance of probiotic strains against SGF at the 95% confidence level.

A reduction in *L. plantarum* counts and *L. casei* Shirota counts changed approximately between 1 and 4 log CFU/g after SGF and between 1 and 2 log CFU/g after BSS. This situation detected that these probiotic bacteria were more resistant to BSS than SGF. Chen et al.[19] reported that that probiotic strains exhibited higher resistance to acidic conditions than to bile salts. However, in another study, probiotic *L. rhamnosus* were found more resistant to bile salts than to acid [34]. This situation considered that resistance of probiotics against SGF and BSS conditions could change according to strains.

Stimulated Gastric Fluid conditions caused a drop from 0.94 to 4 log CFU/g in *L. plantarum* counts and from 1.15 to 4.02 log CFU/g in *L. casei* Shirota counts, respectively. After BSS condition, a reduction in *L. plantarum* and *L. casei* Shirota counts varied from 0.77 to 2.76 log CFU/g and from 0.68 to 2.48 log CFU/g, respectively. As reported in Chen et al.[25], our results indicated that coating material combinations had different effect on resistance of probiotic against SGF and BSS conditions.

As mentioned above, optimum rate of 5 different entrapment substances for microencapsulation of each probiotic cell were calculated from optimization model obtained by using response surface methodology. Concentrations of encapsulation agents changing between 0.5-2% for alginate, 0-1% for gellan gum, 0-1% for gelatin, 0-1% for peptide and 0-2% for FOS were tested. The reason for choosing these concentration ranges in this study is suggestions from previous researchers [28]. Entrapment substances at different type and concentrations were evaluated with regard to the protection of cell viability. Probiotic microcapsules with entrapment substances at 26 different combinations were prepared according to the experimental design shown in *Table 1*. Formulation of optimization model from 26 coating material combinations detected optimum proportion of entrapment substances for each probiotic strain. The viability by the best combination were also tested and found higher than other combinations. These results confirmed our hypothesis that optimum combination of encapsulation materials provide highest protection against gastric conditions and give the highest cell viability [35,36].

Concentrations of alginate used for gelling change between 1.5 and 2.5%. However when alginate was used with other gelatinization agents, concentrations of alginate were tested between 0.5 and 2% [19]. As known from literature, peptides as nitrogen sources improve viability of probiotics [19,25]. The present study confirmed that peptides with their prebiotic effect have synergistic activity on probiotic viability. Incorporation of microcapsules with extra coating materials supported additional protecting of the probiotic bacteria [37,38]. Our results confirmed that. Some researcher reported that microencapsulation applications such as incorporation of different coating materials and the double emulsion protected probiotic strains against simulated gastrointestinal tract conditions [18,34]. Similarly, our study showed that extra coating improved survival of probiotic.

In conclusion, selection of optimum or appropriate coating materials used for microcapsules may improve the survival of probiotic strains in functional food products. Microcapsules with prebiotic may be safely used as protective delivery vehicle for the passage from gastrointestinal tract of probiotic strains. Moreover, the present study results may attract the attention of other researchers to investigate innovative entrapment substances. On this sense, further modification and improvement in microencapsulation technique is necessary for resistance of probiotics against gastric conditions.

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Research Article

The Ability of Electrolyzed Reduced Water to Act as an Antioxidant and Anti-Inflammatory Agent in Chronic Periodontitis Wistar Rats *(Rattus novergicus)*

Rini Devijanti RIDWAN [1,a] Tantiana TANTIANA [1,b] Darmawan SETIJANTO [2,c]
Astika Kinant KUSUMA [3,d] Ananda Firman PUTRANTO [4,e]

[1] Oral Biology Department, Faculty of Dental Medicine, Universitas Airlangga, Oral Biology, Surabaya, INDONESIA
[2] Dental Public Health, Fculty of Dental Medicine, Universitas Airlangga, Public Health, Surabaya, INDONESIA
[3] Faculty of Dental Medicine, Universitas Airlangga, Surabaya, INDONESIA
[4] Orthodontics Department, Faculty of Dental Medicine, Universitas Airlangga, Ortodontics, Surabaya, INDONESIA
[a] ORCID: 0000-0002-0090-9746; [b] ORCID: 0000-0001-5185-1647; [c] ORCID: 0000-0001-7182-2712; [d] ORCID: 0000-0001-7488-9517
[e] ORCID: 0000-0002-0960-8739

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Abstract

The chronic periodontitis was inflammation characterized by macrophage activation which releases various metabolites such as ROS that, in turn, produces malondialdehida (MDA) a biological biomarker of lipid peroxidation. The aim of this research is to analyze the effect of Electrolyzed Reduced Water (ERW) on the levels of malondialdehyde, macrophage and lymphocytes cells in Wistar rats suffering from chronic periodontitis. It constitutes an experimental laboratory study incorporating a random sampling method. Twenty-one Wistar rats were induced with up to 10^6 *Porphyromonas gingivalis* in the proximal area of the mandibular molar and divided into three groups which were administered orally on a daily basis as follows: a control negative group (distilled water); a control positive group (a dose of vitamin C at 1.08 mg/200 g Body Weight/day) and a treatment group (20 mL ERW) and observed between Day 1 and Day 14. Samples of gingival tissue were taken from the subjects for analysis with malondialdehyde and the conducting of macrophage and lymphocyte cell counts on Day 14. Data analysis comprised a One-Way Analysis of Variance (ANOVA) and a Mann Whitney test ($P<0.05$) based on a Saphiro-Wilk normality test and a Levene Test for Equality of Variances or a Kruskal Wallis test ($P>0.05$). ERW can decrease the level of malondialdehyde (4.3±0.7), the number of macrophage cells (19.4±8.6) and the number of lymphocyte cells (8.5±0.8) in the treatment group. The ERW mechanism can, therefore, be said to suppress the occurrence of further tissue damage triggered reactive oxygen species (ROS) in Chronic Periodontitis.

Keywords: *Anti-inflammatory, Reactive oxygen species, Electrolyzed reduced water, Malondialdehyde, Periodontitis*

Kronik Periodontitli Wistar Sıçan *(Rattus Novergicus)*'larda Elektrolize İndirgenmiş Suyun Antioksidan ve Antiinflamatuvar Etkisi

Öz

Kronik periodontitis, ROS gibi çeşitli metabolitleri salan ve böylece lipit peroksidasyonun biyolojik bir biyobelirteci olan malondialdehit (MDA)'in üremesine neden olan makrofajların aktivasyonuyla karakterize bir yangıdır. Bu çalışmanın amacı; kronik periodontitli Wistar sıçanlarda elektrolize indirgenmiş su (EİS)'yun malondialdehit seviyesi ile makrofaj ve lenfosit sayılarına etkilerini araştırmaktır. Çalışma deneysel olup rastgele örnekleme metodu kullanılmıştır. Yirmi bir Wistar sıçanın mandibular molar dişlerinin proksimal bölgelerine 10^6 *Porphyromonas gingivalis* uygulandı ve günlük ağız yoluyla belirtilen uygulamaları gerçekleştirmek için üç gruba ayrıldı; Kontrol negatif grubu (distile su), Kontrol pozitif grubu (1.08 mg/200 g vücut ağırlığı/gün Vitamin C) ve Uygulama grubu (20 mL EİS). Gruplar 1. günden 14. güne kadar gözlemlendi. Deneklerden gingival doku örnekleri 14. günde alınarak malondialdehit analizi ile makrofaj ve lenfosit sayımları yapıldı. Verilerin analizi Saphiro-Wilk normalite testi ve Varyansların eşitliği için Levene Testi veya Kruskal Wallis testine ($P>0.05$) dayanarak Tek yönlü varyans analizi (ANOVA) ve Mann Whitney testi ($P<0.05$) ile yapıldı. EİS uygulaması malondialdehit seviyesi (4.3±0.7) ile makrofaj (19.4±8.6) ve lenfosit (8.5±0.8) sayılarında düşmeye neden oldu. EİS'nin kronik periodontitte reaktif oksijen türlerinden kaynaklanan doku hasarını baskılayarak etki ettiği söylenebilir.

Anahtar sözcükler: *Antiinflamatuvar, Reaktif oksijen türleri, elektrolize indirgenmiş su, Malondialdehit, Periodontitis*

İletişim (Correspondence)
+62 31 5992785
rini-d-r@fkg.unair.ac.id

INTRODUCTION

Chronic periodontitis is a periodontal tissue inflammation characterized by migration of junctional epithelium to the apical, periodontal ligament attachment and alveolar bone loss [1,2]. The chronic inflammation is characterized by macrophage activation which releases various metabolites such as Reactive Oxygen Species (ROS) that, in turn, produces Malondialdehida (MDA) a biological biomarker of lipid peroxidation [3].

The administering of vitamin C to patients suffering from periodontitis represents an adjuvant therapy to accelerate the healing process by decreasing ROS levels in the inflammed area. Nevertheless, excessive amounts of vitamin C can prove harmful to the body and potentially become free radicals [4].

Based on previous research, chronic inflammation stimulates ROS production which must be suppressed. Electrolyzed Reduced Water (ERW) at a pH value of 9.8 has been used as chronic disease therapy for conditions such as gastrointestinal conditions, hypertension, diabetes and cancer [5]. ERW at a pH range between 8.5 and 9, may accelerate the healing process in cases of chronic inflammation [6].

Electrolyzed Reduced Water, which is produced by an electrolysis machine that renders the water pH alkaline, also plays a role in inducing detoxification, antioxidation and hydration, regulating blood pressure, promoting metabolism in addition to maintaining bone health [7]. It reduces chronic inflammation by donating active hydrogen atoms to unpaired electron bonds derived from ROS in the tissues. Consequently, the number of free electrons which are present in the tissues decreases [4].

The aim of this research is to analyze the mechanism effect of ERW on the MDA level and the number of macrophage and lymphocyte cells in Wistar rats suffering from chronic periodontitis.

MATERIAL and METHODS

Ethical Clearance and Study Design

This study received ethical clearance approval, number 174/KKEPK.FKG/VIII/2016, relating to animal subjects from the Ethics Research Committee, Faculty of Dental Medicine, Universitas Airlangga. The research constituted analytical observation involving the use of a laboratory experiment and a cross-sectional methodology.

Animal Model

This research was conducted at the Microbiology Laboratory, Faculty of Dental Medicine, Universitas Airlangga and the Biomedic and Parasitology Laboratory, Faculty of Medicine, Brawijaya University. The samples, consisting of 21 male Wistar rats selected by random sampling and weighing between 150 and 200 grams, were assigned to one of three groups: a control negative group, a control positive group and a treatment group. Each of these three groups was itself further sub-divided into three groups which were fed orally and subjected to observation on a daily basis between day 1 and day 14. The seven members of each group were fed from a small container twice a day, once in the morning and again in the afternoon. They drank up to 20 mL (4x5 mL) per day via a small pipe attached to a bottle of water. In order to induce chronic periodontitis in members of the treatment group, they were inoculated with 10^6 CFU of *P. gingivalis* ATCC 33277 bacteria in 30 mL (0.03) of PBS. The bacteria were administered to the proximal area of the mandibular molars once every three days over a period of two weeks by means of a disposable 0.5 cc syringe. The symptoms of chronic periodontitis induced by *P. gingivalis* ATCC 33277 included inflammation of the gingival, gingival hyperplasia, pocket formation and periodontal attachment loss within the affected area. The negative control group was provided with 20 mL (4x5mL) of distilled water per day, while the positive control group received a daily 1.08 mg/200 g BW/dose of vitamin C solution. The treatment group received a daily dose of 20 mL (4x5 mL) of ERW at a pH of 9.8.

Histopathology Examination

Hematoxylin Eosin (HE) staining was performed to determine the number of macrophages and lymphocytes present. In addition, gingival tissue from the left side of the jaws of the subjects was reduced to a powder. MDA levels were subsequently examined by homogenizing the tissues with cold phosphate buffer (50 mM) before inserting them into the assay tubes and adding 10 µL of 31 Probucol. 200 µL of the existing standard samples were also introduced into the assay tubes. R1 reagents of MDA-586 kit (MDA BIOXYTECH 586) were added before being vortexed. 150 µL of R2 reagent was subsequently added to each tube prior to re-vortexing.

Measurement of Malondialdehyde

The next stage consisted of incubating all control and treatment group tubes at a temperature of 45ºC for 60 min after which the samples were centrifuged at 10.000x for ten min to separate the supernatants. These were subsequently inserted into a cuvette in order to permit reading of the absorbance values at a wavelength of 532 nm. The MDA levels were observed and compared using a spectrophotometer at the same wavelength. The absorbance data derived was then calculated using alogarithm formula.

Statistical Analysis

Data was analyzed using Statisitical Package for Social Science (SPSS) version 17.00 (IBM SPSS, Chicago, USA). Analysis of Variance was performed ($P<0.05$) based on

Levene's variance of homogeneity test and Saphiro-Wilk normality test result (P>0.05).

RESULTS

The quantitative data obtained confirmed the MDA levels in each group. ERW reduced MDA levels *(Fig. 1)* and, therefore, also the number of macrophages and lymphocytes *(Fig. 2; Fig. 3)*. The results of Histopathlogy examination in terms of the number of macrophage and lymphocytes can be seen in *Fig. 4*. While there were significant differences in macrophage and lymphocyte cell numbers between the groups P=0.000 (P<0.05), those in the MDA level P=0.670 (P>0.05) were insignificant.

DISCUSSION

Based on the statistical test results, there was no significant difference in the MDA levels between groups possibly due to not all of the samples having been placed in the assay

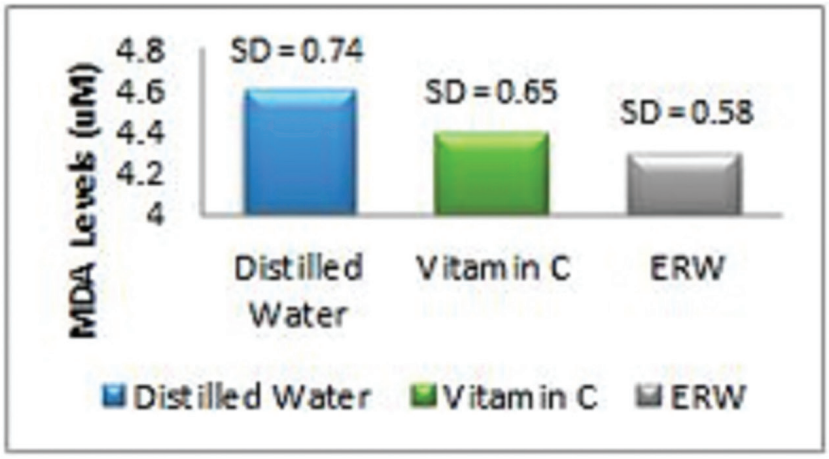

Fig 1. Mean MDA levels in each group

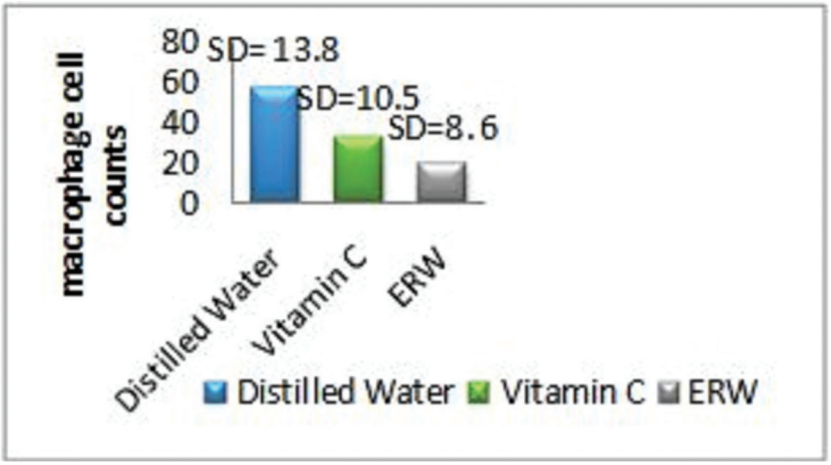

Fig 2. Mean number of macrophage cells in each group

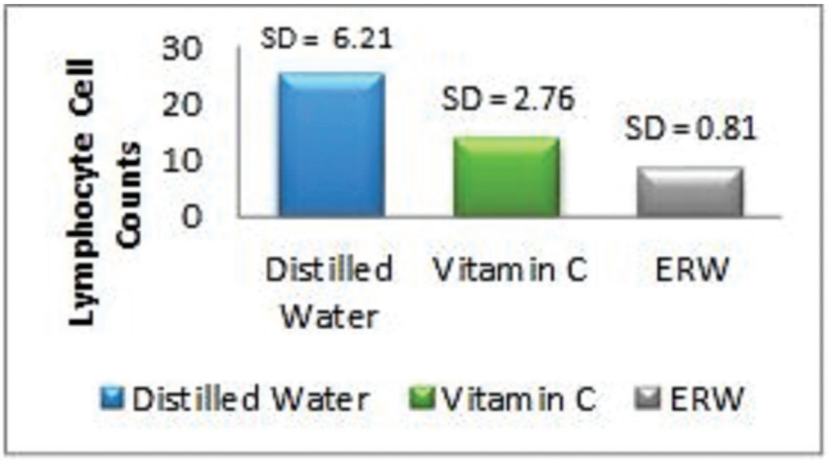

Fig 3. Mean number of lymphocyte cells in each group

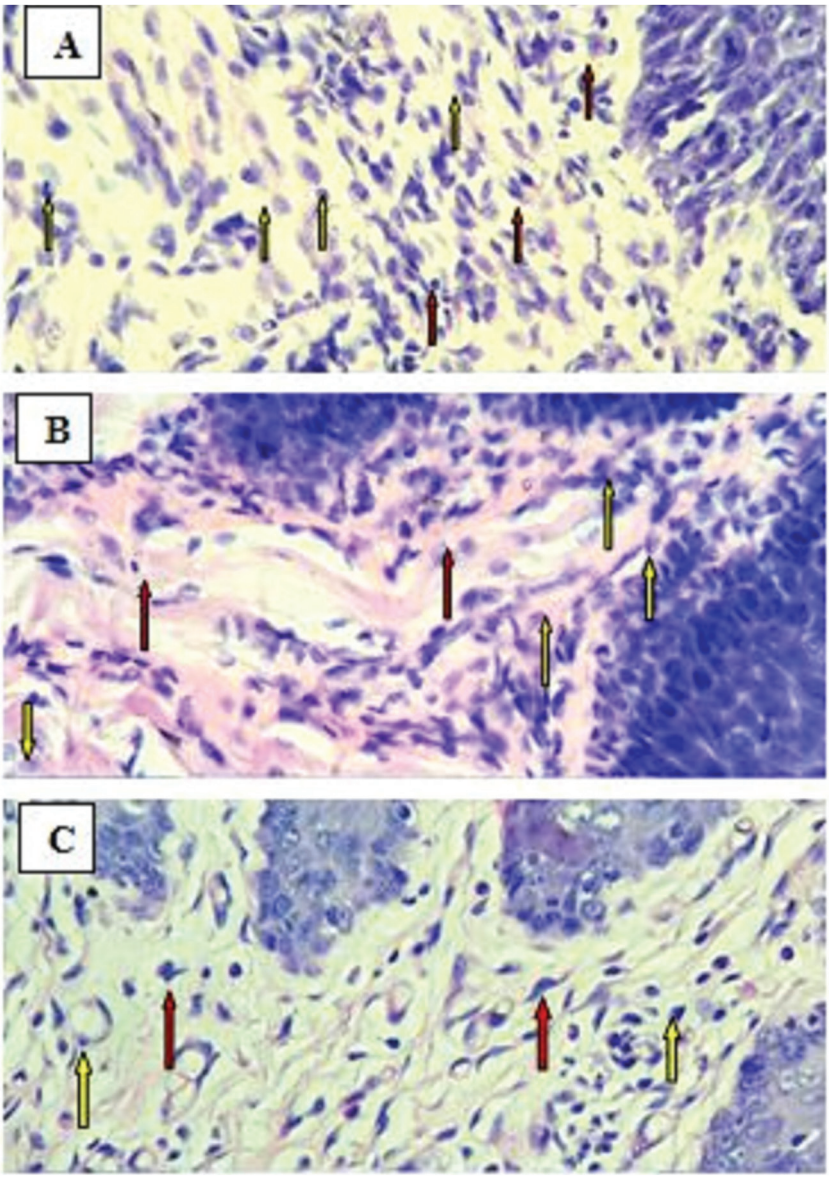

Fig 4. HPA examination results in the gingival tissues at 400x magnification for Macrophage *(Yellow arrows);* Lymphocyte *(Red arrows);* (A) Negative control group; (B). Positive control group; (C) and the Treatment group

tubes. The presence of certain samples differing from the standard color meant that the data obtained was not significant. This insignificant result could have occurred due to a decrease in ERW antioxidant activity. A previous study by Henry and Chambron argued that ERW is affected by heat and, if not consumed immediately, can affect the stability of active hydrogen atoms [4]. Significantly, during the research reported here, ERW antioxidant activity decreased. The data obtained also indicated differences in the mean MDA level. However, in general, the results showed that ERW produced anti-ROS antioxidant activities in cases of chronic periodontitis affecting Wistar rats. These findings concurred with those of research conducted by Freeman [7] which posited that ERW exerts an antioxidant effect.

Shirahata et al.[8], state that ERW, as an antioxidant, contains NaOH with high levels of hydrogen molecules and abundant Pt nanoparticles. At the time that Pt nanoparticles are synthesized, they produce a compound of O_2, OH and H_2O. Pt nanoparticles will activate the hydrogen molecules through catalytic processes and can also demonstrate both antioxidant activity, for example, that of superoxide anions which is identical to the function of superoxide enzymes, and antioxidant enzyme activity such as that of catalases. Moreover, Pt nanoparticles can stimulate the reducibility of antioxidants, although they demonstrate weak auto-oxidant activities [7,9].

The ability of ERW to release hydrogen atoms is reinforced in line with a study by Choi [10] confirming that ERW possesses a hexagonal structure. As a result, ERW can easily provide hydrogen atoms as active components by penetrating the cell components in the tissue. As a compound, ROS has a free electron bond which will stabilize when ERW is applied to the inflamed tissue. Therefore, it can be said that the ERW mechanism is capable of preventing the occurrence of further tissue damage triggered by ROS [8,9].

In this study, the chronic inflammatory cells examined were macrophages and lymphocytes. The statistical test results showed there to be a significant difference in the mean numbers of macrophages and lymphocytes between the groups of Wistar rats suffering from chronic periodontitis. This result indicates that ERW plays an anti-inflammatory function as explained in a previous study conducted by Lee et al.[11] This proves that ERW has a positive effect on immune response through a preventative process against cellular protein breakdown in lymphocytes as inflammatory cells. Similarly, a study conducted by Dovi states that ERW may inhibit COX-2 expression by decreasing proinflammatory cytokines, namely; IL-1 and TNF-α. Decreasing or inhibiting IL-1 and TNF-α synthesis can lead to reduced stimulation of cell membrane phospholipids with the result that arachidonic acid cannot be released from the cell membrane phospholipids by phospholipase activation. This condition triggers both reduced COX-2 protein synthesis and prostaglandin biosynthesis culminating in a decreased inflammatory response [12,13].

Based on the results of the MDA level measurement, the number of macrophages and lymphocytes in the control group was used as a standard to determine more specifically the antioxidant effects of ERW. Vitamin C, which has been used as adjuvant therapy for chronic periodontitis treatment, was employed as another antioxidant source. The blood plasma of patients suffering from chronic periodontitis contained lower vitamin C levels than those under normal conditions [13]. Vitamin C was considered an antioxidant because it can be donated electron thus, prevent the formation of other compounds through the oxidation process by releasing the carbon chain. After donating electrons to the free radicals, vitamin C will be oxidized to semi-dehydroascorbut acid or ascorbyl radicals that are relatively stable, rendering it an antioxidant. In other words, ascorbic acid can react with free radicals, reducing reactive free radicals to non-reactive free radicals. The free radicals experiencing a reduction from reactive ones to non-reactive ones are called scavengers or sequencers [14].

The results of this study showed that ERW had antioxidant and anti-inflammatory capabilities superior to vitamin C, since the pH of ester C used as the third generation of vitamin C is extremely similar to that of water, while ERW has an alkaline pH. A study conducted by Arifah [6] showed an increase in the number of macrophages and lymphocytes treated with ERW because of the balance between the acidity of the tissue and the alkalinity of the drinking water. Inflamed tissue possesses a more acidic pH than normal tissue due to an anaerobic metabolism process which is a response to chronic inflammation involving an increased demand for the energy necessary for homeostasis [5,15,16].

Light visible spectrophotometry (UV-Vis) at a wavelength of 532 nm was employed to observe the binding activity of α-diphenyl-β-picrylhydrazyl (DPPH) as an antioxidant marker. The working principle of UV-VIS spectrophotometry is that light will be absorbed by the solution in the cuvette, although some rays are also transmitted [15]. The light absorbance values produced contained heterogeneous numbers confirming that the statistical test results were not significant. Several factors can influence spectrophotometer measurement results such as poor control during sample processing prior to calculation of the visible spectrophotometer readings. This results in heterogeneous sample colors despite the treatment of the samples being consistent. This error could occur due to differences in sample volumes in the assay tubes before MDA levels were measured by spectrophotometer. ERW can act as anti-inflammatory agent with reduced antioxidant activity in Wistar rats suffering from chronic periodontitis. The ERW mechanism can suppress the occurrence of further tissue damage triggered by ROS. However, further study is required to confirm this conclusion.

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Conflict of Interest

The authors confirm that there is no conflict of interest in this study.

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Research Article

The Effects of Clay Modeling and Plastic Model Dressing Techniques on Veterinary Anatomy Training [1]

Burcu ONUK [1,a] Ahmet ÇOLAK [2] Serhat ARSLAN [3,b] Sedef Selviler SİZER [2,c] Murat KABAK [1,d]

[1] *This study was presented in the 5. International Vet-Istanbul Group Congress & 8. International Sciencefic Meeting Days of Veterinary Medicine, 23/27 September 2018, Ohrid, Macedonia*

[1] Ondokuz Mayis University, Department of Anatomy, Faculty of the Veterinary Medicine, TR- 55200 Samsun - TURKEY
[2] Ondokuz Mayıs University, Graduate School of Health Sciences, TR- 55200 Samsun - TURKEY
[3] Ondokuz Mayis University, Department of Biometry, Faculty of the Veterinary Medicine , TR- 55200 Samsun - TURKEY
[a] ORCID:0000-0001-8617-3188; [b] ORCID:0000-0002-2110-8489; [c] ORCID: 0000-0002-1990-4507; [d] ORCID:0000-0003-4255-1372

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Abstract

In addition to classical anatomy applications, particularly in medicine education, the contribution of different methods (3D modeling, body painting and clay modeling) has been investigated for the purpose of anatomy training. In recent years, clay modeling which is the one of these methods is frequently used. The aim of the present study was to determine the contribution of clay modeling and plastic model dressing methods to veterinary anatomy training. The ruminant forelimb's bones, joints, muscles and nerves were chosen as the topic of this study. Clay material was used for bone models, coloured play dough were used for joints, and Colored Eva Sponges were used for muscles and nerves. Students were divided into two groups as new method group and classical method group. The students were given exams and the results of exams were evaluated at the end of each application. It was detected that the student success rate increased when clay modelling and plastic model dressing methods were employed ($P<0.05$). There was no significant difference for the lesson success rate between male and female students ($P>0.05$). With this study, the clay modeling and plastic model dressing methods applied for the first time in veterinary anatomy training was found to increase student achievement and motivation significantly. Furthermore, we believe that organ models prepared by this method will contribute to education.

Keywords: *Clay modelling, Plastic model dressing, Veterinary anatomy education*

Kil Modelleme ve Plastik Model Giydirme Tekniğinin Veteriner Anatomi Eğitimine Etkisi

Öz

Anatomi eğitiminde, özellikle tıp fakültelerinde klasik anatomi uygulamaların yanında farklı yöntemlerinde (3D modelleme, vücut boyama, vb) eğitime katkısı araştırılmaktadır. Son yıllarda bu yöntemlerden kil modelleme yöntemi sık kullanılmaktadır. Bu çalışma ile kil modelleme ve plastik giydirme yöntemlerinin veteriner anatomi eğitimine katkısının belirlenmesi amaçlandı. Konu olarak ruminant ön bacak kemikleri, eklemleri, kasları ve sinirleri seçildi. Kemik modellemeleri için kil materyal, eklem için renkli oyun hamurları, kas ve sinir için ise renkli eva süngerleri kullanıldı. Öğrenciler yeni yöntem grubu ve klasik yöntem grubu olarak ikiye ayrıldı. Her uygulama sonu sınav yapıldı ve sonuçları değerlendirildi. Başarının, kil ve plastik model giydirme yöntemi ile arttığı saptandı ($P<0.05$). Erkek ve kız öğrencilerin ders başarıları arasında fark gözlenmedi ($P>0.05$). Veteriner anatomi eğitiminde ilk kez uygulanan ve eğitime katkısının araştırıldığı bu çalışmanın öğrenci başarısını ve motivasyonunu oldukça arttırdığı saptandı. Ayrıca bu yöntem ile hazırlanacak olan organ modellerinin de eğitime katkısının olacağını düşünmekteyiz.

Anahtar sözcükler: *Kil modelleme, Plastik model giydirme, Veteriner anatomi eğitimi*

INTRODUCTION

Current research reveals that learning should be considered as a mental activity. Those who use the left hemisphere of the human brain learn by reading, and those who use the right hemisphere actively learn by observation and

İletişim (Correspondence)
+90 362 3121919/3891 Fax: +90 362 4576922
burcuonuk@omu.edu.tr

experience [1]. In general, the primary goal of veterinary anatomy training is to ensure that the learner obtains the necessary knowledge in the most effective way and practices it efficiently. For this, different teaching methods have been used in which the information is given in various ways [2-8]. In anatomy learning, in the teaching-learning process, visual methods are more effective than auditory and other methods [5,9,10]. The foundation of anatomy education is based on the cadaver studies [7,11]. In addition, methods such as book, atlas, model, visualization and simulation are also used in anatomy education [3,5,6,11-13]. In anatomy training, as an addition or alternative to these methods, lectures using new methods have been gaining an increasing importance [2,4,14-17]. Furthermore, the efficiency of the methods used in anatomy education has also been studied [10,14,18,19].

An educational method which has been started to be used in medical anatomy training in the world is clay modeling [4,20,21]. In veterinary anatomy training, no study on clay modeling or plastic model dressing methods has been reported. With this study, clay and plastic models are included in the dressing training process. Thus, it is aimed that students learn and become activate by doing-living. The study has aimed to determine the effects of these methods on learning and to reveal the results obtained from the exams and questionnaires by using statistical analysis and to determine the contribution towards education.

MATERIAL and METHODS

Preparation of Anatomical Materials

In this study, bone, joint, muscle, nerve cadaver and models prepared routinely for anatomy lesson were used. Also bone models of the ruminant forelimb *(Scapula, skeleton brachii, skeleton antebrachii, skeleton manus)* were created using clay by a selected group of students who were taking the course. Colored Play Dough was distributed to the same group in order for them to create joint models *[articulatio (art.) humeri, art. cubiti, art. manus]* on plastic models by using the dressing method. Colored Eva Sponges were used to create muscle *[musculus(m.) infraspinatus, m. deltoideus, m. supraspinatus, m. deltoideus, m. teres minor, m. teres major, m. subscapularis, m. brachialis, m. biceps brachii, m. triceps brachii, m. tensor fascie antebrachii, m. extensor carpi radialis, m. extensor digitorum lateralis, m. extensor carpi ulnaris, m. adductor digiti I longus, m. flexor carpi ulnaris, m. flexor carpi radialis, m. flexor digitorum superficialis, m. flexor digitorum profundus]* and nerve [*nervus (n.) suprascapularis, n. musculocutaneus, nn. subscapulares, n. axillaris, Nn. pectorales craniales, n. thoracicus longus, n. thoracodorsalis, n. thoracicus lateralis, Nn. pectorales caudales, n. radialis, n. ulnaris, n. medianus*] in models.

Application of the Course

This study was approved by Ondokuz Mayis University, Social Sciences and Humanities Research and Publication Ethics Committee (Number: 2018/72-108). It was applied to the students (n:105) who were recently enrolled in the anatomy course. The students did not have anatomical knowledge. Firstly, all students were taught the theoretical courses including ruminant forelimb bones, joints, muscles and nerves for first, second, third and fourth weeks, respectively *(Table 1)*. At the end of each theoretical course of the week, the students took a written exam.

For practical course, the students (n:105) were divided into two groups (Group 1: 56 students; Group 2: 49 students) by regarding their preferences. The students of the Group 1 performed the classical method including re-expression of the subjects described in the theoretical lecture on the cadaver in the laboratory. The students of the Group 2 performed practical courses in accordance with the new method including the clay and plastic materials in a different hall. The theoretical knowledge was repeated in the practical course at each stage. In the new method, students used clay materials during the first two weeks and they studied on the plastic material in the following two weeks. Coloured eva sponges were used for dressing on these plastic materials to explain the structures of muscles and nerves. The plastic materials were 3D plastic models which were previously sculpted by us using real bone model. To 3D models, we injected to silicon molds the liquid casting materials consisting of polyester, calcite (marble powder-0,5per), cobalt and mekperoxide mixture *(Table 1)*. In Group 1 and Group 2, practical courses were carried out interactively. At the end of the courses, both groups took the same written examination every week.

Table 1. *Theoretical and practical course content according to the weeks*

Week	Theoretical Course Lesson Subjects/ Attending Number of Students Participating	Practical Course Lesson Materials/Attending Number of Students Participating	
		Classical Method (Group 1)	New Method (Group 2)
1	Ruminant forelimb bones/85	Ruminant forelimb bones cadaver/41	Clay materials/47
2	Ruminant forelimb joints/91	Ruminant forelimb joints cadaver/35	Clay materials and colored play dough/31
3	Ruminant forelimb muscles/85	Ruminant forelimb muscles cadaver/36	Plastic ruminant forelimb bones and coloured eva sponges/29
4	Ruminant forelimb nerves/83	Ruminant forelimb nerves cadaver/37	Plastic ruminant forelimb bones and coloured eva sponges/31

Table 2. *Evaluation feedback questionnaire form of after practice course for new method group*	
Quesions	**Points***
Schematic drawing and clay modeling practices facilitated my comprehension of the subject	
My interest in the subject gradually increased during the practical course	
Complicated topics got clarity during the practical course	
I performed the applications without any distraction	
My interest and attention were distracted during this practice	
This application brought me in nothing	
Comments	
** 1: Strongly disagree, 2: Rather disagree, 3: No idea, 4: Rather agree, 5: Strongly agree*	

Finally, a feedback form was carried out on students in Group 2 *(Table 2*-lowest score 1 and highest score 5).

Statistical Evaluation

In this study, the data obtained from the exam and feedback forms that were given to the students were recorded using SPSS for Windows (Version 21.0; SPSS Inc., Chicago, IL, USA) software. Independent Samples T test was used for statistical analysis. Comparisons of means were made by converting success rates (number of successful students/ total number of students) of subjects to proportional variables based on their frequencies and analyzed with Chi-square ratio test at P<0.05 level and comparison of differences of groups [22] were done with ODDS Ratio (OR).

RESULTS

The structures consisting of the ruminant forelimb such as bones (3 lesson hours=135 min), joints (3 lesson hours=135 min), muscles (3 lesson hours=135 min), nerves (3 lesson hours=135 min) were taught theoretically. At the end of each theoretical course, students were given written exams prepared in advance. Exams were graded out of 100. The means of student remarks from first week to fourth weeks were obtained as: 0.86±0.38; 11.09±1.25; 2.65±0.79; 8.36±1.85, respectively. *Fig.1* shows the variance of theoretical course success graph of students.

The practice course (4 lesson hours=180 min) was taken by both groups following the theoretical course every week. Group 1 took practical courses in accordance with the classical method. Group 2 used clay and plastic materials according to the new method *(Fig. 2, 3)*. At the end of the courses, both groups took the same written exam which was prepared in advance. According to the exam marks of these two groups, the success rate was demonstrated in *Fig. 4*. Quizzes after theoretical course and practical course were compared and it was determined that both the classical application method used in anatomy and the new method used in this study increased success after application (P<0.01). However, when these two methods of applications were compared with one another, it was determined that the success rate increased more with the use of the new method *(Table 3, Fig. 5)*. The success rates from the first week to the fourth week were 105.15%, 96.23%, 168.87%, 16.74%, respectively. For both groups, the results of the last week's test were statistically unreliable. It was thought that this was caused by the fact that the students were in the period of intensive final exams.

At the end of this study, the successes of male and female students were compared in order to determine

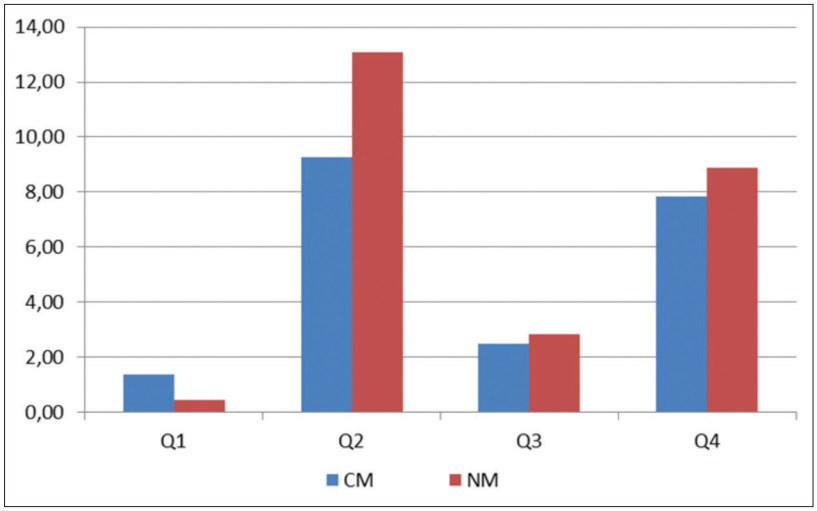

Fig 1. Theoretical course success graph of students who take the classical (CM) (Group 1) and new methods (NM) (Group 2)

Fig 2. Student applications of clay modelling method
A: Operational clay material, B-C-D-E-F: Sculpting steps of the clay models were designed by students

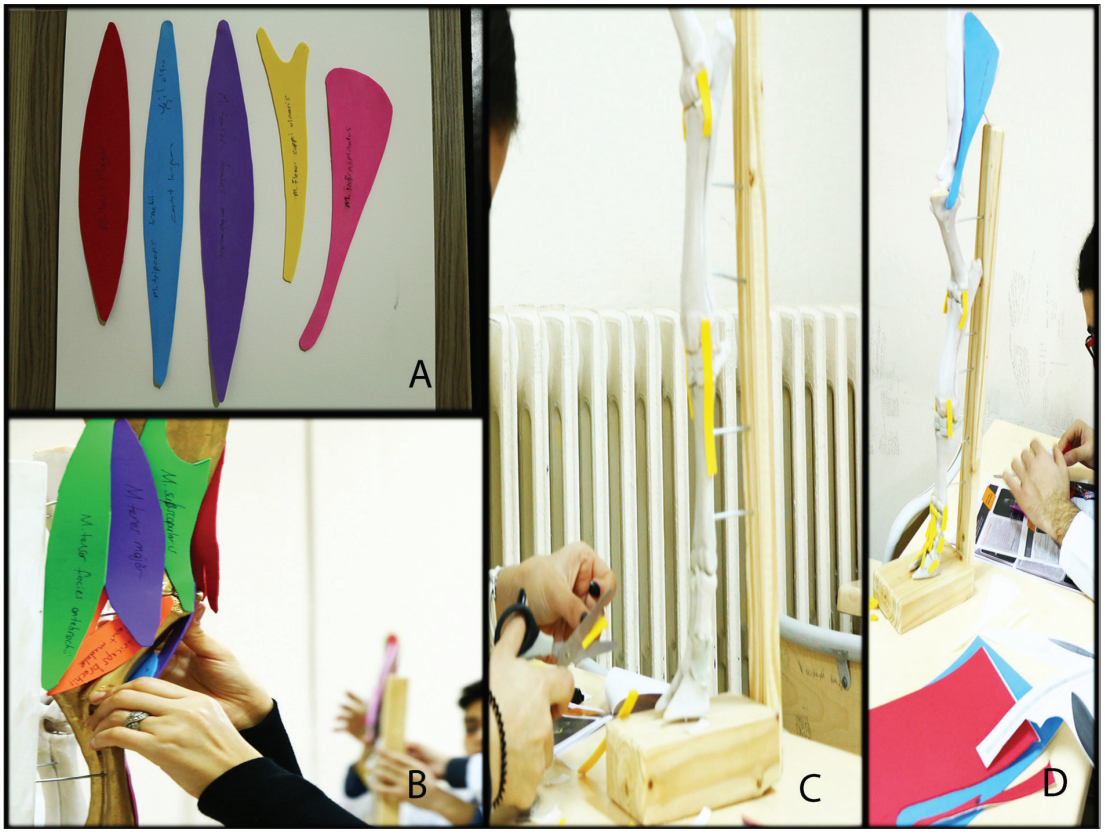

Fig 3. Students applications of plastic model dressing method
A: Preparing of muscle templates using with Colored Eva Sponge, B-C-D: Dressing of muscle and ligament templates on plastic model

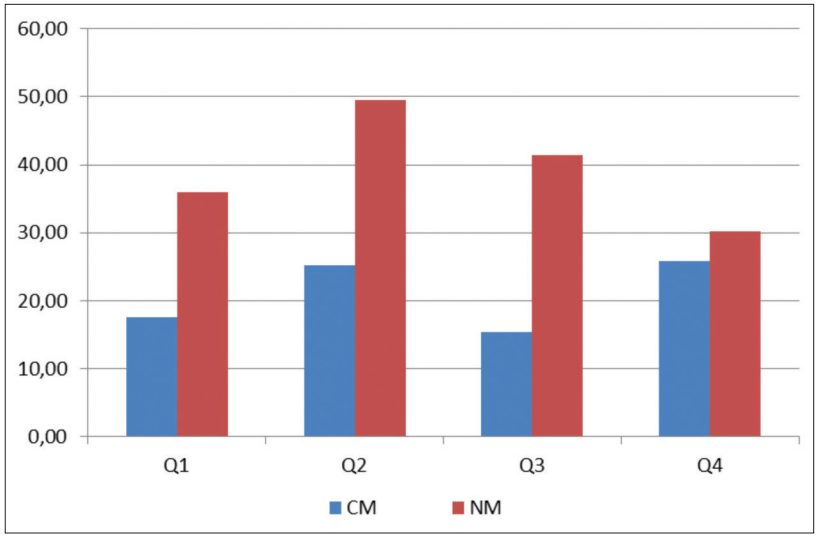

Fig 4. Results of the classical methods (CM=Group 1) and new (NM=Group 2) in the practical course

Table 3. *After practical courses, examination resuts* of the new and classical methods*

Methods	Classical (Group 1)		New (Group 2)	
	Mean	Std. Error	Mean	Std. Error
Q_1	17.55 [b]	2.88	36.00 [a]	3.95
Q_2	25.20 [b]	3.88	49.45 [a]	4.47
Q_3	15.42 [b]	3.18	41.46 [a]	2.73
Q_4	25.86 [a]	5.38	30.19 [a]	6.24

*[a,b] Same letters in same rows is not significant at level α=0.05, * Exam was graded out of 100, Q_i: from Quiz1 to Quiz4*

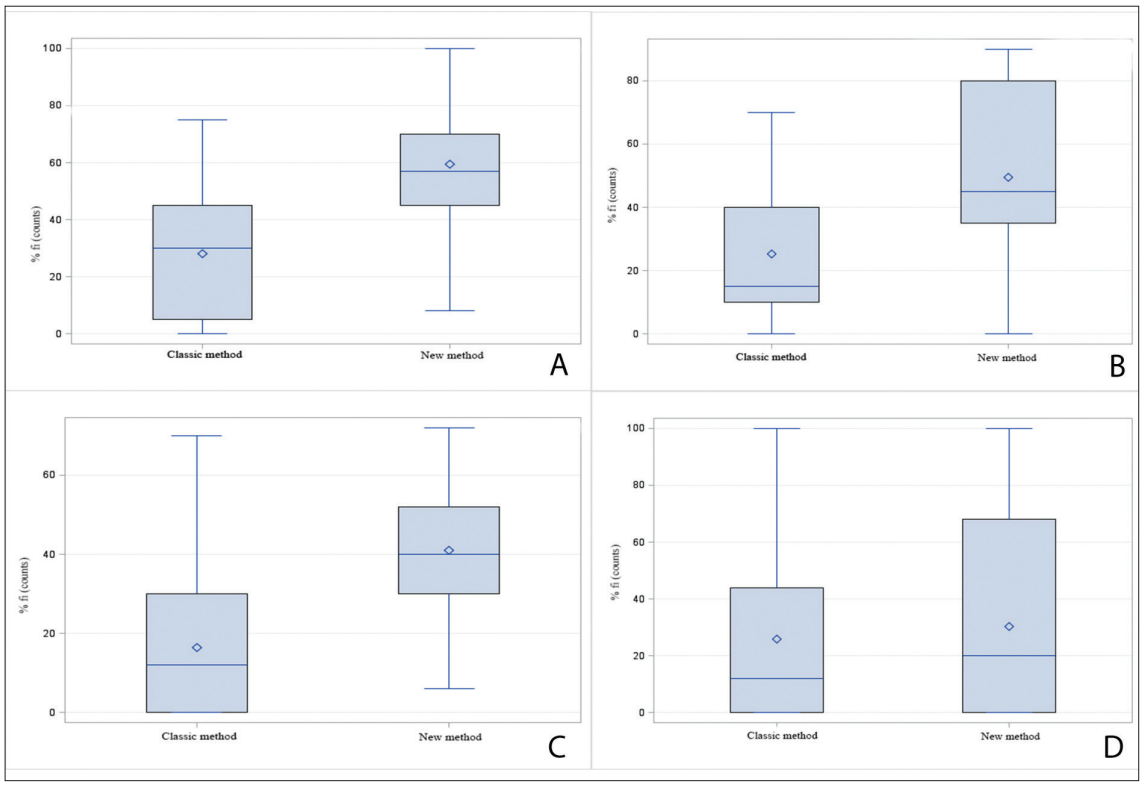

Fig 5. Distribution of the scores for groups at the end ofthe practical course each week (A=1, B=2, C=3, D=4)

Table 4. *Attending number of students participating in theoretical and practical course n=105*

Course Methods	TC1	PC1	TC2	PC 2	TC3	PC 3	TC4	PC 4
Classical Method	38 (18M/20F)	41 (24M/17F)	47 (27M/20F)	35 (22M/13F)	45 (26M/19F)	36 (18M/18F)	42 (24M/18F)	37 (19M/18F)
New Method	47 (23M/24F)	47 (23M/24F)	44 (22M/22F)	31 (12M/19F)	40 (19M/21F)	29 (10M/19F)	41 (20M/21F)	31 (11M/20F)

F: *Number of female students,* ***M:*** *Number of male students,* ***TC:*** *Number of students participating in theoretical course,* ***PC:*** *Number of students participating in practical course*

whether the success of students with the new method of application varied based on gender. It was found that the new method contributed to the success of both genders at similar rates. Moreover, there was no difference in participation rate between the new method groups and the classical method groups according to gender *(Table 4)*. Apart from these, the participation rates of classical method and new method were compared. The success of new method was found to be more than 2 times (ODDS ratio = 2.435) higher compared with the classical method ($P<0.01$) in all application periods.

While this study was being applied, the results of the study were not shared with the students. When the course was completed, a questionnaire was conducted for Group 2. The students who participated to the survey said that the applied lectures were rather satisfactory. ($P=0.1048$).

DISCUSSION

Veterinary Anatomy education is usually taught by (2D) diagrams, photographs and medical tools. Interactive 3D anatomical models and computer-aided learning packages are being developed for the introduction of new models [3,6]. New approaches are tried to facilitate learning mostly in medical anatomy training. In most of these, efficiency is measured by conducting surveys among students [4,17,21,23]. Because of the difficulty of finding cadavers in medical schools, there are new studies examining the effect of clay modeling on learning [24,25]. Alternative haptic simulators have been developed for cadaver use in student applications in veterinary education and positive feedback has been obtained from students [2]. There have also been studies on case-based learning aimed at learning related to clinical anatomy and basic sciences [16]. In this study, we used clay method and plastic model dressing method to make students more active in education process.

In anatomy training, it is reported that plastisin, clay modeling, drawing and body painting are part of the active learning category and when these methods have been applied as "learning by doing and experiencing", the course topics will not be forgotten [3,23,24,26]. There are studies showing that clay modeling is effective in anatomy training [4,20,21,25]. Motoike et al.[20] found that the clay modeling study, in which students were active, increased the success rate of students' exam results and this method was more effective than the cat dissection in the short term on learning about human muscles. DeHoff et al.[25] studied clay modeling in muscles, peripheral nerves and blood vessels in human anatomy and reported that this technique could be used independently in anatomy training. Oh et al.[21] investigated the effects of polymer clay on medical students' understanding of cross-sectional anatomy. Cross-sectional surfaces of organ models such as brain, heart, and lungs made with colored clay were compared with corresponding MRI and CT images of the same section and it was reported that students understood the topic easier. It was stated that there was no difference between classical method and clay group when the permanence of knowledge was measured after a certain period of time. However, it was affirmed that the clay method had an important effect on the learning of complex structures. In this study, in which bone modeling with clay was performed, it was observed that student success rate increased in accordance with the literature [4,20,21-25].

There are also other different methods that are used as alternatives in anatomy training. These are digital, non-digital 3D imaging [15,18], Mobile Augmented Reality [17,27] and methods of painting live material [3]. In this study, apart from these methods, plastic model dressing method has been applied. It was found that plastic model dressing method increased the motivation of the students similar to the other methods mentioned above.

Cevik Demirkan et al.[28] have stated that traditional training tools such as bone, skeletal model and cadaver were very effective in learning about veterinary anatomy and that new alternative approaches could not be an alternative to anatomy education but could be used as an adjunct. The findings obtained in this study are different from those reported by Çevik Demirkan et al.[28]. The results of our study showed that the new method increased the success compared to the classical method. It is thought that the effect of all alternative methods on the success of the course cannot be the same.

In literature reviews, among the studies in which the classical and other methods have been compared, there is no study that looked into the link between the success rate in the course and the gender. In this study, the success rates of the male and female students were compared. Clay and plastic model dressing techniques were shown to increase success rates in both genders.

This study was the first one to evaluate the success of students by using clay method and plastic model dressing in veterinary anatomy training. As a result, it has been found

that clay modeling and plastic model dressing techniques increase student motivation and contribute to learning positively. We think that this method can be expanded to cover all the subjects of anatomy in the future.

Conflict to Interest

We have no conflinct of interest to declare

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Research Article

Drone Semen Cryopreservation with Protein Supplemented TL-Hepes Based Extender [1]

Selim ALÇAY [1,a] Selvinar ÇAKMAK [2] İbrahim ÇAKMAK [2] Emine MÜLKPINAR [1,b]
Mehmed Berk TOKER [1,c] Burcu ÜSTÜNER [1] Hasan ŞEN [2] Zekariya NUR [1,d]

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[1] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Uludag University, TR-16059 Bursa - TURKEY
[2] Beekeeping Development-Application and Research Center, Uludag University, TR-16059 Bursa - TURKEY
[a] ORCID: 0000-0002-2472-8157; [b] ORCID: 0000-0003-0251-987X; [c] ORCID: 0000-0002-7678-3289; [d] ORCID: 0000-0002-1438-221X

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Abstract

The aim of the current study was to determine the optimum concentration of bovine serum albumin for post-thawing quality of drone sperm and this is the first study to evaluate the effect of BSA supplemented TL-Hepes based extenders for drone semen cryopreservation. Sexually mature drones were used for semen collection. Pooled semen was diluted with TL-Hepes based extender supplemented with different concentrations of BSA (1 mg/mL, 3 mg/mL, and 5 mg/mL) or without BSA (control), at a final concentration of $100x10^6$ spermatozoon/mL. Motility, plasma membrane functional integrity (HOST), and defected acrosome (PSA-FITC) were evaluated in the study. At post thaw, the highest sperm motility rates were obtained in the BSA5 group ($P<0.05$). Functional integrity of sperm membrane was better preserved in the BSA3 and BSA5 groups compared to the other groups. The acrosomal integrity rates were higher in BSA5 group than in control group ($P<0.05$). The study shows that bovine serum albumin supplemented TL-Hepes based extenders have beneficial effect on drone semen parameters at post-thaw. The results of the study demonstrated a notable advantage of using 5 mg/mL of BSA in TL-Hepes based extender.

Keywords: *Drone spermatozoa, Bovine serum albumin, Cryopreservation*

Arı Spermasının Protein Eklenmiş TL-Hepes Bazlı Sulandırıcı İle Dondurulması

Öz

Bu çalışmanın amacı, arı spermasının çözdürme sonrası kalitesi için en uygun sığır serum albümin konsantrasyonunu belirlemekti ve bu çalışma, arı spermasının dondurulması için BSA eklenmiş TL-Hepes bazlı sulandırıcıların etkisini değerlendiren ilk çalışmadır. Cinsel olarak olgun arılar sperma toplanması için kullanıldı. Alınan spermalar bir araya getirildikten sonra final konsantrasyonu $100x10^6$ spermatozoon/mL olacak şekilde farklı dozlarda BSA içeren (1 mg/mL, 3 mg/mL, ve 5 mg/mL) ve içermeyen (kontrol) TL-Hepes bazlı sulandırıcılarla sulandırılmıştır. Spermanın değerlendirilmesi amacıyla motilite, plazma membran fonksiyonel bütünlüğü (HOST), ve akrozomal bozukluk (PSA-FITC) değerlerine bakılmıştır. Eritme sonrası en yüksek motilite oranları BSA5 grubunda elde edilmiştir ($P<0.05$). BSA3 ve BSA5 gruplarında fonksiyonel membran bütünlüğü diğer gruplara göre daha iyi korunmuştur. Akrozomal bütünlük oranları BSA5 grubunda kontrol grubuna göre daha yüksek bulunmuştur ($P<0.05$). Çalışma sonucunda, sığır serum albümini eklenmiş TL-Hepes bazlı sulandırıcıların eritme sonrası arı sperm parametreleri üzerinde yararlı etkisi olduğu görülmektedir. Çalışma sonucu elde edilen veriler göz önüne alındığında TL-Hepes bazlı sulandırıcıya 5 mg/mL BSA ilavesinin önemli bir avantaj oluşturduğu görülmektedir.

Anahtar sözcükler: *Arı sperması, Sığır serum albumini, Kriyoprezervasyon*

INTRODUCTION

Cryopreservation is the pillar of the reproductive biotechnology [1]. This reversible process brings sperm metabolism to a standstill, in this way the genetic materials are successfully storaged for a long time [2]. Although

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

cryopreservation is a reversible operation, there are some harmful effects (cold shock, ice crystallization, lipid peroxidation etc.) on spermatozoa [2,3]. These adverse effects may cause irreversible decreases on motility, viability and fertilization ability of spermatozoon [4,5]. Therefore, the achievement of sperm cryopreservation depends on minimizing the adverse effects and maintaining the post-thaw semen quality [1,5].

Freezing of drone semen will improve preservation of the genetic diversity in the honey bee in different regions. For this purpose, genetic diversity of honey bee colony genetic diversity may be explored for the disease resistances [6-10]. Drone semen cryopreservation has been accomplished in recent years. However, the freezing success of drone semen has not reached the desired level nowadays [11,12].

Bovine serum albumin (BSA) has a multifunctional effect on spermatozoa with its macromolecular structure and antioxidant capacity. Therefore, BSA increases the post-thaw sperm motility and protects the plasma membrane against cold shock [13]. In addition, BSA increases the possibility of sperm-zona pellucida interactions and fertility results. For these reasons, various extenders supplemented with BSA are being used for cryopreservation or liquid storage of bull [14], ram [15], goat [16], stallion [17], buffalo [18] and rabbit semen [19]. However, the effect of BSA supplemented TL-Hepes based extender on drone semen cryopreservation has not been examined until now.

The drone semen cryopreservation contributes to the selection and conservation of gene lines in superior genetic characteristics [8,9]. For this purpose, freezing and storage of drone semen which has superior genetic characteristics, allows preservation and widespread of the specified characteristics. The study was conducted to compare the various concentrations of BSA supplemented extenders to freeze of drone spermatozoa.

MATERIAL and METHODS

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

Experimental Design

The study was designed to investigate the efficacy of BSA supplementation to the extender in drone semen cryopreservation. Therefore, different concentrations of BSA (0 mg/mL, 1 mg/mL, 3 mg/mL or 5 mg/mL) supplemented TL-Hepes based extender was used for post-thaw drone semen quality.

Semen Extender Preparation

Hepes based extenders contained 114 mmol NaCl, 3.2 mmol KCl, 2.0 mmol $CaCl_2 2H_2O$, 0.5 mmol $MgCl_2.6H_2O$, 25.0 mmol $NaHCO_3$, 0.40 mmol $NaH_2PO_4.H_2O$, 10 mmol NaLactate (60% syrup), 200 µL Catalase, 10% DMSO, 4 g/L penicillin G, 3 g/L dihydrostreptomycin in distilled water. BSA added to each group of extenders according to experimental design.

Semen Collection and Dilution

Strong and healthy colonies were used to produce honey bee drones that were collected from 5 colonies. Sexually mature drones (15 days or more) were used for sperm collection. Ejaculation was triggered by exerting pressure on the thorax and then gently squeezing the abdomen [12]. Drone semen was taken up into the Schley syringe under a stereo microscope. The collected semen volume per drone was approximately 1 µL in the experiment. The drone semen was pooled (five times) in order to eliminate individual differences. The average volume of each pooled semen was 100 µL.

Each pooled semen were divided into five equal aliquots and diluted separately for a final concentration of approximately $100x10^6$ (spermatozoon/mL) with control or BSA supplemented TL-Hepes based extenders. Diluted samples were cooled to 5°C in an h. After cooling, the sperm samples were equilibrated for further 120 min at 5°C.

Semen Freezing and Thawing

Equilibrated semen was placed into straws (0.25 mL) and frozen in liquid nitrogen vapor. The straws were stored in a liquid nitrogen tank. Three straws were used for post-thaw semen parameters in each group.

Semen Evaluation

Semen evaluation was carried out via thawing the straws in a water bath that has 37°C warmth. Sperm cells were assessed for motility, functional integrity of the cell membrane (hypoosmotic swelling test) and the integrity of the acrosomes with FITC conjugated Pisumsativum agglutinin. The same person conducted the processes and measurements along the research. Semen motility was determined using a phase-contrast microscope (Nikon Alphaphot YS, Japan) (400×) with a warm slide that heated to 37°C and the motility results were expressed in percent [12].

The hypoosmotic swelling test (HOST) was assessed based on coiled tails at drone semen. Semen (10 µL) was incubated with host solution (100 µL of 100 mOsm) at 37°C for 30 min. At least 200 sperm cells were evaluated and spermatozoa with coiled tail were recorded [20].

Acrosomal integrity was evaluated with using Fluorescein lectin staining assay (PSA-FITC). Briefly, spermatozoa (10 µL) were suspended in 100 mL phosphate buffered saline (PBS) and centrifuged at 100 RCF (g) for 5 min. The sperm pellet was resuspended in 100 mL PBS. Spermatozoa were smeared on glass microscope slides using another slides and fixed with acetone at 4°C for 15 min. Spermatozoa were stained with the solution of PSA-FITC in a dark chamber at

37°C for 1 h. At least 200 drone spermatozoa were assessed at per smearunder a fluorescence microscope [21].

Statistical Analysis

The results were analyzed using SPSS (SPSS 23.0 for Windows; SPSS, Chicago, IL, USA) and presented as mean ± standard deviation. Shapiro Wilk test was used as normality test. Semen parameters were analyzed using one-way ANOVA followed by Tukey. Pearson correlation coefficient was used to evaluate the relationships among the values of motility, plasma membrane functional integrity and acrosomal integrity. P<0.05 were considered to be statistically significant.

RESULTS

Sperm motility, plasma membrane functional integrity and defected acrosome rate of pooled semen were 88.00±2.73%, 89.40±3.36% and 6.40±1.67%, respectively. The percentages of sperm motility, plasma membrane functional integrity and defected acrosome of post-thawed drone semen from BSA and control groups, were indicated in *Table 1*.

Motility of drone spermatozoon was progressively decreased through the process of freeze-thawing (P<0.001). The motility rates better preserved in the BSA groups than the control groups (P<0.05). The highest percentage of motility was obtained in BSA5 group at post-thaw (P<0.05).

Plasma membrane functional integrity *(Fig. 1)* rates were reduced after thawing procedure (P<0.001). BSA3 and BSA5 groups had better results than BSA1 and control groups in terms of the functional integrity of the cell membrane (P<0.05).

Sperm acrosome *(Fig. 2)* was negatively affected by the freeze-thaw process (P<0.001). The percentage of defected acrosome in BSA5 group was lower than control group (P<0.05). Defected acrosome rates were not found significant among BSA groups (P>0.05).

The Pearson correlation test values are shown in *Table 2*. Motility was positively correlated with membrane integrity but negatively correlated with defected acrosome rates (P<0.01). In addition, there was a negative correlation between membrane integrity and defected acrosome rates (P<0.01).

DISCUSSION

Evidences suggest that, cryopreservation has a destructive effect on spermatozoon because of temperature change, cold shock and ice crystallization. These adverse effects provoke to decrease of motility, acrosomal integrity, and

Table 1. *The mean results of studied sperm post-thaw parameters on different extender groups*

Group	Motility (%)	HOST (%)	Defected Acrosome (%)
Control	39.33±3.71^{a}	59.20±3.58^{a}	24.20±3.82^{a}
BSA1	43.66±4.41^{b}	61.53±4.15^{a}	21.34±2.79^{ab}
BSA3	50.00±4.62^{c}	66.06±3.15^{b}	21.17±2.97^{ab}
BSA5	54.33±3.71^{d}	67.73±4.07^{b}	18.80±3.44^{b}

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

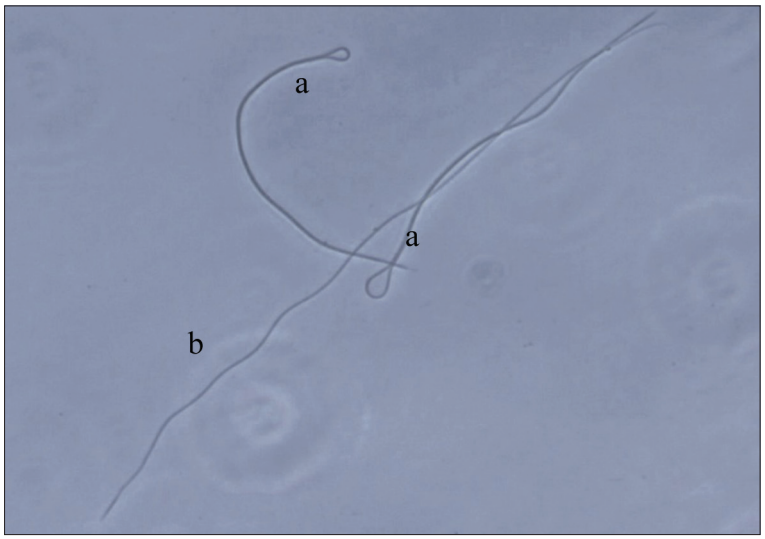

Fig 1. Membrane integrity (a) and damaged membrane (b) by HOS test

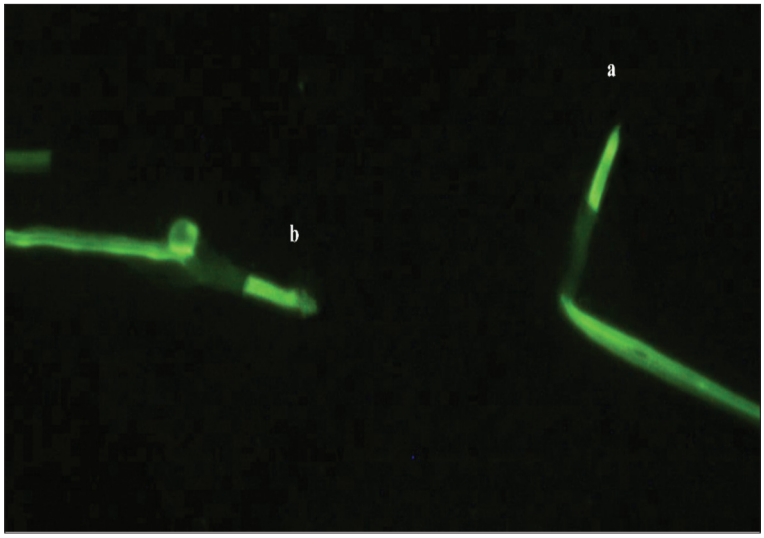

Fig 2. FITC-PSA stained spermatozoa with intact (a) and defected acrosome (b)

Table 2. *Correlation coefficient (r) of studied drone semen parameters*

Spermatological Parameters	HOST (%)	Defected Acrosome (%)
Motility	0.797**	-0.801**
Host (%)		-0.762**

*** Significance of the correlation is at the P<0.01*

fertilizing ability of spermatozoa [5,21-23]. Various extenders were used to minimize the adverse effect of freeze thaw process [5,8,22-24]. In the current study, we compared the effect of exogenous addition of BSA in TL-Hepes based extender on drone semen quality at post thaw. This is the first study to evaluate the effect of BSA supplemented TL-Hepes based extenders for drone semen cryopreservation.

After artificial insemination of queen bees, only motile spermatozoa could arrive to the spermatheca over a 48 h period [24,25]. In the study, BSA5 group yielded higher motility rates than other groups at post thaw time point (P<0.05). The post-thaw motility values of drone semen cryopreserved with different extenders ranged between 25%-62% [8,11,12,24]. After thawing, the motility rate in BSA5 group has a common point with the findings of other studies. In the study; BSA supplementation prompted to clear increase on motility at post-thaw. In addition, increasing doses of BSA positively affected sperm motility.

Plasma membranes have an important role in spermatozoon metabolism [19]. Therefore, integrity of the plasma membrane is essential for capacitation, acrosome reaction and oocyte fusion of sperm [26]. However, plasma membrane could lose its selective permeability because of the cold shock [27]. The protection against cold shock is possible with increasing the fluidity of cell membrane [28]. The protective effect of BSA against cold shock is based on this expected impact. BSA attaches to the sperm membrane and then changes sperm membrane lipid composition and decreases to phospholipid concentration [29]. In our study, the HOST rates in BSA3 and BSA5 group were higher than in the other groups (P<0.05). The HOST values have common points for the results with the previous research [12].

Acrosomal integrity is related with sperm penetration and fusion to zona pellucida. Therefore, this is another important factor in the fertilization process. The other adverse effect of cryopreservation is the acrosomal damage [5,21,23]. Bovine serum albumine succesfully protects the integrity of acrosome [15,19]. In the study, there was no statistical difference among BSA groups. Additionaly, BSA5 group preserved acrosomal integrity better than control group. The statistical difference of acrosome integrity between the groups of control and BSA5 by evaluating with PSA-FITC staining assay will brighten the path of further studies for this area.

In the study, a positive correlation was obtained between the post thaw sperm motility and plasma membrane functional integrity rates (P<0.01). As this is an expected result; the motility partly depends on the transport of compounds across the cell membranes [23], there are previous reports with similar findings [5]. In addition, motility negatively correlated with non-intact acrosome rates (P<0.01). These results showed the same understanding that pointed with the other reports [20].

The outcomes of the study indicated that BSA5 group preserved sperm motility better than other groups. Considering to all sperm parameters (motility, plasma

membrane functional integrity and acrosomal integrity); BSA5 group was the optimum for drone semen cryopreservation among studied doses. Beneficial effect of BSA supplementation looked promising to increase the utility of TL-Hepes based extender for drone spermatozoa. Further studies should take place to improve the effects of the BSA supplemented TL-Hepes based extender concerning with sperm fertility.

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Research Article

Evaluation of Early and Late Period Results of Polyester Film Use for the Repair of Ventral Hernias: An Experimental Study on Rabbit Models [1]

Engin KILIÇ [1,a] Sadık YAYLA [1,b] Vedat BARAN [1,c] Berna ERSÖZ KANAY [2,d]
Celal Şahin ERMUTLU [1,E] Kemal KILIÇ [3,f] Mustafa GÖK [4,g] Serpil DAĞ [5,h]

[1] *This study was presented orally in the 14th National Veterinary Surgery Congress, October 23-26, 2014, Antalya, Turkey*

[1] Kafkas University, Faculty of Veterinary Medicine, Department of Surgery, TR-36100 Kars - TURKEY
[2] Dicle University, Faculty of Veterinary Medicine, Department of Surgery, TR-21280 Diyarbakır - TURKEY
[3] Tuzla State Hospital, Clinic of General Surgery, TR-34947 İstanbul - TURKEY
[4] Adnan Menderes University, Faculty of Medicine, Department of Radiology, TR-09010 Aydın - TURKEY
[5] Kafkas University, Faculty of Veterinary Medicine, Department of Pathology, TR-36100 Kars - TURKEY
[a] ORCID: 0000-0001-8126-3918; [b] ORCID: 0000-0001-6734-421X; [c] ORCID: 0000-0003-3466-5345; [d] ORCID: 0000-0001-5165-0618
[e] ORCID: 0000-0002-8923-7682; [f] ORCID: 0000-0003-1070-6886; [g] ORCID: 0000-0001-7021-0984; [h] ORCID: 0000-0001-7667-689X

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Abstract

The purpose of this study is to assess clinical, MR, USG and histopathological findings of early and later results of polyester film use in experimental ventral hernia model in rabbits. In this study, 16 New Zealand rabbits were divided into 2 groups of 8 rabbits each. After xylazine HCl usage as a sedative, the surgery is conducted under general anaesthesia by ketamine HCl. Upon median skin incision, a 2 cm diameter round defect on linea alba level was formed in rabbits lying in the supine position. The defect was repaired with 4 cm diameter polyester film which had been prepared beforehand in the shape of a disc. After clinical follow up of 15 days for rabbits in group I and 60 days for group II, USG and MR images were taken, by re-laparotomy the presence and extent of peritoneal adhesion was examined visually. All data from the study were statistically assessed via Minitab-16 package program. Tissue samples from the graft area were stained through hematoxylin-eozin and Crossman's triple dye and assessed by usage of light microscope. During histopathological examination, it was observed repair by fibrous tissue in the area where surgery was performed for all the animals in 15 day group. Neither foreign body giant cell reaction nor necrosis was observed in two groups. When clinical, USG, MR findings along with re-laparotomy, macroscopic and histopathological results are taken into account, it was seen that polyester film bore successful results with regards to ventral hernia therapy in rabbits. Based on the data provided by this study, it is decided that the mentioned material should be tried for large hernia defect repair in domestic animals.

Keywords: *Graft, Ventral hernia repair, Polyester film, Rabbit*

Ventral Hernilerin Onarımında Polyester Film Kullanımının Erken ve İleri Dönem Sonuçlarının Değerlendirilmesi: Tavşan Modellerinde Deneysel Bir Çalışma

Öz

Bu çalışmada, tavşanlarda deneysel olarak oluşturulan ventral herni modelinde, polyester film kullanımının erken ve ileri dönem klinik, MR, USG ve histopatolojik sonuçlarının değerlendirilmesi amaçlanmıştır. Çalışmada kullanılan 16 adet Yeni Zellanda tavşanı 8'erli 2 gruba ayrıldı. Operasyonlar xylazin HCl sedasyonunu izleyerek ketamin HCl ile elde edilen genel anestezi altında gerçekleştirildi. Operasyon masasına sırtüstü pozisyonda yatırılan tavşanlara median deri ensizyonu yapıldıktan sonra linea alba düzeyinde 2 cm çaplı daire şeklinde bir defekt oluşturuldu. Defekt önceden disk şeklinde hazırlanan 4 cm çaplı daire şeklindeki polyester film ile onarıldı. I. Gruptaki tavşanların 15, II. gruptakilerin ise 60 günlük klinik takibin sonunda USG ve MR görüntüleri alındıktan sonra bu tavşanlara relaparotomi uygulanarak peritoneal adezyon varlığı görsel olarak değerlendirildi. Çalışmadan elde edilen tüm veriler Minitab-16 paket programı kullanılarak istatistiksel olarak değerlendirildi. Greft uygulanan bölgeye ait doku örnekleri Hematoksilen-Eozin (HE) ve Crossman'ın üçlü boyama yöntemi ile boyanarak ışık mikroskobunda değerlendirildi. Histopatolojik incelemede 15 günlük gruptaki hayvanların tamamında uygulama yapılan alanda fibröz doku ile onarım gözlendi. Ayrıca her iki grupta da yabancı cisim dev hücre reaksiyonu ve nekroz oluşumu gözlenmedi. Klinik, USG ve MR bulguları ile relaparotomik makroskopik ve histopatolojik sonuçlar polyester filmin tavşanlarda ventral herni sağaltımında başarılı sonuçlar verdiğini ortaya koymuştur. Sonuç olarak çalışmadan elde edilen veriler dikkate alındığında bu materyalin evcil hayvanlarda görülen geniş defektli fıtıkların onarımında kullanılabilceği sonucuna varıldı.

Anahtar sözcükler: *Greft, Ventral herni onarımı, Polyester film, Tavşan*

İletişim (Correspondence)
+90 546 2626007
sahinermutlu@hotmail.com

INTRODUCTION

Ventral hernias are hereditary or acquired as a result of various reasons. Hernias occurring following abdominal surgical interventions have an important place within the acquired ones [1,2]. Regardless of the reason, the treatment option is operation and primary repair should be considered first. However, in cases where the tissue is not strong enough or the hernia hole is too large, various graft materials are used for support [3-5]. Nonabsorbable meshes such as polyester, silicone, polyester weave, polyvnyl weave, and polypropylene are used while absorbable materials such as polyglactin 910, polyglactic acid can also be preferred for this purpose [6-8]. Nowadays, polytetra filament mesh, sepra-mesh, marlex, prolene, surgipro, mersilene, and goro-tex are amongst the most preferred materials [4,8-14]. All these graft materials can have superior characteristics over each other while in practice, the choice of graft material can change to begin with according to the habit of the operator, the general characteristics and the cost of the material. In hernioplasties, adhesion is the most commonly encountered complication along with foreign body reaction, inflammation, fibrosis, calcification, thrombosis, seroma, graft migration, chronic pain, and infection [1,4,14-17]. An ideal graft material is desired to not interact with the tissue fluids, be inert, not cause inflammation and foreign body reaction, be resistant against mechanical tension, not be carcinogenic, not cause allergy or hypersensitivity, be producible in needed sizes and forms, be easily sterilisable, and be economical [1,2,4,18].

In this study, the objective was to clinically, radiologically (MRI, USG), and histopathologically examine the early and late period results of the use of x-ray film (polyester film), which has almost no cost, in ventral hernia models formed experimentally in rabbits and to evaluate this used material regarding its compatibility with general characteristics of a graft material.

MATERIAL and METHODS

After obtaining the Dicle University Animal Experiments Local Ethics Committee approval (DÜHADEK-2013-16), the animal material of the study which started as planned was formed by 16 adult male rabbits *(New Zealand)* weighing average 3450 g (3120-3860 g). The rabbits were separated into two groups consisting of 8 rabbits each as Group I to be monitored for 15 days (to see if the peritoneal wall is fully closed) and Group II to be monitored for 60 days (for evaluation of late-term results). Rabbits were fed with standard rabbit pellet feed and water *ad-libitum* until the end of the study. Before the beginning of the experiments, the rabbits' adaptation to the environment was provided for one week. During the adaptation period and throughout the experiment, the rabbits were kept in light for 12 h and in darkness for 12 h at 20-22°C constant temperature and 45-55% humidity rate.

For the operations, following xylazine HCl (10 mg/kg, IM, Rompun® 2%, Bayer, Germany) sedation, dissociative anaesthesia produced by ketamine HCl (30 mg/kg, IM, Ketasol® 10%, Richterfarma, Australia) was preferred. After shaving and disinfecting the abdominal region, the region was prepared aseptically. The rabbits were placed on the operation table in dorsal position and medial skin incision was performed *(Fig. 1 a)*. Afterwards a 2 cm diameter circle shaped defect was formed at the linea alba level *(Fig. 1 b)*. The defect was repaired in-lay with a 4 cm diameter round

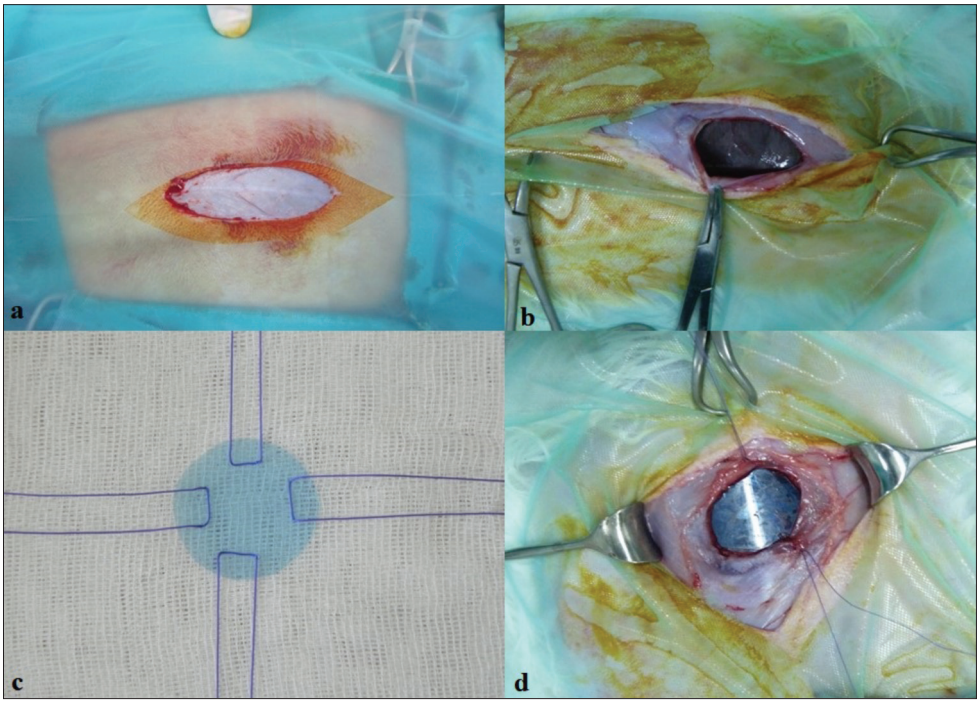


Fig 1. Repair of hernia line with polyester graft. **a-** Medial skin incision, **b-** Forming a 2 cm diameter circular defect at the linea alba level, **c-** Polyester film previously prepared in disc shape, **d-** Insertion of polyester film to defect

shaped polyester film (Fuji medical X-Ray film) previously prepared in disc shape *(Fig. 1 c,d)*. The polyester film used as the graft material was acquired by scratching the previously used x-ray film until revealing the support layer and then subjecting to the sterilization process with ethylene oxide *(Fig. 2)*. The x-ray film left in a 1% benzalkonium chloride (Zefiran forte, İlsan-İltaş, Turkey) solution for 30 min. Then the film (polyester graft) was scraped with the spatula until only the support layer remained. Disc-shaped grafts with a diameter of 5 cm were obtained and these were sterilized by ethylene oxide. The area was routinely closed, and each rabbit was kept in their own separate cages. In order to provide postoperative analgesia, metamizole sodium (30 mg/kg, IM, Novalgin-Sanofi, Turkey) was applied for 3 days and in order to provide prophylaxis cephazolin sodium (20 mg/kg, IM, Iespor 250 mg, İbrahim Etem Ulugay İlaç Sanayi Türk A.Ş., Turkey) was applied for the same period of time. The care of the operation areas of the rabbits was given daily and their feed-water intake was monitored. USG (Toshiba Aplio XG Ultrasound Scanner, Japan, 1-8 MHz) and MRI (Magnetom Essenza, 1.5 Tesla MR Scanner, Siemens, Healthcare, Erlangen Germany) images of the rabbits in Group I were taken at the end of the clinical follow up of 15 days while the images of the rabbits in Group II were taken at the end of the clinical follow-up of 60 days. Relaparotomy was performed on the rabbits and abdominal cavity was reached with an incision of approximately 5 cm diameter from 1 cm lateral of the graft and firstly the peritoneal surface of the graft was evaluated in terms of adhesion *(Fig. 3)*. Existence of peritoneal adhesion and its degree was recorded according to Jenkins et al.[19] visual adhesion scale. After necessary tissue samples (5 cm diameter from 1 cm lateral of the graft) were taken for histopathologic examination, the laparotomy gaps of the rabbits were closed with the same method and similar care and feeding conditions were provided in the postoperative period for their survival. Tissue samples of the grafted region were fixed in 10% buffered formalin. Paraffin blocks of the tissues were prepared following routine laboratory procedures. The sections of 5 µm thickness taken from these blocks were stained with Hematoxylin-Eosine (HE) and Crossman triple staining method and evaluated under light microscope (Luna 1970). The sections stained with Crosmann triple staining method were examined in terms of fibrous tissue formation and organization. The sections stained with HE were evaluated in terms of leucocyte infiltration, foreign body giant cells, fibrosis, capillarization, and necrosis. In the samples taken for histopathological examination, the graft material was stripped from the tissues in order to prevent any problems that might occur during the tissue

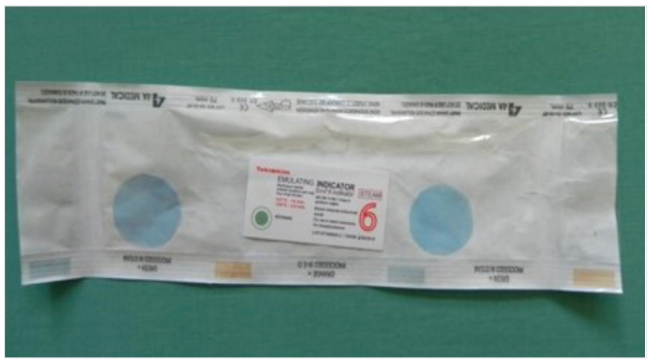


Fig 2. Sterilized polyester graft with ethylene oxide

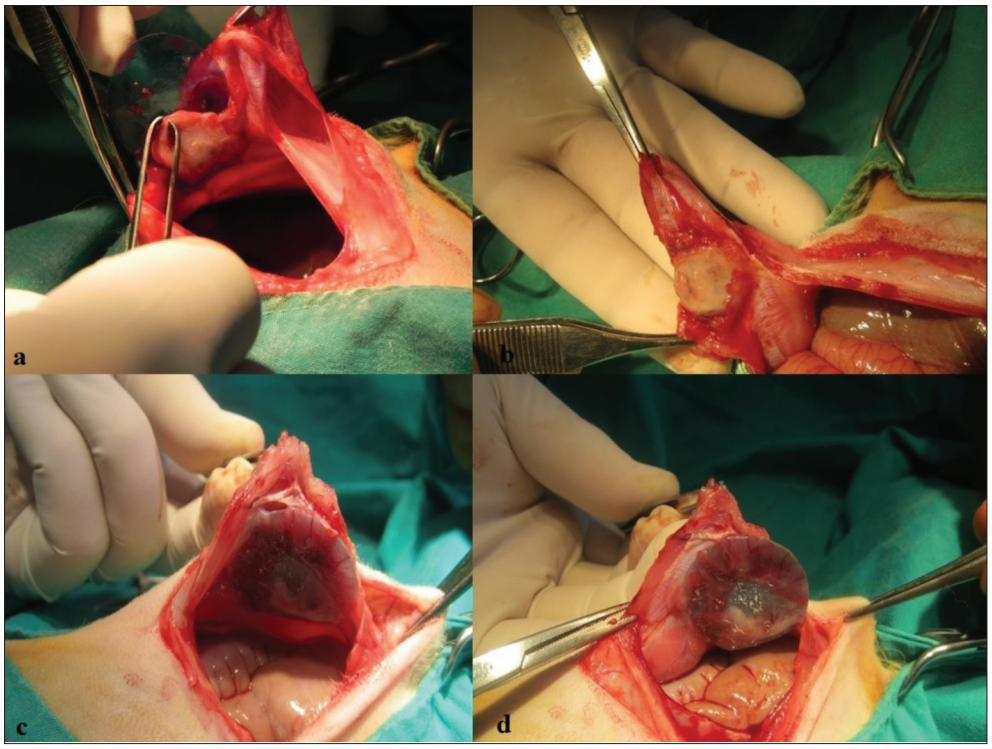


Fig 3. Macroscopic evaluation of the peritoneal surface of the graft. **a-** Removal of the graft from the pouch between the peritoneum and the abdominal muscles, Group II; **b-** Peritoneal graft site, Group II; **c-** The peritoneum completely covered the abdominal surface of the graft, Group I; **d-** No adhesion between graft and abdominal organs, Group I

administration procedures. All data acquired from the study were statistically evaluated by the use of Minitab-16 package programme.

RESULTS

During the daily routine follow-up of the rabbits, there were no problems encountered such as serositis, haematoma, and increase in body temperature. It was observed that 6 h after waking up from anaesthesia, the rabbits were willing in feed and water intake and were able to move inside the cage with comfort. In the ultrasonographic examination conducted on the 15th day, it was observed that in all of the rabbits in Group I, the graft kept its position, there was an increase of thickness in cutaneous and subcutaneous tissues. It was detected that there was a clear difference between Group I and II in terms of tissue thicknesses. In the MRI results of Group I, it was observed in the transverse fat saturated (fat-sat) T1AG and the sagittal fat saturated (fat-sat) T1AG that there was an increase of thickness in cutaneous and subcutaneous tissues (compatible with granulation tissue) in the area the graft was placed. It was observed especially in the fat saturated images that this granulation tissue did not extend to the mesenteric fat tissue *(Fig. 4)*. A minimal level of thickness increase was observed in the cutaneous and subcutaneous tissues in the graft applied region of Group II in the MRI results. But, these thickness increases were not measured. It was observed that the granulation tissue became thinner and did not extend to the mesenteric fatty tissue or abdominal fascia *(Fig. 5)*. In the macroscopic evaluation of the hernia region, adhesion was not observed in any of the rabbits in Group I and II *(Table 1)*. It was detected by only observation that the surface of the graft material facing the abdominal cavity was covered with peritoneum compatible layer. It was observed that the surface of the peritoneal membrane covering the graft

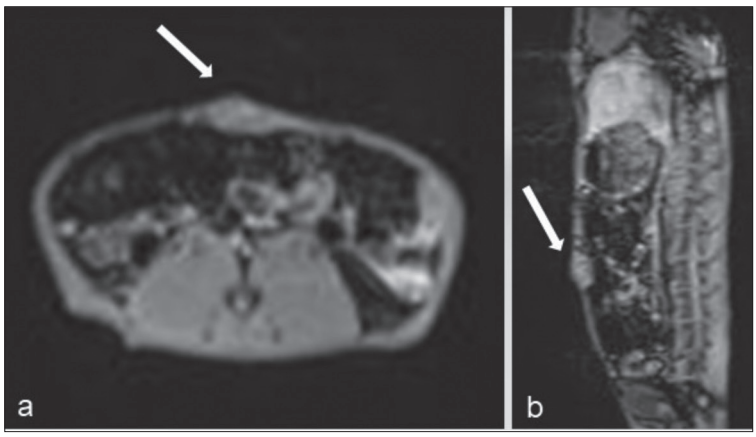


Fig 4. MRI of one case in Group I. **a-** Transverse fat-suppressed T1AG; **b-** Saggital fat-suppressed T1AG

Fig 5. MRI of one case in Group II. **a-** Transverse fat-suppressed T1AG; **b-** Saggital fat-suppressed T1AG

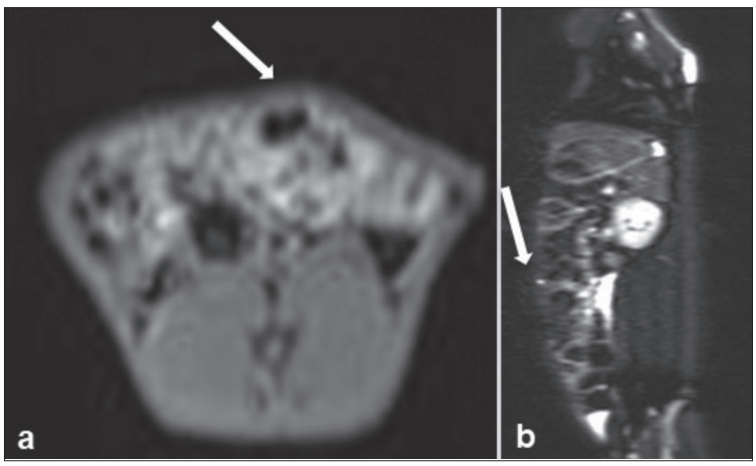


Table 1. *Distribution of adhesion grades in group I and group II*

Adhesion Grades		**Group I**	**Group II**
No adhesion	0	n: 8	n: 8
Adhesion allocated with mild blunt dissection	1	n: -	n: -
Adhesion allocated with aggressive blunt dissection	2	n: -	n: -
Adhesion allocated with sharp dissection	3	n: -	n: -

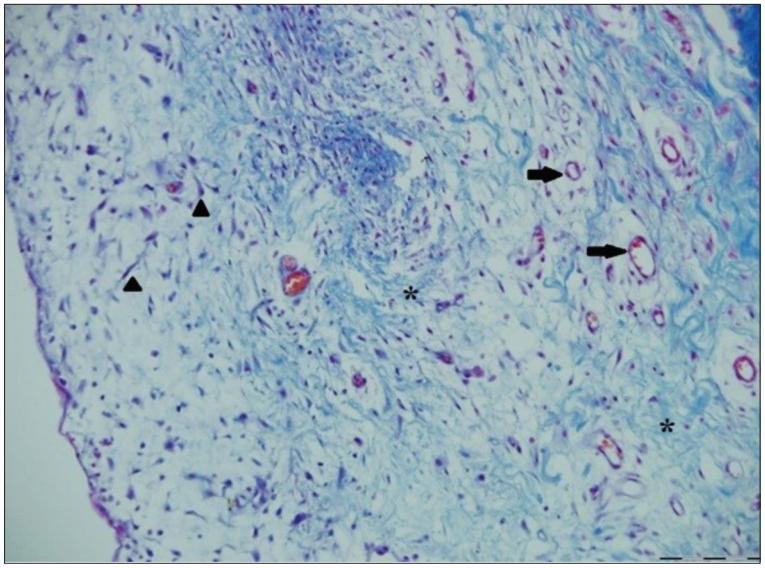
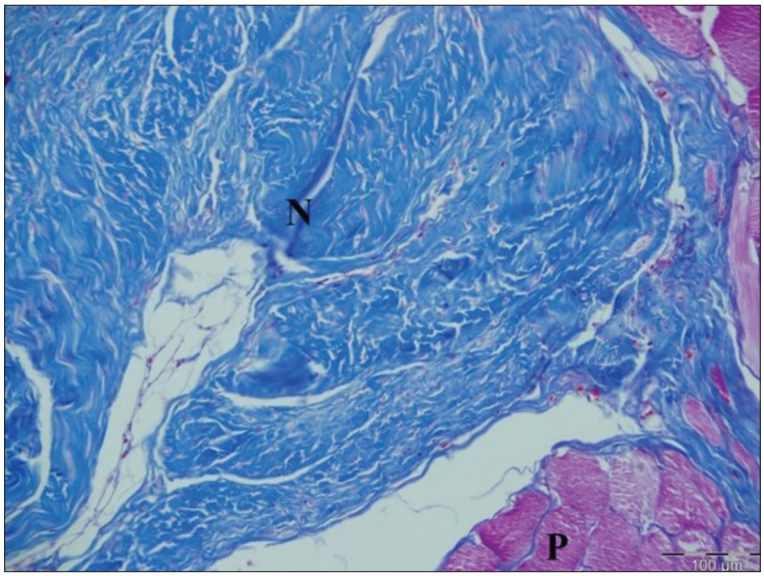


Fig 6. Histopathologic evaluation of a case in Group I. Collagen fibers *(stars)*, vascularization *(arrows)*, Fibroblast/ fibrocits *(arrowheads)*, Crosmann's triple stain, Bar: 100 µm

Fig 7. Histopathologic evaluation of a case in Group II. Scar tissue formation (N) Collagen fibers, normal peritoneal surface (P), Crosmann's triple stain, Bar: 100 µm



was smooth. According to the visual adhesion scoring, adhesion level in all cases was recorded as "0".

In the histopathologic examination of the tissue samples taken from the abdominal wall of the area where the graft is applied, it was observed that the graft was covered with fibrous tissue in all of the animals in the 15-day group. New capillary formations, many fibroblasts and fibrocytes, collagen fibres and a small number of mononuclear cell infiltration was observed in the forming granulation tissue *(Fig. 6)*. Foreign body giant cell reaction and necrosis were not observed in any of the rabbits. Organization characterized with fibrous tissue formation was observed in the application region in Group II of the study. Vascularization, decrease in cellularity, and increase in collagen fibres were observed in the application region of the rabbits in this group. Similar to the other group, there were no foreign body giant cell reaction and necrosis observed in this group as well *(Fig. 7)*.

DISCUSSION

Regardless of the reason, operation is mandatory for the treatment of ventral hernias [18,20]. In cases where primary repair is inadequate, herniorrhaphy can be performed using various graft materials with organic or synthetic characteristics [12,17]. For this purpose, for many years until today, many graft materials have been used by different researchers. Although some improvements can be provided in the general characteristics of the graft materials parallel to the scientific and technological developments, it is difficult today to talk about a graft material with ideal characteristics. Studies regarding introducing different graft materials into clinical practice by taking into consideration postoperative complications are continuing today as well. In this study, the polyester support layer acquired by separating from the top, emulsion, and bottom layers of the idle, used x-ray films were used as graft material. It was aimed to contribute to veterinary medicine

a new graft material by evaluating together the early and late period clinical, MRI, USG, and histopathologic results of polyester film use for the purpose of hernioplasty. One of the most important complications developing after hernioplasty operations is foreign body reaction [4,10,14]. Not encountering any foreign body reactions in and around the hernia region with graft application during the postoperative examinations and histopathologic examinations of all the rabbits (n=16) in Groups I and II evaluated in the context of the presented study has given us hope that the polyester films are biocompatible and can be used in clinical cases safely after being subjected to an appropriate sterilization operation.

In the postoperative process following hernioplasty operations, it has been reported that complications can develop such as inflammation, fibrosis, calcification, thrombosis, seroma, graft migration, chronic pain, and infection [4,21-23]. Complications such as seroma, inflammation were not encountered in the wound region during the daily clinical examinations of the rabbits from the first day until the end of the study. It was determined that the graft kept its first applied position in the USG and MRI examinations and it was detected that the granulation tissue was relatively thick on the 15th day but the granulation tissue became thinner on the 60th day. Calcification or fibrosis were not observed in the area during re-laparotomy. These complications reported to have developed in some cases where ventral hernias are repaired with graft [1,11] were not encountered in any of the rabbits included in the study. This result was interpreted that a used polyester film may be in inert structure.

It has been reported that adhesions are at the top of the most prevalently encountered complications in hernioplasty operations [11,24] and adhesions can cause many negative results from infertility to death [5]. It is known that adhesions can develop with the effect of evaporation even in the cases where just the abdomen is opened without any surgical intervention to intraabdominal organs [3,22,25,26]. Thus, it is suggested for the incision line to be kept as limited as possible during laparotomy operations and to humidify intraabdominal organs throughout the operation. Physiologically, the lubrication of the peritoneal cavity is provided with peritoneum fluid. Since most of the synthetic and organic graft materials used for hernioplasty have a permeable structure, peritoneum fluid and inflammation cells required for healing leak through the hernia line to the subcutaneous region or outside the skin. As a result of the contamination forming related to this, fibrosis and adhesion form between the graft, the surrounding tissues, and the abdominal organs. Adhesion was not encountered at the examination during relaparotomy performed both on the 15th day of the study and the 60th day of the study. Histopathologic results also showed that there was no inflammatory reaction which can provide basis for adhesion. It is considered that the polyester film used in-lay not being permeable, its surface being smooth, and it being biocompatible were effective in the non-development of adhesion.

During the relaparotomy conducted both on the 15th day and the 60th day, it was observed that the surface of the graft material facing the abdominal cavity was covered with a thin, shiny, and smooth layer in all cases. The studies showed that this area is closed in a short time with the peritoneal epithelisation mechanism regardless of the size of the peritoneal loss. It was detected with the histopathologic evaluation of the presented study that the thin membrane covering the graft was of normal peritoneum structure.

An ideal graft material is desired to be inert, not interact with tissue fluids, not cause inflammatory and foreign body reactions, not be carcinogenic, not cause allergy and hypersensitivity, be resistant against mechanical tensions, be produced in required forms, and be sterilisable [2,18,23,26].

There are different sizes of commercial forms of x-ray films such as 13-18, 18-24, 24-30, 30-40, 35-35 cm [11]. Thus, it can be said that the polyester film prepared by cutting before the operation or during the operation in appropriate sizes according to the size of the hernia hole provides an advantage. Since the hernia size was formed as determined in the experimentally planned study, the graft materials were prepared in advance by cutting them to the appropriate size (5 cm). Moreover, suture materials were attached in four different directions to the prepared graft material in advance and application ease was provided. In the meantime, it was observed that the spearhead needles damage the grafts during suturing as the film is of semi-rigid structure and at the same time, since the line the needle passes through is of sharp characteristics, it can damage the thread. From this experience, it is suggested that the needles to be used in grafting should be tubular body needles.

It has been reported that there are different graft materials in the polyester structure such as mersilene, paritex, dacron, biomesh used in the treatment of hernia. However, all of these materials need to go through some processing to be converted into graft. These processes also require a certain amount of cost. In this material, which we prefer as autograft, there is no such cost.

A statistical comparison was not performed since adhesion was not observed in any of the cases (n=16) used for the study during the re-laparotomies performed for adhesion scoring.

In conclusion, when clinical, MRI, USG, and histopathologic results are evaluated together, since it was reached the opinion that the polyester film meets most of the expected characteristics from an ideal graft material in experimental ventral hernia model, it gave hope to the researchers that

the same material can be safely used in the clinical cases as well.

Authors' Contrubution

EK, CŞE, VB designed and executed the study, SY, BEK, KK did study sampling, MG did radiological examination, SY arranged and analyzed the statistical data, SD processed the samples, and histopathological examination, EK and CŞE wrote the manuscript. All authors reviewed and approved the manuscript for submission.

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Research Article

Computer-Assisted Automatic Egg Fertility Control

Mustafa BOĞA [1,a] Kerim Kürşat ÇEVİK [2,b] Hasan Erdinç KOÇER [3,c] Aykut BURGUT [4,d]

[1] Niğde Ömer Halisdemir University Bor Vocational School, TR-51700 Bor/Niğde - TURKEY
[2] Akdeniz University, Faculty of Social Sciences and Humanities, TR-07600 Manavgat/Antalya - TURKEY
[3] Selçuk University, Faculty of Technology, TR-42130 Selçuklu/Konya - TURKEY
[4] Çukurova University, Directorate of Research and Application Farm, TR-01230 Sarıçam/Adana - TURKEY
[a] ORCID: 0000-0002-2845-4528; [b] ORCID: 0000-0002-2921-506X; [c] ORCID: 0000-0002-0799-2140; [d] ORCID: 0000-0002-5335-5070

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Abstract

This research aimed to determine the fertilization control of the eggs in an incubator between 0th and 5th days by image processing techniques via low-priced tools. Three different datasets that were composed of eggs whose images taken at different times in the incubator were prepared. Several filtering and morphology methods, gray level conversion and dynamic thresholding were utilized to process the 15 egg images. Moreover, the original processing codes based on the problem were given. White and Black percentages of binary images were utilized to determine the egg control. According to the test results, for the first dataset; 73.34% of fertility accuracy was achieved on the third day; 100% of fertility accuracy was achieved on the fourth day, for the second dataset; 93.34% of fertility accuracy was achieved on the third day; 93.34% of fertility accuracy was achieved again on the fourth day; for the third dataset, 93.34% of fertility accuracy was achieved on the third day; 100% of fertility accuracy again was achieved on the fourth day. When the results were evaluated, it was seen that egg fertility has been determined successfully automated with low cost tools.

Keywords: *Egg incubator, Poultry production, Egg fertility control, Image processing, Dynamic thresholding*

Bilgisayar Destekli Otomatik Yumurta Döllülük Kontrolü

Öz

Çalışmada kuluçka makinesinde yumurtaların 0-5 gün aralığında döllülük kontrolünün kolay elde edilebilen ve az maliyetli araçlar kullanılarak görüntü işleme teknikleri ile tespit edilmesi amaçlanmıştır. Denemede, ev tipi standart kuluçka makinesi içine farklı zamanlarda görüntüleri alınan 15 yumurtadan oluşan üç farklı veri seti hazırlanmıştır. Yumurta görüntülerinin işlenmesinde çeşitli filtreleme ve morfoloji yöntemleri, gri seviye dönüşümü ve dinamik eşikleme yöntemi kullanılmıştır. Ayrıca probleme dayalı özgün görüntü işleme kodları yazılmıştır. Elde edilen binary görüntülerin beyaz/siyah oranları döllülük kontrolünü belirlemede kullanılmıştır. Deneysel sonuçlara göre ilk veri setinde 3. gün %73.34, 4. gün %100, ikinci veri setinde 3. gün %93.34, 4. gün %93.34 ve üçüncü veri setinde 3. gün %93.34, 4. gün %100 doğrulukla döllülük durumları tespit edilmiştir. Elde edilen sonuçlar değerlendirildiğinde, yumurta döllülük kontrolünün az maliyetli ve edinilebilir araçlar ile başarılı bir şekilde otomatikleştirilebileceği görülmüştür.

Anahtar sözcükler: *Kuluçka makinesi, Kanatlı hayvan üretimi, Döllülük kontrolü, Görüntü işleme, Dinamik eşikleme*

INTRODUCTION

The egg industry is one of the main industries in the food chain as well as it plays a significant role in meeting the protein need of the world. Hatchability is essential in the egg industry. Even though the hatchability is affected from various factors such as the quality of eggs, breeding ratio, survival rate, and poultry quality; the most important factor is being sure about the eggs in the incubator are the fertile ones [1,2]. Durmuş pointed out that using quality hatching eggs is pretty significant besides providing optimum incubation conditions to keep hatching at high levels. However, Kamalı and Durmuş pointed out that there are a lot of factors that affect the chick quality as well as the chick quality will increase based on these factors to reach the optimum level [3]. Generally, the qualified personnel manually control the eggs which have fertility before putting them into the egg incubator. However, the fertility control is performed during the preliminary development phase and the final phase (18th day for chicken egg) to

İletişim (Correspondence)
+90 242 7427025 Mobile: +90 536 8379550
kcevik@akdeniz.edu.tr

keep the temperature and humidity values of the egg incubator. Performing the fertility control by manual and being based on the expert control increase the error rate. The control process is effort consumption; the qualified employees who control thousands of eggs per day are not productive because of tiredness and optical aberrations. Therefore, only some of the eggs are randomized to determine the fertilized eggs; this means that many of the unfertilized eggs will remain in the incubator [4]. Moreover, the unfertilized egg that remains in the incubator will spoil and there will be gas emission that negatively affects other healthy embryos. This is because; performing control and pulling out the unfertilized eggs from the incubator will increase the hatchability [1]. Developing an error-free, rapid and low priced computer-aided system to identify the unfertilized eggs at the right time will provide an advantage for the incubation system by purifying the system from the man-made errors.

Many of the investigators tried to determine the fertility control, embryo development, and fracture-crack control by using image processing methods with the help of the computer. Das and Evans [5,6] obtained egg images by using backlighting and high intensity candling lamp and get 100% classification result on 4th day. Lawrence et al.[7], Smith et al.[8], Smith et al.[9] Liu and Ngadi [4] and Islam et al.[10] used hyperspectral NIR imaging method and get success rates 100% (4th day), 91%-83% (3rd days), 100% (1st day) and 100% (4th day) respectively. Zhu and Ma [11] use halogen lamp for taking the egg images and achieved 92.5% success rates on 6th day. Lin et al.[12] used thermal imaging and 96% success rates are obtianed. Hashemzadeh and Farajzadeh [1] preferred light emitting diode based imaging method and get 98.25% classification rates on 5th day. Önler et al.[13] used ultrasound based imaging technique and get 86% success rates.

We can see screening method and enlightening have a significant effect on success rates of fertilization control. However, many of those methods are expensive and do not contain easy-to-use systems. Our research offered a cheap and easy-to-use approximation. Therefore, our research aimed to determine the fertilization control of eggs between zero and fifth days in the egg incubator by using derivable and low priced tools with the help of the image processing techniques.

MATERIAL and METHODS

The system is composed of three main elements. The first of them is the incubator system; the second of them is the screening system and the third one is the computer software that provides to be processed the images obtained. Since the goal of the study was to actualize the eggs fertilization control by the cheap and derivable methods, the materials to be used was selected in accordance with the method. A professional household type incubator with 48 egg capacity was used in the system actualized. 10 Watt 24 Volt 350mA Power LED lightened the egg incubator. A mechanism was designed in which an egg can be put on in and the lightning remains in the lower part. Color camera with 16 mm lens at 2048x1536 pixels resolution recorded the images. Moreover, for constituting a dark environment, there was designed a box with a hole for the camera to monitor inside. Box designing is absolutely optional for the dark environment. It is enough to take the pictures in a dark environment even if a box is not available. The first two stages of the system designed are shown in *Fig. 1*.

The images of the eggs put in the incubator were recorded by putting in the imaging system at the appointed times. Those images recorded were transferred into computer-aided fertilization control software. The control success of the software is based on operating these two systems successfully. As the accuracy and noiselessness of the images increase, the possibility of obtaining a result with an easy algorithm increases at the same time. Afterward, the development in the environment with 37.8°C temperature and 50-55% humidity was let for 18 days; the eggs were transferred into the exit machines with 37.5°C temperature, 65-70% relative humidity. The assembly process was automatically actualized so as to be once every h. The incubation machine was settled within the boundaries that are determined by Kamanlı and Durmuş [3]. Since 10W Power LED dissipated a great deal heat, egg shooting time was taken short (1 s) to avoid eggs from the heat. Moreover, the boxes that were designed for a dark environment was used so as not to affect the backgrounds of the images.

There is computer-aided fertilization control software in the third stage. *Fig. 2* shows the block diagram of the software created.

The first stage in computer-aided fertilization control software is to take pictures. The images are transferred into the software after the imaging process is actualized. Those pictures are turned into a binary image by a

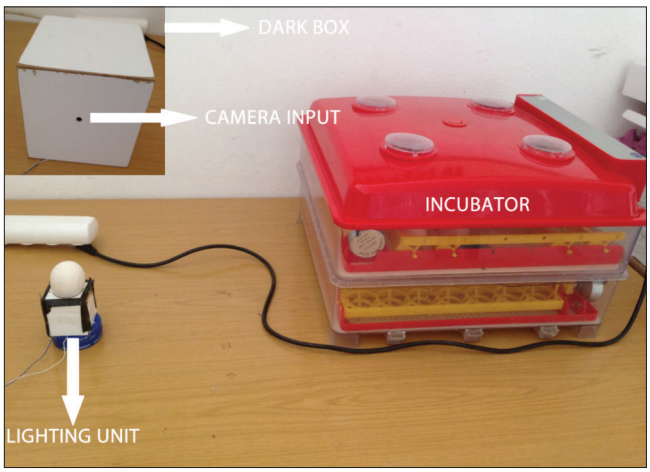

Fig 1. Designed system

specific threshold value to specify the boundaries of the eggs. Since the different eggs have a different color, size and blood vessels in the embryo and also these values change in different days; the attributes of the image was considered to determine the threshold value. In other words, the threshold values of the images each of the eggs in different days are distinct. This transaction is named as dynamic thresholding in the literature [1]. The errors that emerge by a constant threshold value will be avoided by the dynamic thresholding. The noise images arising from the enlightening may occur after the thresholding. Dilation and erosion methods from the morphological processes were used to eliminate the noise. *Fig. 3* shows the turning process of the image of the camera to the binary image by applying dynamic thresholding.

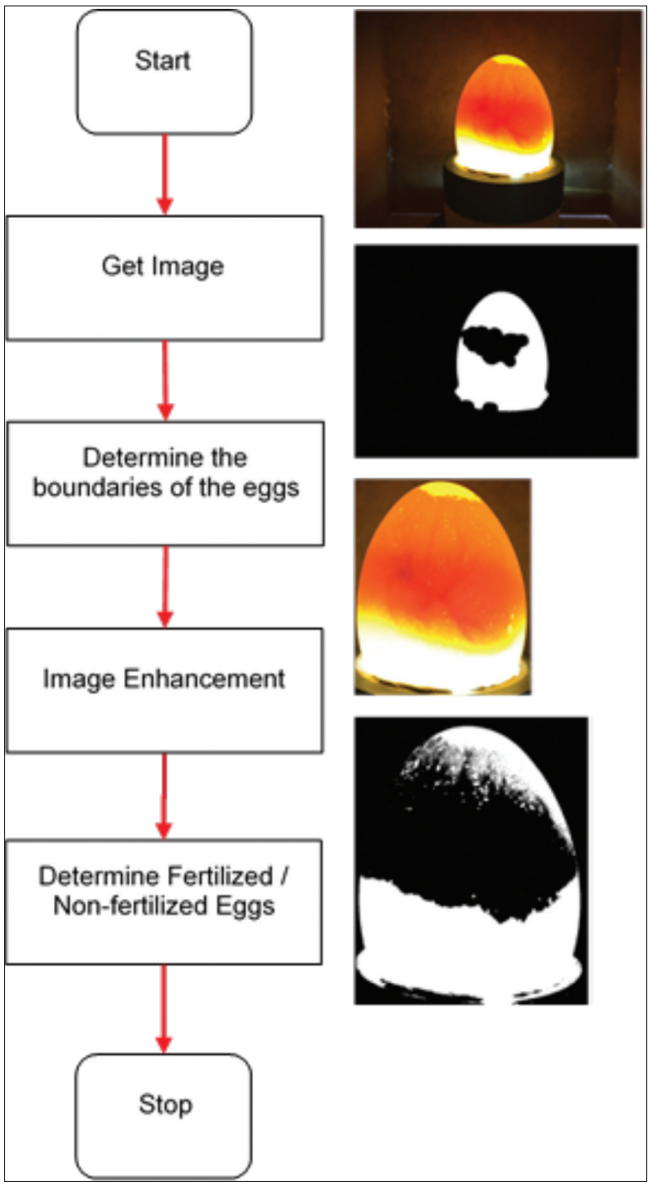

Fig 2. Flow diagram of the software designed

In the next step, the boundaries of the egg were determined by applying dynamic thresholding and morphological processes. The boundaries of the white area in the binary image were determined (top-left/right, bottom-left/right). The boundaries of the eggs were determined by taking the values of the points. The egg image was separated from the background at the end of this process.

There is a need for applying image enhancement methods to actualize the fertilization control on the egg image separated from the background. This circumstance may occur because of the camera, shooting method or the person that takes the picture. Median filter (5x5) was used in our research to enhance the egg images and also purify the images from the probable noises.

The area of the embryo was computed for the fertilization control after the filtering application. As is mentioned in literature, this area shows an alteration from starting the zero day to 21st day. This change was observed in the images obtained. It is enough to use proper incubation, lightning, and imaging for this observation. Our research used the images obtained by actualizing the conditions. *Fig. 4-a* shows the change of fertilized eggs between zero and fourth days; *Fig 4-b* shows the change of unfertilized eggs between zero and fourth days.

As is seen in *Fig. 4-a,b*, the embryo that develops in fertilized eggs grows in the egg and its area increases at the same time. This condition for the fertilized eggs is consistent when all the images are analyzed. Since an embryo development does not form in the unfertilized eggs, any area increase cannot be seen as well. However, some eggs spoil in incubation environment over time, the yellow area in them becomes darker. This situation may reveal the errors in the test results. Starting from this point, the binary images that can be obtained by a threshold

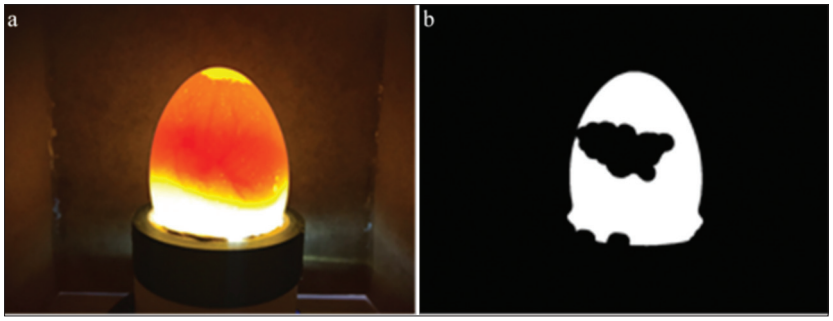

Fig 3. Obtaining binary image by dynamic thresholding **a)** raw image **b)** binary image

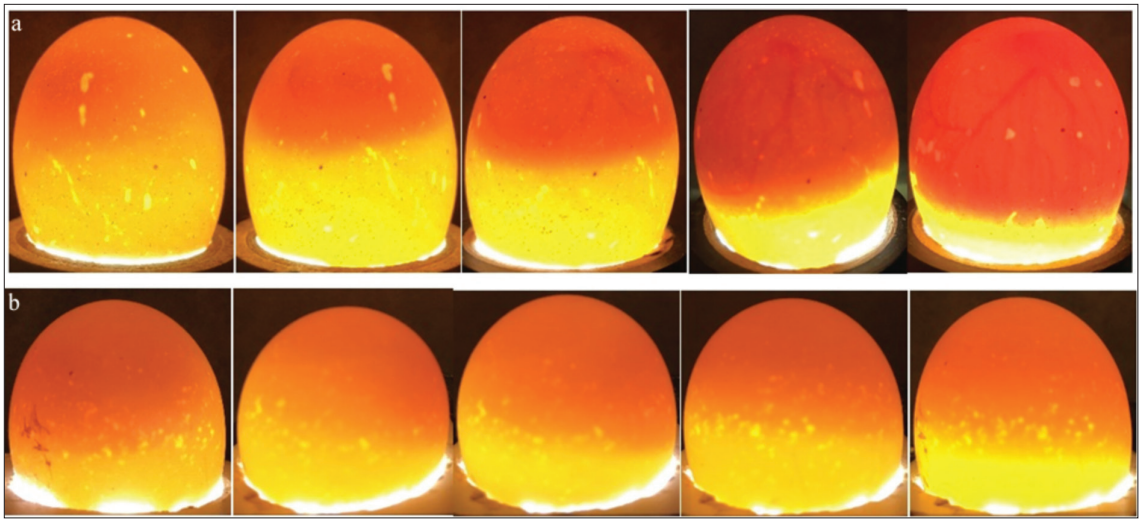

Fig 4. Changes of an eggs **a)** change of a fertilized egg between 0 and 4 days, **b)** change of an unfertilized egg between 0 and 4 days

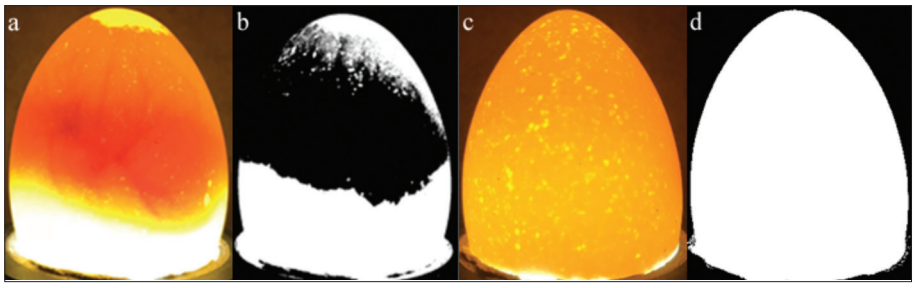

Fig 5. Fertilized and unfertilized eggs' binary images obtained by the dynamic threshold **a)** the Third day fertilized egg, **b)** Binary image of A **c)** the Third-day unfertilized egg, **d)** Binary image of C

value (dynamic threshold) was applied to the enhanced egg pictures in the fertilization control. It is observed at the end of this process that the white areas of fertilized eggs are narrowest; the white areas of unfertilized eggs are large. Furthermore, it is seen when the change between the images taken between zero and fourth days are reviewed that the fertilization control can be easily actualized. *Fig. 5* shows the image of the picture by the dynamic thresholding by separating from the noises.

Matlab R2014a version was used at the stage of actualizing image processing software. Image processing codes peculiar to the egg fertilization control problem were utilized besides the standard function of Matlab.

Dataset Preparation: Our research used three different datasets that were composed of eggs whose pictures were taken at different times. There were 45 eggs so as to be 15 eggs in each of the datasets that were decided by the qualified personnel. The eggs whose effects (broken, cracked, porous, etc.) are less for affecting the fertility were selected. Fifteen Light brown shelled eggs (10 fertilized, 5 unfertilized) from ATAK-S race were used in the first dataset. Again, 15 Light brown shelled eggs (10 fertilized, 5 unfertilized) from ATAK-S race were used in the second dataset. Fifteen Light brown shelled eggs (14 fertilized, 1 unfertilized) from ATAK-S race were used in the third dataset. When the quality characteristics of ATAK-S eggs are examined, it is seen that egg weight is 65.21 g, shape index is 75.59%, shell thickness is 0.33 mm and shell weight is 7 g [14]. The images of the eggs were recorded so as to be taken an image in every 24 h from the moment (zero hour, zero day) that is placed in incubation to 120th h. The recorded images are in JPEG format. Entirely, 75x3=225 images (15x5=75 for each of the dataset) were recorded. The eggs were kept in incubation for 21 days to be completed during the incubation process. The eggs which did not incubate at the end of the 21st day were analyzed by the expert and it is pointed out that there was not a problem about the fertilization control; the problem resulted from the incubator and environmental conditions.

When the materials and methods used in this study are listed, a professional household type incubator with 48 egg capacity, 10 Watt 24 Volt 350mA Power LED, Color camera with 16 mm lens at 2048x1536 pixels resolution, ATAK-S race eggs and dynamic thresholding method with Matlab R2014a Image Processing ToolBox.

RESULTS

The images of 15 eggs (10 fertilized, 5 unfertilized) between zero and fourth days were given as an entrance to the system designed. The images in software separated from the background by processing; the percentage ratio of white and black areas was found in the egg image. The

higher the white pixel rate, the less the fertility rate. In the same way, the less the white pixel rate, the greater the fertility rate. *Table 1* shows the white pixel ratios of 15 eggs between zero and fourth days.

As is seen them of pixel changes values, the eggs which do not change throughout the period (0-4 days) or change at a low ratio are unfertilized ones. The eggs whose value at the end of the period is far lower than the value at the start of the period are fertilized ones. For example, egg 13; initial value: 80, end value: 14, result: egg 13 fertile. Egg 3; initial value: 79, end value: 73, result: egg 3 infertile.

Sum difference between two days was considered to evaluate these ratios (the difference between zero and first day; the difference between zero and second, the difference between zero and third day, the difference between zero and fourth day). *Table 2* shows the values.

As is seen in *Table 2*, if the difference of white pixel ratios of the eggs changes in an instant at a high rate, the egg is likely to be fertilized. This change ratio is accepted as 2% for 0-1; 4% for 0-2; 8% for 0-3; 20% for 0-4. This change does not exceed 20% between 0 and 4 days, the result is selected as unfertilized. The information on the growth ratio of the chick in the egg was used. The success rates obtained according to this data are given under each column.

The success rate at the end of the first day was 53.34%; 66.67% for the end of the second day; 73.34% for at the end of the third day; 100% for the end of the fourth day. As is seen in *Table 3*, the effect of the white pixel ratio change to the fertility value is quite a little between zero and the first day. This is because using data of 1st and 5th day's leads to the correct conclusion rather using the data belong to zero and fifth days.

The images of 15 eggs (10 fertilized, 5 unfertilized) between the first and fifth days were given as an entrance to the system designed. The *Table 3* shows the white pixel ratios of 15 eggs between first and fifth days.

As is seen them of pixel changes values, the eggs which do not change throughout the period (1-5 days) or change at a low ratio are unfertilized ones. The eggs whose value at the end of the period is far lower than the value at the start of the period are fertilized ones. For example, egg 28; initial value: 75, end value: 22, result: egg 28 fertile. Egg 24; initial value: 72, end value: 66, result: egg 24 infertile.

Sum difference between two days was considered to evaluate these ratios (the difference between the first and the second day; the difference between the first and the third day, the difference between the first and the fourth day, the difference between the first and the fifth day). *Table 4* shows the values.

As is seen in *Table 4*, if the difference of white pixel ratios of the eggs changes in an instant at a high rate, the egg is likely to be fertilized. This change ratio is accepted as 4% for 1-2; 8% for 1-3; 20% for 1-4; 30% for 1-5. If this change does not exceed 20% between zero and fourth days, or the change takes a negative value, the result is selected as unfertilized. The information on the growth ratio of the chick in the egg was used

The success rate for the second dataset was 73.34% at the end of the second day; 93.34% at the end of the third day; 93.34% at the end of the fourth day; 93.34% at the end of the fifth day.

The images of 15 eggs (14 fertilized, 1 unfertilized) between the first and fifth days were given as an entrance to the system designed in the third dataset. *Table 5* shows the

Table 1. *Number of white pixel ratio for dataset 1 for 0th and 4th days (%)*

Egg Number	Day					Expert Assessment
	0th day	1st day	2nd day	3rd day	4th day	
1	70.05218	74.7298	74.19153	65.37102	17.75795	Fertile
2	73.0347	72.62266	71.44976	35.76302	25.62944	Fertile
3	79.73458	78.43909	75.59067	69.04541	73.83005	Infertile
4	72.60527	71.53416	58.64252	37.23622	29.57277	Fertile
5	70.90953	65.08063	70.98815	53.69586	51.42568	Infertile
6	75.98155	70.81526	63.63636	30.83919	29.09275	Fertile
7	73.25327	70.70671	61.06334	42.71911	28.00998	Fertile
8	74.77697	72.29558	60.86581	38.3648	23.08489	Fertile
9	77.3723	70.24388	78.0647	64.69531	60.64912	Infertile
10	80.20004	74.46872	79.76624	60.4134	60.7817	Infertile
11	68.97427	66.83322	54.21348	33.66571	32.0993	Fertile
12	79.0481	75.03264	75.27244	58.69613	59.58343	Infertile
13	80.02702	67.60631	56.60874	28.253	14.84707	Fertile
14	60.56951	58.25204	58.78945	46.65361	30.3672	Fertile
15	71.86004	65.10965	64.02399	48.62033	34.39325	Fertile

Table 2. *White area changing table between the days (%)*

Egg Number	Differences by Days				Software Assessment	Expert Assessment
	0-1 Difference ≥2% (fertile)	0-2 Difference ≥4% (fertile)	0-3 Difference ≥8% (fertile)	0-4 Difference ≥20% (fertile)		
1	-4.67	-4.13	4.68	52.29	Fertile	Fertile
2	0.41	1.58	37.27	47.40	Fertile	Fertile
3	1.29	4.14	10.68	5.90	Infertile	Infertile
4	1.07	13.96	35.36	43.03	Fertile	Fertile
5	5.82	-0.07	17.21	19.48	Infertile	Infertile
6	5.16	12.34	45.14	46.88	Fertile	Fertile
7	2.54	12.18	30.53	45.24	Fertile	Fertile
8	2.48	13.91	36.41	51.69	Fertile	Fertile
9	7.12	-0.69	12.67	16.72	Infertile	Infertile
10	5.73	0.43	19.78	19.41	Infertile	Infertile
11	2.14	14.76	35.30	36.87	Fertile	Fertile
12	4.01	3.77	20.35	19.46	Infertile	Infertile
13	12.42	23.41	51.77	65.17	Fertile	Fertile
14	2.31	1.78	13.91	30.20	Fertile	Fertile
15	6.75	7.83	23.23	37.46	Fertile	Fertile
Success rate	53.34%	66.67%	73.34%	100%		

Table 3. *Number of white pixel ratio for dataset 2 for 1st and 5th days (%)*

Egg Number	Day					Expert Assessment
	1st day	2nd day	3rd day	4th day	5th day	
16	62.89813	71.27311	78.88054	76.23356	77.21532	Infertile
17	62.48269	51.5847	30.56968	19.40743	14.91582	Fertile
18	56.85506	52.23822	34.70326	50.5552	42.58216	Fertile
19	54.96044	49.9108	36.8949	25.05271	15.01342	Fertile
20	72.31574	82.1813	78.18832	68.6549	63.36109	Infertile
21	63.21411	56.53763	40.92532	19.11876	11.55453	Fertile
22	59.17022	52.65631	39.00066	22.67896	11.30802	Fertile
23	55.64947	43.04546	33.24711	18.53764	11.29753	Fertile
24	72.22282	51.68187	62.22455	67.82684	66.80628	Infertile
25	57.04524	61.20489	39.50373	29.52043	26.61711	Fertile
26	49.0064	40.1006	28.68986	17.23708	10.34289	Fertile
27	48.58799	45.78101	27.86972	17.70482	22.59436	Fertile
28	75.29982	62.32376	40.22897	19.06477	19.06477	Fertile
29	41.12498	53.78965	47.32667	52.08565	50.06751	Infertile
30	43.65557	55.23351	47.89443	46.40716	43.31085	Infertile

white pixel ratios of 15 eggs between first and fifth days.

As is seen them of pixel changes values, the eggs which do not change throughout the period (1-5 days) or change at a low ratio are unfertilized ones. The eggs whose value at the end of the period is far lower than the value at the start of the period are fertilized ones. For example, egg 36; initial value: 76, end value: 14, result: egg 36 fertile. Egg 32; initial value: 71, end value: 75, result: egg 32 infertile.

Sum difference between two days was considered to evaluate these ratios (the difference between the first and the second day; the difference between the first and the third day, the difference between the first and the fourth day, the difference between the first and the fifth day). *Table 6* shows the values.

As is seen *Table 6*, the success rate for the third dataset was 60% at the end of the second day; 86.67% at the end of the third day; 93.34% at the end of the fourth day; 100% at the end of the fifth day.

DISCUSSION

In this paper, there was performed a situation assessment as fertilized/unfertilized of the eggs by making an image-

Table 4. *White area changing table between the days (%)*

Egg Number	Differences by Days				Software Assessment	Expert Assessment
	1-2 Difference ≥4% (fertile)	1-3 Difference ≥8% (fertile)	1-4 Difference ≥20% (fertile)	1-5 Difference ≥30% (fertile)		
16	-8.37	-15.98	-13.34	-14.32	Infertile	Infertile
17	10.90	31.91	43.08	47.57	Fertile	Fertile
18	4.62	22.15	6.30	14.27	Infertile	Fertile
19	5.05	18.07	29.91	39.95	Fertile	Fertile
20	-9.87	-5.87	3.66	8.95	Infertile	Infertile
21	6.68	22.29	44.10	51.66	Fertile	Fertile
22	6.51	20.17	36.49	47.86	Fertile	Fertile
23	12.60	22.40	37.11	44.35	Fertile	Fertile
24	20.54	10.00	4.40	5.42	Infertile	Infertile
25	-4.16	17.54	27.52	30.43	Fertile	Fertile
26	8.91	20.32	31.77	38.66	Fertile	Fertile
27	2.81	20.72	30.88	35.99	Fertile	Fertile
28	12.98	35.07	56.24	56.24	Fertile	Fertile
29	-12.66	-6.20	-10.96	-8.94	Infertile	Infertile
30	-11.58	-4.24	-2.75	0.34	Infertile	Infertile
Success rate	73.34%	93.34%	93.34%	93.34%		

Table 5. *Number of white pixel ratio for dataset 2 for 1st and 5th days (%)*

Egg Number	Day					Expert Assessment
	1st day	2nd day	3rd day	4th day	5th day	
31	61.48384	65.76606	54.1254	29.72434	30.62046	Fertile
32	71.24564	68.02674	70.05505	75.39222	75.40167	Infertile
33	62.16644	65.04775	56.84631	32.06525	29.84828	Fertile
34	73.97004	55.07065	52.86121	41.95793	36.86408	Fertile
35	49.74073	46.3681	40.28495	20.06469	10.3954	Fertile
36	76.629	66.53882	52.30564	17.72862	14.06614	Fertile
37	64.16081	47.61415	40.44904	26.43988	19.40788	Fertile
38	67.84067	62.05111	39.7178	30.64041	22.47425	Fertile
39	74.53868	64.07787	48.70116	21.99707	20.60232	Fertile
40	56.10094	49.67823	33.35632	28.53916	19.93295	Fertile
41	71.26095	57.1348	45.10106	36.09442	31.20518	Fertile
42	75.32446	73.28786	67.29159	47.35383	38.64619	Fertile
43	65.79996	54.47365	56.1797	41.19385	32.47017	Fertile
44	75.30359	71.54523	48.6836	26.07057	20.89791	Fertile
45	58.17535	57.69725	48.3054	40.76475	20.89839	Fertile

based analysis between the zero and the fifth days. Pictures of the eggs were taken on a daily basis by using a power led camera. The fertility status was controlled by the image processing software. There was guessed right at 100% at the end of the study conducted on three datasets that were composed of 15 eggs.

Outer shell boundaries of the eggs were determined first. After, the region of interest (ROI) was separated from the image obtained by various filtering and morphological processes. The color-gray level conversion was actualized and the black-white image was received by using an adaptive thresholding method. The white pixels in the black-white image were counted; the number was proportioned to the whole of the egg. The dark areas which are marked as white are the areas that are constituted by the chick which grows in the egg.

Daily changes of the percentage values were computed by taking the arithmetic difference. These proportional differences were compared with the threshold values determined (≥2% between zero and first day, ≥4% for the zero and second day, ≥8% for the zero and the third day, ≥20% for the zero and the fourth; ≥30% for zero and fifth). The results with fertilized and unfertilized were obtained. Experts' information on the growth rate of the chick in the egg was effective in determining these threshold values.

Table 6. *White area changing table between the days (%)*

Egg Number	Differences by Days				Software Assessment	Expert Assessment
	1-2 Difference ≥4% (fertile)	1-3 Difference ≥8% (fertile)	1-4 Difference ≥20% (fertile)	1-5 Difference ≥30% (fertile)		
31	-4.28	7.36	31.76	30.86	Fertile	Fertile
32	3.22	1.19	-4.15	-4.16	Infertile	Infertile
33	-2.88	5.32	30.10	32.32	Fertile	Fertile
34	18.90	21.11	32.01	37.11	Fertile	Fertile
35	3.37	9.46	29.68	39.35	Fertile	Fertile
36	10.09	24.32	58.90	62.56	Fertile	Fertile
37	16.55	23.71	37.72	44.75	Fertile	Fertile
38	5.79	28.12	37.20	45.37	Fertile	Fertile
39	10.46	25.84	52.54	53.94	Fertile	Fertile
40	6.42	22.74	27.56	36.17	Fertile	Fertile
41	14.13	26.16	35.17	40.06	Fertile	Fertile
42	2.04	8.03	27.97	36.68	Fertile	Fertile
43	11.33	9.62	24.61	33.33	Fertile	Fertile
44	3.76	26.62	49.23	54.41	Fertile	Fertile
45	0.48	9.87	17.41	37.28	Fertile	Fertile
Success rate	60%	86.67%	93.34%	100%		

With reference to the experimental results, the fertilization was found as 73.34% for third day and 100% for the fourth day in the first dataset; as 93.34% for the third day and 93.34% for the fourth day in the second dataset; 93.34% for the third day and 100% for the fourth day in the third day in the third dataset. Taking the pictures via nonprofessional apparatus is one of the significant reasons that negatively affect the success. Success rate can be increased by improving the imaging mechanism. Moreover, the circumstances like shell color, shell thickness affect the success of the system because of the types of eggs. It is thought that there can occur success difference in different egg types.

When we compare the proposed method with the previous works, it can be seen that many of the previous works achieved 100% success rates [4,7,8,10] in determination of the fertilization of the eggs. However, many of those methods are expensive (halogen lighting and NIR sensing system, Near-Infrared Hyperspectral Imaging system and visible transmission spectroscopy screening technique) and do not contain easy-to-use systems. Our research also achieved 100% success rate and offered a cheap (LED illumination), easy-to-use and derivable method to ideally perform the fertilization control of the eggs.

The success of the system can be tested by controlling fertilization status of the eggs in different characteristics (white-brown-dirty-crack etc.) in the next part of the research.

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Research Article

Age-related Changes in the Cloacal Microbiota of Bar-headed Geese *(Anser indicus)*

Wen WANG [1,a] Kirill SHARSHOV [2] Yuhui ZHANG [3] Linsheng GUI [1,4]

[1] State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xi'ning 810016, Qinghai Province, CHINA
[2] Research Institute of Experimental and Clinical Medicine, Novosibirsk 630117, RUSSIA
[3] College of Eco-Environmental Engineering, Qinghai University, Xi'ning 810016, Qinghai Province, CHINA
[4] College of Agriculture and Animal Husbandry, Qinghai University, Xi'ning 810016, Qinghai Province, CHINA
[a] ORCID: 0000-0002-3156-7929

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Abstract

The gastrointestinal microbiota played an important role in animal health by acting as a barrier against pathogens, exerting multiple metabolic functions and stimulating the development of the host immune system. To better understand the age-related dynamic changes in gut microbiota, we used 16S rRNA genes sequencing to investigate the cloacal microbial communities of the adult and chick bar-headed geese *(Anser indicus). Fusobacteria, Firmicutes, Proteobacteria, Actinobacteria* were the main components shared by adults and chicks. The former had more *Proteobacteria* and *Cyanobacteria* and the latter had more *Fusobacteria* and *Actinobacteria*. At the genus level, most of the dominant genera found in chicks were different from those in adults. In addition, adults had richer and more diverse bacterial communities than chicks. Our analysis of the composition of cloacal microbiota at the OTUs level also showed very large overlap existed in the bacterial assemblages between chicks and adults. These overlapped microbes were considered as the major microbes in the gastrointestinal tracts of bar-headed geese throughout their whole life span. Taken together, the results of this study provided a first inventory of the gut microbiotas of chick bar-headed geese and represented a first step in a wider investigation of the sequential changes in gut microbiotas with ages in bar-headed geese.

Keywords: *Bar-headed goose, Cloacal microbial community, 16S rRNA genes*

Hint Kazı *(Anser indicus)*'nın Kloaka Mikrobiyotasında Yaşa Bağlı Değişiklikler

Öz

Gastrointestinal mikrobiyota, patojenlere karşı bir engel görevi görerek, çoklu metabolik fonksiyonları yerine getirerek ve konakçı bağışıklık sisteminin gelişimini uyararak hayvan sağlığında önemli bir rol oynar. Bağırsak mikrobiyotasında yaşa bağlı dinamik değişimleri daha iyi anlayabilmek için, yetişkin ve civciv Hint kazlarının *(Anser indicus)* kloakal mikrobiyal yapısını araştırmak amacıyla 16S rRNA gen sekanslaması kullanıldı. F*usobacteria, Firmicutes, Proteobacteria* ve *Actinobacteria* yetişkin ve civciv kazlar tarafından ortak paylaşılan bileşenlerdi. Yetişkinlerde *Proteobacteria* ve *Cyanobacteria* civcivlerde *Fusobacteria* ve *Actinobacteria* daha fazlaydı. Civcivlerde bulunan baskın genusun çoğu yetişkinlerinkinden farklıydı. Ayrıca, yetişkinler civcivlerden daha zengin ve daha farklı bakteriyel mikrobiyotaya sahipti. OTU seviyesinde kloakal mikrobiyotanın analizi, civcivler ve yetişkinler arasında bakteriyel topluluklar bakımından büyük ölçüde örtüşmenin olduğunu gösterdi. Bu örtüşen mikroorganizmaların, Hint kazlarının hayatları süresince gastrointestinal kanallarının ana mikroorganizmaları olduğu düşünülmektedir. Çalışma, Hint kazlarının bağırsak mikrobiyotasını belirlemiş ve yaş ile bağırsak mikrobiyotasında gelişen değişiklikleri belirlemek amacıyla yapılacak detaylı çalışmalar için bir adım atmıştır.

Anahtar sözcükler: *Hint kazı, Kloakal Mikrobiyota Bileşenleri, 16S rRNA geni*

INTRODUCTION

In animals, microorganisms occur both externally (e.g. skin and feather) and internally (e.g. gastrointestinal and reproductive tracts) of their hosts [1]. The majority of the microorganisms associated with animals inhabit the gastrointestinal tracts at an abundance of potentially trillions of cells whose collective genome named "gut microbiome" [2]. A wealth of studies have shown that gut microbiome plays an important role in several fundamental and crucial

İletişim (Correspondence)
+86-971-5201533; Fax: +86-971-5201533
linshenggui2018@163.com

processes such as development [3], immune homeostasis [4], nutrient assimilation [5,6], vitamins synthesis and sterols metabolism in the host [7], and diseases (e.g., obesity, diabetes, and cancer) in humans and other animals [8].

Birds are endothermic, feathered amniotes with 10.659 described species and more than 20.413 subspecies [9]. Compared to other mammalian vertebrates, several characteristics make birds the most interesting and useful model for studying gut microbiome. First, unlike other mammals where host genetics have shown a clear influence on the colonizing process of gut microbiota [10], birds are more likely to acquire microbes after hatching from the nest environments or food. For example, birds brood parasites offer a unique and powerful model to investigate these questions because genetic and environmental transmission of microbes are naturally decoupled. A study of Brown-headed cowbirds *(Molothrus ater)*, a brood parasite that relies on other species to hatch and raise their young, found that gut microbiome was not related to host species, but rather to environments [11]. Secondly, many birds regurgitate food to their young, thus provides a mode of vertical transmission of gut microbiome across generations, whereas mammals acquire important maternal microbes during the birth process [12]. Thirdly, birds possess a cloaca, which serves the dual functions of excretion and sexual copulation. Thus, gastrointestinal tract microbiotas of birds provide another avenue for exploring the potential exchange of components of the endogenous microbiome during reproduction. For example, Kreisinger et al.[13] described the cloacal microbiomes in free living barn swallows *(Hirundo rustica)* and found that nesting pairs had significantly more similar microbiomes within pairs than between nonbreeding individuals. Lastly, in the fieldwork, we have noticed that some bird species lived in mouse holes. This phenomenon, birds and mouse share the same living environments, provides another opportunity to understand the coevolution of the gut microbiotas with different hosts (birds and mammals) in the same environments.

In general, compared to other mammalian vertebrates, we know much less about the gut microbiota of wild birds [14]. The majority of avian microbiome studies have focused on economically important species such as chicken [15], turkey, duck and ostrich [16]. The reasons for this are various but may relate to the collection of biological samples (especially feces) from several groups of wild birds is a difficult task. Similar to other vertebrates, the gut microbiota of birds are dominated by the four major phyla [17]: *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*.

The bar-headed goose *(Anser indicus)* is endemic to Asia, breeding in the Mongolia plateau of central Asia and the Qinghai-Tibet plateau in China, wintering in the south-central Tibet and South-Asian subcontinent [18]. As one of the dominant waterfowl species in wetland areas in Qinghai-Tibetan Plateau, bar-headed geese are increasingly being reared in several provinces of China since year 2003 for the purpose of both conservation and economic development [19]. In the early stage of industrialization of this bird, a limited number of wild eggs were collected and then artificially incubated using an incubator. The gastrointestinal tracts of newly-hatched chicks are immediately colonized by microorganisms present in the surrounding artificial environments. By contrast, in the wild, the gastrointestinal tracts of newly-hatched chicks are rapidly colonized by members of the gut microbiomes from its mother's feces and nest environments. In many bird species, the chicks' gut microbiotas are dynamical changing communities that gradually develops toward the adult community structure [20]. Therefore, figuring out the gut microbiomes of wild chick bar-headed geese is important for the management of the artificially reared chicks. In our previous studies [21], we have found that the core gut microbiomes of wild bar-headed geese were dominated by *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*. Furthermore, *Bacteroidetes* were found to be higher in artificially reared bar-headed geese compared to wild ones [22]. However, it remains unclear how the normal gut microbiome changes between young and adult wild bar-headed geese.

Here, we describe for the first time the cloacal microbiota in bar-headed geese comparing both adults and chicks and to analyze the similarities and differences between them. Cloacal swabs were selected for high-throughput sequencing of 16S rRNA V3-V4 regions in this study, because it was not feasible to obtain naturally passed feces from wild chicks. Cloacal swabs are believed to at least partially reflect the microbiota present in the gastrointestinal tracts [23] and do not require invasive sampling. Our results form an important basis for understanding changes in gut microbiota compositions and patterns with host age in wild birds.

MATERIAL and METHODS

Ethics Statement

This study conformed to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398). The research protocol was reviewed and approved by the Ethical Committee of Qinghai University. Samples collection was authorized by the officer Yubang He from the Administration Bureau of Qinghai Lake National Nature Reserve, Qinghai Province, China. All wild bar-headed geese were released at the capture site immediately after cloacal samples collection.

Samples Collection

A total of 5 wild bar-headed geese, 3 chicks (abbreviation: C group) and 2 adults (abbreviation: A group), were used in this study. These birds were captured using mist nets in

a farmland (N: 37° 01′ 39.3″, E: 99° 44′ 21.8″, Elevation: 3,200 m) adjacent to Bird Island of Qinghai Lake National Nature Reserve. Every day only one captured bird was randomly selected for cloacal sampling to minimize the overlap in bacterial assemblages between two individuals due to, for example, a shared nesting environments. Cloacal samples were collected using sterile DNA-free microbiological nylon swabs inserted about 10 mm inside the cloaca for approximate 20 s and gently twisted by 360 degrees. Swabs were placed into DNA-free sterile tubes and initially kept in car-refrigerator (-20°C), then shipped to the laboratory stored at -80°C until samples processing.

DNA Extraction, PCR Amplification, and Illumina HiSeq 2500 Sequencing

DNA extraction was performed on samples by using E.Z.N.A.® stool DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's protocol. The purity and concentration were checked using NanoPhotometer (Implen, Westlake Village, CA USA) and Qubit2.0 Flurometer (Life Technologies, Carlsbad, CA, USA). The V3-V4 regions of the bacteria 16S rRNA genes were amplified by PCR (95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min) using primers 341F (5›-barcode-CCTACGGGNGGCWGCAG-3›) and 805R (5›-barcode-GACTACHVGGGTATCTAATCC-3›), where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate, 20 µL mixture containing 4 µL of 5 X FastPfu buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 mM), 0.4 µL of FastPfu polymerase, and 10 ng of template DNA. PCR products were then run on 1% agarose gel and bands of appropriate size were extracted from the gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions using 30 µL of buffer in the elution step. Concentration of the purified PCR product was measured using a QuantiFluor™ - ST (Promega, Madison City, WI, USA). Purified amplicons were pooled in equimolar and paired - end sequenced (2 x 250) on an Illumina HiSeq2500 platform according to the standard protocol.

Data Accessibility

The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (accession number: SRP090120).

Data Analysis

The raw fastq files were demultiplexed based on the barcode and primer sequence with the following criteria: (i) exact barcode matching, (ii) 2 nucleotide mismatch in primer matching, (iii) reads containing ambiguous characters were removed. Then paired - end reads for each sample were run through Trimmomatic (version 0.33) [24] to remove low quality base pairs using these parameters [SLIDINGWINDOW: 50: 20 MINLEN: 50]. Trimmed reads were then further merged using FLASH program (version 1.2.8) [25] with the parameters [-m 10 -x 0.2 -p 33 -r 300 -f 450 -s 150].

The 16S sequences were analyzed using a combination of software UPARSE (usearch version v8.0.1517, http://drive5.com/uparse/) [26], QIIME (version 1.9.1) [27], and R (version 3.2.3). The cleaned reads were clustered at 97% sequence identity into operational taxonomic units (OTUs) using the UPARSE pipeline (http://drive5.com/usearch/manual/uparse_cmds.html). The OTU representative sequences were aligned against to the greengenes reference template set (http://greengenes.lbl.gov/Download/Sequence_Data/Fasta_data_files/core_set_aligned.fasta.imputed) based on PyNAST (version 1.2.2) [28]. The phylogenetic tree was constructed using FastTree (version 2.1.3) [29] with the filtered alignment. The Ribosomal Database Project (RDP) Classifier (version 2.2) [30] was employed for taxonomy assignment against Greengenes (version gg_13_8) [31] with confidence score > = 0.8.

For the alpha-diversity metrics, Chao1 and observed species were calculated by mothur (version 1.36) [32] and Rarefaction plots were generated with iterations of 10 at each sampling depth 3000 and increments of 100. Differences between two independent groups were evaluated by the Welch's t-test. P-values<0.05 were considered to be significant. All figures were generated with customized R scripts.

RESULTS

After alignment, gap removal, and potential chimera removal, nearly 512,818 valid clean reads were generated for cloacal bacteria, representing 256,388 assembled sequences with a median length of 450 bp from our dataset *(Table 1)*. These assembled sequences yielded a total of 916 distinct OTUs, ranged from 46 to 574, with a 97% sequence similarity threshold. *Table 2* showed the number of OTUs assigned to different taxonomic levels (from phylum to genus) in each sample.

A total of 9 different bacterial phyla were identified in the cloacal microbiotas of chicks *(Fig. 1A)*. The results showed that *Fusobacteria* predominated (48.29%) among chicks followed by *Firmicutes* (22.21%), *Proteobacteria* (22.07%), *Actinobacteria* (5.02%) and *Tenericutes* (1.93%) *(Table 3)*. A total of 17 different bacterial phyla were identified in the cloacal microbiotas of adults *(Fig. 1A)*. The top 5 most abundant phyla identified were: *Proteobacteria* (64.69%), *Firmicutes* (23.92%), *Cyanobacteria* (8.48%), *Actinobacteria* (1.43%) and *Fusobacteria* (0.56%) *(Table 3)*. Comparison at the phylum level showed that *Fusobacteria* (P=0.239) and *Actinobacteria* (P=0.125) abundances tended to increase in chicks, while *Proteobacteria* (P=0.211) and *Cyanobacteria* (P=0.136) abundances tended to increase in adults *(Table 3)*.

At the genus level, the sequences from the samples represented 18 and 24 genera in chicks and adults, respectively *(Fig. 1B)*. The sequences that could not be

Table 1. *Raw data before and after standard quality control filters*

Samples	Raw Reads	Raw Bases (bp)	Clean Reads	Clean Bases (bp)	Assembled Reads
C1	114.432	28.608.000	105.086	26.271.500	52.540
C2	114.860	28.715.000	103.966	25.991.500	51.978
C3	111.432	27.858.000	102.416	25.604.000	51.205
A1	112.320	28.080.000	101.044	25.261.000	50.517
A2	110.510	27.627.500	100.306	25.076.500	50.148

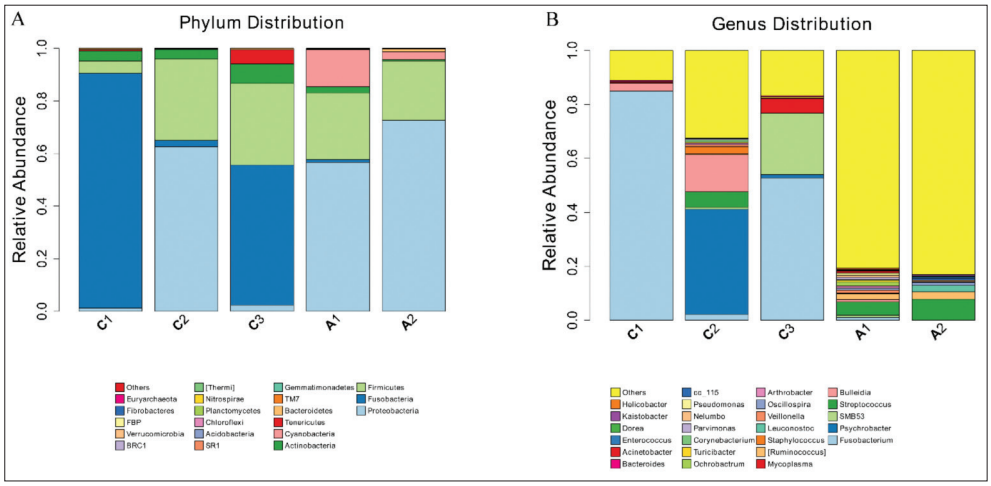

Fig 1. The phylum (A) and genus (B) level microbial compositions of each sample

Table 2. *The number of identified OTUs and taxonomic units in each sample*

Samples	OTUs	Number of Taxonomic Units				
		Phylum	Class	Order	Family	Genus
C1	46	7	13	14	13	11
C2	189	9	16	21	21	18
C3	133	7	15	21	21	15
A1	574	16	25	25	25	25
A2	509	18	24	24	24	23

Table 3. *Comparison of the top 5 most abundant phylum in each group*

Phylum	C Group	A Group	P Value
Fusobacteria	48.29%	0.56%	0.239
Firmicutes	22.21%	23.92%	0.890
Proteobacteria	22.07%	64.69%	0.211
Actinobacteria	5.02%	1.43%	0.125
Tenericutes	1.93%	-	0.454
Cyanobacteria	-	8.48%	0.136

classified into any known genus were assigned as "others". The proportions of these genera varied between 11.10 and 80.64% among the different samples. The top 6 most abundant genus of each group was shown in *Table 4*. These dominant genera in chicks and adults accounted together for an average of 80.04% and 12.04%, respectively *(Table 4)*. Most of the dominant genera (4/6) found in chicks were different from those of adults except the genera *Streptococcus* and *Fusobacterium*.

We employed Chao1 index and observed species curve to estimate the alpha diversity of the chicks and adults cloacal samples. The Chao1 index and observed species curve were lower in chicks than in adults samples *(Fig. 2)*, and there were significant differences ($P<0.05$) between the groups, according to Welch's t-test statistics. These results suggested that the diversity of the cloacal microbiota of adult bar-headed geese was higher than in chicks.

Bray-Curtis clustering and Venn diagrams were used to explore similarities and differences between adults and chicks *(Fig. 3)*. Analyses based on Bray-Curtis distances revealed strong clustering of the samples by ages *(Fig. 3A)*. At the OTU level, there were 186 OTUs shared between the samples from adults and chicks, whereas the other 640 OTUs and 90 OTUs, were specific to the adults and chicks, respectively *(Fig. 3B)*. These results indicated that

Table 4. *The top 6 genera in each group*

Phylum	Genus	C Group	A Group	Genus	Phylum
-	Others	20.11%	81.85%	Others	-
Fusobacteria	Fusobacterium	46.64%	6.31%	Streptococcus	Firmicutes
Proteobacteria	Psychrobacter	13.39%	2.34%	[Ruminococcus]	Firmicutes
Firmicutes	Bulleidia	8.33%	1.19%	Leuconostoc	Firmicutes
Firmicutes	SMB53	7.87%	0.88%	Oscillospira	Firmicutes
Tenericutes	Mycoplasma	1.93%	0.77%	Ochrobactrum	Proteobacteria
Firmicutes	Streptococcus	1.88%	0.55%	Fusobacterium	Fusobacteria

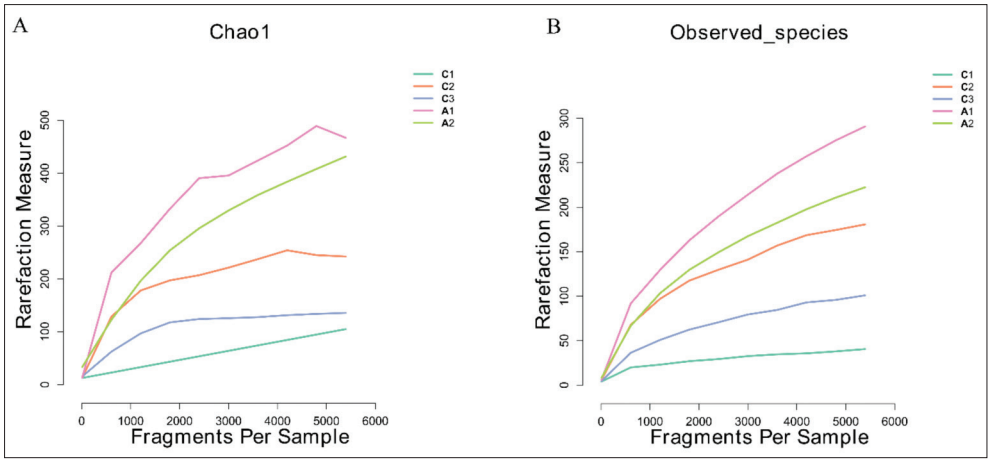

Fig 2. Comparison of the Chao1 based rarefaction curves (A) and Observed species curves (B)

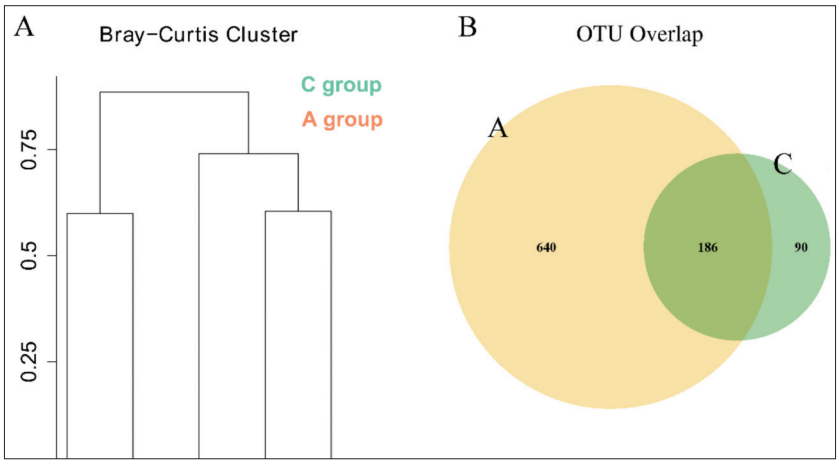

Fig 3. Hierarchical clustering of each sample (A) and the Venn diagrams (B) of both two groups

majority of OTUs (67.39%) presented in the chicks were also presented in the adults. The top 25 most abundant OTUs at the genus level shared by both adults and chicks were shown in *Fig. 4*.

DISCUSSION

In this study, we for the first time characterized and compared the cloacal bacterial microbiotas of adult and infant bar-headed geese, thus providing new insights into the impact of ages on alterations of the gut microbiotas. Our results showed that chicks had a lower and less diverse cloacal microbiota than adults. These results were consistent with earlier studies on the cloacal bacterial assemblages of both adults and chicks in a wild population of black-legged kittiwakes [33]. Our results also supported findings of newly published works by Barbosa with older penguins showing a higher diversity than younger ones [34]. There might be several reasons for age-related variations in diversity. First, the physical and chemical properties of the gastrointestinal tracts in chicks differ from adults. For example, the early colonization of the gut by facultative anaerobes, which then created the anaerobic conditions required for colonization by obligate anaerobic gut microbes [3]. Similarly, it is expected that young bar-headed geese enrich their gut microbiomes as their state of gut

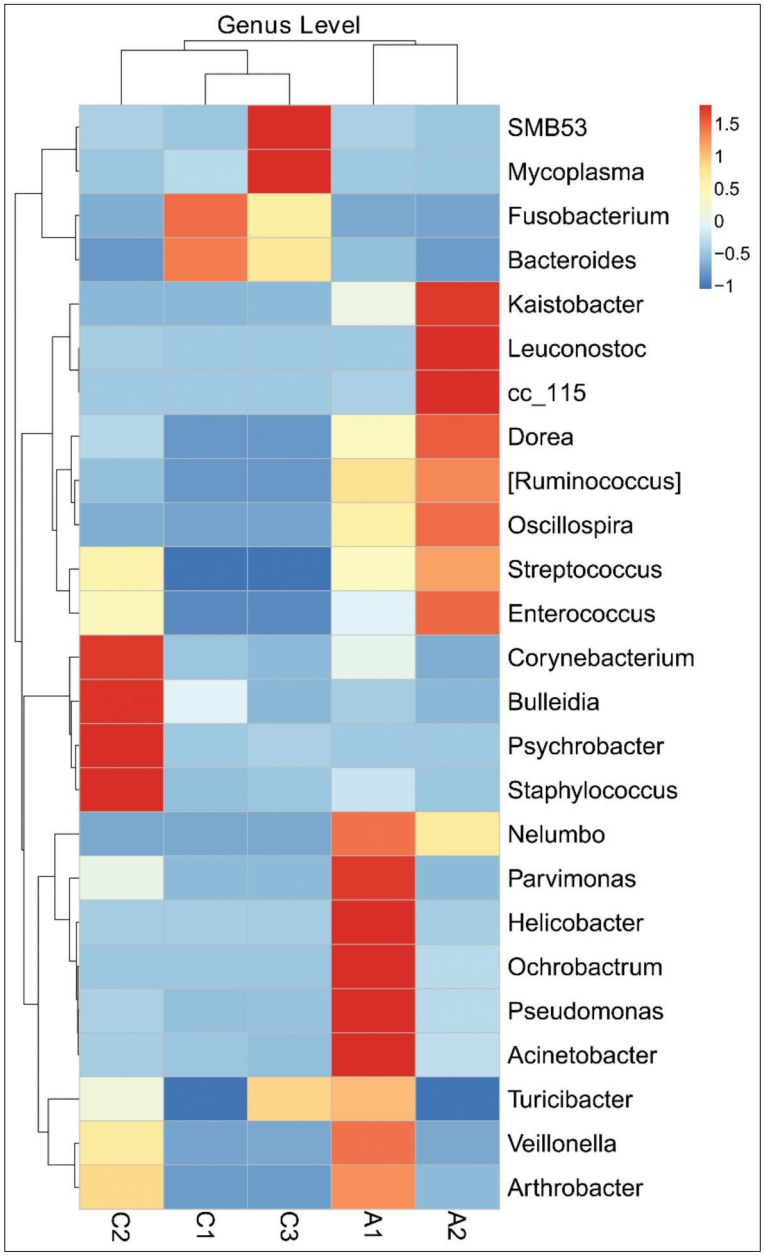

Fig 4. The heat map of the top 25 most abundant genus in each sample

transition to a stable adult state. Second, the low mobility of chicks resulted in a restricted environments from which to obtain bacteria. Therefore, their decreased microbial diversity could be related to the lower capabilities to contact with natural environments. Last, immune system was found to be one of the strongest environmental factor shaping gut microbiotas in animals [10]. Adults had developed more adaptive immune system to establish symbiotic relationship with the microbiotas compared to chicks [35]. Therefore, more microbiotas presented in the cloacal of adult bar-headed geese.

The present results clearly showed some differences in cloacal microbiotas between chicks and adults. Relative abundances of *Fusobacteria* (48.29%) and *Actinobacteria* (5.02%) in cloacal of chicks were greater than those in adults. An increase in the prevalence of *Fusobacteria* had also been reported in other birds' microbiomes such as penguins [36], emus [37] and vultures [38]. *Fusobacteria* phyla were found to be the producers of butyrate, which was known to enhance the body fat accumulation and the immune function of bird hosts [39]. In this context, we can expect that the greater relative abundance of butyrate-producing *Fusobacteria* may be helpful to increase the survival rates of chick bar-headed geese by enhancing their fat accumulation. In the case of penguins, *Actinobacteria* were found to be higher abundant in the gastrointestinal microbiotas of Adélie penguins due to the capability to degrade chitin in their diets [34]. This indicated that higher abundance of *Actinobacteria* in chick bar-headed geese may be related to their food digestion capabilities required. However, the diet composition of bar-headed geese during their early developmental stage are virtually unknown. Therefore, the diet and the microbiota of chick bar-headed geese should be integrated in future prospective studies.

Proteobacteria and *Cyanobacteria* were found to be higher in adult bar-headed geese than in chicks. The *Proteobacteria* is the largest bacterial phylum in terms of the number of culturable bacteria and is abundant in the gastrointestinal tracts in the majority of birds [14]. The observed *Cyanobacteria* correspond to the chloroplasts from the plant-based diets. As an herbivorous species, the nourishment of bar-headed geese is composed of highly fibrous plant material, mainly grass, leaves, twigs and seeds [40].

Although there were many differences between chicks and adults, our analysis of the compositions at the OTUs level in each group showed very large overlap existed in the bacterial assemblages between chicks and adults. The establishment of gastrointestinal microbiotas of young birds is characterized by a high turnover of many transient species and large changes in community structure over short periods of time [41]. For example, van Dongen et al.[33] found that, chick and adult black-legged kittiwakes shared only seven OTUs, resulting in pronounced differences in microbial assemblages. In contrast to this findings, our results showed that 67.39% of the OTUs in chicks also presented in the cloacal microbiotas of adult bar-headed geese. Given that chick bar-headed geese were fed exclusively on food regurgitated by adults, the shared OTUs could be related to the feeding habits of this bird species. Another reason might be that chicks and adults shared the same nesting environments. These 186 OTUs

shared between chick and adult bar-headed geese may be beneficial or commensal for the host, and therefore retained in the gastrointestinal tracts.

We acknowledged that our study had limitations. The sample size of adult bar-headed geese was relatively small. As such, the inability to collect required duplicate samples may reduce the accuracy of partial results. To describe the sequential changes of the cloacal microbiotas, more time points should be set in the future work.

In conclusion, this study is an elementary characterization and comparison of cloacal microbiotas in both chick and adult bar-headed geese. Future studies should include broader sampling of chicks for more detailed comparative analyses, thus help in the development of strategies to guide the formation of health-promoting microbiotas that could then be used for the artificially reared chick bar-headed geese.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Salivary and Serum Levels of Serum Amyloid A, Haptoglobin, Ceruloplasmin and Albumin in Neonatal Calves with Diarrhoea [1]

Ekin Emre ERKILIÇ [1,a] Oğuz MERHAN [2,b] Ali Haydar KIRMIZIGÜL [1,c]
Metin ÖĞÜN [2,d] Enes AKYÜZ [1,e] Mehmet ÇİTİL [3,f]

[1] Kafkas University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-36100 Kars - TURKEY
[2] Kafkas University, Faculty of Veterinary Medicine, Department of Biochemistry, TR-36100 Kars - TURKEY
[3] Erciyes University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-38039 Kayseri - TURKEY
[a] ORCID: 0000-0003-2461-5598; [b] ORCID: 0000-0002-3399-0667; [c] ORCID: 0000-0002-6660-2149; [d] ORCID: 0000-0002-2599-8589
[e] ORCID: 0000-0002-3288-2058; [f] ORCID: 0000-0001-9839-7533

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Abstract

This study was aimed at the determination of the serum levels of serum amyloid A (SAA), haptoglobin (Hp), ceruloplasmin (Cp) and albumin (Alb), and the salivary levels of Hp and Cp in neonatal calves with diarrhoea. Male and female neonatal Simmental calves, 15 of which were sick and 10 of which were healthy, constituted the study material. Five mL blood samples from the jugular vein and saliva samples absorbed with swabs were collected once from all animals. While serum SAA ($P<0.001$), Hp ($P<0.001$) and Cp ($P<0.001$) levels were found to be significantly higher in the sick calves, compared to the healthy calves, no significant difference was detected for the serum Alb levels ($P>0.05$). Furthermore, the salivary Hp ($P<0.05$) and Cp ($P<0.001$) levels of the sick animals were higher than those of the healthy animals. In result, it was ascertained that serum Hp, SAA and Cp levels and salivary Hp and Cp levels significantly altered in diarrhoeic animals. Thus, it is suggested that in studies involving the measurement of Hp and Cp levels, saliva samples could be collected non-invasively as an alternative to blood samples.

Keywords: *Calf, Serum amyloid A, Haptoglobin, Ceruloplasmin, Saliva*

İshalli Neonatal Buzağılarda Salya ve Serumda Serum Amiloid A, Haptoglobin, Seruloplazmin ve Albumin Seviyeleri

Öz

Bu çalışma ishalli neonatal buzağılarda serumda serum amiloid A (SAA), haptoglobin (Hp), seruloplazmin (Cp) ve albumin (Alb), salyada ise Hp ve Cp seviyelerinin belirlenmesi amacıyla yapılmıştır. Çalışmanın materyalini neonatal dönemdeki farklı cinsiyette 15 hasta, 10 sağlıklı simental ırkı buzağı oluşturdu. Çalışmaya dahil edilen buzağıların *vena jugularis*'lerinden bir kez 5 mL kan ve swaplar yardımı ile salya örnekleri alındı. Serum SAA ($P<0.001$), Hp ($P<0.001$) ve Cp ($P<0.001$) seviyeleri hastalarda sağlıklılara göre istatistiksel olarak önemli seviyede yüksek belirlenirken serum Alb ($P>0.05$) seviyesinde önemli bir değişiklik olmadığı görüldü. Hasta hayvanlarda salya Hp ($P<0.05$) ve Cp ($P<0.001$) seviyeleri sağlıklılara göre yüksek olduğu belirlendi. Sonuç olarak ishalli hayvanlarda serum Hp, SAA ve Cp seviyeleri ile salyada Hp ve Cp seviyelerinde önemli değişikliklerin olduğu belirlendi. Hp ve Cp'nin değerlendirileceği çalışmalarda serum alınmasına alternatif olarak non-invaziv bir yöntem olan salya alınmasıyla da ölçülebileceği kanısını uyandırdı.

Anahtar sözcükler: *Buzağı, Serum amiloid A, Haptoglobin, Seruloplazmin, Salya*

INTRODUCTION

Diarrhoea in neonatal calves is a major cause of economic loss [1]. The risk of diarrhoea is highest during the first month of life and decreases with the advance of age. Diarrhoea may result from the single infection of various agents or from mixed infections caused by multiple infectious agents. The severity and prognosis of the disease are influenced by several factors. These include, among others, nutrition, management and environmental factors [2].

İletişim (Correspondence)
+90 474 242 6836/5235
ekin_emre_24@hotmail.com

The levels of acute-phase proteins, which are synthesized in the liver, are very low in healthy animals and alter very rapidly in the event of inflammation [3]. Acute-phase proteins are classified as either negative or positive. Those, the levels of which rapidly decrease after infection, are classified as negative acute-phase proteins [such as albumin (Alb)], whilst those, the levels of which rapidly increase after infection, are classified as positive acute-phase proteins [such as serum amyloid A (SAA), ceruloplasmin (Cp), and haptoglobin (Hp)] [4].

Saliva, which is a continuously produced secretion, is being used for analyses in human medicine [5]. In veterinary medicine, saliva samples have been used for the measurement of total sialic acid levels and oxidative stress parameters (malondialdehyde (MDA), glutathione, nitric oxide (NO)) in cattle [6], SAA [7,8], Hp [8-10], cortisol [8], interleukin-18 (IL-18) [11], and C-reactive protein (CRP) [9,10,12] levels in pigs and CRP levels in dogs [5]. However, veterinary research in this field is scarce. To our knowledge, no previous study has been conducted in diarrhoeic calves using saliva samples. This study was aimed at the determination of the serum levels of SAA, Hp, Cp and Alb, and the salivary levels of Hp and Cp in neonatal calves with diarrhoea.

MATERIAL and METHODS

Animals

This study was conducted pursuant to the approval of the Local Ethics Board for Animal Experiments of Kafkas University (KAU-HADYEK, Research Code: KAU-HADYEK/2017-021). Male and female neonatal Simmental calves, 15 of which suffered from diarrhoea and 10 of which were healthy, constituted the study material. The sick animals included in the study showed clinical signs such as diarrhoea, decrease or absence of the sucking reflex, lateral recumbency, the inability to stand up, dehydration and coldness of the extremities. Five mL blood samples were taken once from the jugular vein of each animal, and were centrifuged at 3000 rpm for 10 min for the seperated of sera. The serum samples were transferred into eppendorf tubes and stored at -20ºC until being analysed.

Each animal was also sampled for saliva. For this purpose; cotton-tipped swabs were brushed mouth mucosa until the cotton tip became wet with saliva. This sampling method was repeated until a sufficient amount of saliva (approximately 0.3 mL) was collected. Once collected, the saliva samples were centrifuged at 3000 rpm for 10 min, transferred into eppendorf tubes and stored at -20°C until being analysed. SAA and Hp levels in serum samples determined with a enzyme-linked immunosorbent assay kit (Tridelta®, Ireland). Serum Alb levels were determined colorimetricly (Biolabo®, France) in accordance with the manufacturers' instructions. Serum Cp levels were determined as described by Colombo and Richterich [13]. The colorimetric measurement of salivary Hp and Cp levels was performed using the methods described by Skinner et al.[14] and Colombo and Richterich [13], respectively.

Statistical Analysis

The results obtained in the present study were applied a normality test using the SPSS 18 software package, and it was determined that the data showed a normal distribution. The results were compared using the t-test. All results were given as mean ± standard deviation.

RESULTS

All of the sick and healthy animals included in this study were in the neonatal stage of life (0-28 days). Clinically, the sick animals manifested diarrhoea, decrease or absence of the sucking reflex, dehydration, enophthalmos, coldness of the extremities, lateral recumbency, and the inability to stand up.

The serum SAA, Hp and Cp levels of the sick animals were determined to be 42.23±6.14 µg/mL, 0.30±0.02 g/L, and 19.40±2.70 mg/dL, respectively, whilst the same parameters were 14.43±3.29 µg/mL, 0.068±0.014 g/L, and 13.25±2.71 mg/dL, respectively, in the healthy animals *(Table 1)*. Accordingly, it was observed that the serum levels of these parameters were significantly higher in the sick animals, compared to the healthy animals (P<0.001). The serum Alb levels of the healthy and sick animals were measured as 2.95±0.25 g/dL and 2.85±0.25 g/dL, respectively *(Table 1)*. The serum Alb levels of the sick animals were lower than those measured in the healthy animals, but this difference was statistically insignificant (P>0.05).

The salivary Hp and Cp levels of the sick animals were determined as 7.90±2.65 µg/mL and 2.38±0.41 mg/dL, respectively. Furthermore, the salivary Hp and Cp levels of the healthy animals were measured as 5.28±1.76 µg/mL and 1.66±0.36 mg/dL, respectively *(Table 2)*. The

Table 1. *The serum Hp, SAA, Cp and Alb levels of the sick and healthy animals*

Parameter	Control Animals	Sick Animals	P Value
SAA (µg/mL)	14.43±3.29	42.23±6.14	P<0.001
Hp (g/L)	0.068±0.014	0.30±0.02	P<0.001
Cp (mg/dL)	13.25±2.71	19.40±2.70	P<0.001
Alb (g/dL)	2.95±0.25	2.85±0.25	P>0.05
P<0.05 is statistically significant			

Table 2. *The salivary Hp and Cp levels of the sick and healthy animals*

Parameter	Control Animals	Sick Animals	P Value
Hp (µg/mL)	5.28±1.76	7.90±2.65	P<0.05
Cp (mg/dL)	1.66±0.36	2.38±0.41	P<0.001
P<0.05 is statistically significant			

salivary Hp (P<0.05) and Cp (P<0.001) levels of the sick animals were found to be significantly higher than those of the healthy animals. Moreover, it was observed that the salivary levels were rather low in comparison to the serum levels.

DISCUSSION

Diarrhoea cases of various aetiology are one of the main causes of morbidity and mortality in animal holdings. The retarded growth of diarrhoeic animals, treatment costs, human labour and mortality all cause economic loss [15]. The sick animals included in the present study showed clinical signs of diarrhoea, decrease or absence of the sucking reflex, dehydration, enophthalmos, coldness of the extremities, lateral recumbency and the inability to stand up. The clinical signs observed in this study are similar to those observed and reported by Özkan and Akgül [16].

The levels of acute-phase proteins alter in the event of inflammation, trauma, stress and infection [17]. Serum Alb levels decrease with acute phase reactions [18]. In previous research carried out in calves with diarrhoea [19] and pneumonia [20,21], the Alb levels of the sick animals were found to be lower than the levels measured in the control animals. Similarly, in the present study, it was determined that serum Alb levels were lower in the sick animals, when compared to the healthy animals, yet this difference was statistically insignificant (P>0.05).

Haptoglobin and SAA are acute-phase proteins, which are significant for cattle [22]. In healthy cattle, Hp is found at very low levels [23], but its blood levels increase with inflammation, trauma and infection [3]. As this increase in Hp levels occurs before clinical symptoms appear, it is suggested that Hp levels could be measured for the purpose of early diagnosis [24]. In a study carried out in calves, it was observed that SAA levels increased with stress [25]. Similarly, in studies performed in calves with pneumonia, the SAA levels of the sick animals were found to be higher than those of the control animals [20,26]. Reports indicate that, in comparison to healthy animals, diarrhoeic calves presented with significantly increased Hp levels [2,19,27] and SAA levels [2,27]. Likewise, calves infected with *Cryptosporidium* have also been reported to have significantly higher levels of Hp and SAA, in comparison to healthy animals [28]. In agreement with previous research, in the present study it was determined that diarrhoeic calves had serum Hp (P<0.001) and SAA (P<0.001) levels higher than those of healthy animals.

Ceruloplasmin is used less frequently for diagnostic purposes in comparison to other acute-phase proteins [4]. It protects cells against oxidative damage and has cytoprotective activity [17]. In previous research, it was demonstrated that the Cp levels of diarrhoeic calves were significantly higher than those measured in control animals [1,19]. Similarly, the present study showed that the serum Cp levels of the diarrhoeic calves were significantly higher than those of the healthy control animals (P<0.001).

Blood sampling is an invasive method, which causes pain and stress and makes it difficult to collect samples when repeated sampling is required [7]. Gómez-Laguna et al.[10] suggested that, for the measurement of acute-phase protein levels, saliva and meat juice could be used as an alternative to serum samples. Saliva is a biological fluid and can be collected non-invasively [5]. Uzlu et al.[6] measured some oxidative stress parameters (MDA, glutathione and NO) and total sialic acid levels in serum and saliva samples taken from bulls infected with foot and mouth disease, and determined that significant alterations had occurred in both the salivary and serum levels of these parameters. In a previous medical study performed in patients with oral squamous cell carcinoma, the measurement of salivary interleukin-1 alpha, interleukin-6, interleukin-8 and granulocyte macrophage-colony stimulating factor levels demonstrated that these parameters significantly differed between the patients and the control group [29]. In another study carried out in humans with urticaria, the salivary C-reactive protein (CRP) levels of the patients were found to be higher than those of the healthy subjects, and this difference was statistically significant [30]. While research on the evaluation of stress in pigs showed that the salivary SAA level was a potential biomarker [8], in another study, salivary and plasma cortisol levels were determined to display parallel alterations, and were found to be correlated with each other (r=0.60) [31]. Parra et al.[5] ascertained that the salivary CRP levels of healthy dogs and sick dogs with various diseases significantly differed from each other. In another study carried out in pigs, the comparison of the salivary and serum CRP concentrations of healthy and sick animals revealed a statistically significant positive correlation, and the salivary CRP levels of the sick animals were observed to be higher than the levels measured in the healthy animals [12]. In the present study, the salivary levels of the acute-phase proteins Hp (P<0.05) and Cp (P<0.001) were found to be significantly higher in the diarrhoeic animals, in comparison to the healthy calves.

In conclusion, significant alterations were determined to have occurred in the serum Hp, SAA and Cp levels and the salivary Hp and Cp levels of diarrhoeic neonatal calves. The Hp and Cp levels measured in the saliva were lower than those measured in the serum samples, yet the alterations observed in the salivary levels were statistically significant like alterations detected in the serum levels. Furthermore, in view of salivary sampling being a non-invasive method and Hp and Cp levels being able to be measured in saliva samples, it is considered that in future research, saliva samples could be used as an alternative to serum samples for the measurement of these parameters.

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ERRATUM

Interrelationships of Serum and Colostral IgG (Passive Immunity) with Total Protein Concentrations and Health Status in Lambs [1]

Erhan GÖKÇE [1] Onur ATAKİŞİ [2]

[1] *This project supported by TUBITAK (Project Code: TOVAG 108 O 847) and Scientific Research Projects Coordination Unit of Kafkas University*

[1] Departments of Internal Diseases, Faculty of Veterinary Medicine, Kafkas University, TR-36300 Kars - TURKEY
[2] Department of Chemistry, Faculty of Art and Science, Kafkas University, TR-36300 Kars - TURKEY

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ERRATUM

In this article some authors who participated to the study at all stage were erreneously forgetten to be included. Therefore the article was corrected as below;

Interrelationships of Serum and Colostral IgG (Passive Immunity) with Total Protein Concentrations and Health Status in Lambs [1]

Erhan GÖKÇE [1] Onur ATAKİŞİ [2] Ali Haydar KIRMIZIGÜL [1] Hidayet Metin ERDOĞAN [1]

[1] *This project supported by TUBITAK (Project Code: TOVAG 108 O 847) and Scientific Research Projects Coordination Unit of Kafkas University*

[1] Departments of Internal Diseases, Faculty of Veterinary Medicine, Kafkas University, TR-36300 Kars - TURKEY,
[2] Department of Chemistry, Faculty of Art and Science, Kafkas University, TR-36300 Kars - TURKEY

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İletişim (Correspondence)
+90 474 2426807/5237 Fax: +90 474 2426853
erhangokce36@hotmail.com

INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given in the title page only.

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