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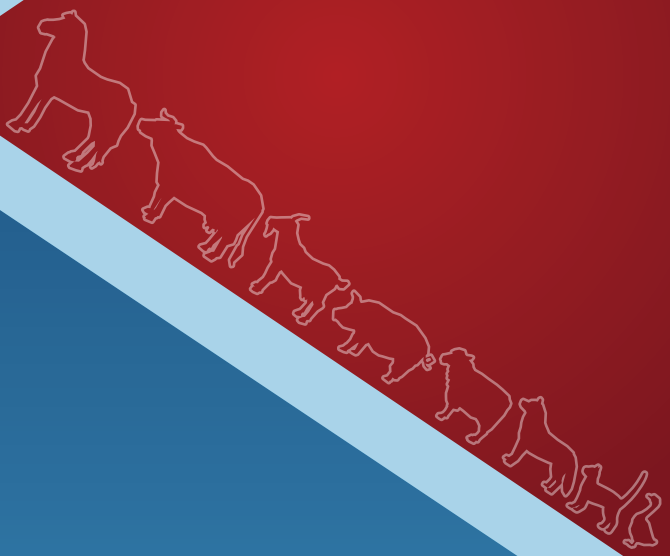

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Address for Correspondence

Kafkas Üniversitesi Veteriner Fakültesi Dergisi Editörlüğü 36040, Kars - TÜRKİYE
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RESEARCH ARTICLE

Milk Non-Enzymatic Immunofiltration Assay "mNERIFA": An Alternative Rapid Bovine Milk Test for Anti-*Brucella* Antibody Detection [1]

Evrim GENÇ [1,a,†] Gülnur SERDAR [2,b,†] Yunus KILIÇOĞLU [3,c] Oktay GENÇ [1,d (*)]

[1] *This study was presented as a poster presentation at the 4th International Eurasian Conference on Biological and Chemical Sciences held on 24-26 November, 2021 in Ankara, Türkiye*

† *These authors contributed equally to this work*

[1] Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Microbiology, TR-55139 Samsun - TÜRKİYE

[2] Samsun Veterinary Control Institute, Department of Rabies Diagnostic, TR-55200 Samsun - TÜRKİYE

[3] Samsun Veterinary Control Institute, Department of Serology, TR-55200 Samsun - TÜRKİYE

ORCIDs: [a] 0000-0002-6405-6076; [b] 0000-0002-3756-4606, [c] 0000-0002-6788-9284; [d] 0000-0003-0777-6824

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Abstract: Rapid milk tests for the indirect diagnosis of brucellosis are limited and generally not preferred due to insufficient diagnostic performances and inappropriate handling process. In this study, a rapid immunofiltration assay known as Non-Enzymatic Immunofiltration Assay (NERIFA) was evaluated based on diagnostic performances and applicability for the detection of anti-*Brucella* antibodies in bovine milk samples as milk NERIFA (mNERIFA). For this purpose, wheys, that were obtained from rennin precipitated milk constituted with reference sera, were used as test material. Besides, an enzyme-linked immunosorbent assay (ELISA) was also developed for the evaluation of mNERIFA. After approval, the ELISA was evaluated as important (P<0.001) by Receiver Operating Procedures (ROC) analysis based on the diagnostic index (J) score specified as the Youden index (J: 0.963) and the Area Under the Curve (AUC) value (0.996), its results compared with mNERIFA by kappa statistical analysis. ELISA's comparison with mNERIFA based on diagnostic performances indicated that ELISA's performance was in perfect agreement with mNERIFA (κ=0.97) and the agreement was 0.97% (0.92-0.99%) with CI 95%. In the optimization process of mNERIFA, it was found that there was no significant difference between the individual and pooled whey samples (P>0.05), and a pool of 5 samples can be used instead of individual testing. In this study, it was concluded that mNERIFA may be recommended as a rapid test for anti-*Brucella* antibody detection in bovine milk samples based on the diagnostic performances, applicability, and pooling capacity.

Keywords: *Bovine brucellosis, Milk NERIFA, Milk test, Rapid test, Serology*

Süt İmmünofiltrasyon Testi "mNERIFA": Anti-Brusella Antikor Tespiti İçin Alternatif Hızlı Süt Testi

Öz: Hızlı süt testleri brusellozun indirekt teşhisi amacıyla sınırlıdır ve yetersiz teşhis özellikleri ve uygun olmayan işleme süreci nedeniyle genellikle tercih edilmez. Bu çalışmada, Non-Enzimatik İmmünofiltrasyon Testi (NERIFA) olarak bilinen hızlı bir immünfiltrasyon testi, süt NERIFA olarak tanısal performanslarına ve anti-*Brucella* antikor tespiti yönünden sığır süt örnekleriyle değerlendirildi. Bu amaçla referans serumlardan oluşturulan rennin ile çökeltilmiş sütten elde edilen süt serumu test materyali olarak kullanıldı. Ayrıca, mNERIFA'nın değerlendirilmesi için bir enzim immünosorbent testi (ELISA) geliştirildi. ELISA'nın, Receiver Operating Procedures (ROC) analizi ile Youden endeksi olarak bildirilen tanı indeksi (J: 0.963) ile Eğri Altındaki Alan (AUC) (0.996) değerine dayalı olarak uygunluğu (P<0.001) onaylandıktan sonra, mNERIFA ile kappa istatistiksel analizi ile karşılaştırıldı. ELISA'nın tanısal performanslara dayalı olarak mNERIFA ile karşılaştırması, ELISA'nın performansının mNERIFA ile mükemmel uyum içinde olduğunu (κ=0.97) ve bu uyumun %95 güven aralığında %0.97 (%0.92-%0.99) olduğu belirlendi. mNERIFA'nın optimizasyon sürecinde, bireysel ve havuzlanmış süt serumu örnekleri arasında anlamlı bir fark olmadığı (P>0.05) ve bireysel testler yerine 5 örneklik bir havuzun kullanılabileceği belirlendi. Bu çalışmada, mNERIFA'nın, tanısal performanslarına, uygulanabilirliğine ve havuzlama kapasitesine bağlı olarak sığır sütü örneklerinde anti-Brusella antikorlarının tespiti için hızlı bir test olarak önerilebileceği sonucuna varılmıştır.

Anahtar sözcükler: *Hızlı test, Seroloji, Sığır brusellozisi, Süt NERIFA, Süt testi*

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(*) Corresponding Author

Tel: +90 362 312 1919-1571
E-mail: ogenc@omu.edu.tr (O. Genç)

Introduction

Brucellosis is one of the seven neglected prevalent zoonotic diseases that affect animals and human beings worldwide [1]. It is a costly, highly contagious disease that affects many farm animals [2,3]. The disease is usually manifested by abortion, retention of the placenta, stillbirth, infertility with the scattering of the organism in uterine discharges and milk. Diagnosis is based on isolation and detection of *Brucella* spp. from abortion material, mammary secretions, or postmortem tissues [4-6]. *Brucella* culture is accepted as a reference as the gold standard and it may give rise to false negative results in serological evaluation. Although serological tests are used for the screening and monitoring in surveillance and control and eradication phase of the disease [7], but no single valid serological test is available to detect every stage of infection with *Brucella* spp. [8] with higher accuracy. Because of this, bovine brucellosis diagnosis is performed with serial or parallel tests in blood sera and generally, Rose bengal test (RBT) and Complement fixation test (CFT) are preferred in combination to confirm the diagnosis in many countries [7,9]. ELISAs being more sensitive and specific, are recommended in place of both CFT and RBT and other conventional tests [5] by OIE as a suitable screening test [7,10]. However, a limited number of tests mostly Milk Ring Test (MRT) and ELISA [11-13] and less frequently chromatographic tests [14] are being used for brucellosis screening based on testing anti-*Brucella* antibodies in milk.

Rapid tests are preferable in field conditions due to higher sensitivity, specificity, rapidity, and applicability [15,16]. Among these tests, immunochromatographic [14,16] and immunofiltration [17-19] based formats have been suggested for serological detection of bovine brucellosis, but their applicability in milk is very limited. Immunochromatographic rapid tests are more common [20-22] compared with immunofiltration based formats. However, there is no information with immunofiltration tests to detect anti-*Brucella* antibodies in milk.

In rapid tests, either in immunochromatographic or immunofiltration, nanoparticles as detector molecules are conjugated to antibodies that recognize the antigens and antibodies [23], and in some cases, proteins such as proteins A, and G are preferred in place of antibodies due to their higher affinities [13]. Recently, the gold nanoparticles conjugated with proteins have been used commonly in different species for the detection of different antibody isotypes. The affinity of protein G is better for the detection of bovine IgG isotypes and the strong optical absorption of gold nanoparticles with protein G enables its colorimetric detection by the naked eye [23,24]. These properties of protein G conjugated with gold nanoparticles make it special for rapid testing for both antigen and antibody detection. This study aimed to determine the diagnostic performance of mNERIFA based on detector Gold/Protein G (G/PG) nanoparticles for brucellosis detection as an alternative test in bovine milk samples.

Material and Methods

Ethical Statement

Ondokuz Mayıs University Animal Experiments Local Ethics Committee provided an ethics report for this study (E-68489742-604.02.03-255675).

Blood Sera

A total of 87 reference sera was included in the study. Of these, 60 were obtained from certified *Brucella*-negative herds and 27 were from aborted dairy cows. The sera were also confirmed serologically by iELISA. All positive references were from aborted cows and in which *Brucella abortus* biotype 3 isolates were isolated from vaginal swabs and milk samples.

Milk Samples

Milk samples obtained from a certified *Brucella*-negative herd was tested for the presence of anti-*Brucella* antibody by Milk Ring Test (MRT) (MRT Antigen Institute Pourquier, France), and then it was used for preparation of whey for ELISA and mNERIFA testing.

Milk Whey Preparation

Since milk samples were not suitable for the evaluation of mNERIFA, wheys were prepared from the milk samples. For this purpose, all control reference sera were transferred into the milk samples at a ratio of 1: 2 considering the probability of binding of the antibodies to milk fat globules in place of transferring directly the reference sera to wheys. Briefly, milk samples were prepared by the reference sera. The mixture was then incubated with microbial coagulating solution (Turkish rennet, Yayla) at room temperature for 30 min. After centrifugation at 3.000xg for 10 min at 4°C, the wheys were collected [25] and used to determine the diagnostic performance of the ELISA.

Reference Sera

An OIE serum containing 1000 Complement Fixation Test Unit (CFTU) anti-*Brucella* antibody was used for the optimization of CFT, ELISA, and mNERIFA. Monoclonal anti-*Brucella* LPS antibody (LPS-mAb, clone 4B5A) was used as a reference antibody for checking the LPS consistency of the crude LPS antigen.

Antigens, Reagents and Bacteria

Antigens and other reagents such as complement and amboceptor were obtained commercially and used in CFT (Virion Serion, Germany). Crude lipopolysaccharide

(LPS) antigen used in ELISA and mNERIFA was prepared from *B. abortus* S19 vaccine strain by the hot phenol method described by OIE [10]. Ring test antigen was used for the selection of the milk samples to be tested with mNERIFA.

Pooling Samples

The pooling process was performed with 2 to 5 combinations of positive and negative wheys to determine how the pooling process visually affected the test results. For this purpose, milk wheys prepared from negative and positive references were pooled with the combination of 2, 3, 4, and 5 samples, and compared for background development and visual efficiency.

Complement Fixation Test (CFT)

Sera from brucellosis-free dairy cows used in the study as reference standard was confirmed by CFT and it was performed according to the method described by OIE [7].

Indirect Enzyme-Linked Immunosorbent Assay (iELISA)

The iELISA procedure was carried out following the method [26] for testing the wheys with some modifications. Briefly, microplates were coated with *B. abortus* crude LPS antigen prepared in carbonate buffer (0.1 M, pH 9.6) and kept overnight at +4°C. The microwells blocked with 1% fish gelatine in phosphate-buffered saline (PBS) containing 0.2% Tween 20 (FG-PBST) were incubated at 37°C for 1 h. After washing, wheys and reference sera were diluted 1:50 and 1:100, respectively, in FG-PBST and transferred to microwells. Then, conjugate (alkaline phosphatase conjugated sheep anti-bovine IgG, Novus Biologicals, NB776) diluted 1: 4 000 were added to wells and incubated for 1 h at 37°C. After pNPP (p-Nitrophenyl Phosphate, Amresco), the plate was left 1 h at 37°C for the reaction development. The absorbance was read at 405 nm in ELISA reader (Multiskan EX, Fisher Scientific, Shanghai) after stopping with 1 N NaOH. The assay was carried out in duplicate and results were assessed by ROC analysis.

Milk Non-Enzymatic Rapid Immunofiltration Assay (mNERIFA)

The NERIFA [18] was developed for the detection of anti-*Brucella* antibody was performed with milk wheys. Briefly, the test cassettes shown in *Fig. 2* was prepared to contain the control and test dots. Two kinds of material known as absorbant pad and laminated nitrocellulose membrane (Schleicher & Schuell BioScience GmbH, Dassel, Germany) were inserted into casette respectively and two sides of the membrane were dotted with control and the respective antigen. Each test contained bovine IgG (1 mg/mL, Sigma-Aldrich) as control and crude LPS (1:200) as the test antigen. In this test procedure, laminated nitrocellulose membrane was firstly regenerated with a blocking solution (1% PBST/FG). Then, wheys generated from milk were added to the cassette after 1:2 dilution in blocking solution. Following flow-through it was washed 3 times with the washing solution. Then, the progression was maintained by CG- protein G conjugate (gifted, Abcam ab270696). One minute later, the stopping solution was added and the results were interpreted by comparing to the control side and checking with respective control sera of 2 and 20 CFTU/mL by their color intensity. In negative sample, the interpretation of the result was carried out as only control dot visible, while in positive sample two dots were visible. The cassettes presenting the test results were shown in *Fig. 2* with representative samples. A total of 27 positive and 60 negative wheys both individually and pooled were tested by mNERIFA and the results were evaluated by eye inspection.

Determining the Limit of Detection

The detection limit of mNERIFA was defined by OIE reference serum containing 1000 CFTU/mL. Two-fold dilutions of the reference serum from 8 U to 0.125 U was prepared for determining the limit of detection in mNERIFA.

Statistical Analysis

The accuracy of the ELISA was determined by ROC analysis and optimal cut-off selection was performed with Youden Index by MedCalc statistic [27]. The diagnostic performance of mNERIFA and the degree of agreement between ELISA and mNERIFA was estimated by kappa statistic with 95% CI using Medcalc. Statistical differences between individual and pooled whey samples in mNERIFA were determined by P-value and a P-value less than 0.05 was considered statistically significant. The significance level of ROC analysis (AUC values) was evaluated ($P<0.001$) as important.

Results

The ELISA results obtained from control standards and reference serum were optimized and evaluated for diagnostic performance and accuracy. An optimal cut-off value was selected based on the Youden index (J) score (0.963, cut-off>0.45) and evaluated by ROC analysis *(Fig. 1)*. Based on the cut-off value, sensitivity and specificity of the test were determined as 96.3% (CI 95%, 81-99.9%), 100% (CI 95%, 94-100%), respectively. As the accuracy of the test being 0.996, the ELISA was accepted to be accurate ($P<0.001$) for testing of the wheys for mNERIFA *(Table 1)*.

mNERIFA was performed with reference wheys and compared to ELISA by Medcalc statistics based on

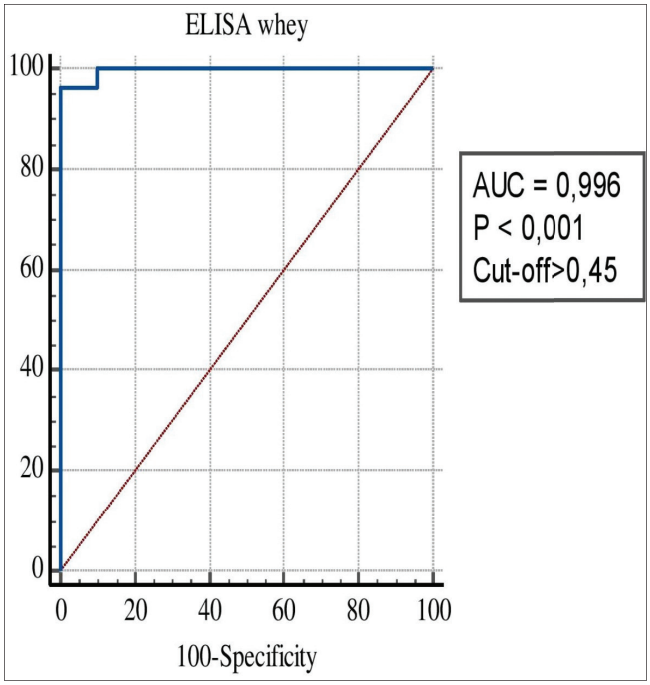

Fig 1. ROC analysis results of ELISA. Cut-off>0.45, Youden Index (J): 0.963, sensitivity: 96.3%, specificity: 100%

Table 1. *Diagnostic performances of ELISA and mNERIFA relative to control reference sera*

Diagnostic Criteria	ELISA Positive (n=26) Negative (n=59)	mNERIFA Positive (n=26) Negative (n=58)
Sensitivity	96.3% (81.03-99.91%)	96.3% (81.03-99.91%)
Specificity	98.3% (91.06-99.96%)	96.7% (88.47-99.59%)
Accuracy	97.7% (91.94-99.72%)	96.5% (90.25-99.28%)

diagnostic criteria. These results indicated that ELISA's performance was in perfect agreement with mNERIFA (κ=0.97) and the overall proportion agreement was 0.97% (CI 95%, 0.92-0.99%). Sensitivity, specificity and accuracy results were 96.3% (CI 95%, 81.3- 99.91%), 96.7% (CI 95%, 88.47-99.59%) and 96.5% (CI 95%, 90.25-99.28%) respectively *(Table 1)*.

mNERIFA results were also investigated by pooling up to 5 samples and some representative results are shown in *Fig. 2*. No difference was observed in terms of both individual and pooled samples with mNERIFA (P>0.05).

The detection limit of the mNERIFA was determined as 2 CFTU/mL and it was accepted as the cut-off level in the test. In addition, different reference sera were evaluated based on OD values of the ELISA compared with OIE reference serum quantitatively, and accordingly 20 CFTU was selected as the optimal low OD sera unit. Therefore, 2 CFTU/mL and 20 CFTU/mL sera as cut-off and low concentration control sera were included respectively in the panel of mNERIFA testing.

Fig 2. mNERIFA results with representable whey samples. mNERIFA results are visualized representatively. The first line numbered 1,2 and 3 show negative, 2, and 20 CFTU antibody results, respectively. The second line illustrates pooling negative results (no 4, 5 and 6) and 3rd and the bottom line show pooling (7,8,9) and individual positive sera (10,11,12) of different OD values selected respectively

Discussion

Various rapid serological tests have been developed to accurately determine the test performance based on sensitivity, specificity, sensitivity + specificity (performance index), accuracy in milk [8,9,28,29]. Most of these rapid tests are antibody detection tests such as lateral flow assays (LFAs) and agglutination-based MRT [13,20,21,30]. Different Lateral Flow tests and ELISAs have been developed and improved considerably for the detection of anti-*B. abortus* antibodies in milk [5,11,30,31]. In ELISAs, anti-immunoglobulin isotype enzyme conjugates are generally used for the detection of anti-species immunoglobulins against reactive antigens [5,9,23,29]. However, Protein G's affinity for IgG subclasses instead of detecting only one isotype antibody has replaced anti-immunoglobulin antibodies in these tests. Therefore, peroxidase and alkaline phosphatase-labeled Protein Gs substituted for enzyme-labeled secondary antibodies in ELISA, LFA, and ERIFA [17,31,32] and different LF tests and ELISAs for the detection of anti-*B. abortus* antibodies in milk have been developed and improved considerably [18]. Although the same mechanism works with NERIFA, only LFA is used for bovine brucellosis detection in milk. In this study, we hypothesized that NERIFA, which is also compatible with bovine, ovine, and

human brucellosis detection [18,19], may be an alternative test for milk screening. Specific antibodies are detected by the binding of the protein G/gold conjugate (protein G/GC) to the reacting *Brucella* LPS in lieu of secondary antibodies on laminated nitrocellulose membrane in mNERIFA. Due to the clogging of the membrane by milk and fat globules, an ELISA was optimized in which a whey-dependent assessment could be performed. The ELISA results were determined by its performance by ROC analysis. ELISA was found to be significant (P<0.001) depending on the results of the diagnostic index (J) score specified as the Youden index (J: 0.963) and the Area Under the Curve (AUC) value (0.996) for testing whey samples and comparing mNERIFA *(Fig. 1)* in the study.

mNERIFA was performed with the reference wheys and compared to ELISA based on diagnostic performances by Medcalc statistics. Wheys were prepared from control and OIE reference sera after being treated with rennin and a total of 87 including 27 positive and 60 negative wheys were tested for mNERIFA evaluation. Based on diagnostic properties; sensitivity, specificity and accuracy were found 96.3% (81.3-99.91%), 96.7% (88.47-99.59%) and 96.5% (90.25-99.28%), by mNERIFA respectively. These results are concordant with the ELISA and lateral flow assays but higher than MRT [21,33]. Positive sera used in the study were from the aborted cattle and *B. abortus* biotype 3 was the etiological agent in all events. Therefore, it was evaluated as gold standard sera with the negative sera which were from *Brucella*-negative herd. Although all positive sera were from gold standards, only one in 27 was found to be negative in both ELISA and CFT, it was evaluated as positive and accepted as false negative in ELISA and mNERIFA. Since the sensitivity of serological tests based on bacterial detection is low [4,14,29], that resulted in a decrease from 100% to 96.6% in sensitivity in the study. Although the decrease in the sensitivity of the test, these results indicated that diagnostic performances of the mNERIFA were compatible with those studies [29-32] for the milk testing. We may suggest this prototype alternative testing of brucellosis in milk samples considering the diagnostic performances. Protein G has a strong binding activity to bovine IgG and its isotypes, but its affinity to IgM and IgA in milk are not cited [15,32]. Therefore, detection of antibodies other than IgG in milk may be a disadvantage for milk testing with mNERIA.

The detection limit and weak positive antibody unit detected by quantitative ELISA (data not shown) have been accepted as the criteria for evaluating mNERIFA. In the study, we have shown that 2 and 20 CFTU/mL sera would be necessary for the evaluation of the test results *(Fig. 2)*. We, therefore, suggest these control sera should be included in each test panel.

In order to investigate pooling effect into test performance, wheys were pooled with 2 through 5 positives and negatives. There was no difference (P>0.05) between the individual and pooled whey results.

According to the data obtained in the study, our results show that mNERIFA may be an alternative test for anti-*Brucella* antibody detection in milk samples. Although the whey preparation process is a disadvantage in mNERIFA, its diagnostic performances, rapidity, evaluation in 5 min, pooling capacity with 5 samples make mNERIFA an alternative for rapid milk test. Although this study was simulated in the lab from wheys generated from the control sera, large-scale field studies are required where mNERIFA can be standardized before being adapted for field use.

In conclusion, based on the diagnostic performances and applicability presented here mNERIFA may be recommended as an alternative serological test for bovine brucellosis milk testing.

Availability of Data and Materials

Data sets analyzed during the current study are available from the corresponding author (O. Genç) on reasonable request.

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Ethical Statement

Ondokuz Mayıs University Animal Experiments Local Ethics Committee provided an ethics report for this study (E-68489742-604.02.03-255675).

Competing Interests

The authors declared that there is no conflict of interest.

Authors' Contributions

EG, GS and YK were ranked based on the contribution rates for performing lab studies and literature search and the corrections. EG and GS contributed equally to this work. The determination of the subject and the writing stages were planned and carried out by the corresponding author OG. All authors have contributed to the revision and final proof-reading of the manuscript.

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Research Article

Proteases and Collagenase Enzymes Activity After Autologous Platelet-Rich Plasma, Bio-Physically Activated PRP and Stem Cells for the Treatment of Osteoarthritis in Dogs

Mustafa ARICAN [1,a (*)] Kamil ÜNEY [2,b] Kurtuluş PARLAK [1,c]
Elgin Orçum UZUNLU [1,d] Gonca SÖNMEZ [3,e]

[1] Department of Surgery, Faculty of Veterinary Medicine, Selçuk University, TR-42130 Konya - TÜRKİYE
[2] Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Selçuk University, TR-42130 Konya - TÜRKİYE
[3] Department of Genetic, Faculty of Veterinary Medicine, Selçuk University, TR-42310 Konya - TÜRKİYE
ORCIDs: [a] 0000-0001-8180-135X; [b] 0000-0002-8674-4873; [c] 0000-0002-8656-037X, [d] 0000-0001-5356-8968, [e] 0000-0002-0399-7788

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Abstract: The aim of this study was to evaluate the treatment of osteoarthritis (OA) in dogs with intra-articular injection of an (autologous platelet-rich plasma (PRP), Mesenchymal stem cells (MSCs), bio-physically activated platelet-rich plasma (B-PRP/plasma-poor platelets-PPP) and their combinations and to determine the changes in the levels of synovial fluid samples (inflammatory mediator, proteases (MMP-2 and MMP-9) and collagenase enzymes (MMP-1, MMP-8 and MMP-13) obtained from the joints. Total of 36 different breeds, sex, age and weight of dogs with osteoarthritis were used as the material. The groups were divided as Group I (PRP), Group II (MSCs), Group III (PRP + MSCs), Group IV (B-PRP), Group V (B-PRP + MSCs) and group VI (saline control). Only single injection was given. Metalloproteinases were measured by using the ELISA in preoperative and postoperative treatment of 0, 15th, 30th, 60th, and 90th days in synovial fluid samples. Clinical and radiographic examinations were performed on the 0, 15th, 30th, 60th, and 90th days. The results obtained from the PRP + MSCs combination group were more successful. It was also noted that successful results could be obtained with PRP alone or in combination with stem cells, especially if repeated intraarticular injections are required for osteoarthritis. New studies are needed to understand the effectiveness of B-PRP. Only B-PRP or stem cell combination was effective on many enzymes, but varying results were obtained with each case.

Keywords: *Anti-inflammatory effect, Collagenase, Gelatinase, Lameness, Pain scores, Dog*

Osteoartritli Köpeklerde Otolog Trombositten Zengin Plazma, Biyo-Fiziksel Olarak Aktive Edilmiş TZP ve Kök Hücre Tedavilerinin Proteaz ve Kollajenaz Enzim Aktivitesine Etkisi

Öz: Bu çalışmanın amacı, köpeklerdeki OA'in tedavisinde eklem içi enjeksiyonu yapılan, otolog trombositten zengin plazma (PRP), Mezenkimal kök hücreler (MSC'ler), biyo-fiziksel olarak aktive edilmiş trombositten zengin plazma (B-PRP/plazmadan fakir trombositler-PPP) ve kombinasyonlarının etkinliğinin, sinovyal sıvı örneklerinde (yangısel mediator, proteazlar (MMP-2 ve MMP-9) ve kollajenaz enzimlerinin (MMP-1, MMP-8 ve MMP-13) düzeylerindeki değişiklikleri değerlendirmektir. Farklı cins, cinsiyet, yaş ve kiloda OA teşhisi konulan 36 köpek kullanıldı. Gruplar; Grup I (PRP), Grup II (MSC'ler), Grup III (PRP + MSC'ler), Grup IV (B-PRP), Grup V (B-PRP + MSC'ler) ve Grup VI (kontrol) oluşturdu. Sadece tek intra artiküler enjeksiyon yapıldı. Operasyon öncesi ve tedavi sonrası 0, 15, 30, 60 ve 90. günlerde metalloproteinaz seviyeleri ELISA ile ölçüldü. Sinovyal sıvı örneklerinde klinik ve radyografik muayeneler 0, 15, 30, 60 ve 90. günlerde yapıldı. PRP + MSC kombinasyon grubundan elde edilen sonuçların başarılı olduğu gözlendi. Özellikle osteoartritin tedavisinde tekrarlayan intraartiküler enjeksiyonlar gerekiyorsa, PRP tek başına veya kök hücrelerle kombinasyon kullanımı halinde başarılı sonuçlar alınabileceği kaydedildi. B-PRP'nin etkinliğini anlamak için yeni çalışmalara ihtiyaç olduğu gözlendi. Genelde B-PRP veya kök hücre kombinasyonu birçok enzim üzerine etkili olduğu kayıt edidi. Ancak vakalar arasında farklı sonuçlar elde edildi.

Anahtar sözcükler: *Anti-inflamatuar etki, Kollajenaz, Jelatinaz, Topallık, Ağrı skorları, Köpek*

Introduction

Osteoarthritis (OA) is one of the most common joint diseases in dogs. It is observed in about a quarter of the dog population. Joint disease occurs in 20% of the dog population over the age of one [1]. In the pathogenesis of OA,

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(*) Corresponding Author
Tel: +90 332 223 8698 Cellular phone: +90 532 367 5586 Fax: + 90 332 241 0063
E-mail: marican@selcuk.edu.tr (M, Arıcan)

cartilage destruction and remodeling in the bone, active chondrocytes in the articular cartilage and inflammatory cells around are effective.

Metalloproteinases (MMPs) can degrade all components of the extracellular matrix which are central role in the disease process of arthritis. The mechanism involved in cartilage degradation is not fully understood. Macrophages, neutrophils and chondrocytes effective in the destruction of the cartilage matrix. Especially, MMPs expressed in connective tissue degrade collagen [2]. Proteases and collagenases (collagenase-1 (interstitial collagenase (MMP-1); collagenase-2 (neutrophil collagenase (MMP-8) and collagenase-3 (MMP-13) have an active role in the destruction of the cartilage structure (collagen and proteoglycan) in osteoarthritis. In addition, MMP-13 serves as a major mediator of type II collagen cleavage and matrix degradation [3]. Increase of MMPs has been demonstrated in synovial fibroblasts, chondrocytes and inflammatory cells [4,5]. MMP-2 and MMP-9 have an active role in cartilage degradation [5]. It was shown that MMPs triggers the release of growth factors that independently affect the extracellular matrix and contribute to all stages of wound healing [2].

PRP has been used frequently in human patient with OA [6,7]. It contains different types of growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGFs) and other cytokines that stimulate healing of soft tissue. Growth factors are effective for healing tissues in cell proliferation and regeneration of matrix metabolism. PRP has begun to be used as a source of autologous growth factor accelerating the healing process in articular cartilage, intervertebral disc and bone [6]. In recent years, intra-articular PRP has also been used in the treatment of OA in dogs [8-12].

Bio-physically activated PRP (Platelet poor plasma) is a biologic treatment that is derived from blood. It is similar to PRP except that it has a lower concentration of platelet cells but number of growth factors are high. B-PRP contains a concentration of growth factors that can help stimulate healing and also provide extended anti-inflammatory relief. Before PRP is applied, the process of revealing the healing and growth factors within the platelets is called the activation of thrombocytes. The activation of platelets is possible by chemical and physical methods. In the chemical method, bovine thrombin or calcium chloride ($CaCl_2$) is added to PRP to activate platelets before application. It has been shown that 70% of the growth factors from α-granules are released within 10 min and almost all within 1 h after the addition of activator [9,13].

The aim of this study was to evaluate the treatment of OA in dogs with intra-articular injection of an (autologous platelet-rich plasma (PRP), Mesenchymal stem cells (MSCs), bio-physically activated platelet-rich plasma (B-PRP/ plasma-poor platelets-PPP) and their combinations and to determine the changes in the levels of synovial fluid samples (inflammatory mediator, proteases (MMP-2 and MMP-9) and collagenase enzymes (MMP-1, MMP-8 and MMP-13) obtained from the joints.

Material and Methods

Ethical Statement

This study was approved by the Selcuk University Animal Experiments Local Ethics Committee (Approval no: 2016/14).

Design of the Study

Thirty-six dogs (different age, breed, gender, weighing 25 to 50 kg (mean 32.1), mean age 5.1 years) diagnosed with unilateral stifle OA were used as the study material. Randomized, controlled trial was designed for study in Small Animal Hospital, Faculty of Veterinary Medicine, University of Selcuk, Türkiye. The groups were divided as Group I plasma rich plasma platelets (PRP), Group II (Mesenchymal stem cells [MSCs]), Group III (PRP + MSCs), Group IV (bio-physically activated platelet-rich plasma (B-PRP/PPP), Group V (B-PRP + MSCs) and group VI control (0.9% isotonic saline). In particular, the dogs with no systemic disease were used as a material. In the last three months, no surgical application or steroid applications were made in the dogs participating in the study. Osteoarthritis was evaluated as primary and secondary according to their cause. A diagnosis of secondary OA was made when abnormalities causing joint instability (e.g., cranial cruciate ligament rupture) were evident. Cases with suspected meniscus damage were excluded from the study. Only dogs who had not received any treatment or nutritional supplement before were included in the study. Clinical examination, radiographic examinations and joint fluid analysis were performed on days 0, 15th, 30th, 60th, and 90th in each group.

Clinical Examination

The diagnosis was made by clinical examination while sitting, walking, running, climbing stairs, and jumping activities especially by tests performed on the affected joint. Pain scoring tests (Canine Brief Pain Inventory, CBPI) and walking scoring tests (Hudson Visual Analog Scale, HVAS) were performed [14,15]. The repeated walking and pain rating tests were examined by the same veterinarian.

Radiographic Examination

Radiographic examination was done before treatment and after at 4, 8, and 12 weeks (Regius 110; Konica Minolta, Tokyo, Japan). The radiograms were checked for enthesophte formation, periarticular osteophytes, subchondral bone

sclerosis, fat pat displacement, intra-articular mineralization, articular surface narrowing and soft tissues were evaluated. Classification of OA was mild (1), moderate (2), moderate to severe (2 to 3), or severe (3) as previously described by according to the Kellgren-Lawrence scoring [16]. The repeated radiological examination was also performed and evaluated by the same veterinarian.

Platelet-Rich Plasma Preparation

Genesis Autologous Cell System 2 (Neo Genesis, Seoul, Republic of Korea) branded PRP (30 mL) preparation kits were used in the study to obtain the standardized platelet counts at the desired level. The acid citrate (3 mL) from the kit was added to a 50 mL injector in order to prevent blood clotting and 27 mL of the blood was taken from the jugular vein. A 30 mL mixture of the blood and acid citrate was injected into the Genesis tube. The tubes were centrifuged in a Genesis centrifuge instrument at 1700 x g for 5 min. The anticoagulated blood revealed three layers after centrifugation: bottom layer (red blood cells, density = 1.09); middle layer (platelets and white blood cells (buffy coat), density = 1.06); top layer (plasma, density = 1.03). After centrifugation, the bottom lid of the tube was replaced with a buffy coat controller cap and the pusher was assembled. The buffy coat controller was turned counter-clockwise until the layer of the red blood cells reached the "0" line. The platelet poor plasma portion was removed, the pusher was turned until the layer of the red blood cells reached the top, and the PRP portion (3-5 mL) was pushed into a Luer lock injector. The platelet count obtained from the Genesis Autologous Cell System has been reported to be 4-8 times greater than the normal. The number of platelets injected for each dog ranged between 1.200.000 and 1.500.000. The injection was given immediately when PRP was prepared [8,9].

Preparation of Bio-Physically Activated Platelet-Rich Plasma

After the PRP was prepared, it was placed on one end of the Biophysics activator instrument. An empty Luer lock injector was mounted on the other end of the instrument. The activation process was achieved by injecting platelets from one syringe to the other via the activator a total of 30 times [9]. The platelet concentrate was injected immediately. The concentrate (PRP and B-PRP) was injected intraarticularly until sufficient resistance was obtained to push back the syringe plunger.

Allogeneic Adipose Mesenchymal Stem Cell (MSCs) Isolation and Expansion

Allogeneic adipose stem cells preparation was produced by Biovalda Ltd. (Ankara, Türkiye). MSCs were isolated from adipose tissue allogeneic origin and reproduced. Adipose tissue donors were recruited from dogs (n = 3) included in the study, which were determined to have no infectious or infectious diseases. Approximately 1000.000-1.300.000 cells were injected intraarticularly.

Joint Fluid Sampling

After centrifugation for 5 min (10.000 G), the joint fluid samples were frozen and stored at -80°C until analysis. The joint fluid was dissolved shortly before the analysis. Various MMPs that work as metalloproteinases (MMP-1, MMP-2, MMP-8, MMP-9, MMP-13) were measured by ELISA.

Lameness and Pain Evaluations

Pain and lameness evaluations were performed on the 1st, 3rd, 5th, 7th, 15th day and at weeks 4, 8 and 12 after the PRP and saline injections. Canine Brief Pain Inventory (CBPI) and Hudson and Visual Analog Scale (HVAS) [14] scoring system were used for lameness and pain assessment [15,17]. The study was conducted in a blind manner. The same veterinarian was assessed for dogs. The same scoring system was used throughout the 12 weeks for all time points.

Pressure Analyzer (Pet Safe Stance Analyzer)

A pressure analyzer (Pet Safe Stance Analyzer, Kruuse, Germany) was used to measure the unequal weight distribution for extremities in dogs. It is based on the principle of proportionally calculating the body weight falling at four different measurement points and transferring them to the computer. In practice, the analyzer was made ready by weight calibration before the cases were examined. Subsequently, the subjects were placed on the analyzer one by one and fixedly held with one extremity in each square compartment. Meanwhile, by taking weight measurements (at least 15 times) from an auxiliary analyzer control, the average pressures were automatically recorded in the computer.

Protease and Gelatinase Zymography

Gelatin zymography technique was used for gelatinase activity levels using a modification of a previously described [4,8,9]. Synovial fluid samples collected on day 0, 30, 60, and 90 after PRP or saline injection were used for analysis.

Enzyme-Linked Immunosorbent Assay (ELISA) for Protease and Gelatin-Degradation

Synovial fluid samples were taken from dogs before the application of groups and on the post-op. 15th, 30th, 60th, and 90th days. ELISA was used to measure degree of disintegration of gelatin. Double-antibody sandwich ELISA was used for measuring MMP-1 (cat.no: 201-15-0945); MMP-2 (cat.no: 201-15-0159); MMP-8 (cat.no: 201-15-0887); MMP-9 (cat.no: 201-15-0038) and MMP-13 (cat.no: 201-15-1978) Sunred Biological Technology Co., Ltd. (Shanghai, China). The intensity of the reaction product was measured at 450 nm in an ELISA plate

reader (MWGt Lambda Scan 200, Bio-Tek Instruments, Winooski, VT, USA). All samples were assayed in duplicate. The calculation was made following the manufacturer's recommendations.

Statistical Analysis

SPSS 20.0 (IBM, USA) was used for The HVAS and the CBPI scoring tests. The data is analyzed by Non-parametric the Mann Whitney U test. The results of the joint fluid ELISA tests run on the six groups were evaluated by Tukey's test.

Results

Results of Clinical Examination

Medium and large breed dogs were evaluated. Their age ranged was from 4 to 8 years. No complications related to platelet concentration, activated platelet concentrate, stem cells or saline were observed.

Finding of Radiographic Examination

Thirty dogs had moderate OA (radiographic score 2), four had moderates to severe OA (radiographic score 2-3), and two had severe OA (radiographic score 3). At the end of the 12th week, the same procedures were performed as before the treatment. Considering the radiological results, no difference was found between the groups.

Preparation of PRP, Bio-Physically Activated PRP, Allogeneic Adipose Mesenchymal Stem Cell (MSC)

The platelet concentration (mean 1.420.000 platelets/μL) was significantly (P<0.001) higher than the platelet counts in the blood samples, representing a 4.0- or 5.0-fold increase in the platelet count. The White Blood Cell count (1.09 s/μL) was significantly decreased. Hematocrit was also significantly lower than Hct for the blood samples.

Lameness and Pain Evaluations

The HVAS and CBPI scores were not affected by the clinicians who evaluated the cases because of blind manner. For the dogs in the PRP group, activated PRP, MSCs scores for all components of the HVAS (mood, attitude, activity, comfort, exercise, playfulness, walking comfort) and all components of the CBPI (general activity, pain, rise, walk, run, climb and the ability to enjoy life) were significantly different between pre-treatment and week 4 (P<0.05), week 8 (P<0.05), and week 12 (P<0.05). Improvements in treatment outcomes were noted in almost all groups.

Pressure Analysis Findings

In the study, unequal weight distributions in the extremities were evaluated in the compression analysis and recorded all. Pre-op and post-op 15th, 30th, 60th, and 90th day results were compared individually. The problematic extremity of the 36 dogs were easy to distinguish from the other limbs as their compression force was insufficient due to the inadequate weight usage. As a result of the treatments, no difference was observed between in the PRP group, activated PRP, MSCs groups. Pressure analysis findings did not support clinical findings in some cases.

Protease and Gelatinase Zymography

MMP-2 activity of 70 kDa enzyme in pre-treatment synovial fluid samples was increased. Enzyme activity was seen at 204 kDa (MMP-9 pro-dimer) and 257 kDa (MMP-9 dimer). MMP-1 was partially inhibited in MSCs, MSCs + PRP and MSCs + B-PRP day 90th. The MMP-2, MMP-8 and MMP-9 concentration was inhibited in the B-PRP group and these enzymes were inhibited partially all groups. MMP-13 concentration was inhibited in the MSCs, MSCs+PRP and MSCs + B-PRP. The effects of 0, 50, and 100 mM EDTA (MMP inhibitor) were also investigated. Enzyme bands were partially inhibited by 50 mM EDTA and totally inhibited at 100 mM.

Metalloproteinases ELISA Analysis Results in Joint Fluid Samples

Gelatin degradation of synovial fluid demonstrated as measured by ELISA. The effects of single and combined administration of PRP, B-PRP and MSCs on joint fluid for MMP-1 *(Table 1, Fig. 1)*, MMP-2 *(Table 2, Fig. 2)*, MMP-8 *(Table 3, Fig. 3)*, MMP-9 *(Table 4, Fig. 4)*, and MMP-13 *(Table 5, Fig. 5)*, levels in dogs with osteoarthritis were presented. MMP-1 levels were started to decrease statistically in B-PRP+MSCs group on the 15th and enzyme

Table 1. *The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-1 (ng/mL) levels in dogs with osteoarthritis (Average ± SS)*

Group	Day 0	Day 15	Day 30	Day 60	Day 90
Control	46.00±18.98	60.91±21.90^{ab}	69.36±32.51^{a}	47.46±25.93	81.16±27.62^{a}
PRP	41.11±15.76	52.96±18.65^{ab}	10.07±9.72^{b*}	43.87±23.05	43.82±19.05^{ab}
BPRP	63.66±14.97	31.13±22.12^{ab}	67.47±44.77^{a}	50.53±27.47	49.05±17.97^{ab}
MSC	58.35±25.15	57.93±21.85^{ab}	68.83±36.26^{a}	52.12±32.71	32.61±15.83^{b}
PRP+MSC	42.15±28.03	27.63±15.34^{b}	44.07±19.47^{ab}	41.66±24.04	25.33±13.83^{b}
BPRP+MSC	38.63±15.57	71.73±35.35^{a}	90.70±19.36^{a*}	69.80±25.40*	62.31±29.78^{ab}

^{ab} *Different letters in the same column are statistically significant (P<0.05); * Statistically significant compared to day 0 (P<0.05)*

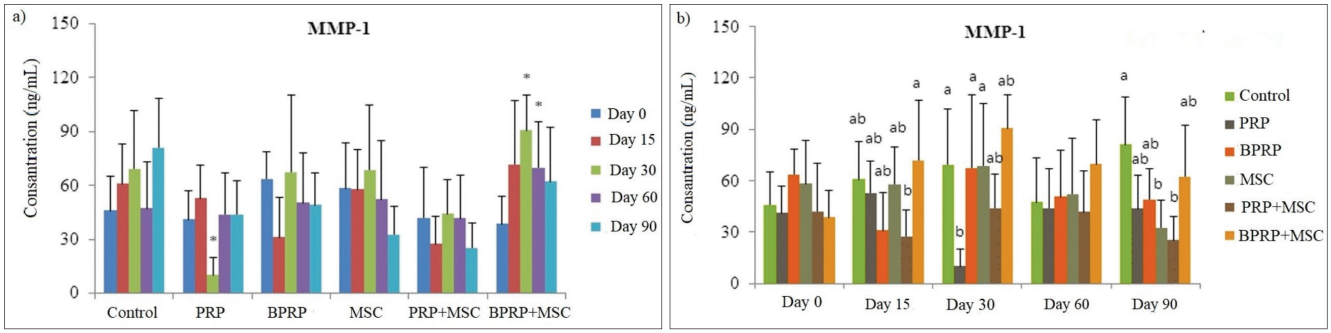

Fig 1. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-1 (ng/mL) levels in dogs with osteoarthritis. (a) In-group, (b) Shows differences between groups. ab- Different letters in the same column are statistically significant (P<0.05), * Statistically significant compared to day 0 (P<0.05)

Table 2. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-2 (ng/mL) levels in dogs with osteoarthritis (Average ± SS)

Group	Day 0	Day 15	Day 30	Day 60	Day 90
Control	165.75±36.13	157.08±45.02^{ab}	159.06±48.19	142.99±44.78^{ab}	254.83±36.09^{a*}
PRP	178.90±58.16	167.34±62.93^{a}	165.82±47.77	196.13±43.97^{a}	143.16±33.94^{b}
BPRP	198.53±34.46	227.70±56.87^{a}	152.07±57.30	105.61±37.08^{b*}	81.99±31.62^{bc*}
MSC	144.46±50.99	145.95±59.13^{ab}	136.18±60.97	81.99±49.86^{b}	87.34±46.57^{bc}
PRP+MSC	141.93±65.51	64.47±52.87^{b}	115.91±48.01	71.86±41.95^{b}	47.42±40.41^{c*}
BPRP+MSC	149.18±29.76	139.03±48.24^{ab}	132.72±28.36	71.13±44.76^{b*}	93.61±42.01^{bc*}

*^{ab} Different letters in the same column are statistically significant (P<0.05); * Statistically significant compared to day 0 (P<0.05)*

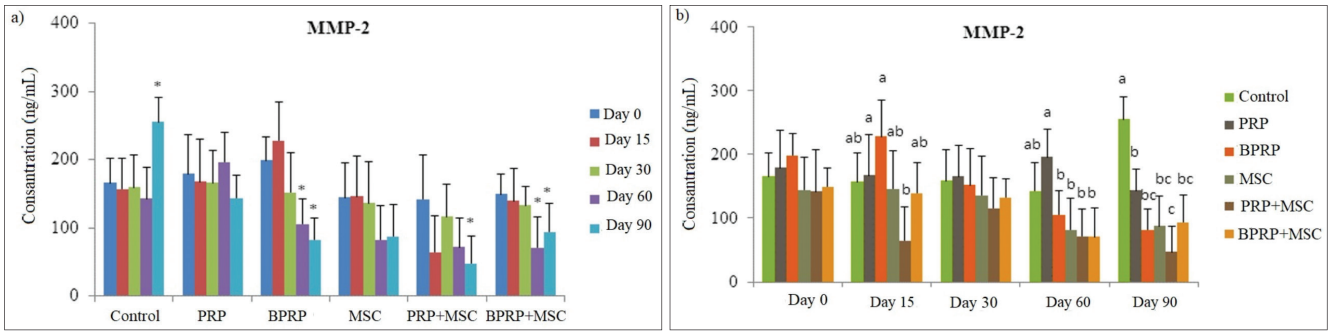

Fig 2. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-2 (ng/mL) levels in dogs with osteoarthritis. (a) In-group, (b) Shows differences between groups. ab- Different letters in the same column are statistically significant (P<0.05), * Statistically significant compared to day 0 (P<0.05)

Table 3. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-8 (ng/mL) levels in dogs with osteoarthritis (Average ± SS)

Group	Day 0	Day 15	Day 30	Day 60	Day 90
Control	36.00±17.11	44.58±19.44^{a}	36.58±15.38	42.99±14.98^{ab}	63.06±13.88^{a*}
PRP	42.07±22.77	40.87±22.87^{ab}	40.05±9.98	51.87±19.46^{a}	31.30±20.04^{b}
BPRP	39.88±9.85	34.15±8.52^{abc}	23.68±15.28*	27.14±5.22^{bc}	19.33±8.07^{b*}
MSC	25.12±10.85	26.00±7.78^{abc}	31.04±13.37	17.73±9.22^{c}	22.47±7.36^{b}
PRP+MSC	29.34±13.19	15.10±8.08^{c*}	20.97±7.73	17.77±11.13^{c}	11.14±8.18^{b*}
BPRP+MSC	25.11±9.44	19.94±8.63^{bc}	35.56±4.70	23.17±9.12^{bc}	24.40±9.09^{b}

*^{ab} Different letters in the same column are statistically significant (P<0.05); * Statistically significant compared to day 0 (P<0.05)*

levels decreased in PRP + MSCs on the 30th (P<0.05). MMP-2 level decreased significantly in B-PRP and PRP + MSCs group on the 60th and B-PRP + MSCs 90th day. However, MMP-2 level decreased after treatment in all groups. MMP-8 level decreased significantly in the B-PRP and PRP + MSCs group on the 60th and in the B-PRP +

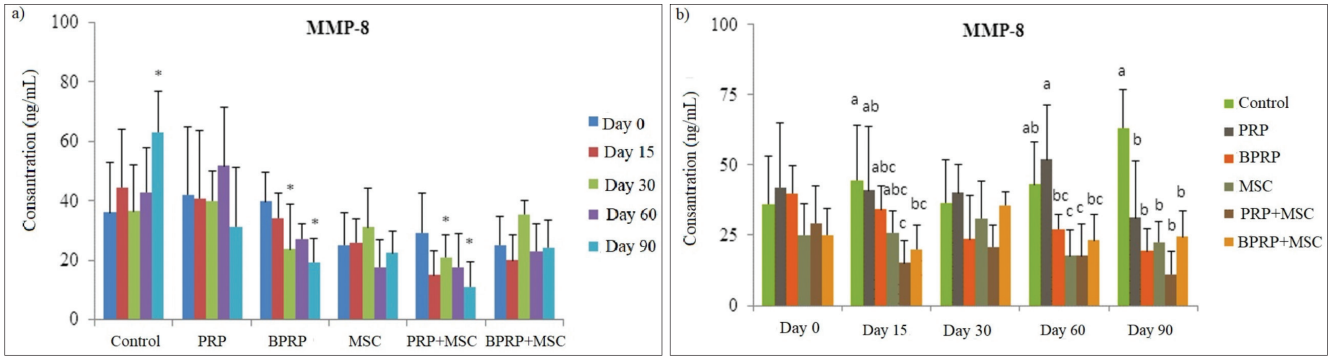

Fig 3. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-8 (ng/mL) levels in dogs with osteoarthritis. (a) In-group, (b) Shows differences between groups. ab- Different letters in the same column are statistically significant (P<0.05), * Statistically significant compared to day 0 (P<0.05)

Table 4. *The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-9 (ng/mL) levels in dogs with osteoarthritis (Average ± SS)*

Group	Day 0	Day 15	Day 30	Day 60	Day 90
Control	172.42±70.37	297.52±94.04^{a*}	257.58±124.22^{a}	284.07±105.03^{a*}	417.01±116.97^{a*}
PRP	217.90±45.43	210.63±82.25^{ab}	223.02±57.79^{ab}	240.77±62.05^{ab}	189.62±58.77^{b}
BPRP	227.69±22.25	172.26±81.02^{b}	165.75±20.65^{ab*}	163.30±39.57^{bc*}	128.37±46.10^{b*}
MSC	182.96±71.41	173.88±26.06^{ab}	169.06±40.43^{ab}	121.97±58.78^{c}	143.75±32.65^{b}
PRP+MSC	166.02±61.56	119.11±74.72^{b}	138.41±64.43^{b}	74.59±33.28^{c*}	85.27±68.52^{b}
BPRP+MSC	150.64±58.15	139.99±43.02^{b}	242.70±38.50^{ab*}	161.78±52.78^{bc}	167.49±56.94^{b}

*^{ab} Different letters in the same column are statistically significant (P<0.05); * Statistically significant compared to day 0 (P<0.05)*

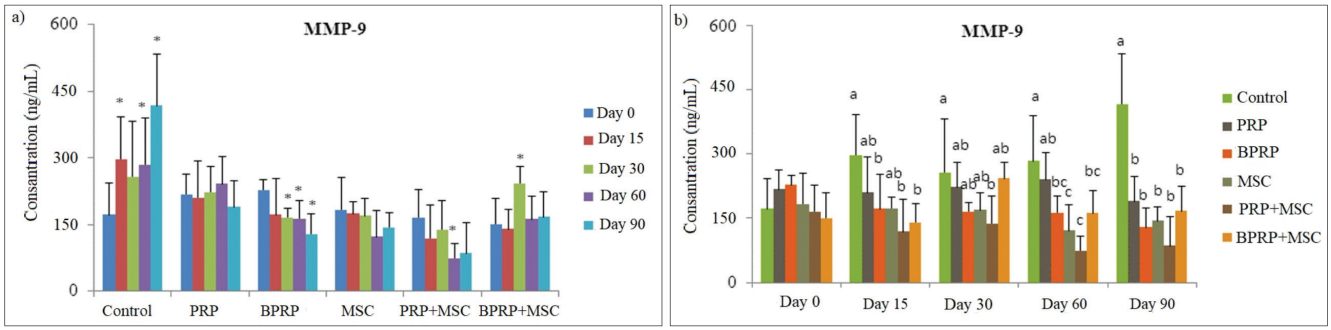

Fig 4. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-9 (ng/mL) levels in dogs with osteoarthritis. (a) In-group, (b) Shows differences between groups. ab- Different letters in the same column are statistically significant (P<0.05), * Statistically significant compared to day 0 (P<0.05)

Table 5. *The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-13 (ng/mL) levels in dogs with osteoarthritis (Average ± SS)*

Group	Day 0	Day 15	Day 30	Day 60	Day 90
Control	15.98±9.34	26.83±11.31^{ab}	23.91±11.20	21.51±10.09	35.85±8.71^{a*}
PRP	24.49±11.96	24.44±11.15^{ab}	21.77±8.82	33.32±11.18	22.53±8.69^{ab}
BPRP	23.92±10.02	18.87±12.29^{ab}	20.00±10.61	18.20±8.73	15.46±13.17^{b}
MSC	19.71±10.76	32.10±15.00^{a}	30.69±15.14	19.18±14.36	23.64±11.19^{ab}
PRP+MSC	24.12±10.62	9.21±5.44^{b*}	17.07±6.41	13.79±13.08	7.95±6.10^{b*}
BPRP+MSC	22.16±7.32	25.25±14.25^{ab}	31.71±5.16*	22.87±11.53	16.49±3.58^{b}

*^{ab} Different letters in the same column are statistically significant (P<0.05); * Statistically significant compared to day 0 (P<0.05)*

MSCs on 90th day. MMP-8 levels also decreased in all groups at the end of the 90th day. MMP-8 level increased significantly in the control group on the 90th day. B-PRP + MSCs groups were significantly lower than the control

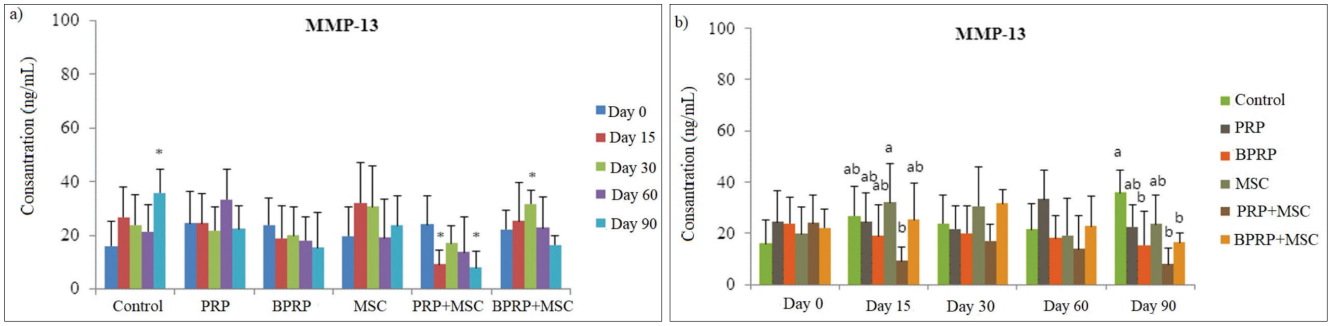

Fig 5. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-13 (ng/mL) levels in dogs with osteoarthritis. (a) In-group, (b) Shows differences between groups. ab- Different letters in the same column are statistically significant (P<0.05), * Statistically significant compared to day 0 (P<0.05)

group (P<0.05). MMP-9 level decreased significantly in the B-PRP group, PRP + MSCs group on the 60th and B-PRP + MSCs in 90th day. MMP-9 levels decreased after treatment in all groups. Especially, B-PRP+MSCs groups were significantly lower than the control group (P<0.05). Nevertheless, MMP-9 level increased significantly in the control group on the 90th day. And also, MMP-13 levels decreased after treatment in all groups.

Discussion

Treatment selections for dogs with osteoarthritis are varied. Despite this diversity, the clinical results of the treatment options are not satisfactory. However, studies conducted in recent years support different treatment alternatives such as weight control, NSAID, neutrocetic drugs and prosthesis applications [18]. The use of intraarticular, autologous PRP in the current treatment of osteoarthritis, which is common in dogs, has been demonstrated in our previous study [8,9,11,19]. Especially PRP has been shown to inhibit MMP-9 enzymes that are effective in the pathogenesis of osteoarthritis. Protease enzymes and cytokines play an important role in osteoarthritic pathogenesis. In particular, it causes the destruction of proteoglycan (PG) and collagen structure in cartilage tissue at the onset of the disease. In recent discussions, PRP and MSCs combination has been reported to provide faster recovery and positive results in clinical and gait analysis [19]. However, enzyme analyses are not available in any clinical studies presented. In addition, there is limited studies on the use of PRP with bioactivators [12]. In the current study, with a broad perspective on current treatment methods of osteoarthritis, clinical findings to be obtained as a result of joint fluid analysis for protease and collagenase enzymes (MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13) were evaluated.

Particularly, in the radiographic evaluation, 30 of 36 cases were diagnosed with 1 degree osteoarthritis, 4 cases moderate (2nd degree), and 2 cases severe (3rd degree) osteoarthritis. Evaluations were made based on Innes et al.[20]. In this study, it was considered that no examples could be provided in terms of breed, sex, weight and the degree of osteoarthritis. Therefore, it was not possible to ensure homogeneity between animal materials. Synovial fluid samples were taken from animals with osteoarthritis before and after treatment, in this way, it would provide the opportunity to be evaluated not only as a group but also individually. At the end of the treatment, it was observed that the cases generally improved. However, in some groups, the recovery process and a comfortable life started faster such as PRP + MSCs combination group.

The Hudson Visual Analog Scale-HVAS HVAS and Canine Brief Pain Inventory CBPI have been evaluated in previous studies [14,15,17] and are known as subjective methods of assessing pain and lameness. The results indicated that clinical efficacy of PRP + MSCs combination therapy. According to the subjective HVAS, statistical (P <0.05) changes were observed in the walking and pain tests of dogs on day 0 and 15th, 30th, 60th, and 90th (P<0.05) changes were observed in all groups. Lameness assessment was used with force plate analyzer in the study. The results were compared individually pre-op and post-op 90th day. According to the force plate findings, there is an improvement in (85%) cases. But force plate findings did not support clinical findings in 5 cases. As a result of the treatments, no difference was observed between the groups.

It has been shown that the amount of gelatinase enzymes (MMP-2 and MMP-9) increases in the joint fluid in osteoarthritis cases in dogs [4]. Increases in the amount of MMP-2 and MMP-9 enzymes have been demonstrated as an indicator of destruction in the joint cartilage and distinguishing the early stage of OA [20,21]. MMPs are catabolic mediators responsible for the degeneration of joint cartilage. An increase in the amount of MMP-2 and MMP-13 was determined especially in the advanced stages of the disease [21]. It has been reported that the levels of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 increase in case of joint damage [10,19,22]. Endogenous factors are effective in inhibiting MMP. α2 macroglubine is the most important regulator of collagenolysis to play active role in the inhibition of MMP-2, MMP-9 and MMP-

14 enzymes. The release of MMPs is controlled by TIMP. When MMP/TIMP is in equilibrium in normal tissue, the balance is disrupted in OA and MMP level increases [23]. It has been stated that MSCs and PRP application increase TIMP level. In the presented study, proMMP-9 (inactive form) has been demonstrated with different molecular weights on zymography gels. While 204 kDa was seen as the inactive form of MMP-9, 257 kDa was displayed as the active form of MMP-9. The destruction of the cartilage structure begins with the arrival of the enzyme in the active form [24]. ELISA results were also shown that, MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 levels increased in the control group. But MMP-2, MMP-8 and MMP-9 levels statistically significantly decreased in the B-PRP group and in addition MMP-13 levels decreased in the PRP-MSCs group. Furthermore, MMP-1 level also decreased in PRP-MSCs, MSCs and B-PRP groups on day 90th compared to day 0, but there was wide variation within the cases. No statistical difference was observed. The levels of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 decreased in all groups compared to the control group. One of the aims of the study here is to determine which group is more effective. According to the results obtained from the study, it has been shown that MMP activation is suppressed in the PRP group within one month. According to the zymography results, it was observed that enzyme activation continued in the control group. This situation has been determined as encouraging about the anti-inflammatory activity of PRP. In this study, MMP-13 level decreased in the 90th day of PRP, B-PRP, MSCs, MSCs + PRP, MSCs + B-PRP groups, while the most successful group was recorded as the MSCs + PRP combination group. MSCs + PRP tries to regulate the pathological effects of OA, for this purpose, it has been reported to have a role such as creating less inflammation, pain and providing lubrication in the joint. PRP reduces cartilage destruction in the formation of osteoarthritis by increasing endogenous HA production as well as its anti-inflammatory effect [25]. PRP is showing a similar effect to HA, inhibits the inflammatory mediator in the synovial membrane and articular cartilage structure [26]. In addition, PRP contains abundant stem cells, which enables the stem cell to be directed to the tissue to be treated [27]. In this respect, MSCs + PRP combination group showed statistically significant changes [18]. It has been also reported that PRP reduces MMP-13 expression, which has an important role in the development and destruction of OA, by almost 100% [5]. The combination use of PRP and MSCs causes more collagen II to proliferate, while reducing chondrocyte apoptosis [28]. Using a combination of PRP and MSC, both symptomatic and structural improvement was also noted [28,29]. Therefore, in the study, MSCs + PRP group was found to be the most successful in its activity against in terms of inhibition of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 enzymes. There is no study on the efficiency of autologous bioactivator platelet-poor plasma (B-PRP) in enzyme inhibition. In recent years, it is thought that bioactive proteins released from platelets and high concentrations of growth factors affect the regeneration of tissues such as ligaments and cartilage, which have limited healing capacity under normal conditions. It has been explained that uncovering the growth factors in the platelet without injecting it into the joint will further increase its effectiveness. However, MMP-1, MMP-2, MMP-8, MMP-13 were decreased in the B-PRP group, at the end of the 90th day after treatment. But there was no change in MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 levels between day 0 and day 90th in the stem cell combination of B-PRP. These results show us that the growth factors coming out of the platelet did not increase its effectiveness. Although the cases are seen well clinically, no change was observed between the pre-treatment and post-treatment radiological analyzes between the groups. When the radiological examination results were examined, it was determined that the treatments performed had no effect on the reduction of osteophytic growths too.

As a result, the results obtained from the PRP+MSCs combination group were more successful. It was noted that successful results could be obtained with PRP alone or in combination with MSCs, especially, repeated intraarticular injections are required in osteoarthritis. First, PRP has an anabolic effect on chondrocytes and synoviocytes, resulting in increased cell proliferation and hyaluronic acid release. Second, PRP could be acted as a bioactive cytoskeleton for cartilage destruction and to increase cartilage regeneration. Thirdly, PRP has potential for anti-inflammatory effect and alleviate OA symptoms. PRP has an anabolic effect on the proliferation of chondrocytes and MSC cells, as well as suppresses deregulation and development in the matrix. Although B-PRP is new, more studies need to be made to understand its effectiveness. Only B-PRP or MSCs combination was effective on many enzymes, but varying results were obtained between each case.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (M. Arican).

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Ethical Statement

This study was approved by the Selcuk University Animal Experiments Local Ethics Committee (Approval no: 2016/14).

Conflict of Interest

There was no conflict of interest in regards to authors reporting their findings.

Authors' Contributions

Study design, survey development, data analyses and manuscript preparation performed by all authors. MA: conception and design of the study, acquisition, analysis and interpretation of data, drafting and critical revision of the article; KÜ: analysis of the data and critical revision of the article. KP; acquisition of the data, EOU: acquisition of the data; GS: interpretation of data.

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Research Article

The Clues of the Discovery of New Pathogens Eventuated by the Horizontal Gene Transfer Among the *Bacillus cereus* Group Members

Fatih BÜYÜK [1,a (*)] Özgür ÇELEBİ [1,b] Mustafa Reha COŞKUN [1,c] Mitat ŞAHİN [1,d]

[1] Kafkas University, Faculty of Veterinary Medicine, Department of Microbiology, TR-36100 Kars - TÜRKİYE
ORCIDs: [a] 0000-0003-3278-4834; [b] 0000-0002-3478-008X; c 0000-0002-1441-3995; [d] 0000-0003-0106-5677

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Abstract: Genes, which constitute the biological basis of heredity, are DNA segments that give various structural and functional properties to the organism they are found in. Genes have become differentiated and highly conserved among microorganisms that have completed their evolutionary development and completely distanced from each other. However, horizontal gene transfers (HGT) can occur between microorganisms even though they have high structural similarities in the environment, especially in soil. An example, HGT can occur between the members of the *Bacillus cereus* group, and thus other species can develop into new life-threatening pathotypes by taking some virulence structures of the most pathogenic species-*Bacillus anthracis*. This study aimed to investigate some virulence genes (protective antigen-PA and capsule-cap) of *B. anthracis* in *B. cereus* group members isolated from soil, which is known as the reservoir of this group of bacteria and can easily trigger the genetic material exchange. The study material has consisted of 15 soil samples taken from 5 different locations in the Kars region (Turkey) contaminated with cattle carcasses that died with suspected anthrax. *B. cereus* group bacteria were obtained from the soil by *in vitro* cultural method, and the agent identification was performed by phenotypic and molecular methods (PCR). As a result, a total of 1350 *Bacillus* species was identified from the soil, including 123 *B. anthracis*, 303 *B. mycoides*, 348 B. thuringiensis and 576 *B. cereus*. DNAs obtained from all isolates other than *B. anthracis* were pooled at a final concentration of 4 ng/mL and as an average of 10 extracts and analyzed by PCR for the relevant virulence genes (PA and cap). While the PA gene was found in 6 (4.88%) of 123 DNA pools, the cap gene was not detected in any of the pooled DNA. All *B. anthracis* isolates were evaluated as fully virulent strains as they did not lose the PA and cap genes. The DNA pooling method has been an obstacle to the selection of gene positivity individually, however, it roughly offered some clues indicating the HGT. Thus, further studies with sampling and methodological diversities are needed that will enable the proof of HGT, which mediates the emergence of new pathotypes among the *B. cereus* group members.

Keywords: *Bacillus cereus group, Bacillus anthracis, Horizontal gene transfer, PCR*

Bacillus cereus Grubu Üyeleri Arasında Yatay Gen Transferi İle Ortaya Çıkan Yeni Patojenlerin Keşfine İlişkin İpuçları

Öz: Kalıtımın biyolojik temelini oluşturan genler, bulundukları organizmaya çeşitli yapısal ve işlevsel özellikler kazandıran DNA parçalarıdır. Evrimsel gelişimini tamamlamış ve birbirinden tamamen uzaklaşmış mikroorganizmalar arasında genler farklılaşmış olup yüksek oranda korunaklıdır. Ancak çevrede, özellikle de toprakta yüksek yapısal benzerliklere sahip olsalar bile mikroorganizmalar arasında horizontal gen transferleri (HGT) meydana gelebilmektedir. Bunun bir örneği, *Bacillus cereus* grubunun üyeleri arasında meydana gelebilmekte ve bu nedenle diğer türler, grubun en patojen tür olan *Bacillus anthracis*'in bazı virülans yapılarını HGT ile alarak yaşamı tehdit eden yeni patotiplere dönüşebilmektedir. Bu çalışmada, bu bakteri grubunun rezervuarı olarak bilinen ve genetik materyal değişimini kolaylıkla tetikleyebilen topraktan izole edilen *B. cereus* grubu üyeleri arasında *B. anthracis*'in bazı virülans genlerinin (koruyucu antijen-PA ve kapsül-cap) araştırılması amaçlandı. Çalışmanın materyalini, Kars yöresinde şarbon şüphesiyle ölen sığır karkaslarıyla kontamine olmuş 5 farklı lokasyondan alınan 15 toprak örneği oluşturdu. *B. cereus* grubu bakteriler topraktan eldesi *in vitro* kültür yöntemiyle gerçekleştirildi ve etken tanımlaması fenotipik ve moleküler yöntemlerle (PCR) yapıldı. Çalışma sonucunda topraktan 123 *B. anthracis*, 303 *B. mycoides*, 348 B. thuringiensis ve 576 *B. cereus* olmak üzere toplam 1350 *Bacillus* türü tanımlandı. *B. anthracis* haricindeki tüm izolatlardan elde edilen DNA'lar, son konsantrasyonda 4 ng/mL ve ortalama 10 ekstrakt olacak şekilde havuzlandı ve ilgili virülans genleri (PA ve cap) yönünden PCR ile analiz edildi. Havuzlanan 123 DNA'nın 6 (%4.88)'sında PA geni saptanırken, DNA havuzlarının tümü cap geni yönünden negatifti. PA ve cap genlerini kaybetmemiş oluşuyla *B. anthracis* izolatlarının tümü tam virülent suşlar olarak değerlendirildi. DNA havuzlama yöntemi bireysel gen pozitifliğinin seçimine engel teşkil ederken, HGT'yi kabaca gösterecek bazı ipuçları sundu. Bu nedenle, *B. cereus* grup üyeleri arasında yeni patotiplerin ortaya çıkmasına aracılık eden HGT'nin kanıtlanmasını sağlayacak örneklem ve metodolojik çeşitlilik içeren ileri çalışmalara ihtiyaç vardır.

Anahtar sözcükler: *Bacillus cereus grup, Bacillus anthracis, Horizontal gen transfer, PCR*

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(*) Corresponding Author

Tel: +90 474 242 6836 Fax: +90 474 242 6853
E-mail: fatihbyk08@hotmail.com (F. Büyük)

Introduction

Horizontal gene transfer (HGT) is known as the process of transferring part or complete DNA from a donor to a recipient bacterium or host genome [1]. Gene transfer occurs more easily between bacteria or DNA sequences that are very close to each other or between different microorganisms with high structural similarity. Genetic material transfer can occur in natural conditions, especially in environments such as soil, and very rarely (10^{-7} - 10^{-9}), as well as through artificial techniques such as transfection, microinjection and vectorial processes, and the frequency of transmission can thus be increased (10^{-3} - 10^{-4}) [2]. As a result of HGT, the recipient bacterium turns positive in terms of the features encoded by the genes transferred by the donor bacterium, and thus new phenotypes and pathotypes can emerge. HGT can occur by three different mechanisms (transformation, conjugation and transduction) under normal conditions among the bacteria. HGT is more like the transfer of circular plasmids that can replicate independently of a pathogen's main genetic material (chromosomal DNA) [1,2].

Bacillaceae is a Gram-positive, aerobic and endospore-forming bacterial family commonly found in soil with approximately 94 different species. The family divides into 6 large groups according to their physiological, biochemical and morphological characteristics, and pathogenic species are found in the *Bacillus cereus* group. In this group, apart from *Bacillus anthracis*, there are some species such as *Bacillus cereus, Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomycoides* [3]. Members of the *B. cereus* group have a significant impact on human and animal health, agriculture and the food industry [4]. In addition, various enzymes and metabolites secreted by the *B. cereus* group can exhibit probiotic properties and are used in the destruction of environmental pollutants [5,6].

Bacillus anthracis, which is the agent of anthrax, differs from other species of the *B. cereus* group with its unique phenotypic (non-motility, presence of capsule, Gamma phage and penicillin susceptibility) and genotypic (presence of pXO1 and pXO2 plasmid) features [7]. However, this difference has recently come into question with the discovery of *B. cereus* and *B. thuringiensis* isolates that have genetic structures similar to the *B. anthracis* plasmids. Many of these mutant bacteria, called "*B. anthracis*-like organisms", exhibit potential or lethal pathogenic features and may lead to clinical pictures that do not comply with the defined classical anthrax [8-13]. Another risk of these mutant strains, most of which are detected from environmental sources such as soil, is that they have the potential to be used as biological agents instead of *B. anthracis*.

Soil is the main source of exposure to anthrax spores, for which the tolerable level in humans and many susceptible hosts is not yet known [14]. Considering that a cattle dying from anthrax sheds approximately 10^{12} spores [15], soil containing spore-laden untreated animal burials represents a significant accumulation of *B. anthracis* genetic material as well as being a serious source of infection. Genetic material transfer is much higher among the members of the *B. cereus* group compared to that in natural conditions (10^{-7} - 10^{-9}). Soil is seen as a favorite environment for the transfer of *B. anthracis* specific genetic structures with HGT alternatives and thus the evolution of *B. cereus* group bacteria and the emergence of new phenotypes [16].

This study aimed to investigate *B. anthracis* specific virulence genes (protective antigen-*PA*, capsule-*cap)* by PCR in *B. cereus* group bacteria isolated from soil, which is a reservoir of these bacteria and highly promotes the horizontal gene transfer.

Material and Methods

Study Material

Soil contaminated with *B. anthracis* spores was used as study material. For this purpose, soils were taken from animal burials where cattle that died with suspected anthrax, soil ground areas contaminated with blood and body fluids, and restricted open pasture areas where their carcasses were left were sampled in Kars Province of Turkey. Totally, 500 g soil was taken from the upper 20 cm section (rhizosphere) of a 4 m^2 width area representing the spore contamination. These samples were used for the isolation of both *B. anthracis* and other members of the *B. cereus* group in order to demonstrate reciprocal gene transfer.

Bacterial Isolation

The isolation of *B. anthracis* and other *B. cereus* group bacteria from soil samples was carried out by modifying the method applied by the World Health Organization [17]. In this method, 40 g of soil sample was diluted with 200 mL of sterile distilled water in plastic cups, and left for 30 min at room temperature to obtain the supernatant by shaking gently for about 30 s. Then, 1 mL of supernatant was taken and heated in a water bath at 62.5-63°C for 15-20 min. Ten-fold serial dilutions of the heat-treated samples were prepared with distilled water, and 150 µL of the dilutions were plated onto 7% sheep blood agar (SBA) plates. Bacterial colonies formed after 24-48 h of incubation at 37°C in an aerobic environment were evaluated for specific agents, and the number of uniform colonies specific for each bacterium was noted for use in future spore and frequency calculations.

Phenotypic Identification

Non-hemolytic, 2-4 mm in diameter, white or grayish-white, medusa-head rough colonies on the SBA plates

were evaluated for *B. anthracis*. Techniques such as penicillin and Gamma phage sensitivity (sensitive to both), capsule presence/synthesis ability (azure-eosin staining and mucoid growth in bicarbonate agar medium), motility examination (nonmotile in semi-solid medium) were applied for identification of the suspected colonies [18,19]. In the identification of *B. cereus* and *B. thuringiensis*, phenotypic test features such as hemolysis on the SBA plates, resistance to penicillin and Gamma phage, lack of capsule and presence of motility were taken into account. The presence of insecticidal crystals formed during sporulation of *B. thuringiensis* was also investigated [20]. For this purpose, smears were prepared after incubation of *B. thuringiensis* suspected isolates in spore media at 37°C for 48 h. Smears stained with malachite green were analyzed by phase-contrast microscopy for the presence of parasporal crystal structure next to the bacterial spores. The identification of *B. mycoides* included typical rhizoid or hair-like colony production, as well as being immobile and encapsulated, and penicillin and Gamma phage resistance [21].

Molecular Identification

DNA extraction from *Bacillus* isolates was performed by boiling method. For this purpose, 1-2 copy of freshly cultured bacterial colonies were taken and diluted in 100 µL nuclease free water. The colonies were kept in the block heater at 95°C for 10 min, and then heat shock was applied by keeping them on ice blocks for 10 min. Supernatant, obtained by centrifugation at 7500 rpm for 10 min, was checked for sterility (no growth was observed after 48 h of incubation of 5 µL DNA plated on the SBA plates). For the confirmation of phenotypically identified *B. anthracis* isolates, PCR techniques with a couple of primer pairs, PA5/8 and CAP6/103, were used [18,19]. The PA5/8 primer pair targets the amplification of the *pag* gene which is originated from the pXO1 plasmid and encodes the protective antigen (PA). The CAP6/103 primer pair targets the amplification of the *capB* gene, which is one of the genes that originated from the pXO2 plasmid and encodes the capsule. *B. anthracis* vaccine strain (Sterne) containing *pag* gene and not *capB* gene was used as a control in both PCR. PCRs targeting the amplification of *gyr* gene were applied to identify *B. cereus*, *B. thuringiensis* and *B. mycoides* isolates [22]. All PCRs were performed with the components and thermal cycles stated in *Table 1*.

PCR Analysis of the Obtained Isolates in Terms of the *Bacillus anthracis* Virulence Genes

Bacillus anthracis specific plasmid and virulence genes were investigated with the primer PA5/8 and Cap6/103 in *B. cereus*, *B. thuringiensis* and *B. mycoides* isolates that were identified by phenotypic and molecular methods [18,19]. DNA extraction from each isolate was carried out separately by boiling method. After the DNA extraction, in order to reduce the heavy workload that will be encountered during the PCR processes, the "pooling method" was applied to the DNA extracts [23]. In this context, the concentrations of individual DNA extracts were measured with Microplate Spectrophotometer (BioTek, Take3 Mikro-Volume-Plate, SN243903). The concentration of the extracts was adjusted to 40-80 ng/µL with nuclease-free water. Then, 2 µL of each DNA extract was taken, with a final concentration of 4 ng/µL, and 123 DNA mixes were created in a total volume of 20 µL consisting of an average of 10 different DNA samples. While creating the DNA pools, care was taken to bring together the isolates in the same SBA plates. The individual DNAs of the isolates were stored in separate tubes at -20°C to be used in further extractions. PCR components and thermal cycle were created as in *Table 1*. Analysis of the PCR products was performed in horizontal gel electrophoresis containing 1.5% agarose.

Table 1. *Primers and PCR characteristics used for identification of the Bacillus cereus group members and virulence genes*

Species	Primer	Sequence (5'—3')	Target	PCR Component	Thermal Cycle	Amplicon (bp)	Reference
B. anthracis	PA8 PA5	GAGGTAGAAGGATATACGGT TCCTAACACTAACGAAGTCG	pXO1	• 2.5 µL PCR buffer (x10, with $MgCl_2$) • 0.5 µL dNTP (10 mM) • 1 µL primer F (10 pmol) • 1 µL primer R (10 pmol) • 0.5 µL Taq polymerase (5 U) • 2.5 µL template DNA (40-80 ng/µL) • Nuclease free water	**One cycle** • Denaturation at 94°C for 2 min **Thirty cycles** • Denaturation at 94°C for 1 min • Annealing at 55°C for 1 min • Elongation at 72°C for 1 min **One cycle** • Final elongation at 72°C for 5 min	596	[18,19]
	CAP6 CAP103	TACTGACGAGGAGCAACCGA GGCTCAGTGTAACTCCTAAT	pXO2			1035	
B. thuringiensis	BTJH-IF BTJH-R	GCTTACCAGGGAAATTGGCAG ATCAACGTCGGCGTCGG	*gyrB*		**One cycle** • Denaturation at 94°C for 5 min **Thirty cycles** • Denaturation at 94°C for 30 sec • Annealing at 63°C for 30 sec • Elongation at 72°C for 30 sec **One cycle** • Final elongation at 72°C for 5 min	299	[22]
B. cereus	BCJH-F BCJH-IR	TCATGAAGAGCCTGTGTACG CGACGTGTCAATTCACGCGC	gyrB			475	
B. mycoides	BMSH-F BMSH-R	TTTTAAGACTGCTCTAACACGTGTAAT TTCAATAGCAAAATCCCCACCAAT	gyrB			604	

Results

Isolation and Identification Findings of the *Bacillus cereus* Group Bacteria

In the study, a total of 15 soil samples were taken from 5 different locations in the Kars region contaminated with dead cattle carcasses with suspected anthrax. A total of 123 *B. anthracis* isolates were obtained from the soil samples, one representative of each SBA plate, after cultural examination. *B. anthracis* load in the soil samples ranged from $1.01x10^2$ to $1.24x10^8$ spores/gram *(Table 2)*. The organic matter contents of the soil ranged from 1.5% to 5.7%, and thus they were ranked as loamy sandy soil and clayey soil.

In order to detect possible horizontal gene transfer, all SBA plates showing *B. anthracis* colony morphology following the cultural analysis were evaluated. Isolate collections were created by collecting all of the colonies of individual *Bacillus* species on the SBA plates. The distribution of the collected isolates was as follows; 924 *B. cereus* and/or *B. thuringiensis* and 303 *B. mycoides* *(Table 2)*.

Molecular Identification Findings

All 123 *B. anthracis* isolates phenotypically identified were confirmed by PCR with the presence of the *PA* and *cap* genes *(Fig. 1-B)*. Therefore, with the presence of these genes, which are critical for pathogenicity, all isolates were found to be fully virulent *B. anthracis* (without gene loss). As a result of PCR analysis of 924 isolates characterized phenotypically as *B. cereus* and/or *B. thuringiensis*, 567 were identified as *B. cereus* and 348 as *B. thuringiensis*. All 303 *B. mycoides* isolates phenotypically characterized were confirmed as *B. mycoides* by PCR *(Table 3)*.

PCR Findings of the *Bacillus* Isolates in Terms of the *Bacillus anthracis* Virulence Genes

As a result of PCR analysis with the PA5/8 primer of 123 DNA pools created from different *Bacillus* isolates, *B. anthracis* specific *PA* gene was found in 6 (4.88%) of the pools *(Fig. 1-A)*. The *PA* gene positive isolate combinations were as follows: *B. cereus* alone in 21 samples, *B. cereus* and *B. thuringiensis* in 28 samples, *B. cereus*, *B. mycoides* and *B. thuringiensis* in 10 samples. In the present study, in which *B. cereus* was detected to be significantly higher ($P<0.05$), the total distribution of these *PA* gene positive isolates was as follows; 39 *B. cereus*, 19 *B. thuringiensis* and 1 *B. mycoides*. As a result of PCR analysis with the Cap6/103, no *cap* gene was found in any of the DNA pools *(Table 4)*. Therefore, no *B. anthracis* specific *cap* gene transfer was observed among the *B. cereus*, *B. thuringiensis* and *B. mycoides* isolates. On the other hand, due to the presence of the *PA* and *cap* gene regions in all isolates, no simultaneous loss of related genes was observed in the *B. anthracis* isolates.

PCR was performed separately with the PA5/8 primers on all individual bacterial DNAs constituting each of

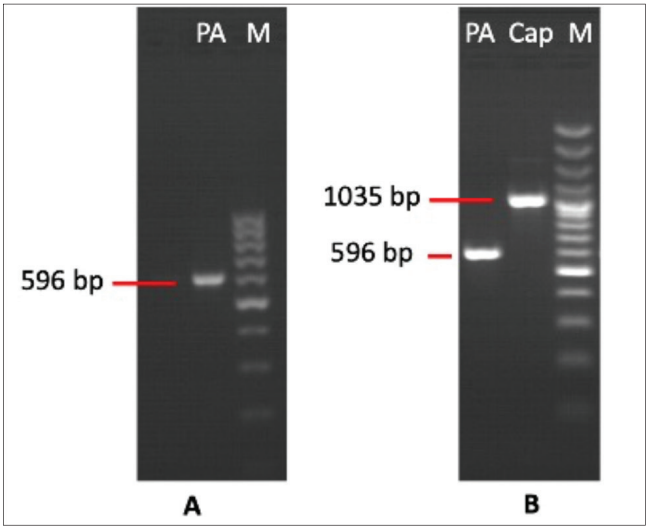

Fig 1. Gel electrophoresis image of amplified products of the PCRs. **A:** 596 bp size amplified product of the *PA* gene region detected in DNA pools from *Bacillus* isolates *(B. cereus*, *B. thuringiensis* and *B. mycoides)*, **B:** Amplified products of the *PA* (596 bp) and *cap* (1035 bp) genes of fully virulent *B. anthracis* isolates. PA: Protective antigen specific PCR, Cap: Capsule specific PCR, M: Marker: (DNA Marker 100bp Ladder, MBS657623, MyBioSource for *Fig. 1-A* and GeneRuler 100 bp Plus DNA ladder, SM0321, Thermo Fisher Sci for *Fig. 1-B*)

Table 2. *Sample locations and culture results of the soil in terms of the Bacillus cereus group bacteria*

Location	Contamination Date	Contaminant Type	*B. anthracis* Spore Load (spore/soil per gram)	Number of Soil Samples	Culture Results				
					Number of Isolates	Distribution of Isolates*			
						B. anthracis	*B. cereus*	*B. mycoides*	*B. thuringiensis*
Digor-Hanevler District	2009	Cattle	$1.01x10^2$	2	180	15	75	45	45
Selim-Center	2002	Cattle	$1.32x10^3$	4	360	30	155	80	95
Dikme Village	2010	Cattle	$1.24x10^8$	4	360	32	150	81	90
Çamurlu Village	2011	Cattle	$3.03x10^3$	2	180	20	76	39	46
Külveren Village	2011	Cattle	$3.46x10^4$	3	270	26	120	58	72
Total				15	1350	123	576	303	348

** This distribution was formed after the phenotypic analysis findings and the identification of the bacterial species was confirmed by PCR*

Table 3. *Phenotypic and molecular identification test findings of the Bacillus isolates*

Species	Phenotypic Identification Tests						PCR
	Colony Type on SBA	Hemolysis on SBA	Motility	Capsule	P	γ	
B. anthracis (n: 123)	Rough	Non-hemolytic	Nonmotile	+	+	+	*B. anthracis* (n: 123)
B. cereus and *B. thuringiensis* (n: 924)	Rough	*β- hemolytic*	Nonmotile	-	-	-	*B. cereus* (n: 576) *B. thuringiensis* (n: 348)
B. mycoides (n: 303)	Rhizoid	*β*- hemolytic	Nonmotile	-	-	-	*B. mycoides* (n: 303)

SBA: *%7 sheep blood agar,* ***P:*** *Penicillin sensitivity,* ***γ:*** *Gamma phage sensitivity*

Table 4. *DNA samples that were detected positive in terms of the presence of the Bacillus anthracis plasmid and virulence genes*

Number of DNA Pool	Number of Isolates			PCR Findings		P-value
	B. cereus	*B. mycoides*	*B. thuringiensis*	PA5/8	Cap6/103	
Pool 4	3	-	5	Positive	Negative	P<0.05
Pool 9	11	-	-	Positive	Negative	
Pool 11	3	-	7	Positive	Negative	
Pool 12	10	-	-	Positive	Negative	
Pool 26	3	1	6	Positive	Negative	
Pool 28	9	-	1	Positive	Negative	
Total	39	1	19			

the 6 DNA pools in which the *PA* gene was detected. However, all individual DNAs were found to be negative for the presence of the *PA* gene.

Discussion

Toxins [a cell-binding protein known as protective antigen (PA), enzymes known as edema factor (EF) and lethal factor (LF)] and capsule are the main virulence factors in the pathogenesis of *B. anthracis* [24]. The genetic materials originating from these virulence factors are plasmids pXO1 and pXO2, respectively. Although the horizontal transfer of these plasmids is greater in clinical specimens [9,12,25], few have been reported in environmental isolates [26,27]. Hu et al.[26] found the horizontal transfer rate of the pXO1 and pXO2 plasmids to be 6.6% and 7.7%, respectively. Simultaneous transfer of both plasmids among the environmental isolates was determined at a rate of 0.46% [26]. Compared to this [26], Cooper et al.[27] reported a lower rate (1.75%) of pXO2 loss among the *B. anthracis* isolates obtained from soil, but on the other hand, no gene gain was reported among the *B. cereus* group members. In many studies, compared to the pXO1 plasmid, the loss of the pXO2 plasmid was higher due to its sensitivity to the environment or fragility to the laboratory storage [18,26-31].

In the trace of *B. anthracis* plasmids, the PA gene, which provides the integration of the pXO1 plasmid originated toxins (EF and LF) into the host cell, and the pXO2 originated *cap* genes confer the antiphagocytic ability to the bacteria, are frequently investigated. For this purpose, primer pair PA5/8 for the amplification of *PA* and primer pair Cap6/103 for the amplification of the region between *cap B* and *cap C* gene, are widely used [18,27,32]. In this study, *PA* gene positivity was obtained in 6 (4.88%) of a total of 123 DNA pools, and these findings are quite similar to the rates found in *B. cereus* group isolates obtained from the environmental sources by Hu et al.[26]. However, since the *PA* gene positivity was detected only in DNA pools and not in the individual DNAs in the present study, the bacterial origin of the HGT and the *PA* gene prevalence specific to bacterial species could not be determined. Nevertheless, *B. cereus* and *B. thuringiensis* appear to be *Bacillus* species with the easier horizontal gene transfer capability (P<0.05). On the other hand, the absence of loss of these genes in *B. anthracis* isolates obtained from the same SBA plates containing *PA* gene positive isolates was interpreted as the presence of more than one copy of the relevant genes in donor bacteria *(B. anthracis)* [33] and as the absence of simultaneous gene exchange among the *B. cereus* group members.

The plasmid pXO2, which is smaller (96.2 kb) in size than pXO1 [34], is more common in transferability especially among the environmental isolates obtained from the soil [18,26,27]. High organic matter and exudate requirement for the conjugation, rhizosphere repellency for the transformation, and the presence of the specific phages for the transduction are the main factors affecting the gene acquisition pathways in the soil [35,36]. On the other hand, plasmid losses mostly depend on the soil type which differs according to matter, sand, silt and clay concentration [35-37].

As a matter of fact, Salgado et al.[37] obtained a *B. anthracis* strain with the pXO2 plasmid loss from a clay loam soil, while a *B. anthracis*-like organism with the pXO1 plasmid gain was obtained from a loamy sand soil. Contrary to the reports, none of the isolates were found positive for this plasmid in this study, even though the same gene losses have been found previously among the *B. anthracis* isolates obtained from the soil with divergent (1.5-5.7%) organic matter contents in the same study area [18,27]. It is still more likely that the pXO2 plasmid transfer may have been adversely affected this time by the factors such as the nutritional, biotic and abiotic structures of the soil which are assumed favorable for the HGT due to the substantial biosynthetic and energetic requirements of the conjugation, DNA uptake and lytic cycle. Furthermore, there is a biosynthetic gene operon *(capBCAD)* on the related plasmid that contains many genes encoding the capsule [34,38], and screening of only the *capB* and *capC* genes in this study may have been insufficient as an indicator of the HGT.

In large-scale studies, the "DNA pooling method" gives promising results in terms of reducing the workload and cost and adding practicality to the study [23]. It has even been reported that the "pooling method" is more useful in detecting gene alleles with low frequency [39,40]. In the "DNA pooling" process, the DNA concentration used, the methodology (double pooling, multiple pooling, etc.) and the order of the application (pooling before DNA extraction, pooling before PCR amplification, etc.) change the effectiveness of the method [23]. In addition to these advantages of the "DNA pooling method", there are also some disadvantages that reduce sensitivity and minimum detection limit of the method [41,42]. In this study, individual DNA extracts of average 10 isolates *(B. cereus, B. thuringiensis* and *B. mycoides)* were combined with "multiple-pooling" with a final concentration of 4 ng/µL and this process was done just before the PCR amplification [23]. One of the reasons for the PA gene positivity detected in pooled DNAs could not be detected in individual DNAs is thought to be the variational conditions listed above for the "pooling method". In addition, the use of DNA mixes of different isolates *(B. cereus*, *B. thuringiensis* and *B. mycoides)* taken simultaneously and randomly representing the same SBA plate may have caused the DNAs to act as complements to each other during the PCR amplification among the bacteria of the *B. cereus* group with very high DNA homology (>99%), and may have ultimately led to mismatch amplifications [43].

In conclusion, spore-contaminated soil representing the significant accumulation of the genetic material of *B. anthracis* appear to be favorite environments for transfer of plasmid or various virulence genes via HGT. As a matter of fact, in this study, which included PCR-based analytic methods, *B. anthracis* specific *PA* gene positivity was found to be 4.88% among the soil-borne *B. cereus* group bacteria *(B. cereus, B. thuringiensis* and *B. mycoides),* but this positivity did not be differentiated on an individual basis. Except for the *B. anthracis*, no horizontally transferred *cap* gene was found among the other *B. cereus* group members. By increasing the diversity of samples and methods that will enable the HGT evidence, such transfers of genetic material, which are seen as possible among the *B. cereus* group members and may mediate the emergence of new pathogens, will be able to detect more comprehensively.

Availability of Data and Materials

The datasets and analyses done during the current study are available from the corresponding author (F. Büyük) on reasonable request.

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Conflict of Interest

The authors did not report any conflict of interest related to this article.

Author Contributions

FB, ÖÇ, MRC and MŞ planned, designed, and supervised the research procedure, FB and MRC carried out the experiments and the analytic tests and all authors wrote the article.

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Research Article

Comprehensive Effects of Fetal Calf Serum in Soybean-Lecithin Based Goat Semen Cryopreservation Extenders and Impacts on Incubation Resilience

Mehmed Berk TOKER [1,a (*)] Selim ALCAY [1,b]

[1] Bursa Uludag University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, TR-16059 Bursa - TÜRKİYE
ORCIDs: [a] 0000-0003-4033-9749; [b] 0000-0002-2472-8157

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Abstract: The aim of this study was to investigate the effects of various fetal calf serum (FCS) doses on %1 soybean lecithin-based semen extenders for goat semen cryopreservation and the impacts on spermatological parameters over post-thaw and post-incubation (6 h) time periods. Sperm samples collected via electro-ejaculation method were pooled to overcome the individual differences and were used in the study. The samples then were split into four equal aliquots to create study groups as; 0.25%, 0.50%, 0.75% FCS supplemented and a sample of FCS-free control group. Each sample group was diluted to approximately 150 x 10^6 spermatozoon/mL final concentration and two step dilution method was used for cryopreservation. Study groups were examined for sperm motility, plasma membrane functional integrity with hypoosmotic swelling test (HOST), acrosome integrity by FITC-*Pisum sativum* agglutinin (PSA-FITC) and DNA damage by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL). All samples were incubated for further 6 h in a humidified air chamber with 5% CO_2 at 39°C. The results indicated that FCS supplementation in soybean lecithin-based extenders for goat sperm cryopreservation had significant effects on post-thawing time point motility ($P<0.05$), plasma membrane integrity ($P<0.05$) and acrosomal integrity ($P<0.05$) parameters. Subsequently to 6 h of incubation period, DNA integrity results yielded better scores comparing to control group in addition with other spermatological parameters ($P<0.05$).

Keywords: *Cryopreservation, Fetal calf serum, Goat semen, Incubation resilience, Soybean lecithin*

Lesitin Bazlı Teke Sperma Sulandırıcılarına Eklenen Fötal Buzağı Serumunun Spermanın Dondurulma ve İnkübasyon Direnci Üzerine Etkileri

Öz: Bu çalışmanın amacı teke spermasının dondurulmasında kullanılacak %1 soya lesitini bazlı sulandırıcıya eklenen çeşitli dozlarda fötal buzağı serumunun (FBS) dondurma-çözdürme ve 6 saatlik inkübasyon süreci sonrası dönemde spermatolojik parametrelere etkilerini araştırmaktır. Sperma örnekleri elektro-ejakülatör ile alınmış ve bireysel farklılıkların üstesinden gelinmesi amacıyla birleştirilmiştir (pooling). Birleştirilen örnekler dört eşit gruba bölünmüş ve %0.25, %0.50, %0.75 FBS eklenmiş ve FBS eklenmemiş kontrol olacak şekilde çalışma grupları oluşturulmuştur. Her çalışma grubu dondurulma amacıyla 150 x 10^6 spermatozoon/mL final konsantrasyon olacak şekilde iki aşamalı olarak sulandırılmıştır. Çalışma grupları sperma motilitesi, hipo-ozmotik şişme testi ile ölçülen plazma membran fonksiyonel bütünlüğü, FITC-*Pisum sativum* agglutinin (FITC-PSA) ile ölçülen akrozomal bütünlük ve terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL) testi ile ölçülen DNA bütünlüğü açısından incelenmiştir. Tüm gruplar çözdürme sonrası dönemde inkübasyon direncinin ölçülmesi amacıyla 6 saat boyunca %5 CO_2 ve 39°C sıcaklıklığa ayarlanmış bir ortamda inkübe edilmiştir. Çalışmanın sonuçları soya lesitini bazlı sperma dondurma sulandırıcılarına FBS eklenmesinin çözdürme sonrası motilite ($P<0.05$), plazma membran bütünlüğü ($P<0.05$) ve akrozomal bütünlük üzerine istatistiki anlam düzeyinde fark yarattığını göstermiştir. Buna ek olarak, gerçekleştirilen 6 saatlik inkübasyon sürecinin sonucunda FBS'nin adı geçen spermatolojik parametrelere ek olarak DNA bütünlüğü sonuçlarında da pozitif etki yarattığı tespit edilmiştir ($P<0.05$).

Anahtar sözcükler: *Sperma dondurma, Fötal buzağı serumu, Teke sperması, İnkübasyon direnci, Soya lesitini*

Introduction

Cryopreservation of domestic ruminants' semen is an excellent method to procure better and desired gene merits to obtain greater yield numbers within next generations [1]. To achieve the aim of enhanced success rates in terms of

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(*) Corresponding Author

Tel: +90 224 2941350 Cellular phone: +90 530 038 8394
E-mail: berktoker@uludag.edu.tr (M.B. Toker)

cryopreservation, various ways and methods have been investigated in different species throughout years [2-4]. Despite goat semen can be freezable like other ruminant semen, some of its' physiologic specialities need to be taken into consideration to reach the best out of it [5]. The interaction between egg-yolk in freezing media and egg-yolk coagulating enzyme (EYCE) produced in bulbo-urethral gland of bucks' as an enzyme is one of the biggest challenges for cryopreservation of semen [6]. Sperm cells are exposed to various effects within cryopreservation process that hampers the well-being of the cells [7,8]. Soybean lecithin contains low-density lipoprotein fractions as it is in the composition of egg-yolk [9]. Phosohatidylcholines have a great role in the defense systems of sperm membrane throughout the freeze-thawing process and soybean lecithin can be used as an extender component in regards of this matter [4]. Since there have been researches done and published about using soybean lecithin, it can be a better choice instead of egg-yolk in goat semen cryopreservation extenders [10-12].

Cold shock and ice crystal formations that happen in the process of cryopreservation of semen affect spermatological parameters and functions, and hampers the success of fertility [13]. Various substances have been used in freeze-thawing processes to protect sperm cells and prevent the effects of physical and chemical stress sources to increase the achievement of the method [14]. Through freezing and thawing process, fetal calf serum (FCS) acts as a buffer to osmotic shock, protects membranes and reduces the impacts of ice formation [15]. FCS contains many elements that cells need such as hormones, growth factors, sugars, amino acids, lipids and trace elements to contribute continuation of a tissue [16]. FCS has also been used widely as a protein to stabilize the cell culture mediums for a while [17,18].

This study aimed to supplement vital low-density lipoprotein (LDL) resources by adding soybean lecithin as the main component of the sperm cryopreservation extender. In addition to that, supplementing the extender with 0.25%, 0.50% and 0.75% FCS to overcome the negative effects of cryopreservation process and to find out the effects over spermatological parameters on post-thaw and 6 h of incubation time periods is one of the main aims of the research. To carry out the study, Saanen bucks were chosen as the sperm donors and the incubation resillience of the sperm samples assessed for 6 h. To evaluate the sperm samples; percentage of motility, sperm membrane funcitonal integrity with hypo-osmotic swelling test (HOST), FITC conjugated *Pisum sativum* agglutinin (PSA FITC) for acrosomal integrity and Terminal Deoxynucleotidyl Transferase-Mediated Fluorescein (TUNEL assay for DNA damage were investigated in the study at two time points (post-thaw 0 h and 6 h).

Material and Methods

Ethical Statement

Experimental designs and studies have been approved by Bursa Uludağ University Scientific Ethical Committee (Approval no: 2016-13/03).

Chemicals

The chemicals that have been used throughout the study were purchased from Merck KGaA (Darmstadt, Germany) unless otherwise indicated.

Animals, Collection and Processing of Semen

Study was conducted with five Saanen bucks (3 to 5 years old) during non-breeding season that were housed at a farm in Bursa (latitude 40°11'N, longitude 29°04'E, altitude 155 m above sea level), Türkiye. Animals were maintained with similar feeding and housing conditions and water consumption was up to will. Semen samples were gathered from animals by electro-ejaculation method (Minitüb GmbH, Germany) for five times with at least three-day intervals [15]. After collection, gathered samples were immersed to a 32-34°C water bath in sterile glass tubes for evaluation of spermatological parameters. Collected semen samples were evaluated for volume (measured with conical tubes that graduated at 0.1 mL intervals), consistency, wave motion (3-5 on a 0-5 scale), concentration (at least 1 x 10^9 spermatozoa/mL, measured with haemocytometer) and motility (at least 75%) and pooled afterwards [10].

Extenders and Dilution of Semen

Extenders were freshly prepared prior to the study day. Extenders consisted of 223.7 mmol/L Tris, 55.5 mmol/L fructose, 66.6 mmol/L citric acid, 100.4 mmol/L Trehalose, 4.03 mmol/L EDTA, 4 g/L penicillin G, 3 g/L dihydro-streptomycin and 1% lecithin in distilled water and relevent concentrations of FCS were supplemented to the groups. Lecithin was dissolved by a magnetic stirrer in room temperature to create a homogeneous extender solution beforehand adding FCS to the relevant groups. Experiment groups were arranged as; control (without FCS supplementation), FCS0.25 (with 0.25% FCS added), FCS0.50 (with 0.50% FCS added) and FCS0.75 (with 0.75% FCS added) [10]. Pooled samples were divided into four equal aliquots in every repetition of the study and diluted with the relevant extender to approximately 150 x 10^6 spermatozoon/mL final concentration.

Semen Cooling, Freezing, Thawing and Incubation

Diluted semen samples were gradually cooled in a water bath by the aid of ice cubes within an h to 4°C and equilibrated for another two h at the same temperature [19]. Upon equilibration, diluted samples were loaded into 0.25 mL French straws. Freezing procedure was conducted

as; 3°C/min from +4°C to -8°C and 15°C/min from -8°C to -120°C in liquid nitrogen vapor, by Nicool Plus PC gamete freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France). After -120°C point, straws plunged into -196°C liquid nitrogen for storage [10]. Three sperm straws from each investigation group were thawed at 37°C for 30 sec in a water bath for spermatological parameter evaluations. Thawed samples were then incubated for 6 h in a humidified air chamber with 5% CO_2 at 39°C to examine the impacts of incubation effects over semen characteristics [20].

Evaluation of Semen

Spermatological parameters were analysed by a person throughout the study at two different time points; post thawing (0 h) and post-incubation (6 h). Motility and HOST assessments were conducted with a phase-contrast microscope (Olympus BX51-TF - Olympus Optical Co., Ltd., Japan) over a warm slide of 37°C. Fluorescent spermatological parameters (PSA-FITC and TUNEL) were evaluated by a fluorescent attachment on the same microscope. Spermatological motility parameter was subjectively assessed at 400x magnification with a warm slide (37°C).

Hypo-osmotic swelling test (HOST) was conducted to analyse the functional integrity of sperm membrane. The method based on curled and swollen tails of sperm cells. 10 µL of semen sample were incubated for 60 min at 37°C with 100 µL HOST solution (9 g fructose and 4.9 g sodium citrate per liter of distilled water - 100 mOsm). After 60 min of incubation, 20 µL of mixture was examined over a warm slide that was covered with a slip. At least 200 cells were evaluated under 1000x magnification and the recorded results were presented as percentiles [21].

Acrosomal integrity of sperm cells were evaluated by FITC conjugated *Pisum sativum* agglutinin (PSA-FITC). The method was carried out with slight modifications of Toker et al.[20]. Basic changes of the method from the indicated citation are; 20 µL of semen sample was resuspended in 500 µL of PBS and centrifuged at 100 g for 10 min. After discarding the supernatant, sperm pellet was suspended in 250 µL PBS for the second time and the method was applied as the same herehence.

DNA fragmentation rates were obtained by using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Germany) was used as Ustuner et al.[19] indicated.

Data of the examinations were analyzed using SPSS (SPSS 20.0 for Windows; SPSS, Chicago, IL, USA). Normality of the distribution was tested by using Shapiro Wilk. Semen parameters were analyzed according to distribution and Kruskal Wallis test then Mann Whitney U test used to determine the significances between study groups.

Results

The impacts of FCS on post-thaw and post-incubation semen parameters throughout the study were analysed by 4 spermatological evaluation methods. The results of sperm motility percentages, status of plasma membrane functional integrity, acrosomal integrity and sperm DNA damage were presented in *Table 1* for both time periods (post-thaw and 6 h of incubation).

Motility

Sperm motility was affected and decreased as expected throughout the thawing and incubation procedures. Despite presenting a higher value than the control, 0.25% group didn't differentiate significantly on post-thaw. But, 0.50% and 0.75% FCS groups were better with a statistically significant value on post-thawing time point ($P<0.05$). After incubation period, status did not change in terms of motility parameter. 0.50% and 0.75% FCS groups were presented significantly better results for motility ($P<0.05$).

Plasma Membrane Functional Integrity (HOST)

Hypo-osmotic swelling test results presented similar statistical effects with motility throughout the study. At

Table 1. *The mean (X±Sx) of evaluated sperm resilience parameters on different extender groups*

Incubation Period (h)	Group	Motility (%)	Plasma Membrane Integrity (%)	Acrosome Integrity (%)	DNA Damage (%)
0 h	Control	48.67±2.97^{a}	59.00±2.55^{a}	69.20±3.11^{a}	4.60±0.40
	0.25	50.33±3.52^{a}	59.80±2.68^{a}	73.40±2.30^{a}	4.00±0.55
	0.50	53.67±2.97^{b}	65.00±1.22^{b}	74.40±3.21^{a}	3.80±0.37
	0.75	58.00±2.54^{c}	71.60±2.51^{c}	78.40±2.19^{b}	3.20±0.37
6 h	Control	4.00±2.07^{x}	14.00±1.58^{x}	56.60±5.50^{x}	7.80±0.37^{x}
	0.25	4.00±2.07^{x}	14.40±1.95^{x}	60.40±4.67^{xz}	7.40±0.25^{x}
	0.50	9.00±3.87^{y}	22.40±4.72^{y}	63.60±5.13^{xy}	5.60±0.40^{yz}
	0.75	17.00±2.54^{z}	29.80±2.39^{z}	67.20±4.44^{yz}	5.20±0.37^{yz}

^{a,b,c} and ^{x,y,z}: Values with different superscripts in the same column for each of incubation time are significantly different ($P<0.05$)

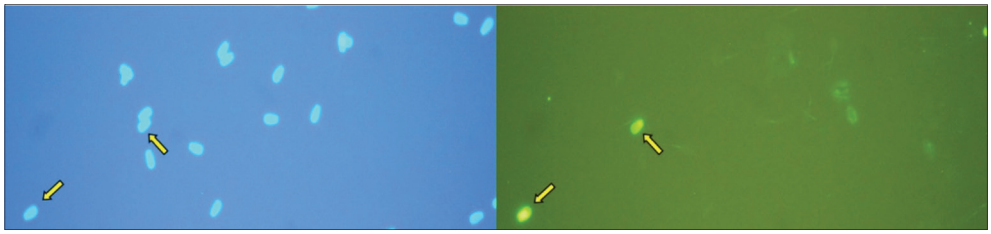

Fig 1. DNA fragmentation by TUNEL indicated with arrows

post-thaw and post-incubation time point, there was no statistically significant difference between the control and 0.25% FCS groups. Yet, the groups containing 0.50% and 0.75% FCS were yielded better and higher integrity scores than the control (P<0.05).

Acrosomal Integrity (PSA-FITC)

The effects of freezing on sperm acrosomal integrity presented itself by post-thaw and incubation time points inevitably. The group containing 0.75% FCS yielded the best and statistically significant result comparing to the control and the other two study groups on post-thaw (P<0.05). Though 0.75% FCS group kept presenting better results comparing to the control group after incubation (P<0.05), no significance amongst FCS groups were seen.

DNA Damage (TUNEL)

The results of TUNEL assay presented no significant difference amongst the study groups in post-thaw time point (P<0.05). After incubation period, 0.50% and 0.75% FCS containing groups yielded significantly better results (P<0.05) and showed the effects of FCS on DNA damage by the time *(Fig. 1)*.

Discussion

The hampering effect of cryopreservation over spermatological parameters and the preventive scientific experiments have been shown by many studies throughout the years [20,22]. Sperm motility, acrosome and plasma membrane integrities, as well as DNA integrity values are some of the values shown to be affected in previous studies [23-25]. The aim of this study was to inhibit these negative effects of cryopreservation process. For this purpose, soybean lecithin (1%, v/v) was added to the semen freezing extenders as the main component. Control (without FCS), FCS0.25 (with 0.25% FCS added), FCS0.50 (with 0.50% FCS added) and FCS0.75 (with 0.75% FCS added) groups were designed for investigating the effects of FCS throughout the cryopreservation process. These effects were analysed at two different time points; at post-thaw (0 h) and after 6 h of incubation to have a better understanding for the survivability of the cells.

Soybean lecithin is a great source for lipoproteins that contains phosphatidylcholine and many fatty acids. These fatty acids offer a great amount of stabilty for mammalian cells [26,27]. Soybean lecithin is shown to be an alternative to egg-yolk for goat semen cryopreservation in previous studies [4,12,28]. Fetal calf serum (FCS) is a great source of mixed elements and nutrients, and has been used in cell cultures [29]. FCS is a mixture that consists of low and high molecular weight biomolecules, many factors regarding the subject of growth, hormones, amino acids, trace elements, vitamins and matters for antioxidant properties that are vital for cellular growth and survivability [30,31]. To our understanding, there is no report about cryopreservation goat semen with FCS in a soybean lecithin-based extender yet. Sandal et al.[32] reported a study about cryopreservation of goat semen with various methods containing FCS but used egg-yolk in semen cryopreservation extenders. Sariözkan et al.[17] investigated the effects of 10% FCS in long term (72 h) liquid storage (5°C) of rabbit semen and did not report positive effects in terms of motility and acrosomal integrity. Blank et al.[16] studied the influence of different concentrations of FCS in cryosurvival of chicken spermatozoa and found out that FCS may be a viable supplement for chicken semen during freezing and thawing process.

Motility values decreased as expected on post-thaw and post incubation time points, respectively. At post-thaw, 0.50% and 0.75% FCS added groups yielded better results comparing to the control (P<0.05). After incubation, the effects of time over motility were observed but the status of the results did not change and presented the same statistical difference as it is on post-thaw time point (P<0.05). Mata-Campuzano et al.[7] indicated the similar effects of incubation and the results on a study that was conducted on rams. Sandal et al.[32] reported less effective results in terms of motility by adding FCS to extenders comparing to the non-added groups. This result may be originating from adding a high dose of FCS (10%) comparing to our study.

Plasma membrane integrity and the functionality has vital impacts on maturation of cell and ability of penetration to ovum for semen [9]. It was naturally affected by cryopreservation process [10]. Our study showed that, as the amount of FCS increased more the integrity and the functionality of plasma membrane was protected. At post-thaw and post incubation time points 0.50% and 0.75%

FCS groups presented statistically significant results comparing to the control (P<0.05). Although it has been done in different topics, our findings were consistent in terms of post-incubation membrane integrity concept with Alcay et al.[9] and presented higher post-thaw values than the study Salmani et al.[1].

Acrosomal integrity of goat sperm cells carry of great importance in terms of penetration ability thereby the potential of fertility [33]. The protective effect of 0.75% FCS study group on both time points of the study indicated the success of the component with regards to acrosomal integrity (P<0.05). Despite, both 0.25% and 0.50% FCS groups yielded better scores comparing to the control, neither of it was statistically significant in two time points (P>0.05). Memon et al.[34] and Sun et al.[35] indicated the decreasing effect of cryopreservation process in terms of acrosomal integrity in their studies. Anakkul et al.[36] had investigated the effects of incubation on acrosomal integrity and found the same reducing effect of the process on goat semen.

Effects of FCS on DNA integrity presented the importance by incubation period. Although there is not a significant difference observed on post-thaw time point amongst groups (P>0.05), by the end of the 6 h incubation time, 0.50% and 0.75% FCS yielded statistically significant results comparing to the control (P<0.05). As DNA integrity is a great indicator of early embryo development maintenance for semen [10], presented results refer the importance of adding FCS to the goat semen cryopreservation extenders especially over time. Regarding the topic of DNA damage, Sandal et al.[32] published similar results with current study by adding FCS to the sperm extenders.

As a conclusion, supplementing various doses of FCS to soybean lecithin-based goat semen cryopreservation extenders indicated its importance within post-thaw and 6 h of incubation periods. FCS has long been a well-known additive for embryonic development, but the effects on sperm cryopreservation of it requires more attention with further evaluations.

Availability of Data and Materials

The dataset generated during the current study is available from the corresponding author (M.B. Toker) on reasonable request.

Ethical Statement

Experimental designs and studies have been approved by Bursa Uludağ University Scientific Ethical Committee (Approval no: 2016-13/03)

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Conflict of Interests

The authors declared that there is no conflict of interest.

Author's Contribution

MBT and SA designed and performed the experiments. MBT analyzed the data, made tables and wrote the paper. Both authors reviewed and approved the final manuscript.

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RESEARCH ARTICLE

Effects of Adding Different Dosages of *Saccharomyces cerevisiae* in Diet on Growth Performance, Carcass Characteristics, Intestinal Morphology, and Gut Microflora of Broilers

Željko MAKSIMOVIĆ [1,a] Marija STARČEVIĆ [2,b (*)] Dragan ŠEFER [3,c] Jelena JANJIĆ [2,d]
Anita RADOVANOVIĆ [4,e] Stamen RADULOVIĆ [3,f] Dejan PERIĆ [3,g] Radmila MARKOVIĆ [3,h]

[1] Feix Nutrition D.O.O., Bulevar Kralja Petra I 55/20, 21000 Novi Sad, SERBIA
[2] Department for Food Hygiene and Technology, Faculty of Veterinary Medicine, University of Belgrade, Bulevar Oslobodjenja 18, Belgrade 11000, SERBIA
[3] Department of Nutrition and Botany, Faculty of Veterinary medicine, University of Belgrade, Bulevar Oslobodjenja 18, 11000 Belgrade, SERBIA
[4] Department of Histology and Embryology, Faculty of Veterinary medicine, University of Belgrade, Bulevar Oslobodjenja 18, 11000 Belgrade, SERBIA
ORCIDs: [a] 0000-0002-4269-857x; [b] 0000-0002-0857-5218; [c] 0000-0002-4394-6336; [d] 0000-0003-3351-7199; [e] 0000-0001-9195-7147
[f] 0000-0002-7250-537X; [g] 0000-0002-4752-7489; [h] 0000-0002-4479-5007

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Abstract: The aim of this study was to evaluate the effect of adding different dosages of live yeast *(Saccharomyces cerevisiae)* in diet on growth performance, carcass characteristics, intestinal morphology, and gut microflora of broilers. The study was conducted on 270 one-day old chickens (Ross 308) during a 42-day period. Broilers were randomly allocated to one of three dietary treatments that differed in the content of added yeast: no additional yeast; with 0.25 g/kg; and with 0.65 g/kg of added yeast. Each experimental group contained 90 animals. The results from our study showed that diet supplemented with 0.25 g/kg of yeast resulted in better growth performance and carcass quality (P<0.05), improved intestinal morphology (P<0.001), higher development of beneficial microflora *(Lactobacillus* spp.) (P<0.01), and reduction of pathogenic bacteria *(Escherichia coli)* in guts of broilers than in the group fed only with the basic diet (P<0.05). On the other hand, the group with the highest inclusion of yeast in diet (0.65 g/kg) achieved similar results for the examined parameters as did the group with no added yeast. Therefore, the adequate dose of *S. cerevisiae* in diet was 0.25 g/kg of yeast, while higher dose of live yeast in broiler diet (0.65 g/kg) did not achieve better results.

Keywords: *Broiler, Live yeast, Performance, Carcass characteristics, Intestinal morphology, Intestinal microflora*

Rasyona Farklı Dozlarda *Saccharomyces cerevisiae* İlavesinin Etlik Piliçlerin Büyüme Performansı, Karkas Özellikleri, Bağırsak Morfolojisi ve Bağırsak Mikroflorası Üzerine Etkileri

Öz: Bu çalışmanın amacı, etlik piliçlerde rasyona farklı konsantrasyonlarda canlı maya *(Saccharomyces cerevisiae)* ilavesinin büyüme performansı, karkas özellikleri, bağırsak morfolojisi ve bağırsak mikroflorası üzerine etkisini değerlendirmektir. Çalışma, 42 gün süre boyunca 270 adet bir günlük civcivler (Ross 308) üzerinde gerçekleştirildi. Etlik piliçler sırasıyla; maya içermeyen, 0.25 g/kg maya içeren ve 0.65 g/kg maya içeren üç farklı rasyon uygulama grupları içerisine rastgele dağıtıldı. Her deney grubunda 90 adet hayvan yer aldı. Çalışmamızdan elde edilen sonuçlar, sadece temel rasyonla beslenen gruba göre 0.25 g/kg maya ilaveli rasyon ile beslenen etlik piliçlerin, daha iyi büyüme performansı ve karkas kalitesi sergilediğini (P<0.05), gelişmiş bağırsak morfolojisine (P<0.001) ve daha yüksek oranda faydalı mikrofloraya *(Lactobacillus* spp.) sahip olduklarını (P<0.01) ve bağırsaklarında daha az oranda patojenik bakterilerin *(Escherichia coli)* yer aldığını gösterdi (P<0.05). Diğer taraftan, rasyonunda en fazla (0.65 g/kg) maya bulunduran grup, incelenen parametreler yönünden rasyonunda maya bulundurmayan grupla benzer sonuçlar verdi. Bu nedenle, broiler rasyonlarında *S. cerevisiae*'nin yeterli konsantrasyonu 0.25 g/kg iken, daha yüksek konsantrede (0.65 g/kg) canlı maya kullanımı daha iyi sonuçlar vermemiştir.

Anahtar sözcükler: *Broiler, Canlı maya, Performans, Karkas özellikleri, Bağırsak morfolojisi, Bağırsak mikroflorası*

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(*) Corresponding Author

Tel: +381 11 3615 436; Fax: +381 11 2685 936
E-mail: marijadok@gmail.com (M. Starčević)

Introduction

In the past twenty years, annual poultry meat production has increased from 40 mt to 132 mt (annual increase 3.5% - 4.7%) and the average annual consumption of poultry meat in the world is 15 kg per capita [1]. Reasons for increasing world poultry production could be found in the high nutritional value and favorable content of protein and fat in meat, the meat's acceptability by many cultures and regions, and low cost. Moreover, poultry meat is easily affected by modifications in feed ingredients that influence the health and growth performance of poultry, gaining meat with beneficial effects on human health [2,3]. Recently, probiotics and prebiotics have been introduced to poultry feed in order to achieve many beneficial effects in poultry. Several studies indicated the importance of probiotics and prebiotics as potential substitutes for antibiotics in order to improve bacteria-related immune dysfunction, intestinal morphology disruption, and growth performance in broilers [4-8]. Probiotics comprised of live yeast contain many biologically valuable proteins, functional nucleic acids, vitamin B-complex, mannanoligosaccharide, immune enhancers such as β-glucan, and growth promoting factors [8,9]. Therefore, live yeast *(Saccharomyces cerevisiae)* has been reported to have several beneficial effects on poultry, such as achieved gut microbial balance, improved humoral immune response and intestinal morphology, and favorable growth performance results [10,11].

However, various researchers have reported different effective doses of yeast *(S. cerevisiae)* in broiler feed on poultry health, growth performance results, and carcass characteristics [8]. Therefore, the aim of this study was to evaluate the effect of adding different dosages of live yeast *S. cerevisiae* in diet (no additional yeast; with 0.25 g/kg; and with 0.65 g/kg of added yeast) on growth performance, carcass characteristics, intestinal morphology, and gut microbiota of broilers.

Material and Methods

Ethical Statement

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 (Resolution number: 01-511-2/2020/07/23).

Animals, Housing, and Feeding

The study was conducted on 270 one-day-old chickens of both sexes and the same origin (Ross 308) during a 42-day period. At the beginning of the study, broilers were randomly allocated to one of three dietary treatments. Each experimental group contained 90 animals housed in groups of 15 birds per pen in six repetitions (stocking density = 0.15 m^2/head). Conditions in the facility (ventilation, heating, lighting, and relative humidity) were according to the technological standards and recommendations for this hybrid [12]. Pens were bedded with straw and provided with fresh water and feed *ad libitum*. At the beginning of the trial, the temperature of the room was 32°C and then was gradually lowered to 22°C, which was maintained until the end of the study. During the trial, relative humidity was 60-70%. A continuous period of six hours of dark was provided during the night, and for the rest of the day, artificial light was uniformly distributed throughout the house. Broilers were not identified by sex at any time during the study, so we assumed an approximately equal ratio of males and females was distributed in the experimental groups.

From the start of the trial, each group of broilers was fed with one of three experimental diets which comprised the same basal diet, but differed only in the content of added *S. cerevisiae*. Basal diet was formulated according to the recommendations for Ross 308 strain [13] *(Table 1)*. Diets were fed from days 1 to 42 including starter (days 1-10), grower (days 11-24), and finisher (days 25-42). All components of the diets were analyzed for dry matter [14] (ISO 6496, 1999), crude protein [15] (ISO 5983-1, 2005), crude fat [16] (ISO 6492, 1999), crude fiber [17] (ISO 6865, 2000), ash [18] (ISO 5984-1, 2002), calcium [19] (ISO 6490-1, 1985), and phosphorus [20] (ISO 6491, 1998) *(Table 1)*. Yeast was added to the diets in the microspherule form of live yeast concentrate *(S. cerevisiae*, new strain, 1 x 10^{10} CFU/g, produced by Lesaffre, France) at different levels: the first group with no additional yeast (background only); the second with 0.25 g/kg; and the third with 0.65 g/kg of added yeast. Levels of active yeast in the three experimental diets were: 0 CFU/kg (group with no additional yeast), 2.5 x 10^9 CFU/kg (group with 0.25 g/kg of added yeast), and 6.5 x 10^9 CFU/kg (group with 0.65 g/kg of added yeast).

Growth Performance and Carcass Characteristics

To study the effect of added yeast in the diet on growth performance of broilers, all animals were weighed on days 1, 10, 24, and 42 to obtain average body weight and daily weight gain. Feed consumption per pen was recorded during periods 1-10 days, 11-24 days, 25-42 days, and for the overall study duration (1-42 days). Feed conversion ratio (FCR) was calculated as the ratio between feed intake and weight gain.

At the end of the study, broilers were transported to the slaughterhouse, electrically stunned and immediately slaughtered by severance of the jugular veins. Subsequently, animals were processed following standard industrial techniques. Carcasses were stored in a ventilated cold room at 2°C. After 24 h of chilling, 30 carcasses from each

Table 1. Ingredients and chemical composition of diets

Ingredient (g/kg)	Starter (0-10 days)	Grower (11-24 days)	Finisher (25-42 days)
Maize	458.70	494.10	548.00
Rye	50.00	50.00	50.00
Soybean meal (CP 44%)	226.50	147.80	67.40
Full fat soya	218.90	266.70	293.10
Monocalcium phosphate	13.20	13.50	11.80
Limestone	14.00	11.50	12.70
Salt	3.50	3.10	3.10
Lysine L-79	3.00	1.40	1.70
Methionine DL-99	2.20	1.90	2.20
Vitamin-mineral premix[a]	10.00	10.00	10.00
ME (MJ)	12.50	12.97	13.39
Chemical Composition (g/kg)			
Dry matter	898.20	901.40	896.30
Crude protein	224.60	215.00	178.40
Ether extract	59.30	64.90	74.90
Crude fiber	33.10	34.90	29.30
Ash	64.10	59.30	48.60
Calcium	9.80	9.70	9.50
Total phosphorus	7.90	8.40	6.90

[a] Vitamin-mineral premix provided per kg of diet: Vit. A: 10.000 IU; Vit. D_3: 4.000 IU; Vit. E: 55 mg; Vit K_3: 3 mg; Vit B_1: 3 mg; Vit. B_2: 8 mg; Vit. B_3: 65 mg; Vit. B_5: 20 mg; Vit. B_6: 5 mg; Vit. B_7: 0.3 mg; Vit B_9: 2 mg; Vit. B_{12}: 0.02 mg; Iron ($FeSO_4$): 80 mg; Copper ($CuSO_4$): 8 mg; Manganese ($MnSO_4$): 60 mg; Zinc ($ZnSO_4$): 40 mg; Iodine (KI): 0.33 mg

group were measured to calculate dressing percentage on cold carcass weight. Furthermore, carcasses were separated into breasts and drumsticks with thighs that were weighed, and their percentage of total cold carcass weight was calculated.

Morphological and Histological Analyses

For morphological and histological analyses, tissue samples of the ileum and cecum were collected from 12 birds in each experimental group. Samples were fixed in 10% buffered formalin saline, dehydrated by immersing through a series of alcohols of increasing concentrations (from 70% to absolute), and then infiltrated with xylene and embedded in paraffin. Paraffin sections of 2 µm thickness were cut with a rotary type microtome and placed on glass slides. Sections were stained with Mayer's hematoxylin and eosin (HE) and with a combination of periodic acid Schiff stain and Alcian blue (PAS-AB) [21,22]. Histological sections were examined using a light microscope (Olympus BX53) with the objective magnifications x 4 and x 10. Morphometric examinations were carried out using Olympus cellSens software *(http://www.olympusamerica.com)*. For parameters of gut integrity (villus height, crypt depth, villus height/crypt depth ratio), 15 measurements for each parameter per animal were made (five villi per slide; three slides per animal). Villus height was measured over the vertical distance from the villus tip to villus-crypt junction level. Crypt depth was measured over the vertical distance from the villus-crypt junction to the lower limit of the crypt. Goblet cells were enumerated on 10 different villi along 500 µm of each villus surface, and goblet cell density was calculated.

pH and Microbiological Analyses

After evisceration, contents from the ileum and cecum were collected in sterile bags by squeezing the intestine gently. The pH of these contents was measured using a hand-held pH-meter Testo 205 (TestoAG, Lenzkirch, Germany). Collected ileum and cecum contents were held under the cold conditions (2°C) until microbiological analyses of total aerobic bacterial count, *Lactobacillus* spp. count, *Enterococcus* spp. count, and *Escherichia coli* count were conducted. A sterile stick was used to put 1 g of intestinal content into a sterile test tube together with buffered peptone-water, and this was resuspended by vortexing. Each pooled sample (0.1 mL) was serially diluted via 10-fold dilutions (from 10^{-1} to 10^{-9}). Duplicate plates of selective media were inoculated with 0.1 mL of each dilution to determine the bacterial species defined by standard laboratory methods. The total aerobic count was determined using standard plate count agar medium (OXOID). The plates were incubated at 30°C, aerobically for 24-48 h. Total *Enterococcus* spp. and *E. coli* were counted on UTI agar (urogenital tract infections agar) (HiMedia) after incubation for 24 h at 37°C, while total *Lactobacillus* counts were determined on selective MRS agar (de Man, Rogosa, and Sharpe) agar supplemented with 20 mg/mL vancomycin (Sigma Aldrich) and 2 mg/mL cefotaxime (Sigma Aldrich), after incubation for 72 h at 30°C. Micro-aerophilic atmospheres, used for lactobacilli growth on agar plates, were produced using the AnaerocultR C (Merck KgaA, Darmstadt, Germany). Bacterial colonies were counted immediately after removing plates from the incubator, and the bacterial numbers were expressed as $\log_{10}$ CFU per gram of digesta.

Statistical Analyses

Statistical analysis of the results was elaborated 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 pooled standard error of means (SEM). One-way ANOVA with Tukey's post hoc test was performed to assess the significance of differences among experimental groups. Statistical significance was considered at a level of $P<0.05$.

Results

Growth Performance of Broilers

Body weight of broilers did not differ on days 1 and 10 across the treatment groups *(Table 2)*. On days 24 and 42,

body weight of the group with 0.25 g/kg of added yeast was higher (P<0.05) than the group with no added yeast (0 g/kg). For the overall period of the trial, higher daily weight gain (P<0.05) was found in the group with 0.25 g/kg of added yeast than in the group fed with basic diet. During the third period (days 25-42) and for the overall period of the study, feed intake was higher (P<0.05) in the group with 0.25 g/kg of added yeast than in the two other experimental groups. Moreover, broilers fed only with basal diet had higher (P<0.05) feed conversion ratios during the second period (days 11-24) and for the overall period of the study compared to those supplemented with 0.25 g/kg and 0.65 g/kg of added yeast.

Carcass Quality of Broilers

A higher dressing percentage (P<0.05) was determined in the group with 0.25 g of added yeast/kg than in the group with no added yeast in diet *(Table 2)*. Supplementation of yeast significantly improved yields and the proportion of carcass cuts (breast and drumsticks with thighs), where the group with 0.25 g of added yeast/kg had higher (P<0.05) weight of breast and drumsticks with thighs compared to the control group. In addition, the third group (0.65 g/kg of yeast) did not differ in carcass quality parameters compared to the two experimental groups (0 g/kg and 0.25 g/kg of added yeast).

Intestinal Morphology of Broilers

In ileum, villus height was significantly higher in broilers supplemented with yeast (0.25 g/kg and 0.65 g/kg of added yeast) than in broilers fed with basic diet *(Table 3)*. Lower crypt depth (P<0.05) was found in the group supplemented with 0.25 g/kg of yeast compared to the control group of birds (0 g/kg of added yeast). In ileum, the villus height to crypt depth ratio was the highest in the experimental group with 0.25 g/kg of added yeast. Goblet cell density in ileum was higher in the group supplemented with 0.25 g/kg of yeast than in the control group. Supplementation of yeast with 0.25 g/kg resulted in higher villus height in cecum than in birds fed with basic diet. Moreover, crypt depth was lower and the villus height to crypt depth ratio was higher (P<0.05) in cecum of broilers with supplemented yeast (0.25 g/kg and 0.65 g/kg of added yeast) than in broilers with no yeast supplement. Goblet cell density in cecum did not differ among experimental groups.

Table 2. *Growth performance and carcass characteristics of broilers receiving diets containing different levels of yeast (Saccharomyces cerevisiae)*

Parameter	Day	Level of Yeast in Diet			SEM	P Value
		0 g/kg	0.25 g/kg	0.65 g/kg		
Body Weight (g/bird) (n=90	1	42.12	42.88	42.12	0.55	0.1561
	10	277.70	274.50	280.70	5.38	0.6708
	24	1071.00^{A}	1150.00^{B}	1113.00^{AB}	24.56	0.0005
	42	2499.00^{A}	2611.00^{B}	2548.00^{AB}	55.84	0.0511
Daily Weight Gain (g/bird) (n=90)	1-10	23.56	23.16	23.86	0.85	0.6039
	11-24	56.65^{A}	62.57^{B}	59.45^{C}	1.18	<0.0001
	25-42	84.00	85.94	84.41	2.01	0.4770
	1-42	58.50^{A}	61.14^{B}	59.64^{AB}	1.33	0.0532
Daily Feed Intake (g/bird) (n=6	1-10	34.50	33.78	34.31	1.18	0.7428
	11-24	80.14^{A}	82.43^{B}	81.14^{AB}	0.93	0.0288
	25-42	166.94^{A}	170.65^{B}	166.18^{A}	1.15	0.0005
	1-42	103.33^{A}	104.52^{B}	102.52^{A}	0.56	0.0018
Feed Conversion Ratio (n=6)	1-10	1.46^{A}	1.42^{B}	1.44^{AB}	0.02	0.0148
	11-24	1.42^{A}	1.32^{B}	1.36^{C}	0.01	<0.0001
	25-42	1.99	1.98	1.97	0.01	0.1694
	1-42	1.76^{A}	1.71^{B}	1.73^{C}	0.01	<0.0001
Carcass Weight (g) (n=30)	42	1899.00	2005.00	1959.00	19.19	0.1076
Dressing Percentage (%) (n=30)	42	73.99^{A}	74.90^{B}	74.49^{AB}	0.12	0.0175
Breast (g) (n=30)	42	737.60^{A}	805.30^{B}	758.60^{AB}	10.27	0.0175
Breast (%) (n=30)	42	38.33^{A}	39.27^{B}	38.59^{AB}	0.14	0.0323
Drumsticks with Thighs (g) (n=30	42	525.60^{A}	577.00^{B}	553.00^{AB}	7.25	0.0265
Drumsticks with Thighs (%) (n=30)	42	28.03^{A}	28.74^{B}	28.19^{AB}	0.11	0.0314

^{A,B,C} *Means within a row with the different superscript letter significantly differ at P<0.05; Tukey's tests were applied to compare means, SEM=Standard error of the mean*

Table 3. *Intestinal morphology of broilers receiving diets containing different levels of yeast (Saccharomyces cerevisiae)*

Segment of Intestine	Intestinal Morphology	Level of Yeast in Diet			SEM	P Value
		0 g/kg	0.25 g/kg	0.65 g/kg		
Ileum (n=12)	Villus height (μm)	1192.00^{A}	1448.00^{B}	1349.00^{B}	60.45	<0.0001
	Crypt depth (μm)	138.30^{A}	114.80^{B}	126.00^{AB}	6.18	0.0002
	Villus height/Crypt depth ratio	8.72^{A}	12.70^{B}	10.80^{C}	0.79	<0.0001
	Goblet cell density (cells/500 μm)	89.90^{A}	100.60^{B}	96.40^{AB}	4.47	0.0205
Cecum (n=12)	Villus height (μm)	198.00^{A}	225.60^{B}	212.80^{AB}	7.47	0.0004
	Crypt depth (μm)	42.20^{A}	32.20^{B}	31.40^{B}	1.66	<0.0001
	Villus height/Crypt depth ratio	4.74^{A}	7.06^{B}	6.81^{B}	0.34	<0.0001
	Goblet cell density (cells/500 μm)	49.50	54.20	53.60	2.99	0.1252

^{A,B,C} *Means within a row with the different superscript letter significantly differ at P<0.05; Tukey's tests were applied to compare means, SEM=Standard error of the mean*

Table 4. *Bacterial counts (log_{10} cfu/g) and pH value in ileal and caecal digesta of broilers receiving diets containing different levels of yeast (Saccharomyces cerevisiae)*

Parameter	Segment of Intestine	Level of Yeast in Diet			SEM	P Value
		0 g/kg	0.25 g/kg	0.65 g/kg		
Total Aerobic Bacterial Count (n=12)	Ileum	6.84	6.69	6.70	0.19	0.5763
	Cecum	8.23^{A}	7.65^{B}	7.69^{AB}	0.27	0.0227
Lactobacillus spp. (n=12)	Ileum	6.99	7.16	7.06	0.26	0.7341
	Cecum	7.65^{A}	7.98^{B}	7.88^{AB}	0.12	0.0042
Enterococcus spp. (n=12)	Ileum	6.66	7.08	7.31	0.33	0.0664
	Cecum	7.65	7.60	7.52	0.18	0.4356
E. coli (n=12)	Ileum	6.50^{A}	5.95^{B}	6.09^{AB}	0.25	0.0281
	Cecum	7.84	7.34	7.36	0.26	0.0363
pH value (n=12)	Ileum	6.80^{A}	6.45^{B}	6.47^{B}	0.14	0.0066
	Cecum	7.20^{A}	6.78^{B}	6.73^{B}	0.09	<0.0001

^{A,B} *Means within a row with the different superscript letter significantly differ at P<0.05; Tukey's tests were applied to compare means, SEM=Standard error of the mean*

Bacterial Counts and pH Value in Ileal and Caecal Digesta of Broilers

Supplementation of yeast did not affect counts of total aerobic bacteria, *Lactobacillus* spp. or *Enterococcus* spp. in ileum *(Table 4)*. On the contrary, a higher (P<0.05) number of *E. coli* was found in ileum of the control group than in the group of broilers supplemented with 0.25 g/kg of yeast. Broilers supplemented with yeast (0.25 g/kg) had lower count (P<0.05) of total aerobic bacteria and higher count of *Lactobacillus* spp. (P<0.01) in cecum than control broilers (0 g/kg of added yeast). Moreover, experimental groups did not differ in the number of *Enterococcus* spp. or *E. coli* in cecum. In addition, the pH of digesta in ileum and cecum of birds fed with basic diet was higher (P<0.05) than in groups supplemented with yeast (0.25 g/kg and 0.65 g/kg of added yeast) *(Table 4)*.

Discussion

In our study, the addition of live yeast in diet improved growth performance results in broilers. The group supplemented with 0.25 g/kg of added yeast achieved higher body weight than the group with no added yeast. Similar results were found for daily weight gain, feed intake, and feed conversion ratio. Our results are consistent with those previously reported by other authors [7,23-26]. Live yeast could enhance growth performance in animals by reducing adverse effects of pathogen bacterial colonization in gut [27], but also by improving nutrient digestibility in broilers [11]. Live yeast is a good source of small peptides, free amino acids, and nucleotides that are necessary for animal growth and have a high rate of digestion and absorption [24,28]. Moreover, live yeast contains growth factors like pro-vitamins and/or micronutrients that stimulate broiler growth [24]. Although in the previously mentioned studies, rates of improved growth performance results were proportional to their viable yeast count, in our study the group with the highest level of live yeast (0.65 g/kg) achieved similar growth performance results to the group with no added yeast. Similarly to our results,

Wang et al.[5] and He et al.[11] used the same concentration of *S. cerevisiae* as we did (1 x 10^{10} CFU/g), but added in higher levels in feed (0.5 g/kg, approximately 5 x 10^{9} CFU/kg; 1 g/kg, approximately 1 x 10^{10} CFU/kg; and 5 g/kg, approximately 5 x 10^{10} CFU/kg), and did not find any effect of supplemented yeast on broiler growth. It seems that high levels of live yeast adversely affect growth performance [29]. According to Reisinger et al.[30], high inclusion of live yeast in diet decreases broiler performance because of a potential over-reaction of the immune system.

Regarding carcass quality parameters, higher dressing percentage, yield, and participation of the most valuable carcass parts (breast and drumsticks with thighs) were determined in the group with the medium level of added yeast in feed (0.25 g/kg) than in the group fed only with basic diet. Similarly to growth performance, in our study, the carcass quality parameters of the group with the highest dietary yeast level (0.65g/kg) did not differ from the control group. As previously mentioned, this could be a consequence of detrimental effects of higher dietary yeast levels on animal growth and consequently on carcass quality [29]. Moreover, different doses of probiotics result in variations of growth performance and carcass quality, suggesting that the optimal level of probiotics depends on the microorganisms added into broiler feed [31].

In our study, adding live yeast to broiler diet notably improved intestinal morphology, both in ileum and cecum, in terms of higher villus height, lower crypt depth, and higher ratio of villus height to crypt depth. Our results are consistent with those found by other authors [10,11,29]. Probiotics induce the formation of short-chain organic acids that stimulate the proliferation of epithelial cells and lead to greater villus height [32]. The intestinal structure influences the absorptive capacity of gut, since greater villus height allows better contact with the digesta and absorption of nutrients. These changes are associated with better growth performance because of the larger absorption surface [33]. Moreover, chickens supplemented with yeast had higher expression of genes involved in differentiation of epithelial cells preferentially towards absorptive cells than secretory cells, resulting in an increase of villus height [29]. Moreover, in our study, goblet cell density in ileum was higher in the group supplemented with 0.25 g/kg of live yeast than in the control group. On the other hand, supplementation of live yeast did not affect goblet cell density in cecum of our broilers. Regarding yeast supplementation, other authors reported higher density of goblet cells in broilers fed diets supplemented with yeast or yeast products, suggesting that yeast induces proliferation of goblet cells as a defense mechanism [29,30,34]. In fact, Pascual et al.[29] found that higher density of the goblet cells was associated with their lower size, since they were constantly exposed to stimulus, producing and releasing mucin. A greater number of goblet cells and higher mucin production have protective effects under any challenging condition, protecting the epithelial cells from pathogenic microorganisms and mechanical damage [30,35]. Mucin produced by goblet cells is a key factor for normal intestinal function and the interaction between the immune system and intestinal microbiota [36]. Contrary to expectations, our group with the highest inclusion of yeast (0.65 g/kg) did not show any difference in goblet cell density compared to the control group, suggesting the high dose of *S. cerevisiae* (6.5 x 10^{9} CFU/kg) impaired goblet cell reproduction, which we speculate could reduce the broilers' resistance to pathogenic microorganisms.

In our study, supplementation of live yeast had a positive effect on the intestinal microbiota. We found lower total aerobic bacterial count and *E. coli* count, as well as higher *Lactobacillus* spp. count in both the ileum and cecum of the group with 0.25 g/kg of added yeast than in the control group. Beneficial changes of intestinal microflora after yeast supplementation in feed were also observed by other authors [5,34,37]. Other studies showed that supplementation of yeast improved the broiler performance due to inhibition of pathogen growth, seen for *E. coli* [10], *Salmonella* [38-40] and *Campylobacter* [38]. Beneficial effects of live yeast on intestinal microbiota could be ascribed to yeast's important components, mannanoligosaccharides and β-glucans. In fact, β-glucans could adsorb or bind toxins, viruses, and pathogenic bacteria, while mannanoligosaccharides act as prebiotics, providing nutrients for beneficial microbes in the gastrointestinal tract [28]. Recent studies have revealed direct binding of *S. cerevisiae* cell wall to pathogenic bacteria, *E. coli*, *Salmonella*, and *Listeria* [27]. Moreover, our broiler group with the highest level of added yeast achieved similar results, in terms of intestinal microbiota, as the control group, indicating this high dose of *S. cerevisiae* did not positively affect the intestinal microbiota. This could be a consequence of the very high concentration of yeast in the gut that compromised the growth of other beneficial microorganisms by competing for the same nutrients. However, the added yeast did increase the number of *Lactobacillus* in the gastrointestinal tract and reduced the pH in both ileum and cecum of our yeast-supplemented groups. One of the advantages of lower pH in guts is it creates a more unsuitable environment for the growth of pathogenic bacteria such as *E. coli* and *Salmonella* spp. [41].

In conclusion, the results from our study show that diet supplementation with yeast at a level of 0.25 g/kg results in better growth performance and carcass quality, improved intestinal morphology, and greater development of beneficial microbiota in guts of broilers than in the group fed only with the basic diet. We emphasize the importance of an appropriate level of *S. cerevisiae* in diet, since the

higher dose of live yeast used in our study (0.65 g/kg) produced broilers with similar growth performance, carcass quality, intestinal morphology, and gut microbiota as the group with no added yeast.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (M. Starčević).

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Ethical Statement

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 (Resolution number: 01-511-2/2020/07/23).

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Competing Interests

The authors declare no conflict of interest.

Author Contributions

Experimental design was conceived by RM, DŠ and AR. Data were collected by ŽM, SR, and DP. Statistical analysis was conducted by JJ and MS. Original draft was written by MS and ŽM. All authors have contributed to the revision and final proof-reading of the manuscript.

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Research Article

Evaluation of the Effect of Naringenin Liposomal Formulation on Retinopathy in an Experimental Rabbit Model

Anayatollah SALIMI [1,2,a] Behzad SHARIF MAKHMALZADEH [1,2,b]
Mostafa FEGHHI [3,c] Anahita REZAI [4,d] Farid BAGHERI [2,e (*)]

[1] Nanotechnology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IRAN
[2] Department of Pharmaceutics, Faculty of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IRAN
[3] Department of Ophthalmologo, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IRAN
[4] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, IRAN
ORCIDs: [a] 0000-0003-1505-7969; [b] 0000-0002-0000-01000; [c] 0000-0002-0000-02000; [d] 0000-0003-1505-7969; [e] 0000-0003-1505-7969

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Abstract: Neovascularization is a hallmark of diabetic retinopathy (DR). Naringenin has protective effect against DR. Liposomes are suitable Nano-carriers for ocular drug delivery. Hence, we prepare and evaluate the effect of naringenin liposomal formulation (NLF) on DR in a rabbit model. NLF was prepared by thin lipid film method. NLF characteristics were evaluated by Scanning Electron Microscope (SEM), Dynamic Light Scattering (DLS) and zeta potential. NLF release pattern and stability were assessed. Thirty-six rabbits were divided into six groups (control, placebo, bevacizumab and NFL (200, 500 and 800 µg/mL)). Intravitreal injection of Alpha-aminoadipic acid (α-AAA) induced retinopathy. NFL was administered for three weeks. Neovascularization scoring was done by an eye examination. Furthermore, histological evaluation of retina was performed for angiogenesis and dilation of vessels. SEM imaging revealed successful NLF preparation. The particle size obtained 148 to 215 nm. Encapsulation Efficiency were 43% and 66% which is good for naringenin. Zeta potential was 15mV. Two formulations of NLF showed suitable release (% drug released after 1 h (%D1) was 5.5% and 3.1% and % drug released after 24 h (%D24) was 72.93% and 52.01%). NLF exhibited an acceptable stability. Histological findings confirmed neovascularization. Treatment with bevacizumab and three doses of NLF significantly decreased neovascularization score, respectively. Furthermore, histological results revealed that three doses of NLF especially 800 µg/mL improved damage caused by α-AAA in the retina. NFL showed protective effects against neovascularization. We showed that NFL is capable of nanoformulation with a great attenuating effect against neovascularization. Finally, the findings revealed that NFL is an anti- neovascularization agent.

Keywords: *Liposome, Naringenin, Neovascularization, Rabbit, Retinopathy*

Deneysel Tavşan Modelinde Naringenin'in Lipozomal Formülasyonunun Retinopati Üzerine Etkisinin Değerlendirilmesi

Öz: Neovaskülarizasyon, diyabetik retinopatinin (DR) bir özelliğidir. Naringenin, DR'ye karşı koruyucu bir etkiye sahiptir. Lipozomlar, oküler ilaç dağıtımı için uygun nanotaşıyıcılardır. Bu nedenle, bir tavşan modelinde naringeninin lipozomal formülasyonunu (NLF) hazırladık ve DR üzerindeki etkisini değerlendirdik. NLF, ince lipid film yöntemiyle hazırlandı. NLF özellikleri, Scanning Elektron Mikroskobu (SEM), Dinamik Işık Saçılımı (DLS) ve zeta potansiyeli ile değerlendirildi. NLF salınım modeli ve stabilitesi değerlendirildi. 36 tavşan; kontrol, plasebo, bevacizumab, 200 µg/mL NFL, 500 µg/mL NFL ve 800 µg/mL NFL olmak üzere altı gruba ayrıldı. Alfa-aminoadipik asit (α-AAA)'in intravitreal enjeksiyonunu takiben retinopati oluşturuldu. Üç hafta boyunca NFL uygulandı. Göz muayenesi ile neovaskülarizasyon skorlaması yapıldı. Ayrıca anjiyogenez ve damarların dilatasyonu için retinanın histolojik değerlendirmesi yapıldı. SEM görüntüleme, NLF hazırlığının başarılı olduğunu ortaya çıkardı. Elde edilen partikül boyutu 148 ile 215 nm arasındaydı. Kapsül oluşturma verimliliği %43 ve %66 olup naringenin için oldukça iyiydi. Zeta potansiyeli 15mV idi. NLF'nin iki formülasyonu da uygun salınım gösterdi (1 saat sonra salınan ilaç yüzdesi (%D1) %5.5 ve %3.1 ve 24 saat sonra salınan ilaç yüzdesi (%D24) %72.93 ve %52.01 idi). NLF, kabul edilebilir bir kararlılık sergiledi. Histolojik bulgular neovaskülarizasyonu doğruladı. Sırasıyla bevacizumab ve üç doz NLF ile tedavi, neovaskülarizasyon skorunu önemli ölçüde azalttı. Ayrıca histolojik sonuçlar, üç doz NLF'nin özellikle de 800 µg/mL'nin retinada α-AAA'nın neden olduğu hasarı iyileştirdiğini ortaya koydu. NFL, neovaskülarizasyona karşı koruyucu etkiler göstermiştir. NFL'nin neovaskülarizasyona karşı büyük bir zayıflatıcı etki ile nanoformülasyon yapabildiğini gösterdik. Son olarak, bulgular NFL'nin bir anti-neovaskülarizasyon ajanı olduğunu ortaya koydu.

Anahtar sözcükler: *Lipozom, Naringenin, Neovaskülarizasyon, Retinopati, Tavşan*

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(*) Corresponding Author

Tel: +98 916 6707016
E-mail: faridbagheri85@gmail.com (F. Bagheri)

Introduction

Retinopathy is called any damage to the retina of the eye [1]. Diabetes [2], hypertension [3], and prematurity of infants [4] develop retinopathy. Diabetic retinopathy (DR) is a chronic complication that affects almost all diabetic patients [5]. DR is the fifth leading cause of blindness and visual disorders. Global prevalence of DR is 22.27% and 103.12 million adults live with DR worldwide in 2020 [6]. The retinal microvascular changes in DR. The permeability of the arteries increases and the blood vessels in the retina proliferate abnormally in response to the lack of blood supply [7]. Moreover, an increase in the thickness of the basement membrane of capillaries occurs in DR, which is related to the deposition of advanced glycation end products (AGEs). As the thickness of the basement membrane increases, the capillaries become blocked and the retina becomes ischemic [8]. AGEs also increase vascular endothelial growth factor (VEGF) expression in microvascular endothelial cells [9]. Retinal cells need high oxygen, so hypoxia is caused by vascular damage in the retina [10]. Hypoxia stimulate VEGF production [11]. VEGF reduces blood retinal barrier (BRB) damage and increases vascular leakage by reducing the expression of occludin, as binding proteins and protector of the BRB [12]. Thus, VEGF plays important role in neovascularization in retina [13]. Alpha-aminoadipic acid (α-AAA) is used for induction of neovascularization model similar to retinal injury in DR. α-AAA as the analog of amino acid, L-glutamic acid exhibits gliotoxic effects in the retina [14]. Hence, inhibition of VEGF production reduces capillary vascular leakage. Therefore, the use of anti-VEGF therapies could treat retinopathy [15]. Bevacizumab is a recombinant human monoclonal antibody that affects all VEGF isoforms. Bevacizumab could treat many retinopathies due to increasing VEGF activity including DR [16].

Despite the effectiveness of synthetic drugs, their side effects have raised concerns [17]. Studies have turned their attention to active compounds derived from plants [18-22]. Flavonoids, as the largest group of polyphenols, exhibits many therapeutic effects [23]. Naringenin is a flavanone exerts antioxidant, free radical scavenging, anti-inflammatory and immunomodulatory properties [24]. Naringenin could ameliorate diabetic retinopathy through the exhibition of antioxidant and anti-inflammatory effects [25]. Moreover, naringenin has an inhibitory effect against angiogenesis and VEGF production [26]. Medications used to treat eye diseases often have a short shelf life and little eye contact. One way to increase the shelf life, solubility and bioavailability of drugs is to use nanoparticles for drug delivery [27]. The use of nanoparticles for drug delivery to treat various diseases is increasing rapidly. Loading of drugs and effective plant compounds in nanoparticles has been considered due to the increase in drug shelf life [28]. Because of their biocompatibility and lack of toxicity, nanoliposomes are recommended as promising nanocarriers for ocular administration [27]. Furthermore, it has reported that liposomal nanoformulation could enhance solubility and bioavailability of naringenin and participate in controlled delivery of naringenin (a,b). Previous studies have shown the therapeutic effect of NLF against various diseases including nonalcoholic fatty liver disease (NAFLD) (c). Hence, we decided to prepare and evaluate of the therapeutic effect of naringenin liposomal formulation (NLF) on retinopathy in a rabbit model.

Material and Methods

Ethical Approval

The Animal Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (AJUMS) approved all of the experimental protocols (IR.AJUMS.ABHC.REC.1399.064).

Chemicals

The following chemicals: Cholesterol (Merck Company, Germany), Lecithin (Merck Company, Germany), Naringenin (Merck Company, Germany), DL-Alpha-aminoadipic acid (Sigma Company, Germany), Stearylamine (Gattefossé Company, France), Ethanol (Merck Company, Germany), Methanol (Samchun Company, Korea), Chloroform (Samchun Company, Korea) were used in the current study.

Preparation of Liposome

The liposome nanoparticles were prepared by the thin-film hydration method. Briefly, phospholipids and drugs including lecithin, cholesterol, stearylamine and naringenin were dissolved in 50 mL of organic solvent containing chloroform and methanol (2:1) and the chloroform and methanol solution was evaporated by Rotary Evaporator (120 rpm) at 60°C to prepare thin lipid film. The sample was vacuumed overnight to remove the remaining solvent. In the next step, the volumetric flask was heated to 45°C and after adding the aqueous phase including the phosphate buffer solution, the mixture was vortexed by hand and stirred for 20 min.

Liposome Physicochemical Characterization

Liposome Size: Particle size was measured at 25°C by particle size analyzer. The mean droplet size of samples was determined by SCATTER SCOPE 1 QUIDIX (South Korea) based on photon correlation spectroscopy with a wide measurable size range (1-7000 nm). Each sample was measured triplicate.

Zeta Potential: Zeta Analyzer was used to check the surface charge. The Zeta Potential of samples was determined using a Malvern Zetasizer Nano-range instrument (Malvern Instruments Ltd., Malvern, UK).

Morphologic Characterization

Scanning Electron Microscopy (SEM): To perform the SEM imaging, the shape and structure of dried samples of the liposome was examined at 15 kV with a 6300 field emission scanning electron microscope (Hitachi, S-4160).

Naringenin Loaded Liposome Calculation

For this purpose, liposomal formulations were centrifuged at 12.000 rpm for 30 min. Then we separated the liposomes from the surface of the solution and centrifuged the aqueous solution containing the unloaded drug again, and this time we separated the aqueous solution and the liposomes again for 15 min. To calculate the amount of unloaded drug, the underlying liquid was isolated and the amount of drug was measured. The amount of loaded drug was calculated by differentiating the amount of unloaded drug from the total drug used. On the other hand, to confirm the test results, the upper liposomal phase was dissolved in methanol to break the liposomes. Then the amount of loaded drug was calculated. In this way, both loaded and unloaded drugs were calculated and the percentage of loaded drugs was calculated based on the following equation:

Entrapment efficiency = Trapped drug/Total drug x100

Naringenin Loaded Liposome Release Pattern

Static diffusion cells were used for this purpose. After separating the liquid phase and the liposomal phase after centrifugation, 2 mL of the liposomes were separated and placed in the donor phase. The amount of drug passing through the cellulose membrane that enters the acceptor phase, which is the artificial tear environment of the eye with a pH of 7.4, was then measured for 24 h. By plotting the cumulative amount of drug passed over time, the pattern of drug release from the liposome was determined.

Liposome Stability Evaluation

In order to evaluate the stability of liposomal formulations, the samples were kept at room temperature for three months. After this period, the formulations were evaluated for drug loading capacity, suspension stability and particle size. Decreased loading capacity or cake formation in the suspension and particle size change are considered as signs of instability.

Naringenin Amount Determination

The amount of drug was determined by HPLC with c18 column at a wavelength of 288 nm.

pH Test

pH was measured by a digital pH meter [29].

Animals and Study Design

The experimental procedures were conducted at Animal Laboratory, affiliated to Lorestan University of Medical Sciences, Khorramabad, Iran. A total of thirty-six adult male albino New Zealand rabbits weighing 1.5 to 2 kg were chosen for this investigation based on previous studies and kept in standard cages (d). The rabbits were housed under appropriate environmental conditions that included a temperature of 22°C, 12 h light-dark cycles, and with free access to standard laboratory food and tap water.

Induction of Retinopathy: By injecting alpha-amino adipic acid, which is an angiogenic agent, into adult male New Zealand albino rabbits, we caused angiogenesis and retinopathy, and the retinopathy caused by alpha amino adipic acid injection was similar to diabetic retinopathy. Eight weeks after the injection of the mentioned compound, the occurrence of retinopathy was confirmed by the ophthalmologist using clinical examination and microscopic images. The animals' health status and detailed eye examination was done before the study by a physician. Intraocular injection of Alpha-Aminoadipic acid (α-AAA) was used to induce retinopathy. Fifty µL of 0.025 M α-AAA was injected into a rabbit's eye using a 27-gauge (27 µm) needle. Lidocaine eye drops alone were used for anesthesia before injection and tetracycline eye drops was used to prevent infection after injection of Alpha-Aminoadipic acid. A 27-gauge needle was inserted into the vitreous cavity, 1.5 mm posterior to the superotemporal limbus, and the needle tip was directed into the midvitreous under direct visualization with external illumination of indirect ophthalmoscopy. Ophthalmoscope was used to evaluate the retina for the development of retinopathy.

Grouping: The rabbits were randomly divided into the following six groups (six rabbits per group):

Group 1: NLF treated group with a concentration of 200 µg/mL

Group 2: NLF treated group with a concentration of 500 µg/mL

Group 3: NLF treated group with a concentration of 800 µg/mL

Group 4: Selected placebo formulation treated group (received vehicle = liposomal formulation without naringenin)

Group 5: Bevacizumab treated group with intravitreal injection of a concentration of 1.25 mg/50 µL using a 27-gauge needle

Group 6: Healthy non-diabetic animals.

Two months after induction of retinopathy, the treatment continued by administration of drops of different concentrations of the selected liposomal formulation twice daily for 5 days a week for three weeks. The administration of drops was carried out in the right eye and the left eye was considered as negative control (untreated retinopathy).

Neovascularization Scoring: After induction of retinopathy on the 8th weeks following injection of α-AAA, neovascularization evaluation was performed using ophthalmoscopic examination by a physician according to the scoring between zero and one as follow:

Index1: Complete normal retinal neovascularization to the end of zone III (Zero=The lowest amount of angiogenesis and 1= The highest amount of angiogenesis)

Index2: Change the color of the edges from pink to white (Zero=The lowest amount of angiogenesis and 1= The highest amount of angiogenesis)

Index3: No increase in disease severity (Zero=The lowest amount of angiogenesis and 1= The highest amount of angiogenesis)

Index4: Blood vessels cross from the line demarcation border (Zero=The lowest amount of angiogenesis and 1= The highest amount of angiogenesis)

Index5: Start the process of replacing active lesions with zero-grade scar tissue (Zero=The lowest amount of angiogenesis and 1= The highest amount of angiogenesis) [15,30].

It is necessary to mention that mydriatics (Tropicamide 1% w/v, eye drops) (MYDRAX®, Sinadarou Labs Co., Iran) used prior to eye examinations for neovascularization scoring.

Histological Evaluation

To conduct histological evaluation, 0.5 mL of 2% sodium fluorescein solution was injected intraperitoneally, and then the rabbits were euthanized with sodium phenobarbital at a dose of 200 mg/kg. In histological evaluation, the aim was to detect neovascularization and observe dilated vessels. For this purpose, the removed eye was placed in a mixture of 8% formaldehyde, 30% ethanol and 10% glacial acetic acid for 24 h at 4°C and then transferred to a 70% ethanol solution. Next, tissue was embedded in paraffin and cut into a 5 µm thick sections. Finally, the sections were stained with hematoxylin and eosin (H&E) and tissue changes and neovascularization were examined in all samples [31].

Statistical Analysis

Data analysis was carried out using SPSS 24 analytic software (SPSS, Inc., Chicago) and Graph Pad Prism (Version 8.01). Statistical analysis was performed on the effect of independent variables on physicochemical properties of formulations (Particle Size, Naringenin amount and pH parameters) by one-way ANOVA using Minitab software. Kruskal-Wallis non-parametric statistical test was used for neovascularization parameter and Mann-Whitney was used for pairwise comparison. All data were displayed as mean ± standard deviation (SD). Differences with p-value ≤0.05 were considered significant.

Results

Liposome Characterization

Different liposome formulation's components are based on factorial design and formulation characters including the Particle size and Encapsulation Efficiency (EE%) demonstrated in *Table 1*. Through this experiment, particle sizes were between 148 to 215 nm. Low particle size provided high surface area and so high drug partitioning into the eye. Also, EE% values were between 43% and 66% so it can be considered a good value for a lipophilic compound such as naringenin. Due to the need for a particle size below 200 nm for the appropriate ocular formulation, formulations No. 5 and 7 were selected for release studies and the selection of the final formulation.

Zeta Potential

As shown in *Fig. 1*, Zeta potential was obtained as 15 mV for NLF.

Morphology of NLF

As displayed in *Fig. 2*, the SEM image showed vesicular structures with an average diameter of 150 nm, that is, the following results reported by the particle size analyzer in this study.

Release Study

The percentage of released drugs is an important characteristic of the formulation which plays an important role in formulation effectiveness. Drug release profiles

Table 1. *Liposomal formulation's components and liposome characterization*

Formulations	%Loading (EE %)	PZ	Factorial	Aqueous Volume	%Lipid	Drug	Blocks	PtType	RunOrder	StdOrder
1	55.0±2.65	204±14.4	NNP	10	5	0.15	3	1	17.15.1	13.11.9
2	46.0±4.36	207±17.9	NPN	10	10	0.1	3	1	19.9.2	5.13.21
3	51.0±4.00	214±15.6	NNN	10	5	0.1	3	1	22.13.3	1.9.17
4	55.3±9.02	202±6.00	PNP	15	5	0.15	3	1	26.14.4	4.12.20
5	66.0±2.65	147±21.4	NPP	10	10	0.15	3	1	24.11.5	7.15.23
6	51.0±6.56	206±8.15	PPN	15	10	0.1	3	1	21.12.6	6.14.22
7	64.0±6.00	168±6.51	PPP	15	10	0.15	3	1	20.16.7	8.16.24
8	43.0±2.65	215±19.55		15	5	0.1	3	1	18.10. 8	2.10.18

EE: Encapsulation efficiency, PZ: particle size; N0. 1-8 show liposomal formulations; No. 5 and 7 were selected finally because of their particle size (<200 nm)

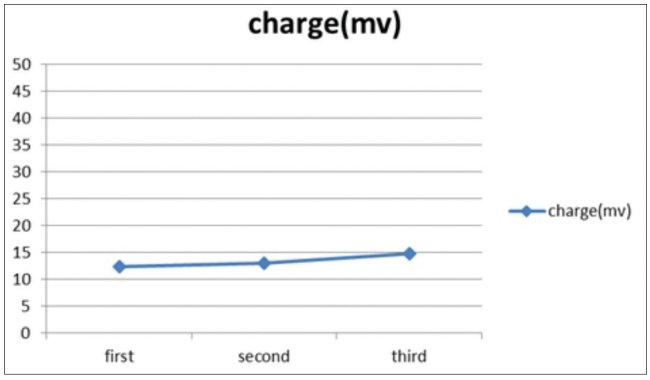

Fig 1. Zeta potential of NLF

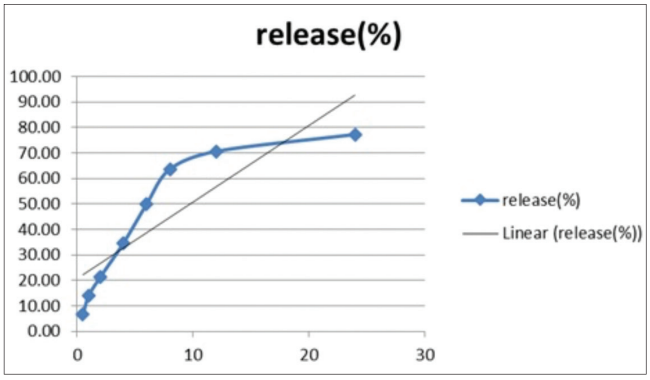

Fig 3. Percentage of naringenine release in liposomal formulation No. 5.

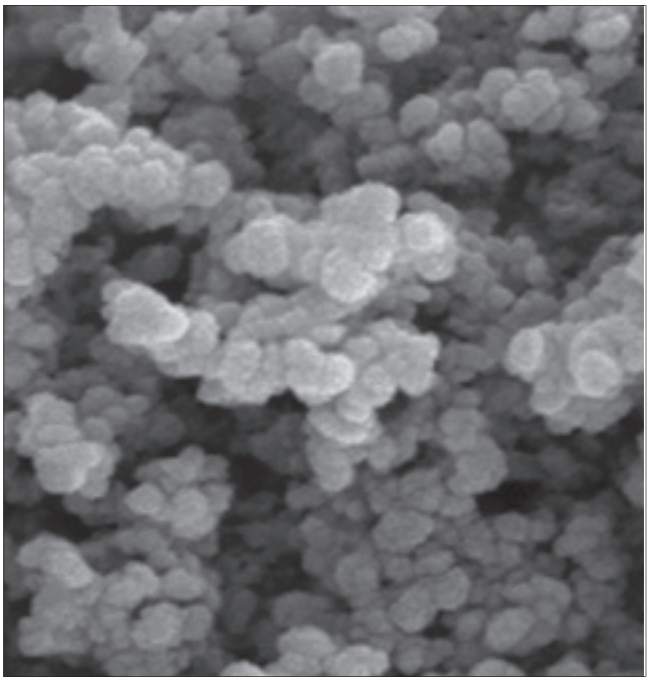

Fig 2. Scanning electron microscopy (SEM) image of liposomal formulation No.5 (as selected formulation) represents particle size as 147 nm

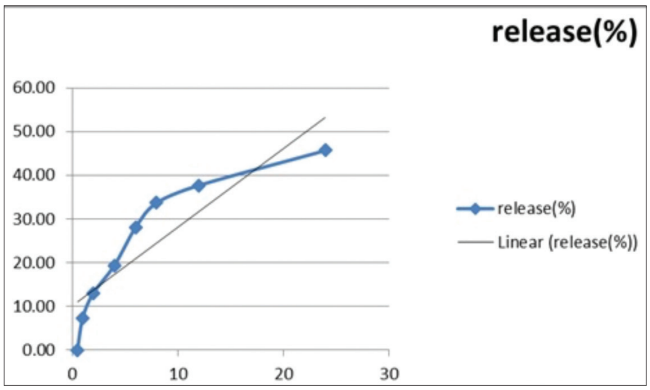

Fig 4. Percentage of naringenine release in liposomal formulation No. 7

Table 2. *Percentage of naringenine release after 1 h (%D1) and 24 h (%D24) in d liposomal formulations*

Liposomal Formulations	Percentage of Naringenine Release	
	%D1 (mean±SD)	%D24 (mean±SD)
No. 5	6.67±0.42	77.3±6.13
No. 7	7.33±0.23	53±3.26

Naringenin release through liposomal formulations NO.5, 7

for each formulation were provided in buffer phosphate. The percentage of drug released after 1 h (%D1) and 24 h (%D24) as a sign of fast and sustained release, respectively, are shown in *Table 2*.

It was observed that %D1 for batch No. 5 was 5.5% and batch No. 7 was 3.1%. Also, %D24 values were 72.93% and 52.01% belongs to batch No. 5 and 7.

The release profiles of naringenin through selected liposomes are illustrated in *Fig. 3, Fig. 4*.

Formulation No. 5 was selected for clinical studies at the specified concentrations due to the cumulative release rate as well as the higher loading rate.

Stability of Selected Liposomes

The stability of naringenin-loaded optimized liposome was assessed by checking the change in EE%, particle size, and cake formation of optimized liposomes stored at room temperature for 3 months. Results revealed no cake formation and change in EE%. The change in particle size for 3 months is illustrated in *Fig. 5*.

As shown in *Table 3*, no significant difference was seen in the average particle size of the optimized liposomes for 3 months after storage at room temperature (P=0.979).

Naringenin Amount Determination

The amount of naringenin in liposomal formulation has been shown in *Table 4*. Significant difference was seen in the amount of naringenin in liposomal formulation between different concentrations (P<0.001).

The pH of NLF has been shown in *Table 5*. No significant difference was observed in the pH of naringenin liposomal formulation between different concentrations (P=0.765).

Neovascularization Scoring

As shown in *Table 6* and *Fig. 6*, clinical scoring of neovascularization reached to peak significantly four and especially eight weeks after injection of α-AAA (P<0.001). It is necessary to mention that injection of α-AAA did not show any complications *(Fig. 7)*. On the other hand, treatment with NLF at the doses of 200, 500 and 800 µg/mL could significantly decrease clinical score of neovascularization compared to the treated group with placebo (P<0.001). This means that significant reducing clinical scoring of neovascularization was associated with increasing the duration of treatment (1, 2 and 3 weeks) and increasing the dose of NLF (200, 500 and 800 µg/mL).

Histological Findings

As shown in *Fig. 8*, prepared sections showed that the eyes of the placebo rabbits have numerous and dilated blood vessels and many erythrocytes in the retinal stroma. This indicated the occurrence of extensive angiogenesis in the retina compared to control group [31]. In the bevacizumab group, the retinal stroma had a limited number of vessels and was very similar to the control group. It was also seen that the number of blood vessels reduced in the NLF treated group with a concentration of 200 µg/mL, but a number of dilated vessels were still observed. Furthermore, the number of blood vessels also reduced in the NLF treated group with a concentration of 500 µg/mL and a much smaller number of dilated blood vessels were seen. In the NLF treated group with a concentration of 800 µg/mL, the microscopic structure showed a high improvement and was very similar to the bevacizumab and control groups.

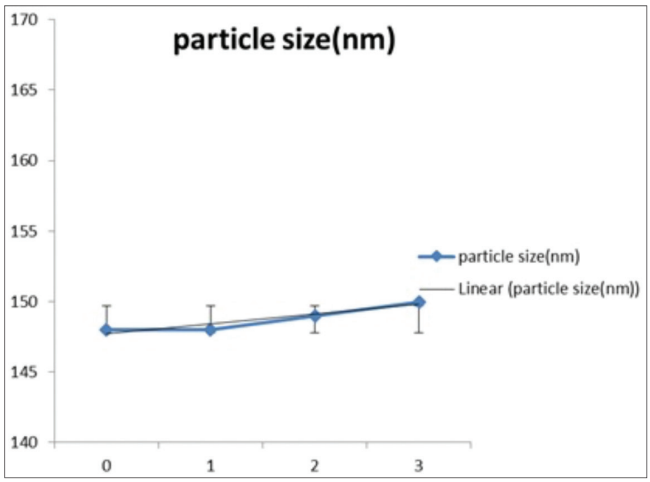

Fig 5. The changes in particle size during 3 months in selected liposomal formulation (No. 5)

Discussion

Nanoparticles are suggested for drug delivery in wide range of diseases including ocular diseases. The selection of a suitable nanocarriers with appropriate drug loading rate, slow release, high shelf life and low toxicity has a great importance for drug delivery. Nanoliposomes are one of the most widely used nanocarriers used in clinical applications for drug delivery in various diseases. Liposomes are conventional nanocarriers utilized to ocular drug delivery. Liposomes displayed several benefits such as the ability to elevate the concentration of drug in ocular tissues, enhancing drug penetration, controlling drug release, lack of toxicity or irritation to the eyes [32,33]. Hence, we decide to prepare, and evaluate of the therapeutic effect of NLF on retinopathy in animal model.

Table 3. *The changes in particle size during 3 months in selected liposomal formulation (No. 5)*

Parameter	Time of Stability				P-value
	1st Day	1st Month	2nd Month	3rd Month	
Particle size (nm)	148±2.00	148±8.72	149±6.24	150±7.81	0.979

Table 4. *The amount of naringenin in liposomal formulation*

Parameter	Concentration			P-value
	200 µg/mL	500 µg/mL	800 µg/mL	
Naringenin amount	0.1811±0.0229[c]	0.37494±0.00882[b]	0.4898±0.0497[a]	<0.001

[a-c] *Dissimilar letters show a significant difference*

Table 5. *The pH Evaluation of Selected Liposomal Formulation (No.5)*

Parameter	Concentration			P-value
	200 µg/mL	500 µg/mL	800 µg/mL	
pH	6.87±0.252	6.9±0.1	6.8±0.1	0.765

Table 6. Clinical scoring of neovascularization based on the time course of treatment in different treated groups

Intervention	Group						P-value
	Control	Treated with Bevacizumab	Treated with Placebo	Treated with NLF 200 µg/mL	Treated with NLF 500 µg/mL	Treated with NLF 800 µg/mL	
Before injection of α-AAA	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	1.000
Four weeks after injection of α-AAA	0.00±0.000^{b}	3.91±0.12^{a}	3.67±0.18^{a}	3.88±0.21^{a}	3.83±0.16^{a}	3.66±0.3^{a}	<0.001
Eight weeks after injection of α-AAA	0.00±0.000^{b}	4.86±0.09^{a}	4.86±0.15^{a}	4.8±0.1^{a}	4.9±0.14^{a}	4.83±0.02^{a}	<0.001
One week after treatment with NLF	0.00±0.000^{e}	1.1±0.2^{d}	4.9±0.2^{a}	3.4±0.20^{b}	3.2±0.1^{b}	1.8±0.1^{c}	<0.001
Two weeks after treatment with NLF	0.00±0.000^{c}	0.8±0.1^{bc}	5.00±0.2^{a}	2.7±0.2^{b}	1.8±0.2^{bc}	1.2±0.1^{bc}	<0.001

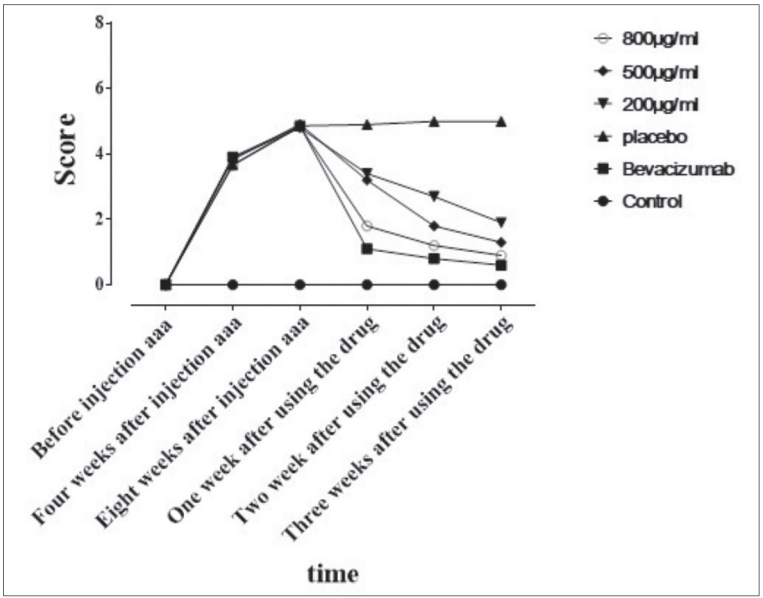

Fig 6. Clinical scoring of neovascularization based on the time course of treatment in different treated groups. Symbols shows clinical scoring of neovascularization in different groups before and after tretment

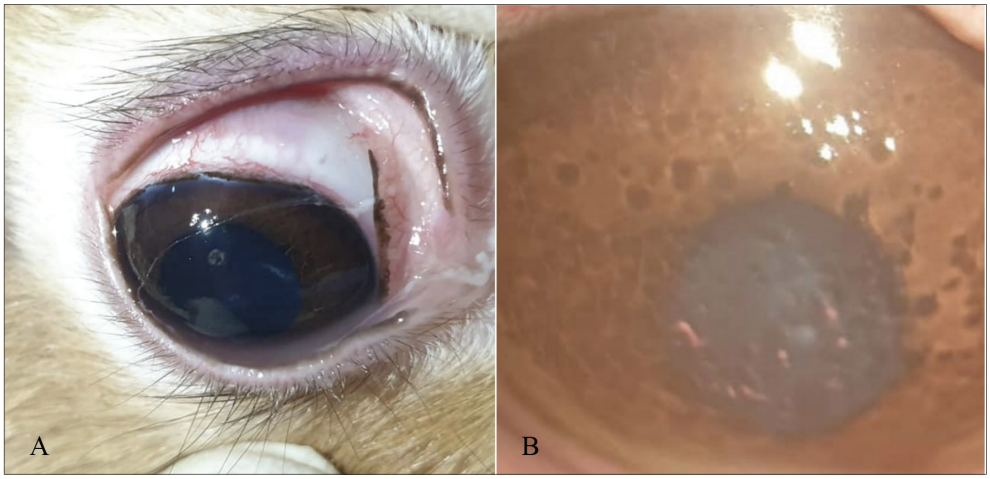

Fig 7. View of clinical examination of the rabbit eye after injection of α-AAA (A), Ophthalmoscopic view of the rabbit eye after injection of α-AAA (B)

The results of our study revealed that particle sizes of NFL were obtained between 148 to 215 nm. Moreover, the SEM image showed vesicular structures with an average diameter of 150 nm. Particle size is one of the most important absorption parameters. Smaller particles can provide more surface area for absorption. Reducing the particle size to the nanometer scale, especially below 100 nm, increases the desired properties such as stability and transparency of the system. The change in particle size occurs due to the phenomena of aggregation

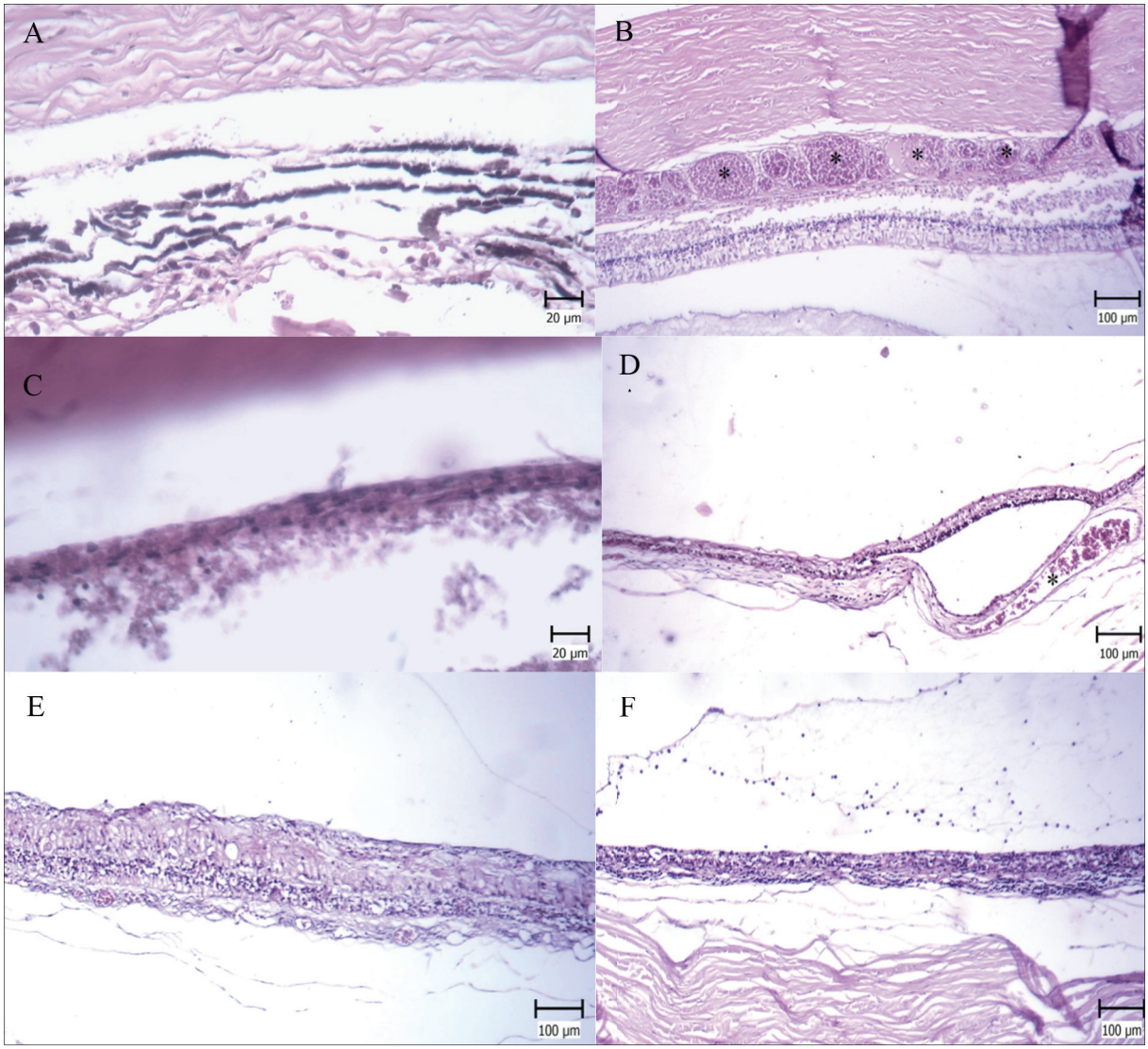

Fig 8. Histological findings of neovascularization. Control group (A), Placebo group (B), Bevacizumab (C), NLF 200 µg/mL (D), NLF 500 µg/mL (E) and NLF 800 µg/mL (F). Placebo group showed numerous and dilated blood vessels and many erythrocytes in the retinal stroma. Bevacizumab and NLF 800 µg/mL showed retinal stroma similar to control group. X40, H&E

and miscibility [34]. Basically, lecithin acts as the main skeleton in the formulation of liposomes and cholesterol is added to the formulation to increase the stability and stabilization of the liposomes. Thus, lecithin-rich liposomal membranes and the arrangement of acyl chains in one direction reduce the spaces created by the large polar groups in the lipid head, which in turn increases the contact and interaction between the chains. Cholesterol, on the other hand, may reduce the binding of hydrophobic molecules in bilayer membranes [35]. There have been various reports on the effect of cholesterol on liposome particle size, in which the liposome preparation method seems to have been very effective. The formation of complex colloids is accompanied by an increase in the particle size distribution. According to research, changes in physical properties such as particle size and particle size distribution of this liposomal formulation may be due to the accumulation of liposomes [34]. Malheiros et al.[36] concluded that the addition of cholesterol increased the diameter of vesicles in liposomes containing nisin. But, Gopinath et al.[37] reported that the increase in cholesterol concentration did not cause a significant change in particle size.

We showed that EE% values were between 43% and 66% so it can be considered a good value for a lipophilic compound such as naringenin. High EE% is recognized as an important factor utilized to state the liposomal formulation quality. Wang et al.[38] understood that Naringenin solubility and bioavailability could increase via Liposomal Nanoformulation. They obtained EE% of this formulation as 72.2±0.8%. The results of their study were consistent with our study.

Zeta potential was obtained as 15 mV for NLF. Zeta

potential is an important physical property of liposomes that is an important factor in determining the stability of colloidal systems and is the best indicator for determining the surface electrical status of dispersions. This factor indicates the amount of charge accumulation in the immobile layer and the intensity of adsorption of opposing ions to the particle surface, resulting in electrostatic stability. Zeta potential values of about 25 V m (positive or negative) are considered a criterion for separating the surfaces of particles with high and low electric charge. Colloidal systems containing particles with low zeta potential (positive or negative) have a high tendency to accumulate in the absence of other inhibitory factors such as high viscosity and spatial inhibition [39]. Cholesterol is a neutral molecule and the negative charge of the particles can be caused by the formation of a hydrogen bond between the choline group in phosphatidylcholine and the hydroxyl group in the cholesterol head. As a result of the formation of this bond, the positively charged choline group is drawn into the membrane and the negatively charged phosphatidyl group is drawn to the membrane surface, thus increasing the negative charge of the particles and the electrostatic repulsion between them [40]. Malheiros et al.[36] showed that the addition of cholesterol to the niacin-containing liposome increased the zeta potential.

It was observed that %D1 for batch No. 5 was 5.5% and batch No. 7 was 3.1%. Also, %D24 values were 72.93% and 52.01% belongs to batch No. 5 (selected formulation) and 7. Results revealed no cake formation and change in EE%. Moreover, no significant difference was seen in the average particle size of the optimized liposomes for 3 months after storage at room temperature. Release of drug from nanoliposomes depends on various factors including composition, synthesis method, type of drug and environmental conditions (temperature and pH). In the study of Tohidlou et al.[41] which used thin lipid film for preparation of liposomes, the %D1 was 5.8% and %D24 was 30%.

Another part of our result showed that clinical scoring of neovascularization enhanced significantly after injection of α-AAA. In addition, treatment with bevacizumab and NLF at all doses significantly decreased clinical score of neovascularization.

The rabbit model of neovascularization is recognized as a typical model for preclinical studies comparing the anti-angiogenic effects of various compounds. Emerging data have revealed that Bevacizumab could prevent neovascularization as an effective anti-angiogenic agent. It showed that bevacizumab-loaded albumin nanoparticles played role in the treatment of corneal neovascularization. The results of their study exhibited that bevacizumab-loaded albumin nanoparticles could decrease fibrosis, inflammation and edema in the rat model of neovascularization.

Naringenin is a flavonoid which has been shown protective against retinopathy in several studies [42,43]. Al-Dosari et al.[44] exhibited that naringenin could attenuate oxidative stress and apoptosis in diabetic retinopathy. In other studies, it has been determined that the administration of naringenin and other natural compounds in new drug delivery system [45,46] in the form of eye drops helps to inhibit corneal neovascularization through its anti-inflammatory and antioxidant properties [47,48]. Because effective antioxidant compounds [49] have antimicrobial, anti-inflammatory and restorative properties [50-52]. Naringenin has a low solubility which may lead to limitation in its bioavailability [38]. Researchers have been recommended liposomal nanoformulations for increase in enhanced solubility and bioavailability of naringenin [38]. Hence, the use of naringenin liposomal nanoformulations regarding to the useful effects of naringenin as a retinoprotective agent and liposomes as ocular drug delivery nanoparticles can have a beneficial function in the treatment of retinopathy.

Our results also demonstrated an extensive angiogenesis in the retina of placebo group compared to control group. However, treatment with bevacizumab and three doses of NLF especially 800 μg/mL could reverse retinal damage caused by injection of α-AAA. Researchers have shown anti-angiogenesis effect of naringenin for treatment of malignant melanoma [53]. It has also shown that naringenin could inhibit corneal neovascularization by anti-inflammatory and antioxidant mechanisms.

In the bevacizumab group, the retinal stroma had a limited number of vessels and was very similar to the control group. It was also seen that the number of blood vessels reduced in the NLF treated group with a concentration of 200 μg/mL, but a number of dilated vessels were still observed. Furthermore, the number of blood vessels also reduced in the NLF treated group with a concentration of 500 μg/mL and a much smaller number of dilated blood vessels were seen. In the NLF treated group with a concentration of 800 μg/mL, the microscopic structure showed a high improvement and was very similar to the bevacizumab and control groups.

The strength of the current study can be seen as its novelty. This means that the preparation of liposomal nanoparticles from naringenin and its effect on retinopathy has been done for the first time. Also, choosing the rabbit model as a standard model for investigating neovascularization was another strength of this study. However, limitations such as not investigating the cytotoxicity of prepared nanoparticles are also observed in this study.

Preparation of bioactive nanoformulation of naringenin

loaded into liposome was successfully carried out. SEM imaging confirmed successful formulation of NFL. The particle size, zeta potential and encapsulation efficiency of NFL revealed a good value for a lipophilic compound such as naringenin. The release and stability of NFL was reasonable. NFL exhibited ameliorative effects against neovascularization caused by α-AAA through reduced clinical score of neovascularization. Taken together, the present study demonstrated that the NFL suggested a capable nanoformulation with the peaked attenuating effect against neovascularization. Finally, the findings revealed that NFL could be proposed as a potential anti-neovascularization agent in retinopathy.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (F. Bagheri). The data are not publicly available due to privacy or ethical restrictions.

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Ethical Approval

The Animal Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (AJUMS) approved all of the experimental protocols (IR.AJUMS.ABHC.REC. 1399.064).

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Competing of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Author Contributions

AS, BSH and MF conceived and supervised this study. BSH, SHM and MF completed the main experimental content. AS, AR and FB collected and analyzed data. FB and AS wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Research Article

The Meta-Analysis of Conception Rates of Dairy Cattle Treated with the Ovsynch Protocol in Türkiye

Aytaç AKÇAY [1,a (*)] Murat ABAY [2,b] Elif ÇELİK [3,c]

[1] Ankara University, Faculty of Veterinary Medicine, Department of Biostatistics, TR-06110 Ankara - TÜRKİYE
[2] Erciyes University, Faculty of Veterinary Medicine Department of Obstetrics and Gynecology, TR-38039 Kayseri - TÜRKİYE
[3] Erciyes University, Faculty of Veterinary Medicine, Department of Biometrics, TR-38039 Kayseri - TÜRKİYE
ORCIDs: a 0000-0001-6263-5181; b 0000-0003-2457-1919; c 0000-0002-5073-1907

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Abstract: This study was aimed at the meta-analysis of the conception rates to the first insemination of dairy cattle treated with the Ovsynch protocol, and the determination of the sources of heterogeneity between the studies included in the analysis. The study material comprised of 46 primary studies conducted between 1999-2019 on the use of the Ovsynch protocol in Türkiye. The heterogeneity between these studies was assessed by meta-analysis using the random-effects model and the Der Simonian-Laird method. Accordingly, the common conception rate to the first insemination calculated for the primary studies, in which dairy cattle were treated with the Ovsynch protocol, was 0.412 (95% CI: 0.384-0.442) (P<0.001). The source of heterogeneity between the primary studies was determined by subgroup analyses. Conception rates were calculated for subgroups, which were established for geographical region, cattle breed, year of publication and parity. While the conception rates significantly differed for geographical region, the differences observed for cattle breed, year of publication and parity were statistically insignificant. When assessed for geographical region, the lowest common conception rate was determined in the Eastern Anatolia region (26%), whilst the highest common conception rate was determined in the Black Sea region (51.6%). The common conception rates calculated for cattle breed were 41.3% for Holstein cattle and 43.6% for Brown Swiss cattle. It was observed that the conception rates had decreased by 10% in the last 20 years to a level of 40.2% in the period between 2015 and 2019. The common conception rates calculated for parity were 39.4% for heifers and 41.9% for multiparous cows. It is considered that the results obtained in this study will contribute to the development of new strategies for a rational production in the dairy sector.

Keywords: *Conception rate, Dairy cattle, Meta-analysis, Ovsynch protocol, Synchronization*

Türkiye'de Ovsynch Protokolü Uygulanan Sütçü Sığırlarda Konsepsiyon Oranlarının Meta-Analizi

Öz: Bu çalışmada Ovsynch protokolü uygulanmış olan sürülerdeki ilk tohumlamadaki konsepsiyon oranlarının meta-analizi ile değerlendirilmesi ve çalışmalar arasındaki heterojenliğin kaynaklarının belirlenmesi amaçlanmıştır. Çalışma materyalini, 1999-2019 yılları arasında Türkiye'de yürütülmüş ve Ovsynch protokolü ile yapılmış 46 primitif çalışma oluşturmuştur. Çalışmalar arasında belirlenen heterojenlikten dolayı uygulanan meta-analizinde rastgele etki modeli altında Der Simonian-Laird yöntemi kullanılmıştır. Bu yöntem sonucunda Türkiye'de sütçü sığırlarda ovulasyon senkronizasyon için ovsynch protokolü uygulanmış tüm çalışmalarda ilk tohumlama sonucunda elde edilen ortak konsepsiyon oranı 0.412 (%95 CI: 0.384-0.442) hesaplanmıştır (P<0.001). Çalışmalar arası heterojenliğin kaynağını belirlemek için alt grup analizi yapılmıştır. Oluşturulan alt gruplara göre (coğrafi bölgeler, ırk, yıl ve parity) konsepsiyon oranları hesaplanmıştır. Konsepsiyon oranları arasındaki farklılık coğrafi bölgelere göre önemli bulunurken; ırk, yıl ve pariteye göre önemli bulunmamıştır. Coğrafi bölgelere göre Türkiye'de ortak konsepsiyon oranı en düşük Doğu Anadolu Bölgesinde (%26), en yüksek Karadeniz Bölgesinde (%51.6) belirlenmiştir. Irklara göre ortak konsepsiyon oranları Holştayn'larda %41.3, İsviçre Esmer'lerinde %43.6 olarak hesaplanmıştır. Yayının yapıldığı yıllara göre konsepsiyon oranı son yirmi yılda %10'luk düşüş göstererek son yıllarda (2015-2019) %40.2 'ye gerilemiştir. Pariteye göre ortak konsepsiyon oranı düvelerde %39.4, multipar ineklerde %41.9 hesaplanmıştır. Bu çalışmada elde edilen sonuçların süt sığırcılığında rasyonel üretim için gerekli olan yeni stratejilerin geliştirilmesine katkı sağlayacağı düşünülmektedir.

Anahtar sözcükler: *Konsepsiyon oranı, Meta analizi, Ovsynch protokolü, Senkronizasyon, Sütçü sığır*

Introduction

Recently, dairy holdings are faced with the problem of significantly reduced fertility in parallel with increased milk yields. Due to the difficulty of detecting estrus behavior after parturition or the development of postpartum anestrus

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(*) Corresponding Author

Tel: +90 312 3170315/4475 Cellular phone: +90 5055025853 Fax: +90 3123164472
E-mail: aytacakcay@gmail.com (A. Akçay)

problems in high-producing dairy cattle, performing artificial insemination within the economically viable time interval has become even harder. In 80% of dairy cattle, the first postpartum ovulation occurs within 50 days after parturition, and of these animals, only 54-68% maintain cyclicity. Both estrus detection and timely insemination directly affect the reproductive performance of animals [1].

In dairy cattle, estrus or ovulation synchronization techniques are used to increase reproductive yields, detect estrus, perform timely artificial insemination and avoid postpartum anestrus problems. Synchronization also allows for ease of application, reducing human errors and decreasing labor costs. When applying estrus synchronization techniques, estruses and/or ovulations not being able to be induced timely in all animals, and factors such as ovulations spreading over a one-week period prevent the achievement of the intended conception rates [2,3]. Ovulation synchronization methods synchronize follicular development and corpus luteum regression, such that fixed-time artificial insemination can be performed without the need for observing estrus signs. Cattle with a known ovulation time can be inseminated within the targeted time period to achieve the conception rate required for the intended reproductive yield. These protocols, which mimic the natural estrus cycle, involve the use of several hormones including progestogens, prostaglandin (PG) F2α, gonadotropin-releasing hormone (GnRH), luteinizing hormone, follicle-stimulating hormone and estrogen [4,5].

A fixed-time artificial insemination program has been developed, based on ovulation synchronization (Ovsynch) through the combined use of GnRH and PGF2α. The Ovsynch protocol involves injections of GnRH (on days 0 and 9) and PGF2α (on day 7) and the performance of artificial insemination 16-24 h after the last GnRH injection. This protocol is aimed at achieving follicular development in parallel with luteal regression, and thereby, synchronizing ovulations [6]. The Ovsynch protocol has found common use in dairy holdings, yet the conception rates achieved with this protocol have been reported to be lower than those achieved with inseminations based on estrus detection [7,8]. Furthermore, significant differences have been observed between the conception rates achieved with the use of the Ovsynch protocol in different studies [9].

The conception rates achieved in lactating dairy cattle with the use of the Ovsynch protocol have been reported to range from 32% to 76.9% [6,10-12].

While literature reviews bring together different results obtained from studies conducted on a particular subject, the meta-analysis method has been developed with an aim to combine different results for the generation of a common result. Meta-analysis is a statistical method used to combine and synthesize independent individual studies conducted on a particular subject with an aim to provide interpretation through the conversion of their results into a common measurement unit [13,14].

The present study was designed to perform a meta-analytical assessment of the conception rates achieved with the use of the Ovsynch protocol for ovulation synchronization in dairy cattle raised in Türkiye. For this purpose, firstly the heterogeneities between the primary studies were determined and pooled conception rates were calculated for these studies included in the meta-analysis. Next, subgroups were established for the year of publication, geographical region, cattle breed, and parity (heifers and cows) to perform meta-regression analyses with an aim to determine the sources of heterogeneity between the studies.

Material and Methods

The study material comprised of 46 primary studies, which were selected in view of predetermined inclusion criteria, among 266 studies listed by a database scan *(Table 1)*. Literature searches were performed using "Ovsynch" and "cattle" search strategy in PubMed and Web of Science databases. The inclusion criteria were the study having been conducted in Türkiye, having been published between 1999-2019, having investigated the use of the Ovsynch protocol in dairy cattle for ovulation synchronization, and having reported conception rates achieved with the use of the Ovsynch protocol. Considering the PICO criteria, the problem was determined as the rate of conception with the Ovsynch protocol, intervention was the application of the Ovsynch protocol, the comparison criterion was the comparison of the rates of conception by years and regions, and the result was the effect of the Ovsynch protocol on the conception rates. The PRISMA Statement guidelines were followed in determining the studies to be included in the meta-analysis and the flow chart diagram is presented in *Fig. 1* [15].

Begg and Mazumdar's rank correlation test was used to determine the publication bias of the included studies, and a funnel plot was built as a graphic representation of the effect size and sample size. The funnel plot, where the "x" axis showed the conception rates and the "y" axis showed the standard error of the effect size, was drawn in the reverse scale (zero at the top with downward increasing values). An assessment was made of the heterogeneity between the primary studies for effect size, with an aim to select the meta-analysis method (fixed-effects or random-effects models) to be used for combining the results of the primary studies and calculating the common rates. The degree of heterogeneity was assessed visually using a forest graph and by means of some statistical measures. An increased distance between the point estimates and a

Table 1. Characteristics of studies included in the meta-analysis

No	Study	Number of Animal	Number of Conception	Conception Rate	Breed	Region	Animal Parity
1	**Kaya A, Çoyan K, Semacan A:** GnRH ve PGF2a kombinasyonunun ineklerde östrüs senkronizasyonu ve gebelik üzerine etkisi. *Vet Bil Derg* 15 (1): 121, 1999	10	6	0.600	Brown Swiss	Central Anatolia	Cow
2	**Çoyan K, Ataman MB, Erdem H, Kaya A, Kasıkcı G:** Synchronization of estrus in cows using DoublePGF2α, GnRH-PGF2α and hCG-PGF2α combination. *Revue Méd Vét* 154 (2): 91-96, 2003	10	6	0.600	Brown Swiss	Central Anatolia	Cow
3	**Aral F, Çolak M:** Reproductive performance and synchronization of the ovulation and estrus in brown Swiss Cows and heifers using the protocol GnRH- PGF2 alpha-GnRH and PGF2 alpha. *Turk J Vet Anim Sci*, 28 (1): 179-184, 2004	13	5	0.385	Brown Swiss	Central Anatolia	Cow
4	**Aral F, Çolak M:** Esmer ırk inek ve düvelerde GnRH- PGF 2 alfa-GnRH ve PGF 2 alfa ile östrus ve ovulasyon senkronizasyonu ve döl verim performansı. *Turk J Vet Anim Sci*, 28: 179-184, 2004	13	6	0.462	Brown Swiss	Central Anatolia	Heifer
5	**Nak Y, Nak D, İntaş KS, Tek HB, Keskin A, Tuna B:** Ovsynch, PRD + PGF2α + PMSG ve norgestomet içeren kulak implantı + PGF2α + PMSG ile sağıtılan siklik ve asiklik anöstrüslü sütçü ineklerde kızgınlık ve gebelik oranlarının karşılaştırılması. *Uludag Univ J Fac Vet Med*, 24 (1-2-3-4): 33-39, 2005	109	46	0.422	Holstein	Marmara	Cow
6	**Nak Y, Nak D, İntaş KS, Tek HB, Keskin A, Tuna B:** Ovsynch, PRD + PGF2α + PMSG ve Norgestomet içeren kulak implantı + PGF2α + PMSG ile sağıtılan siklik ve asiklik anöstrüslü sütçü ineklerde kızgınlık ve gebelik oranlarının karşılaştırılması. *Uludag Univ J Fac Vet Med*, 24 (1-2-3-4): 33-39, 2005	34	20	0.588	Holstein	Marmara	Heifer
7	**Adataş TY:** İneklerde Ovsynch ve Co-synch yöntemleri ile ovulasyonun senkronizasyonu. Yüksek lisans tezi, Ankara Üniversitesi Sağlık Bilimleri Enstitüsü, 2006	20	9	0.450	Holstein	Central Anatolia	Cow
8	**Kacar C, Yildiz S, Pancarci SM, Kaya M, Oral H, Gurbulak K, Gungor O:** Administration of GnRH treatment prior to Ovsynch protocol to stimulate ovarian cycle in cows with functional anoestrus. *Bull Vet Inst Pulawy*, 50 (4): 497-501, 2006	24	5	0.208	Crossbred	Eastern Anatolia	Cow
9	**Cirit U, AK K, Ileri IK:** New strategies to improve the efficiency of the Ovsynch protocol in primiparous dairy cows. *Bulletin of the Veterinary Institute in Pulawy*, 51 (1): 47-51, 2007	18	9	0.500	Holstein	Marmara	Cow
10	**Gumen A, Keskin A, Tek HB, Yilmazbas G, Seyrek İntaş K;** Sütçü ineklerde Ovsynch yönteminde yapılan modifikasyonla gebelik oranının artırılması. *II. Veteriner Jinekoloji Kongresi*,122-123, Antalya, 2006	38	17	0.447	Holstein	Marmara	Cow
11	**Kaçar C, Kamiloğlu NN, Uçar Ö, Arı UÇ, Pancarcı ŞM, Güngör Ö:** İneklerde β-karoten + E vitamini uygulamasıyla kombine edilen Ovsynch ve Cosynch senkronizasyon programlarının gebelik oranı üzerine etkisi. *Kafkas Üniv Vet Fak Derg*, 14 (1): 45-50, 2008.	57	10	0.175	Crossbred	Eastern Anatolia	Cow
12	**Bülbül B, Kırbaş M, Köse M, Dursun Ş, Çolak M:** İneklerde östrüs siklusunun farklı dönemlerinde başlatılan Ovsynch protokolünün östrüs senkronizasyonuna etkileri, *İstanbul Üniv Vet Fak Derg*, 35 (1): 7-17, 2009	41	20	0.488	Brown Swiss	Central Anatolia	Cow
13	**Çelik HA, Avcı G, Aydın İ, Bülbül A, Bülbül T**: Effect of β-carotene on ovarium functions and Ovsynch success in repeat breeder cows. *Kafkas* Üniv *Vet Fak Derg*, 15 (1): 87-94, 2009	11	3	0.273	Holstein	Aegean	Cow
14	**Elibol E, Uçar M, Yılmaz O:** Ovsynch uygulanan ineklerde sun'i tohumlama sonrası 12. günde yapılan GnRH enjeksiyonunun gebelik oranına etkisi. *Kocatepe Vet J*, 2 (1): 13-18, 2009	20	11	0.550	Holstein	Aegean	Cow
15	**Aksu EH, Bozkurt T, Türk G:** Farklı senkronizasyon uygulamaları ile senkronize edilen ineklerde üreme performansı üzerine vitamin E'nin etkisi. *FÜ Sağ Bil Vet Derg*, 24 (2): 71-76, 2010	13	3	0.231	Holstein	Eastern Anatolia	Cow
16	**Abay M, Bekyürek T, Demiral O, Atabay Ö**: Holştayn ırkı primipar ineklerde post partum dönemde Cosynch ve Ovsynch uygulamalarının gebelik oranları üzerine etkisi, *III: Veteriner Jinekoloji Kongresi*, Antalya, 2008	16	6	0.375	Holstein	Central Anatolia	Cow
17	**Çevik M, Selçuk M, Doğan S:** Comparison of pregnancy rates after timed artificial insemination in Ovsynch, Heatsynchand CIDR-based synchronization protocolin dairy cows. *Kafkas Univ Vet Fak Derg*, 16 (1): 85-89, 2010	13	10	0.769	Holstein	Black Sea	Cow
18	**Doğruer G, Sarıbay MK, Karaca F:** Laktasyondaki sütçü ineklerde ovsynch ve çift doz PGf2 alfa+ GnRH uygulamaları sonrası elde edilen gebelik oranlarının kıyaslanması. *IV. Veteriner Jinekoloji Kongresi*, 162-163, Antalya, 2010	42	15	0.357	Holstein	Mediterranean	Cow
19	**Keskin A, Mecitoğlu GY, Karakay E, Taşdemir U, Alkan A, Okut H, Gümen A**: Siklik ve siklik olmayan sütçü ineklerde Ovsynch protokolüne verilen yanıtın karşılaştırılması. *Uludag Univ J Fac Vet Med*, 29 (2): 27-34, 2010	250	94	0.376	Holstein	Marmara	Cow
20	**Nak Y, Tuna B, Nak D, Karakaş E:** Kızgınlıkları gözlenemeyen inek ve düvelerde Ovsynch, Ovsynch+Progestin ve Ovsynch+Progestin+çift suni tohumlamanın gebelikler üzerine etkisi. *IV. Veteriner Jinekoloji Kongresi*, 18-19, Antalya, 2010	173	69	0.399	Holstein	Central Anatolia	Heifer
21	**Pancarcı ŞM, Güngör Ö, Lehimcioğlu NC, Kaçar C, Öztürkler Y:** Sağmal ineklerde Ovsynch protokolü sırasında farklı CIDR uygulamarının follikül dalgaları ve aksesuar CL oluşumuna etkileri. *IV. Veteriner Jinekoloji Kongresi*, 242-243, Antalya, 2010	6	2	0.333	Brown Swiss	Eastern Anatolia	Cow
22	**Yıldız A:** Effect of administering Ovsynch protocol plus postbreeding infusion on first service pregnancy outcome in cows. *J Anim Vet Adv*, 9 (9): 1345-1350, 2010	12	3	0.250	Holstein	Eastern Anatolia	Cow

Table 1. Characteristics of studies included in the meta-analysis (continued)

No	Study	Number of Animal	Number of Conception	Conception Rate	Breed	Region	Animal Parity
23	**Kara U, Ayaşan T, Hızlı H, Gök K:** Ovsynch protokolünün inek ve düvelerin gebelik oranı üzerine etkisi. *Erciyes Üniv Vet Fak Derg*, 8 (1): 1-8, 2011	24	12	0.500	Holstein	Mediterranean	Cow
24	**Kara U, Ayaşan T, Hızlı H, Gök K:** Ovsynch protokolünün inek ve düvelerin gebelik oranı üzerine etkisi. *Erciyes Üniv Vet Fak Derg*, 8 (1): 1-8, 2011	24	7	0.292	Holstein	Mediterranean	Heifer
25	**Abay M:** Holştayn ırkı düvelerde uygulanan ovulasyon senkronizasyonunda iki farklı GnRH analoğu ve beta karoten + E vitaminin etkinliği. Doktora tezi, Erciyes Üniversitesi Sağlık Bilimleri Enstitüsü, 2010	40	18	0.450	Holstein	Central Anatolia	Heifer
26	**Keskin A, Mecitoğlu GY, Karakay E, Taşdemir U, Alkan A, Okut H, Gümen A:** Siklik ve siklik olmayan sütçü ineklerde ovsynch protokolüne verilen yanıtın karşılaştırılması, *Uludag Univ J Fac Vet Med*, 29 (2): 27-34, 2010.	347	178	0.513	Holstein	Marmara	Cow
27	**Yılmaz C, Yılmaz O, Ucar M:** Effect of PGF2α and GnRH injections applied before Ovsynch on pregnancy rates in cows and heifers. *Kafkas Univ Vet Fak Derg*, 17 (4): 641-644, 2011	37	14	0.378	Holstein	Aegean	Cow
28	**Yılmaz C, Yılmaz O, Ucar M:** Effect of PGF2α and GnRH injections applied before Ovsynch on pregnancy rates in cows and heifers. *Kafkas Univ Vet Fak Derg*, 17 (4): 641-644, 2011	80	26	0.325	Holstein	Aegean	Heifer
29	**Abay M, Akçay A, Bekyürek T, Gürbulak K, Canooğlu E:** Sütçü ineklerde Ovsynch protokolünde iki farklı GNRH analogunun epidural ve intramuskuler uygulamalarının gebelik oranı üzerine etkisi. Erciyes Üniversitesi Bilimsel Araştırma Projesi Sonuç Raporu (TSA-12-4057), Kayseri, 2012	400	148	0.370	Holstein	Central Anatolia	Cow
30	**Çınar M, Güzeloğlu A, Erdem H:** Effect of presence of corpus luteum at the beginning of Ovsynch protocol on pregnancy rates in lactating dairy cows. *Kafkas Univ Vet Fak Derg*, 18 (3): 513-516, 2012	140	44	0.314	Holstein	Central Anatolia	Cow
31	**Emre B, Zonturlu AK, Korkmaz Ö:** Sütçü ineklerde Ovsycnh protokolünü takiben uygulanan Fluniksin Meglumin'in gebelik oranı üzerine etkisi. *Harran* Üniv *Vet Fak Derg*, 1 (2): 88- 91, 2012	26	14	0.538	Holstein	Southeastern Anatolia	Cow
32	**Gumen A, Keskin A, Mecitoglu GY, Karakaya E, Alkan A, Okut H, Wiltbank MC:** Effect of presynchronization strategy before ovsynch on fertility at first service in lactating dairy cows. *Theriogenology*, 78 (8): 1830–1838, 2012.	126	59	0.468	Holstein	Marmara	Cow
33	**Emre B, Korkmaz Ö, Zonturlu AK:** Sütçü İneklerde Ovsynch protokolünde ikinci GnR uygulamasının geciktirilmesinin gebelik oranı üzerine etkisi. *Atatürk* Üniversitesi *Vet Bil Derg*, 9(3): 187-193, 2014	40	22	0.550	South Anatolian Red	Southeastern Anatolia	Cow
34	**Karakaya E, Yilmazbas-Mecitoglu G, Keskin A, Alkan A, Tasdemir U, Santos J, Gumen A:** Fertility in dairy cows after artificial insemination using sex-sorted sperm or conventional semen. *Reprod Dom Anim*, 49 (2): 333-337, 2014	156	63	0.404	Holstein	Marmara	Cow
35	**Karyağdı SS, Demiral ÖO, Abay M:** Sütçü ineklerde klasik ovulasyon senkronizasyonu protokolünde progesteron ve östrojen uygulamalarının gebelik oranlarına etkisi. *Erciyes* Üniv *Vet Fak Derg*, 11 (3): 175-182, 2014	43	19	0.442	Holstein	Black Sea	Cow
36	**Köse M, Tekeli T:** İsviçre Esmeri düve ve laktasyonda olmayan ineklerde ovaryum fonksiyonlarının östrüs senkronizasyonu ve gebelik oranı üzerine etkisi. *Eurasian J Vet Sci*, 30 (2): 53-58, 2014	20	8	0.400	Brown Swiss	Central Anatolia	Cow
37	**Köse M, Bülbül B, Dursun Ş, Kırbaş M:** Düvelerde östrüs siklusunun folliküler ya da luteal evresinde başlatılan Ovsynch protokolünün folliküler ve luteal senkronizasyon üzerine etkisi. *YYU Vet Fak Derg*, 25 (1): 7-10, 2014	28	8	0.286	Brown Swiss	Central Anatolia	Heifer
38	**Yilmazbas-Mecitoglu G, Karakaya E, Keskin A, Gumen A, Koc V, Okut H:** Comparison of synchronisation and fertility after different modifications of the ovsynch protocol in cyclic dairy cows. *Acta Veterinaria Hungarica*, 62 (1): 64-73, 2014	105	51	0.486	Holstein	Marmara	Cow
39	**Abay M, Bekyürek T, Akçay A, Ata S:** Sütçü ineklerde farklı senkronizasyon protokollerinin gebelik oranı üzerine etkisi. *VI. Türk Veteriner Jinekoloji Derneği Ulusal Kongresi*, 132-133, Muğla, 2015	185	76	0.411	Holstein	Central Anatolia	Cow
40	**Salar S, Baştan A:** Erken postpartum dönemde subklinik ketozisli ineklerin ovsynch protokolüne yanıtlarının incelenmesi. Ankara Üniversitesi Bilimsel Araştırma Projesi Sonuç Raporu (14L0239002), Ankara, 2016	156	48	0.308	Holstein	Central Anatolia	Cow
41	**Çakırcalı R, Gümen A, Karakaya Bilen E, Orman A, Mecitoğlu Z, Keskin A:** Sütçü ineklerde Ovsynch protokolü süresince uygulanan Propilen Glikol'ün fertilite üzerine etkisi. *VII. Ulusal II. Uluslararası Türk Veteriner Jinekoloji Derneği Kongresi,* 170-176, Antalya, 2019	72	22	0.306	Holstein	Marmara	Cow
42	**Karaca F, Dogruer G, Sarıbay MK, Yasar E, Ateş C:** The effect of the reduced dose of GnRH on conception, ovulation and ovarian structures in Ovsynch program of lactating dairy cows. *Animal Review*, 3 (3): 66-72, 2016	20	8	0.400	Holstein	Mediterranean	Cow
43	**Karakaya-Bilen, E, Yilmazbas-Mecitoglu G, Keskin A, Guner B, Serim E, Santos J, Gümen A:** Fertility of lactating dairy cows inseminated with sex-sorted or conventional semen after Ovsynch, Presynch-Ovsynch and Double-Ovsynch protocols. *Reprod Dom Anim*, 5 (2): 309–316, 2019	50	21	0.420	Holstein	Marmara	Cow
44	**Küçük N, Tuna B, Peker C, Uçar EH:** Farklı ovsynch protokolleri ile senkronize edilen Holstein düvelerde ovaryum dinamiklerinin ve gebelik oranlarının araştırılması. *Uluslararası* Çiftlik *Hayvanları Hekimliği Kongresi*, Muğla, 2019	32	19	0.594	Holstein	Aegean	Cow
45	**Shahzad AH, Sattar A, Ahmad N, Ahmad I, Nak D, Nak Y:** Evaluation of Ovsynch and CIDR + Ovsynch protocols to improve reproductive efficiency in lactating dairy cows. *Pakistan J Zool*, 51 (5): 1607-1614, 2019	58	23	0.397	Holstein	Marmara	Cow
46	**Topçu E, Binli F, Ay SS:** Sütçü ineklerde Progesteron (PRID®) ile desteklenen Ovsycnh yönteminin gebelik oranı üzerine etkisi. *Dicle* Üniv *Vet Fak Derg*, 11(2): 71-76, 2018.	30	16	0.533	Holstein	Black Sea	Cow

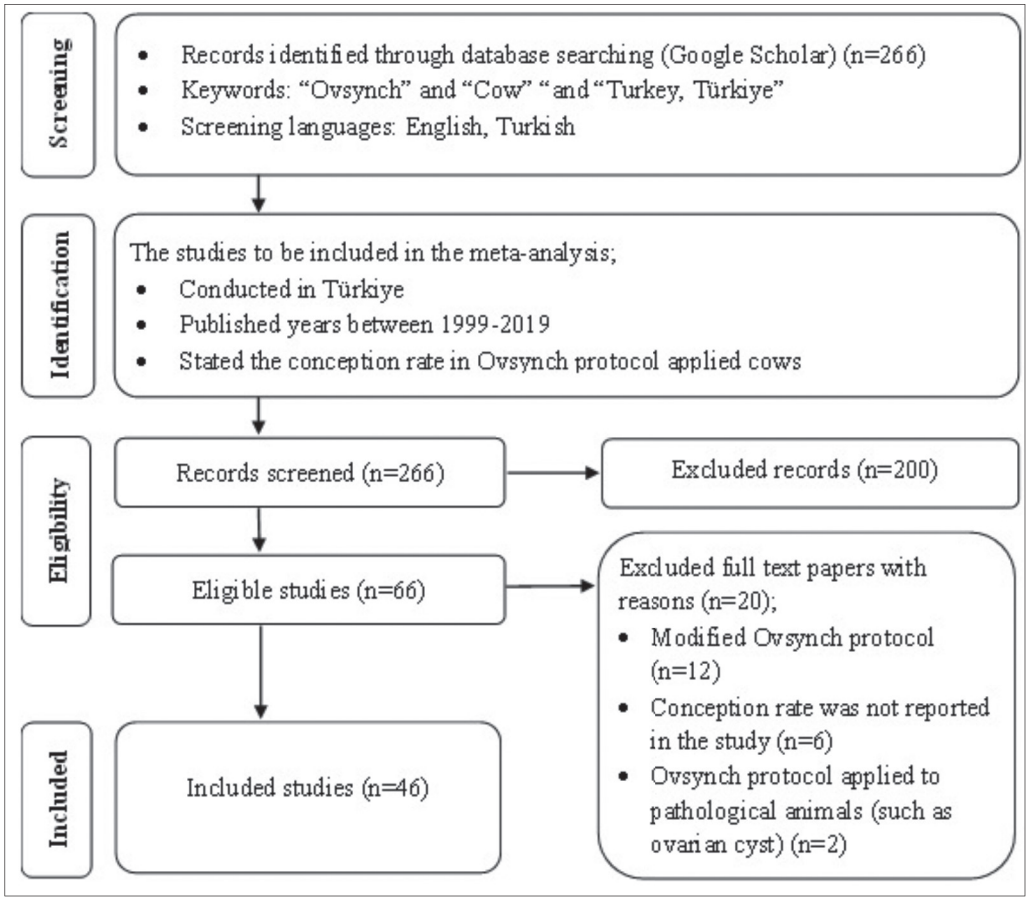

Fig 1. Flow diagram of the literature search selection for studies that Ovsynch protocol applied, and conception rate determined in Türkiye

low intersection of the confidence intervals of studies in a forest graph point out to a high degree of heterogeneity.

Cochran's Q test was used to determine the heterogeneities between the studies, I^2 statistics were used to determine the level of heterogeneity and τ^2 statistics were calculated to determine the true between study variances. As analyses demonstrated a heterogeneity between the primary studies for effect size, the random-effects model (the Der Simonian-Laird method) was chosen. The random-effects model considers variances both within and between the included studies and assumes that there are differences between all studies for effect size.

Subgroup analyses were performed to determine the sources of heterogeneity between the studies. Conception rates were calculated for the subgroups, which were established for geographical region, cattle breed, year of publication and parity. Furthermore, in view of the heterogeneity between the studies, to ensure an effective, reliable and valid parameter estimation with minimum variance, a univariate meta-regression analysis was performed, and a comparison was made of the conception rate alterations observed within the subgroups. The moments method was used for the meta-regression analysis, and models were established according to the random-effects model. A low number of studies being included in the analysis is a major disadvantage for meta-regression analyses. According to the criterion set for the number of studies required for establishing a subgroup, it was not possible to include 4 studies that were conducted in southeastern Anatolia in hybrid and South Anatolian Red cattle. Thus, it was not possible to assess the interaction of multiple factors. Subgroups were established for the geographical region of the study location (Mediterranean, Eastern Anatolia, Aegean, Central Anatolia, Black Sea and Marmara regions), cattle breed (Holstein and Brown Swiss), publication year of the study (1999-2004, 2005-2009, 2010-2014 and 2015-2019) and parity (heifers and multiparous cows). Thereby, common conception rates were calculated for the subgroups and the correlation of these rates were assessed. The statistical significance of the meta-regression models was assessed with the Z test. Meta-analyses were performed using the CMA (Comprehensive Meta Analysis) and R 4.2.1 *(www.r-project.org/)* software and "metaphor" package. The significance level of the Cochran Q heterogeneity statistics was set at $P<0.10$ and the significance level of the effect size and coefficients was set at $P<0.05$.

Results

In the present study, the conception rates of a total of 3182 cattle, which were treated with the Ovsynch protocol in 46 studies conducted between 1999-2019, were included in the meta-analysis. The conception rates reported in these previous studies are shown in the *Table 1*.

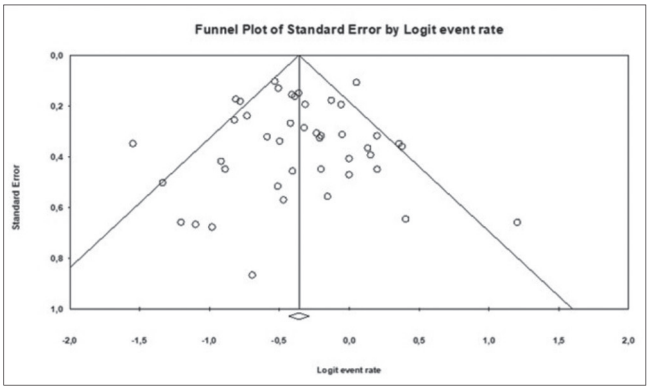

Fig 2. Funnel plot of study sample regarding conception rates with Ovsynch protocol

Begg and Mazumdar's rank correlation test was performed to determine the publication bias of the studies included in the meta-analysis. Kendall's tau b coefficient was calculated. This coefficient is expected to be close to 1 and the p value is expected to be larger than 0.05. Begg and Mazumdar's rank correlation test showed that the study sample was not biased (Kendall's tau=0.04, P=0.670). In the funnel plots, the scattering of the studies included in the meta-analysis within a triangle with a downward-facing base indicated that there was no asymmetry in the study sample *(Fig. 2)*.

The heterogeneity observed between the primary studies for the conception rates was assessed with Cochran's Q, I^2 and τ^2 test statistics (Q=92.392, P<0.001, I^2=51.295, τ^2 =0.067). I^2 (%) calculations showed that there was a moderate level of heterogeneity between the studies for the conception rates. The forest plot, which was built as a graphic representation of this heterogeneity, showed the

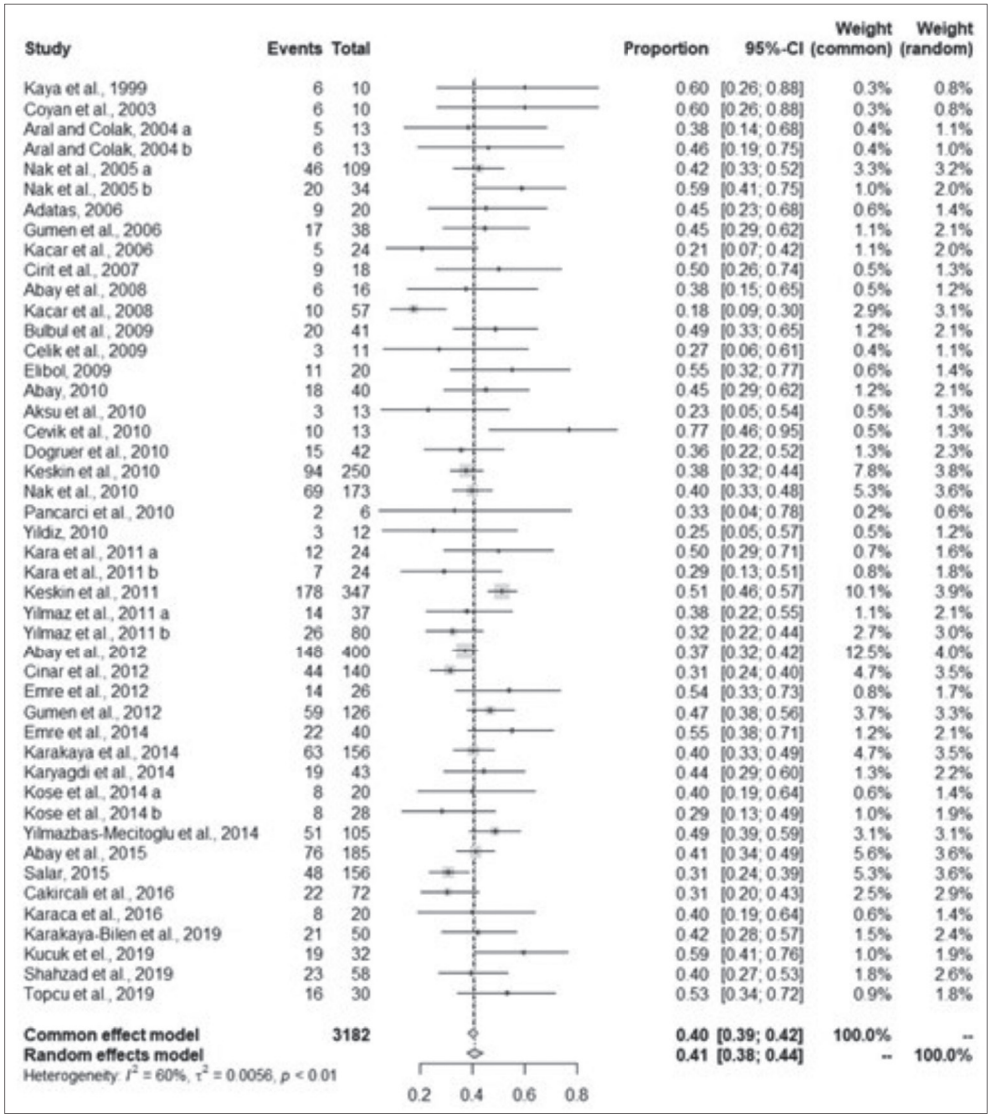

Fig 3. Forest plot regarding conception rates with Ovsynch protocol

Table 2. *Subgroup analysis results by region, breed, year and animal*

Item	Subgroups	Overall Rates of Conception Rates					Heterogeneity		
		Number of Study	Number of Cows	Number of Conception Cows	Conception Rate	95% Confidence Interval of Conception Rate	Cochran's Q	I^2 %	P Values (Cochran's Q)
Region	Mediterranean	4	110	42	0.384	0.297; 0.479	2.342	0.01	Q=10.463, df=5, **P=0.063**
	Aegean	5	180	73	0.425	0.309; 0.549	9.186	56.46	
	Central Anatolia	15	1265	477	0.378	0.352; 0.405	15.653	10.56	
	Black Sea	3	86	45	0.516	0.408; 0.622	3.974	49.68	
	Marmara	12	1363	603	0.438	0.397; 0.480	22.649	51.433	
	Eastern Anatolia	3	31	8	0.260	0.135; 0.440	0.230	0.0	
Breed	Holstein	34	2894	1187	0.413	0.384; 0.443	66.272	50.21	Q=0.244, df=1, **P=0.621**
	Brown Swiss	8	141	61	0.436	0.354; 0.520	5.567	0.0	
Year	1999-2004	4	46	23	0.500	0.357; 0.643	1.546	0.0	Q=4.263, df=3, **P=0.234**
	2005-2009	9	307	141	0.460	0.405; 0.517	5.683	0.0	
	2010-2014	21	2079	851	0.403	0.366; 0.441	44.397	54.95	
	2015-2019	8	603	233	0.402	0.340; 0.466	14.858	52.89	
Animal Parity	Multipar Cow	35	2643	1094	0.419	0.389; 0.449	61.699	44.89	Q=0.326, df=1, **P=0.568**
	Heifer	7	392	154	0.394	0.347; 0.444	9.856	39.12	

Cochrane's Q: Weighted sum of squares of observed effect sizes; I^2 %: The rate of true variance to observed variance; df: Degree of freedom

point estimates and confidence intervals of the studies included in the meta-analysis. Using the Der Simonian-Laird method in association with the random-effects model, the common conception rate for all of the studies, which were included in the meta-analysis and in which dairy cattle raised in Türkiye were treated with the Ovsynch protocol, was calculated as 0.412 (0.384-0.442) (P<0.001) *(Fig. 3)*.

The results of the subgroup analyses performed to determine the source of heterogeneity between the primary studies are shown in *Table 2*. Statistically significant differences were detected between the subgroups established for the geographical regions of the study locations (P<0.10). Accordingly, the lowest conception rate was determined in the Eastern Anatolia Region (26%). No statistically significant difference was determined between the subgroups established for cattle breed (P>0.1). The common conception rates for Holstein and Brown Swiss cattle were calculated as 41.3% and 43.6%, respectively. The subgroups established for the publication year of the studies also showed no statistically significant difference (P>0.1). The lowest common conception rate (40.2%) was determined for the most recently conducted studies (2015-2019). Likewise, the subgroups established for parity did not show any statistically significant difference (P>0.1). The common conception rates for heifers and multiparous cows were calculated as 39.4% and 41.9%, respectively.

The statistical significance of the heterogeneity sources was calculated with univariate meta-regression analyses. In the meta-regression model, the conception rate was the dependent variable and geographical region, cattle breed, year of publication and parity were the independent variables. The use of the hypothesis test with all coefficients as zero in a model assessing conception rates for independent variables of breed, year of publication and parity showed that there was no statistically significant difference (P>0.1). However, it was determined that the geographical regions of Türkiye led to statistically significant differences in the conception rates and was a factor influential on the heterogeneity observed between the primary studies (Q=9.63; P<0.1). In the meta-regression model established for geographical regions, the R^2 analogue value was calculated as 0.34. Therefore, the conception rates for geographical region were determined to account for 34% of the heterogeneity between the studies. Accordingly, the model used for meta-analysis demonstrated that, with reference to the Eastern Anatolia Region, the common conception rate for the Black Sea Region was calculated 1.140 fold higher (P=0.020) *(Table 3)*.

Discussion

In recent years, studies have been conducted on the assessment of the effects of different synchronization methods on the conception/pregnancy rates of dairy cattle by meta-analysis [16-20].

Borchardt et al.[17], upon making a meta-analytical comparative assessment of studies reporting conception rates achieved with the use of different synchronization

Table 3. *Univariate meta-regression models regarding conception rates with Ovsynch protocol*

Item	Variables	Coefficient of Meta-regression Model of Conception Rates					Test of the model (Simultaneous Test that All Coefficients are Zero)	Univariate meta-Regression Models and R^2 Analog Values
		Coefficient	%95 Confidence Interval of Coefficient	Standard Error	Z Values	P Values		
Region	Intercept	-1.046	-1.878; -0.213	0.425	-2.46	**0.014**	Q = 9.63 P = 0.086	CR_{Region}= -1.046 + 0.575(MeR) + 0.705(AR) + 0.579(CAR) + 1.140(BSR) + 0.797(MaR) R^2 analog = 0.34
	Mediterranean	0.575	-0.361; 1.511	0.478	1.20	0.229		
	Aegean	0.705	-0.199; 1.609	0.461	1.53	0.127		
	Central Anatolia	0.579	-0.270; 1.429	0.433	1.34	0.181		
	Black Sea	1.140	0.176; 2.105	0.492	2.32	**0.020**		
	Marmara Region	0.797	-0.050; 1.644	0.432	1.84	0.065		
	Eastern Anatolia (Reference)							
Breed	Intercept	-0.260	-0.642; 0.122	0.195	-1.33	0.182	Q = 0.20 P = 0.656	CR_{Breed}= -0.260 + 0.091(HF) R^2 analog = 0.01
	Holstein	0.091	-0.491; 0.309	0.204	-0.45	0.656		
	Brown Swiss (Reference)							
Year	Intercept	-0.412	-0.649; -0.174	0.121	-3.40	**0.001**	Q = 3.86 P = 0.277	CR_{Year}= -0.412+0.417(Y_1) + 0.268(Y_2) + 0.020(Y_3) R^2 analog = 0.01
	1999-2004	0.417	-0.252; 1.085	0.341	1.22	0.222		
	2005-2009	0.268	-0.100; 0.637	0.188	1.43	0.153		
	2010-2014	0.020	-0.259; 0.299	0.143	0.14	0.888		
	2015-2019 (Reference)							
Animal Parity	Intercept	-0.423	-0.709; -0.137	0.146	-2.90	**0.004**	Q = 0.35 P = 0.552	CR_{Animal}= -0.423 + 0.095(C) R^2 analog = 0.01
	Multipar Cow	0.095	-0.217; 0.407	0.159	0.60	0.552		
	Heifer (Reference)							

Q: *Cochrane's Q (Weighted sum of squares of observed effect sizes),* ***CR:*** *Conception Rates,* ***MeR:*** *Mediterranean Region,* ***AR:*** *Aegean Region,* ***CAR:*** *Central Anatolia Region,* ***BSR:*** *Black Sea Region,* ***MaR:*** *Marmara Region,* ***HF:*** *Holstein-Friesian,* Y_1***:*** *1999-2004,* Y_2***:*** *2005-2009,* Y_3***:*** *2010-2019,* ***C:*** *Cow*

protocols, calculated common conception rates of 41.7% for the Presynch + Ovsynch protocol and 46.2% for the double Ovsynch protocol. The common conception rate calculated in the present study for the use of the Ovsynch protocol in dairy cattle from Türkiye is similar to the common conception rate previously reported for the Presynch + Ovsynch protocol, and lower than that reported for the Double Ovsynch protocol [17].

Rabiee et al.[19] assessed conception rates reported to have been achieved with the use of the Ovsynch, Presynch and Selectsynch protocols by meta-analysis, whilst Borchart et al.[18] assessed conception rates reported to have been achieved with the use of the Ovsynch protocol and the modified Ovsynch protocol by meta-analysis [18,19].

In the present study, with an aim to produce meta-analysis results closer to the population parameter, conception rates achieved with the use of the prostaglandin-based Ovsynch protocol, which is known to have common use in Türkiye, were assessed. In the past 20 years, many individual studies have been conducted in Türkiye for the investigation of the effects of the Ovsynch protocol, applied to dairy cattle for ovulation synchronization, on conception rates. Conception rates ranging from 17.5% to 76.9% were reported in the 46 primary studies included in the meta-analysis performed in the present study. The heterogeneity observed between these primary studies for conception rate are considered to arise, to a large extent, from the results of the studies conducted by Çevik et al.[10], Kaçar et al.[21], Kaçar et al.[22] and however, heterogeneity cannot be simply attributed to consistent differences observed between individual studies.

The heterogeneity between the primary studies included in the meta-analysis was determined by Cochran's Q, I^2 and τ^2 test statistics. In view of the high level of heterogeneity between the studies, the random-effects model, and due to it being least affected by the outlier, the Der Simonian-Laird method were used for the calculation of the common rates. The common conception rate calculated for the studies, which were included in the meta-analysis and involved the application of the Ovsynch protocol to dairy cattle raised in Türkiye, was 41.2% (%95 CI: 38.4-44.2). Out of the 46 primary studies included in the meta-analysis, 10 had reported conception rates that fell within the 95% confidence interval of the common conception rate (38.4-44.2), whilst 17 studies had reported rates below and 19 had reported rates above the CI of the common conception rate. Common conception rates were calculated for the subgroups (geographical region, breed, year of publication and parity) that were established to determine the source of heterogeneity between the studies included in the meta-analysis.

According to meta-regression analyses, among the subgroups established for geographical region, the lowest conception rate was determined for the Eastern Anatolia Region (26%), whilst the highest conception rate was determined for the Black Sea Region (51.6%). The conception rates calculated for the other geographical regions were similar and fell within the confidence interval of the common conception rate. The conception rate calculated for the Eastern Anatolia Region being 1.140 fold higher than that calculated for the Black Sea Region was attributed to the harsh climatic conditions of Eastern Anatolia. Literature reports have also pointed out to the significant effect of geographical region, month/season, and geographical region-month/season interaction on conception rates in different countries [23-25].

Although it has been reported that cattle, which are of different genetic structure and are raised in different geographical regions, would differ for fertility [26,27], the common conception rates calculated for the two breed subgroups in the present study demonstrated similar values for Holstein (41.3%) and Brown Swiss (43.6%) cattle. This result agrees with previous studies suggesting similar fertility characteristics for the Holstein and Brown Swiss breeds, compared to some dual-purpose cattle breeds [28,29].

In parallel with the improvement of management systems in the dairy industry in recent years, better nutrition and extensive genetic selections have resulted in a steady increase in milk yields per cow. However, increased milk yields have brought about decreased reproductive yields [30,31].

In the present study, no statistically significant difference was observed between the subgroups established for the year of publication of the studies with respect to the conception rates of dairy cattle raised in Türkiye (P>0.10). However, it was determined that conception rates had decreased by 10% in the last 20 years.

The present study showed that heifers and multiparous cows included in the subgroups established for parity showed no statistically significant difference for conception rates. It has been reported that the conception rate to the first insemination is either higher [32,33] or similar [21,22,34] in heifers, compared to multiparous cows.

In conclusion, with the meta-analysis conducted in this study results close to the population parameter were obtained for the use of the prostaglandin based Ovsynch protocol, which is known to have common use in Türkiye. It is considered that the results of the present study will contribute to the development of new strategies for rational production in the dairy cattle sector. The main limitation of this study is prostaglandin-based synchronization protocols other than Ovsynch, and progesterone-based synchronization protocols having not been included and assessed. The conduct of a network meta-analysis for the overall assessment and relative comparison of all available synchronization methods in the future would provide more detailed results. The generation of reliable results with a meta-analysis depends on the careful selection of the primary studies to be included in the analysis, the careful assessment of the included studies, the selection of the proper statistical model for use, and the accurate interpretation of the results of the analysis.

The meta-analysis method may produce more reliable and valid results than the individual studies included in the analysis if utmost attention is shown at each phase of the process, from the selection of the studies to be included in the analysis to the interpretation of the results of the analysis. Combining the results of small-sample studies with an aim to make valid, consistent and adequate parameter estimations with minimum variance requires adherence to certain rules as well as planned and disciplined conduct. Thus, in view of the vast amount of data required to be dealt with in meta-analyses, these assessments should be performed by a team of relevant experts with extensive knowledge.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author (A. Akçay) on reasonable request.

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Conflict of Interest

The authors report no conflicts of interest.

Author Contributions

AA and MA conceived the study. AA, MA and EC collected and analyzed data. AA, MA and EC have approved and read the final version of the manuscript.

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Research Article

Usnic Acid Reduces Colon Cancer Cell Viability and Colony Formation by Affecting Cancer Cell Metabolism

Turgut ANUK [1,a,† (*)] Barış YILDIZ [2,b,†] Ramazan DEMİREL [3,c] Meryem İÇEN [3,d]
Selma YILDIRIM [3,e] Hatice BEŞEREN [4,f] Baycan MOR [5,g] Özkan ÖZDEN [6,h (*)]

† These authors contributed equally to this study

[1] Department of General Surgery, Faculty of Medicine, Kafkas University, TR-36300 Kars - TÜRKİYE
[2] Department of Physiology, Institute of Health Sciences, Kafkas University, TR-36300 Kars - TÜRKİYE
[3] Department of Bioengineering, Institute of Natural and Applied Sciences, Kafkas University, TR-36300 Kars - TÜRKİYE
[4] Department of Pathology, Faculty, Kafkas University, TR-36300 Kars - TÜRKİYE
[5] Department of Molecular Biology and Genetics, Faculty of Science and Letters, Kafkas University, TR-36300 Kars - TÜRKİYE
[6] Department of Bioengineering, Faculty of Engineering and Architecture, Kafkas University, TR-36300 Kars - TÜRKİYE
ORCIDs: [a] 0000-0002-8903-9993; [b] 0000-0002-8432-4656; [c] 0000-0001-8654-5359; [d] 0000-0002-5233-5609; [e] 0000-0003-3521-3383
[f] 0000-0002-4780-540X; [g] 0000-0001-9480-3143; [h] 0000-0002-9467-3761

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Abstract: Today, some natural and plant-based products are of great interest, at least as supplements, in cancer prevention and treatment due to their anti-cancer properties. One of them is usnic acid, which is a secondary metabolite synthesized by the polyketide pathway in Usnea lichen species and is a promising agent for cancer treatment. In this study, the effects of usnic acid on cell proliferation, colony formation, and cancer metabolism-related Sirtuin 2 (SIRT2) and lactate dehydrogenase (LDH) enzymes in COLO-205, a colon cancer cell line, were investigated. COLO-205 cells were exposed to three different doses of usnic acid: 15 µM, 30 µM, and 60 µM for 24 h. Usnic acid treatment reduced colon cancer cell viability at a dose dependent manner. The highest dose of usnic acid treatment (60 µM) decreased cell viability by about 40%. It has been determined that a 15 µM dose of usnic acid reduces cell viability by about half, and a dose as low as 1 µM reduces the colony-forming abilities of cancer cells by about half. It has been determined that usnic acid reduces the anti-cancer effect seen in this cell line, at least in part, by altering SIRT2 and LDH protein expressions, thus affecting cancer metabolism.

Keywords: *Cancer, Cell culture, Colon, LDH, SIRT2, Sirtuin*

Usnik Asit Kanser Hücre Metabolizmasını Etkileyerek Kolon Kanseri Hücre Canlılığını ve Koloni Oluşumunu Azaltır

Öz: Günümüzde bazı doğal ürünler, anti-kanser özelliklerinin bulunmasının nedeniyle, kanser önleme ve tedavisinde, en azından takviye olarak büyük ilgi görmektedir. Bunlardan birisi de Usnea liken türünde poliketid yolu ile sentezlenen bir ikincil metabolit olan usnik asittir ve kanser tedavisi için umut verici bir ajandır. Bu çalışmada, usnik asidin bir kolon kanseri hücre hattı olan COLO-205'te hücre proliferasyonu, koloni formasyonu ve kanser metabolizmasıyla ilişkili Sirtuin 2 (SIRT2) ve laktat dehidrojenaz (LDH) enzimleri üzerine etkileri araştırılmıştır. COLO-205 hücreleri, 24 saat boyunca üç farklı usnik asit dozuna maruz bırakıldı: 15 µM, 30 µM ve 60 µM. Usnik asit tedavisi, doza bağlı bir şekilde kolon kanseri hücresi canlılığını azalttı. En yüksek doz usnik asit muamelesi (60 µM), hücre canlılığını yaklaşık %40 oranında azalttı. Usnik asidin 15 µM dozunun hücre canlılığını yaklaşık yarı yarıya azalttığını ve 1 µM kadar düşük dozlarda ise kanser hücrelerinin koloni oluşturma yeteneklerini yaklaşık yarı yarıya düşürdüğü belirlenmiştir. Usnik asidin bu hücre hattında görülen anti-kanser etkisi, en azından kısmen, SIRT2 ve LDH protein ifadelerini değiştirerek, dolayısıyla kanser metabolizmasına etki ederek azalttığı belirlenmiştir.

Anahtar sözcükler: *Hücre kültürü, Kanser, Kolon, LDH, SIRT2, Sirtuin*

Introduction

Cancer ranks second place on the list of deadliest diseases in the world; however, knowledge about the molecular mechanism of cancer is still lacking. Statistical studies show that the disease is increasing exponentially and is

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(*) Corresponding Author

Tel: +90 532 697 4498 (T. Anuk), +90 536 564 5229 (Ö. Özden)
E-mail: turgutanuk@gmail.com (T. Anuk), ozden@kafkas.edu.tr (Ö. Özden)

becoming significantly dangerous [1,2]. Colorectal cancer is one of the most common types of all cancer kinds [2]. Modern treatment methods, such as chemotherapy, radiation therapy, and immunotherapy are widely applied to treat many types of cancer; however, the importance of herbal extracts and phytochemicals complementing to conventional treatments is increasing in the fight against cancer [3,4]. One of the reasons that lead researchers to seek alternative treatments is the high side effects and resistance of some cancer cells to chemotherapy. In the light of recent studies, it has been claimed that therapeutic metabolites obtained from organisms existing in nature are an unlimited source for future drug production [5]. One of the alternative treatment methods to treat cancer could be applications with usnic acid obtained from lichens.

Complex organisms formed by the combination of fungi, algae, and cyanobacteria with a symbiotic bond are called lichens [6]. These organisms, which are known to have more than 17.000 species and are resistant to harsh living conditions, live on the soil surface or in the bark of trees. The most famous lichen secondary metabolite is usnic acid. Usnic acid can be easily isolated in high product purity and has various properties, such as anti-inflammatory, analgesic, wound healing, antioxidant, anti-microbial, anti-protozoal, and anti-viral [7,8]. Lichens synthesize hundreds of metabolites and these metabolites have been widely used due to their therapeutic effects [9]. Its promising effects on cancer treatment were first identified in studies on mice for the treatment of Lewis lung cancer [10]. In addition, cytotoxic and mutagenic activities of usnic acid were detected on normal and malignant human cell lines [11]. However, it is poorly known the molecular mechanisms of how usnic acid shows its therapeutic effect on cancer cells.

Studies have shown that the sirtuin (SIRT) protein family has important effects on the occurrence of several age-associated diseases, including cancer [12,13]. SIRTs regulate a variety of cellular functions, including metabolism, aging, inflammation, differentiation, stress response, and mitochondrial biogenesis [14,15]. SIRT2 is mainly found in the cytoplasm and plays important roles in the cell cycle and regulation of metabolism [16,17]. SIRT2 deacetylates and increases lactate dehydrogenase (LDH). LDH is an important enzyme for energy metabolism and catalyzes the conversion of pyruvate to lactate in the anaerobic metabolic pathway. LDH might be up-regulated in serum and tissues of patients diagnosed with cancer and is believed to associate with higher cancer cell proliferation rate and survival [18].

This study aimed to observe the effects of usnic acid on SIRT2 and LDH in colon cancer cell lines to understand the mechanism of action based on its known positive effects on cancer.

Material and Methods

Cell Cultures

COLO-205 colon cancer cell lines were maintained in RPMI-1640 (Roswell Park Memorial Institute-1640) (Gibco, USA) growth medium which contains 10% fetal bovine serum (FBS) and 1% antibiotic at 37°C, in a sterile incubator including 5% CO_2.

Cell Viability Assays

Cancer cells ($1x10^3$) were seeded in sterile 96-well microplates which contain a 100 µL growth medium in each well. After 24 h, cells were treated with 15 µM, 30 µM, and 60 µM doses of usnic acid (Santa Cruz Biotechnology, U.S.A.) for 24 h. The doses were selected according to a previous report [19]. To determine the cell viability, the 10 µL cell viability test kit (ECOTECH, CVDK-8, Turkey) was added to each well-containing 100 µL medium, and absorbance was measured at 450 nm with a spectrophotometer. In addition, the cells were photographed via an inverted microscope (Invitrogen, USA).

Homogenization of Usnic Acid-Treated Colon Cancer Cells

At the end of treatments, cells were collected and stored at -80°C until the homogenization procedure. A lysis buffer containing 1:1000 phenylmethylsulphonyl fluoride (PMSF) proteinase inhibitor was added to each cell sample. Finally, samples were centrifuged at 1000 rpm for 5 min and the supernatant portion was taken, and the total protein concentrations of the samples were determined by the Bradford assay. This assay is a spectroscopic procedure to measure total protein concentration in our homogenates. The assay solution includes Coomassie Brilliant Blue G-250 dye, and upon binding to proteins, the color of the dye and its absorption changes from 465 to 595 nm in response to various concentrations of protein. BSA standards were prepared using 1:1 serial dilutions from 2 to 0.0625 mg/mL.

Determination of Protein Expression with Western Blotting

TGX Stain-Free Fast Acrylamide SDS gel (10%) is prepared following the manufacturer's protocols (BIO-RAD, USA). Forty micrograms of each sample including 4x loading dye were heated at 95°C for 5 min. These samples were loaded into the wells and run at 120V for 120 min. Afterward, the transfer of membranes from the gel to a PVDF membrane was performed at 150 mA. Following the transfer procedure, the blotted PVDF membrane was blocked with 5% dry milk for 30 min, and then incubated with 1:1000 diluted SIRT2 (Sigma, USA), and LDH (Cell Signaling) primary antibodies for 16 h at 4°C. After the overnight incubation period,

the membrane was washed with three times phosphate-buffered saline including 0.1% Tween 20 (PBS-T). The secondary antibody incubation was performed at a 1:10000 dilution (Abcam, USA) at room temperature for 30 min. After secondary antibody treatment, membranes were washed again three times and were visualized with the Immobilon ECL Ultra Western HRP Substrate kit (Merck, USA).

Colony Formation Assay

To examine the effects of usnic acid doses on colony formation in COLO-205 colon cancer cells, cells were seeded on sterile 6-well plates with a 2 mL growth medium with 200 cells per well (n=6) as described previously [20]. At the end of 72 h, the growth medium in each well was withdrawn and replaced with a fresh growth medium containing usnic acid at doses of 1 µM, 5 µM, and 15 µM. The experiment was continued for 14 days in total by replacing the media in the wells with fresh medium containing usnic acid at the indicated doses at three-day intervals. The reason why the amounts of this dose determined in colony formation are different from the dose amounts used in the study is due to the fact that no cell colony was observed in the 15 µM dose application determined in the study. In our colony formation preliminary study, it was observed that these doses destroyed the colony formation, and therefore it was deemed appropriate to use low doses. At the end of the experiment, the medium in each well was discarded to determine the colony formations, and each well was washed with PBS and incubated with 1 mL of the fixative solution (1 acetic acid: 7 methanol) for 2 min in room temperature. At the end of the period, the fixative solution was removed from the wells and the samples were stained with crystal violet. At the end of the staining, the dye residues in the wells were removed and colony staining was obtained with the help of an imaging system. Transferring the colony formations to numerical data was performed with the Particle Tool in ImageJ 1.53m software.

Statistical Analysis

The data set was created by recording all the numerical data obtained with IBM SPSS 26.0 software. All the data in the data set were first evaluated according to the consistency of the parametric test assumptions and then the results of the homogeneity tests. In line with the obtained significance values, One-Way ANOVA was used to analyze the data. The probability value $P<0.05$ was accepted as a significance level in all statistical tests. Graphs were created by using the mean and standard deviation values.

Results

Usnic Acid Reduced the Cell Viability of Colon Cancer Cells

The effects of 15 µM, 30 µM, and 60 µM usnic acid doses applied to COLO-205 colon cancer cells for 24 h on cell survival are shown in *Fig. 1*. The data we obtained showed that the percentage of cell survival decreased in a dose-dependent manner of usnic acid, and all these reductions were significant at the $P<0.001$ level compared to the control and DMSO groups. Almost 50% of colon cancer cells died in response to as low as 15 µM usnic acid.

The images of COLO-205 colon cancer cells, which were exposed to usnic acid for the specified time, are shown in *Fig. 2*. The growth of COLO-205 significantly decreased especially at 60 µM usnic acid. Some cellular damage was detectible after 24 h of treatment of usnic acid in a dose-dependent manner. Non-treated, control cells had a round shape and their sizes were very similar. Treatment of usnic acid killed the cells and some debris were more common around the cells.

Protein Expressions of SIRT2 and LDH After Treatment with Usnic Acid

The effects of the indicated usnic acid doses on SIRT2 and LDH protein amounts in COLO-205 colon cancer cells

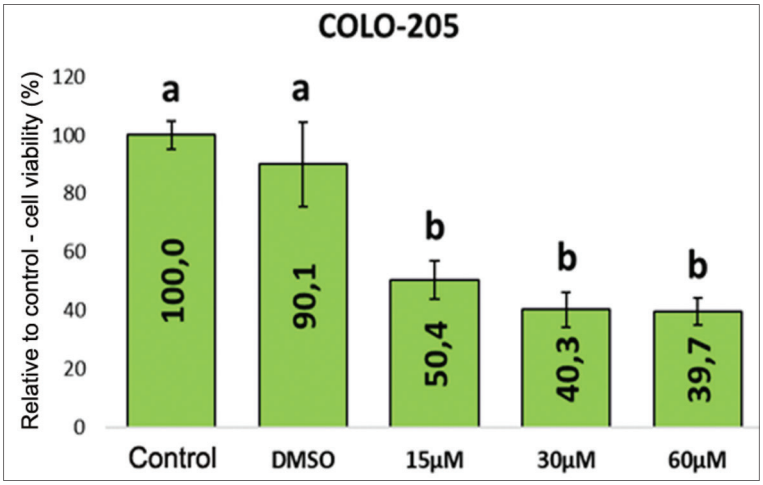

Fig 1. Effects of usnic acid on COLO-205 colon cancer cell survival after 24 h (One-Way ANOVA: $P<0.001$, posthoc Bonferroni: $P<0.001$). The experiment was repeated at least three times

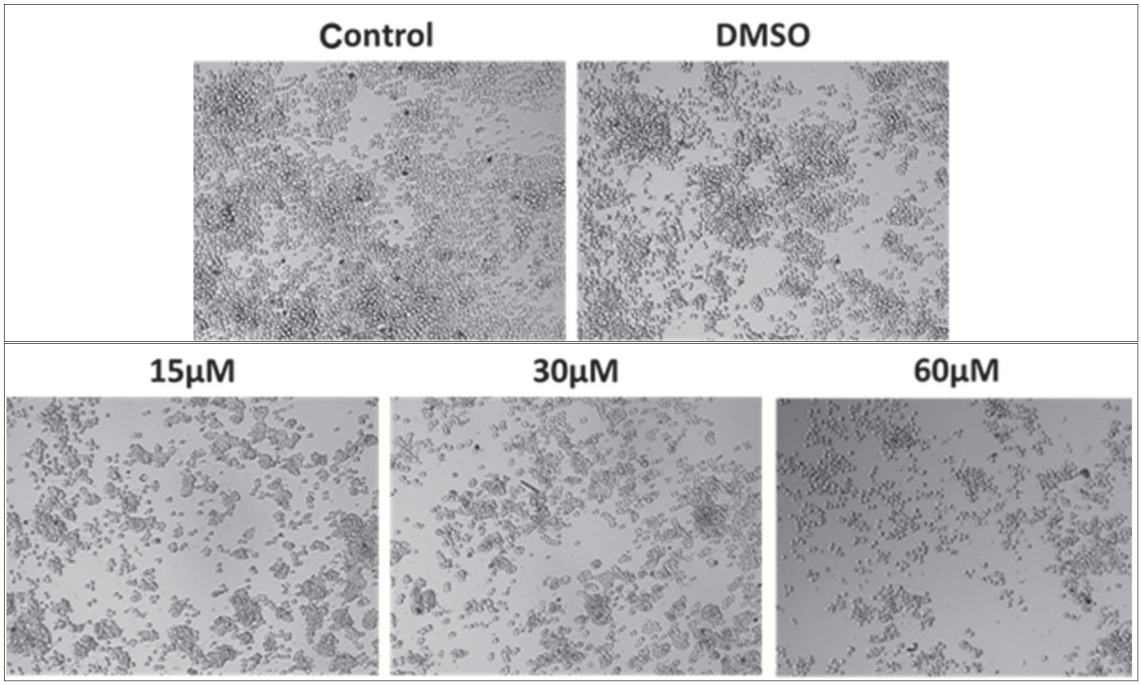

Fig 2. Inverted microscope images (40X magnification) were obtained at the end of the 24 h of COLO-205 colon cancer cells exposed to 15 µM, 30 µM, and 60 µM usnic acid. Representative images are shown

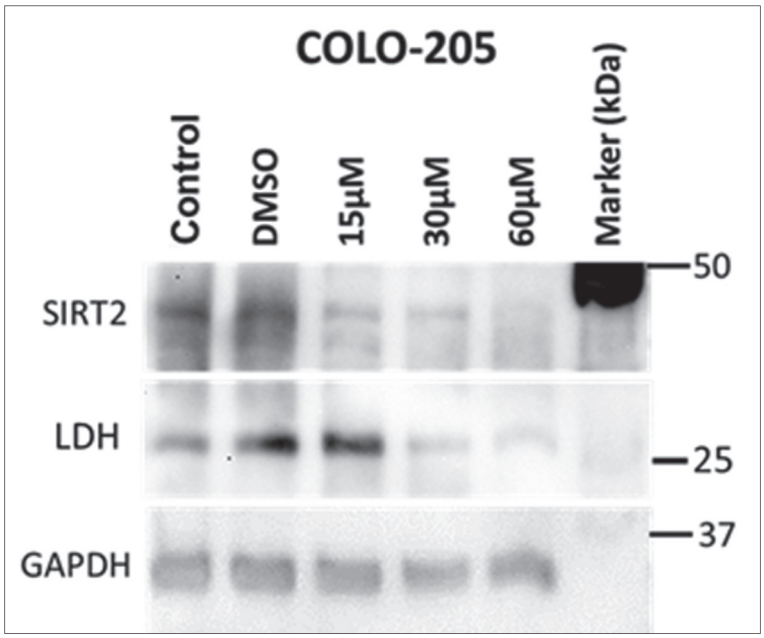

Fig 3. The effects of 15 µM, 30 µM, and 60 µM usnic acid doses were applied to COLO-205 colon cancer cells on SIRT2 and LDH protein amounts at the end of the 24 h. GAPDH was used as the loading control. The experiment was repeated at least three times

were investigated. The GAPDH was used as a loading control *(Fig. 3)*. The data obtained as a result of the Western blotting analysis showed that SIRT2 protein expression decreased with usnic acid application compared to the control group. In addition, it was observed that usnic acid decreased SIRT2 protein amounts inversely with increasing dose administration. It was observed that 60 µM usnic acid dose application dramatically reduced the amount of SIRT2 protein in COLO-205 cells. Similarly, the amount of LDH protein in COLO-205 colon cancer cells reduced drastically in response to both 30 and 60 mM usnic acid.

The Effect of Usnic Acid on Colony Formations of Colon Cancer Cells

With this experiment, it was aimed to measure the anti-proliferative and transformation potential of usnic acid. The effects of 1 µM, 5 µM and 15 µM usnic acid doses applied to COLO-205 colon cancer cells on the colony formation numbers of the cells at the end of the 14 days

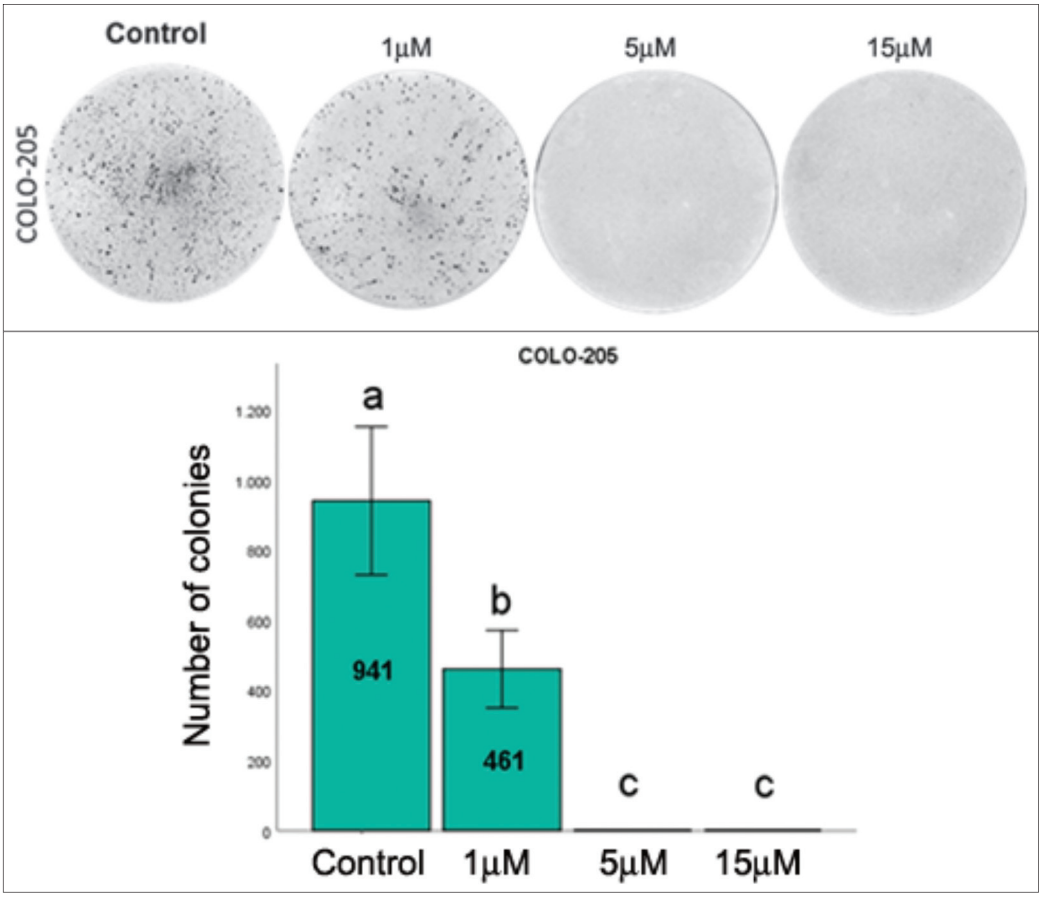

Fig 4. The effects of 1 µM, 5 µM, and 15 µM usnic acid application on COLO-205 cells on colony formation numbers at the end of the 14 days (One-Way ANOVA: P<0.001, PostHocTamhane's T2: pab, ac, bc<0.01)

are shown in *Fig. 4 (lower panel)*, and the images of the colony formations obtained at the end of the experiment are shown in *Fig. 4 (upper panel)*. The reason why the doses of usnic acid used in colony formation differ from the hypothetical experimental doses is that these doses (15 µM, 30 µM, and 60 µM) completely prevented the formation of colony formation in COLO-205 colon cancer cells in the preliminary study. For this reason, the dose densities used in our colony formation experiment were redesigned to include the lowest of the hypothetical dose densities, 15 µM. The data obtained as a result of the colony formation analysis show that there is statistical significance at the P<0.01 level between all administered dose groups and the control group *(Fig. 4)*. In addition, it was observed that COLO-205 colon cancer cells could not form any colony formation at 5 µM and 15 µM usnic acid doses *(Fig. 4)*. These results showed that an usnic acid concentration as low as 1 µM significantly reduces the ability of a single COLO-205 cell to develop into a colony.

Discussion

Previous studies have reported that usnic acid displays some anti-cancer activities by inducing apoptosis and decreasing apoptosis *in vitro* and *in vivo* [6,19]. The data obtained with this study showed that usnic acid lowers cell viability and colony formation ability of the colon cancer cells. In COLO-205 cell lines, usnic acid decreases the amount of both SIRT2 and LDH proteins in a dose-dependent manner.

The roles of SIRT2 in cancer are context-dependent, and both tumor suppressor and tumor-promoting activities of SIRT2 were reported [12,17,21]. Some studies have reported that low expression of SIRT2 as a deacetylase protein with anti-cancer properties is associated with poor prognosis in cancer and this protein is downregulated in many cancer types [22]. In addition, mice with the SIRT2 gene silenced spontaneously develop tumors of different organ origins [23]. On the contrary to these studies, some studies have reported that SIRT2 is up-regulated in various cancer types [24].

Cancerous cells gain many advantages by producing hypoxia signals, and in this respect, promoting aerobic glycolysis in energy production [25]. It has been proposed that SIRT2 protein modulates the Warburg effect, which has an important incident in cancer cell proliferation, by increasing the amount of intermediate products involved

in the energy production system [16]. In the data obtained, it was seen that at high concentrations of usnic acid, COLO-205 cells had a reduced expression of SIRT2 in colon cancer cells. SIRT2 has several non-histone substrates associated with diverse signaling pathways and cellular events. One of the substrates of SIRT2, which plays an important role in cancer, is LDH. LDH is one of the key enzymes for the Warburg effect since most tumors display increased aerobic glycolysis and lactate production [20,26]. High levels of LDH stimulates cancer cells to proliferate at a higher rate by increasing angiogenesis and epithelial to mesenchymal transition (EMT). It has been reported that SIRT2 deacetylates and increases the enzymatic activity of LDH; consequently, promoting cell proliferation [27]. In our study, we determined that LDH protein expression is reduced in response to as low as 30 μM usnic acid treatment. Reducing LDH protein levels by usnic acid treatment possibly decreased the rates of aerobic glycolysis and decreased the survival of cancer cells. According to our cell viability assay, usnic acid kills almost half of the colon cancer cells when 15 μM usnic acid is used. Moreover, as low as 1 μM usnic acid is sufficient to significantly decrease the number of colonies. This means that in addition to LDH, some other mechanisms, maybe some other SIRT2 substrates, might play a role to exert the anti-proliferative activity of usnic acid.

In conclusion, in the light of all these findings, it cannot be said that usnic acid achieves fully its anti-cancer properties through its effects on LDH expression, but whether it might change the activities of other SIRT2 substrates or other sirtuins should be investigated with detailed studies. In addition, the effects of usnic acid on the sirtuin-mediated anti-cancer pathway may be different in different cancer cells and different cancer types should also be taken into account.

Availability of Data and Materials

The data sets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of Interest

The authors report no conflicts of interest.

Author Contributions

Study conception and design: T.A., B.Y., O.O.; acquisition of data: B.Y., R.D., O.O., M.I., S.Y., B.M.; analysis and interpretation of data: T.A., B.Y., O.O., R.D., H.B. M.I., B.M.; drafting of manuscript: O.O., B.Y., T.A., S.Y., B.M.; critical revision: T.A., B.Y., R.D., M.I., S.Y., H.B., B.M., O.O. Authors give final approval of the version: T.A., B.Y., R.D., M.I., S.Y., H.B., B.M., O.O.

Ethical Approval

Not necessary.

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Research Article

Molecular Detection of Picornaviruses in Diarrheic Small Ruminants at a Glance: Enterovirus, Hunnivirus, and Kobuvirus in Türkiye

Ilke KARAYEL-HACIOGLU [1,a (*)] Selda DURAN-YELKEN [2,b] Feray ALKAN [1,c]

[1] Ankara University, Faculty of Veterinary Medicine, Department of Virology, TR-06110 Ankara - TÜRKİYE
[2] Siirt University, Faculty of Veterinary Medicine, Department of Virology, TR-56100 Siirt - TÜRKİYE
ORCIDs: [a] 0000-0003-1566-630X; [b] 0000-0003-0633-3132; [c] 0000-0003-3854-6503

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Abstract: Enteric diseases are considered an important factor affecting the livestock industry; however, there are limited data on viruses that cause enteric diseases in small ruminants in our country. In this study, we focused on enterovirus (EV), hunnivirus (HuV), and kobuvirus (KoV), which are members of the family of *Picornaviridae*. In order to provide further information on the prevalence and the molecular epidemiology of these viruses, a total of 73 fecal samples or gut contents from diarrheic sheep and goats were screened for EV, HuV, and KoV. For this purpose, RT-PCRs were performed by using the specific primers for each virus. The prevalence rate determined in the sampled population was 2.7% (2/73) for each virus. In detail, EVs (2/60, 3.3%) and HuVs (2/60, 3.3%) were each detected in only sheep samples while KoVs were identified in a goat sample (1/13, 7.6%) and a sheep sample (1/60, 1.6%). There was no evidence of coinfection with these viruses in the tested animals. According to the results of the molecular analyzes, our EVs were clustered in caprine/ovine-specific EV-G and HuV strains retrieved in this study were grouped along with the other caprine/ovine origin sequences in Hunnivirus A2 genotype. Moreover, it was observed that the detected KoVs clustered in distinct species: Aichivirus B and Aichivirus C. In conclusion, this study, which reported the detection of EVs and HuVs from sheep as well as KoVs from sheep and goats in our country, provides valuable data on the epidemiology and molecular characteristics of these viruses.

Keywords: *Enterovirus, Goat, Hunnivirus, Kobuvirus, Sheep*

Bir Bakışta İshalli Küçük Ruminantlarda Picornavirusların Moleküler Tespiti: Türkiye'de Enterovirus, Hunnivirus ve Kobuvirus

Öz: Enterik hastalıklar, hayvancılık endüstrisini etkileyen önemli bir faktör olarak kabul edilmektedir, ancak ülkemizde küçük ruminantlarda enterik hastalıklara neden olan viruslara ilişkin veriler sınırlıdır. Bu çalışmada *Picornaviridae* ailesinin üyeleri olan enterovirus (EV), hunnivirus (HuV) ve kobuvirus (KoV) üzerinde durulmuştur. Bu virusların prevalansı ve moleküler epidemiyolojisi hakkında daha fazla bilgi elde etmek amacıyla, ishalli koyun ve keçilerden toplam 73 dışkı örneği veya bağırsak içeriği EV, HuV ve KoV için test edilmiştir. Bu amaçla, her bir virus için spesifik primerler kullanılarak RT-PCR'lar yapılmıştır. Örneklenen popülasyonda tespit edilen prevalans oranı her bir virus için %2.7 (2/73) olarak hesaplanmıştır. Detaylı olarak değerlendirildiğinde, EV (2/60, %3.3) ve HuV (2/60, %3.3) yalnızca koyun örneklerinde saptanırken, KoV ise bir keçi (1/13, %7.6) ve bir koyun örneğinde (1/60, %1.6) tespit edilmiştir. Bununla birlikte, test edilen hayvanlarda bu viruslarla herhangi bir koenfeksiyon saptanmamıştır. Moleküler analizlerin sonuçlarına göre, EV suşlarının keçi/koyuna özgü EV-G'de kümelendiği ve bu çalışmada elde edilen HuV suşlarının ise Hunnivirus A2 genotipinde diğer keçi/koyun kökenli dizinlerle birlikte gruplandığı ortaya konulmuştur. Ayrıca tespit edilen KoV suşlarının Aichivirus B ve Aichivirus C olmak üzere farklı türlerde kümelendiği gözlemlenmiştir. Sonuç olarak, ülkemizde koyun ve keçilerde KoV'ların yanı sıra koyunlarda EV ve HuV'un tespitini bildiren bu çalışma, bu virusların epidemiyolojisi ve moleküler özelliklerine ilişkin değerli veriler sunmaktadır.

Anahtar sözcükler: *Enterovirus, Keçi, Hunnivirus, Kobuvirus, Koyun*

Introduction

Picornaviruses, which belong to the family *Picornaviridae*, are icosahedral, non-enveloped viruses with a single-stranded positive-sense RNA genome [1]. Despite the fact that the great majority of picornavirus infections are asymptomatic, several picornaviruses cause disorders of the central nervous system, respiratory and gastrointestinal

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(*) Corresponding Author
Tel: +90 312 317 0315/4222
E-mail: ilkeekarayel@gmail.com (İ. Karayel-Hacıoğlu)

tracts, as well as some other organs, such as the heart and liver in humans and animals [2].

The picornavirus genomic RNA (6.7-10.1 kb), commonly contains a single large open reading frame (ORF) flanked by 5'- and 3'-UTRs. A large polyprotein precursor produced by the single ORF is post-translationally cleaved into three distinct P regions (P1-P3), which encode the structural proteins and the non-structural proteins. In detail, P1 encodes the viral capsid proteins, while proteins involved in protease processing and genome replication are encoded by P2 and P3. Also, in many viruses, such as kobuviruses, P1 is preceded by a leader protein (L) [1].

The genus *Enterovirus* consists of 12 species of enterovirus (A-L), and three species of rhinovirus (A-C) [3]. Out of them the three enterovirus (EV) species, EV-E, EV-F, and EV-G are most closely associated with the diseases affecting the livestock industry [4-6]. EV-E and EV-F, previously known as bovine enterovirus A and B, are the causative agents of infections in cattle that display clinical indications ranging from respiratory diseases to enteritis, reproductive disease, and infertility [7-9]. In addition, they have also been detected in asymptomatic animals as well as in the environmental samples [10-12]. EV-G, which was previously referred to as porcine enterovirus B (PEV-B), comprises viruses isolated from pigs, wild boars, and small ruminants such as sheep, goats, and Sichuan takins [4,13]. Previous studies revealed that natural infection with EV-G can cause severe diarrhea with high morbidity and mortality rates as well as neurological disorders, fertility disorders, diarrhea, and dermal lesion [4,10,14,15].

Hunnivirus is a novel picornavirus genus that was established by the International Committee on Taxonomy of Viruses (ICTV) in 2013 [16]. This genus is comprised of a single species, which is known as Hunnivirus A and it has been classified into at least nine genotypes: hunnivirus A1 (formerly bovine hungarovirus 1) [2], hunnivirus A2 (formerly ovine hungarovirus 1) [2], hunnivirus A3 (isolated from sheep cell cultures) [17], hunnivirus A4 (Norway rat hunnivirus) [18], and hunnivirus A5-A9 [3,19,20]. After the first discovery of hunnivirus (HuV) in sheep cell cultures in 1965 [17], it was detected in cattle, sheep, goats, water buffalo, rats, and cats [2,19-21]. However, *Hunnivirus* genus is poorly understood, and it is unknown what clinical signs they can cause in which animal species and its potential risks to human health.

Kobuvirus (KoV) was first reported in a fecal sample from a human with gastroenteritis in 1989 [22]. Subsequently, an increasing number of novel KoVs have been frequently detected in humans and a wide variety of domestic and wild animals with or without clinical signs [23-28]. The genus *Kobuvirus* is grouped into six species, Aichivirus A to F, and 20 genetic types. In addition to these, there are three unclassified KoVs [3]. There have been several reports of KoVs in sheep and goats, and so far, KoVs detected in sheep have been grouped into the Aichivirus B and Aichivirus D species [27,29,30] whereas goat KoVs have been classified into the Aichivirus B and Aichivirus C species [24,31,32]. However, the data of their pathogenicity and epidemiological distribution is still limited.

Considering the circulation of mentioned viruses worldwide among many animal species and the detection of them in some other animal species except small ruminants in Türkiye, fecal samples or gut contents from diarrheic sheep and goats were screened for EV, HuV, and KoV in order to fill the gap in this regard and to provide further information on the molecular epidemiology of these viruses.

Material and Methods

Ethical Statement

The study was approved by the Ankara University Animal Experiments Local Ethics Committee (Decision No: 2022-10-93).

Samples

A total of 73 fecal samples or gut contents from small ruminants (60 sheep and 13 goats) with diarrhea from herds in several provinces of Türkiye were used in this study *(Fig 1)*. Samples were collected by the field veterinarians and sent to our laboratory for routine diagnosis.

Viral RNA Extraction and RT-PCRs

The viral RNA was extracted from sample suspensions (1:10, w/v) using QIAamp Cador Pathogen Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -80°C. Reverse transcription was performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. For the detection of KoV and EV, the generic KoV primer pair UNIV-kobu-F/R [33] and EV primer pair targeting the conservative 5' UTRs (Non-HumanEntero-5' UTR-R/F) [13] were used, respectively. In order to identify HuV, a generic primer pair (Hungaro-3D-F/R) [2], which were designed based upon the nucleotide sequences of the 3D region of hunnivirus A1 (formerly bovine hungarovirus 1), was used. The RT-PCRs were performed using DreamTaq DNA polymerase (Thermo Fisher Scientific, USA) with the following thermal conditions: denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 60 sec, annealing at (48°C for KoV, 51°C for HuV, and 57°C for EV) for 30 sec, extension at 72°C for 60 sec, and final extension at 72°C for 10 min. The products were run in 1% agarose gel stained with SafeView Classic (ABM, Canada), and visualized under UV light.

Sequencing and Phylogenetic Analysis

Nucleotide sequencing of the amplicons with expected sizes (~220 bp for EV, 465 bp for HuV and 217 bp for KoV) were performed using the same primers used in the RT-PCRs. The MUSCLE algorithm as implemented in Aliview Software was used to create multiple sequence alignments [34,35]. Cognate sequences of each viruses representing different genotype/serotype were retrieved from GenBank through the BLAST engine. The identities of nucleotides (nt) and amino acids (aa) were determined using the SIAS online program *(http://imed.med.ucm.es/Tools/sias.html)*. Phylogenetic analyses were conducted using MEGA X software by applying the maximum likelihood (ML) method [36]. Measuring the best fit model with the "Find Best DNA/Protein Model" feature of the MEGA X software was performed before applying the ML method and Bayesian information criteria (BIC) was used to determine the nucleotide substitution model. Accordingly, the ML phylogenetic trees were constructed using the T93 (Tamura Nei) + G (for EV and KoV) and T92 (Tamura-3) + G (for HuV) nucleotide substitution models. The nucleotide sequences of viruses detected in this study were deposited into the GenBank database under the following accession numbers: ON316762-ON316767.

Results

In this study, a total of 73 samples from diarrheic small ruminants, containing 60 sheep and 13 goats, were screened for EV, HuV and KoV. A total of six samples (8.2%) were confirmed to be positive for any one of the viruses studied. Overall, in our survey, the prevalence rate determined in the population was 2.7% (2/73) for each virus. Specifically, EVs (2/60, 3.3%) and HuVs (2/60, 3.3%) were each detected in only sheep samples while KoVs were identified in a goat sample (1/13, 7.6%) and a sheep sample (1/60, 1.6%). No coinfection with these viruses was detected in tested animals. A map of the sample collection sites and the locations of samples positive for the viruses are shown in *Fig. 1*.

In this study two samples from sheep produced the expected size amplicons of the 5'UTR of EVs while there was no positive sample from goats. These sheep were from farms located in different provinces of Türkiye, Cankiri (A1G) and Eskisehir (KRM33). Sequence comparison for the partial 5'-UTR between the A1G and KRM33 strains revealed 92.61% nt identity to each other. Both strains also shared the highest nt identity (A1G 92.21% and KRM33 90.47%) to the corresponding region of ovine enterovirus 2019-00927 isolate [14]. The phylogenetic tree based on the partial 5'-UTR sequences showed that our EVs were clustered in caprine/ovine-specific EV-G *(Fig. 2)*.

Both HuVs reported in this study were identified in sheep samples from Ankara (KD6) and Kirsehir (KRM2); however, none was detected in goat samples. The molecular analysis of the partial 3D gene region showed that our HuV strains shared 91.18% nt and 94.83% aa identity to each other. Interestingly, our strains displayed higher genetic identities with previously identified several HuV strains from small ruminants than each other. Specifically, the KRM2 strain was most closely related to Hungarian sheep and Chinese goat and sheep strains (94.62-96.55% nt and 97.41-98.7% aa identity). The KD6 strain had the highest identity to Chinese goat strain (91.39% nt and 96.77% aa identity). Also, the phylogenetic tree showed that HuV sequences retrieved in this study were grouped along with the other caprine/ovine origin sequences in Hunnivirus A2 genotype *(Fig. 3)*.

Partial nucleotide fragments of the 3D gene region of KoVs were detected in two samples, one from sheep (KRM21) and one from a goat (OBI), and both were obtained from different provinces: Bartin and Hatay, respectively. By sequence analysis of the partial 3D gene,

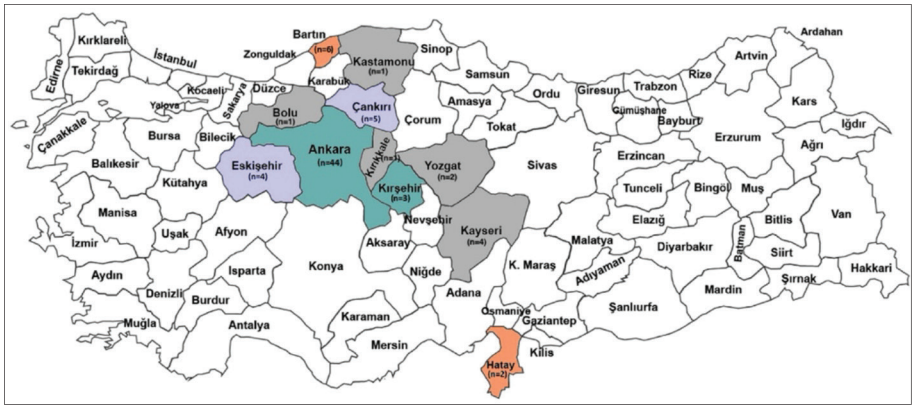


Fig 1. The map showing the distribution of the samples according to sampled provinces. Purple, green, and orange colors indicate the positive provinces EV, HuV, and KoV were detected, respectively. The grey color indicates the provinces of negative samples

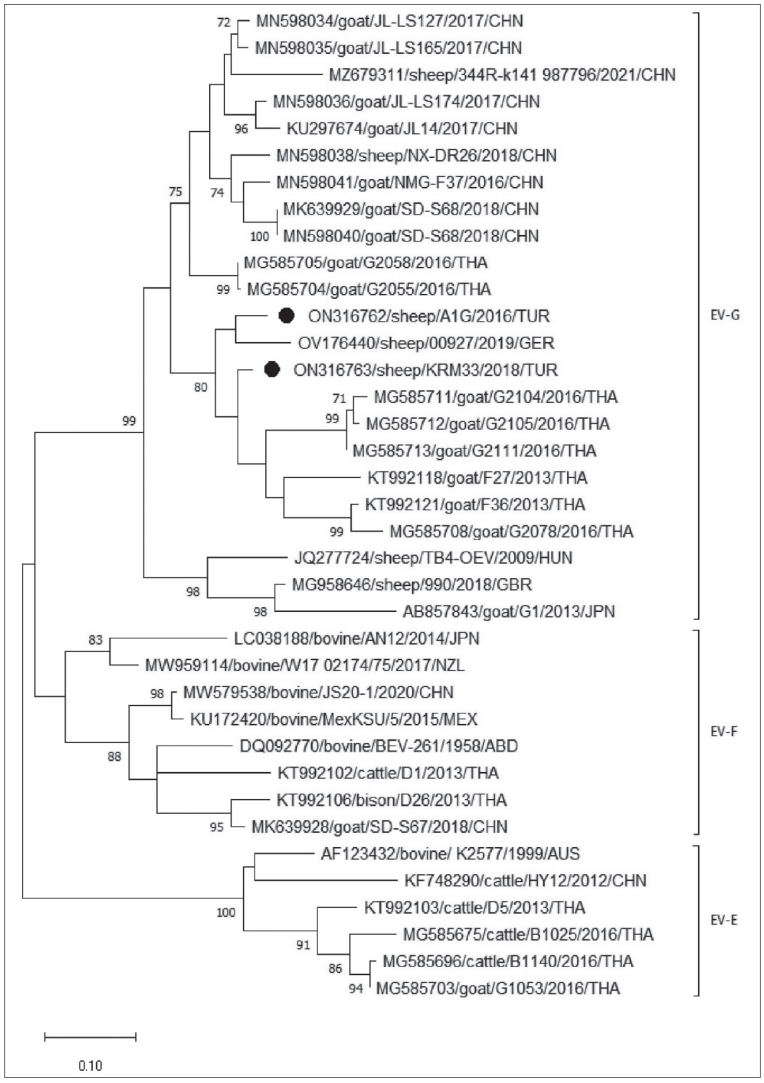
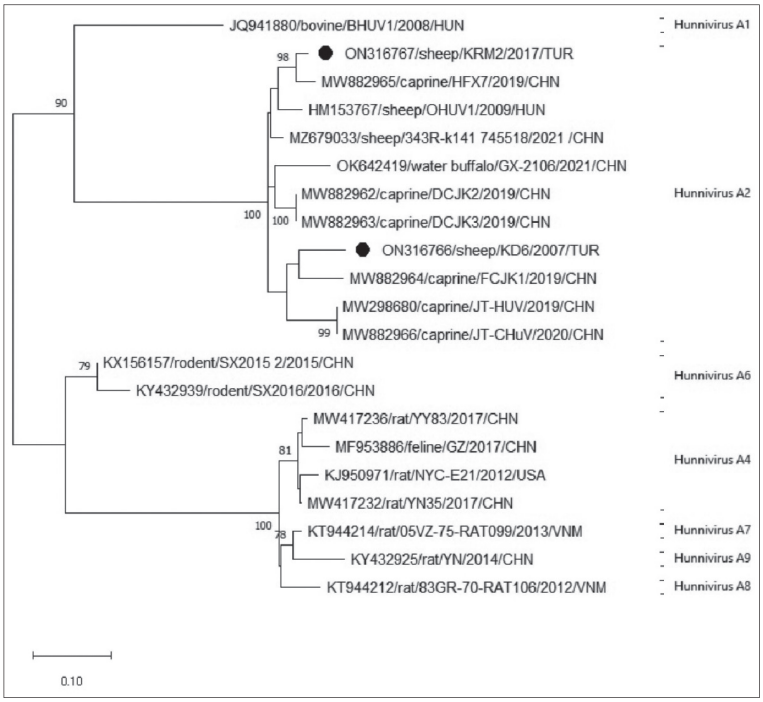


Fig 2. Phylogenetic tree based on the nucleotide of ~270 bp 5'UTR of EV. Our strains are indicated by black dots. The phylogenetic trees were constructed using the Maximum-Likelihood method with bootstrap of 1000 replicates. Numbers to the left of node indicate bootstrap values. Bootstrap values < 70% are not shown. Scale bar indicates nucleotide substitutions per site

Fig 3. Phylogenetic tree based on the nucleotide of 465 bp 3D gene region of HuV. Our strains are indicated by black dots. The phylogenetic trees were constructed using the Maximum-Likelihood method with bootstrap of 1000 replicates. Numbers to the left of node indicate bootstrap values. Bootstrap values < 70% are not shown. Scale bar indicates nucleotide substitutions per site



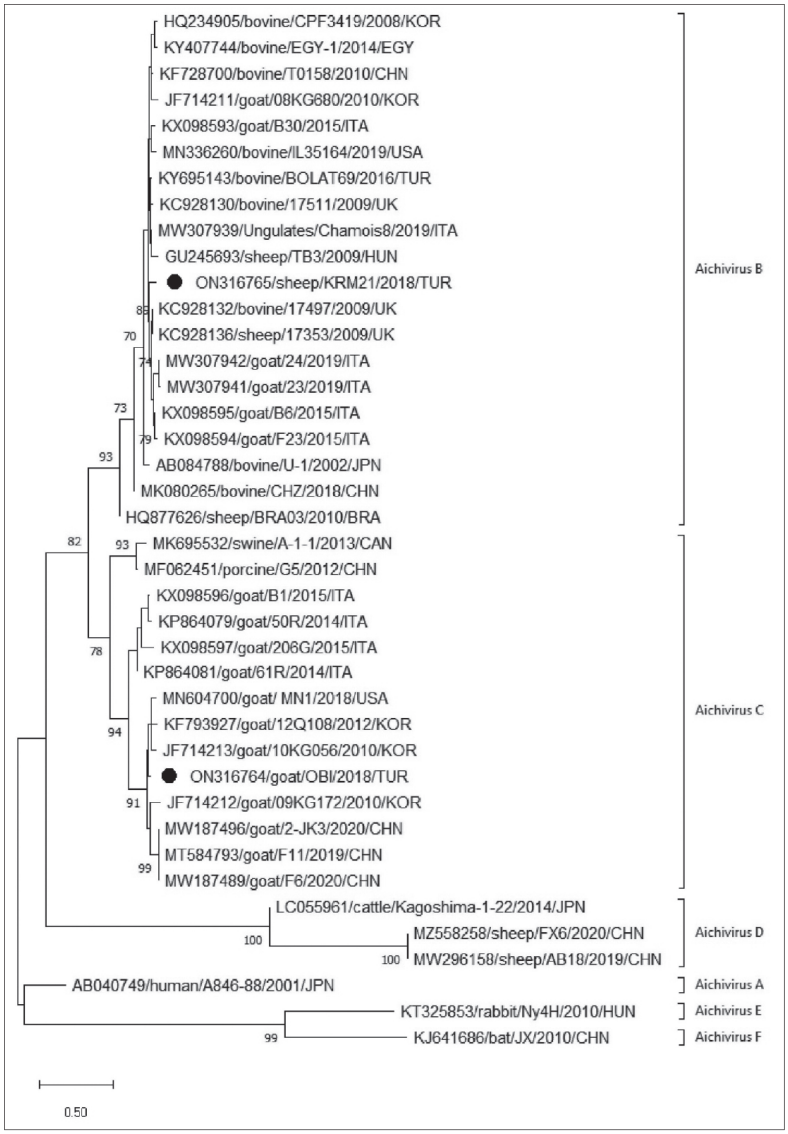


Fig 4. Phylogenetic tree based on the nucleotide of 217 bp 3D gene region of KoV. Our strains are indicated by black dots. The phylogenetic trees were constructed using the Maximum-Likelihood method with bootstrap of 1000 replicates. Numbers to the left of node indicate bootstrap values. Bootstrap values < 70% are not shown. Scale bar indicates nucleotide substitutions per site

our strains shared 76.05% nt and 84.28% aa identity with each other. The phylogenetic tree revealed that the detected both KoVs clustered in distinct species: the KRM21 strain within the species Aichivirus B and the OBI strain in the species Aichivirus C *(Fig. 4)*. The strain KRM21 displayed a close relatedness, 91.82-94.83% nt and 94.28-95.71% aa identity, to KoV sequences clustered in the species Aichivirus B. The strain OBI demonstrated a significant relation to the goat strain detected in USA (94.47% nt and 98.59% aa) and black goat strain from South Korea (94.47% nt and 100% aa identity).

Discussion

According to the data of the Turkish Statistical Institute, as of the end of June 2021, the total number of sheep and goats was determined as approximately 57.4 million (45.2 million sheep and 12.2 million goats), and approximately 10% of the total red meat and milk production were provided from small ruminants [37].

Enteric diseases are considered an important factor affecting the livestock industry and a large variety of viruses could be responsible for causing diarrhea such as rotavirus, coronavirus, picornavirus, and recently identified an increasing number of novel viruses [8]. However, there is limited information on enteric viruses in small ruminants in our country [38-40]. In this study, we focused on picornaviruses considered as another possible etiological agent for diarrhea cases and reported the prevalence and molecular characterization of EVs, HuVs, and KoVs of small ruminants in different provinces in Türkiye.

In this study, two samples from sheep were found positive for EV, and the detection rate of 3.3% was determined. Although EV previously described in Türkiye in cattle [41,42], and a goat [43], there is no report on the detection of EV in sheep. Nevertheless, there are serological studies revealed the detection of antibodies to EVs by neutralization technique using BEV-1 strain in small ruminants at different

rates, 27.6-71.8% in goats, 32.6-46.5% in sheep [44-46]. In several countries, infection with different genotypes/serotypes (EV-E, EV-F, and EV-G) have been reported in sheep and goats [4-6,10,11,13,14,47]. The detection rates of these studies range from 24-60% in goats and 39.1-44% in sheep, which is quite higher than our detection rate.

The molecular analysis of the partial 5'UTR revealed that our strains were most closely related to ovine enterovirus 2019-00927 isolate which is novel EV isolated from a lamb with progressive neurological symptoms [14]. Also, the phylogenetic tree showed that both EVs detected in this study were clustered in caprine/ovine-specific EV-G and grouped with this ovine strain (2019-00927) as well as EVs detected from fecal samples of goats in Thailand *(Fig. 2)*. The 5'UTR is a reasonably conserved genomic region that differs between EVs, making it valuable for detecting and classifying the *Enterovirus* genus into groups [6,48]. However, the current standard classifies EV species, serotypes, and genotypes based on their capsid and polymerase genes, not on the 5'UTR alone [48]. Therefore, more detailed molecular analyses including the capsid and polymerase genes are required to confirm the serotypes/genotypes of the circulating EV strains in our country. Although the viruses detected in this study were most closely associated with a novel EV isolated from a lamb with progressive neurological symptoms; it is not possible to assess whether these viruses might cause any neurological problem since these EV positive animals were reported to us only having diarrhea. This unexpected finding points out that further studies are needed to understand the host range, pathogenesis, and epidemiology of EVs in animals. Especially, considering the recombinant enteroviruses have been reported previously [5,13] it is particularly suggested that more detailed molecular studies should be conducted in the future.

To date HuVs were detected in cattle, sheep, goats, water buffalo, rats, and recently cats [2,19-21]. However, the detailed data related to HuVs in different host species and different geographic locations are quite limited. To the best of our knowledge, only two sequence data of HuV from sheep were deposited to GenBank so far. In this study, we detected two (2/60, 3.3%) HuVs from sheep samples. The detection rate in our study was quite lower than the rate found in sheep, 25%, in Hungary [2]. In the only study on HuV in our country [41], HuV positivity was reported at a rate of 11.2% in cattle.

The phylogenetic tree demonstrated that the KRM2 and KD6 strains belonged to the genotype of Hunnivirus A2 *(Fig. 3)*. Even though we only analyzed the partial 3D gene region in the current study, the present results will provide a valuable information the epidemiology, molecular characteristics, and evolution of HuVs in sheep in Türkiye, since there is limited data on these viruses. Uncertainty exists regarding the host range of HuVs, as well as their pathogenicity in cattle and small ruminants. These viruses were discovered in young animals (cattle and sheep) that appeared to be healthy at the time of discovery [2]. Later, these viruses were identified from diarrheic calves and a cat [21,41]. Because only diarrheic small ruminants were included in this study, similar to those studies, it is not possible to determine how much of a contribution HuV made to diarrhea cases. Consequently, more investigations are required to determine the geographic distribution, the route of transmission and the link between diarrhea in an animal model, as well as to evaluate its zoonotic potential.

Although the presence of KoVs in small ruminants has been confirmed in several countries [23,24,29-31,49,50] there has been no information regarding on this topic in Türkiye. Detection rate of KoVs in this study were 1.6% (1/60) and 7.6% (1/13) in sheep and goats, respectively. The level of KoV detected in sheep was similar to the detection rate (2%) in Northern Ireland [50], however it was quite low compared to the rates of 39.1% and 62.5% in Brazil and Hungary, respectively [27,29]. In this study, 7.6% of diarrheic samples were detected as goat KoV positive which was similar to previous reports in diarrheic goats, 6.5% [32] and 9.3% [49], however, there is a report which determined a much higher detection rate, 87.5% [30]. Despite the fact that a small number of goat samples were examined, the findings verified the presence of KoV in Turkish goats. The common finding of KoV among various wild and domestic animals indicates the widespread nature of these viruses and their potential to cause enteric disease [23]. Globally, goat and sheep KoVs have been detected in both diarrheic and asymptomatic animals [23,24,29-31,49,50]. Therefore, the question of whether there is a link between the presence of KoVs in animals and the development of enteric disease has yet to be fully answered and requires further research into this subject.

On the phylogenetic analysis of the partial 3D sequence, the sheep strain, KRM21, clustered within the species Aichivirus B and shared a branch with the other two Italian goat KoVs [24] detected previously *(Fig. 4)*. Also, the phylogenetic analysis revealed that the goat strain OBI was closely related to the other goat KoVs within the species Aichivirus C, which were previously described as being close to porcine kobuviruses rather than to bovine and sheep KoVs [23,31,49]. This result indicates the possibility of the circulation of different KoVs in small ruminants in our country.

In this study, in order to identify HuVs and KoVs the generic primer pairs, Hungaro-3D-F/R [2] were used, respectively. Indeed, using the primer pair, UNIV-kobu-F/R, new viruses classified in the genus *Kobuvirus* have been identified in various animals [21,29,31] however, detection of HuVs in different animal species with these primers

indicated a more generic nature of them [2]. Reuters et al.[2] reported that the generic KoV primers amplified HuV 3D gene region sequences, with 25% sensitivity. Lu et al.[21] further confirmed that the UNIV-kobu-F/R primers determined HuVs with 30% sensitivity. Unlike these reports, in our study, two amplicons detected by these primers were confirmed as KoV by sequencing and none of the HuV positive samples produced the expected fragment size by RT-PCR using UNIV-kobu-F/R. They were only detected by the primers Hungaro-3D-F/R.

It is known that diarrhea outbreaks are often multifactorial, infection with viruses, parasites, or bacteria, as well as dietary factors might contribute to the severity of diarrhea in animals. In Türkiye, the common pasture usage, breeding of animals by families, and management conditions (quality of barns, feeding, and hygiene) may increase the risk of infectious disease. In our country, as in other countries of the world, studies on diarrhea in small ruminants are quite limited. Although other possible enteric agents causing diarrhea and/or coinfection are not the focus of this article, since some of the samples tested in this study had been used in the previous study [40], it is thought that it would be useful to evaluate the results of both studies together. Considering the samples (n=66) used in both studies, coinfection with rota- and picobirnavirus was determined in two animals, KD6 and KRM21, which were positive for HuV and KoV, respectively. Although our study unveiled the circulation of the mentioned picornaviruses in small ruminant animals, it has several limitations. For instance, any clinically healthy animals were not subjected to this study. In addition, this study was carried out on a relatively limited number of samples, especially in terms of goats, and it mostly consisted of young animals.

In conclusion, our study on the detection and molecular analysis of different enteric picornaviruses from diarrheic small ruminants will contribute significantly to the literature and provide valuable data for understanding their epidemiology, molecular characteristics, and evolution.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (I. Karayel-Hacioglu) upon reasonable request.

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Ethical Statement

The study was approved by the Ankara University Animal Experiments Local Ethics Committee (Decision No: 2022-10-93).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

IKH, SDY, and FA conceived and planned the study design. IKH together with SDY conducted the experiments and performed the molecular biology and bioinformatic analyses (alignments, phylogeny). IKH, SDY, and FA interpreted the obtained data. IKH and SDY drafted and wrote the manuscript; FA reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Research Article

Determination of 25 (OH) D_3, Iron, Free Iron Binding Capacity and D-Dimer Levels in Calf Diarrhea in Neonatal Period

Mustafa Sinan AKTAŞ [1,a] Muhammed Sertaç EROĞLU [1,b (*)] Kerim Emre YANAR [1,c] Emre EREN [1,d]

[1] Atatürk University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-25240 Erzurum - TÜRKİYE
ORCIDs: [a] 0000-0002-7206-5757; [b] 0000-0003-4243-8273; [c] 0000-0001-7302-7077; [d] 0000-0003-3118-7384

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Abstract: In this study, it was aimed to evaluate the relationship between the clinical course of the disease and hematological data, serum 25-hydroxyvitamin D_3 (25 (OH) D_3), iron (Fe), free iron-binding capacity (UIBC), and D-dimer levels in calves with diarrhea in the neonatal period. Within the scope of the study, 10 healthy calves (group-I) and 30 diarrheal calves in the neonatal period of different races, ages and genders were used. Calves with diarrhea were divided into mild (group-II, n=10), moderate (group-III, n=10) and severe (group-IV, n=10) groups. Blood samples were taken from calves in all groups at once. Hematological analyzes were performed using a veterinary-specific hematology analyzer device. In serum samples, 25 (OH) D_3, Fe and UIBC levels were determined with an autoanalyzer, and D-dimer levels were determined with an automatic immunoassay analyzer. In the hematological analysis, an increase was observed in the number of LYMs (lymphocytes) in group-II (5.04±1.3) and III (5.2±3.3) compared to group-I (4.47±1.2), and a decrease was observed in group IV (2.76±0.9) ($P<0.05$). Fe levels in group-II (59±56), group III (56±52) and group IV (72±63) were found to be decreased compared to group-I (131±66) ($P<0.05$). It was determined that the 25 (OH) D_3 level of group IV (13.4±8.5) was higher than that of group-I (6.12±2.73) ($P<0.05$). D-dimer levels of group-III (1.15±1.13) and group-IV (0.96±0.88) were found to be higher than group-I (0.10±1.46) ($P<0.05$).

Keywords: *Calf, D-dimer, Enteritis, Fe, Neonatal, UIBC, Vitamin D*

Neonatal Dönem İshalli Buzağılarda 25 (OH) D_3, Demir, Demir Bağlama Kapasitesi ve D-Dimer Düzeylerinin Belirlenmesi

Öz: Bu çalışmada neonatal dönem ishalli buzağılarda hastalığın klinik seyri ile hematolojik veriler, serum 25-hidroksivitamin D_3 (25 (OH) D_3), demir (Fe), serbest demir bağlama kapasitesi (UIBC) ve D-dimer düzeyleri arasındaki ilişkinin değerlendirilmesi amaçlandı. Çalışma kapsamında neonatal dönemde değişik ırk, yaş ve cinsiyetteki 10 sağlıklı (grup-I) ve 30 ishalli buzağı kullanıldı. İshalli buzağılar, hafif (grup-II, n=10) orta (grup-III, n=10) ve şiddetli (grup-IV, n=10) olmak üzere gruplara ayrıldı. Tüm gruplardaki buzağılardan bir kereye mahsus olmak üzere kan örnekleri alındı. Hematolojik analizler veteriner spesifik hemogram cihazı kullanılarak yapıldı. Serum örneklerinde 25 (OH) D_3, Fe ve UIBC düzeyleri otoanalizör cihazı ile, D-dimer düzeyleri ise otomatik immunoassay analizör cihazı ile belirlendi. Hematolojik analizlerde LYM (lenfosit) sayılarında grup-II (5.04±1.3) ve III'de (5.2±3.3) grup-I'e göre (4.47±1.2) artış, grup-IV'de ise (2.76±0.9) bir azalış şekillendiği belirlendi ($P<0.05$). Grup-II (59±56), grup III (56±52) ve grup IV (72±63) Fe düzeylerinin grup-I'e göre (131±66) azaldığı belirlendi ($P<0.05$). Grup-IV'ün 25 (OH) D_3 düzeyinin (13.4±8.5) grup-I'e göre (6.12±2.73) yüksek olduğu belirlendi ($P<0.05$). Grup-III (1.15±1.13) ve grup-IV'ün (0.96±0.88) D-dimer düzeylerinin grup-I'e göre (0.10±1.46) yüksek olduğu belirlendi ($P<0.05$).

Anahtar sözcükler: *Buzağı, D-dimer, Enterit, Fe, Neonatal, UIBC, Vitamin D*

Introduction

Neonatal calf diarrhea is one of the most important health problems of the livestock industry and causes serious economic losses due to treatment costs, high rates of morbidity and mortality [1]. Non-infectious factors (herd management and environmental factors) as well as infectious factors *(Rotavirus*, *Coronavirus*, *E. coli* and *Cryptosporidium* spp.) play a role in the etiology of the disease [2,3]. A single or more than one infectious agent can cause disease together [4]. Dehydration, electrolyte deficit, metabolic acidosis, hypothermia, endotoxemia and septic

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(*) Corresponding Author

Tel: +90 442 231 7170 Cellular phone: +90 553 168 5594
E-mail: msertac.eroglu@atauni.edu.tr (M. S. Eroğlu)

shock are common complications in neonatal calf diarrhea and are associated with death [5]. Therefore, rapid diagnosis of etiology and determination of physiological changes are important factors for the appropriate treatment and control of the disease [6]. Furthermore, hematologic and biochemical changes in the disease are also considered important indicators of physiological and pathological status [7].

Vitamin D (Vit-D) plays an important role in the development of organisms, autoimmunity, calcium, phosphorus and parathormone metabolism. Its relationships with diabetes, cancer and most of the cardiovascular diseases were demonstrated by Erdem and Akbaş [8]. Vit-D is also used as an inflammatory biomarker [9]. Furthermore, 25 (OH) D_3 levels with a long half-life are measured in order to evaluate the Vit-D level and its effects [8] 25 (OH) D_3 has been reported to be important in terms of intestinal diseases and, in particular, can limit the occurrence of diarrhea and increase resistance to diarrhea [10].

Iron (Fe) is the fourth most abundant element on earth and is needed by most of the organisms, including bacteria, which is one of the infectious agents. The most important function of Fe in humans and animals is the transport of oxygen to tissues [11]. In addition, Fe is necessary for many metabolic processes, and changes in its level are used in both human and veterinary medicine to monitor the inflammatory process as a biomarker [12]. The sum of serum Fe level and free iron binding capacity (UIBC) makes the total iron binding capacity (TIBC) [13].

Dehydration and metabolic acidosis can occur in calves with diarrhea in the neonatal period, and especially in severe cases. It is known that inflammation, endotoxemia and septic shock accompanying metabolic acidosis disrupt the coagulation mechanism. Metabolic acidosis has been reported to alter the balance of procoagulant, anti-coagulant, and fibrinolytic factors that maintain the homeostasis of the coagulation system and triggers disseminated intravascular coagulopathy (DIC) [14]. Many parameters are used to evaluate this impaired mechanism, and D-Dimer analysis has been reported to be one of the most reliable tests for DIC detection [14]. Clinical findings in neonatal diarrheal calves are highly variable and range from mild watery diarrhea to severely dehydrated and acidotic animals, depending on the severity of diarrhea and inflammation [15]. Different clinical scoring methods have also been developed to evaluate clinical status [16]. Therefore, evaluating the changes in the hematological and biochemical components of neonatal calf diarrhea together with the clinical severity of the disease is a more realistic approach to determine the prognosis of the disease and to establish an appropriate treatment protocol.

In this study, it was aimed to evaluate the relationships between the clinical status of the disease (mild, moderate, severe) and hematologic data, serum 25 (OH) D_3, Fe, UIBC, and D-dimer levels in calves with diarrhea in the neonatal period.

Material and Methods

Ethical Statement

This study was conducted by the approval of Atatürk University Local Ethics Committee of Animal Experiments Erzurum, Türkiye (Decision Number: 2021/5).

Animals

The study material includes 40 calves of different breeds and both sexes, 2-30 days old, brought to the Animal Hospital of the Faculty of Veterinary Medicine (Atatürk University, Erzurum, Türkiye) for examination and treatment. Calves were divided into healthy calves and diarrheal calves according to clinical examination and complete blood count findings. 30 calves with diarrhea and 10 healthy calves were included in this study. The rectal temperature (RT), heart rate (HR) and respiratory rate (RR) of all calves were measured during the clinical examination. Healthy animals consist of calves of the same age, breed and characteristics that are brought to our faculty clinic under the same barn conditions as the sick animals for control purposes. Healthy animal criteria were determined according to Walker et al.[17] (Eyeball recession into orbit, skin elasticity and sucking reflex= 0). The study was conducted in accordance with animal welfare principles. Informed consent form was obtained from the owner before the animals were examined.

Groups

A total of 4 groups, one control and three experimental groups, were used in the study, each group consisting of 10 calves. The calves with diarrhea were classified as mild, moderate and severe according to the criteria for clinical dehydration and depression [17].

Group I (control, n=10): This group consisted of 10 healthy calves with no health problems according to their clinical and hematological data.

Group II (n=10): Considering the data in the scoring system, this group was formed by calves with mild diarrhea with 0 eyeball retraction, 0 skin elasticity, 0 sucking reflex and 3 stool consistency points.

Group III (n=10): This group consisted of calves with moderate diarrhea with eyeball retraction 1, skin elasticity 1, suckling reflex 1 and stool consistency score 3.

Group IV (n=10): This group consisted of calves with severe diarrhoea that had an eyeball retraction of 2, skin elasticity of 2, sucking reflex of 2 and stool consistency of 3 according to the scoring system.

Collecting Blood Samples

Blood samples were taken from the *Vena jugularis* of the calves in all groups in 4 mL EDTA tubes (EDTA K3, Pty Ltd., Adelaide, SA, Australia) for hematological analysis, 8 mL serum tubes (Vacutainer, Becton Dickinson Co. USA) with gel for biochemical analysis, and 1.8 mL 3.2% buffered sodium citrate using standard glass vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) for the determination of coagulation factor. Hematological analyzes and D-dimer measurements were performed immediately in the blood samples taken. Blood samples taken in gel serum tubes were allowed to coagulate for 30 min at room temperature and then centrifuged at 10 min/3000 rpm in a refrigerated centrifuge (Bechman Coulter, USA). After the serums obtained were placed in Eppendorf tubes, they were stored in a deep freezer at -80°C until analysis. In addition, stool samples were taken from the rectum in sterile and locked-lid sample containers for agent isolation in calves with enteritis.

Laboratory Techniques

The isolation of agents in stool samples taken from calves with diarrhea in sterile stool containers was performed in according to the manufacturer's instructions using a commercial immunochromatographic diagnostic kit (Rainbow Calf Scours-BIO K 306 Ag Test Kit, Biox Diagnostics, Belgium) containing enteropathogens of *Rotavirus*, *Coronavirus*, *Cryptosporidium, Cl. perfringens* and *E. coli* (F5-K99).

Hematological Analysis

Analysis of white blood cell (WBC), lymphocyte (LYM), neutrophil (NEU), erythrocyte (RBC), hemoglobin (HGB) and hematocrit (HCT) parameters in blood samples taken from calves in all groups was performed using Abacus Junior Vet 5® device (Diatron MI, Hungary), Veterinary-specific hematology analyzer.

Biochemical Analysis

25 (OH) D_3, Fe and UIBC levels in serum samples were determined with Beckman Coulter® AU5800 (Beckman Coulter, Inc., ABD) autoanalyzer according to the operating procedures recommended by the manufacturer.

Statistical Analysis

The Kolmogorov-Smirnov test in SPSS statistical program version 22.0 was used to test the normality of data of this study. Since the data had normal distribution, the SPSS General Linear Model procedure was used for statistical analysis of the data belonging to the hematological analysis, 25 (OH) D_3, Fe, UIBC and D-dimer levels. Statistical comparisons among group means were carried out by Duncan's Multiple Range test available in the SPSS statistical program when the F-test for the groups was statistically significant.

Results

Information Concerning Breed, Age and Gender of the Calves

The information about breed, age and gender of the calves in all groups within the scope of the study are presented in *Table 1*.

Etiological Findings

Etiological findings obtained from stool samples taken from calves in all groups are tabulated in *Table 2*. According to the microbiological results, while none of the pathogens were found in healthy calves in group-I, in the rest of the experimental groups alone or various combinations of *E. coli*, *Rotavirus*, *Coronavirus*, *Cryptosporidium* and *Cl. perfringens* were identified.

Clinical Findings

The body temperature, respiration and pulse rates of the calves in all groups are presented in *Table 3*. When the body temperature, respiration and pulse rates of the calves in group-I, group II, group III and group IV were compared, it was found out that the differences among the groups were not statistically different.

Hematological Findings

Hematological findings of calves in all groups are provided

Table 1. *Breed, age and gender information of calves in group I, group II, group III and group-IV*

Breed, Age and Gender	Group-I	Group -II	Group -III	Group -IV
Simmental		6	6	6
Brown Swiss	8	2	3	3
Holstein	2			1
Mix		2	1	
Age (days)	8.1±3.1	7.9±5.9	3.9±2.3	4.4±3.2
Female	5	6	2	4
Male	5	4	8	6
n= 40	10	10	10	10

Table 2. *Etiological findings of calves in group-I, group-II, group-III and group-IV*

Etiology	Group-I	Group-II	Group-III	Group-IV
E. coli	-	3	4	4
Rotavirus	-	1	1	1
Coronavirus	-	1	-	1
Cryptosporidium	-	-	-	1
Cl. perfringens	-	1	-	-
E. coli + Rotavirus	-	-	1	1
E. coli + Coronavirus	-	-	1	-
Rotavirus + Coronavirus	-	1	-	-
Rotavirus + Cl. perfringens	-	-	1	1
Giardia spp.	-	1	-	-
Giardia spp. + *Cryptosporidium*	-	-	1	1
Negative	10	2	1	-
n= 40	10	10	10	10

Table 3. *Body temperature, respiration and pulse rates of calves in group-I, group-II, group- III and group-IV*

Parameters/Groups	Group-I Mean±SE	Group-II Mean±SE	Group-III Mean±SE	Group-IV Mean±SE
T (°C)	39.0±0.2	38.1±0.8	37.2±1.6	35.9±1.3
P (Beats/min)	113±11	91±46	115±22	107±27
R (Num/min)	31±5	34±5	48±23	42±11
n= 40	10	10	10	10

T: *Body temperature;* ***P:*** *Pulse rate,* ***R:*** *Respiration rate;* ***SE:*** *Standard Error*

Table 4. *Hematological findings of calves in group-I, group II, group III and group-IV*

Parameters/Groups	Group -I Mean±SE	Group -II Mean±SE	Group -III Mean±SE	Group -IV Mean±SE	P Value
WBC (10^3/µL)	9.3±4.3	13.3±9.4	11.27±5.01	9.22±3.78	
LYM (10^3/µL)	4.47±1.2^b	5.04±1.3^a	5.2±3.3^a	2.76±0.9^c	<0.05
NEU (10^3/µL)	4.5±3.3	7.8±8.8	5.89±5.56	6.3±3.3	
RBC (10^6/µL)	7.3±0.9^b	9.6±1.9^a	9.3±2.58^a	9.8±1.96^a	<0.05
HCT (%)	21.7±2.6^b	36.4±6.47^a	36.4±10.4^a	39.3±6.43^a	<0.01
HGB (g/dL)	8.05±1.15^b	11.25±2.32^a	11.2±3.5^a	12.08±2.45^a	<0.01
n= 40	10	10	10	10	

SE: *Standard Error;* ***WBC:*** *White blood cell;* ***LYM:*** *Lymphocyte;* ***NEU:*** *Neutrophil;* ***RBC:*** *Red blood cell;* ***HCT:*** *Hematocrit;* ***HGB:*** *Hemoglobin;* ***a, b*** *The means shown in different lowercase letters between the groups (on the line) are statistically significant.* ***P<0.05:*** *Statistically significant*

in *Table 4*. The LYM numbers of the calves in the group II and group III were significantly higher than those of the calves in group-I. The LYM numbers of calves in the group-IV were significantly lower than those of group-I. Furthermore, the RBC counts of the calves in group-II, group-III and group-IV were significantly higher than those of the calves in group-I. Similarly, the HCT numbers of the calves in group-II, group-III and group-IV were significantly higher than those of the calves in group-I. The HGB numbers of the calves in the group-II, group-III and group-IV were significantly higher than the calves in the group-I.

Biochemical Findings

Fe, UIBC, 25 (OH) D_3 and D-Dimer levels of calves in all groups are given in *Table 5*. The Fe values of the calves in group-II, group-III, and group-IV were determined to be significantly lower than the calves in group-I. While the 25 (OH) D_3 level in group-IV was found to be significantly higher than group-I, D-dimer levels in group III and IV were significantly higher than in group-I and grup-II.

Table 5. *Fe, UIBC, 25 (OH) D_3 and D-dimer and levels of calves in group-I, group II, group III and group-IV*

Parameters/Groups	Grup-I Mean±SE	Grup-II Mean±SE	Grup-III Mean±SE	Grup-IV Mean±SE	P Value
Fe (µg/mL)	131±66[a]	59±56[b]	56±52[b]	72±63[b]	<0.05
UIBC (µg/mL)	386±125	348±120	339±98	342±94	
25 (OH) D_3 (ng/mL)	6.12±2.73[b]	6.08±4.16[b]	8.8±7.2[ab]	13.4±8.5[a<]	<0.05
D-dimer (mg/L)	0.10[b]±1.46	0.26±0.12[b]	1.15±1.13[a]	0.96±0.88[a]	<0.01
n= 40	10	10	10	10	

SE: *Standard Error,* ***25 (OH), D_3:*** *25-hydroxyvitamin D_3,* ***Fe:*** *Iron,* ***UIBC:*** *Free iron-binding capacity;* ***a, b:*** *The means shown in different lowercase letters between the groups (on the line) are statistically significant.* ***P<0.05:*** *Statistically significant*

Discussion

Neonatal calf diarrhea occurs due to infectious and non-infectious causes [2]. In the present study, 27 of the 30 neonatal calves used in the experimental groups were found to be of infectious origin, and 3 of them were of non-infectious origin.

Clinical findings of diarrhea in neonatal calves range from mild watery diarrhea to coma, depending on the severity of diarrhea and inflammation [15]. Calf diarrhea can be clinically classified as mild, moderate, severe, and fatal by interpreting parameters such as clinical status, mobility, interest in the environment, sucking reflex, food intake, eye condition/dehydration [16]. In addition, eyeball recession into orbit, skin elasticity, sucking reflex and fecal consistency can be evaluated and scored as mild, moderate and severe [17]. In the present study, the scoring of Walker et al.[17] was used to classify calves with diarrhea as mild, moderate and severe. In calves with diarrhea in the neonatal period, body temperature, respiration and pulse rates may be normal as well as increased or decreased [18]. Similarly, in this study, it was determined that there were no significant differences between body temperature, respiration, and pulse rates.

Hematological changes may occur in calves with diarrhea in the neonatal period. The most important of these changes is an increase in the number of WBC, LYM and NEU, namely leukocytosis [19]. In the present study, leukocytosis characterized by lymphocytosis occurred in groups-II and III, and leukopenia characterized by lymphopenia occurred in group-IV. The cause of leukocytosis in groups-II and III is likely to be due to the severity of inflammation and infection and hemoconcentration, as demonstrated in a study [19]. The probable cause of leukopenia in group-IV could be due to immunosuppression, as reported by Cho et al.[20]. Although some studies in neonatal diarrheal calves stated that the level of RBC did not change [21], the level of RBC may also have increased due to the increase in hemoconcentration [19,22]. Similarly, the HCT and HGB levels may also raise due to the increase in hemoconcentration [19,22]. In the current study, levels of RBC, HCT and HGB levels of groups-II, III and IV were higher than those of group-I. The probable cause of this could be due to the increase in hemoconcentration as aforementioned. Likewise, according to the data obtained in the present study, the highest increase in hemoconcentration was observed in group-IV.

In addition to well known classical effects of Vit-D, this vitamin has recently been used as an inflammatory biomarker in human medicine [23], and its relations with various diseases have even been revealed [9]. It has been determined that 25 (OH) D_3, a Vit-D metabolite, has a bactericidal effect in children with bacterial enteritis [24]. Vit-D has also been reported to protect against intestinal surface infections and prevent leaky gut syndrome [25]. In a study conducted on children, it was reported that 25 (OH) D_3 regulates the inflammatory response and activates immune cells in this process [10]. Different results have been obtained in studies in which the Vit-D levels and/or its metabolites were assessed in animals. In a study, It was indicated that the 25 (OH) D_3 level of lambs with diarrhea originating from *Giardia duodenalis* was lower than the healthy ones [26]. In another study, 25 (OH) levels of D_3 were found to be low in goat kids naturally infected with *G. duodenalis* and this was associated with enteritis-induced malabsorption [27]. In this study, the serum levels of 25 (OH) D_3 of the calves were found to be lower than those of the control group, and it was concluded that the level of 25 (OH) D_3 was parallel to the disease findings of the calves with diarrhea and could be considered a negative acute phase biomarker [28].

In the current study, as the severity of the disease increases, serum 25 (OH) D_3 level is expected to decrease. However, interestingly, while there was no difference between group-II and group-I, it was determined that it was significantly higher than group-I in calves in groups-III and IV. In a study conducted in people with Behçet's Disease, which is an inflammatory disease, serum 25 (OH) D_3 levels were found to be higher in patients than in healthy control, which was defined as immunomodulatory and downregulate inflammatory pathways commonly associated with Behçet's Disease [29].

Similarly, the increase in 25 (OH) D_3 levels in this study is likely due to downregulation of inflammatory pathways, as in Behçet's Disease. Also as a result of the increase in the severity of the infection, sepsis occurs in the host, and if the host cannot maintain the inflammatory balance, the systemic inflammatory response syndrome is followed by the multi-organ failure syndrome [4,18]. On the other hand, it is stated that during neonatal calf diarrhea, damage to liver functions, and even severe necrotic and dystrophic lesions can occur in the liver [30]. In addition, biologically inactive precursors of Vit-D are converted to active forms by the kidney. In the kidney, 25 hydroxyvitamin D_3 is converted to 1,25 dihydroxyvitamin D by the hydroxylation reaction [31]. Diarrhea and dehydration in calves have been reported to cause acute renal failure [32]. In the current study, 25 (OH) D_3 was believed to be stored in the liver as the severity of the disease increased, and the reason for the increase in the serum 25 (OH) D_3 level was thought to be caused by the liver and/or due to kidney damage. We thought that high 25 (OH) D_3 levels might be associated with liver and kidney damage.

In addition to carrying oxygen to tissues, Fe is also used as a marker of acute inflammation and infection in humans and animals [33]. The reason for the low Fe level may be malabsorption, anorexia, and increased need, as well as serum Fe concentrations that decrease rapidly in response to inflammation [34]. The decrease in response to inflammation is explained by the host defense mechanism, bacterial virulence, and the need for Fe for replication [11]. In a study conducted on cattle, it was determined that serum Fe level decreased in cases of respiratory tract infection, mastitis and reticulo peritonitis traumatica [35]. Similarly, serum Fe level is low in calves diagnosed with SIRS due to diarrhea and it can be used as a marker for the inflammatory process [36]. In another study in which experimental infection was created with *Salmonella dublin* in calves, it was reported that TIBC increased moderately within 24 h Piercy and serum Fe level decreased [37]. In the present study, Fe levels in diarrheal calves in groups-II, III, and IV were determined to be significantly lower than in group-I. According to clinical findings, the serum Fe levels of calves in group IV with severe diarrhea were higher than those of calves in groups-II and III, suggesting that the reason for this low level was not related to the severity of the infection/inflammation. The reason for this is likely to be malabsorption and anorexia as a result of diarrhea, as stated by other researchers [34,38] There was no statistically significant difference between the UIBC levels of the groups. However, numerical differences are similar to serum Fe levels. This similarity was again associated with malnutrition.

The D-dimer arises when the fibrin clots formed by cross-links are dissolved by plasmin as a result of a disruption in the coagulation profile for various reasons [39]. It is also a biomarker used for diagnosis and measurement of response to treatment by the organism in various clinical conditions other than venous thromboembolism [40]. The high D-dimer level is due to the excessive fibrinolytic response in thrombotic and embolic conditions that occur in acute or chronic diseases that cause coagulation disorders [41]. Different results were obtained in studies evaluating D-dimer levels in calves with diarrhea. D-dimer levels were found to be higher in calves in diarrhea cases caused by *Cryptosporidiosis* than in the control group. This increase was explained by infection and procoagulant activation, as well as infection-induced receptor signaling, pro-inflammatory cytokine, chemokines, and antimicrobial peptide production [42]. In another study in calves with diarrhea, the reason for the high D-dimer concentration in the diarrheal group was interpreted as an indicator of impaired hemostasis and the development of secondary fibrinolysis associated with DIC [43]. A study on D-dimer levels of calves with diarrhea-induced sepsis found no significant differences. This is explained by the absence of hyperfibrinolysis [44]. In the present study, while there was no significant difference between group-II and group-I D-dimer levels, it was determined that the calves in group-III and IV were significantly higher than the calves in group-I. Therefore, as the severity of the disease increased clinically, D-dimer levels also increased. These data can be evaluated as an indicator of the deterioration of hemostasis as the clinical severity of the disease increases and the development of secondary fibrinolysis associated with DIC, as stated by [43]. As a result, it was determined that as the severity of the disease increased clinically in calves with diarrhea in the neonatal period, Fe and UIBC levels decreased, while 25 (OH) D_3 levels increased and clinical coagulation disorders could occur.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (M. S. Eroğlu).

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Competing Interests

The authors declared that there is no conflict of interest.

Ethical Statement

This study was conducted by the approval of Atatürk University Local Ethics Committee of Animal Experiments

Erzurum, Türkiye (Ethics Committee Decision Number: 2021/5).

Authors' Contributions

MSA and MSE conceived and supervised the study. MSE, KEY and EE collected and analyzed data. The first draft of the manuscript was written by MSE and KEY and all authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Research Article

Comparison of Digestibility, Stool Quality, Preference and Manufacturing Cost of Grain-inclusive and Grain-free Dry Dog Foods

Oğuzhan KAHRAMAN [1,a (*)] Fatma İNAL [1,b] Mustafa Selçuk ALATAŞ [1,c]
Şeref İNAL [2,d] Mustafa ULUDAĞ [3,e] Cafer TEPELİ [2,f]

[1] Faculty of Veterinary Medicine, Selçuk University, Department of Animal Nutrition and Nutrional Diseases, TR-42100 Konya - TÜRKİYE
[2] Faculty of Veterinary Medicine, Selçuk University, Department of Animal Science, TR-42100 Konya - TÜRKİYE
[3] Çamlıca Mah. 133. Cad. 18/23 TR-06200 Yenimahalle, Ankara - TÜRKİYE
ORCIDs: [a] 0000-0002-9315-5276; [b] 0000-0002-5022-1579; [c] 0000-0001-9920-1838; [d] 0000-0003-4746-8930; [e] 0000-0003-1976-162X
[f] 0000-0003-2966-8330

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Abstract: This study aimed to compare nutrient digestibility, preference rate, effects on stool and cost of grain-inclusive and grain-free dry dog foods. Two dry dog foods with and without grain formulated with poultry meal, barley, rice, corn, peas, carrot, potato flour, whey, fat, vitamin and mineral sources were manufactured. Twelve adult Golden Retriever dogs (age 3-4 years, body weight = 22.5±1.7 kg) were divided into two groups for the digestibility trial. A total of 20 Golden Retriever and Kangal (age 4-5 years, body weight = 35.5±1.9 kg) breed dogs were used for a two-pan preference test. Crude fibre digestibility of grain-free food was significantly lower (P<0.05). There was no difference in other nutrient digestibility in foods as determined by total faecal collection method. Presence of grains in food improved the consistency and dry matter of stool(P<0.05). Dogs preferred grain-free food (55.88%) to grain-inclusive food (44.12%) (P<0.05). The manufacturing cost of grain-free food was found to be about three times higher than grain food. The benefits of grain-free dog diets are debated. The effects of grain and grain-free foods on digestibility, stool parameters and canine health should be demonstrated by further studies. Cost must be calculated to produce reliable and suitable quality dog food with different ingredients. Palatability and intake levels, which are among the most important criteria in dog nutrition, should be determined by preference tests.

Keywords: *Digestibility level, Faecal consistency, Grain free dog food, Grain inclusive dog food, Preference test*

Tahıllı ve Tahılsız Kuru Köpek Mamalarının Sindirilebilirlik, Dışkı Kalitesi, Tercih Oranı ve Üretim Maliyetinin Karşılaştırılması

Öz: Bu çalışmanın amacı tahıllı ve tahılsız kuru köpek mamaların besin madde sindirilebilirliği, tercih oranı, dışkıya etkileri ve maliyetini karşılaştırmaktı. Kanatlı unu, arpa, pirinç, mısır, bezelye, havuç, patates unu, peynir altı suyu, yağ, vitamin ve mineral kaynakları ile formüle edilmiş tahıllı ve tahılsız iki kuru köpek maması üretildi. On iki yetişkin Golden retriever köpek (3-4 yaş, canlı ağırlık = 22.5±1.7 kg) sindirilebilirlik denemesi için iki gruba ayrıldı. İki kap tercih testinde toplam 20 adet Golden Retriever ve Kangal ırkı (4-5 yaş, canlı ağırlık = 35.5±1.9 kg) yetişkin köpek kullanıldı. Tahılsız mamanın ham selüloz sindirilebilirliği önemli düzeyde daha düşüktü (P<0.05). Toplam dışkı toplama yöntemi ile belirlenen diğer besin madde sindirilebilirlikleri arasında fark bulunmadı. Mamada tahılların varlığı dışkı kıvamı ve dışkı kuru madde düzeyini iyileştirdi (P<0.05). Köpekler tahılsız mamayı (%55.88) tahıllıya (%44.12) göre daha fazla tercih etti (P<0.05). Tahılsız mamanın maliyeti tahıllıya göre yaklaşık 3 kat daha yüksek bulundu. Tahılsız köpek diyetlerinin faydaları tartışılmaktadır. Tahıllı ve tahılsız mamaların sindirilebilirlik, dışkı parametreleri ve köpek sağlığı üzerindeki etkileri daha fazla çalışmayla ortaya konmalıdır. Farklı ham maddeler kullanılarak güvenilir ve uygun kalitede köpek maması üretimi için maliyet mutlaka hesaplanmalıdır. Köpek beslemede en önemli kriterler arasında olan lezzet ve tüketilebilirlik düzeyi de tercih testleriyle belirlenmelidir.

Anahtar sözcükler: *Dışkı kıvamı, Sindirilebilirlik düzeyi, Tahıllı köpek maması, Tahılsız köpek maması, Tercih testi*

Introduction

Grains such as barley, wheat, corn, rice, sorghum and oats are used as ingredients and economical energy sources in dry dog foods [1]. Most dry foods have carbohydrate levels of 30-60%, which is mainly derived from cereal grains. Although carbohydrates are not one of the essential nutrients for dogs, they are commonly used for economic

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(*) Corresponding Author
Tel: +90 332 223 2674 Cellular Phone: +90 542 591 6524 Fax: +90 332 241 0063
E-mail: vetoguzhan90@gmail.com (O. Kahraman)

reasons. Starch is an important source of carbohydrates, as it increases the release of glucose after digestion [2]. For these reasons, the nutritional value, palatability and digestibility level of the cereal grains have become an important issue in dog care [3]. Dry dog foods are produced by an extrusion method, which results in the gelatinization of starch present in the ingredients. Gelatinization is described as breakdown of all starch granules by moisture, temperature, pressure and mechanical shear. This gelatinized starch in extruded grains is highly digestible and ranges from 89% to 99% in food products [4]. Due to their important role in the majority of dog diets worldwide, interest in the effects of grains is growing [5].

Dogs willingly eat extruded food, which is rich in cereal grain. In some dog food, rice, barley, oats, wheat or millet are marketed as part of the names of dog food. Of these grains, rice is the most preferred for dog foods due to its high digestibility, low fibre content and hypoallergenic properties. Use of rice does not cause any problems related to digestibility, stool consistency or preference [6]. Whole grains also contribute important nutrients, such as vitamins, minerals and essential fatty acids, in pet foods. Various cereal products can also provide more easily digestible source of protein than some animal proteins sources [1].

Grain-free dog foods are available on the market and have been made more popular by the belief that dogs do not eat cereal grains in nature. Today, commercially available, grain-free pet food options represent more than 40% of available dry dog foods in the United States [7]. Many dog owners believe that grain-free food is best for the health of their animals. However, some owners trying to feed their dogs healthier food are focusing too much on advertisements and labels on commercial foods, such as 'grain-free' and 'gluten-free' [8]. Alternative starch sources, such as potatoes, tapioca and legumes (peas, beans, lentils), are also used in the production of grain-free dry dog foods. Grain-free foods are more expensive than regular grain-inclusive foods in the market because they are considered to be premium dog foods. Despite claims of high quality, only limited studies have reported that grain-free foods are more digestible and palatable for dogs. Chiofalo et al.[9] reported that grain free diets are more suitable for active dogs, but in another study, grain-inclusive foods were shown to be more digestible than grain-free foods in terms of dry and organic matter [3].

Despite the fact that grain-free dog foods are becoming increasingly popular, research on the effects of grain-free dog foods on digestibility, preference, stool consistency and manufacturing costs are limited, and there is not well-established data comparing these parameters with grain-inclusive foods. Therefore, this study aimed to evaluate these parameters in grain-inclusive and grain-free dry dog foods.

Material and Methods

Ethical Statement

The research was carried out with the permission of the Selcuk University, Faculty of Veterinary Medicine, Experimental Animals Production and Research Center ethics committee (Approval no: 2016/74) at the Selçuk University, Faculty of Veterinary Medicine, Application and Research Farm Dog research unit.

Animals

Twelve Golden Retriever dogs (eight females and four males) aged 3-4 years old, with the body weights (BW) of 22.5±1.7 kg and eight healthy adult Kangal breed dogs (four females and four males) aged 4-5 years old (35.5±1.9 kg BW) were used in this study. They were housed in individual concrete kennels with a closed (190 x 190 cm) and open area (510 x 230 cm).

Preparation of Foods

Grain-inclusive and grain-free food formulations were prepared and two experimental diets were created. Nutritionally sufficient foods with balanced energy and protein levels were produced for adult dogs according to FEDIAF guidelines [10]. Poultry meal was used as an animal protein source in the composition of both food types. Cereal grain products such as rice, corn, corn gluten, corn starch and barley were included in the grain-inclusive composition. Potatoes, peas and carrots were included in grain-free formula *(Table 1)*.

Foods were produced at a feed facility operating in Ankara. All of the raw materials in the composition of the foods were supplied by the factory. Raw ingredients were weighed according to the formulations and milled to pass through a 0.4 mm sieve. After homogenization in the mixer, water was added into the conditioner to achieve 20-30% humidity. The mixture was then cooked for 4 min at an increasing temperature between 90-135°C. After four stages in a DG-85 double-screw extruder, wet extrudates were dried in the belt dryer at temperatures of up to 140°C for 30-45 min. Vitamins and minerals were added and oils and fats sprayed into the hot dried foods. After cooling, and packing in airtight bags, food samples were taken from each bag for nutrient analysis and the bags were sealed.

Chemical Analysis

Experimental foods and faeces were analysed for dry matter (DM), ash, crude protein (CP), acid hydrolysed ether extract (EE) and crude fibre (CF); starch analyses were performed using the methods reported in AOAC [11]. Using the results of the analysis, the metabolizable energy of the foods was calculated with the equation below [12]:

Table 1. Ingredient and nutrient composition of dog foods				
Food Formulation	**Digestibility Trial**		**Preference Test**	
Ingredients	**Grain-inclusive,%**	**Grain-free,%**	**Grain-inclusive,%**	**Grain-free,%**
Poultry meal	18.00	26.00	20.00	26.00
Whey powder	2.00	2.00	2.00	2.00
Corn gluten meal	10.00		12.00	
Barley	10.00		10.52	
Corn	20.00		18.00	
Corn starch	12.00		12.00	
Rice	20.00		18.00	
Pea flour		18.00		30.00
Carrot flour		10.00		5.82
Potato flour		36.30		29.00
Sunflower oil	3.00	3.00	3.00	3.00
Beef tallow	3.53	3.53	3.53	3.53
Aminovit[1]	0.30	0.30		
Zinc proteinate	0.02	0.02		
Calcium iodate	0.0002	0.0002		
Minesol[2]	0.85	0.85		
Vitamin premix[3]			0.30	0.30
Mineral premix[4]			0.30	0.30
Choline chloride			0.05	0.05
Potassium chloride	0.30	0.30	0.30	
Calculated nutrients in 100 g dry matter				
Crude protein,g	23.26	23.14	25.22	25.27
Crude fiber, g	2.27	3.95	2.25	4.04
Ash, g	4.64	6.14	5.16	6.17
Carbohydrate, g	61.15	55.64	58.29	52.05
Calcium, g	0.76	1.11	0.90	1.15
Phosphorus, g	0.67	0.80	0.64	0.76
Ether extract, g	11.44	11.78	11.55	11.75

[1] ***Aminovit (per liter):*** *Vit. A 20.000.000 IU, Vit. D_3 200.000 IU, Vit. E 10.000 mg, Vit. B_1 2.500 mg, Vit. B_2 2.500 mg, Vit. B_6 500 mg, Vit. B_{12} 5 mg, Vit. K_3 500 mg, Vit. H 15 mg, Pantotenic Acid 2.500 mg, Choline Chloride 70.000 mg, L-Arginine 600 mg, L-Cystine 100 mg, L-Leucine 600 mg, L-Valine 600 mg, L-Isoleucine 200 mg, L-Histidine 200 mg, L-Phenylalanine 500 mg, L-Proline 800 mg, L-Serine 100 mg, L-Tyrosine 200 mg, L-Treonine 500 mg, DL-Methionine 500 mg, L-Triptophane 20 mg, L-Lysine 3.000 mg, L-Glutamic Acid 4.000 mg, L-Alanine 1.000 mg)*

[2] ***Minesol (per liter):*** *Phosphorus 75.000 mg, Calcium 20.000 mg, Sodium 1.600 mg, Manganese 600 mg, Potassium 1.050 mg, Ferrous 1.600 mg, Magnesium 3.200 mg, Zinc 650 mg, Copper 250 mg, Cobalt 250 mg, Selenium 10 mg, Methionine 10.000 mg, Lysine 5.000 mg); Potassium chloride; Zinc proteinate; Calcium iodate; Sodium bicarbonate*

[3] ***Vitamin premix (mg/kg):*** *Biotin 250 mg, Folate 2.000 mg, Niacin 50.000 mg, Pantothenic acid 20.000 mg, Riboflavin 8.000 mg, Thiamine 4.000 mg, vit B_6 5.000 mg, Vit. B_{12} 25 mg, Vit. A 3.600 mg, Cholecalciferol 125 mg, Vit. E 50.000 mg*

[4] ***Mineral premix (mg/kg):*** *Mn proteinate 70.000 mg, Zn proteinate 100.000 mg, Fe proteinate 70.000 mg, Cu proteinate 14.000 mg, Iodine 1.000 mg, Co proteinate 350 mg, Se proteinate 140 mg, Mo proteinate 700 mg, Mg oxide 35.000 mg*

ME, kcal/kg = ((5.7 × CP × 10) + (9.4 × EE × 10) + (4.1 × (NFE × 10 + CF × 10))) x (91.2 – (1.43 × CF))/100 – (1.04 × CP × 10)

NFE, % = 100 – (% crude protein + % ether extract + % crude fibre + % moisture + % ash)

ME: Metabolizable energy, CP: Crude protein, EE: Ether extract, NFE: Nitrogen free extract, CF: Crude fibre

Digestibility Trial

The digestibility of the nutrients of two foods was determined using the total collection method [10,11]. Twelve adult Golden Retriever dogs were divided into two groups with equal body weight and gender. Animals were fed daily at 10:00 am for 15 days. Clean drinking water was provided *ad libitum*. Daily metabolizable energy (ME) requirements of laboratory kennel dogs were calculated according to the recommendation of NRC (kcal/day, 132 * $BW^{0.75}$ kg) [12]. After day 10 of the acclimation period, the freshly excreted faeces of all animals were collected daily for 5 days. The faeces were weighed and stored in a deep freezer (-18°C) until analysis. The collected frozen faeces of each animal were thawed at laboratory environment temperature (23-25°C) and mixed homogeneously. Stool samples were dried in an oven set at 55°C for 60 h. Dry faeces were then ground with a laboratory mill to pass

through a 1 mm sieve (Retsch SM100, Germany) and nutrient analyses of stools and foods were performed. Dry matter (DM) and nutrient digestibility were calculated using the following equations [13]:

DM digestibility, % = [(Consumed DM – Faecal DM)/Consumed DM x 100

Nutrient digestibility, % = [(Nutrient intake (g/day) – Nutrient in faeces (g/day))/Nutrient intake (g/day)] x 100

Preference Test

A two-bowl preference test was carried out over 8 days. Two foods (450 g each for Goldens, 750 g each for Kangals) were presented in stainless steel bowls to 20 dogs (12 Golden Retrievers, 8 Kangals) to choose between; total quantity of food consumed from both bowls during 20 min was recorded [14]. Grain-inclusive and grain-free foods were served in the same bowls, while switching the bowls between sides every day to avoid bias. Foods were relocated and the same food was presented to each dog four times on the right and four times on the left side.

Throughout the preference test, the animals were kept in their compartments and were provided with access to fresh water at all times. Food bowls were placed in the same location of kennels. The dogs were kept in the open area of kennels while the food was placed. After feeding, bowls were collected and the remaining amounts were weighed. The right and left side bias of the dogs was also recorded and calculated. The preference rate was calculated according to the following formula [15]:

Preference rate of grain food (PRG), % = Grain food consumption, g /(Grain food consumption, g + Grain-free food consumption, g) **x** 100

Preference rate of grain-free food, % = 100 – PRG

Stool Consistency Scoring

Stool consistency was scored by three researchers just before fresh faeces were collected in the last 4 days of the total faeces collection experiment. A 1–5 scoring system was used as follows: 1 = pasty and shapeless; 2 = soft, lightly shaped; 3 = soft, shaped, moist and leaves a mark on the ground; 4 = well-formed, non-dispersing, no mark on the ground; 5 = well-shaped, solid, dry [16].

Determination of the Production Cost of Foods

Cost of the prepared foods was calculated by taking into consideration the unit prices of the raw materials as well as operating, waste, depreciation, packaging and shipping costs. After calculating the unit costs of food, daily food costs were calculated according to the daily consumption of an adult dog with 20 kg body weight.

Statistical Analysis

Statistical analysis was performed using the statistical package of SPSS version 22.0 (SPSS, Chicago, IL, USA). A Mann-Whitney U test was performed to compare nutrient digestibility results of the two independent groups of the same dog population. The significance of preference rate was determined using the same statistical test on the 20 dogs. An independent sample test was used to compare stool consistency scores.

Results

Nutrient composition and metabolizable energy levels of the foods fed to dogs in the research are given in *Table 2*.

Results of nutrient digestibility, preference rate, faecal dry matter and stool consistency of dogs and cost of foods are shown in *Table 3*. Digestibility coefficient of crude fibre (CF) of grain-inclusive food was statistically higher ($P<0.05$). Dry matter (DM), organic matter (OM), ether extract (EE) and crude protein (CP) digestibility coefficients of grain-inclusive and grain-free foods were not significantly different ($P>0.05$). Bowls were offered in different directions, i.e., left and right sides of kennels, but the dogs did not show right or left direction preferences The grain-inclusive group had higher quantities of faecal dry matter and stool consistency ($P<0.01$). Manufacturing and production costs of grain-free food were 3 times higher than those of grain-inclusive food *(Table 3)*.

Preference rate of each dog was given *(Fig. 1)*. Grain-free food was significantly more preferred ($P<0.05$). Unlike Golden Retrievers, Kangal breed dogs preferred grain-inclusive food *(Fig. 2)*.

Discussion

Due to possible differences between the calculated and determined nutritional values of ingredients in the composition of the foods, the energy level found in the grain-free food was slightly lower, while that of the grain-inclusive food was slightly higher. The energy levels of the food are important, as food intake is essentially controlled by the energy density of dogs' food or diets. Therefore,

Table 2. *Determined Nutrient composition (DM,%) and metabolizable energy levels of foods (kcal, DM)*

Food Type	DM	Ash	EE	CF	CP	ME*	Starch
Grain-inclusive	92.56	5.25	12.65	4.30	24.23	395.2	42.10
Grain-free	91.50	5.48	12.11	6.67	23.45	375.2	41.40

** kcal, calculated with the NRC 2006 equations;* ***DM:*** *Dry matter;* ***EE:*** *Ether extraction;* ***CF:*** *Crude fibre;* ***CP:*** *Crude protein;* ***ME:*** *metabolizable energy*

Table 3. Digestibility coefficients, production costs, preference rates of foods and stool characteristics of dogs

Parameters		Food Type		Significance
		Grain-inclusive	Grain-free	
Digestibility coefficients	DM	80.93	80.65	-
	OM	84.43	80.65	-
	EE	97.35	96.74	-
	CF	61.70	53.23	**
	CP	78.03	78.77	-
Stool characteristics of dogs	Stool consistency	41	36.59	*
	DM of stool	4.56	4.07	**
Production cost of dog foods	Amount of food required,kg/d	0.320	0.320	
	Cost,$/kg	0.82	2.49	
	Cost,$/d	0.26	0.80	
	Ratio	100	308	
Preference rates	Intake,d/day	267	325.85	**
	Preference rate of 20 dogs,%	44.12	55.88	*
	Food preference rate of Golden retriever dogs (n= 12)	38.83	61.16	*
	Food preference rate of Kangal dogs(n=8)	59.99	40.01	*
	Preference of side bias of 20 dogs, %	right side 50.31	left side 49.69	-

*** P<0.05 (Mann-Whitney U test), * P<0.001, -: not significant*

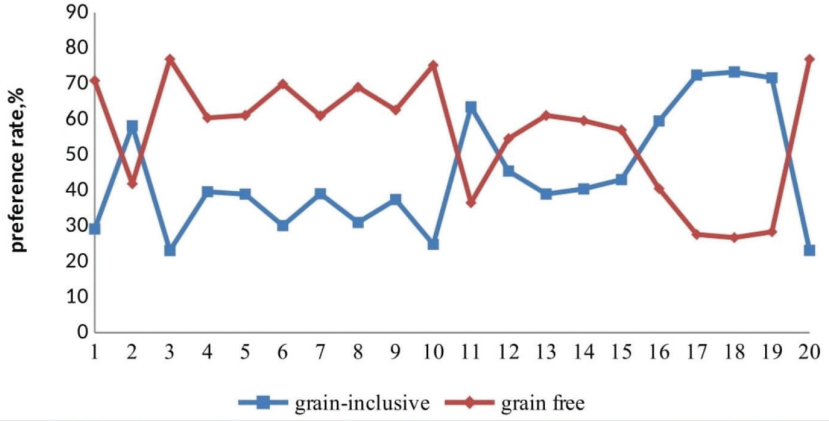
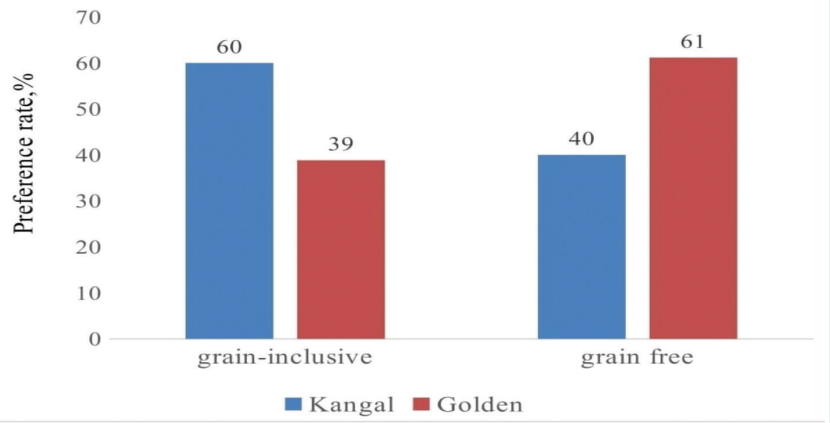


Fig 1. Preference rates of 20 dogs, %

Fig 2. Preference rates of Golden and Kangal breed dogs, %



all nutrients in the food should be relative to the energy content [17]. Modified Atwater factors and NRC equations have moderate accuracy for estimation of the ME for wet pet foods. However, NRC equations are recommended for estimation for dry dog foods [18], and were used to estimate energy level in foods in this study.

Crude fibre (CF) digestion was significantly lower when dogs were fed grain-free food. Contrary to popular belief, grain-free dog foods are generally not low in carbohydrates, with some types even having carbohydrate levels similar to grain-inclusive dog foods [8]. In this study, peas, carrots and potatoes were used as the carbohydrate source in grain-free food, and rice, corn and barley were used in grain-inclusive food. Previously, it has been reported that there is no difference in CF digestibility among dog food based on corn, rice and peas [19]. However, in contrast, one study showed that pea diets had the lowest digestibility level [4]. De-Oliveira et al.[19] reported that CF had the lowest apparent digestibility in dogs because there is a significant correlation between fibre intake and faecal fibre excretion in dogs. Their pea-based diet had a CF level of 8.2%, and this diet had the lowest DM digestibility (76%). In our study, the grain-free diet had 6.67% CF and 80.65% digestibility of DM. The potato flour level was 36% in grain-free food formulation, while the level of carrots was 10%. Although there is no information on the CF digestion of these two vegetables, we believe that the low level of CF digestibility for grain-free foods in this study was caused by the presence of carrots, because carrot peels are generally used as a source of fibre and are composed of more than 75% total dietary fibre level [20]. Kara [21] found that the OM disappearance of carrot-including dog food was 83%, showing one of the lowest coefficients of eight low-priced dog foods he evaluated. Although vegetables are good sources of protein and energy, there is little experimental data regarding their digestibility for domestic dogs [22]. In one study, Kahraman and İnal [3] compared seven grain-free commercial dry foods and 14 grain-inclusive foods, reporting that CF digestion was lower in grain-free foods. Chiofalo et al.[9] found higher protein and fat digestibility, more stable large intestinal fermentation, 13% lower food requirement, reduced stool excretion and higher body condition scores in dogs fed high-protein, low-carb and grain-free food.

Digestibility of DM and OM foods was found to be similar. Due to the formulations of these foods, the nutrient compositions were calculated as similar as possible and DM and OM digestibility results were consistent with those reported by Brambillasca et al.[23]. OM digestibility was close to the average OM digestibility of 38 commercial dry dog foods calculated by Castrillo et al.[24] In this study, OM digestibility found in grain-free food was lower than the figure found for grain-free food by Chiofalo et al.[9]. However, commercial grain-free food used in that study contained 39% CP and 19% EE. Nonetheless, DM digestibility level of their food was quite similar to this study.

In this study, EE digestibility found in both foods was much higher than poultry fat-including foods investigated by Donadelli and Aldrich [25]. This difference could be attributed to the use of sunflower oil and beef tallow in the composition of food in our study. Animal fat and vegetable oils were used in the commercial grain-inclusive and grain-free dry food formulations. Unlike in the current study, Kahraman and İnal [3] reported that fat digestibility was higher for grain-free food than grain-inclusive food. Abd El-Wahab et al.[26] found 87.2-88.0% fat digestibility of 7.7% crude fat-containing food. On the other hand, Kim et al.[27] found 97% fat digestibility in dog food containing 20.5% fat. Here, our foods contained 12% fat and digestibility levels of EE were 96.7-97.3% for grain-free and grain-inclusive foods, respectively. Fat utilization and fat digestibility in dogs is underestimated [28]. Considering that fat level and source could be related to EE digestibility in dogs, this relationship should be investigated in future studies.

Dos Reis et al.[29] found 86% and 87% total tract apparent protein digestibility levels in foods containing 25% and 35% CP, respectively. We report CP digestibility levels of 78.03% for grain-inclusive foods and 78.77% for grain-free foods. The CP digestibility determined here was found to be lower than that found by some previous studies [9,23,24]. This difference could be explained by the fact that CP level can be up to 45% in commercial dry foods. Kahraman and İnal [3] did not find any difference between foods with and without grain in terms of CP digestion. Chiofalo et al.[9] found that CP digestion was significantly higher for grain-free foods compared to grain-inclusive. Because the rate of CP in grain-free food used by Chiofalo et al.[9] was about 60% higher than that in the grain-inclusive dog food of this study. However, high-protein foods do not always have a higher CP digestibility rate. Because high-protein foods can be created with vegetable sources that are rich in protein but low in digestibility.

The consumption rate of a food or diet is the best indicator of overall taste preference [15]. Foods served daily totalled 450 g for Golden Retriever and 900 g for Kangal dogs. On average, 267 g of grain-inclusive food was consumed, while 352.85 g of grain-free food was consumed. In other words, grain-free food was significantly preferred. Dogs had no preference regarding preference for feeding on the right or left side. Various processing techniques and enzyme use affect the preference for canned or cooked meat, soybean or poultry meal in dogs. It is known that previous habits are also important in food preference [27]. However, old habits were not effective here, as the dogs were fed grain-

inclusive food before starting the preference test. Foods were tested for 8 days in this study. In other studies, 4 days of preference testing was reported to be sufficient for dogs to determine palatability [15]. However, increasing the number of days of preference test increases reliability [25]. Callon et al.[22] reported that dogs should be fed a new diet for at least 9 days in order to determine acceptance.

In regard to food preference by dog breed, we saw that 8 Kangal dogs preferred grain-inclusive foods. As Kangal dogs are used in the management of sheep herds in Turkey and traditionally fed with cooked barley or wheat, there may be hereditary reasons that Kangal breed dogs preferred grain foods [30].

Stool consistency scores or faecal DM content affect the stool quality. Although faecal score was considered to be in the optimal range (3-4) for both foods, softer stools were observed in dogs fed grain-free food. This could be explained by the fact that the CF level was higher and CF digestibility was lower for this food type. High fibre levels in the diet limit the interaction between food, enzymes, digestive products and water absorption by increasing transit time through the gastrointestinal tract [23]. Additionally, the stool consistency score (4.07) detected for grain-free food was within the desired intervals for dogs [31]. The result of stool consistency scores of Chiofalo et al.[9] was similar to the current study. Zanatta et al.[32] also reported similar scores and faecal DM in adult dogs fed similar food in terms of nutrient composition. In that study, they fed dogs grain-free and grain-inclusive foods for 84 days. Desired stool consistency scores were observed despite low CF digestibility of grain-free food of this study. This might be the result of insoluble fibre fraction levels of CF in the foods, although this was not determined in this study. Oba et al.[33] found that grain-free dog foods that included potatoes and carrots had higher total insoluble fibre fraction levels, and that these foods had the lowest digestibility. Dhingra et al.[34] also emphasised that most of the total fibre of potatoes and carrots consists of insoluble fibre fraction. The grain-free formula in this study contained potatoes and carrots, and this could be considered to be another cause of the low CF digestibility. Diets prepared in extruded form in this study and by Rashid et al.[35] showed that the insoluble fibre content decreased and the soluble fibre content increased after extrusion. Effects of extrusion on total dietary fibre and insoluble fraction of fibre should be considered in future studies.

In this study, no flavour, digestion, stool consistency enhancers or additives were included in the formulations of the foods. In commercial foods, adsorbent materials can be used to increase faecal consistency or faecal DM. In this study, the faecal DM of dogs was found to be parallel, with faecal scores of 41% (grain-inclusive) and 36.59% (grain-free). Higher stool DM contributed to the stool consistency score.

Commercial grain-free foods are more expensive than grain-inclusive foods on the market. Vegetable flours included in the formula of grain-free food were three times more expensive than grain cereals used in this study. Due to commercial concerns or sales policies, exotic and expensive ingredients are used in the formulations of grain-free foods, and they are generally higher in CP level than regular grain dog foods. Higher dietary protein levels are less sustainable and more expensive. Protein rich diets increase the presence of ammonia by decreasing the number of lactobacilli and enterococci and form carcinogen biogenic amines that decrease digestibility through negative effects on villi height of intestines [36,37].

In conclusion, palatable and nutritionally balanced foods are essential for dog health. There should not be any ingredients or additives that are risky for animals. Regardless of whether a food is grain-inclusive or grain-free, if any nutrient is excessive or deficient, the animal will suffer from it. Grain cereals should not be considered to be unhealthy for dogs. It should always be remembered that dogs can be fed grain cereals, vegetables and animal products. Grains are avoided in foods because owners are trying to protect dogs from allergies or diabetes, but grain-inclusive foods are as digestible as grain-free foods. In grain-free and low-carbohydrate foods, protein and fat levels in particular are considerably higher. Thus, obesity and kidney problems become inevitable. However, grain-free foods can be considered for dogs that are susceptible to grains. Further research on the effects of high-protein and fat-containing grain-free food on obesity, health, digestibility and stool quality parameters should be conducted through long term feeding trials.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (O. Kahraman).

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

OK: the hypothesis of this study; Fİ: dog food formulating; OK, Fİ, MSA, Şİ, MU and CT: experimental procedure follow-up; MU: dog food formulating; OK and Fİ: literature review, assessment of results, final decision.

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SHORT COMMUNICATION

Whole Mitogenome of Golmud Yak *(Bos grunniens)*: Sequencing, Characterization and Phylogenetic Analysis

Guangzhen LI [1,2,a] Shengmei CHEN [1,2,b] Yinlu XIE [3,c] Weixin GUO [1,2,d] Zhijie MA [1,2,e (*)]

[1] Academy of Animal Science and Veterinary Medicine, Qinghai University, Qinghai, Xining, 810016, CHINA
[2] Key Laboratory of Plateau Livestock Genetic Resources Protection and Innovative Utilization, Qinghai, Xining, 810016, CHINA
[3] Golmud Station of Animal Husbandry and Veterinary, Haixi Mongol and Tibetan Autonomous Prefecture of Qinghai Province, Qinghai, Golmud, 816000, CHINA
ORCIDs: [a] 0000-0002-8812-5558; [b] 0000-0002-2955-9719; [c] 0000-0001-5724-9008; [d] 0000-0002-0914-1817; [e] 0000-0001-7544-3533

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Abstract: Golmud yak *(Bos grunniens)* is mainly lived in the southern Qaidam Basin in Qinghai Province, China. Here, the complete mitogenome of the Golmud yak was firstly sequenced using Illumina high-throughput sequencing technique and the corresponding sequence characterization also has been identified. The mitogenome of Golmud yak was 16,324 bp length with an A + T-biased nucleotide composition (60.97%). It includes 22 tRNA genes, 13 protein-coding genes, 2 rRNA genes and a control region (D-loop region), which is consistent with that of other bovine species. The phylogenetic tree showed that Golmud yak was most closely related to Sibu, Huanhu, Zhongdian, Ashdan, Jiulong, Pali, Datong and Bazhou yak breeds, closer to Yushu, Niangya, Qinghai-Gaoyuan, Xueduo, Maiwa and wild yak, but far away from other yak breeds (i.e. Jinchuan, Gannan and Tianzhu white yak). The complete mitogenome sequence reported here would provide valuable information for breeding and improvement of Golmud yak.

Keywords: *Bos grunniens, Mitogenome, Annotation, Phylogeny*

Golmud Yak'ının *(Bos grunniens)* Tam Mitogenomu: Sekans, Karakterizasyon ve Filogenetik Analiz

Öz: Golmud yakı *(Bos grunniens),* çoğunlukla Çin'in Qinghai Eyaletindeki güney Qaidam Havzasında yaşar. Burada, Golmud yakının tam mitogenomu ilk olarak Illumina yüksek verimli sekans tekniği kullanılarak sekanslandı ve karşılık gelen sekans karakterizasyonu gerçekleştirildi. Golmud yakının mitogenomunun uzunluğu %60.97 A + T-taraflı nükleotit bileşimi ile 16.324 bp idi. 22 tRNA geni, 13 protein kodlayan gen, 2 rRNA geni ve diğer sığır türleriyle tutarlı olan bir kontrol bölgesi (D-loop bölgesi) içeriyordu. Filogenetik ağaç, Golmud yakının en yakın olarak Sibu, Huanhu, Zhongdian, Ashdan, Jiulong, Pali, Datong ve Bazhou yak ırkları ile ilişkili olduğunu gösterdi. Bu yaklar, Yushu, Niangya, Qinghai-Gaoyuan, Xueduo, Maiwa and yabani yaklara yakın iken, diğer yak ırklarından (örneğin, Jinchuan, Gannan ve Tianzhu beyaz yakı) oldukça uzaktı. Burada bildirilen tam mitogenom sekansı, Golmud yakının yetiştirilmesi ve geliştirilmesi için değerli bilgiler sağlayacaktır.

Anahtar sözcükler: *Bos grunniens, Mitogenom, Notlama, Filogeni*

Introduction

Yak, an ungulate, provides meat, milk and other necessities for local inhabitants at high altitude area on the Qinghai-Tibetan Plateau (QTP) and other adjacent alpine and subalpine areas [1]. China owns abundant yak genetic resources. At present, there are 22 officially recognized yak breeds (Qinghai-Gaoyuan, Huanhu, Xueduo, Yushu, Niangya, Sibu, Pali, Leiwuqi, Tibet-Gaoshan, Tianzhu, Gannan, Bazhou, Zhongdian, Jiulong, Maiwa, Changtai, Jinchuan, Muli, Pamier, Chawula, Datong and Ashdan) in China. In the paternal genetic study of Qinghai yak, the researcher explored the Y chromosome haplotype diversities and population genetic structure of nine Qinghai yak breeds/populations (Tanggulashan, Tianjun, Qumalai, Qilian, Guoleimude (Golmud), Ganglong, Xueduo,

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(*) Corresponding Author
Tel: +86-971-5318783; Fax: +86-971-531878
E-mail: zhijiema@126.com (Z. Ma)

Huanhu and Datong) and showed that Qinghai Province might be one of the domestication or origin places of yak, in which Golmud yak *(Bos grunniens)* was first described [1]. Golmud yak has excellent properties of meat, milk and reproduction, which is mainly produced for meat in Haixi Mongol and Tibetan autonomous Prefecture of Qinghai province, China. Again, it also owns good adaptability to the cold ecological environment and arid mountainous areas. Recently, based on nucleotide variations of mtDNA, the maternal genetic diversities of Qinghai yak breeds (Qinghai-Gaoyuan, Huanhu, Xueduo and Yushu yak) were also systematically studied. It indicated that Qinghai indigenous yak breeds have rich maternal genetic diversity. And the genetic differentiation between Qinghai indigenous yak breeds was weak, but each Qinghai indigenous yak breed had unique maternal genetic information [2].

Mitochondrial genome (mitogenome) is a kind of circular DNA molecule with fast evolution rate, matriarchal inheritance and rich polymorphism. Therefore, it is considered as an ideal tool in studies on animal matriarchal genetic diversity, population structure and classification [2-7]. At present, D-loop, *Cytb* and mitogenome are widely used in the molecular phylogenetic studies of bovine species [2-8]. In our previous study, based on the D-loop genetic variation, our results showed that the Golmud yak population had rich maternal genetic diversity and owned three maternal lineages [8]. For these reasons, we based on the whole mitogenome sequence genetic variation in-depth study of Golmud yak. Here, we obtained the complete mitogenome sequence of Golmud yak and analyzed its characterization and phylogeny with other yak breeds, which could provide valuable information for breeding and improvement of this population.

Material and Methods

Ethical Statement

This study was conducted according to the guidelines of the Council of China and animal welfare requirements. Based on the recommendations of the Regulations for the Administration of Affairs Concerning Experimental Animals of China and the Institutional Animal Care and Use Committee of Qinghai Province approved all animal experiments.

Sample Collection and Mitochondrial Genome Extraction

The blood sample of one Golmud yak *(Bos grunniens)* was collected in Guoleimude town of Golmud city, Qinghai, China (36°24'51"N, 94°53'42"E). The genomic DNA was extracted using DNA Extraction Kit (Aidlab Biotechnologies Co., Ltd, China) and stored at -20°C for later use. The complete genome of Golmud yak was sequenced using Illumina Nova 6000 platform (Beijing Nuohe Zhiyuan Technology Co., Ltd, China) with sequencing depth of 22.9x. The reads were aligned to the wild yak mitochondrial reference genome (Accession number: NC_006380) using the Burrows-Wheeler Aligner v0.7.15, which were subsequently converted to BAM files. To improve alignment into the circularized genome, the 30 bp of sequence from the end of the mtDNA was attached to the beginning. InDel realignment was performed using the Genome Analysis ToolKit (GATK v3.8) [9], determining the circular mitogenome.

Mitogenome Map Construction, Phylogenetic Tree and Upload Sequence

Mitogenome map of Golmud yak was constructed using OGDRAW v1.3.1 software with default [10]. The corresponding mitogenome sequences of wild yak *(B. mutus),* 16 other domestic yak breeds *(B. grunniens)* and American bison *(Bison bison)* (Acc. no.: NC_12346) in GenBank were used to compare the mitogenome sequence of Golmud yak with Bioedit 7.2.5 software and refined manually. Taking American bison *(Bison bison)* as an outgroup, a phylogenetic tree was constructed based on the Kimura's 2-parameter model using Mega 7.0 by neighbor-joining (NJ) method to explore the phylogenetic relationship between Golmud yak and other yak breeds [11].

Results

The annotated mitogenome sequence of Golmud yak was submitted to GenBank with the accession number OK271109.

The length of Golmud yak's mitogenome was 16,324 bp with an A+T-biased nucleotide composition (61.0%), consisting of 13 protein-coding genes, 22 tRNA genes, two rRNA genes and one non-coding region (D-loop region) *(Table 1; Fig. 1)*.

The similarities of mitogenome sequence between Golmud yak and Pali yak, Qinghai-Gaoyuan yak, Sibu yak, Ashdan yak, Tianzhu white yak, Bazhou yak, Huanhu yak, wild yak, Datong yak, Jinchuan yak, Xueduo yak, Maiwa yak, Yushu yak, Gannan yak, Niangya yak, Zhongdian yak and American bison *(Bison bison)* were 99.9%, 99.8%, 99.9%, 99.9%, 93.1%, 99.9%, 99.9%, 99.9%, 99.9%, 99.3%, 99.8%, 99.8%, 99.8%, 99.3%, 99.8%, 99.9% and 91.3%, respectively *(Table 2)*. The phylogenetic tree showed that Golmud yak was most closely related to Sibu, Huanhu, Zhongdian, Ashdan, Jiulong, Pali, Datong and Bazhou yak breeds, closer to Yushu, Niangya, Qinghai-Gaoyuan, Xueduo, Maiwa and wild yak, but far away from other yak breeds (i.e. Jinchuan, Gannan and Tianzhu white yak) *(Fig. 2)*.

Discussion

Through the prediction of gene structure, we can get detailed information of gene distribution and structure of mitogenome. In this study, the gene composition,

Table 1. Characteristics of the mitogenome of Golmud yak

Gene/ Region	Position		Size (bp)	Base Composition (%)				Start Codon	Stop Codon	Strand
	From	To		A	C	G	T			
D-loop	1	893	893	32.36	25.42	13.77	28.44			H
tRNA^{Phe}	895	961	67	34.33	22.39	19.4	23.88			H
12S rRNA	962	1918	957	36.47	18.18	22.57	22.78			H
tRNA^{Val}	1919	1985	67	38.81	11.94	19.4	29.85			H
16S rRNA	1984	3555	1572	38.1	20.74	17.05	24.11			H
tRNA^{Leu}	3557	3631	75	32.0	17.33	29.08	26.36			H
ND1	3640	4584	945	32.28	29.21	12.28	26.24	ATA	TAA	H
tRNA^{Ile}	4590	4658	69	40.58	15.94	10.14	33.33			H
tRNA^{Gln}	4656	4727	72	36.11	9.72	27.78	26.39			L
tRNA^{Met}	4730	4798	69	27.54	18.84	27.54	26.09			H
ND2	4799	5836	1038	37.19	27.36	8.0	27.46	ATA	TAG	H
tRNA^{Trp}	5841	5907	67	37.31	16.42	20.9	25.37			H
tRNA^{Ala}	5909	5977	69	39.13	10.14	23.19	27.54			L
tRNA^{Asn}	5979	6052	74	31.08	14.86	28.38	25.56			L
OL	6053	6082	30	35.48	25.81	29.03	9.68			L
tRNA^{Cys}	6085	6151	67	29.85	19.4	26.87	23.88			L
tRNA^{Tyr}	6152	6219	68	29.41	16.18	20.59	33.82			L
COX1	6221	7759	1539	28.53	25.54	16.37	29.56	ATG	TAA	H
tRNA^{Ser}	7763	7831	69	24.64	14.49	28.99	31.88			L
tRNA^{Asp}	7839	7906	68	36.76	16.18	17.65	29.41			H
COX2	7908	8588	681	34.21	22.76	14.54	28.49	ATG	TAA	H
tRNA^{Lys}	8595	8661	67	31.34	20.9	17.91	29.85			H
ATP8	8663	8857	195	41.54	23.08	6.15	29.23	ATG	TAA	H
ATP6	8824	9498	675	33.04	26.81	11.41	28.74	ATG	TAA	H
COX3	9504	10286	783	27.64	29.63	15.2	29.12	ATG	TA-	H
tRNA^{Gly}	10288	10356	69	31.88	15.94	20.29	31.88			H
ND3	10357	10701	345	30.92	12.14	28.9	28.03	ATA	TA-	H
tRNA^{Arg}	10704	10772	69	39.13	11.59	10.14	39.13			H
ND4L	10773	11066	294	31.63	23.47	11.9	32.99	ATG	TAA	H
ND4	11063	12430	1368	33.48	26.97	10.09	29.46	ATG	T--	H
tRNA^{His}	12441	12510	70	41.43	8.57	15.71	34.29			H
tRNA^{Ser}	12511	12570	60	31.67	16.67	18.33	33.33			H
tRNA^{Leu}	12572	12641	70	37.14	15.71	20.0	27.14			H
ND5	12648	14444	1797	32.83	10.63	10.63	27.38	ATA	TAA	H
ND6	14452	14970	519	20.23	7.7	29.67	42.39	ATA	TAA	H
tRNA^{Glu}	14974	15042	69	27.54	11.59	21.74	39.13			L
Cytb	15047	16180	1134	31.66	29.01	12.96	26.37	ATG	AGA	L
tRNA^{Thr}	16190	16259	70	35.71	24.59	15.71	24.59			H
tRNA^{Pro}	16259	16324	66	24.24	28.79	13.64	33.33			L

structure and arrangement of mitogenome for Golmud yak are similar to that of most other mammals *(Fig. 1)*. Control region (D-loop) is 893 bp length with an A + T content (60.8%) higher than G + C (39.19%). The two rRNAs *(12S rRNA* and *16S rRNA)* are 957 bp and 1572 bp length, respectively. The 22 tRNAs range in size from 60 bp *(tRNA^{Ser})* to 75 bp *(tRNA^{Leu})* with a total length of 1511 bp. The start codon and stop codon of 13 protein-coding genes of Golmud yak mitogenome are same to that of other bovine species [6,12] *(Table 1)*.

The study on the origin and phylogeny of yak is helpful to reveal its genetic background, evolutionary history and phylogenetic relationship. In previous research reports, there were many studies on the phylogeny of domestic and wild yak maternal lines based on nucleotide variations of mtDNA D-loop region, *Cyt-b* and *COIII* gene [3,6], while there were relatively few studies on the composition and origin of yak maternal lines by using complete mitogenome sequence variation [5]. The wild yak and Qinghai domestic yak breeds were composed of three maternal lineages

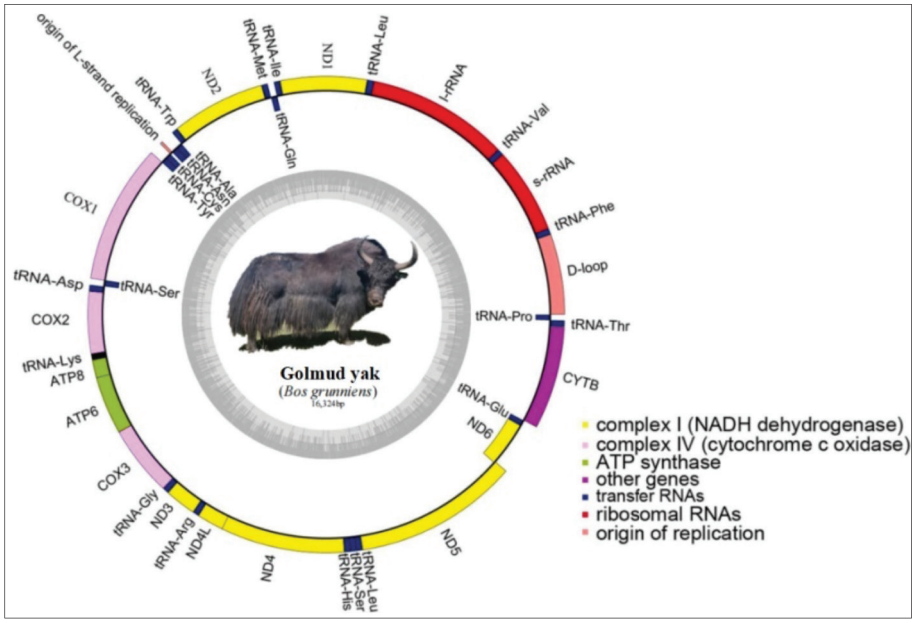

Fig 1. Mitogenome map of Golmud yak *(Bos grunniens)*

Table 2. *Identity of mitogenome sequence alignment among Golmud yak, other 16 yak breeds/populations and American bison (Bison bison)*

Breed	GLM	PL	QG	SB	ASD	TZ	BZ	HH	WY	DT	JC	XD	MW	YS	GN	NY	ZD	AB
GLM	**1**																	
PL	**0.999**	1																
QG	**0.998**	0.998	1															
SB	**0.999**	0.999	0.998	1														
ASD	**0.999**	0.999	0.998	0.999	1													
TZ	**0.931**	0.931	0.931	0.931	0.931	1												
BZ	**0.999**	0.999	0.998	0.999	0.999	0.931	1											
HH	**0.999**	0.999	0.998	0.999	0.999	0.931	0.999	1										
WY	**0.999**	0.999	0.999	0.999	0.999	0.931	0.999	0.999	1									
DT	**0.999**	0.999	0.998	0.999	0.999	0.931	0.999	0.999	0.999	1								
JC	**0.993**	0.993	0.993	0.993	0.993	0.935	0.993	0.993	0.993	0.993	1							
XD	**0.998**	0.998	0.999	0.998	0.998	0.931	0.998	0.998	0.998	0.998	0.993	1						
MW	**0.998**	0.998	0.999	0.998	0.998	0.931	0.998	0.998	0.998	0.998	0.993	1	1					
YS	**0.998**	0.998	0.999	0.999	0.998	0.931	0.998	0.998	0.999	0.998	0.993	0.999	0.999	1				
GN	**0.993**	0.993	0.993	0.993	0.993	0.935	0.993	0.993	0.993	0.993	0.999	0.993	0.993	0.993	1			
NY	**0.998**	0.998	0.999	0.999	0.998	0.931	0.998	0.998	0.999	0.998	0.993	0.999	0.999	0.999	0.993	1		
ZD	**0.999**	0.999	0.999	0.999	0.999	0.931	0.999	0.999	0.999	0.999	0.993	0.998	0.998	0.998	0.993	0.999	1	
AB	**0.913**	0.913	0.912	0.913	0.912	0.971	0.912	0.912	0.913	0.913	0.913	0.912	0.912	0.912	0.913	0.913	0.913	1

GLM: *Golmud yak;* ***PL:*** *Pali yak;* ***QG:*** *Qinghai-Gaoyuan yak;* ***SB:*** *Sibu yak;* ***ASD:*** *Ashdan yak;* ***TZ:*** *Tianzhu white yak;* ***BZ:*** *Bazhou yak;* ***HH:*** *Huanhu yak;* ***WY:*** *Wild yak;* ***DT:*** *Datong yak;* ***JC:*** *Jinchuan yak;* ***XD:*** *Xueduo yak;* ***MW:*** *Maiwa yak;* ***YS:*** *Yushu yak;* ***GN:*** *Gannan yak;* ***NY:*** *Niangya yak;* ***ZD:*** *Zhongdian yak;* ***AB:*** *American bison (Bison bison); The numbers in bold represent the results of identity between Golmud yak and other yak breeds (populations) as well as American bison (Bison bison) in this study*

(lineage I, II and III), and it was speculated that there were three maternal origins in yak. In lineage III, there were only a few wild yak, Xueduo yak and Golmud yak individuals [6,8]. This indicated that Golmud yak and Xueduo yak have significantly unique maternal genetic information, but the phylogenetic analysis and genetic relationship of Golmud yak with other yak breeds were not clarified. In this study, the phylogenetic tree showed that Golmud yak was most closely related to Sibu, Huanhu, Zhongdian, Ashdan, Jiulong, Pali, Datong and Bazhou yak breeds, closer to Yushu, Niangya, Qinghai-Gaoyuan, Xueduo, Maiwa and wild yak, but far away from other yak breeds (i.e. Jinchuan, Gannan and Tianzhu white yak). To some extent, the clustering result among these yak breeds/populations are basically consistent with their differentiation degree, which showing the genetic relationship among them.

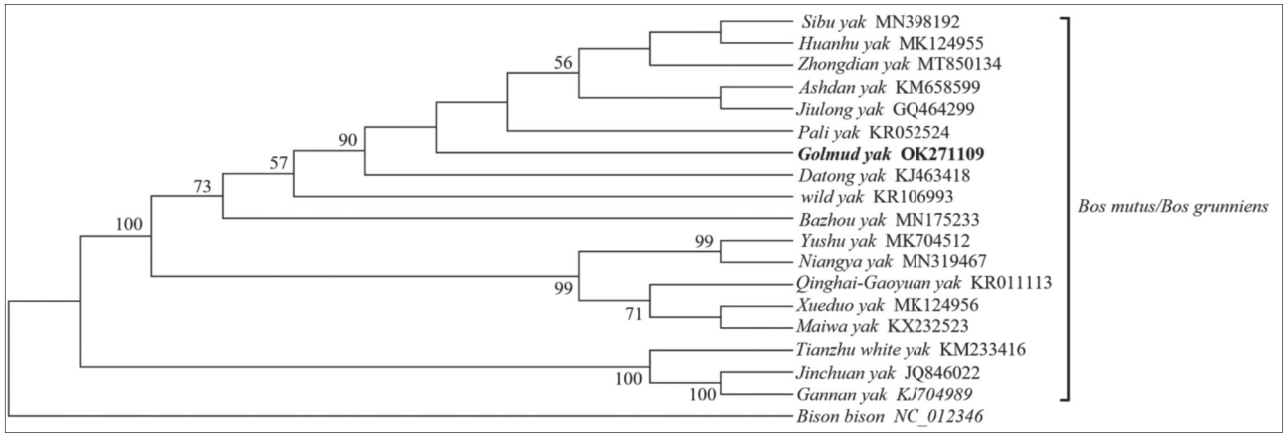

Fig 2. Phylogenetic relationship between Golmud yak and 17 yak breeds/populations in China based on mitogenome sequence variations. The support values (>50) next to the nodes are based on 1000 bootstrap replicates

In this study, the mitogenome characterization and phylogeny of Golmud yak were analyzed for the first time, which laid a foundation for the protection and utilization of this genetic resources and provided a theoretical basis for the breeding and improvement of the yak population.

Availability of Data and Materials

The genome sequence data that support the findings of this study are openly available in GenBank of NCBI at *(https://www.ncbi.nlm.nih.gov/)* under the accession no. OK271109. The associated BioProject, SRA, and BioSample numbers are PRJNA792074, SAMN24377418, and SRS11397677, respectively.

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Not applicable.

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Ethical Statement

This study was conducted according to the guidelines of the Council of China and animal welfare requirements.

Competing Interests

No potential conflict of interest was reported by the author(s).

Author Contribution Statement

ZJ was involved in the conception and design; GZ performed the experiment and data analyses; GZ wrote the original manuscript; ZJ revised the manuscript and approved the final version to be published; GZ, SM, YL, WX and ZJ carried out sampling; all authors reviewed and approved the final manuscript.

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Case Report

A Case Report of Rabies in a Striped Hyena *(Hyaena hyaena)* in Fars Province of Iran

Ehsan SAEIDI [1,a (*)] Foozhan KHERADMAND [2,b]

[1] Collaborator Veterinarian of Fars Provincial Office of Department of Environment, Shiraz, IRAN
[2] Laboratory of Shahin Small Animal Veterinary Clinic, Shiraz, IRAN
ORCIDs: [a] 0000-0003-1582-6553; [b] 0000-0002-4349-4560

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Abstract: Rabies is an infectious, highly fatal and true zoonotic disease. Disease remains endemic and a major public health problem in Iran. In this article, we describe a case report of rabies in a Striped Hyena *(Hyaena hyaena)* in Iran. One Striped Hyena was found in Fars Province Near human communities in a landfill site. Clinical signs were characterized by severe states of depression, blindness, ataxia, anorexia, lethargy and tenesmus. Due to clinical signs and suspected rabies, the animal was euthanized. The brain samples were taken from the Hyena and transported fresh on ice to the laboratory of Pasteur Institute of Iran. Fluorescent antibody technique (FAT) confirmed rabies infection in the Striped Hyena.

Keywords: *: Carnivores, Hyaena hyaena, Iran, Rabies, Striped Hyena*

İran'ın Fars Eyaletinde Çizgili Bir Sırtlanda *(Hyaena hyaena)* Kuduz Olgusu

Öz: Kuduz, bulaşıcı ve son derece ölümcül gerçek bir zoonotik hastalıktır. Hastalık, İran'da endemik ve önemli bir halk sağlığı sorunu olmaya devam etmektedir. Bu çalışmada, İran'da bir çizgili sırtlanda *(Hyaena hyaena)* kuduz vakası ele alınmıştır. Fars Eyaletinde bir çöplük alanında insan topluluklarının yakınında bir adet çizgili sırtlan bulundu. Klinik bulgular şiddetli depresyon, körlük, ataksi, anoreksi, uyuşukluk ve tenesmus ile karakterizeydi. Klinik belirtiler ve kuduz şüphesi nedeniyle hayvana ötenazi uygulandı. Sırtlandan alınan beyin örnekleri buz eşliğinde taze olarak İran Pasteur Enstitüsü laboratuvarına nakledildi. Floresan antikor tekniği (FAT), çizgili sırtlanda kuduz enfeksiyonunu doğruladı.

Anahtar sözcükler: *Karnivorlar, Hyaena hyaena, İran, Kuduz, Çizgili sırtlan*

Introduction

Rabies is one of the oldest known infectious diseases in the world for warm-blooded vertebrates and is caused by viruses in the genus Lyssavirus, family Rhabdoviridae [1]. Experimentally, all warm-blooded vertebrates are prone to rabies, but only mammals are important in the epidemiology of the disease [2]. The causative agent is a neurotropic virus that is primarily replaced and replicated in the central nervous system (CNS) and then transmitted to the saliva and salivary glands. The disease is most often transmitted through the bite of infected animals.

From an epidemiological point of view, two cycles (Urban and Wildlife/Sylvatic cycle) are considered for rabies. In rabies, the term reservoir specifically refers to those species that preserve the disease in the wild [2,3]. Dogs are the main reservoir in the rabies urban cycle in Asia. Wild carnivores are considered a reservoir of rabies in the wildlife cycle. The wildlife cycle usually creates an urban cycle due to frequent contact between wild carnivores and stray dogs [3].

Transmission of rabies virus in animals is almost always the result of the bite of an infected animal [4]. Most dogs and cats become infected through contact with wildlife reservoirs (raccoons, foxes, bats, etc.) [5]. With a few exceptions, the bite of an infected animal is the only route of transmission of rabies in all known cases in humans too. Although rabies is endemic in the wildlife of Iran,

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(*) Corresponding Author
Tel: +98 713 6238421 Cellular phone: +98 939 8453734
E-mail: info_saeidi@yahoo.com (E. Saeidi)

where infection of domestic livestock is frequent, trying to eradicate urban cycle of the rabies is one of the most important priorities of Iran Veterinary Organization.

In the present paper, described a case report of rabies in a Striped hyena that had seen by locals near one of the cities of Fars province.

Case History

On December 10, 2020, at a landfill site 30 km from the city of Dariun, a Striped hyena with the initial signs of weakness and lethargy was Chemical Immobilized by me as a Collaborator veterinarian of Fars Province Environment Organization and a team of rangers Fars Province. The hyena was seen by locals at the landfill site for several days, but there were no reports of attacks on humans or other animals *(Fig. 1-A,B)*.

The hyena was transferred to wildlife rehabilitation center in Bamou National Park. The Striped hyena was male and adult. Clinical examination and observation of behaviors was performed for five days. During these five days, the animal was anesthetized three times with the ketamine (2.5 mg/kg) + medetomidine (0.04 mg/kg) protocol and clinical examination was performed undergo anesthesia. In behavioral observations reclusive and severe states of depression were detectable. In addition to this, the most obvious clinical sign was blindness. Inability to pass Maze test and see objects, absence of PLR Reflex, Menace Response and Dazzle Reflex was detected during behavioral observation and clinical examination. Ataxia as swaying, Anorexia, lethargy and tenesmus was another clinical signs of this Striped hyena. The Striped hyena did not respond to stimuli, had states of depression and was blindness *(Fig. 2)*. Due to clinical signs and suspected rabies, the animal was euthanized with a high dose of sodium thiopental after five days.

Fig 1. The place where the Striped hyena was seen. A- The landfill site, B- Striped hyena among the garbage

Fig 2. Blind striped hyena, with states of depression and no response to stimuli

The brain samples were taken from the Hyena soon after death. The sample was refrigerated and transported fresh on ice to the laboratory of Pasteur Institute of Iran. Impressions of tissue samples from the brain stem, hypothalamus and the hippocampus were examined for rabies infection using Fluorescent Antibody Test (FAT). After three weeks, the results of rabies samples were positive.

Discussion

Rabies is an acute encephalomyelitis of mammals. It is endemic in Iran and has been reported in most provinces. Rabies is the most important zoonotic disease in the country [6]. Although all wild carnivores are considered a reservoir of rabies in the wildlife cycle, reservoirs of rabies vary from region to region. In one study it was reported that in different northern areas of Iran exhibit that dog, fox and jackal are the most common reservoirs of the disease and also wolves as the predominant ones in western parts [7]. On the other hand, in compared to foxes, jackals and wolves, there are fewer reports of rabies in hyenas. In another study, it was reported that although 37% of Serengeti hyenas were exposed to rabies, infection occurred in only 13% of animals, indicating that many animals eliminated the virus from their body after exposure [8].

The most obvious clinical sign of this disease is an acute state of behavioral changes. As a general rule, "the atypicality" is typical sign of rabies [2]. The disease can have a wide range of clinical symptoms, making it difficult to distinguish it from other acute progressive encephalomyelitis syndromes. Because of its public health significance, rabies should be on the list of differential diagnoses considered in every animal with rapidly progressing neurologic dysfunction [9].

In conclusion, all wild carnivores are considered a reservoir of rabies in the wildlife cycle and the disease can have a wide range of clinical symptoms and on the other hand due to frequent contact between wild carnivores and stray dogs the urban cycle of the disease occurs. Therefore, rabies should take precedence over other diagnoses in any wild animal with rapidly developing neurological dysfunction in endemic area like Iran. Furthermore, principled management of human waste, managing the stray dog population as a disease reservoir in the urban cycle to prevent disease transmission to humans, reduce costs of post exposure prophylaxis and efforts to eradicate the urban cycle of disease are essential.

Availability of Data and Materials

The author has provided the required data availability statement, and if applicable, included functional and accurate links to said data therein.

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Competing Interests

The authors declare that there is no conflict of interest

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Author Contributions

ES: Investigator, manuscript preparation and sampling, FK: Sampling and data recording

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LETTER TO THE EDITOR

Canine Distemper Virus Infection in Two Badgers *(Meles meles)* from the Black Sea Region of Türkiye [1]
(Türkiye'nin Karadeniz Bölgesi'ndeki İki Porsukta *(Meles meles)* Kanin Distemper Virüs Enfeksiyonu)

Cagatay ESIN [1,a] Halis CETINER [1,b] Gamze Nur OZKILIC [1,c] Didem PEKMEZCI [1,d (*)]

[1] *Presented in part in abstract form via oral presentation at the International Congress on Biological and Health Sciences, Online, 26-28 February, 2021*

[1] Department of Internal Medicine, Faculty of Veterinary Medicine, University of Ondokuz Mayis, TR-55200 Samsun - TÜRKİYE
ORCIDs: [a] 0000-0002-7029-9066; [b] 0000-0001-8281-0127; [c] 0000-0002-1883-3428; [d] 0000-0002-0631-5471; [e] 0000-0003-2072-8165

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Dear Editor

Canine distemper virus (CDV) belongs to the genus *Morbillivirus* of the family *Paramyxoviridae* and it is responsible for a highly contagious and severe disease, known as distemper, accepted as an emerging multihost pathogen in wild and domestic carnivores, non-human primates and marine mammals [1]. Clinical presentations are generally occurring in immune-compromised animals with two main clinical forms: an acute systemic form and a chronic nervous form characterized by abnormal behavior, incoordination, and convulsions up to paralysis [1]. Several families of wild carnivores are prone to be infected with CDV [2]. However, Mustelidae families are the most affected, but CDV has also been detected in the Felidae, Viverridae, Procyonidae, and Ursidae families [1,2]. Mustelids are worldwide, except in Australia and Antarctica [3]. Moreover, the genus *Meles* (Eurasian badger) is one of the most widespread mustelids in the Palearctic region and the northern part of Türkiye [4]. Available data about diseases in this animal species are generally limited to selected infections, such as bovine tuberculosis in the Eurasian badger *(Meles meles)* [5], rabies in multiple species [6], or canine distemper [7] which also has caused a severe population decline in black footed ferrets *(Mustela nigripes)* [8].

Presence of CDV infection in the wild animal cases is very rare in Türkiye. There is only one report which demonstrated CDV infection in a mink *(Neovison vison)*, sampled from a breeding facility [9]. Therefore, we wanted to present naturally occurring CDV infection in two badgers from the northern part of Türkiye within this letter.

Two badgers *(Meles meles)* which found unconsciously lying in the forest of Samsun at different time intervals, first case in September 2020 and the second in June 2022 were brought to the Veterinary Teaching Hospital by local wildlife conservation officers. The patients were accepted to the Veterinary Teaching Hospital within the protocol numbers 15845 and 20983, respectively. All clinical applications to badgers and reporting of the data obtained in this way were carried out with the permission of the local wildlife conservation authorities and Republic of Türkiye Ministry of Agriculture and Forestry within this 21264211-288.04-6158220 approval number. Clinical manifestations of the badgers, whom were about one year old and males *(Fig. 1-a)*, included severe purulent eye discharges *(Fig. 1-b)*, hyperkeratosis of the nose *(Fig. 1-c)*, hyperkeratosis in paw pads (hard pads) *(Fig. 1-d)*, and tonic-clonic convulsions. It was observed that there was no other abnormality in the CBCs except for lymphopenia. Rectal body temperatures (RBT), pulse per minute (P) and respiratory per minute (R) frequencies were detected in badgers as RBT (37.0°C, 37.5°C), P (120 bpm, 130 bpm) and R (30 rpm, 40 rpm) respectively. Pre-diagnosis CDV infection was suspected at both badgers. Later CDV Ab rapid test kits were performed and according to the test results a high titer (score 6; 1:512) from each sample confirmed the diagnosis. Unfortunately, one of the badgers died while performing diagnostic tests, and the other died after two days during the treatment protocol.

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(*) Corresponding Author
Tel: +90 362 312 1919
E-mail: dkazanci@omu.edu.tr (D. Pekmezci)

Fig 1. A male *Meles meles* **(a)**, severe purulent eye discharges **(b)**, hyperkeratosis of the nose **(c)**, hyperkeratosis in paw pads (hard pads) **(d)**

One study emphasized the circulation of CDV in the northern part of Lombardy (Italy), with a percentage of positive animals of 39.7% in foxes, 50% in badgers, and 14.3% in stone martens [10]. It is believed that domestic dogs or coexisting wild carnivores infected with the virus were the most likely source, therefore serologic tests are needed to perform for definitive diagnosis in such suspicious cases. Prevention with controlling strategies of distemper is quite important to reduce their diffusion in domestic dogs with wild carnivores. Consequently, more effective control mechanisms should be determined and applied to prevent infectious diseases in wild animals.

Availability of Data and Materials

The data in this lettering are available from the corresponding author (D. Pekmezci) in case of a request.

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Competing Interest

The authors declare that they have no conflict of interest.

Author Contributions

C. Esin, H. Cetiner and G. N. Ozkilic participated in the collection and preparation of samples. D. Pekmezci participated in the examination of both patients and drafted the letter to the Editor.

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Review

Recent Advances of Using Polyphenols to Extenuate Oxidative Stress in Animal Production: Evidence from Poultry

Nguyen Hoang QUI [1,a (*)] Nguyen Thi Anh THU [1,b]

[1] Tra Vinh University, School of Agriculture and Aquaculture, Department of Animal Science and Veterinary Medicine, 94000, VIETNAM

ORCIDs: a 0000-0002-3499-0669; b 0000-0002-6778-3631

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Abstract: Oxidative stress has become a very challenging issue to animal production due to its disturbance to animal growth. Under heat stress conditions, the ability of poultry animals to combat oxidation stress is reduced; oxidative stress minimizes the concentration of globulins in plasma, thus lowering poultry's immune status. Many researches have been conducted to solve this problem, and one of the safest solutions is the use of Polyphenols. Polyphenols are widely known as exogenous antioxidants which act as one of the cell's first lines of defence. Thus, the findings on the intracellular antioxidant activity of polyphenol as a plant-based feed supplement, has greatly helped improve poultry antioxidant activity due to the beneficial effects of polyphenols in preventing damage from oxidative stress and removing excessively produced free radicals. This review aims to synthesize information regarding the antioxidant activity of polyphenols and their influence on poultry antioxidant status.

Keywords: *Antioxidant, Monogastric, Oxidative stress, Polyphenols*

Hayvansal Üretimde Oksidatif Stresi Azaltmak İçin Polifenollerin Kullanımında Son Gelişmeler: Kanatlı Hayvan Örneği

Öz: Oksidatif stres, hayvanlarda büyümeyi engellemesi nedeniyle hayvansal üretim için çok problemli bir konu haline gelmiştir. Isı stresi koşulları altında, kanatlıların oksidasyon stresiyle mücadele etme yeteneği azalır; oksidatif stres, plazmadaki globulin konsantrasyonunu en aza indirerek kanatlı hayvanların bağışıklığını azaltır. Bu sorunun çözümü için birçok araştırma yapılmıştır ve en güvenli çözümlerden biri Polifenollerin kullanılması olmuştur. Polifenoller, hücrenin ilk savunma hatlarından biri olarak işlev gören eksojen antioksidanlar olarak bilinir. Bu nedenle, bitki bazlı bir yem takviyesi olarak polifenolün hücre içi antioksidan aktivitesine ilişkin bulgular, polifenollerin oksidatif stresten kaynaklanan hasarı önlemedeki ve aşırı üretilen serbest radikalleri gidermedeki faydalı etkilerinden dolayı kanatlıların antioksidan aktivitesinin geliştirilmesine büyük ölçüde yardımcı olmuştur. Bu derleme, polifenollerin antioksidan aktivitesi ve kanatlıların antioksidan durumu üzerindeki etkileri ile ilgili bilgileri sentezlemeyi amaçlamaktadır.

Anahtar sözcükler: *Antioksidan, Monogastrik, Oksidatif stress, Polifenoller*

Introduction

Due to public health concerns, many countries strictly regulate or even ban to decelerate antibiotic resistance in animals. Therefore, scientists are seeking for alternatives to solve this issue, and the prime alternatives are phytogenic chemicals that are found to be potent feed additives and, too, possess clinical effects [1]. Phytochemicals distribute benefits to, both, animal health and performance, especially, polyphenols that are produced by secondary metabolites [2,3].

Polyphenols, also known as phenolic compounds, is a class of chemical present in various plant species. They are distinguished by the presence of one or more aromatic rings and the presence of one or more than two hydroxyl groups. There are three main groups of polyphenols: Flavonoids, Non-Flavonoids and Tannin, and their biological functions mainly rely on chemical structures [4]. More than 8000 polyphenols have been identified and believed to exhibit immunomodulatory, anti-microbial, anti-inflammatory, antiallergic, antimutagenic, and detoxifying properties which benefit to animals and

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(*) Corresponding Author

Tel: +84 334 933577 Cellular Phone: +84 334 933577
E-mail: nhqui@tvu.edu.vn (N.H. Qui)

humans [2,3,5]. Additionally, polyphenols are potent in combating heat stress through its antioxidant activities since the consequence of heat stress partly causes the oxidative stress in animals. Lykkesfeldt and Svendsen [6] and Chauhan et al.[7] defined oxidative stress occurs once there is an imbalance between Reactive Oxygen Species (ROS) and antioxidant. Administration of antioxidant compounds is necessary to prevent regeneration of free radicals, and polyphenols have been accounted as prominent compounds to take the action. Reactive oxygen species must be removed by administering antioxidant compounds to prevent the further generation of other free radicals, which eventually causes oxidative stress in animals, including poultry. Moreover, some studies proved the positive results of polyphenols via *in vivo* experiment which indicates their potential as natural antioxidants [8-10].

As mentioned previously, this review tries to identify the capability of polyphenols in combating heat stress and oxidative stress of mono-gastric animals, more specifically the poultry animals. Therefore, the objective of this review is to identify the capability of polyphenol compounds to extenuate oxidative stress in poultry which includes modes of action of those compounds in preventing and combating oxidative stress.

Polyphenol's Sources and Modes of Action

Flavonoids are the most abundant polyphenol class, which accounts around 50% of polyphenol class, that have more than 4000 compounds having been identified to date. They have a popular structure consisting of two benzene rings associated with three carbon atoms, resulting in an oxygenated heterocycle. Their role is to protect against free radical damage through the following modes of action: the direct scavenging of ROS occurring in different processes of the body, the activation of antioxidant enzymes, inhibition of oxidases, enhancing antioxidant properties of low molecular antioxidants and mitigation of oxidative stress caused by reduction of α-tocopherol radicals, nitric oxide, metal chelating activity, growth in uric acid levels [4,11]. Flavonoids only occur in plants by biosynthesis via phenylpropanoid pathway, converting phenylalanine into 4-coumaroyl-CoA, which enters the flavonoid biosynthesis pathway. The first enzyme, chalcone synthase, produces chalcone scaffolds from which all flavonoids derive. Flavonoid skeleton modification highly depends on the species, enzymes, such as isomerases, reductases, hydroxylase, and several Fe^{2+}/2-oxoglutarate-dependent dioxygenases which leads to different flavonoid subclasses in plants, and transferases modify the flavonoid backbone with sugars, methyl groups and/or acyl moieties, regulating the physiological activity of the resulting flavonoid by altering their solubility, reactivity and interaction with cellular targets [12]. There are 3 main representative substances to take the action: Flavonols, Procyanidins and Anthocyanins. The functions of Flavonols are to form intramolecular hydrogen bonds by reacting with free radical, improve the activity of antioxidant enzymes such as Glutathione peroxidase (GSH-Px) and Superoxide dismutase (SOD), and increase the mitochondrial membrane potential and decreasing the oxidative damage level of mitochondria. Procyanidins provide hydrogen atoms to react with free radicals chelate with metal ions, downregulate stress-activated Mitogen-activated protein kinase (MAPK) pathway activity, promote Extracellular signal-regulated kinases (ERK) phosphorylation and upregulation the expression of anti-oxidant genes Nuclear factor erythroid 2-related factor 2 (Nrf2) and Heme oxygenase-1 (HO^{-1}), protect Adrenal phaeochromocytoma (PC12) from Hydrogen peroxide (H_2O_2), inhibit lipid peroxidation activity and slow down the enzymatic oxidation of fat. Moreover, Anthocyanins' functions are to regulate oxidase activity by activate Nrf2 and inhibit Nuclear factor kappaB (NF-kB) signaling pathway, chelate with metal ions, inhibit Peroxynitrite (ONOO-) by destroying mitochondrial apoptosis pathway and inhibit Bcl-2-associated X protein (Bax) nuclear translocation [13].

As demonstrated by Molina et al.[14], who performed an experiment on ethanol-induced oxidative stress in mouse liver, pre-treatment of flavonoid may protect oxidative stress by directly quenching lipid peroxides and indirectly by enhancing the production of the endogenous anti-oxidant glutathione (GSH). Moreover, flavonoids exert protective antioxidant effects on bovine mammary cells, according to Perruchot et al.[15], and the finding suggests that flavonoid could be used to prevent oxidative meta-bolic disorders, too. Evidently, flavonoids can be future alternatives of antioxidants in animal production too.

Beside flavonoids, non-flavonoid polyphenol groups are identified as any substance consisting of a benzene ring with one phenol or polyphenol hydroxyl groups with more than one benzene ring such as methyl esters, esters, and other similar compounds which include lignans, stilbenes and phenolic acids [4]. Typically, phenolic acids are divided into hydroxybenzoic and hydroxycinnamic acids. The position and number of methoxy and hydroxyl groups attached to the aromatic ring define the structure of phenolic acids, and they contribute to their antioxidant properties. Phenolic acids are synthesized from aromatic amino acids produced via the shikimate pathway. The shikimic acid is transformed into L-phenylalanine through a chorismic acid intermediate, and then the L-phenyl-alanine is turned into p-oumaric, salicylic, and p-hydroxy-benzoic acids which serve as precursors for other derivatives of phenolic acids [16]. Chlorogenic acid and gallic

acid are the representative substances of phenolic acids. While chlorogenic acid supplies hydrogen atoms to free radicals, chelates with metal ions, prevent NH_2Cl-induced plasmid DNA fragmentation, reduces the expression of Forkhead box O (FOXO) family genes, activates Nrf2 transcription and upregulates the expression of cellular antioxidant enzymes, gallic acid reduces the accumulation of malonaldehyde and nitric oxide (NO^-) to enhance to activities of antioxidant enzymes and clears radicals produced by the Fenton reaction [13]. According to Zhang et al.[17], chlorogenic acid can effectively improve growth performance and enhance antioxidant capacity in chickens challenged with Clostridium perfringens type A. Additionally, Zhao et al.[18] proved that gallic acid supplementation on high weaning weight pigs enhanced plasma antioxidant capacity. Therefore, non-flavonoid polyphenol groups, too, possess high antioxidant properties to improve farm animal health.

Tannins, one of the three main groups of polyphenols, are water-soluble phenolic compounds found in various plant foods, also known as tannic acid, including tea and coffee [4]. Tannins are classified into two groups: hydrolysable and condensed tannins. Physiochemical properties and biogenesis are the indicators of this classification. Hydrolysable tannins result from binding to sugar fragments (mainly to the D-glucose moiety) of gallic, meta-digallic or hexahydroxy diphenic acid residues. Shikimic or dehydroshikimic acids are the precursors of these phenol carboxylic acids. On another hand, condensed tannins are created by oxidative condensation of flavonoids (mostly flavan-3,4-diol monomers, catechins, stilbetes and dihydrochalcones). The precursors of condensed tannins are malonyl-CoA and para-hydroxycinnamoyl-CoA. While condensed tannins are usually stored in heartwood and bark, hydrolysable tannins are stored in leaves, fruit pods and galls [19]. Tannins exert antioxidant effects such as inhibition of lipid peroxidation, scavenging of oxygen radicals, binding and inactivation of pro-oxidative medallions (Fe and Cu), and binding of proteins with suppression of their enzymatic activity (protease inhibition) [20]. Ebrahim et al.[21] suggested that tannins could be potentially applied as a biological antioxidant for poultry nutrition on hot climate condition. Moreover, hydrolysable tannins could be a replacement of Zinc Oxide to reduce diarrhea and improve antioxidant capacity of weaned piglets, too [22]. For that reason, tannins can be used as antioxidant which can be beneficial to animal and human health.

In general, polyphenols protect cells from the free-radical production. Exogenous antioxidants, specifically polyphenols, act as the defenders in the first line of cells combating excessive free radical production, shielding their constituents from oxidation damage [3]. However, polyphenol compounds are not able to be absorbed in its natural forms such as glycosides and polymers, and they have to be hydrolyzed in the host intestine [23]. Some flavonoids can be absorbed at gastric level, as well. The further study and understanding of the role of polyphenol's class in animal diets will be worthwhile.

To sum up, the antioxidant polyphenols have the ability to act on radical scavengers in various ways. Sandoval-Acua et al.[24] defined two ways to scavenge ROS: indirect activity and direct activity of scavenging ROS by inducting the synthesis of reactive oxygen species-removing enzymes. Polyphenols exhibit the indirect activity of antioxidants by activating Nrf2 to reduce oxidative stress [25]. Overall, Polyphenols protect cells against the free radical generation through the following modes of action [2]:

(1) Inhibiting the activities of pro-oxidant enzymes

(2) Activating the enzymes of antioxidant

(3) Scavenging ROS directly through an electron donor participation

(4) Chelation of transition metals which regulates formation of reactive hydroxyl radicals

(5) α-tocopherol radicals' reduction

(6) Alleviation of nitric oxide oxidative stress

(7) Prevents oxidation of low molecular antioxidants such tocopherols and ascorbate which boosts its antioxidant activities.

However, the effects of polyphenol's mechanism on oxidative stress can be different due to the difference in gastrointestinal absorption of various polyphenols, as well as their related interactions with the glucuronide, sulphate, or methyl groups of amino acids, and the type of the circulating metabolites in the blood, which are categorized following polyphenols' chemical forms [3]. Therefore, further studies are required to have proper usage on polyphenol compounds.

Polyphenols in Combating Oxidative Stress

Oxidative Stress

As a result of a disruption in the equilibrium between the accumulation of ROS and the body's antioxidants, cells and tissues are damaged. This results in oxidative stress and a precursor for the entry of various diseases. The reactive species are primarily responsible for generating oxidative stress that could be separated into three categories: free radicals, nonradicals, and redox-active transition metal ions [26]. Free radicals are considered unstable because they contain one or more than two unpaired electrons. In essence, its primary objective

is to achieve stability. Therefore, these molecules either transfer the unpaired electrons to others or simply accept an electron in order to achieve a stable formation [27]. Furthermore, when reactive species are created in excess, stress from oxidation emerges and the systems of antioxidant defense in animals might be overwhelmed while antioxidant enzymes including SOD, catalase, GSH-Px diminishes its activity [28]. Lipiński et al.[2] found that under an oxidative stress environment, the body of animals is unable to effectively counteract the excessively generated free radicals, consisting of reactive nitrogen species (RNS) and/or ROS, which further results to detrimental effects to chromosomes. Additionally, it also modifies the encoded amino acids and consequently many other biological processes [2].

In order to prevent the generation of free radicals, which are known to cause some redox-related diseases and oxidative stress, antioxidant compounds should be administered to remove those [4]. According to Procházková et al.[11], dietary polyphenol's extensive biotransformation such as dietary flavonoids, may modify its bioactive forms in the small intestine and liver, thus affecting their antioxidant activity. In another trial, the diets with higher amounts of polyphenols boosted SOD activity and GSH-Px activity in the blood of poultry species or enhanced the quantities of vitamin E in the plasma of poultry species [29]. The addition of polyphenols to the diet can dramatically boost the activities of GSH-Px, catalase, and SOD in the serum of broiler. Under heat stressed conditions, adding polyphenols to broiler diets boosted antioxidant enzyme activity in broiler serum, such as GSH and SOD, and improved the health status of broilers. Polyphenols supplemented *in vivo* and *in vitro*, at a dose of 0.2 g/litter in water could ameliorate pathological damage and downregulate creatine kinase, lactate dehydrogenase, and creatine kinase-MB isoenzyme levels in cardiomyocytes caused by heat stress [8].

Oxidative Stress by Heat Stress

One of the most common sources of stress affecting animal performance is heat stress, which occurs when temperatures rise above a certain threshold [30]. Heat stress is partly affected by the temperature of the environment, humidity, ventilation, and the quantity of heat exchange due to the density of animals at one place [31]. Recent researches have repeatedly demonstrated the importance of dietary antioxidants in increasing the performance of broilers raised in high temperatures [32]. Under high environmental temperature settings, the activity of diverse plant extracts containing polyphenols showed great results in stabilizing poultry health. Furthermore, stress caused by heat is a significant barrier in poultry industry and a growing concern for many experts studying food safety and global warming [30].

An increase in cellular energy requirement appears to be the initial step in the pathophysiology of heat stress [33]. Yang et al.[34] found that the energy expenditure of cells increases by 2-fold after being exposed to acute heat stress. After 6 h of acute heat stress, mitochondrial transportation as well as β-oxidation of fatty acids were increased, and this was established in the study of Mujahid et al.[35]. To meet the increased energy requirement of the cells for its mitochondrial biogenesis, the production of the enzymatic activity and reducing equivalents of subunits of respiratory chain complexes are enhanced. Furthermore, electron transport is intimately linked to electron transport-linked phosphorylation (oxidative phosphorylation) under normal physiological conditions. Electron transport chain and mitochondrial substrate oxidation activity increases during the early stages of heat stress, thereby resulting in excess superoxide. Down-regulation of animal uncoupling protein increases the stress from oxidation, leading to tissue damage and mitochondrial dysfunction during the later periods of heat stress. The activities of antioxidant enzymes are usually increased. Additionally, acute heat stress causes oxidative capability of mitochondrial metabolism to be reduced, uncoupling protein to be upregulated, the activity pattern of antioxidant enzymes to be altered and stores of antioxidants to be depleted [36]. Furthermore, poultry are unable to sweat, and their body feathers restrict their capacity to expel heat into the intermediate environment greatly [36]. Heat stress is known to inflict severe damage to organisms, as mentioned in all the previous studies above. Physiological and biological changes, such as the development of stress hormones, increased free radical generation, and poorer antioxidant status, as well as diminished resistance and disturbance of homeostasis, are all possible [36].

The Role of Polyphenols in Oxidative Stress Reaction

It has been demonstrated that the application of polyphenol-enriched diets increases the meat's oxidative stability by decreasing the oxidative processes that contribute to meat lipid peroxidation. This is achieved through decreasing the malondialdehyde (MDA) concentration in meat [37]. Therefore, there was a decrease in the level of GSH inside the liver tissue which is an essential antioxidant found inside the living cells and this is a reliable index of the antioxidant activity of the tissue [37]. According to the findings of Rahman et al.[38], broilers fed with diets containing 0.5% - 2% of polyphenols (tea polyphenols) have improved muscle antioxidant activity, growth performance and as well as the meat quality. Furthermore, many studies have shown that polyphenols-based feed could be applied as an alternative for vitamin E in the diet to prevent the oxidative stress for breast meat and thigh meat in poultry without causing any depletion of vitamin E stores in the poultry's tissue reserves. These polyphenols can be used

partially as an alternative to vitamin E or exert antioxidant benefits on their own and it is identified by the amount of α-tocopherol in the diet [9].

The effect of polyphenols is recorded in several studies. Different levels of grape pomace fed for broiler chickens at 28 days of age, which contains an abundant amount of polyphenols, have been demonstrated to reduce and regulate the level of thiobarbituric acid reactive compounds through the scavenging of free radicals [39]. The study of Pirgozliev et al.[40] indicated that broiler poultry animal-fed essential oils (cinnamaldehyde, capsicum oleoresin and carvacrol) had higher hepatic antioxidants, such as coenzyme, carotene, and total vitamin E, than those fed with non-essential oils. Furthermore, using 20 mL/100 kg of rosemary essential oil could increase antioxidant activities in heat-stressed laying hens by raising the activity of glutathione peroxidase, which is a powerful antioxidant enzyme [41]. In addition, 150 mg/kg of oregano powder was utilized in broiler meals as a source of phenolic compounds, and it was discovered that experimental broilers had greater total antioxidant capacity values and lower malondialdehyde values in their serum [42]. Resveratrol have showed a strong antioxidnat capacity in poultry. The supplement of resveratrol as a natural polyphenic compound has an effect on antioxidant activity in poultry species, particularly, Japanese quails [43]. And the results showed that MDA content reduces from 0.75 (mg/dL) in control treatment to 0.23 (mg/dL) in treatment of 400 mg/kg resveratrol. Moreover, the phenolic compound from rosemary volatile oil was recorded a positive effect on GPx from plasma antioxidant parameters in quails [44]. However, plasma SOD activity is not affected by this substance. Sahin et al.[45] also showed that quails given an additional supplement of resveratrol at a dose of 400 mg/kg had a decreased concentration of MDA in their serum. According to the findings of Mazur-Kunirek et al.[46], broilers that were given diets that contained oxidized rapeseed oil and were either supplemented with vitamin E and polyphenols or just polyphenols were found to have higher levels of GSH-Px activity in their blood as well as higher levels of tocopherol and vitamin E in their livers. An increase in the quantities of polyphenols consumed through feed led to an improvement in the antioxidant status of the blood as well as an increase in the amount of non-enzymatic antioxidants found in the liver and breast muscles of broilers. Additionally, Zhang et al.[47] found that resveratrol might protect against the heat stress-induced deterioration of the meat quality of broilers by elevating the total antioxidant capacity and activity of antioxidant enzymes in the muscle tissue (CAT, GSH-PX). According to the findings of Liu et al.[48], providing black-boned broiler chickens with a supplement of either 200, 400, or 600 mg/kg of resveratrol effectively alleviated the effects of heat stress, with the dose of 400 mg/kg demonstrating the most potent antioxidant effect. Curcumin is a yellow polyphenol, and its supplementation improved the resilience of broilers to heat stress, boosting the GSH content and GSH-related enzyme activities and stimulating the expression of Nrf2 and Nrf2-mediated phase II detoxifying enzyme genes [49]. Moreover, it was discovered that green tea has a significant quantity of polyphenol, and it was shown that drinking green tea boosted the total antioxidant activity as well as increased the activity of liver antioxidant enzymes such as GSH-Px and reduced glutathione (GSH). It was shown that increasing the dose of tea polyphenols led to an increase in the concentration of MDA in the livers of Roman laying hens. Additionally, increasing the dose of tea polyphenols led to an increase in GSH-Px activities and GSH-ST levels [50]. Tea polyphenols have the potential to raise the levels of antioxidant enzyme activity while simultaneously lowering the MDA content of Ya'an laying hens [51]. For the influence of polyphenols' compounds on oxidative stress in poultry production under heat stress, Yin et al.[8] also recorded that heat stress increases the total antioxidant capacity, heme oxygenase-1 mRNA, and decreases the superoxide dismutase, while polyphenols help improve these indicators as a powerful antioxidant compound.

Conclusion and Recomendations

Oxidative stress poses a detrimental effect on poultry performance and health which leads to an economic loss to animal production. Evidently, polyphenols from plants can be future potential feed additives. It can be used to improve poultry antioxidant capacity and mitigate oxidative stress by heat stress in intensive production climates. The application of polyphenols could reduce oxidative stress through its mechanism of action and its chemical structures. In order to achieve the advantages from the function of reducing oxidative stress of polyphenols, the dose of polyphenols in the diet and the targeted poultry species should be taken into account.

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Competing Interests

The authors declare that there are no conflicts of interest

Authors' Contributions

NHQ: Made a framework, wrote and format the manuscript, NTAT: correct, complete and revise the manuscript.

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