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Phone: +90 212 217 17 00
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The scope of the journal includes but not limited to; basic and clinical veterinary sciences, raising livestock, veterinary genetics, animal nutrition and nutritional diseases, zooneses, veterinary medicinal products and public health, and food hygiene, technology, exotic animal sicence and laboratory animal sicence.

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Editor-in-Chief: Mustafa Sinan AKTAŞ
Address: Atatürk University, Faculty of Veterinary Medicine, Department of Internal Medicine, Erzurum, Türkiye
E-mail: vetdergisi@atauni.edu.tr

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Address: Atatürk University, Yakutiye, Erzurum, Türkiye

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Address: Büyükdere Cad., 199/6 34394 Şişli, İstanbul, Türkiye
Phone: +90 212 217 17 00
E-mail: info@avesyayincilik.com
Webpage: www.avesyayincilik.com

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Baş Editör: Mustafa Sinan AKTAŞ
Adres: Atatürk Üniversitesi, Veteriner Fakültesi, İç Hastalıkları Anabilim Dalı, Erzurum, Türkiye
E-posta: vetdergisi@atauni.edu.tr

Yayıncı: Atatürk Üniversitesi
Adres: Atatürk Üniversitesi, Yakutiye, Erzurum, Türkiye

Yayınevi: AVES
Adres: Büyükdere Cad., 199/6 34394 Şişli, İstanbul, Türkiye
Tel: +90 212 217 17 00
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Molecular and Pathological Survey of Bovine Viral Diarrhea Virus in the Testis of Bulls

Boğaların Testislerinde Bovine Viral Diyare Virüsünün Moleküler ve Patolojik Olarak Araştırılması

Mustafa ÖZKARACA[1]
Mehmet Özkan TİMURKAN[2]
Yavuz Selim SAĞLAM[3]
Selçuk ÖZDEMIR[4]
Hakan AYDIN[2]
Serdar ALTUN[3]

[1]Department of Veterinary Pathology, Cumhuriyet University, Sivas, Türkiye
[2]Department of Veterinary Virology, Atatürk University, Erzurum, Türkiye
[3]Department of Veterinary Pathology, Atatürk University, Erzurum, Türkiye
[4]Department of Veterinary Genetics, Atatürk University, Erzurum, Türkiye

ABSTRACT

Bovine viral diarrhea virus (BVDV) is an important pathogen that causes diseases in the gastrointestinal, respiratory, and reproductive systems. It also leads to a decrease in reproductive performance and consequently continuous economic losses in cattle management. The presence of BVD virus in bull testes was investigated in this study using indirect immunofluorescence (IF), immunohistochemistry (IHC), and Reverse Transcription polymerase chain reaction (RT-PCR) methods. The tissue distribution of BVDV was examined pathologically and virologically in 100 bull testis tissue samples. For this purpose, IF, IHC, and RT-PCR methods were employed. Positive IF staining was detected in 21 (21%) testis samples using the indirect IF method. In IHC staining, 16 (16%) samples were found to be positive. In RT-PCR, 13 (13%) samples tested positive. The results of the presented study demonstrate that BVDV can infect different cell types in bull testes and that the virus can primarily be transmitted through natural or artificial insemination. Therefore, bulls are an important epidemiological factor in the transmission of the disease.

Keywords: Bovine viral diarrhea virus, immunoflourescence, immunohistochemistry, RT-PCR, testis

ÖZ

Bovine viral diarrhea virus (BVDV), gastrointestinal, solunum ve üreme sistemlerinde hastalıklara neden olan önemli bir patojendir. Ayrıca, üreme performansında azalmaya yol açar ve bu nedenle sığırların yönetiminde sürekli ekonomik kayıplara neden olur. Bu çalışmada, BVDV'nin boğa testisindeki varlığı indirekt immunofloresan (IF), immunohistokimya (IHC) ve ters transkriptaz polimeraz zincir reaksiyonu (RT-PCR) yöntemleriyle araştırıldı. Çalışmada, 100 boğa testis dokusu örneğinde BVD virusunun doku dağılımı patolojik ve virolojik olarak incelendi. Bu amaçla, IF, IHC ve RT-PCR yöntemleri kullanıldı. İndirekt IF yöntemiyle 21 (%21) testis örnek, IHC yöntemiyle 16 (%16) örnek pozitif bulundu. RT-PCR'da ise 13 (%13) örnek pozitif bulundu. Sunulan çalışmanın sonuçları, BVDV'nin boğa testisinde farklı hücre tiplerine enfekte olabileceğini ve virüsün öncelikle doğal veya suni tohumlama yoluyla bulaşabileceğini göstermektedir. Bu nedenle, boğalar hastalığın yayılmasında önemli bir epidemiyolojik faktördür.

Anahtar Kelimeler: Bovine viral diyare virüsü, immunfloresans, immunohistokimya, RT-PCR, testis

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Sorumlu Yazar/Corresponding author: Mustafa Özkaraca
E-mail: mustafaozkaraca@cumhuriyet.edu.tr

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INTRODUCTION

Bovine viral diarrhea (BVD) is a viral cattle disease that occurs worldwide, resulting in significant economic losses.[1] The responsible virus is part of the *Flaviviridae* family of viruses under the genus *Pestivirus*. It is an RNA virus with a positive-strand structure and has 3 classes: type I (BVD1), type II (BVD2), and type III (BVD3).[2] Two distinct biotypes, that is, the non-cytopathic (NCP) biotype and the cytopathic (CP) biotype, are known to be manifested by all genotypes of BVD1 and BVD2 viruses.[3,4] Significant effects of the NCP-BVD become noteworthy in the periods of breeding and pregnancy. Persistently infected (PI) calves are the most important outcomes of this condition.[5,6]

Bovine viral diarrhea can display various clinical manifestations affecting respiratory, gastrointestinal, reproductive, and fetal tissues and manifest in acute, transient, mucosal, or thrombocyt openic/hemorrhagic forms. Persistently infected cattle are also a result of BVD.[7] It is considered that transiently infected (IT) cattle are epidemiologically insignificant compared to PI cattle as they are the prime means of transmission.[8-10] The virus can be found in the testicular tissue and semen of bulls with a BVD infection history.[11] Different approaches can be utilized for the detection of infected specimens. However, the most effective approaches are the precise diagnosis of the acute form and the elimination of PI cattle in a given herd.[12] Cattle worldwide are under the threat of BVD infection.[13] It has been reported that the serological prevalence of bovine viral diarrhea virus (BVDV) ranges between 2.3% and 64.7%.[14,15] It is stated that intensive breeding of dairy cattle generates less than 0.25% PI cattle in establishments.[16] Persistently infected cattle have higher quantities of the virus in their tissues, and established methods can effectively diagnose it, for example, isolation, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), immunofluorescence (IF), and immunohistochemistry (IHC).[17-20] Two objectives of the present study were as follows: (1) comparative detection of BVD virus in testicular specimens using PCR, IHC, and IF methods and (2) determination of viral distribution and localization of antigens in testicular tissue.

MATERIALS AND METHODS

Sample Collection

This study was carried out in accordance with the Declaration of Helsinki. Macroscopically examined 100 bulls' (showing no clinical signs) testes were collected. Collected testis tissues were separated into 2 pieces. Half of the sections were fixed in 10% neutral formaldehyde solution for IHC and IF process. The other half of testis samples that were suspected of having persistent infection were homogenized for reverse transcription (RT)-PCR in a tissue disruptor in phosphate buffer saline (PBS) containing 1% IU of 10 000 IU penicillin/mL, 10 mg streptomycin/mL, and 0.025 mg/mL amphotericin B, and then, tissues were centrifuged at 3000 rpm for 15 minutes at +4°C. This supernatant was passed through a 0.45 µm diameter injector filter in a sterile cabinet and each specimen was transferred to sterile tubes of 2 mL volume. Samples were stored at −80°C until RT-PCR testing was performed.

Nucleic Acid Extractions

A commercial kit (GF-1 Viral Nucleic Acid Extraction Kit (proteinase K included), 100 preps, GF-RD-100, Vivantis, Malaysia) was used for RNA isolation. After frozen testis tissue samples were thawed at room temperature, RNA extractions were performed per the manufacturer's instructions and the samples were passed to RT process.

Reverse Transcription

Because BVD contained a genome in RNA, RT was performed before PCR. Reverse transcriptase enzyme was used for this purpose. First-strand complementary DNA (cDNA) synthesis kit (Thermo Scientific, USA) was used. The kit was applied according to recommendations from the manufacturing company.

Polymerase Chain Reaction

The sample was subjected to PCR process using cDNA template obtained after RT. Bovine viral diarrhea virus-specific primer pair was used to investigate the presence of the BVDV genome in the samples. The heat cycles, primers, and optimization conditions to be used in the reaction were maintained as described in a previous study.[21] Bovine viral diarrhea virus-positive tissue samples, confirmed by sequence reaction, were used as positive control. The amplicons obtained from the PCR were evaluated by gel electrophoresis and 288 bp product reactions were evaluated as positive.

Immunohistochemistry and Indirect Immunofluorescence Examination

Bulls' testis samples were washed with tap water before routine serial treatment of samples with graded alcohol and xylene was performed, and these tissues were embedded in paraffin. After the routine histopathology process, sections of 5 µm in thickness were cut with a rotary microtome and mounted on glass slides that were precoated with poly-L-lysine. After deparaffinization, 3% H_2O_2 solution (hydrogen peroxide; 18304-1L, Sigma, Mo, USA) was applied dropwise on each slide for 10 minutes to inactivate endogenous peroxidase activity. Then, the slides were immersed in antigen retrieval solution (ab96674, Abcam, USA) (pH 6.0) and heated in a microwave for 10 minutes to unmask the antigens. After cooling, sections were incubated for 10 minutes with a protein block solution (Cat. no. ab80436, Abcam) to prevent non-specific binding. Sections were incubated for IF staining with primer antibody (BVDV anti-viral antiserum (Cat. No. 210-70-BVD, VMRD, USA)) at room temperature for 30 minutes. Sections were incubated for IF staining with a secondary antibody—anti-caprine immunoglobulin G-FITC (fluorescein isothiocyanate) (Cat. No. CJ-F-CAPG-10 mL VMRD, USA) in the dark for 45 minutes at room temperature. Sections were then examined with fluorescence microscope at magnification of 40× and closed by fluoroshield mounting medium with 4,6-diamino-2-phenyl indole (DAPI).

In IHC staining, sections were incubated with primary antibody (BVDV anti-viral antiserum, (Cat. No. 210-70-BVD, VMRD) at room temperature for 20 minutes. Labeled Streptavidin-Biotin (LSAB)+System-HRP (Carpinteria, USA) IHC kit was used and sections were incubated with 3,3′-diaminobenzidine and chromogen. The slides were counterstained with hematoxylin, and entellan was dropped on the tissue sections, which were then marked as positive (+) or negative (−) under a light microscope.

Statistical Analysis

The specificity and sensitivity of the tests were calculated using International Business Machine' Statistical Package for Social Science (SPSS) 20.0 software (IBM Corp.; Armonk, NY, USA). The sensitivity and specificity of the test were evaluated using RT-PCR as a relative gold standard. The level of agreement between the results of the PCR, IF, and IHC staining was measured at a 5% level of significance.

RESULTS

Positivities concerning PCR, IHC, and IF are given in Table 1. Pestivirus nucleic acid was detected in 13 (13%) of 100 tissue samples that were suspected of being infected with BVDV (Figure 1). Bovine viral diarrhea virus viral antigens were found in 16 of the

Table 1. Numbers of Positivity of BVDV Antigens Using PCR, Immunohistochemical, and Immunofluorescence

	PCR	IHC	IF
Positive sample number	13/100	16/100	21/100

BVDV, bovine viral diarrhea virus; IF, immunofluorescence; IHC, immunohistochemistry; PCR, polymerase chain reaction.

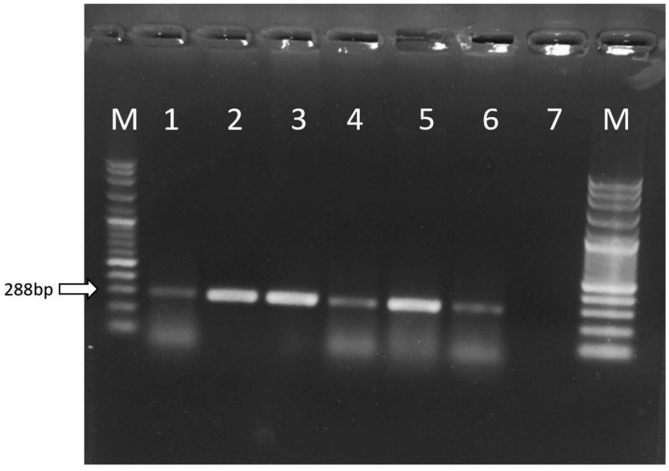

Figure 1. RT-PCR product visualized in agarose gel electrophoresis. Arrows show 288 bp fragments. 1: positive control, 2-6: BVDV-positive samples, and 7: negative control. M = 100 bp ladder. BVDV, bovine viral diarrhea virus; RT-PCR, reverse transcription-polymerase chain reaction.

100 samples examined in IHC staining and in 21 of the 100 samples examined in IF staining. Bovine viral diarrhea virus positivity was detected by IF and IHC methods in all 13 samples that tested positive for PCR. In addition, BVDV positivity was detected in 3 samples with IHC and 8 samples with IF. Bovine viral diarrhea virus positivity determined according to cell types is given in Table 2. Immunohistochemically, BVDV viral antigens were detected in spermatocytes, spermatids, and Sertoli cells in the seminiferous tubules (Figure 2A and C). Immunopositivity was observed as intracytoplasmic at severe level in spermatocytes and spermatids, moderate level in Leydig cells in intertubular areas, and mild level in Sertoli cells. In IF staining, BVDV viral antigen positivity was observed as intracytoplasmic at the severe level in spermatids and Leydig cells in intertubular areas (Figure 2B), moderate in spermatocytes, and mild in Sertoli cells (Figure 2D). Using PCR as the relative gold standard, the IF had a sensitivity of 85.7% and specificity of 87.3% and IHC had a sensitivity of 81.2% and a specificity of 88.1%.

DISCUSSION

With over 70% incidence rate, BVD infection is a significant risk for cattle reproductive health worldwide in all herds. Studies investigating the seroprevalence of BVD disease found seropositivity between 12% and 86% in herds from different countries.[22-26] Seroprevalence studies for BVDV in Türkiye have reported seropositivity between 50% and 94%.[27,28] Different results were seen in the determination studies of BVDV antigen positivity with IHC, IF, or PCR in Türkiye. In the study performed on the testis, BVD virus was not obtained from testicular tissues.[29] In our study, BVDV antigen positivity by PCR, IHC, and IF at 13%, 16%, and 21%, respectively, was seen.

Table 2. BVDV Antigen Positivity Observed in Different Cell Types by IHC and IF Methods

	IHC	IF
Spermatid	16	21
Leydig cells	7	12
Spermatocytes	12	21
Sertoli cells	16	21

BVDV, bovine viral diarrhea virus; IF, immunofluorescence; IHC, immunohistochemistry.

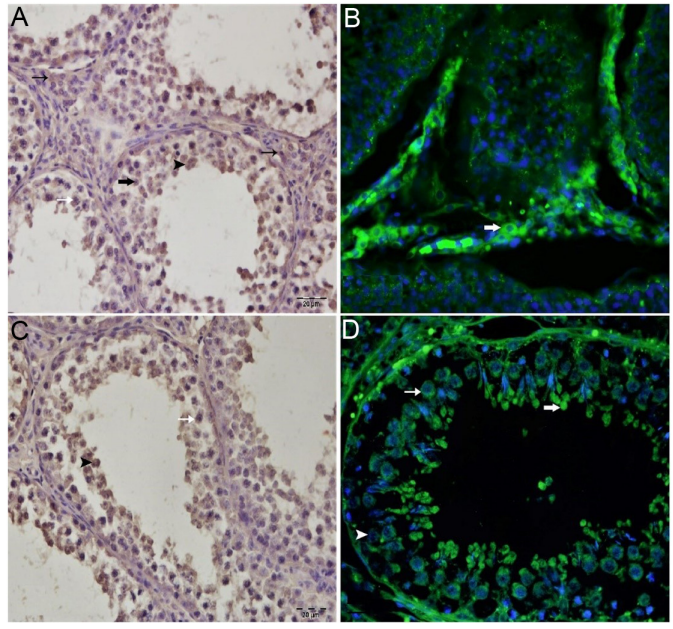

Figure 2. Photomicrograph showing antigenic localization in BVDV-infected testis tissues using immunohistochemistry and immunofluorescence staining (A-D). (A) BVDV antigen positivity in spermatocytes (white arrow), spermatids (arrowhead), Sertoli cells (thick arrow), and Leydig cells (thin arrow) IHC ×20. (B) Intracytoplasmic BVDV antigen positivity in Leydig cells (arrow) IF. (C) BVDV antigen positivity in spermatocytes (white arrow) and spermatids (arrowhead) IHC ×20. (D) BVDV antigen positivity in spermatocytes (thin arrow), spermatids (thick arrow), and Sertoli cells (arrowhead) IF×20. BVDV, bovine viral diarrhea virus

Reproductive results of BVD infection include death in embryonic and fetal stages, defective calves at birth, and PI calves.[30] Again, the BVD virus can be persistent in semen in cases where bulls are significantly seropositive yet non-viremic.[31] It has been observed that PI bulls' entire genital tract, including the testes, has significant amounts of the BVD virus. Acute infection in bulls provided the virus to accessory glands and epididymis but not the testes.[11] However; the presented study found BVDV antigen positivity in bulls' testes. This state shows that the bulls are infected during the formation of blood–testis barriers; so the virus can replicate in the testis and escape from the barrier.[31]

In terms of pathogenesis, persistent infection is formed by transfer of virus from mother to fetus during pregnancy in female animals. Our aim in this study was to investigate the pestiviruses incidentally found in the testis which is one of the places where the virus would persist in male individuals. In this context, pestivirus nucleic acid was searched in testicular tissue of 100 animals cut in a slaughterhouse, and 13 cases (13%) were detected positively. Persistent infection rates were determined between 0.07% and 3.07% in studies conducted.[27,32] The rate, which is quite high compared to these studies, may be due to the fact that our study was carried out in the testis, and RT-PCR was selected as the diagnostic method instead of the Ag-ELISA method used in other studies.[33] The persistence situation could not be fully explained in this study because the animal has to be sampled at least 21 days later again and antigen positivity must be provided for this situation to be revealed.[34] However, since the testicular tissue of the animals that died as the material is selected and single sampling is performed, the status of persistent infection cannot be determined. Antigen positivity in BVDV

infections identifies 2 conditions. Positivity may indicate both acute infection and persistent infection.[12] However, since the findings obtained in the study are the determination of antigen positivity in randomly sampled animals, it is not known whether the animals are in the period of acute infection or persistent infection.

Even though organ-level distribution of virus was present in different research,[35,36] few studies have been performed on prevalence and the localization of virus in the bulls' testis.[37,38] Viral antigens were demonstrated in different tissues in lung, thymus, heart, pancreas, placenta, ovarium, uterine, skin, sinusoids of spleen, and mucosa of the digestive system in many BVD studies.[39-41] In the present study, BVD virus was observed in bulls' testes using IHC and IF methods, and the accuracy of these tests was proven by PCR. Viral antigens were observed to be intracytoplasmic as reported in the literature.[38,42,43] In studies regarding the BVD virus distribution in the testes, Givens et al[44] demonstrated the presence of the antigen in the Sertoli cells but not in Leydig cells and seminiferous tubules adjoining the basal membranes by using 2 bulls that were artificially infected. In studies performed, viral antigen immunopositivity was detected in Sertoli cells and spermatogonia, but not in Leydig cells. In the present study, BVDV immunopositivity was found in Sertoli cells, spermatocytes, spermatogonia, and Leydig cells. Positivity in Leydig cells has been associated with BVDV agent that can settle into Leydig cells, depending on the increased amount of viral antigen. While the results were incompatible with the observations from cell culture, 65 bulls did not provide BVD virus in the following tissues: testes, epididymis, seminal vesicle, and the prostate gland.[29] Positive staining was detected as 21%, 16%, and 13% by indirect IF, IHC, and PCR, respectively. In IHC and IF testing, several factors that may play a role in different outcomes have been reported. One notable drawback of the IF method is its subjectivity in interpretation, even by the experts. It should be noted that the IHC staining enables the detection of virus particles concealed in the regular FA method due to utilizing proteolytic enzymes.[45]

In conclusion, in this study on the investigation of BVDV antigens in bovine abortions in Türkiye where the work was carried out, while 8 (14.28%) samples were detected positive by direct IF method, 6 (10.71%) samples were found to be positive by IHC staining in fetal tissues.[46] Results of the presented study are in accordance with this study and it has determined that bulls are an important epidemiological factor in the transmission of the disease. Bovine viral diarrhea virus-positive bulls are widely used both in natural mating and in artificial insemination; they can cause a significant increase in the percentage of abortions that occur. Bovine viral diarrhea virus antigen positivity was found at 21%, 16%, and 13% by indirect immunofluorescence, immunohistochemistry, and RT-PCR, respectively. Cellular localization of the virus was observed to be testicular cytoplasms. Obtained results indicate that bulls are a transmission source of infection, whether they are persistently infected or in the acute phase, and have an important place in the spread and prevention of the disease.

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Comparison of a Hybrid Intramedullary Pin with External Fixation Procedure and a Tape Splint on Tibiotarsal Fractures in Budgerigars (*Melopsittacus undulatus*): A Retrospective Study

Muhabbet Kuşlarının (*Melopsittacus undulatus*) Tibiotarsal Kırıklarında Intramedüller Pinle Yapılan Eksternal Fikzasyon Hibrit Tekniğiyle Bant Atelinin Karşılaştırılması: Retrospektif Çalışma

Mümin Gökhan ŞENOCAK[1]
Latif Emrah YANMAZ[2]
Elif DOĞAN[3]
Sıtkıcan OKUR[1]
Uğur ERSÖZ[1]
Ferda TURGUT[1]
Ayşe GÖLGELI BEDİR[1]
Ömer Tarık ORHUN[1]
Yakup KOCAMAN[1]
Zafer OKUMUŞ[4]

[1]Department of Surgery, Atatürk University, Faculty of Veterinary Medicine, Erzurum, Türkiye
[2]Department of Surgery, Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Burdur, Türkiye
[3]Department of Surgery, Kastamonu University, Faculty of Veterinary Medicine, Kastamonu, Türkiye
[4]VRM Imaging and Veterinary Health Services, İstanbul, Türkiye

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Sorumlu Yazar/Corresponding Author: Mümin Gökhan ŞENOCAK
E-mail: mgsenocak@atauni.edu.tr

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ABSTRACT

Comparing the short-term clinical outcomes of a tape splint, and the hybrid intramedullary pin with external fixation procedure (IMEF), a surgical approach, in the treatment of tibiotarsal fractures (TTFs) in budgerigars, and to present predictors of TTFs are objectives of this study. A total of 20 budgerigars admitted to the animal hospital with TTFs, which were treated with either the tape splint (n = 11) or IMEF (n = 9) surgery were material of the study. The treatment outcomes of both methods were compared, along with fracture predictors. The success rate of the IMEF surgery and tape splint were 6/9 (66.7%) and 9/11 (81.8%), respectively. The two methods were found to have similar success rates when compared to each other (odds ratio: 0.44, $P = 0.39$). The most common fracture location was the mid-shaft of the tibiotarsus for both treatment groups (IMEF: 6/9, 66.7%; tape splint: 7/11, 63.6%). The most common fracture type was oblique fractures in the IMEF surgery group (5/9, 55.6%), while it was transverse (8/11, 72.7%) in the tape splint group. There was a weak correlation ($r = 0.41$, $P < 0.03$) between the location of the fracture and the fracture type in both groups. In conclusion, both the IMEF surgery and tape splint methods have similar outcomes. The tape splint method should be preferred as the primary treatment option due to its non-invasive nature during TTFs in budgerigars. The IMEF surgery may be considered for the treatment of displaced fractures, but the involvement of the hock and stifle joints should be considered.

Keywords: Avian, budgerigar, fracture, *Melopsittacus undulatus*, osteosynthesis, tibiotarsus

ÖZ

Bu çalışma, cerrahi barındırmayan bir eksternal koaptasyon tekniği olan bant ateli ile cerrahi bir yöntem olan intramedüller pinle yapılan eksternal fikzasyon hibrit tekniğini (IMEF) karşılaştırmayı ve muhabbet kuşlarının tibiotarsal kırıklarına (TTFs) sebep olan öncülleri incelemeyi amaçlamaktadır. Bu çalışmanın hayvan materyalini hayvan hastanesine TTFs şikayetiyle başvuran toplam 20 muhabbet kuşu oluşturmaktadır. Tibiotarsal kırığı bulunan kuşların tedavileri IMEF (n = 9) ya da bant atel (n = 11) yöntemlerinden birisi tercih edilerek yapıldı ve her iki girişim tekniğinin sonuçları ve kırığa neden olan öncüller karşılaştırıldı. Elde edilen bulgular incelendiğinde IMEF cerrahisinin 6/9 (%66,7) ve bant atelinin 9/11 (%81,8) olduğu ve iki yöntemin birbirine kıyaslandığında başarı oranlarının benzer olduğu gözlendi (*Odds oranı*: ,44, $P = ,39$). Her iki yaklaşım tekniğinde de en sık görülen tibiotarsal kırık midşaft kırığıydı (IMEF: 6/9, %66,7; bant ateli (7/11, %63,6). En sık görülen kırık şekli de IMEF cerrahisi ile sağaltılan grupta oblik kırık (5/9, %55,6) ve bant ateli sağaltım grubunda transversal kırık (8/11, %72,7) olduğu gözlendi. Her iki grupta da kırık yeri ile kırık şekli arasında zayıf bir korelasyon olduğu belirlendi ($r = 0,42$, $P < ,03$). Sonuç olarak IMEF ve bant ateli birbirine benzer sonuçları olan iki yöntemdir. Bant ateli yöntemi invaziv olmaması nedeniyle öncelikli olarak tercih edilmelidir. IMEF cerrahisi yalnızca deplase kırıklarda önerilebilir olsa da eklem içerisinde invazyona neden olma potansiyeli göz önünde bulundurulmalıdır.

Anahtar Kelimeler: Kanatlılar, kırık, muhabbet kuşları, *Melopsittacus undulatus*, osteosentez, tibiotarsus

INTRODUCTION

Tibiotarsal fractures (TTFs) are common health issues encountered in budgerigars.[1-3] Trauma, nutritional deficiencies, poor body condition, chronic diseases, stress, and a crowded environment are the leading causes of TTFs.[1,4] The principles of treatment for TTFs generally follow small animal medicine. However, the size sometimes limits the approach with avian patients.[5]

In the case of TTF in birds, the treatment options are usually cage rest, external coaptation, and surgery.[4] The tape splint is a standard external coaptation method for the treatment of minimally displaced fractures in birds.[1] This technique minimizes the compression, rotation, and bending-shearing forces of the fracture site and promotes bone healing.[6] Tape splinting is preferred among clinicians because of its cost, ease of application, good tolerance by the patient, and generally satisfactory results.[7] However, due to poor anatomical alignment and a lack of rigid fixation of fragments, tape splinting can result in complications such as deformity and malunion.[7,8]

Although external coaptation is a good option for minimally displaced fractures, internal fixation in displaced fractures has some advantages, including immediate fracture stabilization, anatomical alignment, potential rapid healing, and minimization of bone healing complications such as malunion and nonunion.[4,8,9]

Although several surgical techniques have been investigated for the fixation of TTFs in birds, including intramedullary interlocking nails,[10] titanium microplates,[11] type II external skeletal fixators,[12] and external skeletal fixator intramedullary pin tie-in,[13] not all of them are suitable for budgerigars. The most common way to treat TTFs in budgerigars is nonsurgical external coaptation. This method has not yet been compared to a surgical method, nor has the use of a surgical technique in the clinical field with budgerigars been described.

The aim of this study was to present the short-term clinical outcomes of a tape splint, a nonsurgical external coaptation technique, and a comparison of the hybrid intramedullary pin with external fixation procedure (IMEF), a surgical approach, and predictors of TTFs in budgerigars.

MATERIALS AND METHODS

The study was performed in a Veterinary Teaching Hospital with the approval of the Atatürk University Local Ethics Council of Animal Experiments (HADYEK decision no: 2021/275).

Animals

Twenty budgerigars (*Melopsittacus undulatus*) were admitted to the Veterinary Teaching Hospital by their owners for TTF treatment and were included in the study.

Study Design

In this study, the cases were randomly assigned to receive either the IMEF or tape splint treatment methods. The inclusion criteria for this study were the presence of a displaced or nondisplaced complete fracture as observed on radiography. Cases with multiple fractures, multi-fragmentary fractures, or open fractures were not included in the study. Patient demographic data, such as age, sex, affected leg, cause of fracture, fracture location, and fracture configuration, were obtained from hospital records.

The operator determined outcomes on the 21st day when pins and splints were removed from both groups owing to weight bearing on the leg.

Preoperative Preparation

Before undergoing IMEF surgery or tape splint treatment, patients were required to fast for 5 hours and have their crop palpated to ensure they were empty. Preanesthetic considerations were assessed through anamnesis, assessment of the patient's awareness and environment, auscultation of the heart and respiratory system, evaluation of hydration and nutritional status, and palpation of the abdominal organs for any enlargement. The feces were also observed for color. Ventrodorsal and lateral orthogonal radiographs in dorsal and lateral recumbency were taken to check for any signs of masses such as eggs, lipomas, granulomas, ingrown feathers, or feather cysts.

Anesthesia was induced using a mask created by attaching a bandage roller to an anesthetic device (Komesaroff Mini-Kom, Kruuse, Langeskov, Denmark) and administering 4% sevoflurane (Sevorane 100% Inhalation Solution, Aesica Ltd, Queenborough, England) in pure oxygen at a flow rate of 2 L/min, while the patient was held wrapped in a towel or by hand (Figure 1A). Once the patient's respiration became regular and flapping movements ceased, it was placed in dorsal recumbency on the surgery table. The wings, feet, and tail were then taped onto the surgery drape (Figure 1B). Anesthesia was maintained using sevoflurane (2.5% in pure oxygen at a flow rate of 1.5-2.5 L/min) through the mask until the surgery or tape splint treatment was completed.

Tape Splint Procedure

A modified Altman's splint,[14] in the form of an external coaptation bandage, was applied to the site of the fracture. This bandage covers both the distal and proximal joints in relation to the fracture line. Radiographs were obtained immediately postfixation and again on the 21st day for all patients.

Hybrid Intramedullary Pin with External Fixation Procedure

The claws were grasped with gauze soaked in povidone–iodine (Dermosept Baticonol, 10%, ALG İlaç Ltd, İstanbul, Türkiye). Feathers were plucked around the leg, and the entire leg was prepared with 10% povidone–iodine followed by 0.4% chlorhexidine (4%, Klorhex, Drogsan, Çubuk, Ankara, Türkiye). The claw was grasped with sterile forceps through an opening on a presterilized transparent oven cooking bag (drape) (Figure 1C). Peripheral intravenous catheter guidewires (Nextech Medical Ltd Company, İstanbul, Türkiye) ranging from 0.4 mm to 0.5 mm were prepared for intramedullary pinning. A 1.5 cm incision was made at the craniomedial side of the affected leg. Fragments were identified between the *m.gastrocnemius medialis* and *m.tibialis cranialis* using Adson forceps. The trocar tip of the pin was advanced into the intramedullary canal of the distal fragment in a retrograde fashion, with the hock joint flexed to > 90° (Figure 1D). The pin was then advanced distally through the joint, and the stifle joint was flexed. The trocar tip was advanced into the proximal fragment and exited the skin through the cranial aspect of the tibial plateau (Figure 1E). The pin was not completely pulled out at this stage; the blunt tip of the pin was inserted into the distal fragment and advanced in a retrograde fashion through the previously created tunnel. The distal tip was pulled out until the pin length exiting distally and proximally was equal (Figure 1G). The proximal and

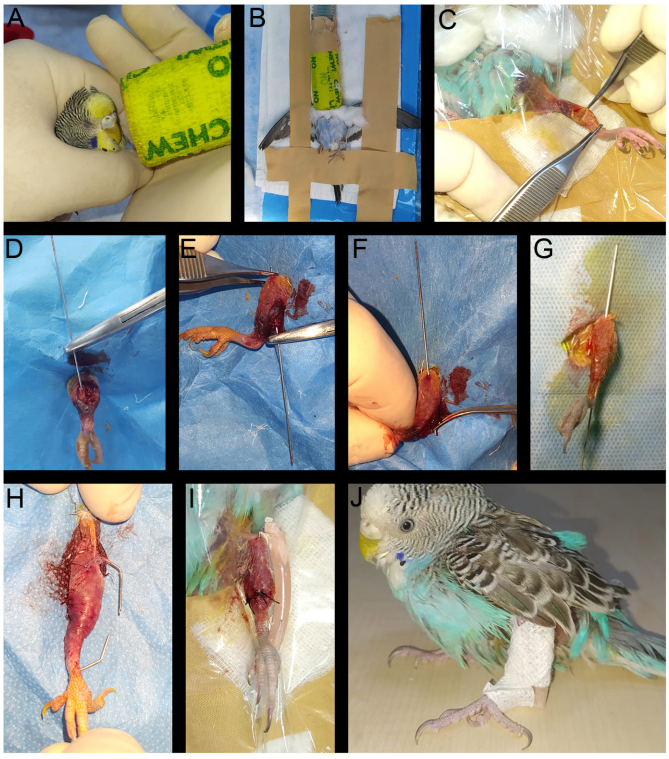

Figure 1. Hybrid intramedullary pin with external fixation procedure (IMEF) in budgerigars. (A) The induction was performed in hand with sevoflurane (4% in pure O_2 with a 2 L/min flow rate) until the excitation phase ended. (B) The bird was placed dorsal recumbency on the surgery table after inducing with anesthesia and taped. (C) A presterilized, transparent commercial oven bag used for under-drape monitoring. (D) Retrograde pin insertion. (E) Care was taken to protect the medial metatarsal vein while the pin was pulled out. (F) Replacement of the pin in the stifle joint. (G) The distal tip was pulled out until the sizes of the outer portion were equal. (H) Proximal and distal tips bent toward each other on the lateral side of the leg. (I) A piece of intravenous set hose was filled with polymethylmethacrylate cement, and pin tips were embedded inside the acrylic cement to fix each other with an acrylic–pin external fixator configuration (APEF). Then the skin was sutured. (J) A piece of adhesive tape was placed around the tibiotarsal and tarsometatarsal skin and secured to the APEF configuration.

distal tips of the pin were bent toward each other on the lateral side of the leg (Figure 1H). A piece of intravenous fluid administration hose was filled with polymethylmethacrylate cement, and the pin tips were embedded inside the cement to fix each other in an acrylic–pin external fixator (APEF) configuration. The skin was then sutured (Figure 1I). A piece of adhesive tape was placed around the tibiotarsal skin and taped to the APEF configuration (Figure 1J), and a secondary piece of tape was taped to the tarsometatarsal skin to prevent the medial rotation of the leg at the pin axis, then placed around the APEF configuration. The surgery was completed. Immediate postfixation radiographs were taken.

On the 21st postoperative day, the patient was anesthetized again for pin removal (Figure 2C). The pin sites were debrided with ethanol (96%, Etil Alkol, Alkomed Kimya, İstanbul, Türkiye) in a water solution (70%) (Figure 2D). The proximal tip of the pin was cut with a wire cutter without crushing or bending and then pulled out from the distal aspect (Figure 2E and F).

Postoperative Period

The following actions were taken after the collection of postoperative radiographs (Figure 2A and B); the patient was wrapped in

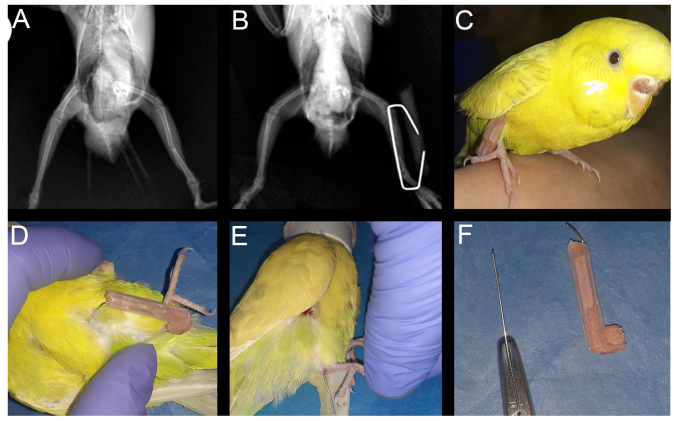

Figure 2. Postoperative management after the hybrid intramedullary pin with external fixation procedure (IMEF) in budgerigars. (A) Preoperative radiograph. (B) A radiograph 21 days after surgery. (C) Patient before the pins were pulled out. (D) Anesthetized patient for pin removal. (E) The proximal tip of the pin was cut without crushing or bending and pulled out from the hock joint. (F) Pulled pin and an acrylic–pin external fixator configuration.

a towel and taken to a prewarmed incubator for a smooth recovery. Butorphanol (1 mg/kg, IM, q12h, ×4, Butomidor, Richterphar Up, Wels, Austria) and Oxytetracycline HCl with vitamin combinations (30 mg/kg, PO, q24h, ×5 days, Vitaform, Vetaş Türkiye) were administered. Meloxicam (1 mg/kg, IM, q12h, ×2, Metacam 2%; Boehringer Ingelheim, Ingelheim, Germany) was used to manage pain.

Statistical Analysis

One-tailed bivariate Pearson correlations were used to compare predictors such as the cause of fracture, fracture location, fracture type, affected leg, age, and sex within treatment groups. The correlations among the predictors were also compared using Pearson correlation without grouping factors. The results of these comparisons were presented as *r* values. In addition, binary logistic regression analysis was performed to compare the predictors between groups. The results of this analysis were presented as odds ratios, *P* values from Fisher's exact test, and lower and upper confidence intervals (95%). Significance was determined by a *P* value of less than .05. All statistical analyses were conducted using the Statistical Package for Social Sciences version 22.0 software (IBM Corp.; Armonk, NY, USA).

RESULTS

A total of 24 budgerigars with TTFs were admitted to the animal hospital for treatment. Four birds were excluded from the study due to not meeting the inclusion criteria, resulting in a sample size of 20 birds. The treatments were administered using either a tape splint (n = 11, 55%) or IMEF surgery (n = 9, 45%). The success rate for the tape splint treatment was 9/11 (81.8%), while the success rate for the IMEF surgery was 6/9 (66.7%). Two patients in both groups experienced mild lameness after the 21st day of treatment. One bird died during surgery due to inadequate monitoring, and resuscitation efforts were unsuccessful.

Both the tape splint (7/11, 63.6%) and IMEF surgery (6/9, 66.7%) groups had a higher proportion of male Budgerigars. The ages of the birds ranged from 4 to 49 months (mean of 19.9 ± 11.9 months) in the tape splint group and from 6 to 48 months (mean of 18.22 ± 14.8 months) in the IMEF surgery group. The left leg was the most

Table 1. Correlation Table of Predictors Between Fracture Location and Cause of Fracture

	Fracture Location			
Cause of Fracture	Proximal	Media	Distal	Total
Door–window trauma	2	6	0	8
Unknown (in cage)	0	4	1	5
Entanglement into cage bars or tulle	0	1	4	5
Children	0	2	0	2
Total	2	13	5	20

There were intermediate bivariate correlations ($r = 0.6$) between fracture location and cause of fracture found ($P = .01$).

frequently affected in both groups (11/20, 55%). Midshaft fractures were common in both the tape splint (7/11, 63.6%) and IMEF surgery (6/9, 66.7%) groups. The most common fracture type in the tape splint group was transverse (8/11, 72.7%), while the most common type in the IMEF surgery group was oblique (5/9, 55.6%). The most common cause of fractures in the tape splint group was door–window trauma (4/11, 36.4%) or entanglement in cage bars or cage cover tulle (4/11, 36.4%). The most common cause of fractures in the IMEF surgery group was door–window trauma (4/9, 44.4%).

There was no significant difference between the 2 treatment methods (odds ratio: 0.44, 95% CI: 0.05-3.50, $P = .39$). A weak correlation was found between fracture location and fracture type ($r = 0.41$, $P < .03$). An intermediate correlation was observed between fracture location and the cause of fracture when the data was not grouped by treatment technique ($r = 0.64$, $P = .01$) (Table 1). A high correlation was found between fracture location and the cause of fracture within the tape splint group ($r = 0.94$, $P < .01$), and an intermediate correlation was observed between fracture location and fracture type in displaced fractures ($r = 0.73$, $P = .01$) (Table 2).

Orthogonal and oblique radiographs revealed apposition in all fractures, with better alignment in the IMEF surgery group. No signs of sclerosis or medullary canal radiodensities were observed on the 21st day radiographs of any patients during the healing process. The distribution of variables such as success rate of treatment method, cause of fracture, fracture location, fracture type, affected leg, age, and sex in the tape splint and IMEF surgery groups are presented in Table 2.

DISCUSSION

This study compared the effectiveness of tape splint and IMEF surgery for treating TTFs in budgerigars and found that IMEF surgery offers several advantages, such as rigid fixation, satisfactory alignment, and apposition. However, it is not a suitable primary fixation option for small birds due to the double risk of anesthesia (for pin replacement and removal), the lack of appropriate monitoring and implants in clinical settings, and the challenges of surgery in small avian patients.[15]

The IMEF surgery was developed as an alternative surgical method for providing rigid fixation of TTFs in budgerigars. To the authors' knowledge, there is limited clinical research on the use of tape splints or surgery for TTFs in budgerigars.

The tape splint method was found to be an easy and inexpensive method that allows fragments to stay together, requires fewer anesthetics, and has a success rate of 81.8% in nondisplaced TTFs in budgerigars. A previous clinical study reported a success rate of 92% for tibiotarsal external coaptation in companion birds.[2] While the tape splint provides satisfactory apposition, it may not provide the same level of alignment as the IMEF surgery. Poor alignment after external coaptation may result in malunion.[15] Orthogonal and oblique radiographs taken immediately postoperatively showed apposition of fractures in the IMEF group but not in the tape splint group. Although poor anatomic reconstruction due to inadequate fracture reduction was observed in patients treated with tape splints, budgerigars were found to tolerate this condition well without obvious lameness.

Surgical techniques are often necessary for proper alignment and apposition of primary bone healing.[4,8] While different surgical

Table 2. The Data of the Cases That Were Treated with Either Hybrid Intramedullary Pin with External Fixation Procedure (IMEF) or a Tape Splint

							Age (Months)		
Treatment, n = 20	Fracture Location	Affected Leg	Sex	Fracture Type	Outcome	Cause of Fracture	<12	12-24	> 12
IMEF surgery (9/20)	P	r	f	O	SR	DH	0	0	1
		l	m	O	SR	DH	1	0	0
	M	r	m	O	SR	DH	1	0	0
		r	m	O	X	CB	1	0	0
		l	m	O	SR	DH	1	0	0
		l	m	T	ML	N/A	0	1	0
		l	m	T	ML	C	1	0	0
		l	f	T	SR	C	0	0	1
	D	l	f	Sp	SR	N/A	1	0	0
Tape splint (11/20)	M	r	m	T	SR	DH	0	1	0
		r	m	T	ML	DH	1	0	0
		r	f	T	SR	CAT	1	0	0
		l	m	O	SR	N/A	0	0	1
		l	m	T	SR	DH	1	0	0
		l	m	T	SR	N/A	0	0	1
		l	f	T	SR	DH	0	1	0
	D	r	m	T	SR	CB	0	1	0
		r	m	T	ML	CB	0	1	0
		r	f	O	SR	CB	0	1	0
		l	f	O	SR	CB	0	1	0

C, children handling trauma; CAT, cat attack; CB, entanglement in the cage bars of cover tulle; D, distal; DH, door hit trauma; f, female; l, left; m, male; M, midshaft; ML, mild lameness; N/A, not answered; O, oblique; P, proximal; r, right; Sp, spiral; SR, successfully recovered; T, transverse; X, died.

techniques have been used to treat TTFs in large birds, not all of them are applicable to small birds.[16,17] There is no previously described surgical fixation method for TTFs in budgerigars, with the exception of one experimental study using intramedullary nailing.[8] The intramedullary nailing method lacks the ability to block rotational forces compared to IMEF surgery. The IMEF surgery can provide rotational and axial stabilization due to the polyaxial surfaces created between pin–bone contact points, percutaneous tapes, and the APEF exoskeleton configuration.

For the conventional tie-in fixation (TIF) configuration, 2 positive threaded-profile pins are placed in the proximolateral and distolateral aspects of the tibiotarsus, along with an intramedullary pin with a size up to 20% of the diameter of the tibiotarsus.[13] The small size of the bone and intramedullary canal limits the use of the TIF configuration in budgerigars. The main disadvantage of the IMEF surgery is that it involves the intraarticular involvement of both the stifle and hock joints, while the TIF technique only affects the stifle joint. The advantage of the conventional TIF technique is that it does not require the insertion of proximal and distal perpendicular pins on the bone axis.

There is limited literature on the use of external fixators for treating TTFs in budgerigars, but there are some reports in larger species. In raptors, the use of an external fixator for treating TTFs has been reported to have a success rate of 84%.[13] The success rate of IMEF surgery in budgerigars (66.7%) was lower than that of raptors. Raptors have a higher success rate than budgerigars because their bones are larger, making the approach easier.

Anesthesia and the challenges of monitoring small patients during surgery were identified as factors that contribute to the lower success rate of IMEF surgery in budgerigars.[18,19] Monitoring a budgerigar with devices is almost impossible due to its size; therefore, the most common method is observational monitoring during surgery. Additionally, monitoring may be complex for the operator if the patient is covered with surgical drapes. These factors negatively impact the success rate of surgery in budgerigars.

There are several causes of TTFs in budgerigars, including trauma from using doors and windows as perches, entanglement in cage wires or cover tulle, improper handling by children during play, and cat attacks. Predictors may lead to a fracture location on the tibiotarsus and may change the position of the fragments.[20]

Midshaft fractures were the most common location on the tibiotarsus in this study, as previously reported in other studies.[2,13] Budgerigars that used doors and windows as perches were more likely to have displaced fractures, likely due to the shearing force of the door or window closing and breaking the bone. Tibiotarsal fractures that were not displaced were more common in patients who became entangled in cage bars or cover tulle.

Oblique fractures were entirely displaced, possibly due to the fragments sliding over each other. Door, window, or cage-related fractures were the most common oblique fractures, likely due to the perpendicular force of the trauma shearing the bone axis. While psittacines tend to have midshaft and distal diaphyseal TTFs,[14] proximal fractures, particularly oblique and displaced ones, are common in budgerigars. The presence of sizable muscles around the proximal fragment, which can cause the fracture surfaces to slide over and collapse, may be the cause of the displacement of proximal oblique fractures.

Anesthesia-related death is a common occurrence in birds due to the lack of proper anesthetic and monitoring equipment.[4,5,14] While there were no complications observed in either treatment group except for one patient who died during surgery, the authors found that IMEF surgery presented more challenges due to anesthesia in budgerigars. These challenges included the lack of intubation and pulmonary resuscitation, the lack of intraoperative ECG monitoring, and the difficulty in approaching small birds. In situations where, appropriate equipment is not available, the use of a tape roll as an induction mask and a presterilized oven bag as a transparent surgical drape for patient monitoring may be helpful during surgery on small avian patients.

There are several limitations to this study. While the IMEF provides satisfactory fixation compared to the tape splint, the level of intraarticular damage to the hock and stifle joints was not evaluated. Additionally, the success rates of the IMEF surgery and tape splint methods were not statistically significantly different from each other, likely due to the small sample size.

In conclusion, the IMEF surgery may be considered an alternative treatment option for reducing displaced TTFs and providing rigid fixation, but the intraarticular involvement of both the hock and stifle joints should be considered when using this technique. Nonsurgical external coaptation with a tape splint, due to its ease of application, noninvasiveness, and lower anesthesia risk, should be considered the primary fixation technique for nondisplaced TTFs in budgerigars.

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Expression of Zonula Occludens-1 and Claudin-1 Proteins in Japanese Quails Testis

Bıldırcın Testisinde Zonula Okludens-1 ve Klaudin-1 Proteinlerinin Ekspresyonu

İlknur ÜNDAĞ
Hasan Hüseyin DÖNMEZ

Department of Histology and Embryology, Selçuk University, Veterinary Faculty, Konya, Türkiye

ABSTRACT

The aim of this study was to evaluate the general histological structure of testis in prepubertal and postpubertal stages of quails and determine the presence and location of claudin-1 and zonula occludens-1 proteins. In this study, testicular tissues obtained from 6 prepubertal and postpubertal stage quails were used. Tissue samples were fixed in 10% formaldehyde and processed for paraffin embedding. Crossman's triple staining method was used for general histological evaluation. Immunohistochemistry and immunofluorescent staining were performed for the expression of claudin-1 and zonula occludens-1 proteins, respectively. It has also been concluded that the proteins of claudin-1 and zonula occludens-1 do not show immunoreactivity because an active blood–testis barrier is not formed yet in seminiferous tubule epithelium in the testis in the prepubertal period as a result of immunohistochemical and immunofluorescence stainings but show immunoreactivity in the basal area in seminiferous epithelium with the blood–testis barrier formed in the postpubertal period. Immunoreactivity wasn't observed in prepubertal quail's testis. The immunoreactivity of claudin-1 has been distinguished as cytoplasmic and membranous in Sertoli cells and spermatogonia in postpubertal quails' testis. The immunoreactivity of zonula occludens-1 has not been observed in seminiferous tubules in prepubertal stage quail's testis. Immunoreactivity has been observed in the basal half of seminiferous tubules in postpubertal stage quail's testis. It has also been concluded that the proteins of claudin-1 and zonula occludens-1 do not show immunoreactivity because an active blood–testis barrier is not formed yet in seminiferous tubule epithelium in the testis in the prepubertal period as a result of immunohistochemical and immunofluorescence stainings but show immunoreactivity in the basal area in seminiferous epithelium with the blood–testis barrier formed in the postpubertal period.

Keywords: Blood–testis barrier, claudin, Japanese quail, zonula occludens

ÖZ

Bu çalışmanın amacı, bıldırcınların prepubertal ve postpubertal dönemlerinde testisin genel histolojik yapısını değerlendirmek ve klaudin-1, zonula okludens-1 proteinlerinin varlığını ve yerleşimini belirlemektir. Bu çalışmada 6 adet prepubertal ve postpubertal dönemlerdeki bıldırcınlardan elde edilen testis dokuları kullanıldı. Dokular %10'luk formaldehitte fikse edildi. Rutin doku takibi işleminden geçirildikten sonra parafin bloklar elde edildi. Genel histolojik değerlendirme için Crossman'ın üçlü boyama yöntemi kullanıldı. Klaudin-1 ve Zonula Okludens-1 proteinlerinin gösterimi için sırası ile immünohistokimya ve immünfloresan boyamaları gerçekleştirildi. Prepubertal bıldırcın testislerinde immünreaktivite gözlenmemiştir. Claudin-1'in immünoreaktivitesi postpubertal bıldırcın testislerinde Sertoli hücrelerinde ve spermatogonyumlarda sitoplazmik ve membransel olarak ayırt edilmiştir. Zonula occludens-1'in immünreaktivitesi prepubertal bıldırcın testisinde seminifer tübüllerde gözlenmemiştir. Postpubertal dönemdeki bıldırcın testisinde seminifer tübüllerin bazal bölgesinde immünreaktivite gözlenmiştir. Sonuç olarak diğer kanatlılarda olduğu gibi bıldırcınlarda da prepubertal dönemde kan-testis bariyerinin gelişiminin tamamlanmamış olduğu; postpubertal dönemde ise kan-testis bariyerinin oluştuğu ve bu bariyerin oluşumunda klaudin-1 ve zonula okludens-1 proteinlerinin katıldığı sonucuna ulaşılmıştır.

Anahtar Kelimer: Bıldırcın, kan-testis bariyeri, klaudin, zonula okludens

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Sorumlu Yazar/Corresponding Author: İlknur ÜNDAĞ
e-mail: ilknur-undag@selcuk.edu.tr

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INTRODUCTION

The reproductive system is one of the most critical factors necessary for the continuity of the generation. The protection of normal male fertility is based on producing healthy sperm. Healthy sperm production requires the differentiation of germ cells in a sheltered environment. The differentiation of the germ cells occurs in the postpubertal period, a long time after the maturation of the immune system. That is why these differentiated germ cells become an inevitable threat to the immune system and are trying to be destroyed by the immune system. The blood–testis barrier occurring with the pubertal period in the testis ensures that the cells are located in a protected medium by keeping the germ cells separate from this adverse situation. The blood–testis barrier among Sertoli cells in seminiferous tubule epithelium was situated around the basal third of the seminiferous tubule. This placement of the blood–testis barrier divides the seminiferous tubule epithelium into 2 regions, apical and basal. Spermatogonia and preleptotene spermatocytes are found in the basal region of the seminiferous epithelium, while primary and secondary spermatocytes, round spermatids, and elongating/elongated spermatids are located in the apical region. Tight junctions have many integral and peripheral membrane proteins. Zonula occludens-1 and claudin-1 proteins are peripheral and integral membrane proteins, respectively.[1-6]

Claudin was discovered by Furuse et al[7] (1998) for the first time. Claudin is a molecule weighing approximately 22 kDa. Claudins are composed of a short amine (NH_2) cytoplasmic area, 2 extracellular areas, 4 transmembrane areas, and a long carboxyl (COOH-) cytoplasmic area. There are 27 different claudin molecules identified in different epitheliums. In the testis, 7 other claudin molecules have been identified as claudin 1, 3, 5, 7, 8, and 11. The cytoplasmic COOH- area of claudin protein binds to Zonula occludens-1's Postsynaptic Density-95 Discs-large zonula occludens-1 (PDZ) in the ratio of 1 : 1.[4,7-11] Claudin-1 is the structural element of epidermal barrier in the epithelium. Claudin-1 takes part in cell motility. The localization of claudin-1 in the testis differs between mammalian species.[12,13]

Zonula occludens, a member of the Membrane Associated Guanylate Kinase homolog protein family, have 3 members zonula occludens-1, -2, and -3. Zonula occludens is structurally composed of 3 different areas: a guanylate kinase–like area, a src-coupling area, and 3 PDZ areas. Zonula occludens control cell reproduction and membrane organization, regulate cell differentiation and polarization, and manage signal transduction pathways. Zonula occludens-1 is synthesized by Sertoli cells but not by germ cells. Zonula occludens-1 provides a mechanochemical binding between the cytoskeleton and integral membrane proteins.[4,14-18]

By detecting these proteins in the structure of the blood–testis barrier, changes in the blood–testis barrier can be detected, and thus, the defects in the blood–testis barrier can be revealed. In the literature review, it was seen that claudin and zonula occludens proteins were not studied in quail testicles before. Therefore, this study aims to conduct the general histological evaluation of prepubertal and postpubertal stage quails' testis and determine the presence and location of claudin-1 and zonula occludens-1 from tight junction proteins forming the basis of the blood–testis barrier.

MATERIALS AND METHODS

This study was approved by the decision no. 2016/54 of the Ethics Committee of Experimental Animals of Reproduction and Research Center of the Faculty of Veterinary of Selçuk University on 29.06.2016. In the study, 30-day-old prepubertal (n = 6) and 70-day-old postpubertal (n = 6) Japanese quails (*Coturnix coturnix japonica*) were used. Testis tissues were obtained from the sacrificed animals. The tissue samples were fixed in 10% formol solution, processed by routine histological techniques, and embedded in paraffin. For the general evaluation, Crossman's triple staining method was used.[19] General histological examinations were made in preparation, and the thickness of the capsule and the diameters of seminiferous tubules were measured.

For the assessment of the claudin-1 protein, the avidin-biotin-peroxidase immunohistochemical staining method was used. Testis samples were left in 1 M citrate buffer (pH = 6) in the microwave (750 W) for antigen retrieval for 15 minutes. Then, the section was incubated with a protein block for 5 minutes to prevent nonspecific antibody binding and ground staining. Then, the section was incubated with claudin-1 primer antibody (Invitrogen, cat. no: 51-900) diluted in the ratio of 1/100 and, after that, with the secondary antibody for 30 minutes. To prevent endogenous peroxidase activity, they were placed into 3% hydrogen peroxidase (H_2O_2) prepared with methanol for 30 minutes. After, they were incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 30 minutes. Finally, they were incubated with diaminobenzidine (DAB). They were counterstained for 2 minutes with Mayer's Hematoxylin. For negative control, phosphate buffered saline (PBS) was dropped on samples instead of primary antibody. Then, the protocol was continued in the same way.[20,21]

For the assessment of the zonula occludens-1 protein, immunofluorescence staining was made. First, the samples were incubated with PBS Triton-X100 normal goat serum for 15 minutes. Then, they were incubated with zonula occludens-1 primary antibody (Abcam, cat. no: ab59720) diluted with antibody dilution solution in the ratio of 1 : 100 and after that with fluorescein isothiocyanate (FITC) conjugate goat anti-Rabbit IgG (Abcam, ab6717) secondary antibody diluted with block solution in the ratio of 1 : 1000 for 3 hours. Finally, the samples were mounted with 4′,6-diamidino-2-phenylindole (DAPI). For negative control, the slides were incubated with PBS instead of primary antibody and the protocol was continued the same way.[22]

Statistical Analysis

Statistical analysis of the data obtained by the measurements was created using the independent sample *t*-test method with MINITAB 14 package program. $P < .05$ was considered as significant.

RESULTS

Macroscopic Results

It was observed that prepubertal and postpubertal stage quails' testis was placed in the abdominal cavity. In addition, it was observed that the ductus deferens of quails were not apparent in the prepubertal period but got apparent in the postpubertal period and opened into the cloaca. In addition, it has been observed that postpubertal stage quails' testis are quite more significant compared to prepubertal stage quails (Figure 1).

Light Microscopic Results

It was observed in tissue sections prepared by Crossman's triple staining method in prepubertal stage quail's testis that

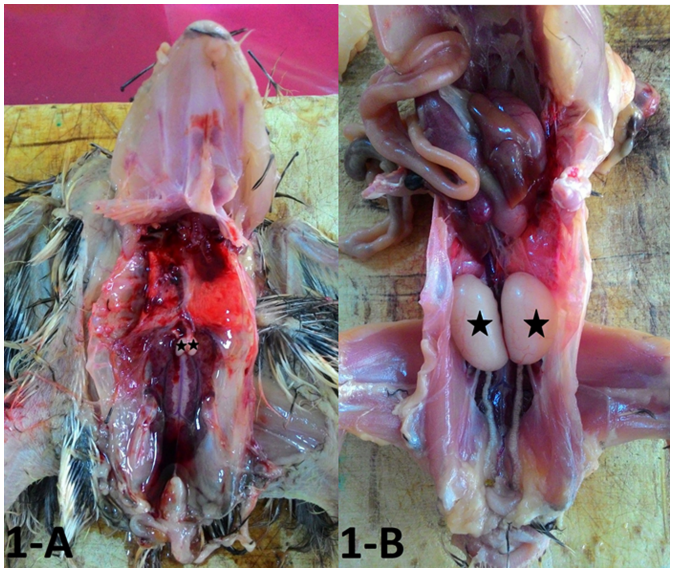

Figure 1. Macroscopic view of the genital organs of man quail. (A) Prepubertal period. (B) Postpubertal period (stars: testis, arrows: ductus deference).

seminiferous tubules are composed of Sertoli cells and spermatogonia. It was observed that Sertoli cells are placed in basal area and in the form of cells having triangle cores sloping toward apical and that there is an apparent basal membrane around seminiferous tubules. Loose connective tissue, blood vessels, and Leydig cells have been encountered in the interstitial area (Figure 2).

In the postpubertal quail testis, it was observed that Sertoli cells and spermatogenic germ cells at various stages of development in seminiferous tubule epithelium were present, and these cells were in the form of columns extending toward the lumen. The presence of sperms in the lumen of the seminiferous tubule has drawn attention. It has been seen that there is an apparent basal membrane around seminiferous tubules. It has been observed that loose connective tissue, blood vessels, and Leydig cells have been encountered in the interstitial area (Figure 3).

Statistical evaluation of the data obtained with measurement of the diameter of seminiferous tubule and thickness of capsule in prepubertal and postpubertal quails' testis has been carried out using the independent sample *t*-test method. It has been found that the diameter of the seminiferous tubule and thickness of the capsule increase in the postpubertal period, and this increase is statistically significant (Tables 1 and 2).

Immunohistochemical Results

As a result of immunohistochemical staining by using a claudin-1 primary antibody, immunoreactivity has not been observed in prepubertal stage quail's testis (Figure 4).

Immunoreactivity in the basal area of seminiferous tubules in postpubertal stage quails' testis sections has been observed. The immunoreactivity of claudin-1 has been distinguished as cytoplasmic and membranous in Sertoli cells and spermatogonia. It has drawn attention that claudin-1 is mainly in the area where the blood–testis barrier is formed. Any immunoreactivity has not been observed in negative control sections (Figure 5).

Immunofluorescence Results

As a result of immunofluorescence evaluation using zonula occludens-1 primary antibody in prepubertal stage quail, the immunoreactivity of zonula occludens-1 has not been observed in seminiferous tubules (Figure 6).

Immunoreactivity has been observed in the basal half of seminiferous tubules in postpubertal stage quail's testis sections

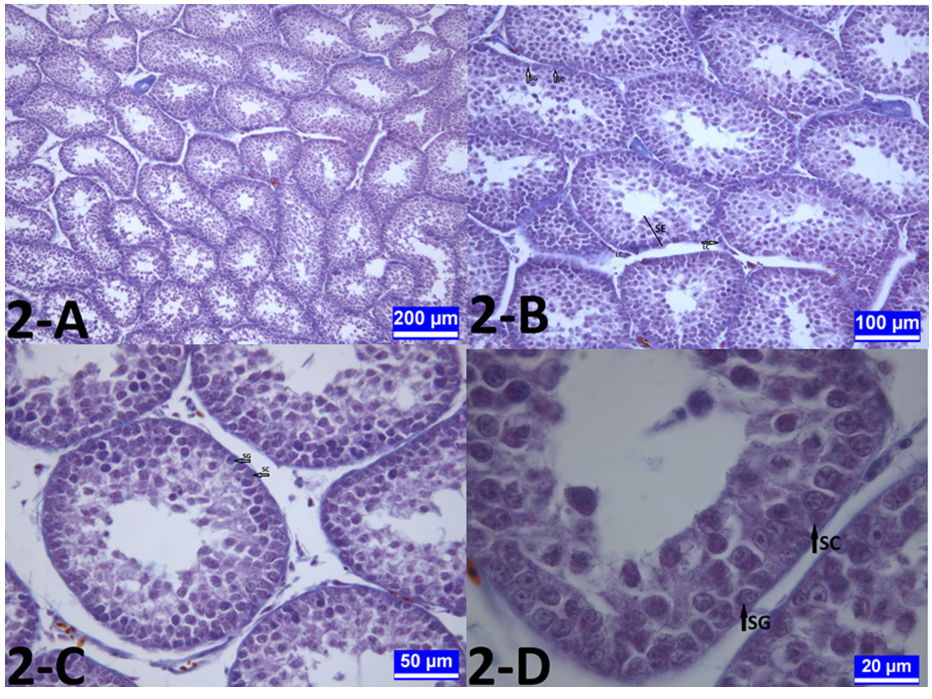

Figure 2. General histological view of prepubertal stage quail's testis in different zoom rates, Crossman's triple staining. LC, Leydig cell; SC, Sertoli cell; SE, seminiferous tubule epithelium; SG, spermatogonia.

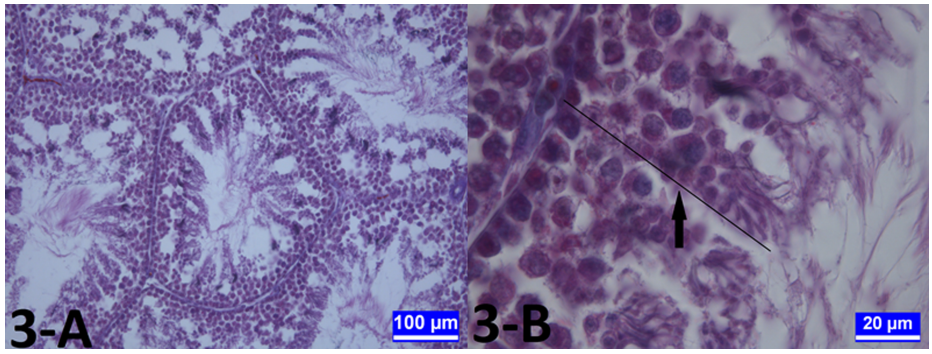

Figure 3. General histological view of postpubertal quail's testis in different zoom rates (arrow: cell cords), Crossman's triple staining.

Table 1. The Diameters of Seminiferous Tubules of Prepubertal and Postpubertal Stage Quails' Testis

	Number of Measurement	Average	Standard Error	Minimum	Maximum
Prepubertal stage	60	158.4	3.7	101.29	234.7
Postpubertal stage	60	294.8[a]	8.9	176.6	472.2

[a]The difference between groups is statistically significant ($P < .05$).

Table 2. The Thicknesses of Capsules of Prepubertal and Postpubertal Stage Quails' Testis

	Number of Measurement	Average	Standard Error	Minimum	Maximum
Prepubertal stage	60	29.04	0.98	16.5	49.75
Postpubertal stage	60	37.92[a]	0.76	26	49,51

[a]The difference between groups is statistically significant ($P < .05$).

(Figure 7-A, B, and C). It has drawn attention that radiation is especially in seminiferous tubules in Sertoli cells. Any immunoreactivity has not been observed in negative control sections (Figure 7-D).

DiscussionThe testis is responsible for reproducing sperms, one of the most critical elements necessary for generation continuity. Sperms are produced in seminiferous tubules, functional units of the testis. There are germ cells and spermatogonia in seminiferous tubules in the prepubertal period.[23] There are specific changes in the postpubertal period. One of these changes is that the germ cells begin to synthesize different surface proteins. Because surface proteins reproduced in germ cells show up a long time after the immune system grows, germ cell is perceived as foreign and want to be destroyed.[1] The formation of the blood–testis barrier occurs in the postpubertal period when germ cells differentiate. This barrier between Sertoli cells keeps spermatogenic cells separate from antigens in systemic circulations and prevents their destruction.[24-26]

Molele et al[27] (2021) reported that spermatogenic germ cells at various stages of spermatogenesis in seminiferous tubule epithelium were present. Correspondingly, in this study, it has been observed that the spermatogenic germ cells at various stages of spermatogenesis in seminiferous tubule epithelium were present.

In their study relating to the determination of the blood–testis barrier in cocks, Bergmann and Schindelmeiser[28](1987) observed that there is no functional barrier in the prepubertal period. Still, an active barrier is formed with the postpubertal period. Osman et al[29] (1980) suggested in their study on matured cocks that there are tight junction ties in the upper part of the area where spermatogonia are between Sertoli cells. They also indicated that

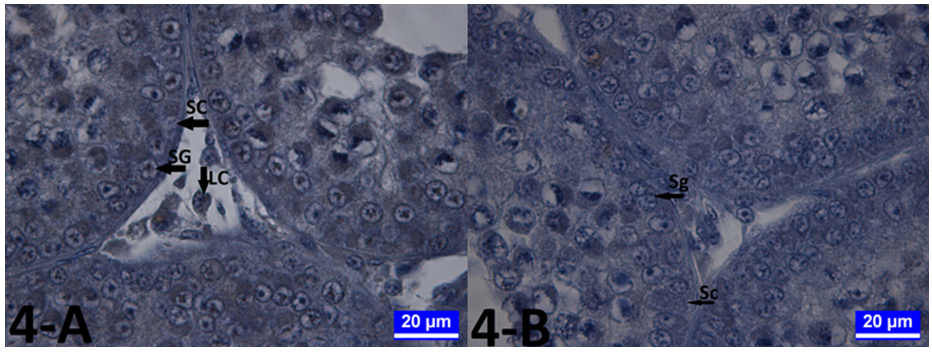

Figure 4. Immunohistochemical evaluation of claudin-1 antibody in prepubertal stage quail's testis. (A) Claudin-1 immunohistochemical staining. (B) Immunohistochemical negative control staining. LC, Leydig cell; SC, Sertoli cell; SG, spermatogonia.

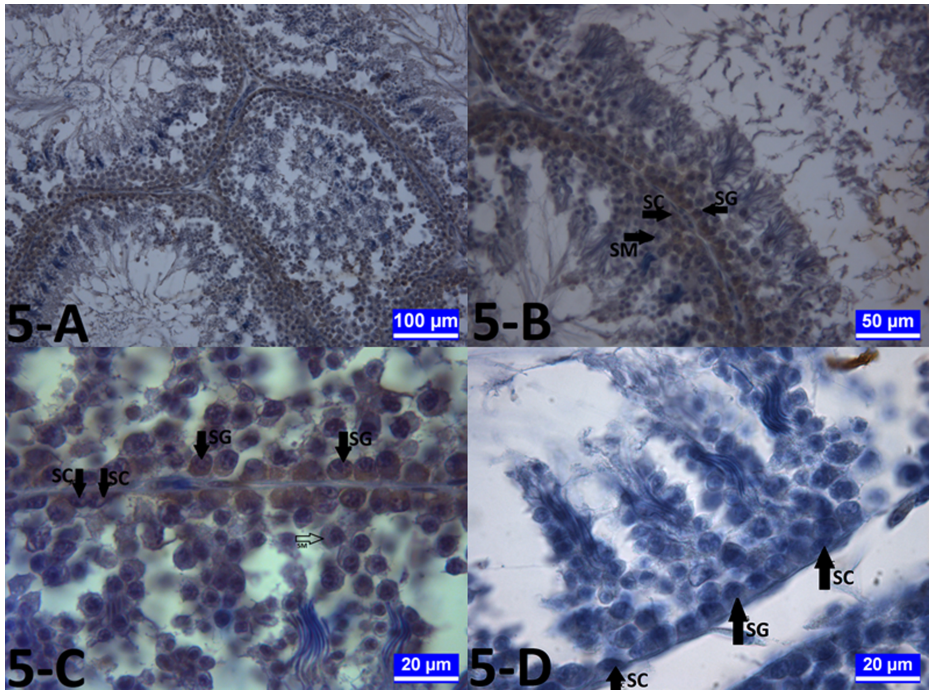

Figure 5. (A, B, C) Claudin-1 immunohistochemical staining of postpubertal stage quail's testis. (D) Negative control. SC, Sertoli cell; SG, spermatogonia; SM, Spermatocyte.

the agent does not go beyond the blood–testis barrier in the testis where coloring agent injection is performed.[14,15] In that study, it has been suggested that claudin-1 and zonula occludens-1 proteins, indicators of the presence of the blood–testis barrier, are present. Therefore, undeveloped blood–testis barrier in the prepubertal period develops in the postpubertal period.

In the study on the testis of a mouse, Gilio et al[30] (2013) reported that the immunohistochemical staining of the claudin-1 protein appears as dark brown in the basal compartment of seminiferous tubules in the testis of mature mice. In the study on the testis of immature and mature pheasants, Park et al[13] (2011) observed that there is weak immunoreactivity in immature (3 and 6 weeks) pheasants and strong immunoreactivity in adult (50 weeks) pheasants as a result of immunohistochemical evaluation. This study also observed that immunoreactivity is in the basal compartment between Sertoli cells in postpubertal stage quail testis by claudin-1's immunohistochemical staining. In Sertoli cells, the immunoreactivity of claudin-1 has been distinguished as cytoplasmic and membranous. It has also been observed that claudin-1 shows immunoreactivity near the basal area of the seminiferous tubule, where the blood–testis barrier is mainly formed. Claudin-1 is a vital protein taking part in the blood–testis barrier, and the presence of this protein couldn't be proven in the prepubertal period when the blood–testis barrier is not formed yet with many studies; it has been shown that the presence of this protein joining the formation of this barrier is seen in the postpubertal period.[282931] In this study, it has not been observed that the immunoreactivity of claudin-1 is in the testis in the postpubertal period. It has been thought to be originated from the fact that the blood–testis barrier is not formed yet in the prepubertal period. The fact that the immunoreactivity of claudin-1 is observed in the area where the blood–testis barrier is formed between Sertoli cells in postpubertal stage quail testis has made us think that the protein of claudin-1 takes part in this barrier. The barrier is formed in this period.

Molele et al[32] (2022) observed that immunoreactivity is in the basal area of seminiferous tubule epithelium. Correspondingly,

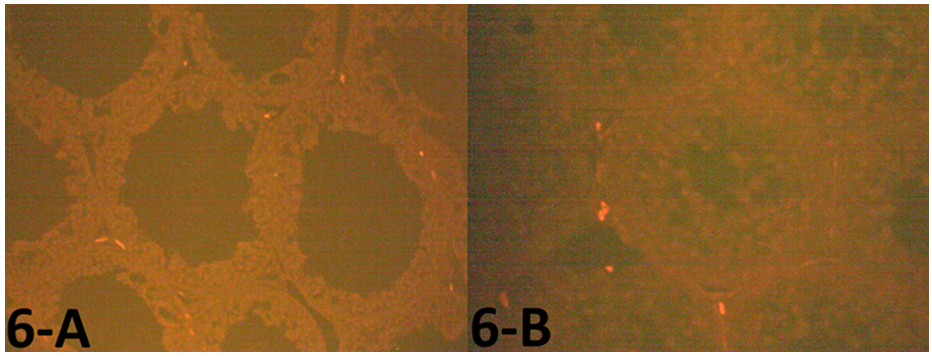

Figure 6. Immunofluorescence designation of prepubertal stage quail's testis. (A) Zonula occludens-1 immunofluorescence staining. (B) Zonula occludens-1 immunofluorescence negative control.

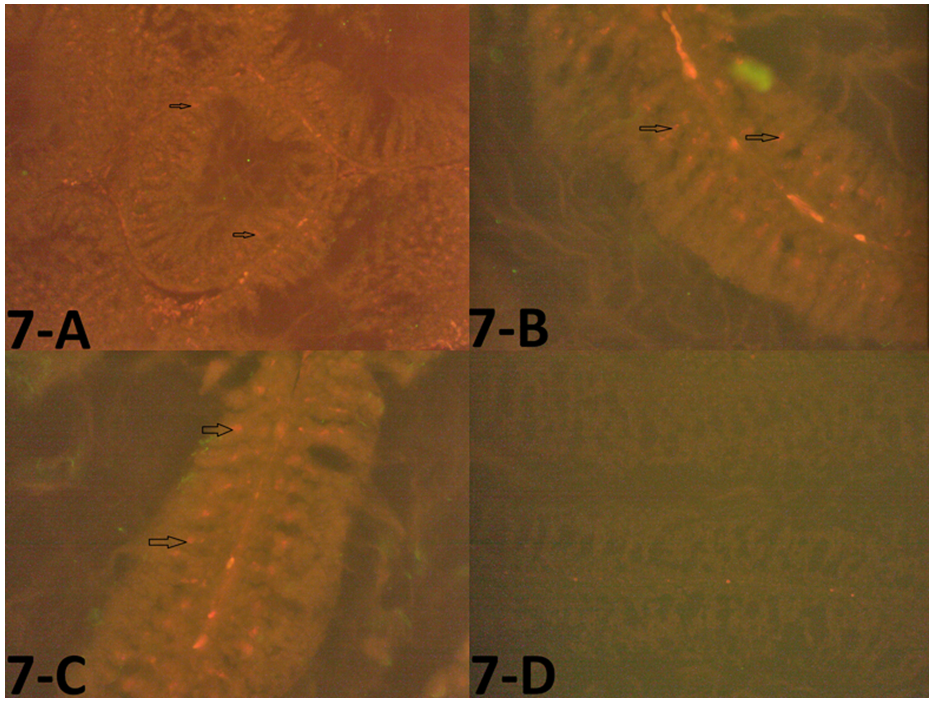

Figure 7. Immunofluorescence designation of postpubertal stage quail's testis. (A, B, C) Zonula occludens-1 immunofluorescence staining. (D) Zonula occludens-1 immunofluorescence negative control (arrow: the immunoreactivity of zonula occludens-1).

in this study, it has been observed that the immunoreactivity of zonula occludens-1 is in the basal area of the seminiferous tubule. Gilula et al[33] (1976) reported in the study on the testis of immature mammals that there is no tight junction in the testis of immature mammals. Stevenson et al[34] (1986) performed immunofluorescence staining of the protein of zonula occludens-1 in the testis of mice. They reported that zonula occludens-1 are immunostained where separated the basal and adlumenal testicular compartments.[34] Fink et al[35] (2006) performed immunohistochemical staining on testis tissues from patients with testicular cancer and healthy people. They suggested that the immunoreactivity of the protein of zonula occludens-1 is present in the blood-testis barrier between Sertoli cells in healthy people. However, they highlighted that the immunoreactivity of zonula occludens-1 decreases in those with testicular cancer and spreads to the cytoplasm. The western blot analyses they performed verified this result, and they suggested that the blood–testis barrier is degraded compared to healthy peoples by lanthanum dye.[35] Byers et al[36] (1991) reviewed the distribution of zonula occludens-1 in the testis of mice and reported that there is no tight junction complex in immature mice. However, they highlighted that zonula occludens-1 is generally distributed as apicolateral in the Sertoli cell membrane in the testis of mature mouse and not in the basal area. In this study, it has been seen that there is radiation in seminiferous tubules in prepubertal stage quail testis as a result of immunofluorescence staining of zonula occludens-1 and in seminiferous tubule epithelium between neighboring Sertoli cells in postpubertal stage quail testis. Based on this result, it has been thought that the blood–testis barrier is not formed or does not complete its formation in the prepubertal period and that the protein of zonula occludens-1 becomes visible as immunofluorescence with the formation of the blood–testis barrier in the postpubertal period.

As a result, diameter of the seminiferous tubule and thickness of the capsule increase in the postpubertal period compared to the prepubertal period. The proteins of claudin-1 and zonula occludens-1 do not show immunoreactivity in the testis in the prepubertal period but show immunoreactivity in the seminiferous epithelium in the postpubertal period.

Prepubertal (30 days) and postpubertal (70 days) stage quails' testis have been evaluated with this study. It has been concluded that general histological views of the testis of quail are similar to those of the testis of poultry and that the diameter of the seminiferous tubule and thickness of the capsule increase in the postpubertal period compared to the prepubertal period.It has also been concluded that the proteins of claudin-1 and zonula occludens-1 do not show immunoreactivity because an active blood–testis barrier is not formed yet in seminiferous tubule epithelium in the testis in the prepubertal period as a result of immunohistochemical and immunofluorescence stainings but show immunoreactivity in the basal area in seminiferous epithelium with the blood–testis barrier formed in the postpubertal period.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Experimental Animals of Reproduction and Research Center of the Faculty of Veterinary of Selçuk University (Date: 29.06.2016, Number: 2016/54).

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Author Contributions: Motivation/Concept – H.H.D.; Design – İ.Ü.; Control/Supervision – H.H.D.; Data Collection and/or Processing – İ.Ü.; Analysis and/or Interpretation – H.H.D., İ.Ü.; Literature Review – İ.Ü.; Writing the Article – İ.Ü.; Critical Review – H.H.D.

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General Beekeeping Structure in Sivas, Türkiye

Arı Yetiştiriciliğinin Genel Yapısı Sivas, Türkiye

Erhan ARSLAN[1]
Metin BAYRAKTAR[2]

[1]Food, Agriculture and Livestock Directorate, Sivas, Türkiye
[2]Department of Animal Science, Faculty of Veterinary Medicine, Fırat University, Elazığ, Türkiye

ABSTRACT

In this study, the level of beekeeping activities in Sivas, which has a wide area, and the technical, economic, and market possibilities of honey production and its by-products were evaluated. As well as, it is foreseen to be a source for studies on beekeeping. A survey was conducted in a total of 325 enterprises out of 2097 beekeeping enterprises existing in Sivas province center and in 16 districts. The data obtained were evaluated by considering appropriate statistical methods and the current situation, problems, and development opportunities of beekeeping in Sivas province were tried to be revealed. It has been observed that breeders have problems such as harsh and long winter conditions, wintering problems, shortage of quality and suitable queen bees, inability to find the market opportunity for the produced honey, and insufficient activities of the organizations. It has been concluded that increasing the number of hives through education, encouraging wandering, and producing mutual projects with government programs will increase honey production per hive. With the increase in honey production and the availability of suitable market conditions, the income of the rural people will increase as well as population migration will be prevented.

Keywords: Beekeeping, honey production, number of colonies, socioeconomic structure, Türkiye

ÖZ

Bu çalışmayla yüzölçümü geniş bir alana yayılan Sivas ilinde arıcılık faaliyetlerinin düzeyi ve buna bağlı olarak bal üretimi ile yan ürünlerinin üretiminin teknik, ekonomik ve pazar-piyasa olanakları değerlendirilmiştir. Bununla birlikte arıcılık ile ilgili çalışmalara kaynak teşkil etmesi öngörülmüştür. Sivas ili merkez ve 16 ilçede halen mevcut olan 2097 arıcılık işletmesinden toplam 325 işletmede anket çalışması yapılmıştır. Elde edilen veriler uygun istatistiksel metotlar göz önünde bulundurularak değerlendirilmiş ve Sivas ilinde arıcılığın mevcut durumu, problemleri ve gelişme olanakları ortaya konulmaya çalışılmıştır. Yetiştiricilerin sert ve uzun geçen kış şartları, kışlatma problemleri, kaliteli ve bölgeye uygun ana arı sıkıntısı, üretilen balın pazar-piyasa olanağını bulamaması, örgütlerin faaliyetlerinin yetersiz olması gibi sıkıntıları olduğu görülmüştür. Bu tespitlerle ilgili kovan sayısının eğitimle artırılması, gezginciliğin teşviki ve örgütlerin hükümet programları ile karşılıklı projeler üretmesi kovan başına bal üretimini artıracağı kanaatine varılmıştır. Bal üretiminin artması ve uygun pazar şartlarının bulunmasıyla kırsal kesimin gelirinde artış olacağı gibi nüfus göçünün de önüne geçilecektir.

Anahtar Kelimeler: Arı yetiştiriciliği, bal üretimi, koloni sayısı, sosyo-ekonomik yapı, Türkiye

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Sorumlu Yazar/Corresponding Author: Erhan ARSLAN
E-mail: erhan.arslan@tarimorman.gov.tr

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INTRODUCTION

Beekeeping production provides very important contributions to the business and therefore to the country's economy. It is an attractive line of business with its features such as using less labor force compared to agricultural branches, low operating costs, easy storage of its products, and selling at value prices. It is accepted that beekeeping provides jobs, income, and healthy nutrition opportunities to the rural population in developing countries.[1]

The geography of Türkiye has a high potential for beekeeping in terms of both its location and the richness of the climate.[2] Since beekeeping is an indispensable element of agriculture and its contribution to pollination will increase the yield and quality of plant production, it will make the producer smile, encourage the profession and make beekeepers economically strong.[3,4]

As in other agricultural activities in Türkiye, new technical developments are being implemented in beekeeping day by day.[5] Turkiye ranks second in the world in honey production with over 6 million colonies. The average honey yield per colony is quite low in Turkiye. One of the most important reasons for this is the lack of technical information such as bee individuals and colonies, their behavior, as well as the lack of sufficient information about diseases and pests, and the lack of timely and accurate combat.[6]

When similar studies on beekeeping are examined, the average age of beekeepers was reported as 40.91 in a study conducted in Bahçesaray district of Van province.[7] In a study conducted in Bingöl province, it was reported that 43% of beekeepers were primary school graduates and 21% were secondary school graduates.[8] In a study conducted in Gaziantep province, it was reported that 54% of beekeepers learned beekeeping as their sole source of income, 34% learned beekeeping from another beekeeper (experienced), and 28% from their father.[9] In the study conducted in Kırşehir province, the loss of colony in the last 3 years was reported as 13%. It has been reported that varroa is seen with the highest rate of 65%, lime disease is encountered secondly with 18%, and foulbrood is encountered with a rate of 9%.[10] In the findings of the research conducted in the Trakya Region, it was reported that 70% of the beekeepers did not care and feed before winter.[11] In the research conducted in the Southern Marmara Region, it was reported that the organization was 70%, 44% did not follow the publications related to beekeeping, and 76% of the beekeepers raised the queen beekeepers themselves.[12]

Beekeeping is one of the most suitable branches of agricultural activity to support the agricultural economy in Sivas, in terms of its geographical location, land structure, climate, and vegetation, as well as the socioeconomic structure of its people. Sivas province, on the other hand, has the second largest area of Turkiye and has more area than some European countries such as Kosovo and North Macedonia. It is also on the transit route of itinerant beekeepers.

Sivas province ranks fourth among the provinces in terms of both the number of colonies and honey production in beekeeping in Turkiye.[13] With this study, which will be carried out in Sivas province and its surroundings, it is aimed to evaluate the beekeeping potential in the region, to raise awareness of the producer mass, to evaluate the positive and negative factors that affect the production of honey and by-products, and to increase the yield per hive by examining the demographic and socioeconomic structure of beekeeping.

MATERIALS AND METHODS

The research material consisted of 325 beekeeping establishments that can best represent the population at 95% confidence level and 10% confidence interval, from 2097 beekeeping enterprises engaged in beekeeping in the center and 16 districts of Sivas, using the proportional sampling method.[14] The data source of the research consisted of face-to-face survey data made with these enterprises selected by random stratified sampling method among the total enterprises. The study was prepared in accordance with the Declaration of Helsinki.

The total number of existing beekeeping enterprises in the center and 16 districts of Sivas province was taken from the records of the Provincial Directorate of Food, Agriculture and Livestock, and the number of beekeeping enterprises to be surveyed was determined in direct proportion to the number of enterprises owned, and these are given in Table 1.

The questionnaire questions used in the research were prepared by making use of similar studies. Before starting the survey, a trial survey was conducted to test the accuracy and comprehensibility of the survey questions, thus minimizing the possible negative effects.

In the research, the Direct Interview method was used during the collection of the material. In this method, the questionnaire forms were prepared in advance and filled in by the researcher under the supervision of the producer.

The questionnaire forms filled with the beekeepers were examined and the necessary controls and arrangements were made, and this information was summarized and thus the analysis was made ready for evaluation. Using the Statistical Package for Social Sciences (SPSS) version 22.0 (IBM Corp.; Armonk, NY, USA) statistical package program from the data obtained, their frequencies, percentages, and honey yield averages were calculated and summarized in the frequency distribution tables.

RESULTS AND DISCUSSION

There is no scientific study that can serve regional beekeeping in Sivas. In the research conducted on beekeepers in Sivas province, the level of knowledge of the breeders on beekeeping and regional problems was collected by interviewing face-to-face. In the research, the issues that will shed light on the studies were emphasized, the problems related to beekeeping were discussed and it was aimed to help the projects to be done. Research findings are gathered under the main headings of socioeconomic characteristics, beekeeping activities, organization and supports, diseases and enemies, and marketing trade.

Socioeconomic Qualifications

Age and Gender

No female breeder could be identified from randomly selected breeders in the surveys. It has been observed that the contribution

Table 1. Number of Existing and Surveyed Beekeeping Enterprises in Sivas Province Districts

District	Number of Existing Businesses	Number of Selected Businesses	District	Number of Existing Businesses	Number of Selected Businesses
Akıncılar	28	4	Kangal	63	10
Altınyayla	18	3	Koyulhisar	136	21
Divriği	173	27	Merkez	241	37
Doğanşar	23	4	Suşehri	111	17
Gemerek	37	6	Şarkışla	61	9
Gölova	23	4	Ulaş	50	8
Gürün	155	24	Yıldızeli	160	25
Hafik	331	51	Zara	302	47
İmranlı	185	29	**TOTAL**	**2097**	**325**

Table 2. Distribution of Beekeepers in Sivas Province by Age and Honey Yield

	Bee Business		Honey Yield
Age Groups	Number	Percent	Avg ± Standard Error
15-25	4	1.23	21.50 ± 2.02
26-35	40	12.31	22.44 ± 1.66
36-45	99	30.46	21.53 ± 1.02
46-55	86	26.46	21.37 ± 1.07
<55	96	29.54	22.23 ± 1.00
Total	325	100	

of women is mostly limited to helping their spouses in beekeeping and honey production activities.

Especially the young population of Türkiye migrates to cities and big cities. Unfortunately, there is an elderly population in rural areas and villages. The most important reasons for migration are unemployment and financial difficulties. Beekeeping is a field of activity that will show itself as a profession and livelihood for the young population, additional income for the elderly population, vitality to the plant product, and contribution to the country's economy as a line of business that can be done in areas where urban development is far away, without the need for large lands. Age distribution and honey yield of beekeepers in Sivas province are given in Table 2.

As can be seen in Table 2, it was determined that most of the beekeepers (86.5%) were breeders over the age of 35. The research shows that the average age is 47 years, and unfortunately, it is seen that the majority of beekeeping activities are carried out by the elderly population. The research reveals the necessity of preventing the disappearance of the young workforce in rural areas or their migration to the cities. The beekeeping profession should be encouraged to young people, they should be informed with courses, and they should be supported with credits or hive incentives.

Education Status

The results of the questions investigating the effect of education on honey yield are given in Table 3.

According to the results of the research, 42.46% of 138 breeders stated that they were primary school graduates. The number of illiterates is low at 8 (2.46%). The beekeepers, who are 23 (7.08%) graduates of college, are retired beekeepers whose beekeeping activities do not go beyond making use of their spare time. It was determined that illiterate people had the least honey yield. Similar results were also seen in studies conducted in Bingöl and Adana. In a study in Bingöl, when the relations between the yield per hive and the education level of the household head were examined, it

Table 3. Distribution of Beekeepers in Sivas Province According to Their Education Level and Honey Yield

	Bee Business		Honey Yield
Education Status	Number	Percent (%)	Avg ± Standard Error
Not literate	8	2.46	16.13 ± 1.59
Primary school	138	42.46	22.10 ± 0.83
Middle school	68	20.92	22.54 ± 1.10
High school	88	27.08	21.92 ± 1.13
Collage/faculty	23	7.08	19.61 ± 2.45
Toplam	325	100	

was seen that the producers who received education at the primary school level obtained more honey per hive. The reason for this is that these people, who are generally above the middle age group, do not see beekeeping as a hobby.

In Türkiye, beekeeping is generally done with ancestral methods and unconsciously. As people who do this job, beekeepers themselves stated that they should be trained practically and theoretically. They stated that some beekeepers emphasized the importance of conscious beekeeping and education in honey yield by giving an example that they had obtained an average of 20 kg honey yield before and increased it to 50 kg as a result of beekeeping training.[15]

Beekeeping Experience

In the study, it was seen that 44.62% of beekeepers have been beekeeping for more than 20 years. In honey yield, it has been observed that they have more honey on average than beekeepers with 6-10 years of experience.

In normal conditions, efficiency is directly proportional to experience. For this, the growers were asked about their honey yield in the last 3 years, and the results are given in Figure 1.

According to the results of the survey, 26% of the growers stated that they bought honey over 30 kg. The costs of people who do not have a level of knowledge with many hives at the beginning will bring a financial burden, and the negativities of inexperience will reduce the excitement of beekeepers. In the survey conducted with the breeders, it was determined that people who do not have beekeeping experience should start their beekeeping activities with 5 hives. The average honey yield of all beekeepers was calculated as 21.82 ± 0.55 kg.

Beekeeping Activities

Bee Breeds Used in Production

Questions were asked about the demands and works of the breeders regarding the bee breeds used in the region. About

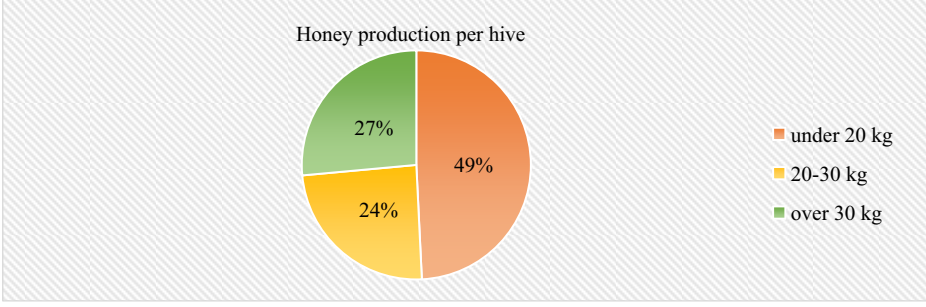

Figure 1. Distribution of beekeepers in Sivas province according to their average honey production

81.23% of the breeders stated that they use Caucasian hybrids and that it is a breed that adapts to the region. While 39.69% were dissatisfied with the queen bee they bought commercially, 28% of the beekeepers stated that there was no change in the yield. Again, beekeeping institutes should be able to reach beekeepers for quality queen rearing and state policies should support queen breeding.

Beekeeping Knowledge Level

As a result of the questions asked about acquiring and increasing beekeeping knowledge, 59.69% of the breeders stated that they received information from experienced beekeepers, others from various publications, courses, and institutions. Similar results were obtained in the study conducted in Tekirdağ province. In the study, it was stated that 26% of beekeepers learned beekeeping from their fathers and 50% from the environment.[16] While there are beekeeping departments in many universities, beekeeping college programs have been opened, and there are people appointed as beekeeping specialists in every Food, Agriculture and Livestock directorate, it is a fact that modern beekeeping techniques still cannot be accepted by producers.[17] Manufacturers should improve themselves and get all the necessary information. For this, they should attend courses, congresses, symposiums, and seminars. They should read books on beekeeping, be in constant dialogue with successful producers, and get help from the relevant programs of universities.[18]

Wandering Status

According to the results of the research, 35.69% of the breeders stated that they did fixed beekeeping, and 12.92% of them stated that they moved their colonies to nearby towns or villages once. In the study, it is seen that the honey yield of those who carry their hives to nectar-rich places once or more than 3 times a year is 22 kg; while the mean yield of less movers is 21 kg and below. In a study conducted in Elazığ, it was determined that 50% of the beekeepers were fixed beekeepers and the other half were nomadic beekeepers.[19]

The application of mobile beekeeping to a large extent is due to the insufficient nectar period in Sivas, since the season starts late and ends early. Accommodation poses a problem for both mobile beekeepers and stationary beekeepers. Wandering beekeepers face difficulties due to features such as rent, suitable and productive vegetation, and water source, while stationary beekeepers complain about the excessive amount of itinerant beekeepers coming to their region, hence the nectar deficiency, in short, the lack of capacity regulation. In this regard, under the leadership of the Ministry of Food, Agriculture and Livestock and with the participation of the unions, areas with suitable flora can be allocated to the wandering beekeepers and they can be accommodated in return for rent.

Beekeeping Costs

The results of the research are in the first place, 40%-80% of the beekeeping costs of 91% are spent on feeding expenses, and secondly, 1%-20% of the 90% are related to transportation, labor, and accommodation expenses, especially medicine and other variables (packaging, honeycomb, etc.) proved to be spent.

Queen Bee Breeding and Replacing

About 78.15% of the beekeepers in Sivas stated that they change their queen bees every 2 years. The results of the research indicated that 36% of the breeders were not satisfied with the commercial queen bees they used in their businesses, while 28% of them stated that it did not affect their yield much. In the interviews, it was stated that the breeder reared his own queen after several unsuccessful commercial queen trials. According to the results of the research, the most important problem that beekeepers want to see as state support is the queen bee. For this, studies should be carried out with the necessary public institutions and private organizations to ensure quality and appropriate queen bee supply. It is also a very important necessity to raise queen bees that have been improved according to the conditions of the country.

Organization and Support

Organization

In the research, while the number of breeders who are members of a union or cooperative related to beekeeping was 284 (87.38%), the number of breeders who are not members of any union was 41 (12.62%). About 68.3% of the members of unions or cooperatives stated that they were dissatisfied with the union they were a member of, whereas 19.8% stated that the unions were sufficient.

The breeders stated that the organizations and unions could not fully fulfill their goals. They stated that the courses and seminars were insufficient, and the cooperatives and unions only required membership due to government support and stated that they did nothing but collect dues, that the produced honey was released to the market very cheaply, and that they could not show a presence in important matters such as branding, cheap input, and quality breeding. The reason why farmer organizations collect monthly and annual dues is that they work for the benefit of their members. It is important to organize courses, seminars, meetings, and organizations for the benefit of beekeepers in their unions and cooperatives, to inform beekeepers, to inform them of innovations, and to help them find the market for their products with cheap and high-quality inputs. According to research done, it has been determined that the producers in Elazığ mostly sell honey at retail. It has been determined that the problems faced by the producers in marketing are that the honey cannot be sold at the value it deserves, foreign products enter the market uncontrollably, hesitations about the naturalness of the products, the absence of cooperatives that will be effective in marketing and the lack of price standards for their products. One of the biggest problems of beekeepers is the marketing problem. The fact that honey prices are not established under free market conditions has created the main problems for the producer. The price issue will be resolved by the producers coming together and providing the organization to market their own products.[20]

Credit Usage

According to the survey results, 72.62% of the breeders stated that they did not use credit. About 15% of the breeders who used loans in amounts higher than 30 000 TL stated that they used the loan they received on behalf of beekeeping not only for beekeeping but also for amortizing other farming activities. For this, it is important to run the monitoring and inspection mechanism of credit institutions and the state.

Disease and Enemies

Bee Diseases

The breeders were asked questions about the diseases they encountered and the solution methods, and the diseases encountered by the beekeepers in Sivas are given in Table 4.

Table 4. Distribution of Beekeepers in Sivas Province According to Bee Diseases They Encountered in Their Farms

	Bee Business	
Disease	Number	Percent
Varroa	256	78.77
Foulbrood	64	19.69
Nosema	0	0.00
Lime disease	5	1.54
Leave the colony	0	0.00
Total	325	100

As can be seen, varroa has a high effect on colony existence and honey production on bees and beekeepers. In the study, the breeders stated that they had problems with the use of drugs, either they could not find an effective drug or they could not use it because it left a residue in the honey. While 224 (68.92%) of 256 of 325 beekeepers surveyed are struggling with varroa, they are fighting against varroa in early spring and late autumn, while 29 (8.92%) are struggling with varroa. In addition to these, one breeder stated that he did not apply any precautions while another breeder struggled for precautions in the spring. In this regard, especially academicians and institutes should work together consciously, and beekeepers should put forward ways of fighting with treatment methods that will help them in their fight against diseases and pests.

Bee Pests

In the dialogues made with the breeders during the survey, they stated that they encountered bee enemies such as bears, bees, and hornets, but they were isolated and did not cause great colony losses.

In a study, it was tried to remove drone and worker bees from closed eyes with oxalic acid, lactic acid, perizin, orange peel, eucalyptus peel, and leaf. As a result, applications that can be an alternative to drug administration and that do not harm human health have been identified.[21] It is certain that with similar studies, beekeepers will be beneficial in combating bee pests and preventing colony losses, thus increasing honey yield.

Marketing Trade

In the research, the breeders were asked about the most important problem of beekeepers, such as high input prices, lack of quality breeders, accommodation and rent, agricultural spraying, lack of education, credit, wrong support policy, high transportation costs, security, and marketing of other bee products other than honey. In the first question, 91 (28.00%) people wrote high input prices for their first choice, while 89 (27.38%) people wrote high input prices for their second choice. The last choice that the breeders do not see as a problem is the marketing of bee products other than honey, with 92 (28.31%), the reason being that there is almost no production of bee products other than honey in the region.

Marketing Problem

In the survey, growers were asked to rank the options related to problems in marketing, such as illegal or imported honey entry, consumer's distrust of honey, lack of standard production, forward sales, and fraud options. According to the results, 256 people (78.77%) attributed the first choice to smuggled or imported honey, the second choice was attributed to the insecurity of 233 (71.69%) consumers, and the third choice was the lack of standard production by 204 people (62.77%).

According to the research, it is seen that the problems of beekeepers is a common problem in Türkiye. Unions and cooperatives with a membership of 87.38% should focus on marketing or branding of honey and implement activities that will provide publications such as films, brochures, and seminars to raise awareness of consumers about the benefits and quality of honey in coordination with the ministry.

As a result, it is important that the Ministry of Food, Agriculture and Livestock carries out policies on providing rich nectar resources to beekeepers, especially training and supervision of beekeepers. Unions and Cooperatives, which are farmers' organizations, are required to create a market for their products with cheap raw materials for their members. Universities and institutes are required to carry out bee breeding studies suitable for their regions along with treatment against bee diseases and pests. Considering the precautions and suggestions for the problems of beekeepers in Sivas province, it has been concluded that honey production per hive can be increased by producing solutions to important problems such as bee losses, marketing, and employment due to conscious beekeeping.

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Microbiological and Cytological Investigation of Clinical Equine Mastitis in Türkiye

Türkiye'de Klinik At Mastitisinin Mikrobioyolojik ve Sitolojik Yönden Araştırılması

Alper METE

İstanbul Veliefendi Racecourse Racehorse Hospital, Jockey Club of Turkey, İstanbul, Türkiye

ABSTRACT

Equine mastitis is an uncommon but may cause some serious clinical conditions including septicemia, arthritis, and pneumonia when transmission of the microbial pathogen to the nursing foal. Mares themselves also may show local and systemic clinical signs associated with mastitis. Little data are available evaluating microbial etiology of clinical equine mastitis associated with cytologic examination in Türkiye. Milk orudder secretion samples, which were admitted to the diagnostic laboratory from a total of 22 clinically mastitic mares, were examined by bacterial-fungal culture and cytological methods between 2016 and 2022. The most common bacterial isolate was found to be *Streptococcus equi* subsp. *zooepidemicus* (54.6%), followed by *Escherichia coli* (27.4%). No fungal pathogen was isolated. Cytologic examinations revealed the presence of strong neutrophilic infiltration (<80%) associated with degenerative changes and the presence of intracellular bacteria. In lactating mares (n = 9), *E. coli* and *S. zooepidemicus* were equally isolated from a total of 6 samples, followed by *S. aureus* (n = 1), *E. cloacae* (n = 1), and *S. maltophilia* (n = 1). On the other hand, in non-lactating mares, *S. zooepidemicus* was the most prevalent agent isolated from 9 samples, followed by *E. coli* from 3 samples and *S. epidermidis* from 1 sample. Further, more comprehensive studies should be conducted regarding subclinical cases and antimicrobial resistance profiles of the agents isolated from equine mastitis cases in horse populations in Türkiye.

Keywords: Bacterial infection, cytology, mare, mastitis

ÖZ

At mastitisi nadir görülen bir durumdur ancak mikrobiyal patojenin emziren taya bulaşması durumunda septisemi, artrit ve pnömoni gibi bazı ciddi klinik durumlara neden olabilir. Kısrakların kendileri de mastit ile ilişkili lokal ve sistemik klinik belirtiler gösterebilir. Türkiye'de klinik at mastitisinin mikrobiyal etiyolojisini sitolojik inceleme eşliğinde değerlendiren çok az veri mevcuttur. 2016-2022 yılları arasında klinik olarak mastitisli toplam 22 adet kısraktan tanı laboratuvarına kabul edilen süt/meme salgı örnekleri bakteriyel-fungal kültür ve sitolojik yöntemlerle incelendi. En yaygın bakteri izolatının *Streptococcus equi* subsp. *zooepidemicus* (%54,6), ardından *Escherichia coli* (%27,4) gelmektedir. Fungal patojen izole edilmedi. Sitolojik incelemeler, dejeneratif değişiklikler ve hücre içi bakteri varlığı ile birlikte güçlü nötrofilik infiltrasyonu varlığını (%80<) ortaya çıkardı. Laktasyondaki kısraklarda (n = 9), *E. coli* ve *S. zooepidemicus* toplam altı örnekten eşit olarak izole edilirken, bunu *S. aureus* (n = 1), *E. cloacae* (n = 1) ve *S. maltophilia* (n = 1). Laktasyonda olmayan kısraklarda ise dokuz örnekte en sık izole edilen etken *S. zooepidemicus* olurken, bunu 3 örnekle *E. coli* ve 1 örnekle *S. epidermidis* izledi. İleride Türkiye'deki at popülasyonlarında subklinik mastitis olguları ve etkenlerin antimikrobiyal direnç profilleri ile ilgili daha kapsamlı çalışmalar yapılmalıdır.

Anahtar Kelimeler: Bakteriyel enfeksiyon, kısrak, mastitis, sitoloji.

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Sorumlu Yazar/Corresponding author: Alper METE
e-mail: alpermete1985@yahoo.com

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INTRODUCTION

Mastitis cases in horses is an uncommon condition unlike in dairy cows because of having been attributed to a short lactation period and the small size of the udder contributes to a more frequent expelling of the udder.[1] Anatomically hidden position of the udders protects them reducing exposure

to trauma and low contact probability to the contaminated ground surface. While mastitis is less prevalent in mares when compared with cows, serious outcomes regarding mastitis can occur in horses as well.[1] In the worst-case scenario, the transmission of the microbial pathogen to the sucking foal can cause septicemia, arthritis, and pneumonia.[1] Agalactia can also lead to subsequent foal malnutrition.[2] Mastitis can also trigger abortion in the mare in case of pregnancy and systemic compromisation, or less commonly, severe infection can cause permanent loss of function in the affected mammary gland due to fibrosis and obstruction.[3] Local swelling or heat in the affected udder, pain, udder asymmetry, firmness, ventral edema with or without concomitant lower limb edema, a congested mammary vein, rejection of the foal, and abnormal purulent and/or serosanguineous secretions are the clinical signs associated with mastitis.[3] Mares can also demonstrate systemic signs such as pyrexia (up to 41°C), anorexia, depression, and hindlimb lameness, but the most common clinical signs are a firm and swollen udder with purulent discharge. Blood analysis of the affected mares often yields unremarkable but may also show neutrophilia and hyperfibrinogenemia. Based on the clinical presentation, mastitis can be encountered as acute, chronic, and clinical or subclinical. Mastitis is mostly caused by bacteria, and less commonly by fungi, nematodes, or non-septic etiologies such as avocado tree poisoning.[4] Over 20 bacterial agents have been associated with mastitis in the equidae family including *Streptococcus* spp. which were the most common isolates reported by different authors.[3,5-7] In California, it was also reported that 42% of the mares suffering from clinical mastitis had Gram-negative bacteria isolated.[6]

In the diagnosis of mastitis, clinical inspection of the udder can be performed to observe typical signs. It is recommended to confirm bacterial isolation by performing a culture associated with the cytology of the milk/udder secretions.[8] The cytologic appearance of mare milk has a proteinaceous background and is either acellular or may contain scarce neutrophils.[9] Horses having mastitis associated with a bacterial agent usually have a high number of neutrophils with a degenerative appearance of the cells.[6]

Equine mastitis may be encountered during early lactation[1]. However, mastitis can be seen in mares at any period of lactation and also in post-lactational regression associated with weaning and is therefore commonly encountered during summer or autumn season.[9] Additionally, mastitis may occur in association with milk build-up relating to illness or loss of a foal and may also be seen in pregnant mares, non-pregnant mares, young fillies, and neonatal foals as well.[9] Different breeds can be affected by mastitis including thoroughbreds, standardbreds, quarter horses, and ponies. Most of the mares are likely to present with unilateral disease, and in some cases, only 1 ductal tree within a mammary can be affected.[9]

One report from Germany predicted that as much as 5% of breeding mares are affected by mastitis.[7] The incidence does not seem to be high in North America.[3,6] However, the true incidence across breeds and countries remains to be determined.[1]

According to the author's knowledge, little data are available evaluating the microbial etiology of equine clinical mastitis in Türkiye. Therefore, this study was designed to evaluate and provide information about the microbial etiology of equine clinical mastitis together with cytological findings.

MATERIALS AND METHODS

Animals

The analysis records of clinical mastitis cases were reviewed for the study. An official consent was taken from general management of Jockey Club of Turkey for publishing the results (Number: 21). A total of 22 mastitic case samples were admitted to Jockey Club of Turkey diagnostic laboratory of İstanbul between 2016 and 2022. Nineteen out of 22 (86.4%) of the mares were English thoroughbreds and 3/22 (13.6%) were Arabian thoroughbreds. The number of non-lactating and lactating mares was 13/22 (59.1%) and 9/22 (40.9%) respectively. The mean age (±SD) of the mastitic mares was 10.6 (±3.6) years old. Initial diagnosis of the cases was based on acute clinical signs associated with mastitis. The clinical signs consisted of 1 or multiple following signs: swollen udder, sensitivity of udder in palpation, and purulent or serosanguineous secretion from the udder. Milk or udder secretions of the mastitic cases were collected by the clinicians after cleaning and disinfection of the udder with standard protocols.[10] Following the discard of the first udder ejections, samples were collected into sterile plastic containers and were sent to the laboratory immediately for microbiological and cytologic analysis with ice packs. Nineteen of the 22 samples were like purulent discharge those had high viscosity with brownish appearance. Three of the samples analyzed were white in color but had a higher viscosity than normal consistency. The analysis of the samples was carried out within 24 hours of sample collection.

Microbiological Analysis

All samples were inoculated into 5% sheep blood agar, Mac Conkey agar for bacterial isolation, and inhibitory mold agar (IMA) for fungal isolation. Bacterial culture plates were incubated at 37°C in both aerobic and microaerophilic conditions for 72 hours. On the other hand, IMA plates for fungal isolation were inoculated and incubated at 25°C in an aerobic atmosphere for 10 days. After incubation, isolated microbial colonies were initially examined based on Gram staining and catalase and oxidase activity. Further identification of the suspected colonies was made by biochemical methods using a commercial bacterial identification system as described by the manufacturer (Diagnostics SK Inc., Galanta, Slovenia).

Cytology

Cytological examinations were carried out by following the standard protocol described previously with some minor changes.[11] Briefly, 50 µL of milk or secretions was radiated on a microscope slide and left to dry at 37°C for 15 minutes. After drying off, prepared smears were stained with May Grunwald–Giemsa quick stain according to the protocol described by the manufacturer (GBL, İstanbul, Türkiye). The stained smears were then examined by visualization of at least 10 different microscopic fields at 100× magnification by using immersion oil. One slide per mare was prepared for the cytologic examination. The cytology results were expressed as the relative number (%) of neutrophils, macrophages, and lymphocytes in the smear. Briefly, it was calculated according to the formula below.

$$\text{Relative number}(\%) = \frac{\text{Number of the individual cell(NEU or MAC or LYM)}}{\text{Number of the total counted cells(NEU} + \text{MAC} + \text{LYM)}} \times 100$$

RESULTS

According to the bacterial isolation results, the most commonly isolated bacterial agent was determined to be *Streptococcus equi* subsp. *zooepidemicus* 54.6% (n=12) followed by *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterobacter cloacae*, and *Stenotrophomonas maltophilia* with the isolation rates of 27.4% (n=6), 4.5% (n=1), 4.5% (n=1), 4.5% (n=1), and 4.5% (n=1), respectively. No fungal agent was isolated from the samples analyzed in the study. In lactating mares (n=9), *E. coli* and *S. zooepidemicus* were equally isolated from a total of 6 samples, followed by *S. aureus* (n=1), *E. cloacae* (n=1), and *S. maltophilia* (n=1). On the other hand, in non-lactating mares *S. zooepidemicus* was the most prevalent agent isolated from 9 samples, followed by *E. coli* from 3 samples and *S. epidermidis* from 1 sample.

Cytological examinations revealed all of the samples had <80% neutrophil with a mean value of 93.5% (± 3,8), which demonstrated degenerative changes and the presence of intracellular bacteria (Figure 1). Degenerative changes were seen as swollen nuclei that partially lose their lobulation (karyolysis) and/or rupture of the nuclear membrane (karyohexis) of the cells probably caused by bacterial endotoxins. On the other hand, mean macrophage and lymphocyte values were found to be 6.0% (±3.3) and 0.6% (±0.9), respectively (Table 1). The distribution of clinical mastitis cases according to the months included in the study is given in Figure 2.

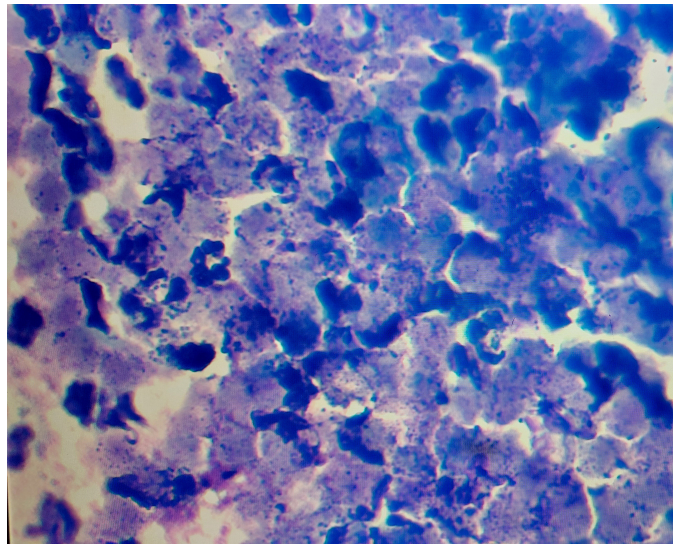

Figure 1. Cytological evoluation of mastitic sample from a mare. Note the NEU cells with degenerative changes and intracellular cocci microorganisms (MGG quick stain, 100x objective).

Table 1. Distribution of Neutrophil (NEU), Macrophage (MAC), and Lymphocyte (LYM) Cells (%) Against Different Bacterial Isolates

Isolate	NEU (Mean ±SD)	MAC (Mean ±SD)	LYM (Mean ±SD)
Gram positive			
Streptococcus. zooepidemicus (n=12)	96.1 (±2.4)	3.9 (±2.1)	0.07 (±0.1)
Staphylococcus aureus (n=1)	92.8	4.6	2.6
Staphylococcus epidermidis (n=1)	84.7	8.2	7.1
Gram negative			
Escherichia coli (n=6)	91.4 (±2.5)	7.8 (±1.5)	1.1 (±1)
Enterobacter cloacae (n=1)	90.2	8.6	1.2
Stenotrophomonas maltophilia (n=1)	88.4	11.4	0.2

DISCUSSION

Mastitis appears to be less prevalent in horses when compared especially with other body site infections. During a 6-year period, only 22 mastitic milk/secretion samples were admitted to our laboratory. On the other hand, 2804 bacterial culture analyses were carried out, which mostly consisted of respiratory system samples during the same time period. The seemingly reduced cases of mastitis in horses can be explained by smaller size and relatively concealed location of the udder, coupled with a smaller storage capacity contributes decreased probability of infection than cows and goats.[12]

A previous study from California demonstrated that 42% of mares were affected by mastitis during the lactation period, another 28% displayed signs within the first 8 weeks of postweaning, and the remaining 30% of the mares were in the non-lactating period.[6] According to the study, in the period of 8 weeks postweaning, drying-off mares were considered to be more likely to suffer from mastitis, which coincides with summer and early fall in the Northern Hemisphere, when insect populations are peaking.[6] Moreover,

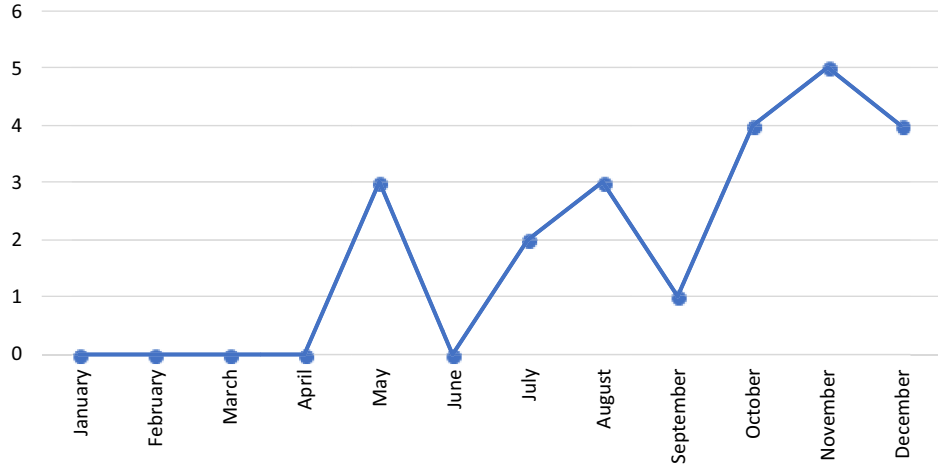

Figure 2. Distribution of clinical mastitis cases according to the months recorded in the study

following the weaning period, udder secretions will accumulate and potentially drip, facilitating the entrance of pathogens to the teat canal.[12] Mares those were presenting mastitis right after parturition usually have a history of dripping milk prefoaling, or may have lost a foal at, or immediately after, parturition.[3] The report from California[6] claimed that 70% of the mares had signs of mastitis from May to September when most mares in reproduction are lactating or have been weaned. In the present study, 40.1% (n = 9) of the mastitis cases were encountered between May and September during lactation period, but 59.1% (n = 13) of the mares were in non-lactating period that coincides October to December. The results of the present study showed to a degree of similarity but mostly revealing that mastitis cases occurred in non-lactating mares. Dry mares may present irregular idiopathic lactation, particularly in autumn, and also improper lactation is encountered in mares with Cushing's disease, possibly due to the secondary increase in blood prolactin level.[13]

In the previous study,[6] the mean age of the affected mares was 13.2 ±6.2 years, ranging from 3 to 24 years old. In the present study, the mean age of the mares was determined to be 10.6 ±3.6 years old that seems ages around 10 years old mares were more likely to suffer from mastitis. But contrary to this, another study demonstrated a broader range of age including a 2-month-old foal and 3 young fillies those were 2-3 years old.[3] The present and previous studies show that mastitis can occur in a broad range of age in mares.

In a previous study, *S. zooepidemicus* was reported to be the most common isolates species (36.8%) in 28 mastitic samples.[6] In the same study, the second most common isolate was determined as *Staphylococcus* spp. and *E. coli* was reported only in 1 case (5.3%), but in the present study, *E. coli* was found to be the second most common agent (27.4%) isolated from clinical mastitis cases. In a different study conducted in Brazil, revealed the most common isolated species were reported as *Streptococcus* spp. (20%) and *S. aureus* (12.73%).[10] *E. cloacae* and *E. coli* were also isolated in 7/55 and 2/55 of the lactating mares, respectively.[10] Böhm et al[7] determined that most bacteria isolated from mastitic samples were also found on the skin of the udder and isolated in the milk of healthy post-partum mares. The present study was in concordance with most of the studies published related to *Streptococcus* spp., especially *S. zooepidemicus* was found to be the most commonly isolated pathogen (54.6%) in the present study. The other agents isolated in the present study were mostly originated from environmental and skin-related bacterial agents. Interestingly, *S. maltophilia* was isolated in 1 mastitic sample in the current study. There were studies reporting *S. maltophilia* isolated from mastitic samples in cows.[14] But no published study or case report could be encountered in mares up to date.

Diagnostic tools widely used in bovine mastitis such as California mastitis test (CMT) have conflicting results when used in mares.[1] CMT is based on somatic cells reacting to a detergent solution a producing a gradable agglutination to the degree of gel formation[15-18]. Waldridge[19] studied CMT and no association was found between CMT results and the presence of clinical or subclinical mastitis and aerobic culture results in mares. Similarly, no association was found between somatic cell count (SCC) and clinical disease.[7,20] In the diagnosis of equine mastitis, it is mostly utilized from clinical signs, culture, and cytology as well.[1] Since the data obtained in previous studies were taken into account, a different diagnostic method like CMT or SCC was not used in the diagnosis of mastitis, other than culture and cytological examination in the current study. Cytology results revealed a strong neutrophil response in clinical mastitic milk samples with bacterial etiology in the present study. Mc Cue and Wilson[6] determined large numbers of neutrophils in the cytologic evaluation of 18 milk samples indicating acute inflammation in 13 (72.2%) and bacteria in 6 (33.3%). Domańska et al[11] also revealed that values of neutrophil, macrophage, and lymphocyte were higher initially than in consecutive examined days after parturition until weaning in non-clinical mastitic mares. After weaning, neutrophil, macrophage, lymphocyte, and bacteriological index in milk increased and did not differ from the mean values in clinical mastitic mares. The results of the present and previous studies indicated that an inflammatory cell response mostly indicating an increase in neutrophil reaching and passing 80% occurs in clinical and non-clinical mare mastitis cases.

In conclusion, to the best of the author's knowledge, the present study is the first that presented a microbiological and cytological evaluation of clinical mastitis cases in mares up to the date in Türkiye. *S. zooepidemicus* was found to be the most common agent followed by *E. coli*. Cytology results yielded strong neutrophilic inflammation and intracellular bacteria with degenerative changes morphologically. More comprehensive studies should be conducted to determine the prevalence of subclinical cases besides clinical cases and also detect antimicrobial resistance profiles of the agents isolated from equine mastitis cases further.

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Research Article *Araştırma Makalesi* DOI: 10.5152/VetSciPract.2023.23107

The Effects of Dietary Supplementation with *Origanum onites* Essential Oil on Growth Performance, Some Blood Parameters, Jejunal Villus Height, and Meat Quality in Broiler Chickens

Etlik Piliçlerde *Origanum onites* Uçucu Yağlı Diyet Takviyesinin Büyüme Performansı, Bazı Kan Parametreleri, Jejunal Villus Yüksekliği ve Et Kalitesi Üzerine Etkileri

Hüseyin Gürkan SARAÇ[1]
Mehmet Akif YÖRÜK[2]

[1]Sample Approval and Reporting Unit, Çorum Food Control Laboratory Directorate, Çorum, Türkiye
[2]Department of Veterinary Medicine, On Dokuz Mayıs University, Samsun, Türkiye

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Sorumlu Yazar/Corresponding Author:
Hüseyin Gürkan SARAÇ
E-mail: gurkansarac1919@gmail.com

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ABSTRACT

The present study investigated the effects of dietary supplementation with *Origanum onites* essential oil on growth performance, some blood parameters, jejunal villus height, and meat quality in broiler chickens. Two hundred chicks were used and allocated to 4 groups, including a control group and 3 treatment groups, which received 3 different levels of dietary *Origanum onites* essential oil (100 ppm, 200 ppm, and 400 ppm). Each study group consisted of 5 subgroups, each including 10 animals. Feed and water were provided *ad libitum*. Light was provided 24 h/day. The ambient temperature was maintained at an optimum level and adjusted on a weekly basis. Dietary *Origanum onites* essential oil was given on a daily basis. The incorporation of different levels of *Origanum onites* essential oil into the diet did not affect body weight, body weight gain, feed intake, feed conversion rate, and carcass yield ($P > .05$). Similarly, dietary supplementation with *Origanum onites* essential oilhad no significant effect on the antioxidant and serum biochemical parameters investigated ($P > .05$). On the other hand, dietary *Origanum onites* essential oilsignificantly affected the spleen weight, jejunal villus height, and meat color (a*) ($P < .05$). No effect of dietary essential oilwas observed on the meat pH value ($P > .05$). Several studies are required to determine the more effective level of *Origanum onites* in broilers.

Keywords: Broiler, meat quality, *Origanum onites*, performance, serum parameters, villus

ÖZ

Bu çalışmada karma yemlere Origanum onites esasnsiyel yağı (OEY) ilavesinin etlik piliçlerde performans, bazı kan parametreleri, jejenum villus uzunlukları ve et kalitesi üzerine etkileri araştırılmıştır. Deneme, kontrol grubu ve 3 farklı seviyede (100, 200 ve 400 ppm) OEY ilavesi yapılan gruplar olmak üzere toplam 4 gruptan oluşturulmuştur. Denemede, her grup kendi içinde ve her birinde 10 hayvan olacak şekilde 5 alt gruba ayrılmıştır. Denemede toplam 200 adet hayvan kullanılmıştır. Su ve yem ad libitum olarak verilmiştir. Aydınlatma 24 sa/gün olarak ayarlanmıştır. Ortam sıcaklığı haftalık olarak optimum değerlerde tutulmuştur. OEY katkısı günlük olarak uygulanmıştır. Etlik piliç rasyonlarına farklı düzeylerde OEY katkısı; canlı ağırlık, canlı ağırlık artışı, yem tüketimi, yemden yararlanma oranı, karkas randımanı değerlerini etkilememiştir (P>0.05). Aynı şekilde serum biyokimyasal ve antioksidan değerler üzerine de OEY katkısının önemli bir etkisi olmamıştır (P>0.05). OEY ilavesi dalak ağırlıkları, jejenum villus uzunlukları ve et rengi (a*) parametrelerini önemli ölçüde etkilemiştir (P<0.05). Et pH değerleri üzerine OEY'nın herhangi bir etkisi olmamıştır (P>0.05). Etlik piliçlerin besi performansını arttırmak için rasyonlara yem katkı maddesi olarak Origanum onites' in kullanılabileceği, ancak kullanım düzeyini belirlemek için konu ile ilgili daha fazla araştırma yapılması gerektiği sonucuna varılmıştır.

Anahtar Kelimeler: Broyler, et kalitesi, Origanum onites, performans, serum parametreleri, villus

INTRODUCTION

Since the ban placed by the European Union (EU) in 2006 on the use of antibiotic growth factors (AGFs) confirmed to pose a risk to human and animal health on the basis of scientific data, researchers continuously seek new feed additives that can replace AGFs.[1] Several recent studies have focused on the use of essential oils (EOs) as substitutes of AGFs in animal nutrition and have investigated the effects of these EOs. It has been demonstrated that EOs have several positive effects on the health and performance of animals, including increased levels of endogenous enzymes.[2] Some feed additives can serve as alternatives of antibiotic substitutes in poultry nutrition, including enzymes, prebiotics, probiotics, manno-oligosaccharides, symbiotics, and phytobiotics.[3] Phytogenic products, including aromatic plants and essential oils, have been reported to show biological activity, when used in animal nutrition, and are considered to be natural products that offer potential use as antibiotic substitutes.[4] EOs are described as natural and non-residual alternative feed additives, which are derived from aromatic plants in various ways, improve the flavor and palatability of feed, and show digestive stimulant and performance-enhancing effects. Depending on the aromatic plants from which they are derived, EOs contain different types and levels of phenolic compounds and, thereby, show a wide range of activities (antimicrobial, antioxidant, anti-inflammatory, antifungal, etc.).[4,5]

Origanum species, classified under the family Labiatae, are among the several alternative feed additives used as performance enhancers for poultry. The main constituents of Origanum are carvacrol and thymol, but the presence of thymoquinone, *p*-cymene, and γ-terpinene have also been reported in the composition of Origanum from different geographical regions.[6] The more widely known Origanum species in Türkiye are *Origanum minutiflorum, Origanum vulgare*, *Origanum syriacum*, *Origanum majorana L.*, and *Origanum onites L.* Among these species, *Origanum onites L.* referred to as "Turkish thyme" or "Izmir thyme," is a well-known herb with common use in medicine and several other areas. This herb is also used for digestive disorders and upper respiratory infections.[7]

Feizi et al (2013) reported that the incorporation of *Origanum vulgaris* essential oil (200/1000cc) into the feed and drinking water of broiler chickens increased body weight (BW), decreased feed intake (FI), and improved the feed conversion rate (FCR).[8] In another study, oregano EOs were determined to show an inhibitory effect against several bacteria, including *Campylobacter jejuni*, *Salmonella enteritidis*, *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*. It has been reported that the purified constituents of *Origanum onites* essential oil (OEO) inhibit HMG-CoA reductase, an important enzyme that regulates cholesterol synthesis, and thereby show a hypocholesterolemic effect.[9]

This study was carried out in order to report the effects of dietary supplementation with essential oil obtained from Izmir thyme (*Origanum onites*) on growth performance, carcass traits, organ characteristics, serum biochemical and antioxidant status, meat quality, and jejunal villi height.

MATERIALS AND METHODS

Animals and Experimental Design

The study was carried out at the premises of the Poultry Unit of the Livestock Research and Application Centre of Atatürk University, Faculty of Veterinary Medicine, using floor cages (121 × 110 × 108 cm). Two-hundred-day-old male Ross-308 chicks were used in the study. After 1 week, the animals were randomly divided to 4 groups, each of 50 chicks, and each group was further divided into 5 replicates, each of 10 animals. The chicks were randomly distributed into 20 compartments, such that the average BWs of the animals in each compartment were equal. The chicks were raised for a period of 42 days, including a 7-day acclimatization period and a 35-day trial period. Throughout the study, water and feed were provided *ad libitum*. The nutrient composition and Near Infrared Spectroscopy (NIRS) analysis results of the feed rations provided to the animals during the different phases of the study are presented in Table 1. Four study groups, including a control group and 3 treatment groups, were formulated. While the control group did not receive any feed supplement, the treatment groups were dietary supplemented with 100 ppm, 200 ppm, and 400 ppm of OEO, respectively. Each day at 17:00 h, the remainder of the feed provided to the animals on the previous day was collected and weighed. Subsequently, the feed was replenished and OEO was incorporated into the feed offered to the treatment groups.

Origanum onites essential oil was stored at +4°C in dark-colored bottles and was incorporated into feed manually, on a daily basis, starting from the lowest supplementation dose. The chemical composition of the essential oil was determined by the supplier (Mahan Cosmetics, Hatay/Türkiye) with an automatic gas chromatograph system equipped with a mass spectrometer and a flame ionization detector (GC-MS/FID) (Table 2). The pen, in which the animals were housed, was illuminated with tungsten bulbs, and light was provided 24 h/day. The temperature of the pen was maintained at 33°C for the first 2 days and was progressively decreased to 24°C by the end of the study. Wood shavings were used as the cage bedding material.

Determination of Performance Parameters

Chicks from each experimental group were weighed on a weekly basis, on the same days and at the same hour, and their BWs were recorded. The difference between 2 consecutive weightings was

Table 1. Nutritional Composition of Feeds and NIRS Analysis Results

Raw Materials	Starter (%) 1–-14 Days	Grower (%) 15-28 Days	Finisher (%) 29-42 Days
Maize	55.48	70.25	62.62
Soybean meal (44%)	22.55	5.05	10.60
Corn gluten (60%)	16.20	20.12	20.55
Limestone	2.35	1.15	2.45
DCP	1.90	1.80	2.10
Salt	0.24	0.23	0.25
Vitamin (K3-A)	0.14	0.15	0.14
Soda	0.10	0.09	0.09
Vitamin E	0.64	0.64	0.65
Lysine	0.35	0.52	0.50
Methionine	0.05	–	0.05
Analysis			
ME (kcal/kg)	3020	3145	3215
Dry matter (%)	89.00	88.00	88.00
Crude protein (%)	24	21.15	20.3
Crude fat (%)	3.45	6.40	3.15
Ash (%)	3.25	2,22	5.12
Methionine (%)	0.78	0.65	0.48
Lysine (%)	1.24	1.13	1.07

DCP, dicalcium phosphate; ME, metabolizable energy.

Table 2. Chemical Composition of *Origanum onites*

No	Component	Quantity (%)	No	Component	Quantity (%)
1	α-pinene	0.56	9	Terpinene-4-ol	0.66
2	α-tujene	0.47	10	Trans-caryophyllene	2.56
3	Myrcene	1.38	11	Borneol	1.08
4	α-terpinene	1.45	12	β-bisabolene	0.53
5	γ-terpinene	7.19	13	Caryophyllene oxide	0.43
6	Cymene	6.12	14	Thymol	5.54
7	1-octen-3-ol	0.43	15	Carvacrol	70.13
8	Linalol	1.47			

recorded as the body weight gain (BWG). Feed was provided to the animals at 17:00 hours each day, in an amount 20% greater than that they could consume. The daily FI of the animals was determined by weighing the leftover feed on the next day and subtracting this value from the amount of feed initially provided. The average daily FI per animal was calculated by dividing the daily FI by the number of animals included in the group. The FCR was calculated by dividing the average FI of the animals in-between 2 consecutive weightings by the average BWG of the animals in the same group in-between the same 2 consecutive weightings.

Determination of Slaughter and Carcass Characteristics

On the last day of the study (day 42), the slaughter weights of the animals were recorded. In total 40 animals, 10 per group (2 animals per subgroup), were randomly selected for slaughter. At slaughter, the chickens were decapitated, plucked, and eviscerated, and their legs were also cut. The carcasses were first weighed to record the hot carcass weight and were weighed for a second time after being kept at +4°C for 24 hours to record the cold carcass weight. The carcass yield was calculated by dividing the carcass weight by the preslaughter weight and multiplying the quotient by 100. The extracted visceral organs were also weighed and their weights were recorded. The weight percentages of the visceral organs were determined by dividing their weights by the BW of the animal and multiplying the quotient by 100.

Determination of Serum Biochemical Parameters

Blood samples were collected from the slaughtered chickens into individually numbered tubes and were centrifuged at 3000 rpm for 10 minutes. The extracted sera were transferred into Eppendorf tubes and stored at −20°C until being analyzed. The biochemical parameters of the samples were determined spectrophotometrically using commercial test kits (Roche) and a Cobas-8000 autoanalyzer.

Determination of Serum Antioxidant Parameters

Serum malondialdehyde (MDA) levels were determined as described by Placer et al (1966) and serum glutathione (GSH) levels were determined as described by Sedlak and Lindsay (1968).[10,11]

Determination of Jejunal Villi Height

At the necropsy of the slaughtered chickens, tissue samples were collected from the jejunum and were subjected to histopathological examination, during which the height of the jejunal villi was determined.[12,13]

Determination of Breast Meat pH and Color Parameters

The pH value of the breast meat samples was determined by placing 10 g of breast meat in 100 mL distilled water and homogenizing the mixture on a homogenizer. The pH value of the homogenates was measured with a pH-meter (SCHOTT L 6880, Lab Star). The color intensities (L*, a*, and b*) of the breast meat samples were analyzed with a colorimeter device (Minolta CR-400).[14]

Statistical Analysis

The data obtained in the present study were analyzed with 1-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) 18.0 software (SPSS Inc.; Chicago, IL, USA). Duncan's multiple comparison test was used for the comparison of the differences detected between the study groups. Statistical significance was set at $P < .05$.[15]

RESULTS

No statistically significant difference was detected for the performance parameters, daily FI, and FCR ($P > .05$). Dietary OEO supplementation caused only numerical alterations in the performance parameters (Table 3). The liveability percentages of the control group and the treatment groups, which received 100 ppm, 200 ppm, and 400 ppm of dietary OEO, were 88%, 90%, 88%, and 96%, respectively.

As shown in Table 4, no difference was determined for the slaughter and carcass characteristics investigated ($P > .05$). Except for the spleen percentage, no statistically significant difference was found for the visceral organ percentages.

No significant differences were detected for the serum total protein, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), glucose, and triglyceride levels among the experimental groups ($P > .05$) (Table 5).

The serum MDA and GSH levels, which are oxidative stress parameters directly related to the life span of cells, and serum antioxidant parameters are shown in Table 6. No statistically significant differences were shown among the study groups for the serum MDA and GSH levels, thus, dietary OEO supplementation was determined to have not affected these parameters ($P > .05$).

Histological analysis demonstrated significant differences for the height of the jejunal villi ($P < .05$) (Table 7). The height of the jejunal villi had increased in the groups that received 200 ppm and

Table 3. Performance Parameters of Broilers as Affected by the Different Levels of *Origanum onites* Essential Oil Ddietary Supplementation

	AFC (g/day)	ADLWG (g/day)	FCR (g/g)	7-42 TLWG (g)	TFI (g)
Groups	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Control	120.79 ± 0.97	61.08 ± 0.86	1.98 ± 0.31	2137.78 ± 30.01	4227.58 ± 33.81
OEO 100	118.97 ± 1.32	61.92 ± 0.98	1.92 ± 0.30	2167.07 ± 34.52	4163.85 ± 46.20
OEO 200	121.45 ± 0.79	62.79 ± 1.38	1.93 ± 0.33	2197.72 ± 48.52	4250.66 ± 27.54
OEO 400	119.22 ± 0.60	64.01 ± 0.33	1.86 ± 0.20	2240.35 ± 11.36	4172.63 ± 21.11
P	0.114	0.070	0.094	0.070	0.114

The difference between the means is significant at the $P < .05$ level. OEO 100, 100 ppm *Origanum onites;* OEO 200, 200 ppm *Origanum onites*; OEO 400, 400 ppm *Origanum onites*. ADLWG, average daily live weight gain; AFC, average feed consumption; FCR, feed conversion rate; TFI, total feed intake; TLWG, total live weight gain.

Table 4 Slaughter and Carcass Parameters of Broilers as Affected by the Different Levels of *Origanum onites* Essential Oil Dietary Supplementation

	Slaughter Weight (g)	Hot Carcass Ratio (%)	Cold Carcass Ratio (%)	Heart (%)	Gizzard (%)	Liver (%)	Spleen (%)
Groups	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Control	2283.00 ± 33.63	72.50 ± 0.52	71.26 ± 0.55	0.53 ± 0.20	0.97 ± 0.06	2.03 ± 0.04	0.13 ± 0.01[b]
OEO 100	2314.00 ± 36.83	71.77 ± 0.21	70.70 ± 0.28	0.53 ± 0.20	1.00 ± 0.06	1.97 ± 0.06	0.15 ± 0.01[a]
OEO 200	2067.00 ± 39.28	72.47 ± 0.32	71.21 ± 0.31	0.53 ± 0.20	1.07 ± 0.08	1.98 ± 0.10	0.16 ± 0.01[a]
OEO 400	2102.00 ± 24.42	71.96 ± 0.29	70.77 ± 0.24	0.56 ± 0.10	0.95 ± 0.02	1.92 ± 0.05	0.12 ± 0.01[b]
P	.544	.197	.330	.188	.182	.284	.050

The difference between the means is significant at the $P < .05$ level. OEO 100, 100 ppm *Origanum onites;* OEO 200, 200 ppm *Origanum onites*; OEO 400, 400 ppm *Origanum onites*.
a,b: Superscripts in a row showed significant differences.

Table 5. Serum Biochemical Parameters (mg/dL) of Broilers as Affected by the Different Levels of *Origanum onites* Essential Oil Dietary Supplementation

	Total Protein	Cholesterol	HDL	Glucose	LDL	TG
Groups	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Control	2.58 ± 0.16	99.10 ± 5.85	67.58 ± 3.68	194.20 ± 11.70	20.00 ± 3.07	57.60 ± 3.94
OEO 100	2.51 ± 0.81	86.60 ± 4.61	62.00 ± 2.90	190.40 ± 8.73	13.42 ± 1.52	55.90 ± 5.82
OEO 200	2.39 ± 0.09	88.50 ± 5.42	62.80 ± 3.56	210.80 ± 13.86	14.07 ± 2.26	58.20 ± 4.79
OEO 400	2.49 ± 0.16	100.30 ± 4.91	69.72 ± 0.11	202.80 ± 13.22	17.72 ± 1.50	64.30 ± 5.37
P	.071	.097	.144	.283	.057	.290

OEO 100, 100 ppm *Origanum onites*; OEO 200, 200 ppm *Origanum onites;* OEO 400, 400 ppm *Origanum onites*. HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides.

400 ppm of dietary OEO, compared to the control group and the group supplemented with 100 ppm of dietary OEO. Jejunal villi height was greatest in the group supplemented with 400 ppm of dietary OEO (994.92 µm).

The results of the color and pH analyses of the breast meat samples are presented in Table 8. As indicated in Table 8, dietary supplementation with OEO did not cause any difference for the L*, b*, and pH values, excluding a* ($P > .05$). The assessment of the color parameters revealed that the a* value had increased in the group that received 400 ppm of dietary OEO, in comparison to the other study groups ($P < .05$).

DISCUSSION

In the present study, the performance values of the animals, based on weekly weightings, did not differ between the study groups. The performance results found in this study are in agreement with those reported in previous research on the effects of various essential oils and organic acids on the performance of broiler chickens (i.e., BW and BWG).[16,17]

In their study on the effects of dietary supplementation with thymol (0.2 g/kg, 0.4 g/kg, 0.8 g/kg) and thymol essential oil (2 mL/kg and 4 mL/kg) on the performance of broiler chickens, Hoffman–Pennesi and Wu (2010) found no effect of dietary supplementation with thymol essential oil on BW.[18] On the other hand, Modeva and Profirov (2003) reported that the incorporation of a commercial plant extract, containing 5% of oregano essential oil, at rates of 0.025% and 0.050% into feed resulted in increased BWG.[19] Similarly, Windisch et al (2008) suggested that phytogenic components could improve nutrient absorption and BWG by increasing the activity of digestive enzymes.[20]

In their investigation on the effects of thyme oil and garlic oil, when administered alone and together, on the performance of broiler chickens, Kırkıpınar et al (2011) determined that, in comparison to the control group, dietary oregano essential oil altered neither FI nor the FCR.[21] In another study on the effects of Origanum essential oil on the performance and immunity of broiler chickens, Mansoub (2011) reported that Origanum essential oil increased FI and improved the FCR, in comparison to the control group.[22]

The results achieved with essential oils are attributed to their appetizing effect, and it is considered that even if they do not increase FI, they may show a positive effect by increasing BWG.[23] It is indicated that Origanum plants increase the FCR by regulating the intestinal microflora and increasing the activation of endogenous digestive enzymes.

Comparison of the carcass characteristics of the animals slaughtered on the 42nd day of the study demonstrated that there was no difference among the 4 experimental groups. The results obtained in the present study for the carcass parameters investigated are similar to those obtained in previous research on the effect of essential oils on performance values.[24,25] In their study on the incorporation of an essential oil blend (thyme, clove, aniseed),

Table 6. Serum Antioxidant Parameters (nmol/mL) of Broilers as Affected by the Different Levels of *Origanum onites* Essential Oil Dietary Supplementation

	MDA	GSH
Groups	Mean ± SEM	Mean ± SEM
Control	2.13 ± 0.19	0.14 ± 0.003
OEO 100	2.39 ± 0.31	0.16 ± 0.003
OEO 200	2.73 ± 0.31	0.16 ± 0.009
OEO 400	2.59 ± 0.24	0.15 ± 0.003
P	.155	.055

OEO 100, 100 ppm *Origanum onites*; OEO 200, 200 ppm *Origanum onites;* OEO 400, 400 ppm *Origanum onites*. GSH, glutathione; MDA, malondialdehyde.

Table 7. Jejunal Villus Heights (µm) of Broilers as Affected by the Different Levels of *Origanum onites* Essential Oil Dietary Supplementation

Groups	Mean ± SEM
Control	858.97 ± 5.27[c]
OEO 100	836.86 ± 14.38[c]
OEO 200	954.73 ± 8.66[b]
OEO 400	994.92 ± 8.78[a]
P	.000

The difference between the means is significant at the $P < .05$ level. OEO 100, 100 ppm *Origanum onites*; OEO 200, 200 ppm *Origanum onites*; OEO 400, 400 ppm *Origanum onites*. SEM, standard error of the mean.
a,b: Superscripts in a row showed significant differences.

Table 8. Breast Meat pH and Color Intensities of Broilers as Affected by the Different Levels of *Origanum onites* Essential Oil Dietary Supplementation

	pH	L*	a*	b*
Groups	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Control	5.89 ± 0.26	52.15 ± 0.36	4.16 ± 0.31[b]	15.66 ± 0.35
OEO 100	5.91 ± 0.20	52.60 ± 0.41	3.88 ± 0.20[b]	15.28 ± 0.34
OEO 200	5.92 ± 0.28	52.48 ± 0.45	4.12 ± 0.19[b]	14.73 ± 0.43
OEO 400	5.88 ± 0.24	52.69 ± 0.44	5.01 ± 0.30[a]	15.69 ± 0.41
P	.283	.408	.048	.111

The difference between the means is significant at the $P < .05$ level. OEO 100, 100 ppm *Origanum onites;* OEO 200 200 ppm *Origanum onites;* OEO 400, 400 ppm *Origanum onites*.
L*, brightness, a*, redness, b*, yellowness. a,b: Superscripts in a row showed significant differences.

at levels of 100 ppm, 200 ppm, and 400 ppm, into the mixed feed of broiler chickens, Şimşek et al (2005) reported to have observed no difference among the carcass parameters with dietary supplementation, when compared to the control group.[26]

In contrast to the present study, several reports have pointed out that dietary EO supplements can improve carcass parameters.[27,28]

In a study on the effects of dietary supplementation with *Thymus vulgaris* powder on the growth and carcass yield values of broiler chickens, El-Ghousein and Al-Beitawi (2009) reported that, when compared to the control group, all treatment groups that received dietary Origanum (at levels of 0.5%, 1%, 1.5%, and 2%) displayed improved carcass yields with the highest yield obtained in the group provided with 2% of *Thymus vulgaris* powder.[29]

In their research on the effects of dietary supplementation with essential oils in broiler chickens, Küçükyılmaz et al (2012) reported hepatic values similar to and splenic values different from those determined in the present study.[30] Liver and gizzard values reported by Çabuk et al (2006) are similar to our values.[31] Furthermore, while the liver, gizzard and heart weights reported by Eleroğlu et al (2016) are similar to those determined in the present study, the spleen weights reported by these researchers differ from those measured in this study.[32] The increase detected in the weight of the spleen in the groups that received 100 ppm and 200 ppm of OEO, when compared to the other study groups, was attributed to the immune system having developed faster, and in return having increased the weight of the spleen in these 2 groups.

Dietary supplementation with OEO having not caused any adverse effect on the serum biochemical parameters investigated in the present study suggests that OEO can be safely used as a feed supplement. In their research on the effects of rosemary, thyme and fennel essential oils on broiler chickens, Belenli et al (2015) determined that thyme essential oil did not alter triglyceride and glucose levels, while it decreased cholesterol and total protein levels.[33]

In the present study, the numerical decrease observed in the cholesterol levels of the groups that received 100 ppm and 200 ppm of dietary OEO can be explained by thymol and carvacrol, both of which are the main constituents of oregano, inhibiting the cholesterol synthesis enzyme HMG-CoA reductase, and thereby, although to a limited level, showing a cholesterol-reducing activity.[34] Gümüş et al (2017) reported that the incorporation of thyme essential oil into quail mixed feed did not affect total protein, glucose, triglyceride, and HDL levels, but decreased cholesterol and LDL levels.[35] In their study on the effects of different levels of dietary pennyroyal essential oil (0.5%, 1%, 1.5%, and 2%) on performance and blood biochemical parameters in broiler chickens,

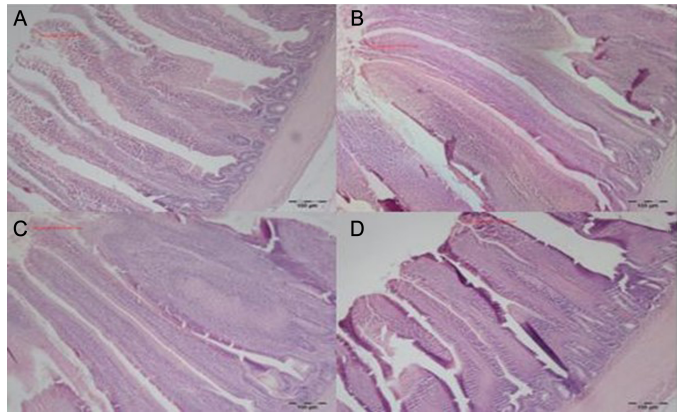

Figure 1. Jejunum hematoxylin and eosin, bar: 100 µm (Control: A; OEO 100: B; OEO 200: C; OEO 400: D).

Nobakht et al (2011) determined no effect on cholesterol, triglyceride and total protein levels, but a decrease in glucose levels with the use of 0.5% and 2% of pennyroyal and an increase in glucose levels with the use of 1% and 1.5% of pennyroyal.[36]

In the present study, according to the results obtained for the antioxidant parameters investigated, dietary supplementation with OEO caused only numerical changes in the GSH and MDA levels. On the other hand, Abdel-Ghaney et al (2017) reported that *Thymus vulgaris* leaves decreased MDA levels and increased GSH levels in broiler chickens.[37] Furthermore, Ölmez et al (2020) determined that dietary supplementation with resveratrol decreased serum MDA levels, but did not affect GSH levels.[5]

The comparative assessment of the jejunal villi heights measured in the present study revealed statistically significant differences among the study groups. It was observed that, when compared to the control group, the height of the jejunal villi had significantly increased values in the groups that received 200 ppm and 400 ppm of dietary OEO ($P < .05$). The rate of increase detected in the height of the jejunal villi in the groups, which received 200 ppm and 400 ppm of OEO, was 11.15% and 15.83%, respectively. It has been reported that, owing to the functional hydroxyl groups found in their composition and the high redox potential of these groups, thymol and carvacrol disrupt the cell wall of pathogenic microorganisms, and thereby, inhibit them with an eventual positive effect on the morphology of the small intestines and significant improvement in the height of the intestinal villi.[34]

The results obtained in the present study for intestinal villus height are in agreement with those reported to have been achieved by Hong et al (2012) with the use of 125 ppm of an essential oil blend (thyme, aniseed, and citrus peel)[38] and by Garcia et al (2007) with the use of 5000 ppm of a plant extract (origanum, rosemary, and sage).[39] On the other hand, Silva et al (2009) determined that dietary supplementation with 0.5 g/kg and 1 g/kg of origanum essential oil did not affect intestinal villus height in broiler chickens.[40]

The myoglobin concentration and hemoglobin level of muscles both affect the color of meat. While the color of meat varies with the amount of these pigments it contains, changes in the pH level of muscles also cause meat color differences. It is reported that post-slaughter meat pH levels are higher in animals exposed to stress reported that the dietary supplementation of broiler chickens with oregano powder (150 mg/kg) did not cause any

statistically significant difference in meat quality parameters.[14,37] The results of the present study are not in accordance with those reported by Aksu et al (2006), suggesting that the incorporation of probiotics into mixed feed decreased the redness (a*) value and increased the yellowness (b*) value of meat.[41] Pirmohamammadi et al (2016) determined that the combined incorporation of thyme (0.5%) and mint (0.5%) into the diet of broiler chickens increased meat pH level.[42]

Higher pH levels increase the color intensity and water holding capacity of poultry meat.[43] Poultry meat is pale, leaky, and soft when the pH level is ≤ 5.8, standard at a pH range of 5.9-6.2 and dark colored, hard and dry when the pH level is ≥6.3. It is also known that the pH level of meat directly affects its shelf life. High pH levels pose the risk of microbial growth in meat, and thus, shorten its shelf life.[44]

Differences between the results of this study and previous studies for meat color intensity (L*: lightness, a*: redness, b*: yellowness) and pH levels are attributed to differences in the type of feedstuff and feed supplements used, the season during which the studies were conducted, the length of the study period, the housing methods applied, and the environmental conditions that prevailed.

In conclusion, no significant improvement was observed in the performance parameters of the broiler chickens that received OEO in their diet. No significant effect having been detected on the performance parameters investigated could be related to either the amount of OEO incorporated into the diet or the absence of stress factors, given that antioxidant feed supplements show a more distinct effect in the presence of stress-inducing conditions.

Based on the results found in this study and in light of those reported in previous research, future studies are required to determine the more effective level of *Origanum onites* toward use as a feed additive in broilers.

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Hypoglycemic and Hypolipidemic Activities of Aqueous Root Extract of *Senna alata* in Alloxan-Induced Diabetic Wistar Rats

Senna alata'nın Aköz Kök Ekstraktının Alloksan ile İndüklenmiş Diyabetli Wistar Sıçanlarında Hipoglisemik ve Hipolipidemik Aktiviteleri

Samuel C. ATTAMA[1,2]
Ngozi OKWELUM[4]
Paul F. EGUNLETI[3]
Solomon C. DAVID[1]
Timothy U. OBETTA[1]
Michael J. AGUIYI[5]

[1]Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka, Nigeria
[2]Department of Veterinary Pharmacology and Toxicology, Federal University of Agriculture, Abeokuta, Nigeria
[3]Department of Veterinary Physiology and Biochemistry, Federal University of Agriculture, Abeokuta, Nigeria
[4]Institute of Food Security, Environmental Resources and Agricultural Research, Federal University of Agriculture, Abeokuta, Nigeria
[5]Department of Veterinary Surgery and Radiology, Universisty of Nigeria, Nsukka, Enugu State, Nigeria

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Sorumlu Yazar/Corresponding Author: Samuel CHIJIOKE ATTAMA
E-mail: attamasc@funaab.edu.ng

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ABSTRACT

In this study, rats with alloxan-induced diabetes were used to examine the hypoglycemic and hypolipidemic effects of *Senna alata* aqueous root extract. Twenty male albino Wistar rats were selected for this study. They were randomly assigned into 4 groups (1-4) of 5 rats in each group. Alloxan monohydrate was administered intraperitoneally to groups 2-4 rats at 160 mg/kg. Forty-eight hours after administration and upon confirmation of diabetes mellitus (fasting blood glucose ≥ 126 mg/dL), group 3 rats were treated with *Senna alata* extract (400 mg/kg) while group 4 rats were treated with glibenclamide (2 mg/kg). Groups 1 and 2 rats received distilled water and were apportioned as normal and negative control groups, respectively. For 21 consecutive days, the treatments were given orally, once daily. Rats in all groups had their fasting blood glucose levels checked after 1 hour, 6 hours, 24 hours, 7 days, 14 days, and 21 days. On the 21 days post-treatment, serum samples were collected from all groups for lipid panel and kidney function examination while pancreases were freshly harvested for histomorphology. In comparison to the diabetic rats in the untreated (negative control) group, the *Senna alata* extract-treated rats had significantly reduced levels of fasting blood glucose, cholesterol, triglycerides, and low-density lipoprotein but higher levels of high-density lipoprotein. Histomorphology of the pancreas of rats treated with *Senna alata* extract revealed more populations of beta-cells compared to that of the diabetic untreated group. This study has demonstrated that aqueous root extract of *Senna alata* has hypoglycemic and hypolipidemic activities and restored pancreatic tissue from injury caused by the alloxan challenge in diabetic rats.

Keywords: Alloxan monohydrate, diabetes mellitus, hypoglycemic, hypolipidemic, *Senna alata*

ÖZ

Bu çalışmada, alloksan ile diyabet oluşturulan sıçanlar kullanılarak *Senna alata*'nın sulu kök özütünün hipoglisemik ve hipolipidemik etkileri incelenmiştir. Bu çalışma için yirmi erkek albino Wistar sıçan seçilmiştir. Sıçanlar rastgele olarak 4 gruba (1-4) ayrılmıştır, her bir grup içinde 5 sıçan bulunmaktadır. Alloksan monohidrat, 2-4 grup sıçanlarına 160 mg/kg dozda intraperitoneal olarak uygulanmıştır. Uygulamanın 48 saat sonrasında ve diabetes mellitus'un teyidi üzerine (açlık kan şekeri ≥ 126 mg/dl), 3. gruptaki sıçanlara Senna alata özütü (400 mg/kg) ile 4. gruptaki sıçanlara glibenklamid (2 mg/kg) tedavisi uygulanmıştır. 1. ve 2. grup sıçanlar ise sırasıyla normal kontrol ve negatif kontrol grupları olarak belirlenmiş olup, distile su verilmiştir. Ardışık 21 gün boyunca tedaviler günde bir kez oral yolla uygulanmıştır. Tüm gruplardaki sıçanların açlık kan şekeri düzeyleri 1 saat, 6 saat, 24 saat, 7 gün, 14 gün ve 21 gün sonra kontrol edilmiştir. Tedavinin 21. gününde, tüm gruplardan serum örnekleri lipid paneli ve böbrek fonksiyonu incelemesi için toplanmış, pankreaslar ise taze olarak histomorfoloji için alınmıştır. *Senna alata* özütü ile tedavi edilen sıçanlar, tedavi edilmemiş (negatif kontrol) grup içindeki diyabetik sıçanlara kıyasla açlık kan şekeri, kolesterol, trigliserit ve düşük yoğunluklu lipoprotein düzeylerinde belirgin bir şekilde azalma göstermiş, aynı zamanda yüksek yoğunluklu lipoprotein düzeylerinde artış gözlenmiştir. Senna alata özütü ile tedavi edilen sıçanların pankreas histomorfolojisi, tedavi edilmemiş diyabetik gruba kıyasla daha fazla beta hücresi popülasyonunu göstermiştir. Bu çalışma, *Senna alata*'nın sulu kök özütünün hipoglisemik ve hipolipidemik etkilere sahip olduğunu ve diyabetik sıçanlarda alloksanın neden olduğu pankreas dokusundaki hasarı onardığını göstermiştir.

Anahtar Kelimeler: Alloksan monohidrat, diabetes mellitus, hipoglisemik, hipolipidemik, *Senna alata*

INTRODUCTION

Diabetes mellitus (DM) is an endocrine and metabolic disorder which manifests as impaired carbohydrate, fat, and protein metabolism.[1] These disorders could be due to a lack of insulin secretion or reduced sensitivity of tissues to insulin.[2] Etiologically, DM is divided into insulin-dependent DM or juvenile-onset DM and non-insulin-dependent DM or adult-onset DM.[3]

Alloxan monohydrate and streptozotocin are the commonest chemicals used in the induction of experimental DM, and both drugs exert their diabetogenic actions when they are administered parenterally.[4] These agents are known to generate free radicals that selectively destroy the insulin-producing pancreatic islets, which are responsible for insulin production.[5] Hyperglycemia usually manifests due to lack of insulin production since insulin is saddled with metabolizing glucose and maintenance of its optimal serum level.[6]

Hyperlipidemia is one of the common long-term complications associated with DM. It's known to occasion various lipid abnormalities including high levels of tryglicerides, low-density lipoproteins (LDL), total cholesterols, and reduced levels of high-density lipoproteins (HDL). These abnormalities predispose to atherosclerosis and cardiovascular diseases. When blood sugar levels are properly maintained, these problems are less frequent and less severe.[7] Pharmacological glycemic control involves using insulin and oral hypoglycemic drugs. There is poor compliance with the use of these agents, especially in developing countries due to their high cost, unavailability, and associated health risks.[8] This has therefore necessitated the search for alternative natural therapeutic agents. Plant sources of drugs are known to possess various advantages over orthodox drugs with regards to their readily availability, low cost, and side effects.[9] The use of herbal medicines in the treatment of ailments is a common practice, especially in developing countries.[10] World Health Organization over the years has advocated the need to explore antidiabetic drugs from natural sources. Therefore, the screening of medicinal plants in each other to identify new and potent hypoglycemic agents is increasing by day among scientific researchers.

Senna alata (SA) is a perennial plant of the Leguminosae family. It is widely dispersed in Africa including Nigeria. It has various common names which include ringworm bush, candle bush, and craw-craw plant, among others. It has been thoroughly screened for biological activities by scientific researchers and has been reported to possess various chemical constituents such as terpenoids, flavonoids, phenols, anthraquinones, and steroids.[11,12] These phytochemicals are known to possess biological activities.[13] Various organs of SA have been investigated for antidiabetic activity.[14,15] However, little is known regarding the hypoglycemic and hypolipidemic properties of SA's aqueous root extract. So, the aim of this study is to determine whether SA's aqueous root extract has any potential hypoglycemic and hypolipidemic effects on alloxan-induced diabetic rats.

MATERIALS AND METHODS

Reagents and Chemicals

Chemicals and reagents used in this study were procured as follows: alloxan monohydrate (Sigma Aldrich, Gallingham, Dorset, UK); glibenclamide (Hovid, Shek Tong Tsui, Hong Kong); creatinine assay kit (Sigma Aldrich); urea assay kit (Sigma Aldrich); total cholesterol assay kit (Cell Biolabs Inc., San Diego, California, USA); triacylglycerides assay kit (Sigma Aldrich); HDL cholesterol assay kit (Elabscience, Houston, Texas, USA). All chemicals used in this study are of optimum analytical grade.

Plant Collection and Preparation of Extract

Senna alata roots were freshly collected at the peak of raining season (July 2022) from the Botanical Garden belonging to Plant Science and Biotechnology Department, University of Nigeria, Nsukka, and were authenticated in the same Department by a botanist. The voucher specimen (*Senna alata*: INTERCEED/2852) was kept at the herbarium. The roots of the plant were washed, sliced into smaller sizes with a clean sharp knife, and dried in a shade room for 28 days. The sliced roots were pulverized into powder using an electric blender. A cold maceration technique with distilled water was used. Two hundred grams of the powdered plant roots were taken and soaked in 400 mL distilled water and allowed for 48 hours with 2 hourly intermittent shaking. To secure the aqueous extract, they were then sieved using No. 1 Whatman filter paper. The aqueous extract was freeze-dried and kept in a rubber screw-cap bottle and preserved in the refrigerator at a temperature of 4°C until needed.

The aqueous root extract of SA produced a yield of 14.5 g (7.25% w/w).

Experimental Animals

Male albino Wistar rats (120-140 g) were procured from a reputable source. The animals were allowed for 2 weeks of acclimatization in a clean wire mesh cage in a controlled environment (temperature 25 ± 2°C 12-hour dark/light cycles) and fed with standard laboratory animal diet ad libitum before the commencement of the study.

The study was carried out after obtaining Institutional Animal Ethical Committee's clearance (Date: 12.08.2022, Number: FVM-UNN-IACUC-2022-0334).

Experimental Protocol

For this study, 20 male Albino Wistar rats were used. They were divided into 4 groups at random (n = 5). Alloxan monohydrate (160 mg/kg) was reconstituted in distilled water and then administered intraperitoneally once to groups 2-4 to induce DM. The remaining 5 rats were normoglycemic (un-induced) rats that formed group 1, 48 hours following alloxan monohydrate administration and upon confirmation of DM (fasting blood glucose (FBG) level ≥ 126 mg/dL), the animals in groups 2-4 (n = 5 per group) were sorted to ensure there were no significant differences in FBG levels among the groups. The treatment agents (SA and glibenclamide) used in this study were reconstituted in distilled water, and all animals were treated as follows:

1 (group 1): uninduced normoglycemic rats in group 1 were given 10 mg/kg of distilled water (normal control); 2 (group 2): negative control group of induced diabetic rats were given 10 mL/kg of distilled water; 3 (group 3): induced-diabetic rats in group 3 were given a 400 mg/kg aqueous extract of SA; 4 (group 4): rats with induced diabetes were placed in group 4 and given 2 mg/kg glibenclamide (standard control). For a total of 21 days, the treatments were given orally once daily. Fasting blood glucose levels were measured using a one-touch ultra-easy glucometer that automatically displays the FBG levels on the screen at intervals of 1 hour, 6 hours, 24 hours, 7 days, 14 days, and

then 21 days. The 400 mg/kg of SA was chosen with respect to the effects observed with its use as reported by previous researchers.[16,17]

Sample Collection

Whole blood was collected for glucose estimation using the tail snip technique during which 1 drop of whole blood from the snipped tail was allowed to fall directly into a glucometer strip. On the last day of the study (day 21), blood samples for serum lipid panels and kidney function tests (triglycerides, total cholesterol, HDL, creatinine, and urea) were collected into heparin-coated bottles through the medial canthus of the rat eye. Thereafter, the animals were humanely sacrificed by inhalation anesthesia using chloroform followed by cervical dislocation. The animals were cut open via the midline of the thoracic and abdominal regions and pancreases were freshly detached for a histopathology examination.

Determination of Serum Biochemical Parameters and Histopathology

The blood samples collected after 21 days of the study were spun in a centrifuge at 10 000 *g* for 10 minutes and the resulting sera were decanted. Triacylglyceride (TAG) value was assayed by quantitative enzymatic method.[18] The cholesterol value was assayed by the enzymatic method.[19] High-density lipoprotein was assayed by using the dextran sulfate-magnesium II precipitation technique.[20] Friedwald formula was applied in calculating the values of LDL and triglycerol values were divided by 5 to get the very-LDL values. The creatinine assay was determined by Jaffe reaction method.[21] Serum urea was assayed according to the Urease-Berthelot method.[22] The histopathological studies of the freshly harvested pancreas were done according to Carlleton's histological technique.[23] The processed slides were viewed with a light microscope at 400× magnification.

Statistical Analysis

One-way analysis of variance was used to analyze the data generated from this study (The Statistical Package for Social Sciences version 23.0 software, IBM Corp.; Armonk, NY, USA). Variable means were separated using Duncan's multiple range test, and differences were deemed significant at $P = .05$. In tables, results were shown as mean and standard error of means.

RESULTS

Effects of Aqueous Root Extract of Senna alata on Fasting Blood Glucose Levels Observed from Preinduction to 21 Days of Treatment in Alloxan-Induced Diabetic Wistar Rats

Findings showed that on day 21 post-treatment, rats treated with SA extract and glibenclamide had FBG levels that were statistically equivalent ($P = .05$), but rats in the untreated (negative control) group had FBG levels that were considerably ($P = .05$) lower (Table 1).

The Percentage Reduction in Fasting Blood Glucose Levels of Alloxan-Induced Diabetic Rats Treated with Aqueous Root Extract of Senna alata

The results also revealed that rats given SA experienced a progressive decline in FBG levels from the first hour to the final 21 days after treatment, while the FBG levels of rats of the diabetic untreated group remained high even with day 21 post-treatment. However, SA and glibenclamide-treated groups recorded 65.25% and 66.36%, respectively, on 21 days post-treatment (Figure 1).

The Effects of Aqueous Root Extract of Senna alata on Lipid Panel of Alloxan-Induced Diabetic Rats

According to the results of the lipid profile, the rats treated with SA had cholesterol levels that were statistically equal ($P < .05$) to those of the rats treated with glibenclamide but significantly ($P < .05$) lower than those of the rats in the diabetic-untreated group. Triglycerol levels were statistically comparable in all groups except for those of the diabetic-untreated group, which were significantly ($P < .05$) higher. High-density lipoprotein values were statistically comparable ($P < .05$) in all groups except those of the diabetic-untreated group, which were significantly ($P < .05$) lower. Rats in the SA and glibenclamide-treated groups had LDL values that were statistically equivalent ($P = 0.05$) but were considerably ($P = 0.05$) lower than rats in the diabetic-untreated group (Table 2).

The Effects of Aqueous Root Extract of Senna alata on Kidney Function of Alloxan-Induced Diabetic Rats

According to Table 3, the urea and creatinine levels of the rats treated with SA and glibenclamide were statistically equivalent ($P = .05$), but they were significantly ($P = .05$) lower than the rats in the untreated diabetic group.

The Photomicrograph of Diabetic Rat Pancreas Treated with Senna alata Extract

Results of the photomicrograph showed that the population of islet cells in both SA-treated rats and those of normal control and glibencamide-treated groups were comparable. However, islet cells were scanty and atrophic in congested pancreatic ducts in rats of the diabetic untreated group (Figure 2).

DISCUSSION

This study investigated the potential hypolipidemic and hypoglycemic effects of SA aqueous root extract in alloxan-induced diabetic rats. Diabetes mellitus, a known chronic disease, is characterized by FBG levels and often associated with certain complications like atherosclerosis, hepatotoxicity, nephrotoxicity, cardiovascular diseases, etc. Alloxan monohydrate is a diabetogenic agent known to induce hyperglycemia by generation of free radicals that damage the beta cells of islets of Langerhans, which in turn occasions low production of insulin.

Table 1. Effects of Aqueous Root Extract of *Senna alata* on Fasting Blood Glucose Levels Observed from Preinduction to 21 Days of Treatment in Alloxan-Induced Diabetic Wistar Rats

	Fasting Blood Glucose Levels (mg/dL)							
Groups	Pre-Induction	48-Hour Pre-Induction	1-Hour Post-Treatment	6-Hour Post-Treatment	24-Hour Post-Treatment	7 Days Post-Treatment	14 Days Post-Treatment	21 Days Post-Treatment
1	81.25 ± 0.56^{a}	81.90 ± 0.35^{a}	82.20 ± 0.80^{a}	80.40 ± 0.55^{a}	78.70 ± 0.07^{a}	78.25 ± 0.15^{a}	79.50 ± 0.55^{a}	76.82 ± 0.72^{a}
2	80.90 ± 0.75^{a}	242.20 ± 1.25^{d}	242.35 ± 1.00^{d}	240.20 ± 1.80^{d}	218.00 ± 1.06^{d}	212.15 ± 1.08^{d}	201.35 ± 1.14^{d}	186.34 ± 1.06^{c}
3	80.40 ± 0.65^{a}	243.00 ± 1.50^{d}	220.50 ± 1.30^{c}	180.45 ± 1.25^{c}	142.70 ± 1.08^{c}	120.55 ± 1.06^{c}	85.70 ± 1.08^{ab}	84.45 ± 1.12^{b}
4	81.30 ± 0.42^{a}	243.75 ± 1.00^{d}	140.66 ± 1.45^{b}	121.60 ± 1.05^{b}	85.40 ± 1.04^{b}	83.68 ± 1.02^{ab}	83.10 ± 1.00^{a}	82.00 ± 1.02^{ab}

Significant differences between groups are denoted by the superscripted letters a, b, c, and d ($P = .05$).

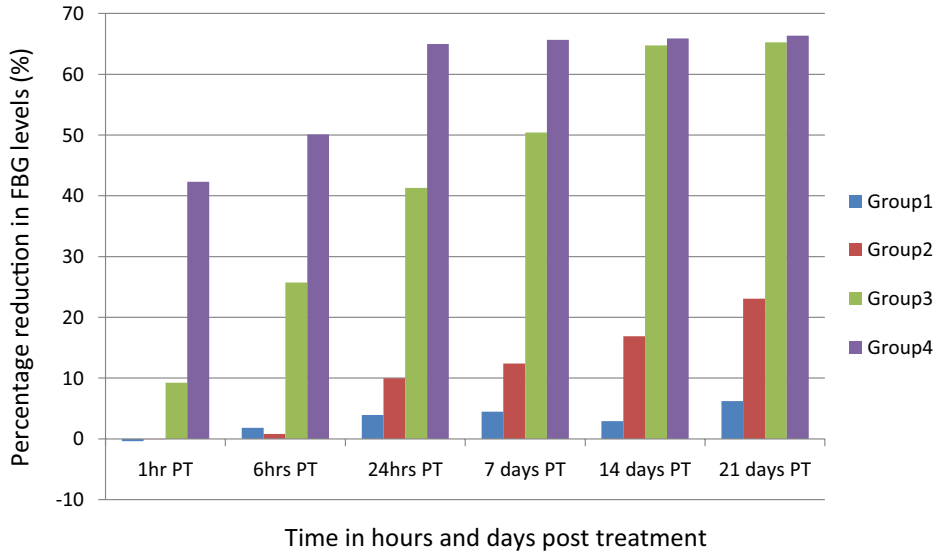

Figure 1. Percentage reduction in fasting blood glucose levels of alloxan-induced diabetic rats treated with aqueous root extract of SA. hr, hour(s); PT, post-treatment; SA, *Senna alata*.

Table 2. Effects of Aqueous Root Extract of *Senna alata* on Lipid Panel of Alloxan-Iinduced Diabetic Rats

	Lipid Panels (mmol/L)				
Groups	Cholesterol	Triglycerol	High-Density Lipoprotein	Low-Density Lipoprotein	Very-Low-Density Lipoprotein
1	4.28 ± 0.01^{a}	1.58 ± 0.02^{a}	2.54 ± 0.04^{b}	1.02 ± 0.07^{a}	0.32 ± 0.01^{a}
2	5.52 ± 0.43^{c}	1.93 ± 0.12^{b}	1.75 ± 0.05^{a}	2.86 ± 0.02^{c}	0.39 ± 0.00^{a}
3	4.61 ± 0.10^{b}	1.64 ± 0.01^{a}	2.36 ± 0.13^{b}	1.50 ± 0.05^{b}	0.33 ± 0.02^{a}
4	4.55 ± 0.06^{b}	1.62 ± 0.03^{a}	2.41 ± 0.08^{b}	1.40 ± 0.08^{b}	0.32 ± 0.12^{a}

The letters a, b, and c in superscripts denote significant differences ($P = .05$) between groups.

Studies showed a significant increase in FBG levels in rats challenged with alloxan monohydrate compared to rats of normal control. However, after 21 days of treatment, animals given the SA root extract had significantly lower FBG levels than rats in the negative control group. This finding corroborates with the work of other researchers [14,15] who reported the hypoglycemic activity of leaf and bark extract of SA in rats.

Dyslipidemia is a complication often associated with diabetes.[24] Findings of this study showed remarkably high concentrations of serum cholesterol, TAG, LDL, and low HDL in untreated diabetic rats, which is consistent with reports from previous studies[25,26] which reported that induction of DM leads to an increase in blood glucose level which also resulted in a commensurate increase in serum lipids. The liver, an organ that depends on insulin and is crucial for maintaining blood sugar and lipid levels, is severely affected by diabetes.[27] Diabetes often leads to lipoprotein abnormalities which are characterized by abnormally high levels of cholesterol, TAG, LDL, and low HDL.[24,28] It is generally known that high serum lipid levels in diabetic patients originate from the enhanced mobilization of free fatty acids from accessory fat depots as a result of insulin's inhibition of hormone-sensitive lipase.[29] It could also be due to certain hormones that enhance lipolysis such as glucagon and catecholamines. The surplus fatty acids produced owing to these effects are usually converted to cholesterol and phospholipids that combine with the excess triglycerols produced simultaneously in the liver and are distributed into the circulation as lipoproteins. Therefore, significant hyperlipidemias ($P > .05$) recorded in diabetic untreated rats could be regarded as an effect of the uninterrupted activity of lipolytic hormones in the fat depots.[30] Treatment of diabetic rats with the SA extract resulted in the alleviation of all dyslipidemia, thereby justifying its antihyperlipidemic potency. These effects, however, could be ascribed to the presence of phytochemicals composed of the SA extract, which may have hindered cholesterol or bile acid absorption. This finding is consistent with the reports of previous researchers.[15,31]

Table 3. Effects of Aqueous Root Extract of *Senna alata* on Kidney Function of Alloxan-Induced Diabetic Rats

Groups	Urea (mg/dL)	Creatinine (mg/dL)
1	61.32 ± 3.11^{a}	2.05 ± 0.04^{a}
2	85.55 ± 0.17^{c}	3.28 ± 0.16^{c}
3	71.20 ± 2.10^{b}	2.65 ± 0.01^{b}
4	69.85 ± 1.00^{b}	2.60 ± 0.02^{b}

The superscript letters a, b, and c indicate significant differences across the groups.

This study observed abnormally high levels of serum urea and creatinine in rats of the diabetic untreated group compared to normal and treated groups. This finding is consistent with the work of a previous researcher[16] who reported a significant reduction to normal levels of serum creatinine, urea, and uric acid in diabetic rats after treatment with SA leaf extract. Renal dysfunction is a common complication associated with diabetes, which increases significantly in diabetic conditions.[32] Due to the kidney's diminished capacity to filter these waste products from the

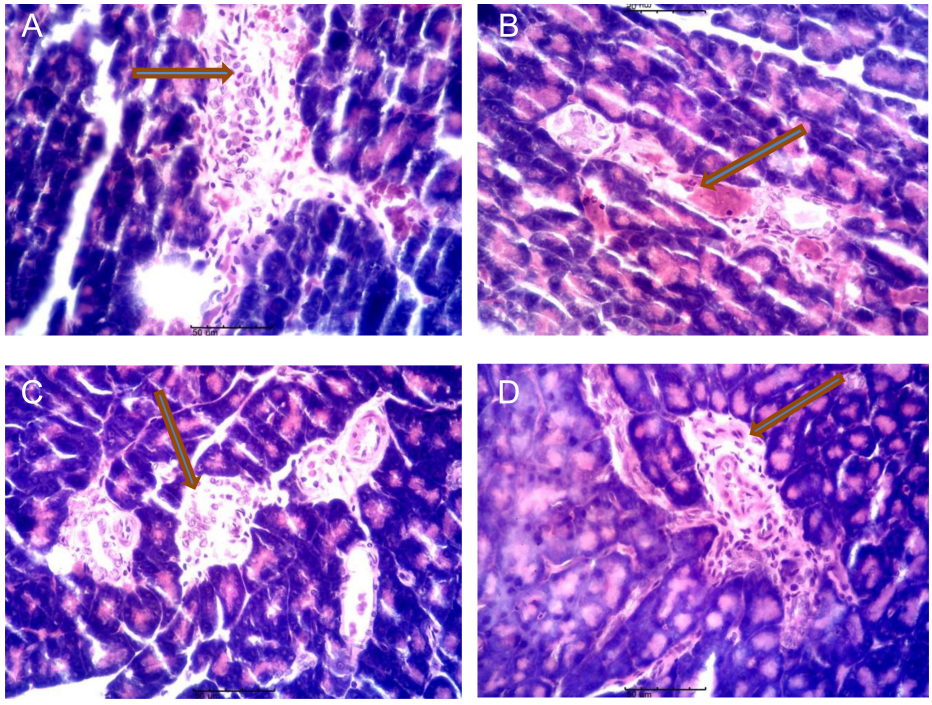

Figure 2. Photomicrograph of the pancreas of rats. (A) Group 1 rats showing well-populated islet cells. (B) Group 2 rats showing severely depleted and atrophic islet cells. (C) Group 3 rats showing relatively moderate islet cell population. (D) Group 4 rats showing well-populated islet cells (arrows) H&E 400×.

blood and ensure that they are excreted into the urine, the significantly increased serum levels of urea and creatinine found in the diabetic untreated group may be explained. Treatment with the SA extract, however, significantly ($P = .05$) decreased serum levels of urea and creatinine in a way that was similar to the standard drug (glibenclamide). This finding implies that SA extract can either directly improve the structural and functional integrity of blood, kidney, and liver cells or may be able to give a protective impact on the kidney.

The findings of this study revealed that induction of DM with alloxan monohydrate caused significant histopathological changes in the pancreas of rats compared to the control group. Previous reports[33] showed that administration of alloxan to experimental rats caused a selective pancreatic beta-cell membrane disruption and intracellular accumulation which resulted in cytotoxicity. Histomorphological observation of the pancreas in the present work confirmed an improvement in rats of the treatment groups compared with the rats of the negative control group. Treatment with SA extract restored the pancreas to normal architecture evident in the regeneration and increased the number of islet cells. This finding is consistent with the reports of previous researchers[34,35] that the plant extract increases the number of beta-cells by reduction of blood glucose.

In conclusion, this study has revealed that the aqueous root extract of SA has hypoglycemic and hypolipidemic activities and restored pancreatic tissue injury caused by the alloxan challenge in diabetic rats. Therefore, aqueous root extract of SA has the potential in the management of DM and its associated complications. Further study to determine the mechanism of action of the SA extract that produced these effects is therefore recommended.

Ethics Committee Approval: This study was approved by the University of Nigeria, Nsukka, Faculty of Veterinary Medicine, Institutional Animal Care and Use Committee (Date: 12.08.2022, Number: FVM-UNN-IACUC-2022-0334).

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ERRATUM

In the article by OKAFOR et al., titled "Detection of Multidrug-Resistant Staphylococci in Beef Processing Line," published in the April issue of Veterinary Sciences and Practices (Vet Sci Pract. 2023; 18(1), 25-30; DOI: 10.5152/VetSciPract.2023.222644), the authorship order has been changed. The authorship order was mistakenly recorded incorrectly during the production stage, and the PDF file has been updated to reflect the existing authorship order at the time of submission. The second last name of Umunna MADUBUIKE has been added to the article. The author's last name has been revised to MADUBUIKE ANYANWU. The institutional information of the co-authors Simeon CHIBUKO OKAFOR and Umunna MADUBUIKE ANYANWU was inadvertently not included during the production stage and has been added to the PDF file as the second affiliation. The added institution is the Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka.

You can access the updated version of the article through the following link:

https://veterinarysciences-ataunipress.org/en/detection-of-multidrug-resistant-staphylococci-in-beef-processing-line-16603

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