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

Address for Correspondence

Kafkas Üniversitesi Veteriner Fakültesi Dergisi Editörlüğü 36040, Kars - TÜRKİYE
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Research Article

Efficacy and Toxicity of Doxorubicin and Cyclophosphamide for the Neoadjuvant Treatment of Locally Advanced Stage Canine Mammary Tumors

Fikriye Ecem KURUOGLU [1,a] Musa Ozgur OZYIGIT [1,b] Deniz NAK [1,c] Zehra AVCI KUPELI [1,d] Zeynep Merve EKICI [1,e] Davut KOCA [1,f] Talha AVCILAR [1,g] Mustafa Eren SAHIN [1,h] Yavuz NAK [1,i] Abid Hussain SHAHZAD [2,j]

[1] Bursa Uludag University Veterinary Faculty, Department of Obstetrics and Gynecology, Gorukle, TR-16059 Bursa - TURKEY
[2] Theriogenology Department, University of Veterinary and Animal Sciences, Lahore, PAKISTAN

ORCIDs: [a] 0000-0002-6715-8529; [b] 0000-0003-0682-8127; [c] 0000-0001-8908-5052; [d] 0000-0003-1853-4679; [e] 0000-0002-6904-7275 [f] 0000-0002-7962-6959; [g] 0000-0001-7034-9644; [h] 0000-0003-1985-2345; [i] 0000-0002-6734-8226; j 0000-0002-9442-0000

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Abstract

The aim of this study was to investigate the effect and toxicity of a doxorubicin cyclophosphamide combination for neoadjuvant use in dogs with locally advanced mammary tumors. A total of 16 dogs with locally advanced mammary tumors aged 8-14 years was used in this study. Samples were taken from all the dogs via Tru-Cut biopsy and fine needle aspiration biopsy of the lymph nodes prior to neoadjuvant chemotherapy. The samples were sent to the pathology laboratory for histopathological examination. Based on the histopathology results, mammary tumors were diagnosed as adenocarcinoma (n=13), tubulopapillary carcinoma (n=2) and malignant mixed tumors (n=1). For the neoadjuvant chemotherapy, doxorubicin (25-30 mg/m^2) and cyclophosphamide (100 mg/m^2) were administered via slow IV injection 4 times at 3-week intervals. Clinical findings such as fever, vomiting, anorexia, lethargy, weight loss, alopecia, enteritis, hematuria, and skin ulceration and laboratory findings such as a decrease in neutropenia and hematocrit values were observed. Based on the longest diameter of the tumors before and after four weeks of chemotherapy, a complete response was detected in 2 dogs (12.5%), a partial response was observed in 9 dogs (56.25%) and the disease was stable in 5 dogs (31.25%). According to results, the toxicity, and efficacy of a doxorubicin and cyclophosphamide combination for neoadjuvant chemotherapy in dogs with locally advanced mammary tumors is acceptable, and the treatment is promising.

Keywords: *Dog, Locally advanced mammary tumor, Neoadjuvant chemotherapy, Doxorubicin, Cyclophosphamide*

Lokal İleri Köpek Meme Tümörlerinde Neoadjuvan Tedavide Doksorubisin ve Siklofosfamidin Etkinliği ve Toksisitesi

Öz

Bu çalışmanın amacı, lokal ileri meme tümörlü köpeklerde neoadjuvan kullanımda doksorubisin ve siklofosfamidin etkinliğini ve toksisitesini araştırmaktır. Bu çalışmada lokal ileri meme tümörlü 8-14 yaş aralığında 16 köpek kullanılmıştır. Neoadjuvant kemoterapi öncesinde tüm köpeklerden Tru-Cut biyopsisi ve ilgili lenf nodundan ince iğne aspirasyon biyopsisi ile örnekler alınmıştır. Numuneler histopatolojik inceleme için patoloji laboratuvarına gönderilmiştir. Histopatoloji sonuçlarına göre, meme tümörlerine adenokarsinom (n=13), tubulopapiller karsinom (n=2) ve malign miks tümör (n=1) tanısı konmuştur. Neoadjuvan kemoterapi olarak, 3 haftalık aralıklarla 4 kez yavaş IV enjeksiyon ile doksorubisin (25-30 mg/m^2) ve siklofosfamid (100 mg/m^2) uygulanmıştır. Yan etki olarak; kusma, anoreksi, uyuşukluk, kilo kaybı, alopesi, enterit, hematüri ve deri ülseri gibi klinik bulgular, nötropeni ve hematokrit değerlerinde azalma gibi laboratuvar bulguları gözlenmiştir. Kemoterapiden önce ve tedavinin bitiminde 4 hafta sonra tümörlerin en uzun çapına dayanarak, 2 köpekte (%12.5) tam cevap, 9 köpekte (%56.25) kısmi cevap ve 5 köpekte (%31.25) stabil hastalık belirlenmiştir. Sonuç olarak, lokal ileri meme tümörlü köpeklerde neoadjuvan kemoterapi için doksorubisin ve siklofosfamid kombinasyonunun toksisitesi ve etkinliği kabul edilebilir ve tedavide umut verici olduğu görülmüştür.

Anahtar sözcükler: *Köpek, Lokal ileri meme tümörü, Neoadjuvan kemoterapi, Doksorubisin, Siklofosfamid*

Correspondence
+90 224 2940873
deniznak@gmail.com

INTRODUCTION

Canine mammary tumors (CMTs) are the most common neoplasms in intact female dogs. Although the prevalence of these tumors decreases in regions where preventive ovariohysterectomy is performed, it remains an important disease entity in veterinary medicine [1]. In female dogs, the probability of mammary tumors is 50%, and half of these tumors have the ability to metastasize [2,3]. Dogs with malignant tumors have been shown to be older than dogs with benign tumors. Malignant tumors were found to be significantly larger than benign tumors [4,5].

The incidence of mammary tumors increases with age and it is most common in dogs over the age of 10-12 years. For mammary tumors, age, hormonal exposure (neutered status/exogenous hormone use), breed and genetic susceptibility and body weight (particularly during puberty) are important risk factors. At the same time, these risk factors may affect posttreatment survival. The entire mamma is exposed to growth factors and sex hormones, rendering it susceptible to carcinogenesis. This was proven to be mainly triggered by progesterone affecting on mammary stem cells from the first estrus cycle on. Consequently, most dogs develop tumors in multiple glands [5].

The therapeutic intent and goals of a given chemotherapeutic regimen are important factors to be considered when a drug is selected or investigated. Neoadjuvant treatment is a treatment performed before surgical removal of the primary tumor to prevent the development of possible postoperative micrometastases and to reduce the size of the tumor to make it suitable for operation. Furthermore, complete regression of the tumor stage and lymph node metastasis was observed after preoperative neoadjuvant chemotherapy. Adjuvant treatment is treatment with chemotherapeutic drugs following surgical removal of the primary tumor [6,7].

Irrespective of the stage of the disease, in studies [1,2,5] conducted to date, the first approach to CMTs is almost always surgically followed by adjuvant chemotherapy with cytotoxic drugs. Adjuvant chemotherapy is recommended particularly in dogs with mammary tumors that are thought to be at risk of metastasis and recurrence. To date, the following chemotherapeutic applications have been conducted in the treatment of CMTs: doxorubicin, docetaxel, paclitaxel, mitoxantrone, gemcitabine, and carboplatin, or combinations of various drug groups such as doxorubicin + cyclophosphamide, 5-fluorouracil + doxorubicin + cyclophosphamide, 5-fluorouracil + cyclophosphamide, mitoxantrone + vincristine + cyclophosphamide, gemcitabine + carboplatin and mitoxantrone + carboplatin [8].

In human medicine, preoperative neoadjuvant chemotherapy is preferred in advanced stage breast tumors and has been accepted as a standard treatment [7,9]. In studies on neoadjuvant chemotherapy in human breast tumors, the complete pathological response rate (pCR) was found to be 13-13.7% as a result of drug administration in the form of a combination of doxorubicin and cyclophosphamide [10,11]. However, despite the advantages mentioned above, there are no studies on the use of neoadjuvant chemotherapy in CMTs. To date, there has been limited study related to the combination of doxorubicin and cyclophosphamide in adjuvant chemotherapy on CMTs. Therefore, this study was aimed at investigating the efficacy and toxicity of a doxorubicin cyclophosphamide combination for neoadjuvant use in dogs with locally advanced mammary tumors.

MATERIAL and METHODS

Patients

This study included 16 intact female dogs with locally advanced mammary tumors (T_{1-3} N_{0-1} M_0). The dogs' ages ranged from 8 to 14 years. The breeds of the dogs included poodle (3), terrier (5), golden retriever (2), Siberian husky (1), German shepherd (2), Rottweiler (1) and Doberman pinscher (2). Of the 16 dogs included in this study, while 4 had one tumor in single mammary tissue, 12 had multiple tumors in different mammary tissues. Mammary tumors were in the inguinal and abdominal mammary lobes in 93.75% of the dogs, while it contained the thoracic lobes in 6.25% in this study. All dogs were fed commercial dog food. Dog's body weights ranged from 5 kg to 48 kg. No dog had a history of pseudopregnancy or hormonal contraception. The approval for conduction of the experiment was obtained from the ethics committee of the Bursa Uludag University (2018-01/03).

Pretreatment Evaluation

All dogs received a general examination, gynecological examination (vaginoscopy, vaginal cytology), complete blood count (CBC) (VetScan HM5, Veterinary Hematology Analyzer, Abaxis, USA), serum biochemistry (VetScan® VS2, Chemistry Analyzer, Abaxis, USA), thoracic radiographs, and intra-abdominal ultrasonography (Mindray, DC-N2, China). If necessary, echocardiography was added to the examination to determine heart pathology. Using the ultrasound guide technique, a 14-gauge core needle biopsy was obtained from the mammary tumors of all dogs. Needle biopsies were taken from the local lymph nodes with a 21 gauge needle. All of the biopsies were then sent to the pathology laboratory. Based on the pathological examination, dogs with malignant mammary tumors were included in the study. All dogs with malignant mammary tumors were evaluated according to the TNM system [5]. The mammary tumors of these dogs were stage II-III-IV (T_{1-3} N_{0-1} M_0) and were identified as locally advanced mammary tumors.

Histopathological Evaluation

The tumor biopsy samples were fixed in 10% buffered formalin and embedded in paraffin. Sections 4 µm thick were obtained from each sample and stained with H&E (hematoxylin and eosin) for histologic examination and were classified according to the World Health Organization criteria for canine mammary lesions [12]. All samples were evaluated with respect to tubule and mammary alveoli formation, pleomorphism, nuclear polymorphism, mitosis index, necrosis, inflammatory infiltration, adjacent tissue invasion, and lymph node metastasis.

Treatment

A combination of 25-30 mg/m^2 doxorubicin (Adriamycin, Saba, Tekirdag, Turkey) (<15 kg, 25 mg/m^2; >15 kg, 30 mg/m^2) and 100 mg/m^2 cyclophosphamide was administered slowly intravenously every three weeks for four cycles. The premedication was given intravenously (I.V) 30 min before each chemotherapy treatment. This premedication included dexamethasone (Dexacure 2 mg/kg IV, Alivir, Ankara, Turkey) and metoclopramide (Metpamid 0.5 mg/kg IV, Zero, Istanbul, Turkey). All dogs were operated 4 weeks later after the last chemotherapy protocol. General anesthesia was performed with xylazine HCl (2 mg/kg) (Alfazyne 2%, Alfasan International BV, Woerden, The Netherlands) injected intramuscularly for premedication. Induction was provided by ketamine HCl (5-10 mg/kg, IV) (Alfamine 10%, Alfasan International BV, Woerden, The Netherlands). An endotracheal tube was inserted and isoflurane (Forane Likid, Abbott, Kent, UK) was delivered in 100% oxygen at 2 L/min as recommended. At the time of anesthetic induction, amoxicillin/clavulanic acid (8.75 mg/kg, IM) (Synulox, Pfizer, Istanbul, Turkey) was administered for prophylaxis and was continued postoperative period for five days. Carprofen (Rimadyl; Pfizer Inc., Zaventem, Belgium) was injected (4 mg/kg, SC) 5 days for postoperative analgesia. All dogs underwent mastectomy.

Evaluation of Response and Toxicity

At the beginning of treatment, tumor volume was measured under ultrasound guidance before each cycle and at 4 weeks after the last chemotherapy. The largest diameter of single tumors and the baseline sum of diameters of 2 and more tumors were evaluated. Complete response, partial response, stable disease, and progressive disease were used to evaluate clinical improvement. Response evaluation criteria are presented in *Table 1* [13].

The side effects observed during treatment as toxicity were classified according to Veterinary Cooperative Oncology parameters v1.1 [14].

RESULTS

The general examination, CBC and serum biochemistry were within normal limits in all dogs. No pathology was found in thoracic radiographs or intra-abdominal ultrasonographic examinations.

Based on the histopathology results, mammary tumors were diagnosed as adenocarcinoma (n=13), tubulopapillary carcinoma (n=2) or malignant mixed tumors (n=1). Whereas 14 dogs had no indication of lymph node metastasis, in two dogs, mammary epithelial tumor cells were noticed in the lymph node via lymph node fine needle aspiration.

The mammary tumors of all dogs were at a locally advanced stage according to TNM classification. Based on the TNM classification, the tumors were grade 2 in 14 dogs and grade 4 in 2 dogs. The two dogs with mammary tumors that showed a complete response were grade 4. Characteristics of dogs and neoadjuvant chemotherapy response rates was summarized in *Table 2*.

The clinical images before and after 4 weeks of treatment for dog with a partial response are shown in *Fig. 1* and *Fig. 2*.

The ultrasound images before and after 4 weeks of treatment for dog with a partial response are shown in *Fig. 3* and *Fig. 4*.

Although no side effects were observed during treatment, clinical findings such as fever, vomiting, anorexia, lethargy, weight loss, alopecia, enteritis, hematuria, and skin ulceration and laboratory findings such as a decrease in neutropenia and hematocrit values were observed after treatment. All of the adverse effects changed between 1 to 2 grades. Three dogs that exhibited alopecia were of the terrier breed. All of the adverse effects were seen after the second neoadjuvant chemotherapy cycle. Five dogs that exhibited vomiting, enteritis, hematuria, and neutropenia were treated symptomatically. These adverse effects were not observed on the third and fourth neoadjuvant chemotherapy cycles. The adverse effects are presented in *Table 3*.

Table 1. *Response evaluation criteria for solid tumors in dogs (v1.0)(13)*

Characterization of Response	
Complete response	Disappearance of all target lesions
Partial response	At least 30% reduction in the sum of diameters of target lesions, taking as reference the baseline sum
Stable disease	Less than 30% reduction (PR) or 20% increase (PD) in the sum of diameters of target lesions
Progressive disease	Either the appearance of one or more new lesions or at least a 20% increase in the sum of diameters of target lesions

Table 2. *Neoadjuvant chemotherapy response rates and characteristics of dogs with locally advanced mammary tumors*

No	Age	Breed	Castrated	Cycle Stage	Tumor No.	Tumor Type	Histologic Tumor Grade		Lymph Node	Tumor Size	Clinical Response
							Before Treatment	After Treatment			
1	10	Poodle	Not Castrated	Anestrus	>1	Adenocarcinoma	I	-	Negative	<3 cm	C.R
2	9	German Shepherd	Not Castrated	Anestrus	1	Adenocarcinoma	I	-	Negative	<3 cm	C.R
3	12	Doberman Pinscher	Not Castrated	Anestrus	>1	Adenocarcinoma	I	I	Negative	3-5 cm	P.R
4	12	Terrier	Not Castrated	Anestrus	1	Schrious adenocarcinoma	III	II	Positive	3-5 cm	P.R
5	13	Terrier	Not Castrated	Anestrus	>1	Tubulopapillary carcinoma	I	I	Negative	3-5 cm	P.R
6	9	Golden Retriever	Not Castrated	Estrus	>1	Schrious adenocarcinoma	II	II	Positive	3-5 cm	P.R
7	10	Terrier	Not Castrated	Anestrus	>1	Adenocarcinoma	II	I	Negative	3-5 cm	P.R
8	9	Rotweiller	Not Castrated	Anestrus	>1	Adenocarcinoma	I	I	Negative	3-5 cm	P.R
9	9	German Shepherd	Not Castrated	Anestrus	>1	Adenocarcinoma	II	I	Negative	3-5 cm	P.R
10	10	Terrier	Not Castrated	Anestrus	>1	Adenocarcinoma	II	I	Negative	3-5 cm	P.R
11	10	Poodle	Not Castrated	Anestrus	>1	Adenocarcinoma	II	I	Negative	3-5 cm	P.R
12	9	German Shepherd	Not Castrated	Anestrus	>1	Adenocarcinoma	II	II	Negative	3-5 cm	S.D
13	7	Terrier	Not Castrated	Anestrus	>1	Tubulopapillary carcinoma	I	I	Negative	3-5 cm	S.D
14	10	Poodle	Not Castrated	Anestrus	>1	Adenocarcinoma	I	I	Negative	3-5 cm	S.D
15	9	German Shepherd	Not Castrated	Anestrus	1	Malignant mixed tumors	I	I	Negative	3-5 cm	S.D
16	9	Siberian Husky	Not Castrated	Anestrus	1	Adenocarcinoma	I	I	Negative	>5 cm	S.D

C.R.:Complete Response; P.R.: Partial Response; S.D.: Stable Disease

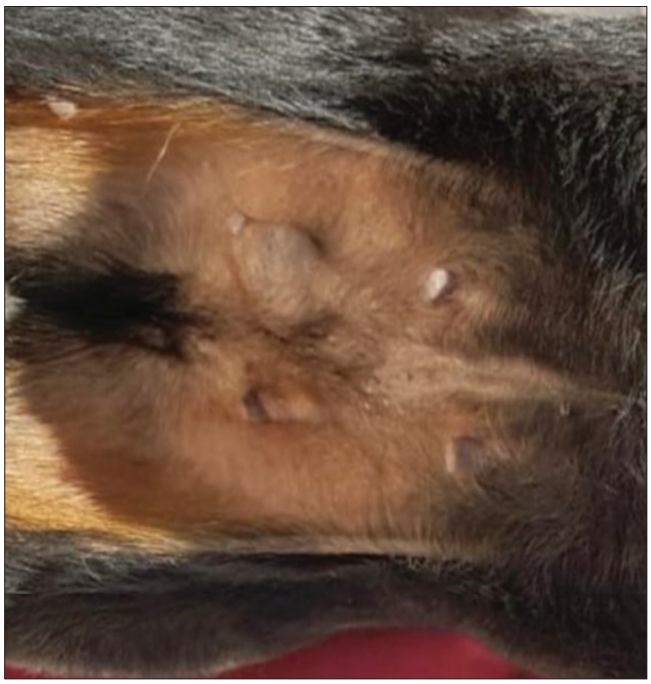

Fig 1. Clinical appearance of mammary tumor on right inguinal mammary gland before treatment

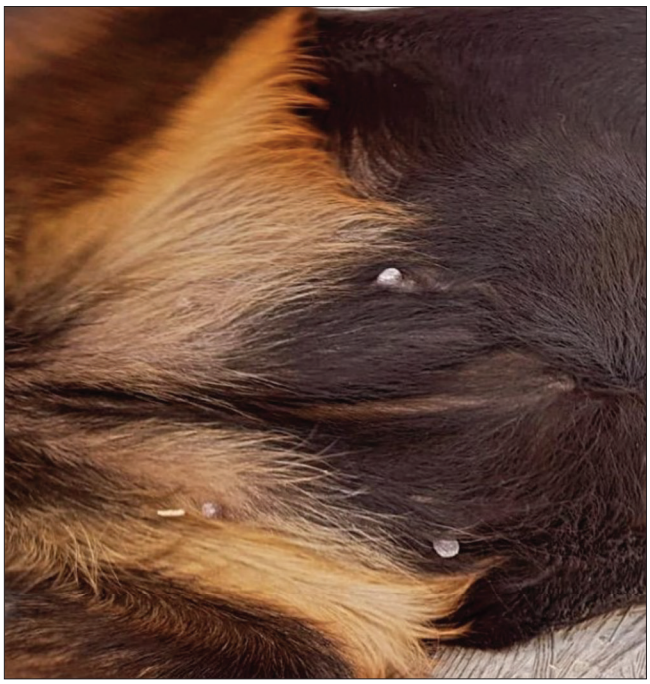

Fig 2. Clinical appearance of partial response in the tumor size after treatment

DISCUSSION

Treatment options for CMTs are limited compared with human breast cancers. However, treatment protocols for human breast tumors can be considered as treatment options for CMTs [1].

In adjuvant treatment, combination chemotherapy is used routinely due to widespread evidence that poly-chemotherapy offers a survival advantage compared with single-agent therapy. Multidrug regimens have generally resulted in higher overall complete response rates with improvement in response durations. When compared

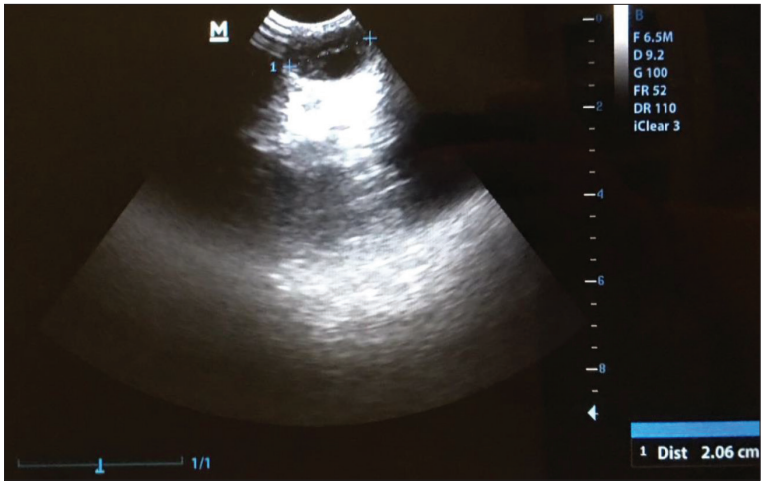

Fig 3. Ultrasound image of a dog before neoadjuvant chemotherapy that produced a partial response

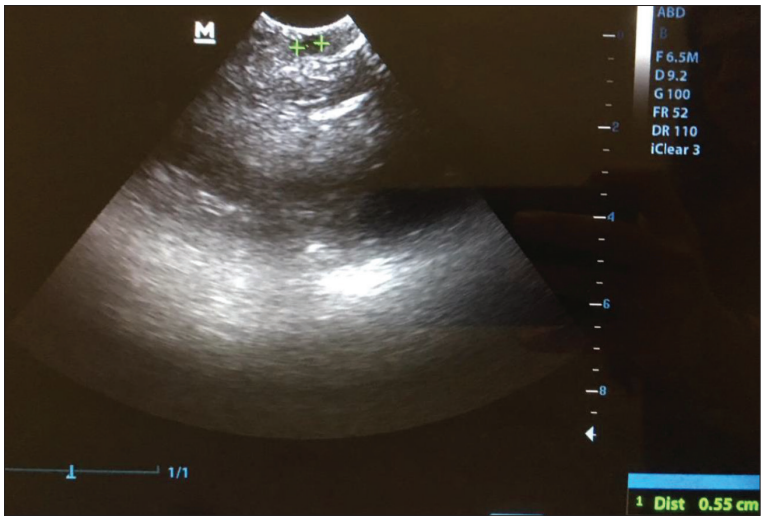

Fig 4. Ultrasound image of a dog after 4 weeks of neoadjuvant chemotherapy that produced a partial response

Table 3. *Adverse effects observed after neoadjuvant chemotheraphy in dogs with locally advanced mammary tumors*

Adverse Effects	Number of Dogs	Stage
Clinical findings		
Fever	1/16	Grade 2
Vomiting	3/16	Grade 1
Anorexia	4/16	Grade 2
Lethargy	4/16	Grade 1
Weight loss	2/16	Grade 1
Alopecia	3/16	Grade 1
Enteritis	2/16	Grade 2
Haematuria	1/16	Grade 2
Skin ulceration	1/16	Grade 2
Laboratory findings		
Neutropenia	2/16	Grade 2 (1) Grade 1 (1)
Hematocrit value ¯	2/16	Grade 1

with patients treated with non-anthracycline-containing regimens, patients treated with anthracycline-containing regimens had a statistically significant reduction in recurrence rates (12%) and mortality rates (11%) [15-17]. Anthracycline-containing regimens provided advantages in overall and recurrence-free survival rates over non-anthracycline-containing regimens. The most commonly used anthracycline-containing adjuvant chemotherapy regimen consists of four cycles of doxorubicin plus cyclophosphamide (AC) administered every 21 days [15,17]. Rastogi et al.[10] conducted a study to determine whether four cycles of doxorubicin and cyclophosphamide (AC) administered pre-operatively improved breast cancer disease-free survival (DFS) and overall survival (OS) compared with AC administered postoperatively in 751 patients. In the preoperative AC group, an objective clinical response occurred in 79% of the assessable patients with a clinical partial response (cPR) in 43% of patients and a clinical complete response rate (cCR) in 36% of patients. In another study, a cCR of 40.1% was found after four cycles of preoperative AC on women with operable breast cancer and a mean tumor size of 4.5 cm [11]. There have been two published studies in veterinary medicine that deal with a doxorubicin and cyclophosphamide combination used on CMTs. In one study, two dogs with inflammatory mammary carcinoma received 1 dose of doxorubicin (30 mg/m^2, IV) on day 1 (day of presentation) and 1 dose of cyclophosphamide (200 mg/m^2, PO) on day 4. Both had presented for re-evaluation on an emergency basis, one at 6 d and the other at 7 d after the initiation of chemotherapy, with clinical signs including severe lethargy (n=2), pale mucous membranes (n=2), melena (n=2), hematemesis (n=1), abdominal hemorrhagic effusion (n=1), and inguinal hematomas (n=1). Additional diagnostic tests were not allowed by the owners and both dogs died on the day of representation. Both dogs were not already dead when re-evaluation was planned or performed [18]. The second study evaluated the effect and toxicity of doxorubicin and cyclophosphamide chemotherapy combined with operative treatment of malignant mammary tumors in 6 dogs. Ten days after the operation, doxorubicin was given intravenously at 20-30 mg/m^2 once weekly for 3 consecutive weeks and cyclophosphamide was given intravenously at 100 mg/m^2 3 days after doxorubicin administration for 3 consecutive weeks. This study showed that doxorubicin and cyclophosphamide chemotherapy effectively suppressed the development of new neoplasms and metastases but was accompanied by general adverse reactions such as lethargy, anorexia, vomiting, hair loss, fever, hypochromic anemia, and strong immunosuppression [19]. The present

study found a complete response in 2/16 (12.5%), a partial response in 9/16 (56.25%), stable disease in 5/16 (31.25%), and progressive disease in 0/16 (0%) of cases after four cycles of neoadjuvant AC protocols on locally advanced CMTs. The mammary tumors size was <3 cm in the two dogs with a complete response. It has been determined that CMTs smaller than 3 cm can be treated with AC treatment and the treatment of small tumors is more successful. Compared with human studies, the complete response rate was lower than but the partial response rate was similar in our study. This difference may be due to species differences and dose differences. The clinical tumor stage is the most important indicator of the pathological complete response rate after neoadjuvant chemotherapy in breast cancer patients. Low clinical tumor stages have significantly higher pCR rates than high tumor stages [20]. In our study, the lymph nodes of the dogs that responded fully were negative (No.1-2 dogs). Liu et al.[21] determined that there a significant relationship between high tumor grade (grade III) and overall response rate. The present study detected that the overall response dogs had grade I-III (unlike the above -mentioned literature). In a study [18] using the high-dose AC protocol, the dogs died, and in another study [19] there were serious side effects. This study documented grade I-II manageable toxicity. The dose used in this study was evaluated as successful both in terms of clinical response and manageable toxicity.

This study presents the first data on response rates and neoadjuvant use of the AC protocol on CMTs. The neoadjuvant AC protocol leads to an improved clinical outcome in canine locally advanced mammary tumors. The AC treatment was well tolerated.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

D. NAK, E. KURUOGLU and Y. NAK, planned and designed the research. Z. M. EKICI, D. KOCA, T. AVCILAR, M. E. SAHIN and A. H. SHAHZAD provided help in the clinic process. M. O. OZYIGIT and Z. AVCI KUPELI made histopathological examinations. All authors discussed the results and contributed to the final manuscript..

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Research Article

Polymorphisms in Some Candidate Genes Associated with Egg Yield and Quality in Five Different White Layer Pure Lines [1]

Eymen DEMİR [1,a] Taki KARSLI [1,b] Hüseyin Göktuğ FİDAN [1,c] Bahar ARGUN KARSLI [1,d] Mehmet ASLAN [1,e] Sedat AKTAN [2,f] Serdar KAMANLI [3,g] Kemal KARABAĞ [4,h] Emine ŞAHİN SEMERCİ [5,i] Murat Soner BALCIOĞLU [1,j]

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[1] Department of Animal Science, Faculty of Agriculture, Akdeniz University, TR-07058 Antalya - TURKEY
[2] Department of Animal Science, Faculty of Agriculture, Isparta University of Applied Sciences, TR-32260 Isparta - TURKEY
[3] Poultry Research Institute, TR-06170 Ankara - TURKEY
[4] Department of Agricultural Biotechnology, Faculty of Agriculture, Akdeniz University, TR-07058 Antalya - TURKEY
[5] Korkuteli Vocational School, Akdeniz University, TR-07800 Korkuteli-TURKEY
ORCIDs: [a] 0000-0003-4507-7426; [b] 0000-0002-2413-1713; [c] 0000-0001-5054-6720; [d] 0000-0002-1762-9847; [e] 0000-0002-7733-1372
[f] 0000-0001-6182-5450; [g] 0000-0003-1936-7750; [h] 0000-0002-4516-6480; [i] 0000-0001-8471-9300; [j] 0000-0002-9424-8319

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Abstract

This is the first comprehensive study aimed to determine polymorphisms in candidate genes (OCX32-exon4, OCX32-exon2, GHR-intron 2, GHR-intron 5, DRD1, DRD2, VIP-501, VIP-I2, VIPR-1, VIPR-2 and MR1C) reported to associated with egg yield and quality in white layer pure chicken lines (Black, Brown, Blue, Maroon and D-229) raised by Ankara Poultry Research Institute by using PCR-RFLP technique. A total of 11 gene regions were amplificated for 192 chickens and were then digested with specific restriction endonucleases to determine the genotypes. Although all chicken lines were monomorphic for VIPR-1/*Hha*I and VIP-501/*Vsp*I gene region, polymorphisms were detected in the rest of nine gene regions. The present study revealed that GHR-intron 2/*Hind*III and GHR-intron 5/*Nsp*I polymorphsims can be integrated with MAS studies to increase egg quality in white layer chicken lines (D-229 excluded) raised in Ankara Poultry Research Institute in the future. In addition, in order to increase egg yield DRD2 and MR1C gene may be used in MAS for all studied chicken lines, while VIP-I2/*Hinf*I polymorphism can be used for Maroon line and VIPR-2/*Taq*I polymorphism for Brown and D-229.

Keywords: *Candidate genes, PCR-RFLP, Polymorphism, Pure chicken lines*

Beş Farklı Beyaz Yumurtacı Saf Hatta Yumurta Verimi ve Kalitesi İle İlişkili Bazı Aday Genlerdeki Polimorfizmler

Öz

Mevcut çalışma Ankara Tavukçuluk Araştırma Enstitüsü tarafından yetiştirilen beyaz yumurtacı saf tavuk hatlarında (Black, Brown, Blue, Maroon ve D-229) daha önce yumurta verimi ve kalitesiyle ilişkili olduğu bildirilen aday genlerdeki polimorfizmlerin PCR-RFLP yöntemiyle belirlenmesini amaçlayan ilk kapsamlı çalışmadır. Toplam 11 gen bölgesi 192 tane tavuk için çoğaltılmış ve genotiplerin belirlenmesi için özgün restriksiyon endonükleazlar ile kesilmiştir. Bütün tavuk hatları VIPR-1/*Hha*I and VIP-501/*Vsp*I gen bölgeleri için monorfik olmasına rağmen geriye kalan dokuz gen bölgesinde polimorfizm belirlenmiştir. Bu çalışma Ankara Tavukçuluk Araştırma Enstitüsü'nde yetiştirilen beyaz yumurtacı saf hatlarda (D-229 hariç) yumurta kalitesini iyileştirmek için gelecekte yapılacak MAS çalışmalarında GHR-intron 2/*Hind*III and GHR-intron 5/*Nsp*I polimorfizmlerinin kullanılabileceğini ortaya çıkarmıştır. Ayrıca, yumurta verimini arttırmak için DRD2 ve MR1C genlerinin çalışılan bütün tavuk hatlarında, VIP-I2/*Hinf*I polimorfizminin sadece Maroon hattında, VIPR-2/*Taq*I polimorfizminin ise Brown ve D-229 hatlarında MAS çalışmalarında kullanılabileceği belirlenmiştir.

Anahtar sözcükler: *Aday genler, PCR-RFLP, Polimorfizm, Saf tavuk hatları*

INTRODUCTION

Although, poultry sector is of the highest intensification rate compared to other sectors related to livestock in Turkey, studies on breeding material production have been carried only by Ankara Poultry Research Institute. The institute

Correspondence
+90 242 3102480
eymendemir@akdeniz.edu.tr

holds a total of 11 pure chicken layer lines composed of 5 white (Black, Brown, Blue, Maroon and D-229) and 6 brown (Rhode Island Red-I, Rhode Island Red-II, Barred Rock-I, Barred Rock-II, Colombian Rock and Line-54) layer lines. Black, Brown, Blue and Maroon lines were imported from Canada in 1995, while D-229 was provided from Czechia in 2010. Since then, selection processes have been carried out to decrease age and body weight at first egg besides feed intake in these chicken lines. Thanks to studies on white chicken layer lines, a hybrid line called ATABEY was obtained [1-3].

In livestock species, traits that are of economic importance show generally quantitative inheritance. Therefore, the expression of these traits in phenotype depends on both numerous loci and environment conditions. In poultry, economically important traits such as egg yield and quality are of low heritability besides showing polygenic inheritance [4,5].

Today, many genes and their receptors such as Gonadotropin Releasing Hormone (*GnRH*) [4], Growth Hormone Receptor (GHR) [6], Prolactin (PRL) [7], Dopamine (DA) [8], Neuropeptide Y (NPY) [9], Vasoactive Intestinal Peptide (VIP) [10], Ovocalyxin-32 (OCX32) [11] and Melatonin [12] were reported to associate with egg yield and quality in chickens. According to previous studies, single nucleotide mutations in these genes are related to egg yield and quality in chickens [6,8].

Today, Restriction Fragment Length Polymorphism (RFLP) based on Polymerase Chain Reaction (PCR) is one of the commonly used molecular techniques to detect known mutations in livestock species [7,13,14]. Using mutations in these candidate genes separately or together in Marker Assisted Selection (MAS) may increase the success in the selection and genetic improvement in chickens [4,9,11].

Hence, this study aimed to determine polymorphisms in a total of 11 candidate genes (OCX32-exon4, OCX32-exon2, GHR-intron 2, GHR-intron 5, DRD1, DRD2, VIP-501, VIP-I2, VIPR-1, VIPR-2 and MR1C) reported to associated with egg yield and quality by using PCR-RFLP technique. The results obtained in the present study may be used by Ankara Poultry Research Institute for traditional selection studies supplemented with MAS in the future.

MATERIAL and METHODS

Ethical Approval

This research was approved by the Ankara Poultry Research Institute Animal Experiments Ethics Committee, Ankara, Turkey (Protocol No: 23.01.2015/03).

Animal Sampling

A total of 192 blood samples were randomly collected from five different white pure layer chicken lines including Black (n=40), Brown (n=40), Blue (n=40), Maroon (n=32) and D-229 (n=40) raised in Ankara Poultry Research Institute. The blood samples were taken from *venous cutenea ulnaris* into vacuum tubes containing EDTA as an anticoagulant. Blood samples were stored at -20°C until the DNA extraction step.

DNA Extraction

A salting-out method described by Miller et al.[15] was used to extract genomic DNA from blood samples. DNA quality and quantity were checked using agarose gel (1%) and spectrophotometer (NanoDrop-SD 1000). DNA concentration was adjusted to 50 ng/µL for PCR-RFLP analysis.

PCR-RFLP Analysis

In total, 11 candidate genes and/or receptors (OCX32-exon4, OCX32-exon2, GHR-intron 2, GHR-intron 5, DRD1, DRD2, VIP-501, VIP-I2, VIPR-1, VIPR-2 and MR1C) reported in previous studies *(Table 1)* were selected. PCR was performed in 20 µL reaction volume containing 1.2 µL HQ buffer (GeneAll), 2 µL 10X buffer (GeneAll), 2.5 mM dNTPS, 10 pM of each primer, 2.5 U *Taq* DNA Polymerase (GeneAll), 50 ng template DNA and 11.4 µL H_2O. PCR amplifications were applied in initial denaturation at 94°C for 5 mins, followed by 30 cycles at 94°C for 45 s, at 50-62°C *(Table 1)* for 45 s and at 72°C for 50 s. The final extension was carried out at 72°C for 5 min.

Amplificated PCR products were digested with restriction endonucleases *(Table 1)* to genotype the animals. Hence, 8 µL of amplified PCR products were mixed with 2.5 U restriction enzymes *(Table 1)* and 8 µL 10X buffer. After incubation, digested RFLP fragments were visualized on agarose gel electrophoresis to detect the genotypes.

Statistical Analysis

Popgene V. 1.32. [17] package program was used to calculate the genotype and allele frequencies in the studied candidate genes. Deviation from Hardy-Weinberg equilibrium was tested by chi-square (χ^2) statistics.

RESULTS

In this study, polymorphisms in a total of 11 candidate genes reported to be associated with egg yield and quality were assessed in five different white layer pure lines by using the PCR-RFLP technique. Primer sets given in *Table 1* were used to amplify studied gene regions. PCR products were digested with specific restriction endonucleases *(Table 1)* after the PCR process in order to detect genotypes. Both amplified and digested products for MR1C gene were given as an example in *Fig. 1*, *Fig. 2*.

All chicken lines were monomorphic for VIPR-1/*Hha*I and VIP-501/*Vsp*I polymorphisms. All individuals were

Table 1. *Some descriptive information about PCR-RFLP process*

Gene	Primers (5'-3'	Chr.	Ann. Temp. (°C)	PCR Size	Restriction Enzyme	Expected Product Size	Ref.
GHR-intron 2	F:GGCTCTCCATGGGTATTAGGA R: GCTGGTGAACCAATCTCGGTT	Z	59	718	*Hind*III	A_1A_1: 428-290 A_2A_2:170-258-290	[6]
GHR-intron 5	F: ACGAAAAGTGTTTCAGTGTTGA R: TTTATCCCGTGTTCTCTTGACA	Z	56	740	*Nsp*I	CC: 550-190, CD:740, 550, 190 DD: 740	[6,16]
DRD1	F:CACTATGGATGGGGAAGGGTTG R: GCCACCCAGATGTTGCAAAATG	13	62	283	BseNI *Cfr*I	AA: 111-172, AG: 111-172-283 GG: 283	[8]
DRD2	F:TGCACATAAAAGCCCACTCACTG R:GCCTGAGCTGGTGGGGGG	24	60	248	*BseG*I	CC: 248, TC: 248-196, TT: 196	[4]
VIP/501	F:GAAACCCATCTCAGTCATCCTA R:ACCACCTATTTTTCCTTTTCTACA	3	55	306	*Vsp*I	II: 306, DI: 306-154, 152, DD: 154, 152	[10]
VIP/I2	F: GCTTGGACTGATGCGTACTT R: GTATCACTGCAAATGCTCTG	3	58	520	*Hinf* I	CC: 480, CT: 520-480 TT: 520	[10]
VIPR-1	F:CCCCGTTAAACTCAGCAGAC R:CCCAAAGTCCCACAAGGTAA	2	58	434	*Hha*I	TT: 434, TC: 434-253-181 CC 253-181	[4]
VIPR-2	F:CTCCTCAGGCAGACCATCATG R:CTTGCACGTATCCTTGGGTAGC	2	58	486	*Taq*I	TT: 486, TC: 486-310-176 CC: 310-176	[4]
OCX32-exon4	F: TGTTTCTGATGAAGAGCCAGA R: CTTTGCCACTCTGTAGGCTGT	9	58	250	*Nco*I	AA: 250 AC: 250-194 CC: 194	[11]
OCX32-exon2	F: GCCCACTGGTCAGAAAAGAA R: CCTGCAGAGGAAAAGAGCTG	9	58	405	*HpyCH4*IV	TT: 237-169, TG: 237-169-151 GG: 237-151	[11]
MR1C	F: GGTGTATCCGTATCCTCTAA R: GACAGTGGGACAATGAAGT	4	50	372	*Mbo*I	AA: 372, AG: 372-333 GG:333	[12]

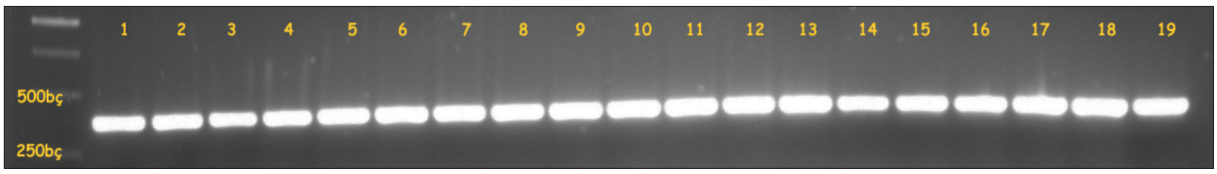

Fig 1. Agarose gel image of PCR products for MR1C gene
(Marker: Thermo, 1kb, Kat. No: SM0311; 1% agarose gel, fragment size 372 bp)

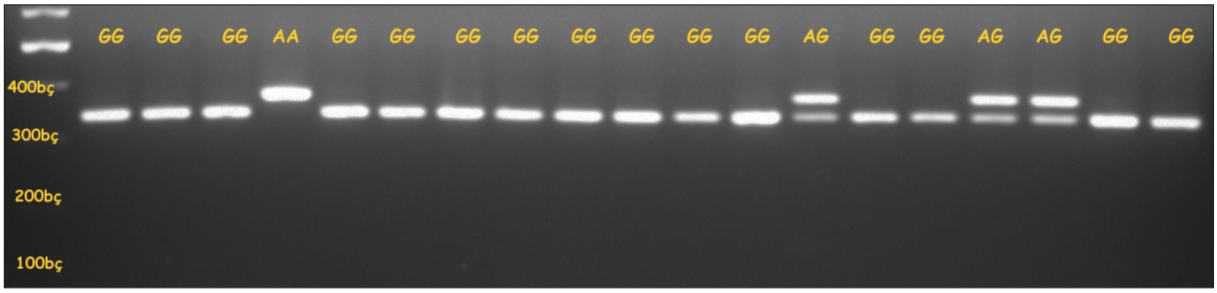

Fig 2. Agarose gel image of digested PCR products of MR1C gene with *Mbo*I restriction enzyme
(Marker: Thermo, 100 bp, Kat. No: SM0241; 2% agarose gel, fragment size GG: 333 bp, AG: 333-372 bp, AA: 372 bp)

with CC and II genotype for VIPR-1/*Hha*I and VIP-501/*Vsp*I polymorphisms, respectively. Except for VIPR-1 and VIP-501, the rest of the nine genes or receptors were found polymorphic. Allele and genotype frequencies for nine polymorphic gene regions are given in *Table 2*.

D-229 line was monomorphic for both GHR-intron 2/*Hind*III and GHR-intron 5/*Nsp*I polymorphisms. A_1A_1 genotype frequency ranged from 0.05 (Blue) to 0.72 (Maroon) in GHR-intron 2/*Hind*III polymorphism, whereas DD genotype frequency varied from 0.43 (Brown) to 0.95 (Blue) in GHR-intron 5/*Nsp*I polymorphism.

Black, Blue, Maroon and D-229 lines were detected to be monomorphic (GG genotype) in DRD1/*BseN*I polymorphism. On the contrary, AA and GG genotype frequencies were 0.10 and 0.90, respectively in Brown lines with significant deviation from HW equilibrium. In DRD2/*BseG*I polymorphism, all chicken lines were polymorphic with significant deviation from HW equilibrium. The lowest

Table 2. *Allele and genotype frequencies of studied genes in five white pure chicken lines*

Chicken Line	Loci	n	Allele Frequency		Genotype Frequency			χ^2
BLACK	GHR-intron2	39	A_1/0.33	A_2/0.67	A_1A_1/0.33	A_1A_2/0.00	A_1A_2/0.67	39.00[b]
	GHR-intron5	38	C/0.30	D/0.70	CC/0.16	CD/0.29	DD/0.55	3.75[a]
	DRD1	40	A/0.00	G/1.00	AA/0.00	AG/0.00	GG/1.00	-
	DRD2	26	C/0.31	T/0.69	CC/0.04	TC/0.54	TT/0.42	1.81[a]
	VIP/I2	40	C/1.00	T/0.00	CC/1.00	CT/0.00	TT/0.00	-
	VIPR-2	39	C/0.96	T/0.04	CC/0.96	CT/0.02	TT/0.02	16.65[b]
	OCX32-exon4	39	A/0.19	C/0.81	AA/0.05	AC/0.28	CC/0.67	0.33[a]
	OCX32-exon2	32	G/0.61	T/0.39	GG/0.56	GT/0.09	TT/0.35	00.64[b]
	MR1C	39	A/0.33	G/0.67	AA/0.05	AG/0.56	GG/0.39	2.83[a]
BLUE	GHR-intron2	40	A_1/0.05	A_2/0.95	A_1A_1/0.05	A_1A_2/0.00	A_2A_2/0.95	40.00[b]
	GHR-intron5	40	C/0.02	D/0.98	CC/0.00	CD/0.05	DD/0.95	0.03[a]
	DRD1	40	A/0.00	G/1.00	AA/0.00	AG/0.00	GG/1.00	-
	DRD2	40	C/0.42	T/0.58	CC/0.15	TC/0.55	TT/0.30	0.63[a]
	VIP/I2	40	C/1.00	T/0.00	CC/1.00	CT/0.00	TT/0.00	-
	VIPR-2	40	C/1.00	T/0.00	CC/1.00	CT/0.00	TT/0.00	-
	OCX32-exon4	40	A/0.40	C/0.60	AA/0.00	AC/0.80	CC/0.20	17.78[b]
	OCX32-exon2	23	G/0.11	T/0.89	GG/0.09	GT/0.04	TT/0.87	13.84[b]
	MR1C	40	A/0.09	G/0.91	AA/0.02	AG/0.12	GG/0.86	1.89[a]
D-229	GHR-intron2	40	A_1/0.00	A_2/1.00	A_1A_1/0.00	A_1A_2/0.00	A_2A_2/1.00	-
	GHR-intron5	40	C/0.51	D/0.49	CC/0.02	CD/0.98	DD/0.00	36.19[b]
	DRD1	40	A/0.00	G/1.00	AA/0.00	AG/0.00	GG/1.00	-
	DRD2	40	C/0.17	T/0.82	CC/0.02	TC/0.30	TT/0.68	0.06[a]
	VIP/I2	32	C/1.00	T/0.00	CC/1.00	CT/0.00	TT/0.00	-
	VIPR-2	40	C/0.75	T/0.25	CC/0.70	CT/0.10	TT/0.20	21.51[b]
	OCX32-exon4	39	A/0.23	C/0.77	AA/0.03	AC/0.41	CC/0.56	0.944[a]
	OCX32-exon2	28	G/0.39	T/0.61	GG/0.29	GT/0.21	TT/0.50	8.49[b]
	MR1C	40	A/0.40	G/0.60	AA/0.17	AG/0.45	GG/0.38	0.16[a]
BROWN	GHR-intron2	40	A_1/0.57	A_2/0.42	A_1A_1/0.57	A_1A_2/0.00	A_2A_2/0.42	40.00[b]
	GHR-intron5	40	C/0.29	D/0.71	CC/0.00	CD/0.57	DD/0.43	6.51[b]
	DRD1	40	A/0.10	G/0.90	AA/0.10	AG/0.00	GG/0.90	40.00[b]
	DRD2	40	C/0.26	T/0.74	CC/0.07	TC/0.37	TT/0.56	0.04[a]
	VIP/I2	40	C/1.00	T/0.00	CC/1.00	CT/0.00	TT/0.00	-
	VIPR-2	40	C/0.99	T/0.01	CC/0.98	CT/0.02	TT/0.50	0.01[a]
	OCX32-exon4	40	A/0.54	C/0.46	AA/0.12	AC/0.82	CC/0.06	17.40[b]
	OCX32-exon2	33	G/0.35	T/0.65	GG/0.21	GT/0.27	TT/0.52	5.26[b]
	MR1C	40	A/0.11	G/0.89	AA/0.02	AG/0.17	GG/0.81	0.61[a]
MAROON	GHR-intron2	32	A_1/0.72	A_2/0.28	A_1A_1/0.72	A_1A_2/0.00	A_2A_2/0.28	32.00[b]
	GHR-intron5	31	C/0.61	D/0.39	CC/0.09	CD/0.34	DD/0.57	0.45[a]
	DRD1	32	A/0.00	G/1.00	AA/0.00	AG/0.00	GG/1.00	-
	DRD2	32	C/0.69	T/0.31	CC/0.37	TC/0.63	TT/0.00	6.61[b]
	VIP/I2	29	C/0.78	T/0.22	CC/0.66	CT/0.24	TT/0.10	2.71[a]
	VIPR-2	40	C/1.00	T/0.00	CC/1.00	CT/0.00	TT/0.00	-
	OCX32-exon4	32	A/0.40	C/0.60	AA/0.12	AC/0.36	CC/0.52	0.89[a]
	OCX32-exon2	24	G/0.60	T/0.40	GG/0.50	GT/0.21	TT/0.29	7.65[b]
	MR1C	32	A/0.40	G/0.60	AA/0.00	AG/0.53	GG/0.47	4.19[b]

χ^2: 0.05; [1] 3.84; [a] Deviation from HWE is non-significant; [b] Deviation from HWE is significant

and the highest C allele frequency were observed in D-229 (0.17) and Maroon (0.69) lines, whereas T allele frequency ranged from 0.31 (Maroon) to 0.82 (D-229). No TT genotype was detected in Maroon line, while it was observed in D-229 line with a high frequency (0.68).

Polymorphism was detected in Black, Brown and D-229 lines, while Blue and Maroon lines were monomorphic (CC genotype) in VIPR-2/*Taq*I polymorphism. The frequency of desired genotype (TT) for the number of egg at 300 days of age were 0.50, 0.20 and 0.02 in Brown, D-229 and Black line, respectively. Only Maroon line showed polymorphism in VIP-I2/*Hinf*I polymorphism. CC, CT and TT genotype frequencies were 0.66, 0.24 and 0.10, respectively in Maroon line. All individuals from other lines were with CC genotype in VIP-I2/*Hinf*I polymorphism.

AA genotype frequency ranged from 0.00 (Maroon) to 0.17 (D-229), whereas GG genotype frequency varied from 0.38 (D-229) to 0.86 (Blue) in MR1C gene. The frequency of desired genotype (AG) for the number of egg at 300 days of age were 0.56, 0.12, 0.45, 0.17 and 0.53 in Black, Blue, D-229, Brown and Maroon line, respectively. In addition, all populations except Maroon line were in HW equilibrium for MR1C gene region.

All studied chicken lines were polymorphic in OCX32-exon2/*Hpy*CH4IV and OCX32-exon4/*Nco*I polymorphisms. G allele frequency ranged from 0.11 (Blue) to 0.61 (Black), while T allele frequency varied from 0.39 (Black) to 0.89 (Blue) in OCX32-exon2/*Hpy*CH4IV polymorphism. The highest GG, GT and TT genotype frequencies were observed in Black (0.56), Brown (0.27) and Blue (0.87) line, respectively. An allele frequency ranged from 0.19 (Black) to 0.54 (Brown), while C allele frequency varied from 0.46 (Brown) to 0.81 (Black) in OCX32-exon4/*Nco*I polymorphism. The lowest and the highest AA genotype frequencies were detected in Blue (0.00) and Brown (0.12) lines, respectively. The highest AC and CC genotype frequencies were detected in Brown (0.82) and Black (0.67) line, respectively. A significant deviation from HW equilibrium was detected in Brown and Blue lines for the OCX32-exon4 gene, while significant deviation from HW equilibrium was observed in all chicken lines for the OCX32-exon2 gene.

DISCUSSION

Li et al.[6] have been reported that GHR-intron 2/*Hind*III polymorphism is related to egg quality in Wenchang chickens, in which individuals with A_1A_1 genotype had higher number of double yolk eggs. Additionally, A_2A_2 genotype frequency was reported as 0.94 in Wenchang chickens by Li et al.[6]. In the present study, no A_1A_1 genotype was detected in D-229 line, whereas its frequency was varied from 0.05 (Blue) to 0.72 (Maroon) in other chicken lines. In addition, Li et al.[16] have been reported that GHR-intron 5/*Nsp*I is associated with eggshell thickness in Wenchang chickens, in which individuals with DD genotype were advantageous in terms of eggshell thickness. In Wenchang chickens, C and D allele frequencies were reported as 0.20 and 0.80 [16], respectively while in Mazandaran native chickens C and D allele frequencies were 0.72 and 0.28 [18], respectively for GHR-intron 5/*Nsp*I polymorphism. In the present study, no DD genotype was detected in D-229 line, while it was observed in other chicken lines with high frequencies (0.43-0.95)

The present study revealed that polymorphisms detected by *Hind*III and *Nsp*I restriction enzymes in intron 2 and intron 5 regions of GHR gene may be used in MAS studies in White chicken lines excluding D-229 raised in Ankara Poultry Research Institute. No desired genotype was detected in D-229 line for both GHR-intron 2/*Hind*III and GHR-intron 5/*Nsp*I polymorphism. It is thought to be due to breeding history since D-229 line was imported from Czechia in 2010, while the other lines were provided from Canada in 1995. Also, the frequency of desired genotypes was variable among the other chicken lines. Although these chicken lines originated from the same genetic resource (White Leghorn), the underlying reason for this situation is thought to be the long-term selection process applied in these lines.

It has been reported that DRD1 gene is associated with total egg production and broodiness frequency, while DRD2 gene is related to egg number at 300 days of age in chicken [4,8]. Chickens with AA genotype reported being shown a superior number of total egg than chickens with GG and AG genotypes in DRD1/*BseNI CfrI* polymorphism [8]. Additionally, individuals with TT genotype reported being shown superior egg number at 300 days of age than individuals with CC and TC genotypes in DRD2/*BseGI* polymorphism [4].

In the present study, the desired genotype (AA) was observed in only Brown line with very low frequency (0.10) in DRD1/*Bse*NI polymorphism, whereas the desired genotype (TT) was detected in all chicken lines with high frequency except Maroon in DRD2/*Bse*GI polymorphism. It was observed that DRD2 gene polymorphism may be used for MAS studies in the future, while DRD1 gene cannot be used in MAS due to a lack of polymorphism.

It has been reported that VIPR-1 and VIPR-2 genes are associated with egg number at 300 days in chickens [4]. Superior values for egg number at 300 days were reported for CC and TC genotypes than TT genotype in VIPR-1/*Hha*I polymorphism and for TT genotype than CC and TC genotypes in VIPR-2/*Taq*I polymorphism [4]. CC, TC and TT frequencies were reported as 0.935, 0.060 and 0.005, respectively in Ningdu Sanhuang chicken [4]; 0.64, 0.29 and 0.07, respectively in Vietnam Voi chicken [19] in VIPR-1/*Hha*I polymorphism. CC, TC and TT frequencies were reported as 0.698, 0.209 and 0.093, respectively in Ningdu Sanhuang chicken [4]; 0.48, 0.33 and 0,19, respectively in Vietnam Voi chicken [19] in VIPR-2/*Taq*I polymorphism.

In the present study, all chicken lines were monomorphic (CC genotype). Although similar CC genotype frequency (0.934) was reported in Ningdu Sanhuang chicken [4], lower CC genotype frequency (0.64) was reported in Wietnam Voi chicken [19]. The main reason of monomorphism could be attributed that these chicken lines were derived from the same genetic origin (White Leghorn). Although all chicken lines were of the desired genotype for egg number at 300 days, VIPR-1/*Hha*I polymorphism cannot be used in MAS studies due to lack of polymorphism.

The desired genotype (TT) frequency for VIPR-2/*Taq*I polymorphism was reported as 0.09 and 0.19 in Ningdu Sanhuang and Vietnam Voi chicken, respectively [4,19]. In the present study, the higher TT genotype frequency (0.50) was detected in the Brown line conserving enough genetic variability. Hence, it is determined that VIPR-2/*Taq*I polymorphism may be used in MAS studies for the Brown line. Although significant deviation from HW equilibrium was observed in D-229 line, TT genotype frequency (0.20) was sufficient. VIPR-2/*Taq*I polymorphism may be used in MAS studies for D-229 line but it is crucial not to decrease genetic variability in this chicken line.

Zhou et al.[10] reported that "AGG" indel was associated with both total and the qualified number of eggs from 90 to 300 days of age in Nindu Sanhuang chickens. In contrast, C+338T was reported to be associated with the duration of broodiness [10]. D allele was reported to be shown superior values for the total number of qualified eggs from 90 to 300 days of age in VIP-501/*Vsp*I polymorphism [10]. CT genotype was reported to be an advantageous genotype for egg production and broodiness traits in VIP-I2/*Hinf*I polymorphism [10]. In the present study, no desired allele (D allele) was detected in five chicken lines of which all individuals were with II genotype in VIP-501/*Vsp*I polymorphism. Similarly, only Maroon line showed polymorphism for VIP-I2/*Hinf*I polymorphism. Hence, it was determined that VIP-501/*Vsp*I cannot be used in MAS studied chicken lines, while *VIP-I2/Hinf*I can be used in MAS for only Maroon line.

Li et al.[12] reported that MR1C/*Mbo*I polymorphism was associated with both age at first egg and egg number at 300 days in chickens, in which individuals with AG genotype showed superior egg number at 300 days than individuals with GG and AA genotypes. A and G allele frequencies were reported as 0.49 and 0.51, respectively, while AA, AG and GG genotype frequencies were 0.17, 0.64 and 0.19, respectively in Wenchang chicken [12]. On the contrary, Padwar and Thakur [20] were reported that Kadaknath and Jabalpur chickens were monomorphich (AA genotype) in terms of MR1C/*Mbo*I polymorphism. In the present study, detected AG genotype frequencies (ranging from 0.12 to 0.56) in five chicken lines were lower than the findings reported by Li et al.[12]. This may be attributed to used different chicken breeds and lines. Significant differences in terms of AG genotype frequency were observed among studied chicken lines, although they were derived from the same breed. This difference is thought to result from the selection process for different purposes.

Uemoto et al.[11] reported that mutations (c.267T>G, c.494A>C and c.381G>C) on OCX32 gene were related to egg production ratio, yellowness and frequencies of meat spot in White Leghorn and Rhode Island Red breeds. G and T allele frequencies were reported as 0.40 and 0.60, respectively in White Leghorn breed for OCX32/*HpyCH4*IV polymorphism [11]. In the present study, similar values were detected in D-229 and Brown lines.

Additionally, C and A allele frequencies were reported as 0.40 and 0.60, respectively in White Leghorn breed for OCX32/*Nco*I polymorphism [11]. In the present study, similar C (0.46) and A (0.54) allele frequencies were detected in the Brown line, while the frequencies were different in other studied chicken lines. This difference could be attributed to many factors such as the selection process and breeding system. This idea was also supported by HW equilibrium values detected for OCX32/*HpyCH4*IV polymorphism. Indeed, a significant deviation from HW equilibrium was detected in only Black lines. Inbreeding and selection processes may cause rapid changes in allele frequencies and deviation from HW equilibrium.

Consequently, this is the first comprehensive study aimed to determine polymorphisms in a total of 11 genes and receptors associated with egg yield and quality in five white layer lines reared by Ankara Poultry Research Institute by using PCR-RFLP. The present study revealed that GHR-intron 2/*Hind*III and GHR-intron 5/*Nsp*I polymorphisms can be integrated with MAS studies to increase egg quality in White chicken lines (D-229 excluded) raised in Ankara Poultry Research Institute in the future. In addition, in order to increase egg yield, DRD2 and MR1C gene may be used in MAS for all studied chicken lines, while VIP-I2/*Hinf*I polymorphism can be used for only Maroon line and VIPR-2/*Taq*I polymorphism for Brown and D-229. It is also important to highlight that association analysis between candidate genes with egg yield and quality should be conducted in five White chicken lines before applying MAS. In addition, the results of the present study showed once again the effects of selection and inbreeding on genetic structure. Despite of deriving from the same genetic origin (White Leghorn), in five chicken lines, allele and genotype frequencies have differentiated due to selection applied for different purposes and inbreeding.

Author Contributions

MSB, TK and SA designed the project. SK provided samples. ED, BAK, MA, HGF and ESS performed DNA extraction and PCR-RFLP stages. ED, TK, MSB, KK and SA performed statistical analysis of data and wrote the article.

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Research Article

Sero-detection of Foot and Mouth Disease Virus Serotypes A and O in One-humped Camels *(Camelus dromedarius)* in the Middle of Iraq

Saad Hashim AL-HUSSEINY [1,a] Qassim Haleem KSHASH [1,b] Asaad JASSIM [1,c]

[1] Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Al-Qadisiyah, IRAQ
ORCIDS: [a] 0000-0002-0966-9515; [b] 0000-0003-4856-625X; [c] 0000-0002-3110-2056

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Abstract

Foot and mouth disease (FMD) is a severe trans-boundary animal disease caused by a foot and mouth disease virus (FMDV) that spreads through Asia and Arabic countries. The current study aimed to investigate the FMDV antibodies in Iraqi dromedary's camels (Camelus dromedaries). A total of 520 serum samples were collected from clinically healthy camels (265 females and 255males) from different areas in the three Provinces of Iraq (Al-Najaf, Al-Muthanna, and Al-Qadisiyah) from February to July 2019 and divided into three groups based on the age of the camels. All sera samples were screened for antibodies against the non-structural protein (NSP) of FMDV using ELISA and further the NSP positive serum samples were screened for antibodies against structural proteins of FMDV serotype O A and C by liquid phase blocking ELISA (LPB-ELISA).The Result indicated that 10% of the sera were positive for NSP antibodies and FMDV serotype A found to be predominant. It was also observed that NSP positive was more in samples collected from female camels (11.30%) than male camels (8.60%), NSP FMDV antibodies were detected in camels of all ages. In summary, our study showed, for the first time in Iraq, that camels are more susceptible to the A and O FMD serotypes.

Keywords: *FMD, NSP, Camels, LPBE, Iraq*

Irak'ın Orta Bölgesinde Tek Hörgüçlü Develerde *(Camelus dromedarius)* Şap Hastalığı Virüsü Serotipleri A ve O'nun Sero-Tespiti

Öz

Şap hastalığı (FMD), Asya ve Arap ülkelerine yayılan ayak ve ağız hastalığı virüsünün (FMDV) neden olduğu ciddi bir sınır ötesi hayvan hastalığıdır. Bu çalışmada Irak tek hörgüçlü develerinde *(Camelus dromedaries)* FMDV antikorlarının araştırılması amaçlandı. Şubat-Temmuz 2019 tarihleri arasında Irak'ın üç vilayetinde (El-Necef, El-Muthanna ve El-Kadisiyah) farklı bölgelerden klinik olarak sağlıklı develerden (265 dişi ve 255 erkek) toplam 520 adet serum örneği toplandı ve develerin yaşına göre üç gruba ayrıldı. Tüm serum numuneleri, ELISA kullanılarak FMDV'nin yapısal olmayan proteinine (NSP) karşı antikor varlığı yönünden tarandı ve NSP pozitif serum örnekleri, sıvı fazı bloke eden ELISA (LPBE) ile FMDV serotipi O, A ve C'nin yapısal proteinlerine karşı antikor varlığının tespiti amacıyla tarandı. Sonuçlar, serumların %10'unun NSP antikorları için pozitif olduğunu ve FMDV serotip A'nın baskın olduğunu gösterdi. Ayrıca NSP pozitiflik oranının dişi develerden (%11.30) toplanan örneklerde erkek develerden (%8.60) daha fazla olduğu, NSP FMDV antikorlarının her yaştan devede bulunduğu belirlendi. Özetle, bu çalışma ile Irak'ta ilk kez develerin A ve O FMD serotiplerine daha duyarlı olduğu tespit edildi.

Anahtar sözcükler: *FMD, NSP, Deve, LPBE, Irak*

INTRODUCTION

Foot and Mouth Disease (FMD) is a highly contagious viral disease that can affect all species of cloven-hoofed animals and wildlife, including cattle, buffaloes, sheep, goat, pigs, elephant, camel, and deer, causing severe economic loss in the livestock industries worldwide [1]. FMD is caused by FMD virus (FMDV) belonging to the genus Aphthovirus, which has seven serotype es namely FMD serotype A, O, C, SAT 1, SAT 2, SAT 3 and Asia1[2]. Although the mortality rate for FMD is below 5%, the disease still leads to a significant loss in livestock productivity and trade. The virus can cause

Correspondence
+964 770 2822276 (Mobile)
saad.ghmeiss@qu.edu.iq

severe lesions in the myocardium of young animals, resulting in death with a high mortality rate among younger animals [3]. Camels are important domestic animals in many areas of the world, such as the Arabian countries and countries in middle and East Asia, North and East Africa, as well as in South America and the disease is endemic in these regions. Camelidae are susceptible to FMD, similar to cattle, sheep, goats, and pigs [4], with clearly distinguishable clinical signs.

Iraq is home to approximately 58000 dromedary camels of two breeds and there is significant utilization of camel milk, meat, leather, and wool. Most camels are pastured in close groups with herds of other large and small ruminants [5]. FMD is endemic in Iraq, and the first notable case of FMD was recorded in 1937, and in 1952 FMD outbreaks are regularly reported among the large and small ruminants in various regions of Iraq [1,5] and the predominant FMDV serotypes detected were O, A, and Asia1. The prevalence rate of FMD in Iraq was 68.7%, 46.6%, and 30% in cattle, buffalo, and small ruminants, respectively. However, there is no information on the prevalence and detection of FMD virus (FMDV) in one-humped camels in Iraq [6]. The viral isolation and ELISA are the gold standards in FMD diagnosis, and the most reliable indicator of FMD viral infection is the detection of antibodies in the serum against polyproteins 3ABC using liquid phase blocking ELISA (LPBE) [7], the Non-structural protein (NSP) enzyme linked immune sorbent assay (ELISA) test discriminates the animals that have been infected from those that have been vaccinated and the test would be able to detect continued viral circulation and would therefore be extremely useful for serological surveys. Given the absence of prior data on the sero-surveillance, in the present study we investigated the serological evidence of the natural exposure of camels, which were reared together with ruminants, to the FMD virus [8].

MATERIAL and METHODS

This study was carried out with the permission of Ethical Committee in the College of Veterinary Medicine, University of Al-Qadisiyah under Ref No. 543/2018.

Animals and Study Area

The study was conducted using 520 Arabian one-humped camels *(Camelus dromedarius)* sampled over a period of six months (February to July 2019) from areas distributed in three Provinces (Al-Najaf, Al-Muthanna, and Al-Qadisiyah Provinces) in the middle of Iraq, which include the central area stretching from the Euphrates to the western frontier, towards the border with Saudi Arabia. This is the region where the majority of Iraqi camels are grazed.

Sampling

In total, 520 blood samples were collected by jugular vein puncture using vacutainer tubes containing serum clot activators from apparently healthy Arabian camels (255 males and 265 females) depending on the clinical examination and case history as well as general conditions and their activities, appetite and close inspection for each lesions or any abnormal behaviors within the herds. Of these, 120 blood samples were collected from slaughtered camels (100 males and 20 females) in an abattoir in the Al-Najaf Province and 400 blood samples were collected from camels (155 males and 245 females) that were in daily contact with ruminants during rearing or grazing. The blood samples were immediately mixed and transported to laboratory on wet ice and refrigerated overnight. The separated serum was centrifuged at 3000 g for 10 min, aliquot, labeled and stored at -20°C until further use. The sera were grouped based on the age of the camels as; under one-year group (<1y), 125 samples, 60 males and 65 females, the1-3y group, (which included 253 samples, 148 males and 105 females) and the >3y group (87 samples, 47 males and 40 females).

Detection of Antibodies Against NSP of FMDV

The sera were screened using the FMDV-ELISA kit (PrioCHECK -prionics, Lelystad B.V., Netherlands) to detect antibodies against the non-structural protein (NSP) of the FMDV. This assay was performed according to the manufacturer's instructions. The optical density was read at 450 nm, and results were expressed as percentage inhibition (PI), which was calculated according to the formula below:

$$PI = 100 - (OD\ test\ sample/OD\ Neg) \times 100$$

Sera with PI >50% were scored as positive [8].

Liquid Phase Blocking Enzyme ELISA (LPBE)

The sera that were scored as positive for NSP by ELISA were further screened using the LPBE kit (FMD World Reference Laboratory, Pirbright Institute, UK) for the detection of antibodies against structural proteins of the three FMDV serotypes (O, A, and C) according to the protocol prescribed by the manufacturer. Optical densities (OD) were read using a microplate reader at 492 nm. A positive reaction was that in which the OD was reduced by more than 50% compared with the OD of the reference antigen controls, as described previously [9].

Statistical Analysis

The obtained data were statistically analyzed for the means and significances between the groups by ANOVA using the SPSS software (IBM SPSS Statistics, version7).

RESULTS

A total of 52 sera from the camels were positive for NSP of FMDV (10%; 52\520), and a higher incidence of FMD infection was observed in female camels (11.3%; 30\265) than in males (8.6%; 22\255) *(Table 1)*.

Table 1. *Sero-positive detection of antibodies against NSP of FMD virus in dromedary camels*

Sex	No. of Tested Samples	No. of Seropositive Samples	%
Male	255	22	8.6[a]
Female	265	30	11.3[b]
Total	520	52	10

Different superscript letters refer to significant variations (P≤0.05)

Table 2. *Sero-positive detection of antibodies against NSP of FMD in camels of different age groups*

Age in Years	No. of Tested Samples	No. of Seropositive Samples	%
< 1	125	14	11.2[a]
1-3	253	22	8.6b
> 3	142	16	11.2[a]
Total	520	52	10

Different superscript letters refer to significant variations (P≤ 0.05)

Table 3. *LPBE results of the serotype analysis in the NSP-positive sera*

Sex	Total	FMD Serotypes							
		A+	%	O+	%	C+	%	-ve	%
Male	22	11	50[aA]	8	36.3[Ba]	-	-	3	13.6[aC]
Female	30	19	63.3[bA]	8	26.6[bB]	-	-	3	10[aC]
Total	52	30	57.6[A]	16	30.7[B]	-	-	6	11.5[aC]

Differences in superscript small letters refer to significant vertical variation and differences in superscript capital letters refer to significant horizontal variation (P≤0.05)

A significantly higher percentage of seropositive samples was recorded in the <1 y and >3 y groups (11.2% in both) than in the 1-3y group (8.6%; 22\253) *(Table 2)*.

The result of LPBE revealed that the FMDV serotypes A and O were detected in the NSP positive serum samples. The predominant FMDV serotype in all NSP positive sera was the A serotype (57.6%; 30\52) and 16 out of 52 sera were positive for the O serotype (30.7%; 16\52).The C serotype was not detected in the screened sera, and 6 out of 52 sera were negative for all three FMD serotypes. Further, the A serotype was predominantly detected in female camels (63.3%), as compared to males (50%) *(Table 3)*.

Higher incidence of infection was observed in camels during April (55.7%; 29\52), then in March (23.07% ;12\52), so in May (17.3%;9\52) but incidence of infection was lower during February (3.8%; 2\52). No infection was detected during June and July.

DISCUSSION

This study was the first to detect antibodies against NSP of FMDV in the sera of dromedary camels in Iraq. The result revealed that 10% of the screened sera collected from Iraqi camels exhibited sero-evidence of NSP FMD indicating the exposure of dromedaries to FMDV infection and these results are consistent with the report of the ability of dromedaries to develop specific antibodies against FMDV [10]. Findings of the present study are in agreement with other published reports on the detection of FMD antibodies in one-humped camels in other countries such as Nigeria [11] , the Kingdom of Saudi Arabia [12] and Egypt [13] using the ELISA test. It was reported that FMD infection in dromedary camels in Egypt acts as the source of infection of other susceptible animals [14]. However, many studies failed to detect FMD antibodies in camels in Sudan [15], and in the United Arab Emirates [16]. The sero-negative FMD detection and failure to isolate FMD from camels have also been previously reported and that suggest these examined camels were not exposure to FMDV, or they developed very low titer of non detectable specific antibodies [17].

In our study, NSP-FMD antibodies were detected more in female camels than in males, and camels less than a year old and more than 3 years old exhibited a higher incidence of FMD infection. Although there are no reports on the effect of age and gender on FMD infection in camels, similar results have been reported in other ruminants, such as cattle [18,19]. The physiological and hormonal differences between both sexes, such as gestation, calving, and lactation in female cattle can act as stressors that influence the immune status and decrease resistance to many microbial infections. The higher incidence FMD virus in female camels may be due to breeding practices in camel

rearing, which permit female camels to come into contact with other camels and other animals in the pasture. This along contact with infected animals in addition with handlers during milking, may underlie more exposure to FMDV caused the increased seropositivity for FMD infection in female camels.

Camel husbandry practices also permit younger camels to associate closely with other susceptible small ruminants, especially sheep (because young camels are smaller in size compared with older camels); this might increase exposure rates to the disease source. The higher incidence of seropositivity for FMD infection in aged camels could be attributed to excessive exposure times to FMD source that increased with age while younger camels are homestead and in less contact with other animals in pasture or in workplace [20].

In our study, the A and O serotypes were detected in serum samples collected from Iraqi camels with the A serotype being the predominant serotype. This is consistent with earlier published reports where FMDV serotypes A and O were detected in camels [11]. The occurrence of two FMDV serotypes A and O in ruminant population was officially registered in Iraq, Iran, Turkey, Saudi Arabia, and Jordan and the O serotype was reported in Kuwait and it was shown that the camels were affected with endemic FMDV serotypes when they are contact with the susceptible ruminants [10]. The LPBE results revealed that six sera that were positive for NSP FMD were negative for the A, O, and C serotypes, suggesting that infected camels are sero-converted but with very low titer of antibodies depending on route of infection or these samples were positive for other FMD virus serotypes that were not checked by the kit, such as SAT1 and Asia, as previously reported in Iraq [21]. On other hand, the antibodies against FMD virus serotype C was not detected in camels sera but A and O serotypes were detectable, our finding is in agreement with the study about FMD infection in ruminants in middle of Iraq [22] which indicates that the A and O FMDV serotypes are predominant and most FMD outbreak was caused by A serotype but not C serotype [23].

Interestingly, among the examined camel sera, we detected FMD virus antibodies in a higher number of samples collected in April and higher seropositive sera in the spring season. This might be because this season provides the ideal micro-environment for the viability and transmission of the FMDV, leading to increased infection among animals.

Moreover, in the spring season, the animals begin grazing on new green pastures in mixed groups and spend long periods of time in close contact with new animal herds among pastures, after the dry winter season, which might lead to higher exposure to FMD infection. The grazing system of camel herds that allows daily contact with other ruminants in the same herd and the process of grazing, buying, selling, and seasonal migration of camels throughout Iraq and into neighboring countries across borders facilitate the transmission of FMDV consequently raising the level of infection in the studied camel herds.

By analyzing the sera of the dromedary camels in three Iraqi provinces, we found that camels are susceptible to FMD infection, particularly to the A and O serotypes, and thus, could play important roles in the transmission of FMD among other domestic animals and in the epidemiology of the disease in this region.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

SHA and QHK designed the study and interpreted results. QHK and AJ were involved in sampling. SHA and QHK carried out sero-detection examination. All authors read and approved the final manuscript.

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Research Article

The Deletion of *Omp19* Gene of *Brucella abortus* 2308 Reduces Its Survival in Mouse Macrophage and in Mice

Yueli WANG [1,a,‡] Zhongchen MA [1,b,‡] Huan ZHANG [1,c,‡] Jihai YI [1,d]
Yong WANG [1,e] Tiansen LI [2,f] Chuangfu CHEN [1,g]

‡ *These authors contributed equally to this work*
[1] College of Animal Science and Technology, Shihezi University, 832000, Shihezi, Xinjiang, CHINA
[2] College of Animal Science and Technology, Hainan University, 570228, Haikou, Hainan, CHINA
ORCIDs: [a] 0000-0001-5320-4953; [b] 0000-0002-3951-4610; [c] 0000-0001-9366-2385; [d] 0000-0002-7027-7668; [e] 0000-0002-4263-8753
[f] 0000-0002-9537-2573; g 0000-0002-1909-9050

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Abstract

The aim of this study is to investigate the role of outer membrane protein *Omp19* of Brucella in its pathogenesis. In this research, the *Brucella* 2308 Δ*Omp19* deletion mutant was constructed and its intracellular survivability was evaluated in murine macrophages RAW264.7 and BALB/c mice. We also analyzed the lysosomal fusion induced by and cytotoxicity of Brucella in murine macrophages RAW264.7. The results showed that the *Brucella* 2308 Δ*Omp19* induced higher levels of immunoprotective cytokines *in vitro* than *Brucella* 2308, and the number of intracellular bacteria in RAW264.7 macrophages was lower after *Brucella* 2308 Δ*Omp19* infection than after *Brucella* 2308 infection. *In vivo, Brucella* 2308 Δ*Omp19* induced protective immune response in mice, and the splenic bacterial load of this deletion mutant was lower than that of *Brucella* 2308. In addition, the Brucella 2308 Δ*Omp19* also promoted the fusion of *Brucella*-containing vacuoles and lysosomes, in macrophages. Therefore, *Brucella* 2308 Δ*Omp19* reduced virulence compared with *Brucella* 2308 and activate the immune response of the host, and is a promising candidate for a live attenuated vaccine.

Keywords: *Brucella, Omp19 gene, Homologous recombination, Viability, Intracellular survival*

Brucella abortus 2308'in *Omp19* Geninin Silinmesi Faregil Makrofajında ve Farelerdeki Sağkalımını Azaltır

Öz

Bu çalışmanın amacı *Brucella*'nın dış zar proteini *Omp19*'un bakterinin patogenezindeki rolünü araştırmaktır. Bu araştırmada, *Brucella* 2308 Δ*Omp19* delesyon mutantı oluşturularak faregil makrofajları RAW264.7 ve BALB/c farelerindeki hücre içi canlılığı değerlendirildi. Ayrıca faregil makrofajları RAW264.7'de *Brucella*'nın neden olduğu lizozomal füzyonu ve sitotoksisite de analiz edildi. Sonuçlar, *Brucella* 2308 Δ*Omp19*'un *in vitro* olarak immünoprotektif sitokin üretimini *Brucella* 2308'den daha fazla uyardığını ve RAW264.7 makrofajlarındaki hücre içi bakteri sayısının *Brucella* 2308 Δ*Omp19* enfeksiyonunda *Brucella* 2308 enfeksiyonundan daha düşük olduğunu gösterdi. *In vivo* olarak, *Brucella* 2308 Δ*Omp19*, farelerde koruyucu bağışıklık tepkisine neden oldu ve bu delesyon mutantının dalak bakteri yükü, *Brucella* 2308'den daha düşüktü. Bunun yanı sıra *Brucella* 2308 Δ*Omp19*, makrofajlarda Brucella içeren vakuollerin ve lizozomların füzyonunu da uyardı. *Brucella* 2308 Δ*Omp19*, *Brucella* 2308 ile karşılaştırıldığında düşük virulansla konağın bağışıklık tepkisini aktive etmiştir ve zayıflatılmış canlı aşı için umut verici bir adaydır.

Anahtar sözcükler: *Brucella, Omp19 geni, Homolog rekombinasyon, Canlılık, Hücre içi sağkalım*

INTRODUCTION

Brucellosis is a zoonotic chronic infectious disease caused by the members of bacterial genus *Brucella*, which infects almost all mammals, causing disease [1]. *Brucella* infection can lead to abortion in pregnant livestock, orchitis in male animals, etc. The main manifestations of infection in humans are fluctuating fever, muscle soreness, and joint swelling and pain [2,3]. However, except for some antibiotic and corticosteroids, there is no safe and effective *Brucella* medicine for humans or animals, so a new treatment method that provides high-level protection is urgently required.

Correspondence
+86-0993-2058002
137716965@qq.com (T. Li), chuangfu_chen@163.com (C. Chen)

The main outer membrane proteins (OMPs) of *Brucella* include Omp10, Omp16, *Omp19*, SP41 and BepC, which are closely associated with the virulence of *Brucella* in previous study [4-6]. The molecular weight of *OMP19* is about 19 kDa. and it plays an important role in the structure of the outer membrane and bridges the outer membrane protein of *Brucella* and polymyxin [7,8]. Recent research shows that *Omp19* and Omp10 do not change their virulence and OM properties in *Brucella ovis* PA mutants, but they have an interchangeable function to maintain the bacterial outer membrane integrity [9]. Moreover, *Omp19* could enable *B. abortus* to evade the antimicrobial activity of proteolytic defense system in host [5]. Several studies have reported that the OMP10 mutant (ΔOmp10) survives less well in infected mice than its parental strain [7,8]. Similar to ΔOmp10, Δ*Omp19* mutant highly weaken virulence in mouse infected by oral route [5]. However, the detailed functions and mechanisms of *Omp19* in host is unknown.

Brucella survives and proliferates in specialized unprofessional phagocytes in animals [10], mainly parasitizing macrophages, dendritic cells, and embryonic trophoblast cells. It invades the cell early, in the form of a membrane-bound vesicle in *Brucella*-containing vacuoles (BCVs). The maturation of BCVs in the mid stage of infection inhibits the binding of lysosomes to *Brucella*, allowing it to escape the killing mechanism of the host cell. The interaction between BCVs and the secretory trafficking of host cell in the later stage of infection causes the BCVs to fuse to the endoplasmic reticulum, triggering autophagy, and facilitating the long-term parasitism and proliferation of *Brucella* [2,11]. *Brucella* also inhibits cell apoptosis and the host immune response by producing different virulence factors, thus promoting its own intracellular survival [12,13]. This study aimed to explore the function of the outer membrane protein *Omp19* of *Brucella*, and tried to clarify its important role in the pathogenesis of *Brucella*, provide a reference for the research and development of *Brucella* candidate vaccine.

MATERIAL and METHODS

Ethical Approval

All animals used were treated humanely and in accordance with institutional animal care guidelines in our experiment. This study was approved by the Animal Care and Use Committee of Shihezi University.

Strains, Cells, and Laboratory Animals

Brucella abortus strain 2308 was provided by the China Center for Disease Control and Prevention (Beijing, China) and cultured in tryptone soya agar (TSA) or tryptone soya broth (TSB) (Oxoid, England). *Escherichia coli* strain DH5α was cultured in Luria–Bertani medium (Difco, Becton Dickinson). The pGEM-7Zf+ plasmid was purchased from Promega Corporation (Madison, WI, USA). Mouse RAW264.7 cells were purchased from the Cell Resource Center, the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences/Peking Union Medical College (Beijing, China), and cultured in Dulbecco's modified Eagle's medium (Gibco, USA) containing 10% calf serum (Gibco) at 37°C under 5% CO_2. A total of 75 six-week-old female BABL/c mice were provided by the Experimental Animal Center of the Academy of Military Medical Science (Beijing, China). We randomly divided them into two groups (a part of 60 mice, a part of 15 mice), and each group was divided into three subgroups (*Brucella* 2308, *Brucella* 2308 Δ*Omp19* and PBS). All experimental procedures and animal care protocols were performed in compliance with Institutional Animal Care Regulations.

Construction of Brucella 2308 Omp19 Gene Deletion Strain

The upstream and downstream homologous primers for the *Omp19* gene were designed with Primer 5.0, based on the gene sequence of international standard strain 2308 of *B. abortus*, published in GenBank. The upstream primers were *Omp19*-N-F GGATCCCGATAAACAGCGTCGGATAGC (*Bam*HI) and *Omp19*-N-R GGGCATGGAACCTCTCTCTGC GGAAACGAGAGAGATAC, and the downstream primers were *Omp19*-C-F CAGTTCTCCATTTGCGC and *Omp19*-C-R GAGCTCGCAGTCTATCGTGTCGGA (*Sac*I). The upstream and downstream target fragments were recovered, amplified with fusion PCR, and then linked to the pMD18-T Simple (TaKaRa, Japan) vector. After screening and sequencing, positive clones of pMD18-T-*Omp19* were obtained. The recombinant plasmid pMD18-*Omp19* was identified by double digestion with *Bam*HI and *Sac*I. The target fragment was linked to the suicide vector pGEM-7zf+. The *sacB* gene is present in *Bacillus subtilis* and can be used as a selective marker for *Brucella*. The *sacB* gene was amplified with primers *SacB*-F GAGCTCGGGGAAGGAAGCACCGCTA (*Sac*I) and *SacB*-R GAGCTCGCTTATTGTTAATTGTCC (*Sac*I). *Sac*I restriction endonuclease was used to digest pGEM-7zf+-*Omp19* and the *sacB* gene, and the target fragments were ligated with T4 ligase. The recombinant homologous suicide vector pGEM-7zf+–*Omp19*–*SacB* was constructed by connecting EM-7zf+-*Omp19* to the *sacB* fragment. Competent *Brucella* 2308 cells were then transformed with the suicide vector using electroporation. Positive clones were screened according to their antimicrobial resistance. Primers *Omp19*-test-FGTCCGCAATGTCGTCAC and *Omp19*-test-R TCCATTCTTCGGCT were used to amplify the positive clones many times until positive clones were isolated.

Determining the Intracellular Viability of Brucella 2308 ΔOmp19 in RAW264.7 Cells

Mouse macrophages were subcultured in six-well plates. *Brucella* 2308 and 2308 Δ*Omp19* were cultured to logarithmic phase. The cells were infected with *Brucella* in a bacterium: macrophage ratio of 100:1. After infection for 1 h, 2.5 μL of gentamicin (50 μg/mL) was added to each well of the six-well plate for 50 min to kill any extracellular *Brucella*. After

4, 12, 24, and 48 h, the cells were collected and counted. Lysozyme (0.2%) was added to release the intracellular bacteria, and the bacterial was diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}, and then used to solid *Brucella* culture medium. The bacteria were cultured in an inverted incubator at 37°C for 3-4 days. The numbers of bacteria in the dishes were recorded.

Measuring Attenuation of Brucella 2308 ΔOmp19 Virulence in BALB/c Mice

To detect the effect of *Brucella* 2308 Δ*Omp19* on the survival of bacteria in mice, the *Brucella* 2308 and *Brucella* 2308 Δ*Omp19* strains were cultured to logarithmic phase and diluted to $5.0x10^6$ CFU/mL. An aliquot (0.2 mL) was intraperitoneally injected into BALB/c mice ($1.0x10^6$ CFU/mouse). The mice in the blank control group were injected with 0.2 mL of phosphate-buffered saline (PBS). There, we used 60 mice which was divided into three groups (*Brucella* 2308, *Brucella* 2308 Δ*Omp19* and PBS). Five mice were selected from each group on days 3, 7, 14, and 28 after injected strains. The mice were killed with CO_2 and their spleens were weighed under sterile conditions. The homogenates were diluted and coated on solid *Brucella* culture medium. The average colony-forming units (CFU) for each group was calculated after 3-5 days.

Cell Cytotoxicity Assay

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme, and the amount of LDH released can be used to calculate the numbers of dead and damaged cells. Macrophages were added to 6 six-well plates and infected with *Brucella* 2308 or the *Brucella* 2308 Δ*Omp19* mutant strain at a multiplicity of infection (MOI) of 10, 100, or 1000. At different times post infection, the supernatant was collected, and the levels of LDH released into it were measured with a Cytotoxicity LDH Detection Kit.

Effect on Lysosomal Fusion

The *Brucella* 2308 and *Brucella* 2308 Δ*Omp19* strains were used to infect mouse macrophages RAW264.7 in a ratio of 200:1. Lysosome staining reagent (250 μL/well) was added to the macrophages after 4, 24, or 48 h, and the cells were incubated at 37°C for 2 h. Paraformaldehyde (4%; 1 mL/well) was added after 30 min, and 0.3% Tranton X-100 (1 mL/well) was added for 10 min to cause cell permeation. Bovine serum albumin (1 mL/well) was added and the cells were incubated for 20 min. A sheep anti-*Brucella* IgG primary antibody (0.5%, 1 mL) was added and the cells were incubated overnight at 4°C. The primary antibody was removed, and 1 mL of a tetramethylrhodamine (TRITC)-labeled donkey anti-sheep IgG secondary antibody (0.2%) was added and the cells were incubated at 37°C for 1 h. The secondary antibody was removed and the cells washed with distilled water and sealed with 50% glycerol. The localization of *Brucella* and the lysosomes was observed in RAW264.7 cell samples under confocal microscopy after post infection 4 h, 24 h and 48 h, and the images were saved. The red spots were TRITC-labeled *Brucella* (BCVs), the green spots were fluorescein-isothiocyanate-labeled lysosomes, and the yellow spots were produced when the red and green signals merged in the figure, indicating the colocalization of BCVs and lysosomes. The fluorescent spots in each field of vision were recorded with a counter. The proportion of yellow signals among the red signals was calculated.

Cytokine Production Assay

After *Brucella* infection, we used an ELISA to evaluate the Cytokines levels in serum samples from mice or RAW264.7 cells. Fifteen mice were used here, also randomly divided into three groups. The cell culture medium from *Brucella*-infected cells (at 4, 12, 24, and 48 h) and serum samples from *Brucella*-infected mice (at 4, 12, 24, and 48 days) were obtained by *centrifugation* (3000 x g, 20 *min*). The interleukin 12 (IL-12) and tumor necrosis factor α (TNF-α) in the medium and the interferon γ (IFN-γ) in the serum samples were detected with enzyme-linked immunosorbent assays (ELISAs; ELISA Quantikine Mouse Kits, R&D Systems), according to the instructions of the manufacturer. For all chemokines, three parallel assays were performed, which were repeated at least three times.

Data Analysis

We looked for information which was benefited and determined the exact changes in the test group by comparing the differences between the test group and the control group. The experimental results for each group were analyzed with Scheffe tests, when finding a significant difference between the averages in each group, check whether there is a difference between one or more pairs of averages. In addition, we also used the One-way ANOVA test method. A P value of <0.05 or <0.01 was considered significant. All results are expressed as means ± SD. Data analysis We use IBM SPSS Statistics software.

RESULTS

Interaction Between BCVs and Lysosomes in Host Cells

The number of yellow spots, indicating the colocalization of the BCVs and lysosomes, was higher when the cells were infected with *Brucella* 2308 Δ*Omp19* than when they were infected with *Brucella* 2308 at 4-48 h. At 4 h, 100% of *Brucella* 2308 Δ*Omp19* (green) colocalized with lysosomes (red) and the patterns were similar at 24 and 48 h post infection (93% and 91%). These fusion rates were all significantly higher than the *Brucella* 2308-lysosome fusion rates at similar time points after infection ($P<0.05$; *Fig. 1*). The detailed data are shown in *Table 1*. Therefore, the knockdown of the *Omp19* gene of *Brucella* 2308 enhanced the fusion of BCVs and lysosomes.

Intracellular Survival of Brucella 2308 ΔOmp19 is Attenuated in Murine Macrophages (RAW264.7)

The intracellular survival of *Brucella* 2308 gradually increased from 4 h post infection. However, the survival of *Brucella* 2308 Δ*Omp19* gradually decreased from 12 h post infection. At 12 h after infection, the numbers of surviving *Brucella* 2308 Δ*Omp19* and *Brucella* 2308 strains differed (P<0.05). By 24 and 48 h after infection, the number of strain *Brucella* 2308 Δ*Omp19* bacteria was significantly lower in the host cells than the number of strain *Brucella* 2308 (P<0.01), the range of decrease by almost 50% of that of 2308 strains *(Fig. 2)*.

Brucella 2308 ΔOmp19 Induced High Levels of IL-12 and TNF-α Release from RAW264.7 Cells

The production of neither IL-12 nor TNF-α differed negligibly in the two strains at 12 h post infection, but at 24 h post infection, the difference was significant that *Brucella* 2308 Δ*Omp19* higher than *Brucella* 2308. The difference in IL-12 was significant that peaked at 24 h post infection, whereas TNF-α production peaked at 48 h post infection *(Fig. 3)*. Therefore, *Brucella* 2308 Δ*Omp19* increased the secretion of immune cytokines IL-12 and TNF-α, which are not conducive to the survival of *Brucella* in cells.

Reduced Virulence of Brucella 2308 ΔOmp19 in Mouse Model

The counts of *Brucella* 2308 Δ*Omp19* in the mouse spleens were significantly lower than those of *Brucella* 2308 on days 3, 7, 14, and 28 after infection (P<0.05; *Fig. 4)*. At 28 days post inoculation, *Brucella* 2308 Δ*Omp19* was virtually cleared from the spleens of the mice. All these results

Table 1. *Fusion rates of Brucella -containing vacuoles and lysosomes*

Strains	4 h	24 h	48 h
2308	10.00%	32.70%	73.20%
2308 ΔOmp19	100%	93.60%	91%

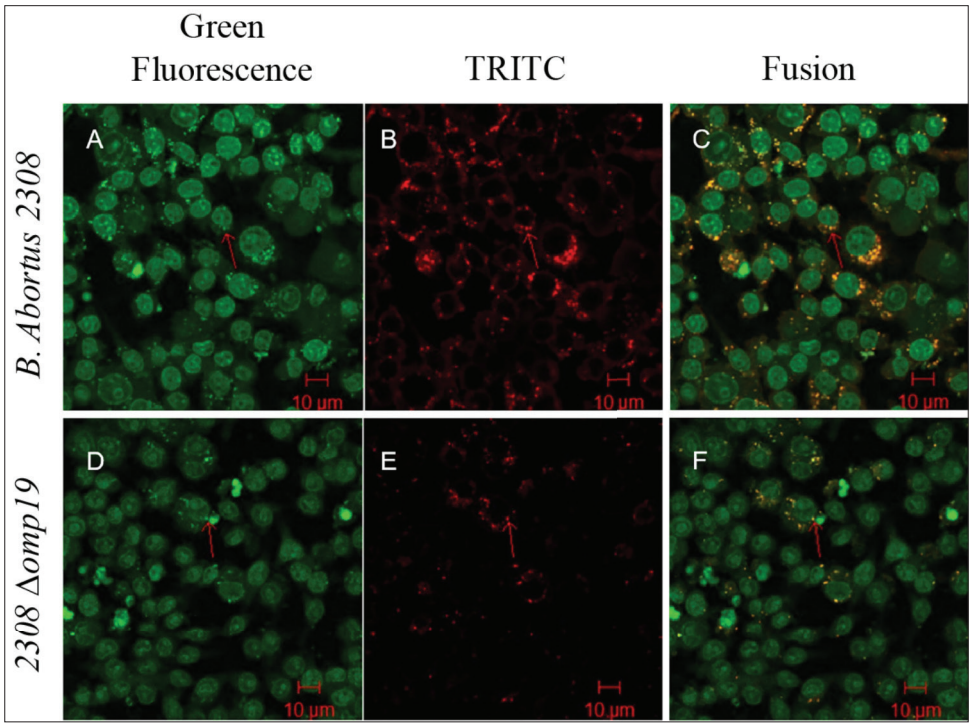


Fig 1. Interaction of *Brucella* 2308 or *Brucella* 2308 ΔOmp19 with RAW264.7 macrophage lysosomes. *Brucella* was labeled with a sheep anti-*B. abortus* IgG antibody and RITC-conjugated donkey anti-*B. abortus* IgG antibody, and lysosome was labeled with fluorescein-isothiocyanate to display green fluorescence. The fixed cells were examined at different times after infection, and the infected cells were imaged with confocal microscopy 4 h after infection. The fusion rate of phagosome-lysosome was calculated from the colocalization of the green fluorescent and TRD markers. The bacteria in each time period were analyzed to determine the fusion rate

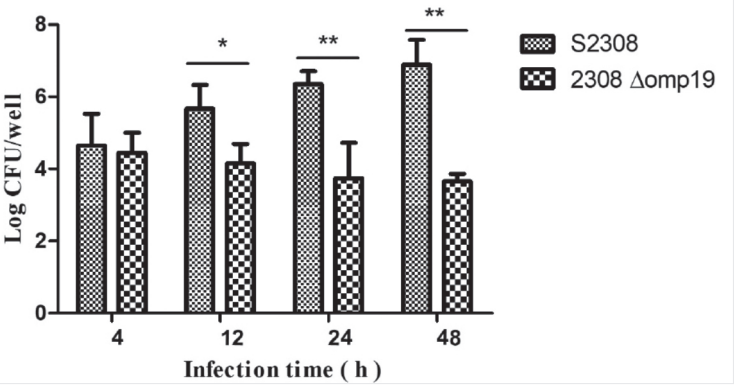


Fig 2. Intracellular viability analysis of *Brucella* 2308 and 2308 ΔOmp19 strains in RAW264.7 mouse macrophages. Macrophages were infected with *Brucella* 2308 or the *omp19*-deleted strain and incubated for specific times. The numbers of bacteria in the RAW264.7 cells were counted at different time points. Each point represents the mean ± standard deviation of three experimental groups. The number of bacteria in the RAW264.7 cells marked with an asterisks (* P<0.05, ** P<0.01) differed significantly in the parental and mutant strains

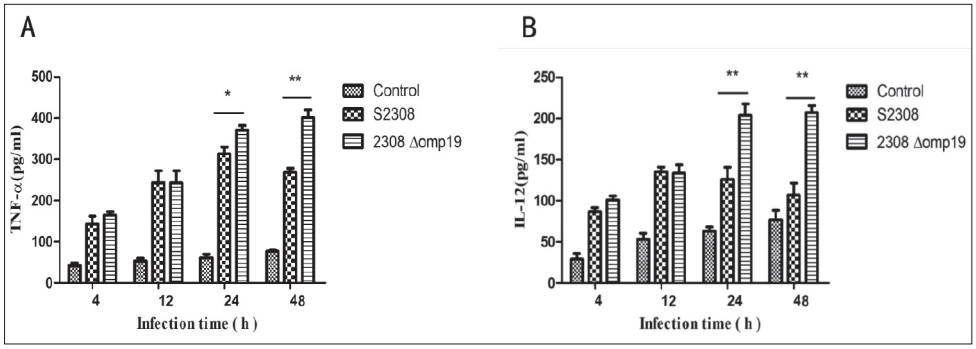
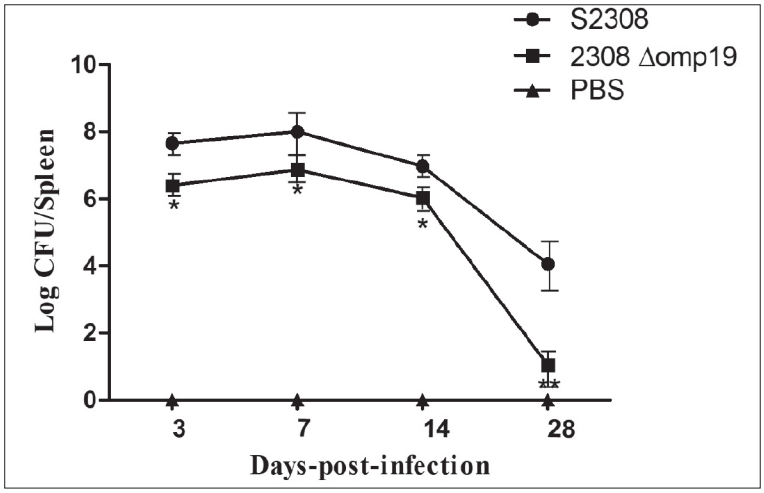


Fig 3. Levels of IL-12 and TNF-α released from RAW264.7 cells infected with *Brucella* 2308 or *Brucella* 2308 ΔOmp19. After bacterial infection, the release of IL-12 and TNF-α was measured at 4, 12, 24, and 48 h with ELISAs. Each point represented the mean ± standard deviation of three experimental groups. Asterisk (* P<0.05, ** P<0.01) indicate significant differences in the release of IL-12 and TNF-α between the parental and mutant strains after infection

Fig 4. Survival of *Brucella* 2308 and *Brucella* 2308 ΔOmp19 in the mouse model. *Brucella* 2308 or *Brucella* 2308 ΔOmp19 ($1x10^5$) was injected into the abdominal cavities of the mice in each group. Values are the averages ± SEM for 20 mice. Each point represents the mean ± standard deviation of three experimental groups. The Asterisks (* P<0.05, ** P<0.01) indicate significant differences in bacterial growth between the parental and mutant strains



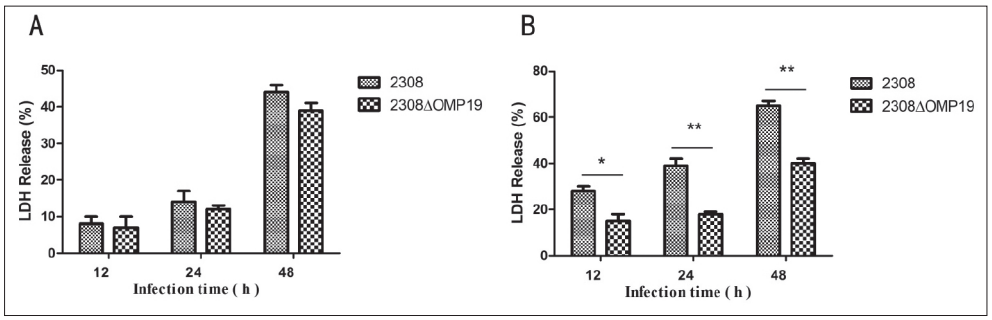


Fig 5. Levels of LDH released from cells infected with *Brucella* 2308 or *Brucella* 2308 ΔOmp19. LDH release was measured at 12, 24, and 48 h after *Brucella* infection. A. MOI = 10; B. MOI = 100. Asterisks (* P<0.05, ** P<0.01) indicate significant differences in LDH release between the parental and mutant strains

indicate that *Brucella* 2308 Δ*Omp19* is less virulent than its parental strain *(Brucella* 2308).

Omp19-Regulated Cytotoxicity of Brucella 2308 for Mouse Cells

To determine whether the cytotoxicity of *Brucella* 2308 for mouse cells is regulated by the *Omp19* protein, we measured the LDH released from mouse cells after *Brucella* 2308 or *Brucella* 2308 Δ*Omp19* infection at an MOI of 10, or 100. At 12, 24, and 48 h post infection, there were no significant differences in LDH release when the cells were infected with *Brucella* 2308 or its mutant at an MOI of 10 *(Fig. 5-A)*, whereas when the cells were infected at an MOI of 100, the LDH released by the differently infected cells differed significantly at 12 and 24 h (P<0.05; *Fig. 5-B)*. At an MOI of 100, *Brucella* 2308 induced high levels of LDH release at 12 and 48 h, but *Brucella* 2308 Δ*Omp19* induced negligible LDH release. Both strains induced high levels of LDH release after infection for 48 h. Therefore, as the MOI increased, the LDH release rate also increased. These results indicate that *Omp19* regulates the cytotoxicity of *Brucella* 2308 for macrophages.

Immune Cytokine Production in Mice after Infection with Brucella 2308 or Brucella 2308 ΔOmp19

The total expression of IFN-γ induced by both *Brucella* strains gradually increased with time relative to that in the PBS-treated control group, and peaked at 14 days post infection. The *Brucella*-2308-Δ*Omp19*-vaccinated mice produced significantly higher IFN-γ levels than the *Brucella*-2308-infected mice (P<0.05*; Fig. 6)* from day 7 to day 28. These results indicate that the *Brucella* 2308-Δ Δ*Omp19* strain induces a high humoral response, which may protect the host against wild-type *Brucella* infection.

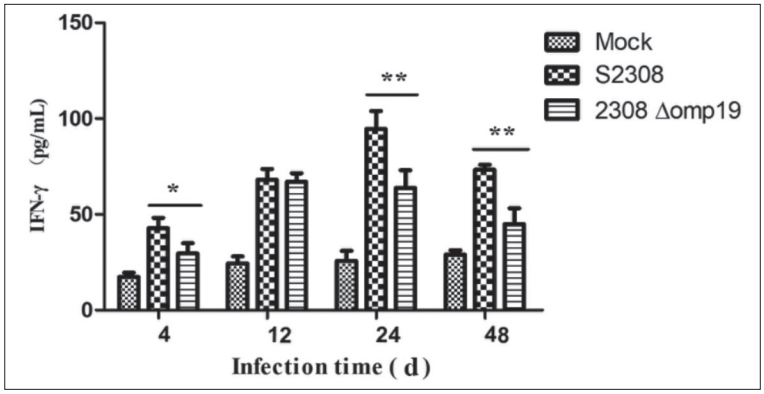


Fig 6. IFN-γ levels in mice infected with *Brucella* 2308 or the *Brucella* 2308 ΔOmp19 mutant. Each mouse was infected with 1x10^5 *Brucella* cells. Serum IFN-γ was measured at 4, 12, 24, and 48 days postinfection. Asterisks (* P<0.05, ** P<0.01) indicate significant differences in IFN-γ expression induced by the parental strain and mutant strain in mice

DISCUSSION

The intracellular survival of *Brucella* requires the participation of virulence factors, including outer membrane proteins, lipopolysaccharide (LPS), virB-encoded type IV secretion system, and virulence regulatory proteins. These virulence factors not only ensure the survival of the bacteria, but also kill the host cell. Vemulapalli et al.[14], reported that the *Brucella* protein BMP18 *(Omp19)* is one of its main virulence factors [14,15], and it is one of the most-studied outer membrane proteins. Silencing *Omp19* can reduce the bacterium's resistance to polymyxin B and deoxycholate [7] because the loss of the protein changes the inherent structure of the LPS in the outer membrane and disrupts the interactions of LPS with other molecules, thus altering the properties of the outer membrane [16]. However, knocking out the *Omp19* gene in *Brucella* RB51 did not alter its invasive capacity because *Brucella* RB51 is a rough vaccine strain in which LPS has no O-side chain [7]. In addition, *Omp19* protein indeed involved in cell invasion by *Brucella* and enhanced its intracellular survival [4,5], but clearly mechanism is unknown.

Therefore, in this study, we constructed an *Omp19* deletion mutant of *Brucella* 2308 and evaluated its virulence in cells and mice. The virulence of *Brucella* 2308 in cells was significantly reduced after the deletion of the *Omp19* gene, which may be attributable to an indirect effect of the deletion on the interaction between the outer membranes of *Brucella*, which would directly affect the invasion capacity and intracellular viability of *Brucella* 2308 Δ*Omp19*. The *cfu* counts of the *Brucella* 2308 Δ*Omp19* mutant in the spleens of mice were also lower than those of the virulent strain *Brucella* 2308, and the mutant was cleared faster than *Brucella* 2308. The ability of the spleen to carry *Brucella* can reflect the virulence of the bacterium, and we demonstrated that the *Omp19* gene of *Brucella* 2308 facilitates its invasion of its host cells and prolongs its intracellular survival. *Brucella* 2308 Δ*Omp19* is also a potential vaccine candidate.

Why is the survival of the *Brucella Omp19*-deleted strain in cells lower than that of its parent strain? Studies have shown that virulent *Brucella* strains inhibit BCV-lysosome fusion in cells after infection, whereas vaccine strains cannot effectively prevent BCV-lysosome fusion [17]. In this study, we showed that the deletion of the *Omp19* gene reduced the virulence of *Brucella* 2308, but that *Brucella* 2308 Δ*Omp19* significantly promoted BCV-lysosome fusion, although it reduced the viability of *Brucella* in cells. Therefore, a lipoprotein may contribute to the intracellular trafficking of *Brucella* in macrophages. Research has shown that *Brucella suis*, which lacks the LPS O side-chain, does not enter the intracellular circulation through the mediation of lipid rafts, and that the BCVs that enter the cell fuse with lysosomes and dissolve [18,19]. Therefore, the knockdown of the *Omp19* gene alters the intrinsic properties of the outer membrane of *Brucella* 2308, and possibly affects the O antigen on LPS, thus reducing its utilization of lipid rafts, and effectively reducing the early survival of *Brucella* in macrophages. However, how the *Brucella* 2308 Δ*Omp19* affects the fusion of BCVs and lysosomes in mouse macrophages requires further study.

Brucella escapes immune surveillance to survive in host cells, a process that depends on the secretion of various effector proteins or virulence factors that interfere with host cell apoptosis [20]. The smooth type of *Brucella* inhibits the apoptosis of host cells [21], mainly through the function of a TNF-*α*-dependent O-side-chain polysaccharide, which is not available in the rough type of *Brucella*. We have shown that the virulence and cytotoxicity of the *Brucella* 2308 Δ*Omp19* mutant strain are reduced compared with those of its *Brucella* 2308 parent, but it does not affect apoptosis (results not shown), and the apoptosis rate was the same as that induced by its parental strain. Therefore, although the *Omp19* virulence protein is a key protein in the intracellular survival of *Brucella*, it does not improve the intracellular viability of *Brucella* by regulating the apoptosis of the host cells. *Brucella* infection can reduce the body's immunity, and *Omp19* is an important protein in the mechanism of *Brucella* infection. Therefore, whether silencing this gene affects the host's immunity has been unclear. A previous study showed that the immune capacity of the host directly affects the replication and internalization of intracellular bacteria [22], and a strong immune response can inhibit the development of a persistent *Brucella* infection [23]. In this study, we found that *Brucella* 2308 Δ*Omp19* induced

higher levels of INF-γ in mice than *Brucella* 2308. IFN-γ, a key Th1-type immune cytokine, is required for the bactericidal activity of macrophages [24]. High levels of INF-γ induce the host immune system to kill and eliminate *Brucella in vivo* and *in vitro*, as shown in *Fig. 6*. Thus, the knockdown of the *Omp19* gene reduces *Brucella* 2308 virulence and activates the immune response of the host, this is a possible that B*rucella* 2308 Δ*Omp19* strain has potential utility as an attenuated vaccine against *Brucella*. The knockdown of gene *Omp19* in *Brucella* changes the intrinsic characteristics of the bacterial membrane, which affects its intracellular viability and induces the Th1 immune response. In further studies in livestock, we will investigate whether the *Brucella* 2308 Δ*Omp19* strain is a possible safe live candidate vaccine against *Brucella* infection.

In this paper, we describe the construction of the mutant *Brucella* 2308 Δ*Omp19* and the preliminary investigation of the mechanisms underlying its interaction with lysosomes. We evaluate the role of *Brucella* 2308 *Omp19* protein in intracellular survival, the host immune response in macrophages and mice. Our results provide information on the roles of the OMPs of *Brucella* in its pathogenic mechanism and may facilitate the development of a candidate vaccine against wild-type *Brucella*.

Acknowledgments

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Conflict of Interest

The authors have declared that no competition interests.

Author Contributions

In the process of writing the article, Wang Yueli, Ma Zhongchen and Zhang Huan are responsible for the provision, integration and writing of the article data, Li Tiansen is responsible for the research conception and design, Chen Chuangfu is the article reviewer, and the other authors provided help in the trial process.

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Research Article

Evaluation of the Protective Effect of Chlorogenic Acid and *Rhabdosciadium anatolyi* Against Cyclophosphamide-Induced Ovarian Toxicity in the Rat with Histopathological and Immunohistochemical Findings

İsmet ALKIŞ [1,a] Suat EKİN [2,b] Serkan YILDIRIM [3,c] Ahmet BAKIR [2,d] Gizem ESER [3,e] Mehmet FIRAT [4,f]

[1] Department of Obstetrics and Gynecology, Faculty of Medicine, Van Yuzuncu Yil University, TR-65080 Van - TURKEY
[2] Department of Chemistry, Division of Biochemistry, Faculty of Science, Van Yuzuncu Yil University, TR-65080 Van - TURKEY
[3] Department of Pathology, Faculty of Veterinary Medicine, Ataturk University, TR-25240 Erzurum - TURKEY
[4] Department of Biology, Van Yüzüncü Yil University, TR-65080 Van - TURKEY
ORCIDs: [a] 0000-0002-5674-4610; [b] 0000-0002-6502-5028; [c] 0000-0003-2457-3367; [d] 0000-0003-0797-285X; [e] 0000-0002-8330-3095
[f] 0000-0001-5814-614X

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Abstract

Cyclophosphamide (CD) has a range of adverse effects on ovarian tissue in humans. It is widely an anticancer drug and used in autoimmune disorders. Also, CD produces reactive oxygen species. In the current study, we evaluated possible protective roles of Rhabdosciadium anatolyi (RA) and chlorogenic acid (CA) on histopathological alterations and immunohistochemical assessment of 8-OHdG in the ovarian tissues of female rats given cyclophosphamide. Female Wistar albino rats were divided into six experimental groups, each consisted of eight rats: control group, CD (200 mg/kg) with i.p. for the first day as single dose, CA (100 mg/kg), RA (300 mg/kg) for each day, RA (300 mg/kg) + CD (200 mg/kg) and CA(100 mg/kg) + CD (200 mg/kg) groups. The rats were administered treatments lasted 7 days for all groups. CA and RA treatment is associated with positive the ovary action CD-induced ovotoxicity in rats. CA and RA could ameliorate the histopathological and immunohistochemical finding restoring which may show moderate levels of primordial follicle, primary, secondary, tertiary and graafian follicles were observed degeneration in germinative cells. Therefore, our results suggest that chlorogenic acid and Rhabdosciadium anatolyi might be a protective effect for CD-induced ovotoxicity.

Keywords: *Cyclophosphamide, Rhabdosciadium anatolyi, Chlorogenic acid, 8-OHdG*

Klorojenik Asit ve *Rhabdosciadium anatolyi*'nin Siklofosfamid İle Ratlarda Oluşturulan Ovaryum Toksisitesine Karşı Koruyucu Etkisinin Histopatolojik ve İmmunohistokimyasal Bulgularla Değerlendirilmesi

Öz

Siklofosfamid (CD), insanlarda ovaryum dokusu üzerinde bir dizi yan etkilere sahiptir. Yaygın bir şekilde antikanser ilacı olarak ve otoimmün hastalıklarda kullanılır. Ayrıca, CD, reaktif oksijen türleri üretir. Bu çalışmada, siklofosfamid verilen dişi ratların over dokularında, *Rhabdosciadium anatolyi* (RA) ve klorojenik asidin (CA) histopatolojik değişiklikler ve immünohistokimyasal olarak belirlenen 8-OHdG üzerine olası koruyucu rolleri değerlendirildi. Dişi Wistar albino ratlar, her grupta sekiz olacak şekilde, altı deney grubuna ayrıldı: kontrol grubu, i.p olarak ilk gün için tek doz CD (200 mg/kg), CA (100 mg/kg) ve RA (300 mg/kg) için her gün, RA (300 mg/kg) + CD (200 mg/kg) ve CA (100 mg/kg) + CD (200 mg/kg) grupları. Ratlara tüm gruplar için işlemler 7 gün süreyle uygulandı. CA ve RA uygulaması, ratlarda CD'nin neden olduğu ovotoksisite, over üzerine olumlu etkisi olması ile ilişkilidir. Germitif hücrelerinde gözlenen dejenerasyonu, aynı zamanda orta düzeyde görülen primordial follikül, primer, sekonder, tersiyer ve graf folliküllerin histopatolojik ve immünohistokimyasal bulgularının düzelmesini CA ve RA sağlayabilir. Bu nedenle sonuçlarımız, CD'nin neden olduğu ovotoksisiteye karşı klorojenik asit ve rabdosciadium anatolyi'nin koruyucu bir etki oluşturabileceğini düşündürmektedir.

Anahtar sözcükler: *Siklofosfamid, Rhabdosciadium anatolyi, Klorojenik asid, 8-OHdG*

Correspondence
+90 532 4420963
ismetalkis@hotmail.com

INTRODUCTION

Cyclophosphamide (CD) is one of the well-known alkylating agents, which is commonly used to treat chronic and acute leukemias, multiple myeloma, lymphoma, retinoblastoma, neuroblastoma, breast and ovarian cancers [1]. CD endures bioconversion by hepatic microsomal P_{450} oxidases that produce two metabolites; acrolein and phosphoramide mustard, which forms covalent bonds with DNA via enzymatic activation that leads to cell death. When enzymatically activated, these cytotoxic metabolites form covalent bonds with DNA and proteins and cause cell death [2]. Although, CD affords employment of it in various applications, the clinical usage of CD is usually restricted due to its side effects and toxicities that include nausea, bone marrow suppression, vomiting, alopecia, nephrotoxicity, hepatotoxicity, ovotoxicity, urotoxicity, immunotoxicity, cardiotoxicity, mutagenicity, teratogenicity, and carcinogenicity [3].

Recent *in vivo* studies regarding chemotherapy-induced destruction of dormant follicles [4] have revealed that chemotherapeutic agents such as cyclophosphamide and cisplatin activate the onset of dormant follicle growth, concurrent with apoptosis of large follicles [5]. As a result of this, destruction of ovarian follicles disrupts ovarian functions and leads to permanent ovarian insufficiency [6]. Cyclophosphamide is mostly linked to the highest risk of female infertility, which is usually assigned to ovarian toxicity and is strongly associated with the cumulative doses of CD. Reproductive functions are deteriorated by rapid depletion of the oocyte reserve that mediates through apoptotic cell death and ovarian atrophy by the disappearance of resting primordial follicles [7].

Phenolic compounds have the aptitude to suppress lipid peroxidation, avoid DNA oxidative damage, and scavenge free radicals. Free radicals cause a decrease in the immune system antioxidants, cause changes in the gene expression, and induce abnormal proteins that lead to degenerative diseases and aging [8]. Chlorogenic acid (CA), which is a kind of phenolic antioxidant, has a certain number of R-OH radicals, that can generate hydrogen free radicals with antioxidant activity, and neutralize free radical activity such as superoxide anion, thus protects tissue cells from oxidative damage [9]. CA has potential activity on lipid peroxidation, for instance 8-hydroxydeoxyguanosine is configured via this inhibitor. It is known that several phenolic compounds have an inhibitory effect on the mutagenesis and carcinogenesis of polycyclic aromatic hydrocarbons [10].

Medicinal plants help us in taking a role of therapeutic alternatives. The enhanced number of these plants and their extracts were proven to have beneficial therapeutic effects, as well as antioxidants, anticancer, anti-inflammatory antimicrobial and immunomodulatory effects [11]. Medicinal herbs have been used to treat different diseases in Turkish traditional medicine. *Rhabdosciadium anatolyi*, also known as endemic taxa of the family Apiaceae, is a flavoring herb widely found in Turkey. *Rhabdosciadium anatolyi* Lyskov & Kljuykov is a wild edible plant with a wide distribution in Hakkari province which is in The Eastern Anatolia Region of Turkey.

Rhabdosciadium anatolyi is particularly located on the wet rocky northern slope near the snowfield of Hakkari [12]. The flowers of this plant are used as an additive spice for pleasant taste in the herbal cheese of Van, salads and yoghurt.

This study was designed to investigate the possible protective roles of *Rhabdosciadium anatolyi* and chlorogenic acid on the effects induced by a single dose of cyclophosphamide on ovarian tissue of Wistar female rats by detecting histopathological alterations and immunohistochemical assessment of 8-OHdG.

MATERIAL and METHODS

Plant Material

Rhabdosciadium Anatoly Lyskov & Kljuykov (Apiaceae) flowers were collected from Yüksekova district, Hakkari province (37°22'41" N, 44°10'08" E, 2356 m 18 July 2018) and dried under the shade. *Rhabdosciadium anatolyi* was identified by Dr. Mehmet Firat. The specimen was stored in Van Yuzuncu Yil University herbarium, Department of Botany, VANF 30400.

Plant Material and Extract Preparation

Rhabdosciadium anatolyi dried flower of plant 35 g were prepared in 350 mL of ethanol (75%) as an extraction solvent for 48 h at room temperature and the resulting extract were filtered through a filter paper. After the evaporation of the solvent under a reduced vacuum at temperatures below 40°C, the filtrate was then dried in an incubator. Dried powder of flower extracts was then prepared in physiologic saline (0.9%), daily.

Experimental Section

- Experimental Animals

In this study, forty-eight female Wistar-albino rats (aged 8 weeks, weighing 200±50 g) were used. Rats were supplied by Van Yuzuncu Yil University, Faculty of Medicine, Experimental Animal Research Center. The rats were housed in clean cages at normal temperature (22±2°C) and normal daily lighting with a 12 h light-dark cycle and supplied with drinking water and food *ad libitum*. Experimental protocols were carried out following the general principles of the animal ethics committee of Van Yuzuncu Yil University (YUHAD-YEK, Date: 25.10.2018; Decision number: 2018-10).

- Experimental Design

The rats were randomly divided into six equal sized groups (eight rats in each group). Group 1: considered as normal control (given 0.9% isotonic saline solution) for 7 consecutive days, calculated according to the body weight. Group 2: cyclophosphamide (CD) was administered (CD 200 mg/kg) intraperitoneally (i.p.) only the first day as a single dose. Group 3: Chlorogenic acid (CA) (100 mg/kg) administered (by an intragastric tube for each day). Group 4: *Rhabdosciadium anatolyi* (RA) administered by an intragastric tube (RA 300mg/kg) every day throughout the experimental period. Group 5: RA (300 mg/kg) were administered orally daily and a single injection of i.p 200 mg/kg CD for the first day. Group 6 rats (CA+CD) were treated with CA (100 mg/kg) for each day and 200 mg/kg CD for the first day as a single dose. The treatment course has lasted 7 days for all groups. At the end of the experimental period, all female Wistar-albino rats were anesthetized and sacrificed; and the ovaries were removed for histopathological and immunohistochemical examinations.

- Histopathological Examination of the Ovaries

Ovarian tissues obtained for histopathological assessment were fixed in 10% (v/v) formalin solution for 48 h before being processed for histopathological analysis and then washed in tap water for 10 h.

With a routine tissue follow up, tissues were passed through alcohol and xylene series, and embedded into paraffin blocks. A 4 µm thick slices were taken from each block, and samples were prepared on slides. Preparations planned for histopathological examination were stained with hematoxylin and eosin and examined using light microscopy (Leica DM 1000, Germany). They were evaluated as none (–), mild (+), moderate (++), and severe (+++) according to the lesions based on histopathological findings.

- Immunohistochemical Examination of 8-hydroxy-2'-deoxyguanosine (8-OHdG)

All sections that were transferred to adherent (poly-L-lysine) slides for immunoperoxidase staining were passed through xylol and alcohol series, deparaffinized and dehydrated. They were washed with phosphate buffer solution (PBS, pH 7.2) for 5 min and stored in 3% H_2O_2 for 10 min, and endogenous peroxidase were inactivated. To prevent the masking of antigen in the nucleus, the sections were heated in an antigen retrieval (citrate buffer, pH 6.1) solution, 5 min each for four times in a microwave (500 watt) oven and then removed from the microwave oven and allowed to cool to room temperature for 30 min. After the incubation (37°C, 60 min), the excess block solution that remained on the tissue sections was removed, and 8-OHdG catalog no. ab183394 Abcam, UK, and PBS for control group) were added prior to the final rinse. Immunohistochemistry procedures were followed according to the manufacturers' (Abcam HRP/DAB Detection IHC kit) instructions. DAP chromogen was added to the sections, and sections were kept for 5-10 min to take in the chromogenic substance. Sections were kept in Mayer's hematoxylin for background staining for 1-2 min and then washed in tap water. After passing alcohol and xylol series, entellan was dropped on the sections and slides were closed with coverslips and examined under light microscopy (Leica DM 1000). The sections were evaluated as no (–), mild (+), moderate (++), and severe (+++) according to their immunopositivity.

Statistical Analysis

The Differences between groups were analyzed using one-way analysis of variance (ANOVA), Duncan test. The statistical analysis was carried out with SPSS®, version 23.0 statistical software (SPSS Inc. Chicago Ill, USA). Statistical significance was considered to be $P<0.05$.

RESULTS

Ovarian samples were evaluated for follicle counts, damage scores. Primordial, primary secondary and Graafian follicle counts were significantly increased in the control, chlorogenic acid and *Rhabdosciadium anatolyi* groups.

Histopathological Findings

The ovaries observed in the control group were in normal histological appearance *(Fig. 1-A)*. In the CD group, necrosis in many of the luteal cells, severe hyperemia and hemorrhage in the vessels, degeneration in germinative cells, very few primordial follicles dominated by luteal structures were observed, while secondary and Graafian follicle structures were not found *(Fig. 1-B)*. When graafian, primary, secondary, and tertiary follicles were examined in terms of counts, a statistically significant difference ($P<0.05$) was detected compared to the control group. In the CA group, it was observed that the primordial, graafian, primary, secondary, tertiary follicles and luteal structures had normal histological appearance *(Fig. 1-C)*. In histopathological examination of ovarian tissues in RA group, it was observed that the primordial, Graafian, primary, secondary, tertiary follicles and luteal structures were in normal histological appearance *(Fig. 1-D)*. In the CD+RA group, moderate levels of primordial, primary, secondary, tertiary and Graafian follicles and degeneration in germinative cells *(Fig. 1-E)*, and CD+RA group a statistically significant difference ($P<0.05$) observed compared to the control group. While there was no significant difference in terms of follicle counts compared to the 6th group, it was found that the count of follicles in this group was higher. Furthermore, CD+CA treated rats showed moderate level in primordial, a small number of primary, secondary, tertiary and Graafian follicles were identified and degeneration in germinative cells *(Fig. 1-F)*. CD+CA group a statistically significant ($P<0.05$) was determined when compared to the control group *(Table 1)*.

Table 1. *Scores of histopathological and immunohistochemical findings*

Parameters	Control	CD	CA	RA	CD+RA	CD+CA
Primordial follicle counts	+++	+	+++	+++	+++	+++
Primer-secondary follicle counts	+++	+	+++	+++	++	+
Graafian follicle counts	+++	–	+++	+++	+	+
Degeneration and necrosis in the ovarian cells	–	+++	–	–	+	+
8--hydroxy-2'-deoxyguanosine	–	+++	–	–	+	+

(-) No change, (+) Mild change, (++) Moderate change, (+++) Severe change

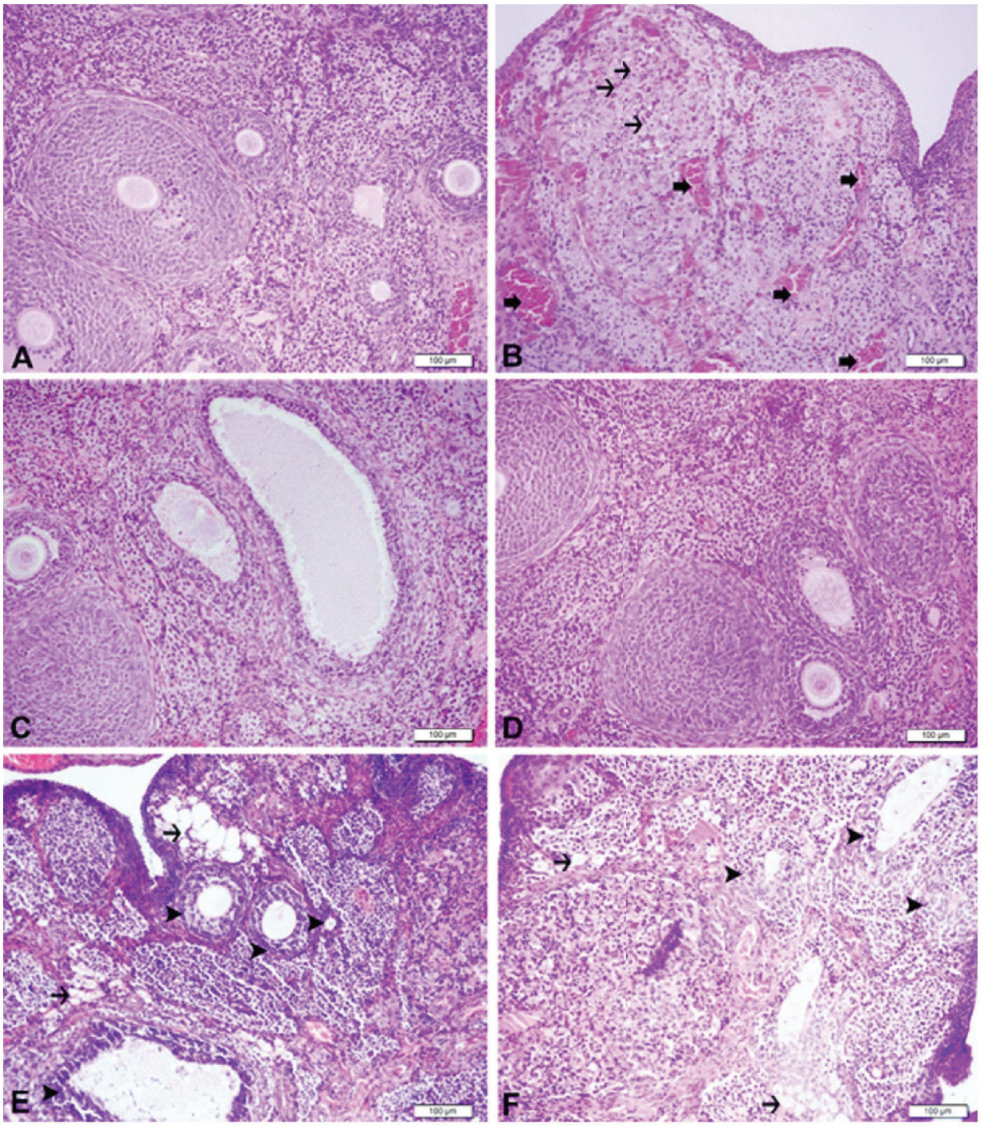

Fig 1. The ovarian tissue, Control group, normal histological appearance (A), CD-treated group, severe level of necrosis in the luteal cell *(thin arrows)*, hyperemia and haemorrhage in the vessels *(thick arrows)*, degeneration in germinative cells, very few primordial follicles dominated by luteal structures (B), CA group, normal histological appearance (C), RA group, normal histological architecture (D), CD+RA group, moderate level in primordial follicle, primary, secondary, tertiary and graafian follicles *(arrow heads)*, and degeneration in germinative cells *(arrows)* (E), CD+CA, moderate level in primordial follicle, a small number of primary, secondary, tertiary and graafian follicles *(arrow heads)*, degeneration in germinative cells *(arrows)* (F), H&E, Bar: 100 µm

Immunohistochemical Results

In the control group, no 8-OHdG expression were determined, as result of immunohistochemical analysis of ovarian tissues *(Fig. 2-A)*. In the CD group, cytoplasmic 8-OHdG expression in the granulosa cells, germinative cells, luteal cells were observed at severe levels in the follicles *(Fig. 2-B)*. Associated to the control group, a statistically significant difference ($P<0.05$) was seen. In the CA group was 8-OHdG expression were not observed *(Fig. 2-C)*. In the RA group, 8-OHdG expressions were observed to be negative *(Fig. 2-D)*. Mild expression of 8-OHdG in the CD+RA *(Fig. 2-E)*, and CD+CA group were observed *(Fig. 2-F)*.

The histopathological and immunohistochemical results in the ovarian tissues were evaluated and the findings scores are shown in *Table 1*.

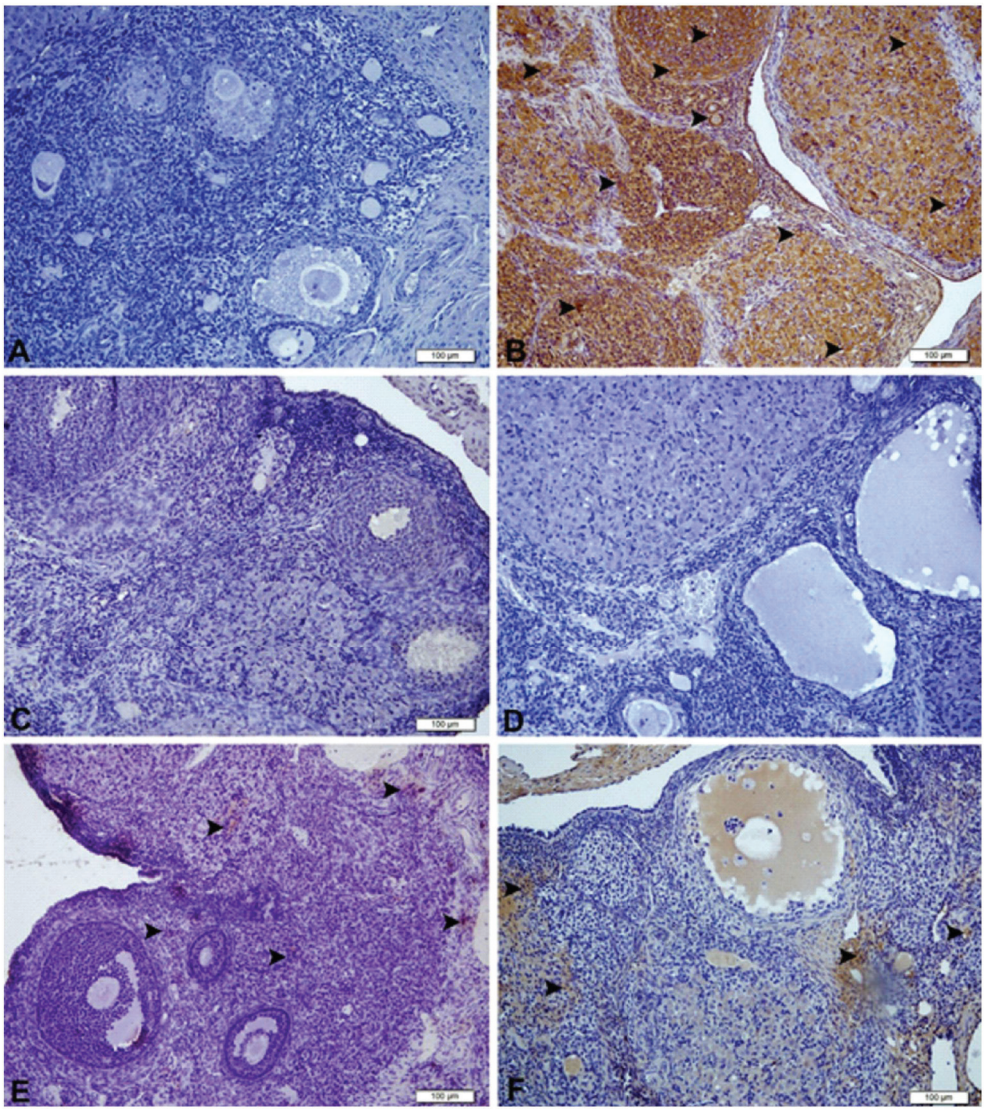

Fig 2. The ovarian tissue, Control group, negative expression of 8-OHdG (A), CD group, cytoplasmic expression of 8-OHdG in the granulosa cells, germinative cells, luteal cells at severe levels follicles (B), CA group, negative expression of 8-OHdG (C), RA group, negative 8-OHdG expression (D), CD+RA group, mild level expression of 8-OHdG (E), CD+CA, mild level expression of 8-OHdG (F), IHC-P, Bar: 100 µm

DISCUSSION

This study was performed to assess the protective roles of *Rhabdosciadium anatolyi* (RA) and chlorogenic acid (CA) cyclophosphamide induced on histopathological alterations. Moreover, current study assayed the possible role of RA flower extracts and CA on immunohistochemical expression of 8-OHdG in ovarian tissue samples in Wistar female albino rats with induced by single dose cyclophosphamide (CD). The usage of high dose CD in cancer treatment is restricted owing to its severe toxicity caused mainly by oxidative stress. The toxic metabolites formed after CD administration are phosphoramide mustard and acrolein. The toxic effect of CD is related to acrolein which is an active metabolite [13]. Acrolein reduces the physiological antioxidant defense, and rises production of reactive oxygen species (ROS) by activating of xanthine oxidase [14].

Himmelstein et al.[15] examined reduced number of follicles in ovarian tissues obtained from patients treated by CD. Alkylating substances such as cyclophosphamide have been reported to inhibit the growth of follicles, destroying small oocytes. Another study by Ataya et al.[16] determined that the mean follicular diameter and number of granulosa cells in each ovary at 16 h and 24 h after CD injection were statistically significantly lower than that of the control group ($P<0.05$ and $P<0.01$). Moreover, significant reductions ($P<0.01$) of primordial and maturing follicles as well as corpora lutea have been observed in cyclophosphamide-treated mice compared to control. In conclusion, granulosa cells appear to be important targets for toxicity in the ovaries of rats treated with cyclophosphamide.

We observed necrosis in many luteal cells, severe hyperemia and hemorrhage in the vessels, degeneration in germinative cells, very few primordial follicles dominated by luteal structures in the rats given single dose CD (CD 200 mg/kg) alone.

In this study, the observation of the significant decrease in primordial follicles and number of follicles in ovarian tissues of rats treated with CD were consistent with those of

other authors [15,16]. Sections of the ovarian tissue obtained from CD (200 mg/kg) + RA (300 mg/kg treated) and CD (200 mg/kg) + CA (100 mg/kg) treated groups have shown near to normal count of primordial follicles.

Primordial follicle counts have been shown to be unfavorably effected in highly concentrated amount of phosphoramide mustard, a toxic metabolite of CD, both *in vivo* and *in vitro* studies. It also destroys rapidly dividing granulosa cells of antral and secondary follicles *in vivo* in mice and, in rats the ovarian stromal cells *in vivo* [7].

Many patients are known to be adversely effected by chemotherapy, via follicular reduction that leads to ovarian failure and infertility. Consequently, chemotherapy destroys proliferation of ovarian follicles throughout treatment, possibly due to the damage triggered by dividing granulosa cells. It has long been suggested that chemotherapeutic agents induce apoptosis of primordial follicles and thus cause ovarian failure. Chemotherapy may lead to vascular damage of certain areas of the ovarian cortex, resulting in depletion of primordial follicles [17].

Immunohistochemical staining of 8-OHdG in the control group, CA and RA groups *(Fig. 2-A, Fig. 2-C,D)* 8-OHdG expression was not detected. Following CD administration, in the CD group, cytoplasmic expression of 8-OHdG in the granulosa cells, germinative cells, and luteal cells was observed at severe levels in the follicles *(Fig. 2-B)*. It was observed that the CD group a statistically important difference compared to the control group ($P<0.05$) was found. In the present study, immunohistochemical analysis clearly demonstrates that the CD+*RA* and CD+CA groups had mild level expression of 8-OHdG *(Fig. 2-E,F)*.

Oxidative stress can be defined as an imbalance between the oxidant and antioxidant resulting from the excessive production of ROS and the ability of antioxidant systems to easily detoxify the ROS or repair the resulting damage [18]. ROS may attack the polyunsaturated fatty acid in the biological membranes and cause free radical chain reactions, which leads to increased lipid peroxidation [19]. Cellular antioxidant system plays an important role in protecting against CD-induced oxidative stress and it's related to toxic manifestations. The recent reports have showed that oxidative stress mediated impairment of tissues redox balance after exposure to CD produces biochemical and physiological disturbances [4].

In this study, we report that the presence of cyclophosphamide affects granulosa cells of ovarian follicles and impairs the follicles by increasing ovarian cytoplasmic 8-OHdG expression in the granulosa cells, germinative cells and luteal cells.

In the groups in which chlorogenic acid and RA were administered simultaneously with CD, immunohistochemical 8-OHdG expression were significantly reduced compared to the CD group. It was found that Chlorogenic acid and RA may have protective roles against CD-induced ovotoxicity and oxidative damage in rats.

With RA and CA treatments, these irregular histopathological results of ovarian tissue have decreased and the ovarian tissue was protected from ovarian toxicity and oxidative stress. The histopathological observations indicated that RA and CA were able to protect the ovarian tissue. Polyphenols can be obtained at high levels in many kinds of foods. These results emphasized the influence of this polyphenol CA and RA flowers for health, possibly preventing toxicity associated with CD. The protective mechanisms of CA are associated with oxidative DNA damage marker 8-OHdG which leads to ovotoxicity. Therefore, CA has the potential to provide cellular protection against CD-induced ovotoxicity.

To our knowledge, there is no published information about CA and RA flowers ethanol extract that protects against histopathological alterations and immunohistochemically 8-OHdG in ovarian tissue samples in Wistar female albino rats with induced ovotoxicity by single dose (200 mg/kg) cyclophosphamide.

In the current study, the histopathological results were confirmed by immunohistochemical studies, which showed that treatment with RA flower extract (300 mg/kg) and CA (100 mg/kg) reduced the necrosis in many luteal cells, severe hyperemia and haemorrhage in the vessels, degeneration in germinative cells, follicles dominated by luteal structures, and primordial follicles and against free radical production by CD-induced ovotoxicity. We found that CA and RA prevent cell damage due to by increased oxidative stress caused by CD. We suggest that CA and RA administered with chemotherapy would reduce side effects of chemotherapy complications on the ovary.

Conflict of Interest

The authors declare no conflicts of interest regarding the present study

Statement of Author Contributions

The sampling collection were made by İA and AB. Histopathological and immubohistochemical examination was performed by SY and GE. The study was designed by İA and SE. MF and AB carried out *Rhabdosciadium Anatoly* flowers collection and identification in the the study. All authors read and approved the final version of the article.

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Research Article

The Effect of Intravenously Paratyroid Cell Xenotransplantation in Sheep: As an Animal Model

Emrah YUCESAN [1,a] Beyza GONCU [2,b] Oguz IDIZ [3,c] Ramazan UCAK [4,d]
Burcu OZDEMIR [2,e] Ebru KANIMDAN [2,f] Yeliz Emine ERSOY [5,g] Erhan AYSAN [6,h]

[1] Bezmialem Vakif University, Faculty of Medicine, Department of Medical Biology, TR-34093 Istanbul - TURKEY
[2] Bezmialem Vakif University, Experimental Research Center, TR-34093 Istanbul - TURKEY
[3] Istanbul Training and Research Hospital, Department of General Surgery, TR-34098 Istanbul - TURKEY
[4] Istanbul Sisli Hamidiye Etfal Training and Research Hospital, Department of Pathology, TR-34360 Istanbul - TURKEY
[5] Bezmialem Vakif University, Faculty of Medicine, Department of General Surgery, TR-34093 Istanbul - TURKEY
[6] Yeditepe University Faculty of Medicine, Department of General Surgery, TR-34734 Istanbul - TURKEY
ORCIDs: [a] 0000-0003-4512-8764; [b] 0000-0001-6026-8218; [c] 0000-0002-8462-7809; [d] 0000-0002-2999-7495; [e] 0000-0003-0288-0470
[f] 0000-0002-7123-4600; [g] 0000-0002-5028-6436; [h] 0000-0002-9563-3761

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Abstract

Parathyroid cell transplantation is an effective approach for the treatment of permanent hypoparathyroidism. Intramuscular and intraperitoneal routes were performed previously but intravenous adminstration has not been conducted previous studies. Our aim is to determine proper homing site for parathyroid cells, therefore we administered parathyroid cells intravenously and observed results. This study is carried out on nine sheep. According to the given substance the sheep were divided into two groups; seven were parathyroid cell injected group and two were isotonic saline solution injected control group. Parathyroid cells were obtained from a patient with chronic kidney failure and were injected intravenously into sheep after cell isolation.: $20x10^6$ cells for two animals, $50x10^6$ cells for two animals, $100x10^6$ cells for two animals, and $200x10^6$ cells for one animal respectively, with no post injection immunosuppresive therapy. Immunosuppresion was not administered. Physical conditions of animals and blood biochemical tests were observed for three months. After sacrifications, kidneys and livers were evaluated histopathologically. In $20x10^6$ and $50x10^6$ cell transplanted groups serum PTH levels increased in the first seven days but in the other groups remained stable. Histopathological evaluations of kidneys and livers revealed fibrosis related to the number of infused cells, however biochemical functional differentiations were not detected. Intravenous parathyroid cell transplantation is considered as an effective and useful technique to perform without immunosuppression. However, further and long term studies need to have more acceptable results in future for clinical purpose.

Keywords: *Xenotransplantation, Parathyroid cell, İntravenous injection, Permanent hypoparathyroidism, Sheep*

Bir Hayvan Modeli Olarak: Koyunlarda Intravenöz Paratiroid Hücre Zenonaklinin Etkisi

Öz

Paratiroid hücre nakli, kalıcı hipoparatiroidizmin tedavisinde etkili bir yaklaşımdır. İntramüsküler ve intraperitoneal yollar daha önce yapılmış, ancak intravenöz uygulama daha önce yapılmamıştır. Amacımız, paratiroid hücreleri için uygun yerleşim bölgesini belirlemekti, bu amaçla paratiroid hücrelerini intravenöz olarak uyguladık ve sonuçları gözlemledik. Bu çalışma dokuz koyun üzerinde gerçekleştirildi. Verilen maddeye göre koyunlar iki gruba ayrıldı; yedisi paratiroid hücresi enjekte edilen grup, ikisi iztonik tuz çözeltisi enjekte edilen kontrol grubudur. Paratiroid hücreleri kronik böbrek yetmezliği olan bir hastadan elde edildi ve hücre izolasyonu sonrası intravenöz olarak koyunlara enjekte edildi. Sırasıyla; $20x10^6$ hücre iki hayvana, $50x10^6$ hücre iki hayvana, $100x10^6$ hücre iki hayvana ve 200x106 hücre bir hayvana hayvana uygulandı ve enjeksiyon sonrası immünsupresyon uygulanmadı. Üç ay süresince hayvanların fiziksel kondisyonları ve kan biyokimyasal testleri gözlemlendi. Sakrifikasyon sonrası, karaciğerler ve böbrekler histopatolojik olarak incelendi. $20x10^6$ ve $50x10^6$ hücre nakledilen gruplarda serum PTH seviyeleri ilk yedi gün yükseldi ancak diğer gruplarda sabit kaldı. Böbreklerin ve karaciğerlerin histopatolojik incelemelerinde, infüze edilen hücre sayısıyla ilişkili fibrozis saptandı, ancak biyokimyasal fonksiyonlarda herhangi bir farklılık tespit edilmedi. İntravenöz paratiroid hücre naklinin, immünsupresyonsuz uygulanacak etkili ve kullanışlı bir teknik olduğu değerlendirildi. Bununla birlikte, klinik amaçla kullanım öncesi ilerleyen dönemde daha kabul edilebilir sonuçları olan uzun süreli çalışmalara ihtiyaç vardır.

Anahtar sözcükler: *Zenonakil, Paratiroid hücresi, İntravenöz enjeksiyon, Kalıcı hipoparatiroidizm, Koyun*

Correspondence
+90 212 5232288/ext.1465 Fax: +90 212 4531870
eyucesan@bezmialem.edu.tr; emrahyucesan@yahoo.com

INTRODUCTION

Permanent hypoparathyroidism (PH) is a clinical condition accompanied by hypocalcemia, hyperphosphatemia, and low parathormone (PTH) levels. The most common etiologic factor of PH is thyroid surgery [1]. The current standard treatment of PH is vitamin D and calcium supplementation. Standard treatment only relieves the symptoms temporarily and may causes several side effects [2]. Recombinant parathormone drugs reveal better results however balancing the dosage, efficacy and safety is not clear. In addition, recombinant parathormone drugs are more expensive than standard treatment [3]. Parathyroid cell transplantation is the most promising technique for the treatment of PH [4]. In the literature, several transplantation approaches have been reported such as autotransplantation [5], allo-transplantation [6], and xenotransplantation [7] (XT). These approaches are used with different methods e.g. direct tissue injection [8], non-treated cultivated cells injection [9], cultured parathyroid cells treated with IFNγ [10], macroencapsulation [11], and microencapsulation [12] Different experimental animal models for the assessment of parathyroid function, morphology, and disease progression have been investigated including, dog [13], rat [14], rabbit [15], and sheep [16]. Among them parathyroid transplantation models were assessed by intramuscular [17] and intraperitoneal routes [7], respectively. Till the time of the research was planned, intravenous parathyroid cell transplantation has not been tried. In the present study, we injected human parathyroid cells to the sheep, and observed the functionality of the cells and their effects on the kidney and liver.

MATERIAL and METHODS

The study was approved by the Bezmialem Vakif University, Local Experimental Animals Ethics Committee (approval number: 2018/12). In the power analysis, the number of subjects was determined in 85% confidence interval and 95% significance level. We studied on nine sheep (12 months-old, mean weight 27.05 kg, weight range 24.5-35.5). The animals were housed and fed *ad libitum* throughout the study. The animals were divided into two groups; intravenous parathyroid cells injected group (n=7) and intravenous isotonic saline solution injected group (n=2). Peripheral blood samples were obtained before XT and continued biweekly after XT for 90 days. Weights and other physical conditions of the animals were examined weekly. After 90 days, the animals were sacrificed. Excisional biopsies were taken from the kidney and liver for histopathological evaluations. Resected tissues were immersed in neutral-buffered 10% formalin and fixed for at least 24 h. Formalin-fixed tissues were processed within paraffin wax and sectioned at 5 µm, mounted on positively charged glass slides, and air-dried overnight. Slides were rehydrated and stained by hematoxylin and eosin. Three to four transects of each slide were counted. Tissues were evaluated by one expert pathologist who blinded the groups, according to fibrosis scoring (Grade 0: No fibrosis for liver and no congestion/fibrosis for kidney, Grade 1: Mild fibrosis for liver and mild congestion/fibrosis for kidney, Grade 2: Moderate fibrosis for liver and moderate congestion/fibrosis for kidney, and Grade 3: Severe fibrosis for liver and severe congestion/fibrosis for kidney) as previously reported by Idiz et al.[18]. Before the XT procedure the Local Human Ethics Committee approval was received (approval number: 71306642-050.01.04). All of the protocols were confirmed according to the ethical guidelines of the Helsinki Declaration and written informed consent was obtained from the donor. The donor patient was a 34 year old man with parathyroid hyperplasia resistant to drug therapy due to chronic renal failure who was referred from the nephrology outpatient clinic to the general surgery department for surgical intervention. Standard subtotal parathyroidectomy procedure was performed and half of each of the resected glands were delivered to the pathology laboratory for histopathological evaluation. The remaining parts of the glands were snap frozen. After the histopathological evaluations were reported as benign parathyroid hyperplasia, the tissues were prepared for the XT process.

Cell Preparation Procedure for XT

In laboratory conditions, the tissue was cut and washed with 1% Phosphate-buffered solution (Thermo Fisher Scientific, MA) and minced in a petri dish on ice. The minced preparation was combined with 2 mL for a total 100 mg/mL bovine serum albumin (Merck Millipore, Germany), 215 mmol collagenase type II (Thermo Fisher Scientific), 0.32 mM DNase I (AppliChem, Gatersleben, Germany), and 1 mL Ham's F10 Supplement (Thermo Fisher Scientific). Samples were transferred to an incubator (CCL-170B-8; ESCO, Singapore) at 37°C with humidified atmosphere containing 5% CO_2, where they were incubated overnight. Each sample was filtered into a 15-mL conical Falcon tube using a sterile cell strainer (70 mm, Falcon; BD Biosciences, NJ). The solution was centrifuged at 306 g for 15 min to obtain a pellet. Cells were suspended in 1 mL of culture medium. Parathyroid cell viability was assessed before cryopreservation using a Muse Cell Analyzer (Merck Millipore) with a Muse Count & Viability Assay Kit (Merck Millipore) [19]. The cells were mixed with 10% DMSO (dimethyl sulfoxide), 10% FBS, and suspended in cryotubes kept at -80°C, and then transferred to a liquid nitrogen tank for storage. The day before XT, cells were removed from the nitrogen tank and cultivated in flasks with McCoy's 5A (Modified) Medium (Thermo Fisher Scientific) with 1% sodium pyruvate, 1% penicillin-streptomycin, and 10% FBS and placed in an incubator (CCL-170B-8; ESCO) at 37°C with 5% CO_2 humidified atmosphere, where they were kept overnight before XT.

XT Procedure

Prepared xenograft cells were administered to seven animals

by a decreased count system: $100x10^6$ cells for two animals, $50x10^6$ cells for two animals, $20x10^6$ cells for two animals, and $200x10^6$ cells for one animal into the 10 mL isotonic saline solution. Injections were performed via external jugular vein catheterization. In the control group, 10 mL isotonic saline solution physiologic was injected intravenously via the external jugular vein.

Blood Biochemical Tests

Serum sheep-PTH and human-PTH levels were measured using Sheep Parathyroid Hormone ELISA Kit® (MyBiSource, CA, USA) and Architect Intact hu-PTH Assay Kit® (Abbott, IL, USA), respectively. A complete blood count (CBC) was measured by Hematology Analyzer Abacus Junior Vet® (Diatron, Budapest, Hungary). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), troponin, creatine kinase (CK), amylase, lipase, urea, total bilirubin, calcium, phosphorus, albumin, and creatinine were measured by Chemistry Analyzer IDEXX VetTest® (IDEXX Laboratories, Maine, USA).

Statistics

All data during the given period were compared with pre-op values. Statistical analyses were performed using SPSS software v22.0 (IBM, Armonk, NY, USA). Data was not normally distributed, thus we used Friedman test as a non-parametric test, and $P<0.05$ was considered statistically significant.

RESULTS

Mean blood biochemical parameter variations, except PTH in the XT group are presented in *Table 1*. No statistically significant changes were detected ($P>0.05$). Mean blood PTH level variations in the XT group is presented in *Fig. 1*. In the $20x10^6$ and $50x10^6$ cell injected groups, PTH increased for the first five days after XT and, then decreased *(Fig. 1-A,B)*. In the $100X10^6$ cell injected group, PTH decreased gradually after XT, but increased slightly between 30 to 60 days *(Fig. 1-C)*. In the $200x10^6$ cell injected group PTH increased gradually after XT, however it did not reach the normal level. According to the non-parametric Friedman test, PTH levels were not detected statistically significant ($P=0.393$). All changes in the PTH levels were compiled in *Fig. 1*. Histopathological fibrosis scores of two groups for the liver and kidney specimens are demonstrated in *Table 2*. According to transplanted cell numbers, fibrosis, inflammatory cell infiltration and necrotic areas were increased in the liver and also the kidney tissue *(Fig. 2, Fig. 3)*.

DISCUSSION

Several therapeutic approaches are available for PH treatment, among them the most effective approach is parathyroid allotransplantation. The first parathyroid allotransplantation was reported in 1911 [20]. Between 1990-2016, 316 allotransplantation cases were published in the literature and most of them were performed via intramuscular routes [5]. Three major routes are defined for cell type transplantations intramuscularly, intraperitoneally,

Table 1. *Blood biochemical parameter differentiations of xenotransplantation (XT) group*

Blood Parameters	Pre XT	Day 30	Day 60	Day 90	Statistical Meaning
AST (72-101U/L)	96.7	95.7	100.4	98.6	P>0.05
ALT (9-22 U/L)	16.0	14.2	21.1	21.0	P>0.05
GGT (33-55 IU)	47.8	51.0	53.1	52.7	P>0.05
ALP (50-228 IU)	88.7	93.2	89.4	90.4	P>0.05
LDH (504-1049 IU)	480.4	513.5	522.5	517.4	P>0.05
CK (8-100 IU)	137.0	150.5	138.4	137.4	P>0.05
Amilase (1-30 IU)	10.7	11.1	10.4	11.7	P>0.05
Lipase(1-71 IU)	11.0	10.4	9.5	10.0	P>0.05
Urea (8-20 mg/dL)	13.1	15.1	12.6	14.1	P>0.05
Creatinine (0.6-1.5 mg/dL)	0.70	0.74	0.77	0.72	P>0.05
T. Bilirubin (0.1-0.4 mg/dL)	0.12	0.14	0.10	0.11	P>0.05
Calcium (9.1-10.8 mg/dL)	9.4	9.5	9.3	9.4	P>0.05
Phosphorus (4.0-8.9 mg/dL)	5.7	5.4	6.0	6.1	P>0.05
Albumin (2.4-3.7 g/dL)	2.7	2.7	3.1	3.0	P>0.05
CRP (<0.03 mg/dL)	<0.02	<0.02	<0.02	<0.02	P>0.05
RBC ($x10^{12}$/L)	9.8	9.6	11.2	10.7	P>0.05
Hemoglobin (8-15 g/dL)	8.3	8.4	8.0	8.1	P>0.05
Platelet ($x10^9$/L)	622	717	684	664	P>0.05

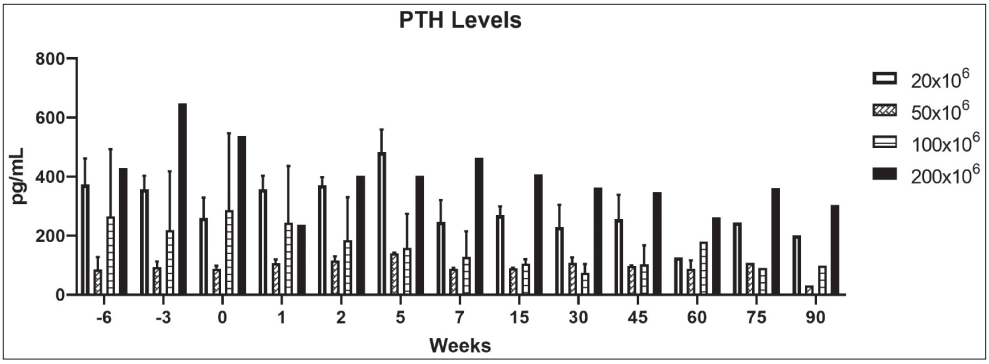

Fig 1. Mean serum PTH (pg/mL) variations of 20 (n=2), 50 (n=2), 100 (n=2) and 200 (n=1) X10^6 cell infused XT groups respectively. Error bars indicated ±SD when at least two animals presented in the same group. PTH: Parathormone

Table 2. *Histopathological fibrosis scores of xenotransplantation (XT) animals for liver and kidney*

Tissue	Grade	Control Group (n=2)	20x10^6 Cells (n=2)	50x10^6 Cells (n=2)	100x10^6 Cells (n=2)	200x10^6 Cells (n=1)
Liver	Grade 0	+/+				
	Grade 1		+/+	+/+		
	Grade 2				+/+	
	Grade 3					+
Kidney	Grade 0	+/+				
	Grade 1		+/+	+/+		
	Grade 2				+/+	
	Grade 3					+

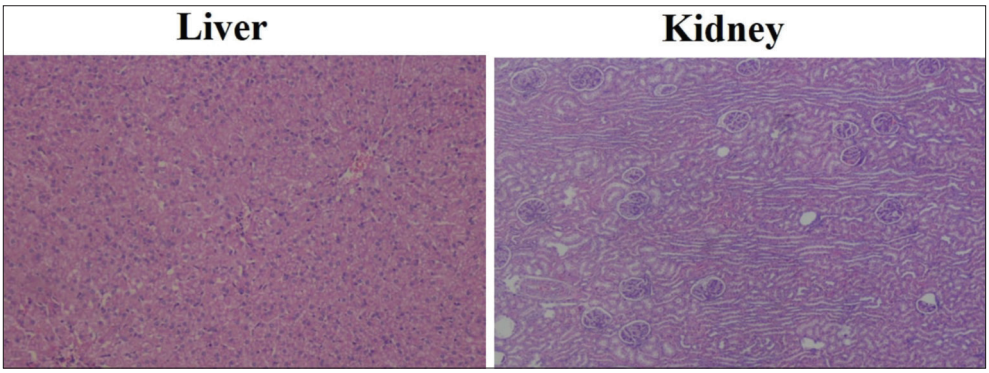

Fig 2. Healthy liver *(left)* and kidney *(right)* histological changes in the control group (H&E X40)

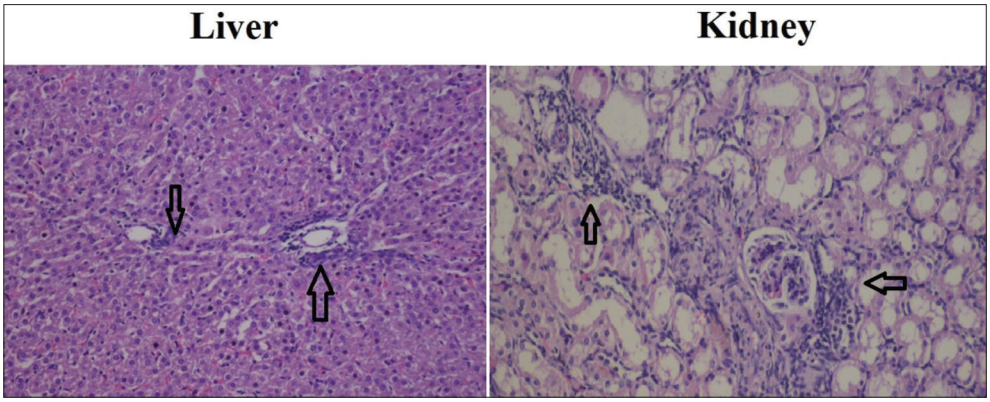

Fig 3. Necrosis in the hepatic central vein area (Grade 3 fibrosis) in liver *(left)* (H&E X100) and the decreased glomerular infiltration, fibrosis and infiltrating mononuclear cells in kidney *(right)* (H&E X200) samples of 200X10^6 parathyroid cell infused XT group

and intravenously [7,21]. The acceptable route for parathyroid cell allotransplantation has not been determined with the best results yet [22]. Intramuscular route is routine way for cell type transplantations because it is easy and quick to perform, but the rate of success varies between the centers [8,23]. Different results could be due to technical preparation of details related to cell manupulation or immunological responses.

On the other hand, according to the Kimura et al.[24], PTH enhances myocyte differentiation by stimulating myotubes and accelerated muscle strength may increase mechanical stress for the transplanted parathyroid allo-graft. Therefore, intramuscular transplantation may increase mechanical stress on transplanted parathyroid cells by muscle strength. Intraperitoneal cell transplantation is a traditional technique but has been popular nowadays [25] and some clinicians use the route for therapy. Several types of cells such as islet cells [26] and Sertoli cells [27] were transplanted via the intraperitoneal route. The main advantages of intraperitoneal adminstration are low intraabdominal pressure and rich vascular structures, as omentum. A disadvantage of the route is that surgical intervention is required under general anesthesia [28]. Intravenous cell transplantation mainly utilize hematopoetic stem cells for bone marrow related hematologic disorders [29]. In addition islet cells [30] and mesechymal stem cells [31] were transplanted intravenously as well. The portal vein is the routine access for transplantation of islet and mesenchymal stem cells [27] but the portal vein route is associated with cell loss and poor engraftment due to instant blood-mediated inflammatory reaction (IBMIR). Once transplanted cells trigger IBMIR, a significant amount of injected cells die or lose their functions [32]. In intravenous transplantation, homing site of transplanted cells is the optimal way for determination of success rate. Whether transplanted cells are positive for CD-34 surface protein, they migrate to the bone marrow directly. CD surface proteins of parathyroid cells have not been detected yet therefore their homing site is uncertain. In this study, we evaluated histopathologically liver and kidney tissue for potential homing sites of parathyroid cells. We postulated that transplanted parathyroid cells may be homing to the liver and kidney due to the membranous protein similarity or joined with their microcapilleries. We detected inflammatory reactions lead to fibrous tissue related to the number of transplanted cells in the liver and kidney, and no parathyroid cells in their tissue histopathologically. So any functional changes were not seen in profile levels of liver and kidney. Serum PTH levels increased during the first seven days only the $20x10^6$ and $50X10^6$ cells in animals recevied XT. Serum calcium levels did not change of all XT animals. The study revealed that, intravenous parathyroid cell administration has not have functional impairement in majority of organs such as the liver and kidney for three months follow-up. We also revealed that, despite human cells being infused in to the sheep without any immunusuppressive therapy, PTH levels increased during the first seven days in the $20X10^6$ and $50X10^6$ cell XT animals. Allotransplantation instead of XT with less number of cells may cause higher serum PTH levels with a longer period of time without histopathological damage to the major organs such as the liver and kidneys. As a conclusion, our results are promising as a new treatment option for the treatment of PH. However, long term follow-up studies with a different number of cells are in need before clinical trial.

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Declaration of Interest Statement

The authors declare no conflict of interest

Author Contributions

OI, YEE, EA were carried out in animal experiments. EY, BG, BO, EK were conducted most of the wet lab experiments. RU was evaluated the pathological specimens. EY, BG, YEE, EA critically read the manuscript. YEE, EA supervised the study.

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Research Article

Impact of Temperature and the Length of Exposure on Morphological Characteristics of Erythrocytes in Antemortem and Postmortem Analysis: Experimental Study on Wistar Rats

Emina SPAHIĆ [1,a] Muhamed KATICA [2,b] Anes JOGUNČIĆ [3,c] Aida KATICA [4,d] Sabaheta HASIĆ [5,e]

[1] Department of Forensic Medicine, Faculty of Medicine, University of Sarajevo, Sarajevo, BOSNIA AND HERZEGOVINA
[2] Department of Pathological Physiology of Domestic Animals, Veterinary Faculty, University of Sarajevo, BOSNIA AND HERZEGOVINA
[3] Department of Epidemiology, Public Health Institute of Canton Sarajevo, Sarajevo, BOSNIA AND HERZEGOVINA
[4] Department of Pathological Physiology of Domestic Animals, Veterinary Faculty, University of Sarajevo, BOSNIA AND HERZEGOVINA
[5] Department of Medical Biochemistry, Faculty of Medicine, University of Sarajevo, Sarajevo, BOSNIA AND HERZEGOVINA

ORCIDs: [a] 0000-0001-8429-0508; [b] 0000-0002-8184-0065; [c] 0000-0001-6926-0079; [d] 0000-0001-9157-6942 ; [e] 0000-0002-4193-9837

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Abstract

Changes in red blood cell morphology can be induced by various stimuli. In order to investigate the effects of different temperature intensities (37°C, 41°C, and 44°C) and exposure time (20 min and until the time of death) on erythrocyte morphology, heat stress was used on a rat model. Thirty-five Wistar rats were divided into three groups: 37°C as control group, 41°C and 44°C as trial groups. The trial groups were classified into antemortem the exposure time of 20 min and postmortem groups exposure time until fatal outcome. The anaesthetised rats were exposed to preheated water using the water bath. May-Grünwald-Giemsa colouring technique was applied on blood samples taken from the abdominal aorta. The light microscopy was performed (Motic Type 102M) to detect poikilocytes. Target cells and anulocytes were predominant in antemortem groups, while anulocytes and spherocytes in postmortem groups 41°C and 44°C, respectively. No difference in poikilocyte number was found between antemortem and postmortem groups 41°C and 44°C. Spherocyte number was significantly higher in postmortem than antemortem group 41°C (P=0.001) and dacryocytes with spherocytes in postmortem group 44°C (P=0.002, P=0.017, respectively). Poikilocytosis is associated with the exposure length and temperature intensity. Spherocytosis and anulocytosis are the most frequent in postmortem groups while target cells and anulocytes in antemortem groups. Following a fatal outcome, spherocytes at 41°C and dacryocytes with spherocytes at 44°C were significantly more than in corresponding antemortem groups.

Keywords: *Heat, Poikilocytosis, Antemortem, Postmortem, Rats*

Antemortem ve Postmortem Analizlerde Sıcaklığın ve Sıcaklığa Maruz Kalma Süresinin Eritrositlerin Morfolojik Özellikleri Üzerine Etkisi: Wistar Ratlarda Deneysel Çalışma

Öz

Kırmızı kan hücresi morfolojisindeki değişiklikler çeşitli uyaranlarla indüklenebilir. Farklı sıcaklık yoğunluklarının (37°C, 41°C ve 44°C) ve maruz kalma süresinin (20 dakika ve ölüm zamanına kadar) eritrosit morfolojisi üzerindeki etkilerini araştırmak için ısı stresi uygulanan bir rat modelinde kullanıldı. Otuz beş Wistar sıçanı üç gruba ayrıldı: kontrol grubu olarak 37°C, deneme grubu olarak 41°C ve 44°C. Deney grupları antemortem 20 dakikalık maruziyet süresi ve postmortem gruplar ölüme kadar maruziyet süresi olarak sınıflandırıldı. Anestezi uygulanan sıçanlar su banyosu kullanılarak önceden ısıtılmış suya maruz bırakıldı. Abdominal aorttan alınan kan örneklerine May-Grünwald-Giemsa boyama tekniği uygulandı. Poikilositleri saptamak için ışık mikroskobu kullanıldı (Motic Tip 102M). Antemortem gruplarında hedef hücreler ve anülositler predominant iken, postmortem gruplarda, 41°C ve 44°C'de sırasıyla anülositler ve sferositler şeklindeydi. Antemortem ve postmortem gruplar arasında 41°C ve 44°C'deki poikilosit sayısında fark bulunmadı. Sferosit sayısı postmortem grubunda, antemortem 41°C grubuna göre (P=0.001) ve dakriyositler sferositlere göre postmortem 44°C grubunda (sırasıyla P=0.002, P=0.017) daha yüksekti. Poikilositozun, sıcaklık yoğunluğu ve maruz kalma süresi ile ilişkili olduğu belirlendi. Postmortem gruplarda en sık sferositoz ve anülositoz görülürken, antemortem gruplarda hedef hücreler ve anülositler görülmekteydi. Ölümcül bir sonucu takiben 41°C'de sferositler ve 44°C'de sferositli dakriyositler, kendilerine karşılık gelen antemortem gruplarından önemli ölçüde daha fazlaydı.

Anahtar sözcükler: *Isı, Poikilocytosis, Antemortem, Postmortem, Rat*

Correspondence
+387 61 031191; +387 33 666545; Fax: +387 33 666545
emina.spahic@mf.unsa.ba; spahica@hotmail.com

INTRODUCTION

Evaluation of haematological parameters enables quick detection of changes in body's homeostasis. It has been known that the original biconcave shape of erythrocytes can be influenced by some unfavourable endogenic and exogenic factors such as exposure to toxic aluminium compounds [1-3], microelements, nutritional deficiency [4], implants placement during surgical interventions [5] as well as effects of electromagnetic field of weak power [6]. Detection of erythrocyte shape abnormality, known as poikilocytosis, in peripheral blood smear reveals the pathophysiology of disease and facilitates patients' early diagnosis and treatment. In forensic medicine, time of death has an extreme importance in homicide or any other instance of un-witnessed death investigation. Despite many researches it remains one of the most challenging variables to establish [7]. Moreover, can some specific or non-specific changes in red blood cell shape be detected under various environmental conditions? It has been known that exposure to higher temperatures for a longer period causes a variety of pathological changes accompanied by multi-organ failure and fatal outcome [8]. The mortality rate associated with high temperature exposure is in the range from 33% to 80% [9,10]. The effects of the body exposure to higher temperature can be identified easily by analysis of biochemical and physiological parameters especially haematological parameters [11]. The changes in blood cell morphology between cadaveric blood collected from corpses at 4°C, stored in test tubes, and blood collected directly from corpses at room temperature, have been studied as a way to determine the post mortem interval [12-15]. Literature data that refer to the correlation between the high temperature effects and the length of body exposure to high temperature with the occurrence of poikilocytic shapes is scarce [16,17].

The aim of the study was to investigate the influence of different water temperatures and length of exposure using heat stress model by water on morphology of erythrocytes.

MATERIAL and METHODS

Ethics Committee Approval

This study was approved by the Ethical Committee of Medical Faculty University of Sarajevo under registration number 02-3-4-1253/20, Bosnia and Herzegovina.

The Study Design and Animals

Prospective, controlled, experimental study has been conducted by using the animal model. The total number of 35 adult, albino Wistar rats, of both sexes and body mass from 250 g to 300 g has been included in the experiment. Animals were kept in polypropylene cages under optimal environmental conditions (temperature 24±2°C, relative humidity of 45% to 65% and a light: dark cycle of 12:12 h), acclimatised for 7 days before the experiment and observed constantly for signs of illness; Commercial food and drinking water were given to animals *ad libitum*.

The Animal Groups

The anaesthetised thirty-five rats were exposed to preheated water using the water bath. The rats were divided into three groups: control group (n=7) exposed to 37°C water temperature, and two trial groups such as group exposed to 41°C water temperature (n=14), and another group exposed to 44°C water temperature (n=14). Each of the trial groups exposed to 41°C and 44°C water temperature was further classified according to the time of analysis, as antemortem group (n=7) that exposure time of 20 min and postmortem group (n=7) that exposure until time of death (n=7).

Experimental Protocol and Sample Collection

Rats were anaesthetised using combination of Ketamine (90 mg/kg of body weight (bw); Ketamine Hydrochloride Injection USP Rotexmedica-Germany) and Xylazin (5 mg/kg bw; 2% Xylazin, Cp Pharma, Bergdorf, Germany) once in dose of 1.2 mL/kg bw +/- 10% before being exposed to specified temperature for the specified time of exposure [18]. Water bath (Memmert GmbH + Co. KG, model size WPE 22) equipped with electronic temperature control was filled with water that was preheated to specified temperature. Experimental protocol was performed for each anaesthetised rats sequentially. Each of them was immersed into preheated water with their heads above the surface. Rats of antemortem groups were sacrificed after the expiration of the designated time of 20 min.

Microscopic Examination and Cell Counting

Blood samples have been collected from abdominal aorta with the objective of making at least two blood smears by using the standard laboratory procedure of May-Grünwald-Giemsa colouring technique. The analysis of coloured blood smears relied on the standard morphology was conducted by two independent researchers; the counting was conducted on representative one-layered visual fields where blood corpuscles just touched one another with their edges and did not overlap [19]. Two thousand erythrocytes were analysed on each coloured blood smear by using light microscope Motic Type 102M and magnification of 1000X in order to determine the possible presence of poikilocytic red blood cells. We took the average value of two independent measurements which has been previously described [19,20]. The most representative visual fields have been saved in the electronic form by using computer software Motic Images Plus 2.0.

The number and type of poikilocytes were recorded as percentages of RBC. Poikilocytosis was classified as semi-quantitatively according to similar research, following the criteria: non-existing (0%), rare (0.05-0.5%), mild (>0.5-3%),

modest (>3-10%), or expressed (>10%) [19].

Statistical Analysis

The normality of data distribution was tested by Shapiro-Wilk test. Categorical data were expressed as a percentage value. To test the differences in numerical values between groups, Kruskal Wallis test and Mann Whitney tests were used. P value <0.05 was considered as statistically significant. All statistical tests were performed using SPSS software version 25.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Fig. 1 shows the results of the poikilocytic shapes frequency in peripheral blood smear of groups. Depending on the temperature to which rats were exposed, target cells and anulocytes were detected in antemortem groups.

In 1000 cell count, there was "mild" number of ovalocytes in Antemortem and Postmortem group at 44°C. "Modest" number of Dacrocytes was detected in Antemortem group at 41°C and in Postmortem groups. Anulocytes were in "mild" number in Control group. Furthermore "moderate" number of anulocytes was detected in Antemortem group at 41°C and in both Postmortem groups. They were "expressed" in Antemortem group at 44°C. Spehrocytes were "expressed" in Postmortem groups. In postmortem groups of rats, the most frequent type of poikilocytosis found at 41°C was spherocyte, while anulocytosis with spherocytosis were detected in rats exposed to 44°C.

Table 1 shows the difference in numbers of poikilocytic shapes between analyzed groups.

Statistically significant differences between antemortem and control groups in number of ovalocytes, dacryocytes,

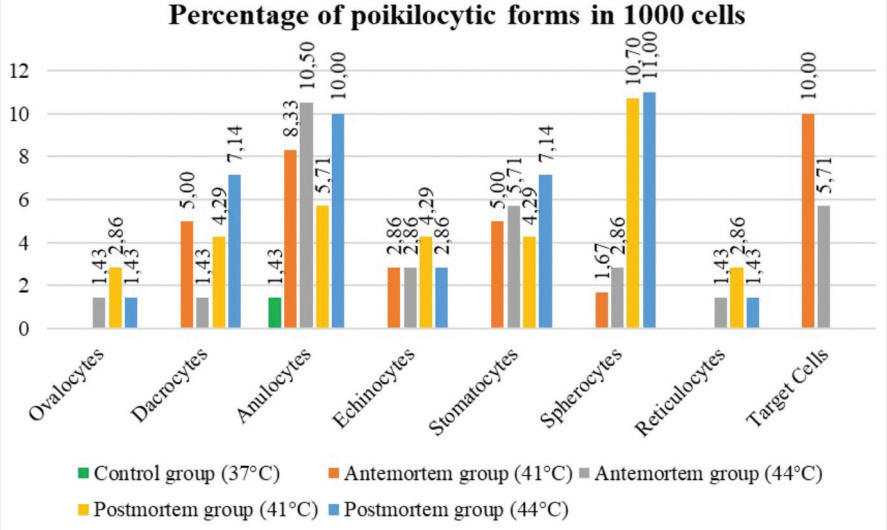

Fig 1. Frequency and types of poikilocytic forms in peripheral blood smear in control and experimental groups. The readings stand for percentage representation of individual poikilocytic types in 1000 cells

Table 1. *Types of poikilocytosis in antemortem and control groups of Wistar rats*

Types of Poikilocytosis	A: Control Group (37°C)	B: Antemortem Group (41°C)	C: Antemortem Group (44°C)	D: Postmortem Group (41°C)	E: Postmortem Group (44°C)
	Median (IQ range)	Median (IQ range)	Median (IQ range)	Median (IQ range)	Median (IQ range)
Ovalocytes	1 (0-2)^{B,C,D,E}	3.5 (1-6)^{A}	3 (2-3)^{A}	9 (4-13)^{A}	3 (1-10)^{A}
Dacryocytes	1 (0-2)^{B,C,D,E}	8.5 (1-12)^{A}	5 (2-9)^{A,E}	7 (5-26)^{A}	16 (8-19)^{A,C}
Anulocytes	1 (0-3)^{B,C,D,E}	39.5 (31-55)^{A}	47 (25-74)^{A}	50 (3-55)^{A}	100 (28-123)^{A}
Echinocytes	0 (0-1)^{B,C,D,E}	2.5 (0-38)^{A}	0 (0-15)^{A}	8 (4-59)^{A}	7 (1-13)^{A}
Stomatocytes	1 (0-2)^{B,C,D,E}	10 (4-22)^{A}	17 (6-35)^{A}	10 (2-51)^{A}	15 (8-26)^{A}
Drepanocytes	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)^{A}	0 (0-1)
Schizocytes	0 (0-2)	1 (1-6)	1 (1-2)	1 (1-2)	1 (1-2)
Leptocytes	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-1)
Acantocytes	0 (0-0)	0 (0-1)	0 (0-0)	0 (0-1)	0 (0-1)
Spherocytes	1 (0-2)^{B,C,D,E}	1 (1-8)^{A,D}	2 (1-15)^{A,E}	47 (40-84)^{A,B}	46 (25-54)^{A,C}
Reticulocytes	1 (1-1)^{B,C,D,E}	1.5 (0-2)^{A}	1 (1-4)^{A}	8 (3-11)^{A}	4 (1-10)^{A}
Target cells	1 (0-1)^{B,C,D,E}	24.5 (20-34)^{A}	12 (3-24)^{A}	2 (1-4)^{A}	1 (1-2)

Data were presented as median with 25th-75th range (IQ); ***p*** *- probability with level of significance P<.05, ^{A} sig. different than A, ^{B} sig. different than B, ^{C} sig. different than C, ^{D} sig. different than D, ^{E} sig. different than E*

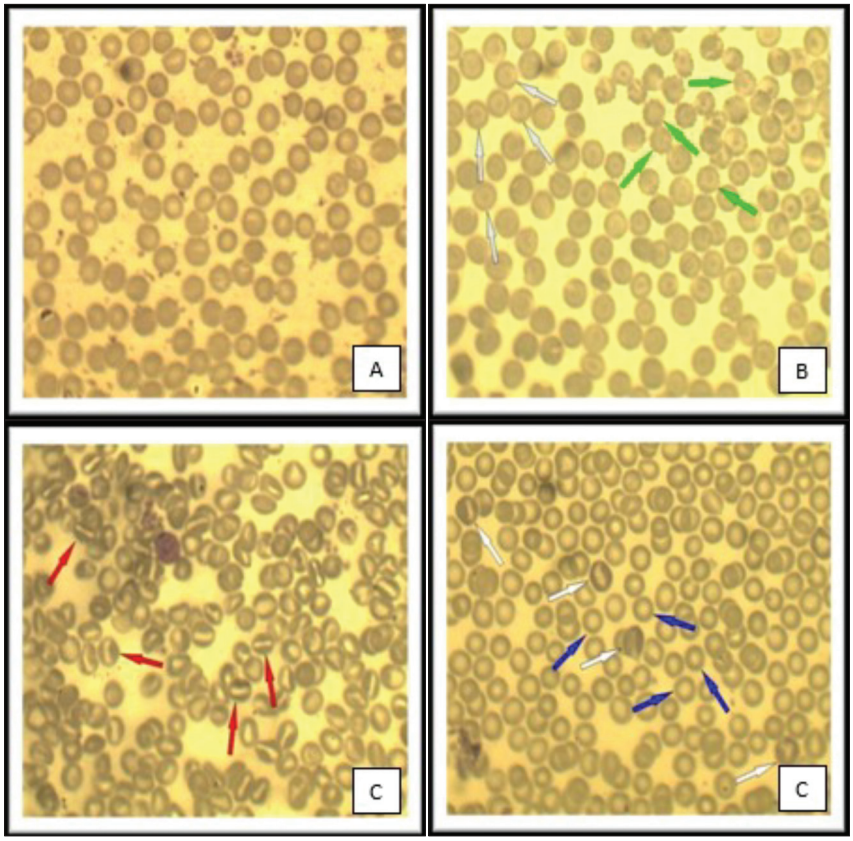

Fig 2. Poikilocytic forms of RBC, peripheral blood smear, magnification 1000x,

A- Normal red blood cells; **B**- *Green arrow:* Echynocytes, **C**- *Red arrow:* Stomatocytes, **D**- *White arrow:* Reticulocytes, *Blue arrow:* Anulocytes

anulocytes, echinocytes, stomatocytes, spherocytes, reticulocytes and Target cells were detected (P<0.05). The ovalocyte, dacryocyte, anulocyte, echinocyte, stomatocyte, spherocyte, reticulocyte and Target cell median values were significantly higher in rats exposed to 41°C than in the control (P<0.05). The difference between the control and antemortem group exposed to water temperature of 44°C was in the median number of ovalocytes, anulocytes, spherocytes, reticulocytes and Target cells (P<0.05). There was no difference in number of specific types of poikilocytic cells between antemortem groups.

Results featured in *Table 1* point towards insignificant difference in the number of abnormally shaped erythrocytes between postmortem groups (P>0.05).

Comparison of the antemortem and postmortem groups of rats exposed to water temperature of 41°C showed a significant difference in the presence of spherocytes (P=0.001). When we compared the rats from antemortem and postmortem groups exposed to water at 44°C, a significant difference in the number of dacryocytes (P=0.02) and spherocytes was observed (P=0.017).

Fig. 2 shows poikilocytic forms of red blood cells. Control group is with non-existing or rare poikilocytotic form. Echinocytes and Target cells are marked in the antemortem group 41°C. Stomatocyte is expressed in the postmortem group 41°C. Anulocytes and reticulocytes are expressed in the postmortem group of 44°C

DISCUSSION

In this study, we investigated the effects of exposures to normal and high water temperatures on erythrocyte morphology of rats blood. Twenty minutes exposure of Wistar rats to water of 41°C and 44°C induced poikilocytosis. Poikilocytosis was associated with the exposure length and temperature intensity. The most distinct shapes of poikilocytes found in the postmortem groups of rats were "expressed" spherocytosis and "expressed" anulocytosis with spherocytosis. In the antemortem group on 41°C poikilocytosis for Target cells was found; while in antemortem group on 44°C anulocytosis was identified. Their presence was "expressed" in our study. In antemortem groups and control group statistically significant difference was in almost all poikilocytic shapes, which indicated that high temperature induces poikilocytosis following 20 min exposure to the high temperature. The present finding was as results of Lucijanović et al.[20], pointing that poikilocytosis was associated with the temperature to which rats were exposed, but also with the length of exposure. Median value in spherocyte number was significantly higher in postmortem than in antemortem group 41°C. Dacriocytes and spherocytes numbers were significantly higher in postmortem than in antemortem group 44°C. The high temperature causes protein denaturation with cell malfunction, loss of membrane integrity and finally cell death. Abnormal erythropoiesis and damage of red blood cells following their formation can result

in poikilocytosis [21]. Among extrinsic factors that affect red blood cell morphology are drugs, chemicals, toxins, heat and abnormal mechanical forces. Poikilocytes of specific shapes suggest different etiologic factors [22,23]. Phagocytes play a significant role in the modulation of local or extended inflammation. In Djaldetti et al.[24] study, a temperature of 40°C significantly increased the number of phagocytic cells, as compared to cells incubated at 37°C. The increased phagocytic capacity of peripheral blood monocytes at high temperature further enlightens the immunomodulatory effect of fever in the immune responses during inflammation [24].

Specific poikilocytes are associated with diseases and noted in the rat, but may also be observed during the evaluation of rat blood in toxicity studies. In these studies the most common forms of poikilocytes are echinocytes and acanthocytes. Acanthocytes also are commonly observed in the blood of rats with severe liver disease like in other species. Red blood cell fragments (schistocytes) are observed in hemolytic processes such as those associated with Heinz body formation, osmotic shock, and disseminated intravascular coagulation. Rarely, red cell shapes such as stomatocytes, target cells, ghost cells, spherocytes, and others were observed by Car et al.[25].

The body temperature elevation caused by body heat exposure, results in significant increase of structurally transformed erythrocytes (echinocytes) and vesicles in blood [24]. Echinocytosis is a morphologic change that is characteristic of thermally injuried red blood cells [26]. The high temperature required for red cell fragmentation is an indicator of membrane abnormality [26]. Increased thermal sensitivity of red cell membrane has been demonstrated for pyropoikilocytes. Pyropoikilocytosis is distinguished by their substantially lower critical temperature of fragmentation. The lowest temperature at which normal red blood cells caused heat-induced fragmentation is 49°C, whereas pyropoikilocytes undergo morphologic changes or fragmentation on lower temperature. Fatality may occur at body temperature >42°C [27]. Our results are different from others, because the lowest temperature we pointed for poikilocytes was at 41°C, and fatality occurred at 41°C water temperature.

The increase in marrow activity is accompanied by a modest local increase in clonogenic marrow stem cells but it cannot be shown for certainty whether the phenomenon of Thermal Marrow Expansion is due to the local effect of elevated temperature on stem or stromal cells. The erythrocytes produced by Thermal Expansion appear normal *in vivo* fragility testing [28].

Hematopoetic stem cells are the only cells capable of producing all blood cell lineages throughout life. Within the bone marrow exists a tightly controlled local microenvironment, that regulates the proliferation and differentiation of stem cells. Regulatory signals emanate from surrounding cells in the form of bound or secreted molecules and also from physical cues such as oxygen tension, stress, contractile forces, inflammation and temperature [29,30]. During homeostasis, the majority of cells in bone marrow can become activated to proliferate and differentiate in response to infectious stress and other extrinsic factors like ambient temperature. Shin et al.[31] has recently discovered intrinsic mechanisms, microenvironmental interactions and communication with surrounding cells involved in stem cells regulation during homeostasis and in regeneration after injury and discussed their implications for regenerative therapy.

It is known that increased body temperature could influence cell volume by direct modification of the transport systems such as Na-K^+ ATP-ase that is sensitive to temperature. Metabolism and cell signalling is accomplished by temperature-sensitive reactions. An increase in body temperature leads to decrease of the erythrocytes volume and increase of phosphatidylserine (PS) exposure at the erythrocyte surface, common hallmarks of eryptosis, or the suicidal death of erythrocytes. Both events are accompanied by an increase of the cytosolic Ca^{2+} activity, which contributes to the cell shrinkage and cell membrane scrambling. The cell shrinkage is induced by activation of Ca^{2+}-sensitive K^+ channels with subsequent exit of K^+ and Cl^- ions with the osmotically obliged water [32]. An increased cytosolic Ca^{2+} has an effect on the cytoskeleton architecture and activates several enzymes such as transglutaminase, phospholipases, calpain, protein kinases, and phosphatases. Calpain degrades cell membrane proteins and thus leads to membrane blebbing, a further hallmark of eryptosis. Erythrocytes that were exposed to PS may adhere to the vascular wall, which results in compromising the micro-circulation in cases of body exposure to high temperature. Higher PS exposure may further activate macrophages to release proinflammatory cytokines such as interleukin-6 (Il-6) and tumour necrosis factor alpha which increase both prostaglandin formation and cortisol secretion [33]. In addition to eryptosis, a long body exposure to high temperature also stimulates haemolysis. However, unlike eryptosis, haemolysis is activated only during body exposure to the high temperatures.

Since there are more and more deaths that occur during bathing, especially in the bathroom or warm ambient in the summer months, the aim was to find out what happens to erythrocytes and their forms during life and after death as a result of high water temperature. We concluded that the length of exposure led to greater changes in erythrocytes morphology than just the 41°C or 44°C water temperature.

Authors' Contributions

E.S and M.K.gave substantial contribution to the conception or design of the article and in the acquisition and interpretation of data for the work. E.S and S.H. had a role in article drafting and in process of revision. A.J and A.K.

gave a substantial contribution to analysis of data for the work. All authors contributed to the critical revision of the manuscript for important intellectual content and have read and approved the final version.

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None

Conflict of Interest

The authors declare no conflict of interest.

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Research Article

Accomplished Management of *Chlamydophila abortus*-induced Enzootic Sheep Abortions: The Case of Şavşat (Turkey)

Fatih BÜYÜK [1,a] Ediz Kağan ÖZGEN [2,b] Emin KARAKURT [3,c] Mustafa Reha COŞKUN [1,d] Eray BÜYÜK [1,e] Murat ÖZMEN [2,f] Serpil DAĞ [3,g] Elif ÇELİK [1,h] Aliye GÜLMEZ SAĞLAM [1,i] Elif KARADENİZ PÜTÜR [2,j] Mustafa ULUCAN [2,k] Hilmi NUHOĞLU [3,l] Mitat ŞAHİN [1,m]

[1] Kafkas University, Faculty of Veterinary Medicine, Department of Microbiology, TR-36300 Kars - TURKEY
[2] Republic of Turkey Ministry of Agriculture And Forestry, Erzurum Veterinary Control Institute, TR-25070 Erzurum - TURKEY
[3] Kafkas University, Faculty of Veterinary Medicine, Department of Pathology, TR-36300 Kars - TURKEY
ORCIDs: [a] 0000-0003-3278-4834; [b] 0000-0002-5665-6864; [c] 0000-0003-2019-3690; [d] 0000-0002-1441-3995; [e] 0000-0003-2071-8901
[f] 0000-0002-6237-1398; [g] 0000-0001-7667-689X; [h] 0000-0003-4531-3863; [i] 0000-0002-7639-5075; [j] 0000-0002-8275-1574
[k] 0000-0001-6316-828X; l 0000-0003-2530-2542; [m] 0000-0003-0106-5677

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Abstract

Infectious sheep abortions caused by bacterial agents such as *Brucella melitensis, Campylobacter* spp., *Listeria* spp., *Chlamydophila abortus* etc. lead significant economic losses in sheep enterprises. Many of these bacteria such as *C. abortus* that causes enzootic sheep abortion are contagious and zoonotic, as well. "Good veterinary practices" performed accurately and timely are extremely important in the management of the outbreak and minimizes the economic losses caused by these infections. This study aimed to diagnose infectious sheep abortions and to manage the outbreaks observed in two enterprises with 850 Hemşin sheep in Şavşat district of Artvin province, Turkey. The disease was diagnosed by conventional and real-time PCRs with detecting *C. abortus* DNA in five aborted fetal tissues. The diagnosis was confirmed immunohistochemically. In the course of the outbreak management, aborted sheep were weed out, treated with 5 mL/sheep I.M. of oxytetracycline for 5 times 24 h apart and got maintained for 3-4 weeks until disposed of by the owners. Pregnant sheep were administered 5 mL/sheep I.M. of oxytetracycline for 3 times as 3 days after the first application and 5 days after the second application. A mineral-amino acid supplement was also administered to the pregnant sheep by adding 15 mL of the drug to 0.5 L water for each sheep for 3 days. For the prophylactic purpose, tetracycline with a dose of 20 mg/kg bw/day was recommended to add to drinking water once a day for 3-5 days following the initial treatment. For biosafety, aborted materials were covered with quicklime and buried in soil depth of 1.5 m and contaminated areas were disinfected with 0.5% bleach once a day for 3 days. A protective immunization could not be done because of the absence of vaccines in the national market and some concerns about the vaccination of late-pregnant sheep. The sheep enterprises were closely monitored for 4-5 weeks until the birth season ended.

Keywords: *Chlamydophila abortus, Hemşin sheep, Outbreak management, Şavşat, Artvin, Turkey*

Chlamydophila abortus Kaynaklı Enzootik Koyun Abortlarının Başarılı Yönetimi: Şavşat Örneği (Türkiye)

Öz

Brucella melitensis, Campylobacter spp., *Listeria* spp., *Chlamydophila abortus* gibi bakteriyel ajanların neden olduğu infeksiyöz koyun abortları, koyun işletmelerinde önemli ekonomik kayıplara yol açarlar. Enzootik koyun abortlarına neden olan *C. abortus* ve sayılan diğer bakterilerin çoğu aynı zamanda bulaşıcı ve zoonotiktir. Doğru ve zamanında yapılan "iyi veteriner hekimlik" uygulamaları salgının idaresinde son derece önemli olup enfeksiyon kaynaklı ekonomik kayıpları minimum seviyeye indirmektedir. Bu çalışmada, Artvin ili Şavşat ilçesinde 850 Hemşin koyunu bulunan iki işletmede gözlenen infeksiyöz koyun abortlarının teşhis edilmesi ve salgının idaresi amaçlanmıştır. Hastalık, atık beş kuzuya ait fötal dokularda *C. abortus* DNA'sının konvansiyonel ve real-time PCR'ler ile saptanması ile teşhis edilmiştir. Teşhis, immunohistokimyasal yöntemlerle doğrulanmıştır. Salgın yönetimi kapsamında, atık yapan koyunlar ayıklanmış, 5 mL/koyun dozda oksitetrasiklin ile İ.M. yolla 24 saat arayla 5 defa sağaltılmış ve elden çıkarılıncaya kadar 3-4 hafta süresince beslenmiştir. Gebe koyunlara, ilk uygulama, 3 gün sonra ikinci uygulama ve 5 gün sonra üçüncü uygulama şeklinde İ.M. yolla 5 mL/koyun dozda oksitetrasiklin uygulanmıştır. Ayrıca bu hayvanlara 3 gün süresince 0.5 L suya 15 mL katılmak üzere aminoasit-mineral takviyesi de yapılmıştır. İlk ilaç uygulamasını takiben profilaktik amaçlı, 20 mg/kg c.a./gün dozda tetrasiklinin günde bir kez olmak üzere 3-5 gün süreyle içme sularına katılması tavsiye edilmiştir. Biyogüvenlik kapsamında, atık materyalleri sönmemiş kireçle kaplanarak toprağa 1.5 m derinlikte gömülmüş ve kontamine alanlar %0.5'lik çamaşır suyu ile günde bir kez olmak üzere 3 gün süreyle dezenfekte edilmiştir. Ulusal pazarda aşının olmayışı ve ileri gebe koyunlarda aşı uygulanması ile ilgili bazı endişelerden dolayı koruyucu bir aşılama yapılamamıştır. Koyun sürüleri doğum sezonu bitene kadar 4-5 hafta süreyle yakından takip edilmiştir.

Anahtar sözcükler: *Chlamydophila abortus, Hemşin koyunu, Salgın yönetimi, Şavşat, Artvin*

Correspondence
+90 544 3760907
fatihbyk08@hotmail.com

INTRODUCTION

Main problems encountered in the sheep raising are perinatal lamb deaths and pneumonia. Among the causes of perinatal lamb deaths, the most prominent are infectious abortions and diarrhea. It is reported that a significant part of sheep abortions in Turkey has an infectious character. Infectious sheep abortions are caused by bacterial, viral and paracetic agents such as Brucellosis, Campylobacteriosis, Chlamydiosis, Listeriosis, Salmonellosis, Bluetongue Virus (BTV), Bovine Viral Diarrhea-Mucosal Disease (BVD-MD), Akabane and *Toxoplasma gondii* [1-4]. Among these, Chlamydial abortions are the infections caused by *Chlamydophila abortus (Chlamydia psittaci*-serotype 1) and lead to reproductive deficiencies, enzootic abortions and related economic losses in sheep [5,6]. Indeed, it has been estimated that Chlamydial agents cause an annual economic losse of 11-48 million pounds in sheep abortions observed in England [7]. Although the prevalence of Chlamydial abortions varies by country, the rate can reach 30% in naive herds. Chlamydial agents are scattered around through the vaginal discharges of aborted sheep and tissues of aborted fetus and transmitted by oral route following the oronasal contact of susceptible animals. The main clinical symptom of *C. abortus* infection is abortion observed in the last 2 or 3 weeks of gestation, which is emerged following an inflammation, thickness and vascular thrombosis in placenta. The abortion can sometimes be seen as an abortion storm, where too many sheep deliver the fetus involuntarily in a short time [8].

As the clinical findings are similar in many infections with abortion, the exact diagnosis of *C. abortus* is made by laboratory analysis. For this purpose, imaging of the elementary bodies on direct smears is agent-specific, however, definitive diagnosis is required an *in vivo* isolation or PCR confirmation of the agent. Immunohistochemical analysis of sections taken from fresh fetal tissues also provides useful information in the diagnosis. Although antibody titer increased 2-3 weeks after the abortion can be detected by Complement Fixation Test (CFT) and ELISA, the possibility of cross-reaction should be considered [9]. In the course of active Chlamydial infection, all animals in the herd, including pregnant sheep, can be treated with long-acting oxytetracycline. On the disease control, the main outbreak management practices that can be applied are early detection of abortion cases, screening of whole herd, isolation of aborted animals, disinfection of contaminated areas and vaccination of healthy animals [10,11].

This study is based on the diagnosis of infectious agents and the management of the enzootic sheep abortions observed in two enterprises where intensive sheep breeding is carried out in Şavşat district of Artvin province.

MATERIAL and METHODS

The study was carried out in two intensive sheep enterprises suffered from infectious abortions in Şavşat district Artvin province (Turkey), in February of 2020. The ethical permission of the study was ensured by the decision of The Kafkas University Animal Experiments Local Ethics Committee with the code of "KAÜ-HADYEK/2020-064". Study material composed of brain, liver, lung, kidney, spleen and stomach (abomasum) contents of five aborted fetuses was obtained from one of the enterprises. As the storage of aborted materials belonging to the other enterprise is not properly maintained, no sample could taken for laboratory diagnosis. All samples were taken following the systemic necropsy of aborted fetuses and maintained properly. For bacterial isolation, approximately 5 g of each fetal tissue and 1 mL of abomasum contents were kept in sterile containers. For molecular analysis, 25-50 mg fetal tissues and 100 µL abomasum content were homogenized in MagNA Lyser Green Beads tubes (Roche, Switzerland). For immunohistochemical analysis, the samples were maintained in 10% buffered formaldehyde solution. All reagents and chemicals were obtained from Fisher Scientific (Loughborough, UK) or Sigma-Aldrich (Dorset, UK) unless otherwise stated in the text.

Cultural Analysis

Cultural analyses were carried out in Microbiology Laboratories of Veterinary Faculty in the Kafkas University and in Erzurum Veterinary Control Institute. *Brucella* spp., *Salmonella* spp. and *Campylobacter* spp. analyzes were done by cultural methods from the samples. For *Brucella* spp. isolation, the samples were plated on Farrell medium plates and incubated at 37°C in microaerophilic conditions for 8 days. Presence of the typical honey-colored smooth colonies with a diameter of 1-2 mm was evaluated on the medium [12]. *Campylobacter* spp. isolation from the samples was done on Skirrow medium. The plated media were incubated at 37°C in microaerophilic condition for 5 days and evaluated for the typical colony presence [13,14]. *Salmonella* spp. isolation from the samples was performed on XLD medium. The plated media were incubated at 37°C in aerobic environment for 5 days and evaluated for the typical colony morphology [15]. Moreover, the fetal tissue samples were plated on 7% sheep blood agar plates and incubated at 37°C for 72 h in aerobic and microaerobic conditions and evaluated for the other bacterial abortive agents which could be culturable *in vitro* conditions.

Molecular Analysis

Molecular analyses were carried out in Molecular Microbiology Laboratories of Veterinary Faculty in the Kafkas University and in Erzurum Veterinary Control Institute.

Conventional PCR and real-time PCR techniques were used for direct diagnosis of the abortive agents. In this context, analysis of bacterial abortive agents such as *C. abortus* and *Leptospira* spp., parasitic agents such as *T. gondii* and viral agents such as Bluetongue Virus (BTV), Small Ruminant

Plague (PPR), Akabane and Bovine Viral Diarrhea (BDV) Virus were performed.

Nucleic acid extraction: Total nucleic acid was extracted through an automated extraction device (Qiacube, Qiagene, Germany) and a commercial kit (Qiamp cador pathogen mini kit, Qiagen, Germany). Tissue samples were placed into sterile homogenization tubes containing 500 µL of PBS, and then lysed at 6000 rpm for 1 min in MagNa Lyser© Instrument (Roche, Switzerland). Tissue samples were then centrifuged at 6000 rpm for 3 min and 200 µL of supernatant was used for extraction.

Conventional PCR analysis: Conventional PCR analysis of *C. abortus* was performed with primers which amplify the polymorphic membrane protein *(pmp)* gene of the bacteria [16] *(Table 1)*. Reaction volume was formed from 5 µL PCR buffer, 0.4 µL dNTP mix, 0.3 µL primer-F, 0.3 µL primer-R, 0.4 µL Taq DNA polymerase, 15.6 µL nuclease-free water and 3 µL template DNA. Cycling conditions were consisted of 5 min of initial denaturation at 94°C, followed by 30 cycles of 30 sec of denaturation at 94°C, 60 sec of annealing at 50°C, and 120 sec of extension at 72°C. The PCR was finalized with an extension at 72°C for 10 min. Amplified products were analyzed by 1.5% agarose gel electrophoresis and 300 bp size products were evaluated.

Real-time PCR analysis: Analysis of bacterial and parasitic agents was performed by LightCycler® Taqman® Master kit (Roche, Switzerland) with using the primers and probes [17-19] specified in *Table 1*. Reaction volume was composed of 9 µL nuclease-free water, 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), 4 µL FastStart mix, 1 µL Taqman probe (4 µM) and 5 µL template DNA. PCR amplification was performed on a real-time PCR machine (LightCycler® 480 Instrument II, Roche, Switzerland). Cycling conditions were consisted of 10 min of initial denaturation at 95°C, followed by 45 cycles of 3 sec of denaturation at 95°C, 30 sec of annealing at 60°C, and 1 sec of extension at 72°C.

Analysis of viral agents was done with one step real-time RT-PCR method. For this purpose, QuantinNova Pathogen + IC Kit (Qiagen, Germany) was used. Reactions were carried out in the presence of primers and probes [20-24] specified in *Table 1*. Nucleic acid extracts were used after 3 min denaturation at 95°C in order to denaturate RNA of the double-stranded BTV. RT-PCR reaction in 20 µL volume was composed of 6 µL nuclease-free water, 5 µL RT-PCR master mix, 2 µL probe assay, 1 µL IC RNA, 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), 1 µL probe (4 µM) and 4 µL template RNA. cDNA acquisition and subsequent amplification were performed on the real-time PCR machine. The one step real-time RT-PCR thermal

Table 1. *Primer and probe sequences used for real-time PCR*

The Agent	Primer and Prob Sequences (5'–3')		References
C. abortus pmp gene	Forward	ATGAAACATCCAGTCTACTGG	[16]
	Reverse	TTGTGTAGTAATATTATCAAA	
Leptospira spp.	Forward	AAGCATTACCGCTTGTGGTG	[17]
	Reverse	GAACTCCCATTTCAGCGATT	
	Probe (FAM/BHQ1)	AAAGCCAGGACAAGCGCCG	
T. gondii B1 gene	Forward	GGAATGAAAGAGACGCTAATGTGTT	[18]
	Reverse	ACAGATACTCATGAATTTCACTTTTCG	
	Probe (FAM/TAMRA)	TTGCAGTCACTGACGAGCTCCCCTCT	
C. abortus ompA gene	Forward	GCAACTGACACTAAGTCGGCTACA	[19]
	Reverse	ACAAGCATGTTCAATCGATAAGAGA	
	Probe (FAM/TAMRA)	TAAATACCACGAATGGCAAGTTGGTTTAGCG	
AKABANE	Forward	GCTAGAGTCTTCTTCCTCAACCAGAA	[20]
	Reverse	AAAAGTAAGATCGACACTTGGTTGTG	
	Probe (FAM/TAMRA)	CCAAGATGGTCTTACATAAGAC	
BVD	Forward	CCATRCCCDTAGTAGGACTAGC	[21,22]
	Reverse	GYGTYGAACTACTGACGACT	
	Probe (FAM/BHQ1)	ACTAGCCGTCGTGGTGAAATCCCTGAGTGG	
BTV	Forward	TGGAYAAAGCRATGTCAAA	[23]
	Reverse	ACRTCATCACGAAACGCTTC	
	Probe (FAM/TAMRA)	ARGCTGCATTCGCATCGTACGC	
PPR	Forward	CACAGCAGAGGAAGCCCAACT	[24]
	Reverse	TGTTTTGTGCTGGAGGAAGGA	
	Probe (FAM/TAMRA)	CTCGGAAATCGCCTCGCAGGC T	

cycle was initiated with a reverse transcriptase step at 50°C for 10 min. The reaction was continued with an initial denaturation at 95°C for 2 min, followed by an amplification step of 45 cycles at 95°C for 5 sec of denaturation and 60°C for 30 sec of annealing.

Immunohistochemical Analysis

Immunohistochemical analyses were carried out in Pathology Laboratories of Veterinary Faculty in the Kafkas University.

Hematoxylin-Eosin staining: Following the systemic necropsy of aborted fetuses, tissue samples (lung, liver) were fixed in 10% buffered formaldehyde solution. After the routine tissue follow-up, sections at 5 µm were taken from paraffin blocks prepared for Hematoxylin & Eosin (H&E) staining. The tissue sections were examined and photographed (Olympus Soft Imaging Solutions GmbH, 3,4, Olympus, Germany) under a light microscope (Olympus, Germany) to detect histopathological changes.

Immunohistochemical staining: Avidin-Biotin Peroxidase method was used as an immunohistochemical method [25]. For this purpose, the sections at 4 µm taken from paraffin blocks were rehydrated. The sections were treated with 3% Hydrogen peroxide solution for 15 min to prevent endogenous peroxidase activity. The microwave method was then applied (Citrat Buffer Solution, pH: 6, for 25 min) to the sections to reveal antigenic receptors. The sections were incubated with non-immune serum (Genemed Biotechnologies, Germany) for 30 min to prevent non-specific staining. Subsequently, the sections were incubated for 1 h at room temperature with an anti-Chlamydia primary antibody (Progen, Germany) diluted 1/100 in Phosphate Buffered Salt Solution (PBS). The sections were washed 3 times for 5 min in PBS solution, and subjected to biotinylated antibody (Genemed Biotechnologies, Germany) for 30 min at room temperature. After washing in PBS for 3-5 min, all the sections were incubated for 30 min with peroxidase-linked Strep Avidin (Genemed Biotechnologies, Germany). 3.3'-Diaminobenzidine tetrahydrochloride (DAB) (Genemed Biotechnologies, Germany) solution was used as the color-producing substrate. The sections were stained with Mayer's Hematoxylin and covered with immune mount. The smears prepared after the covering were examined under a light microscope (Olympus, Germany) and photographed.

Outbreak Management Practices

Outbreak management practices [10,11] were carried out in two different sheep farms, which suffered from the outbreak in the same period and included a total of 850 Hemşin sheep. In this context, since there was no suitable sample flow for the laboratory diagnosis from the farm where there were 150 sheep and 17 aborted lambs, only biosecurity applications and medication treatment procedures were carried out in this herd. In the farm, where 700 sheep and 35 aborted lambs took place, in addition to these applications, diagnosis of the infectious agent was also carried out. All prophylactic and metaphylactic practices were carried out in the farms within the scope of routine veterinary services through the contributions of an authorized veterinary clinic in Şavşat district of Artvin province.

RESULTS

In this outbreak, the enzootic abortion cases were evaluated in two herds consisting of 850 Hemşin sheep. The herds did not have a history of vaccination against *C. abortus* infection. It was reported that there have been no feed changes and drug administrations before the outbreak. It was reported that the herds were exposed to a 2-h open-air trip in a slow rhythm at a temperature of approximately 5°C from a relatively high altitude to a low altitude area before the outbreak begins. The abortions which started the day after the trip occurred as total 52 abortions (35 abortions in 700 head sheep herd and 17 abortions in 150 head sheep herd) within 15 days, including the first abortion, diagnosis and drug administration processes. The vast majority of the abortions (45 abortions) was before the drug administration, and only 7 lamb abortions were observed in two flocks after the drug administration. The abortions were generally observed in sheep in the last 20-30 days of the gestation. It was reported that the symptoms such as loss of appetite, stillness, wet tail and genital discharges were observed just before the abortions. No other findings were observed in aborted fetuses other than generalized edema.

Cultural Analysis Findings

The common bacterial abortive agents, *Brucella* spp., *Campylobacter* spp. and *Salmonella* spp. were investigated in the fetal tissue samples by cultural methods. However, the aforementioned agents were not isolated from any of the five fetal tissues evaluated for this purpose.

Molecular Analysis Findings

Conventional PCR findings: As the results of conventional PCR analysis, the amplified products 300 bp in length specific for *C. abortus* were obtained from all of the three aborted fetal tissues *(Fig. 1)*.

Real-time PCR findings: With the real-time PCR method, two aborted fetal tissues were investigated for the viral, bacterial and parasitic abortive agents and only *C. abortus* nucleic acids were detected in the samples *(Fig. 2)*.

Immunohistochemical Analysis Findings

Hematoxylin & Eosin staining findings: In histopathological examinations, thickness in interalveolar septum was observed in some areas of lung tissue *(Fig. 3)*. Apart from these findings, no significant lesion was observed in the lung. In liver tissue, inflammatory infiltration areas consisting

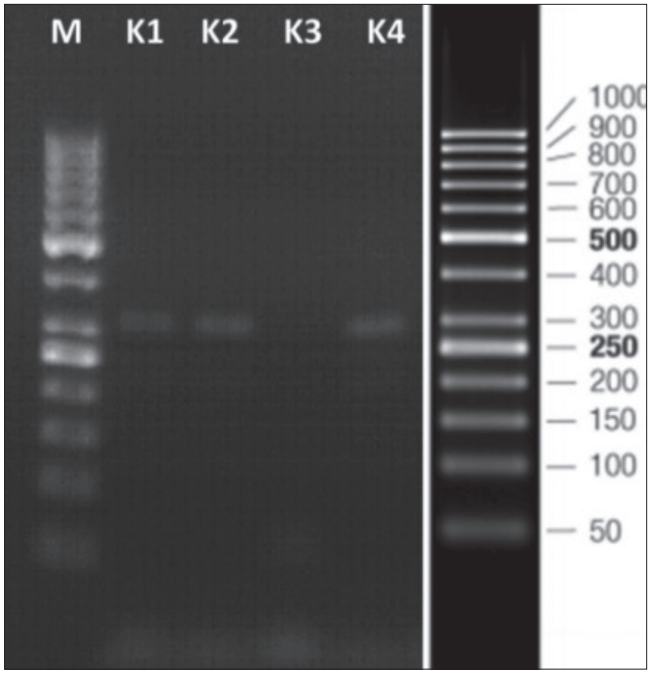


Fig 1. Electrophoresis image of conventional PCR products specific for *pmp* gene of *C. abortus*. M: Marker (Thermo Fisher Sci., SM0371), K1, K2, K4: Fetal tissue samples, K3: *E. coli* OP50

of mostly mononuclear cells and a small number of neutrophil granulocytes were detected in portal area and around vena centralis *(Fig. 4-A,B,C)*. Moreover, focal necrosis and activation in Kupffer cells were among the other important histopathological findings observed in the liver tissue.

Immunohistochemical findings: *Chlamydophila* spp. immunopositivity was detected in brown in the cytoplasm of hepatocytes *(Fig. 5)*.

Outbreak Management Findings

During and after the abortion storm, aborted sheep were identified and separated from the herds due to the risks of spread and transmission of the bacteria with genital excretes and these were housed in an isolated area. These animals were subjected to an intense care and feeding regime for 3-4 weeks until the animals recover from the postpartum period and became butchery. Moreover, these animals received special treatment to minimize the spread of the bacterial agent. In this context, the aborted sheep were treated with 5 mL/sheep I.M. of oxytetracycline (100 mg/mL) (Primavilin® Inj., Vilsan, Turkey) for 5 times 24 h

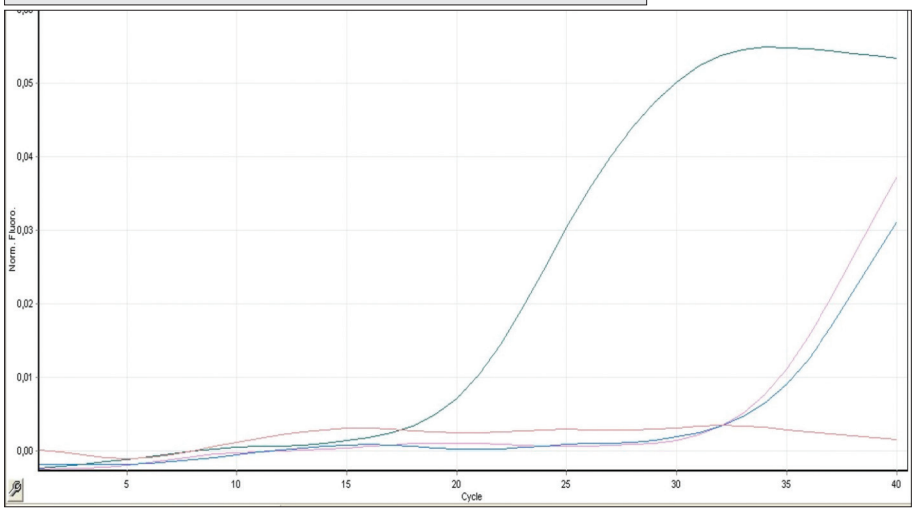
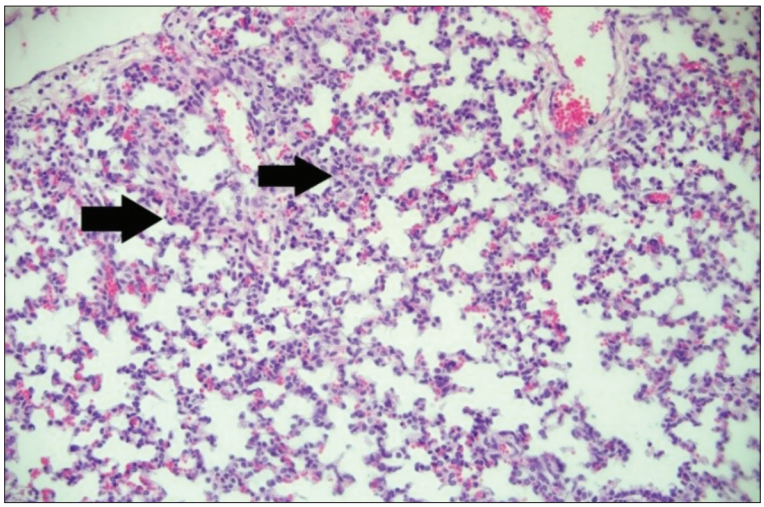


Fig 2. *C. abortus*-specific amplification curve *(pink and blue)* of real-time PCR of nucleic acid samples of two fetuses (Positive control: blue curve, Negative control: horizontal pink curve)

Fig 3. Lung tissue, thickness in the interalveolar septa *(arrows)*, H&E, Bar = 100 µm



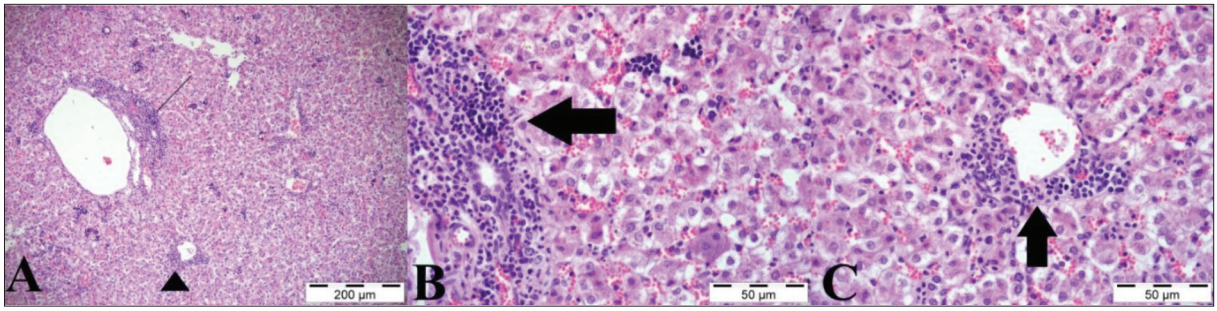


Fig 4. Liver, **A:** Cell infiltration around portal area and vena centralis, H&E, Bar = 200 µm, **B:** Higher magnification, cell infiltration around the portal area, H&E, Bar = 50 µm **C:** Higher magnification, cell infiltration around vena centralis, H&E, Bar = 50 µm

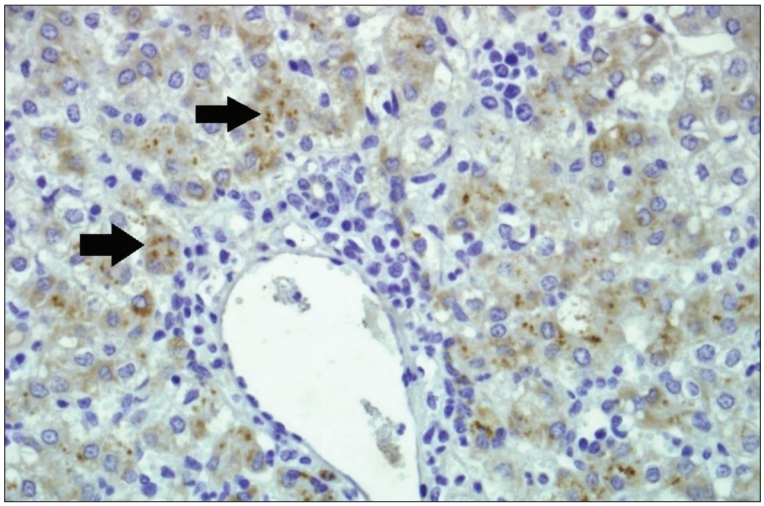


Fig 5. Liver, severe immunopositive reaction of *Chlamydophila* spp. in the cytoplasm of hepatocytes, IHC, Bar = 50 µm

apart. Additionally, the ground of the barn where the aborted sheep were sheltered was decontaminated with 0.5% of bleach once a day for 3 days.

Within the scope of biosafety practices in herds, aborted materials were covered with quicklime and buried in the soil at a depth of approximately 1.5 m and contaminated areas were disinfected with 0.5% bleach once a day for 3 days. During this period, care was taken not to allow the animals to enter and/or exit to the herds.

During the study, only one of the herds could be diagnosed with the infection. Since the story, timing and clinical reflection of the outbreak of the herd consisting of 150 sheep coincided with the other herd, a similar treatment approach was applied to this herd without a diagnosis. In this context, a common treatment regimen was applied to approximately 800 pregnant sheep in both herds to prevent abortion. For this purpose, all pregnant sheep were administered 5 mL/sheep I.M. of oxytetracycline (215 mg/mL) (Primavilin LA® Inj., Vilsan, Turkey) for 3 times as 3 days after the first application and 5 days after the second application. Moreover, a mineral-amino acid supplement (Depomin® Oral Solution, Vetaş, Turkey) was administered to the pregnant sheep by adding 15 mL of the drug to 0.5 L water for each sheep for 3 days. For prophylactic purpose, a commercial tetracycline (500 mg/g) (Tetramed® WSP, Medicavet, Turkey) with a dose of 20 mg/kg bw/day was recommended to add drinking water once a day for 3-5 days following the initial treatment.

In this study, a protective vaccine application could not be performed in the affected herds due to the lack of commercial *C. abortus* vaccine in the national market. Moreover, serological techniques in which disease was diagnosed by measuring the antibody levels to be formed approximately 2-3 weeks after the abortion were not applied on the grounds that they would not be helpful in the diagnosis of this acute course infection. The sheep herds were monitored continuously for 4-5 weeks after the outbreak, while all sheep gave healthy birth and no additional abortion, stillbirth or weak lamb births were reported. The sheep herds will continue to be monitored closely during the next few birth seasons.

DISCUSSION

"Good veterinary practices" for the correct and timely management of the outbreaks caused by the infectious agents in sheep breeding are extremely important. In this context, the biosafety practices such as diagnosis of infectious disease, isolation of infected animals and disinfection of contaminated areas, treatment of infected animals and vaccination of healthy animals, are the main

methods to be applied [10,11]. To detect infected or porter animals and determine the source of infection are the first applications to be made in an outbreak management. The main infection sources in abortive cases are placenta and vaginal discharges of the aborted sheep, and fetal membranes and tissues of the aborted fetus. Isolation and PCR methods can be used to detect *C. abortus* and the other abortive microorganisms in such samples taken freshly and maintained properly. However, *in vitro* culture of the agents with intracellular characteristics such as *C. abortus* cannot be done and thus PCR techniques are used in direct diagnosis of these agents [4,16]. Rapid and reliable diagnosis of the agent in an outbreak is very important in preventing the spread of the disease and thus in reducing economic losses. In this respect, PCR techniques have an important advantage and can identify the genus, species and even subspecies of the microorganisms [26]. The PCR techniques providing the amplification of bacterial outer membrane protein A *(ompA)* and polymorphic membrane protein *(pmp)* genes have been reported to be highly effective in identification of the *C. abortus* from clinical samples [16,19,27]. In this study, PCR techniques were applied in addition to the unsuccessful isolation attempts of the agents from the aborted materials, and direct identification of *C. abortus* from the tissue samples was performed with PCR methods. Appropriate sampling was possible from only one of the herds of sheep where the outbreak was observed and *C. abortus* positivity was achieved in 2 of these aborted lambs with real-time PCR and in 3 with conventional PCR. Tissue samples examined by the cultural methods and PCR were found negative in terms of other abortive agents. In Turkey, the most of the studies of *C. abortus* abortions are serosurvey studies showing the individual or herd-based prevalence of the agent. In these studies, the prevalence of the *C. abortus* was reported between 5.8% and 32% [28-30]. Considering this study, the average abortion rate in two herds was 6.11% (5% in the herd with 700 sheep and 11.33% in the herd with 150 sheep) and the number of abortion diagnosed as *C. abortus* was limited to 5 (9.61%) cases. Although the identification rate of this study is close to some studies, it has not been possible to compare directly with other studies in which only seroprevalence was reported [28-30]. The value of the serological diagnosis, in which a retrospective screening of the Chlamydophila infection can be made by measuring the level of antibodies formed in 2-3 weeks following the abortion, is quite limited in the period when the abortion storms are observed. Therefore, herd screening was not performed with the serological diagnostic tests on the grounds that it would not beneficial during the 15-day period when abortion storms were seen and it was postponed for use in the following season.

Histopathological and immunohistochemical diagnosis of the infection was performed on the fetal tissues of 5 aborted lambs belonging to only one enterprise. Although the findings of histopathological analysis obtained in this study are not diagnostic, they are similar to those [6,31] reported for *C. abortus* infection. Immunohistochemical (IHC) analysis is a proven technique used in confirmation of the diagnosis of *C. abortus* infection [32]. In this study, an immunopositivity was obtained in liver hepatocytes of the samples with the IHC technique using an anti-Chlamydia antibody, which recognizes the common LPS antigen of all *Chlamydia* species. Although the primary location of *C. abortus* in the abortion cases is placenta [31], in this study, IHC positivity obtained from the fetal tissues and the confirmation of the cases with PCR methods reveals the diagnostic value of these tissues in the diagnosis of infection.

Another step of the successful outbreak management is biosecurity practices, including external and internal measures to prevent transmission and spread of the infectious agent [10,11]. In this context, restriction of the animal movements and treatment of contaminated areas are the main applications. In this outbreak, besides bury of the aborted materials covered with quicklime, disinfection of contaminated enterprise areas with 0.5% bleach was also carried out for 3 days. Additionally, free movements of the herds were prevented. In enzootic abortion cases, applications related to retaining or weeding of the aborted sheep in herd are not uniform. Nevertheless, it should be considered these sheep can spread the microorganism despite being immunized following the infection. The practices related to weeding of infected animals vary depending on herd size and especially economic concerns. While it is foreseen that all animals in small herds can be disposed of following the outbreak, this practice is not very economical in large herds [10,11]. In this context, 52 sheep with abortion in these enterprises, which contain 850 sheep in total and have a relatively large herd structure, have been insulated and maintained in a separate area until the postpartum period has passed. These animals, which have been treated with special antibiotics to reduce their active shedding of the bacteria with their genital excretes, were evaluated as butchery after 3-4 weeks, paying attention to the clearance period of the administered drugs from the body. *C. abortus* poses a risk to humans due to its zoonotic feature. The transmission of the bacteria to humans is through especially contact with the infected sheep or aborted materials. *C. abortus* causes respiratory system and cardiovascular diseases in humans, as well as abortion in pregnant women [33]. In this context, in order to prevent possible human transmission and subsequent infections, biosecurity and protection measures have been described in order to fully comply with all individuals at risk in all stages of the outbreak management.

Therapeutic and curative treatment procedures in herds following the arisen of a clinical disease and the diagnosis of infection are the other useful applications in the outbreak management [10,11]. For treatment, tetracycline preparations have widely used in such applications for

Chlamydial infections in veterinary medicine [34]. Metaphylaxis also called control treatment, is the mass medication performed to prevent infection in animals at risk during an outbreak. Moreover, metaphylaxis is carried out to prevent shedding of the microorganism via the animals in incubation period or subclinically infected [35]. In this context, as soon as the infection was diagnosed in a herd, approximately 800 sheep were treated with oxytetracycline in order to prevent possible transmission and abortion in pregnant sheep. Besides, mineral-amino acid supplements were applied to the pregnant sheep. Prophylaxis called preventive treatment is a treatment approach performed in a population for preventive purposes before the infectious disease occurs [35]. In this context, there are some useful applications available regarding to add tetracycline preparations with a dose 150-500 mg/sheep/day to feeds until the end of the lambing season [5,36]. In this outbreak, it was recommended to owners to add a commercial tetracycline preparation to the drinking water once a day for 3-5 days following the preliminary antibiotic treatment. While the metaphylactic and prophylactic applications prevent the spread of Chlamydial agents and additional placental damages in body, they cannot completely eliminate the infection and reduce the existing placental damage. For this reason, new abortions and stillbirths may still be encountered after the drug administrations [5]. There is a similar situation in this outbreak, and fewer abortion cases were encountered in both herds following the drug administrations and the abortion ended within one week.

Vaccination is another method to be applied within the scope of the outbreak management in protection against the infectious diseases [10,11]. Although there are several international trademark vaccines of *C. abortus*, most of them are live vaccines whose have some restrictions such as limited uses in pregnancy and mating season, and these can not be combined with antibiotics, especially tetracyclines. In this study, it was not possible to perform a protective immunization during the outbreak in affected herds due to the absence of vaccines in the national market. Additionally, international vaccination has not been attempted to provide since the herds of late-pregnant sheep had concerns about the above vaccine restrictions. However, due to the need to continue vaccination indefinitely in reducing the incidence of infectious diseases [5], the breeders were advised to vaccinate all healthy animals against *C. abortus* after the birth season in these herds.

In conclusion, in an abandoned sheep herd, the abortion rate can reach up to 30% because of the high contagiousness of *C. abortus* and this leads to significant economic losses in sheep enterprises. The "Good Veterinary Practices" performed accurately and timely during the management of an outbreak such as biosafety measures, treatment, vaccination and close monitoring of the herd throughout this process is extremely important and can reduce the economic losses caused by the infection. In this context, it is hoped that this study, which implements the aforementioned practices that involve an accomplished management of a *C. abortion*-based outbreak in local sheep enterprises, will be beneficial for its stakeholders.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Author Contributions

FB, EKÖ and EK designed the experiment, made the microbiological and histopathological analyses and wrote the manuscript. MRC, EB, MÖ and HN took over the operational stage of the epidemic management. All authors made a substantial contribution to discuss the results and approved the final version of the manuscript.

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Research Article

Early Markers of Mesenteric Artery Ischemia in Rats

Feray AYDIN [1,a] Bilge ASLAN [2,b]

[1] Ankara 29 Mayıs Hospital, Department of Surgery, TR-06105 Ankara - TURKEY
[2] University of Health Sciences, Ankara City Hospital, Department of Anesthesiology and Reanimation, TR-06800 Ankara - TURKEY

ORCIDs: [a] 0000-0002-2847-4780; [b] 0000-0001-6366-7548; [c] 0000-0003-3640-5686

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Abstract

Acute mesenteric ischemia (AMI) is a disease with high mortality (60%-80%). Although it has different etiological causes, the common outcomes are intestinal necrosis and gangrene. The survival rate is reported to be 30-40% on average. Therefore, there is a need for biomarkers to assist the diagnosis. In this study, the early diagnostic values of procalcitonin, lactate, amylase and ferritin levels in AMI were investigated. The rats were divided into two groups:: Control group (Group 1, n=8) consisted of rats which underwent laparotomy only, Study group (Group 2, n=8) consisted of rats who underwent ligation of the superior mesenteric artery (SMA) from the aortic outflow tract to create an experimental ischemia model. Blood samples were taken from both groups at hours 0, 2, 6, 12, and 24, and procalcitonin, lactate, amylase and ferritin levels were compared. Lactate and amylase levels increased in blood in the study group, starting at the 6h after SMA ligation until 24h. Amylase and lactate levels can be used early diagnostic biomarkers in AMI. Increase in procalcitonin level was only significant at the 6h. Ferritin levels did not change in the first 24h and hence can not be considered as an acute phase reactant.

Keywords: *Acute mesenteric ischemia, Procalcitonin, Lactate, Amylase, Ferritin*

Ratlarda Oluşturulan Mezenterik Arter İskemisinin Erken Belirteçleri

Öz

Akut mezenterik iskemi (AMI) potansiyel olarak ölümcül bir hastalıktır (%60-80). Farklı etiyolojik nedenleri olmasına rağmen, mezenterik iskeminin ortak sonuçları bağırsak nekrozu ve gangrendir. Hayatta kalma oranı ortalama %30-40'tır. Bu nedenle tanıda rehberlik edecek biyobelirteçlere ihtiyaç vardır. Bu çalışmada AMI'deki prokalsitonin, laktat, amilaz, ferritin düzeylerinin erken tanısal değerleri araştırıldı. Sıçanlar sadece laporotomy yapılan kontrol grubu (Grup 1, n=8) ve aortik çıkış yolundan superior mezenterik arterin bağlanması ile deneysel bir iskemi modeli oluşturulan deneysel grup (Grup 2, n=8) olarak iki gruba ayrıldı. Sıçanlardan 0., 2., 6., 12. ve 24. saatte alınan kan örneklerindeki PCT, Laktat, Amilaz, Ferritin seviyeleri iki grup arasında karşılaştırıldı. Laktat ve Amilaz'ın kan düzeyleri SMA (Superior Mezenterik Arter) oklüzyonun 6. saatinden başlayarak 24. saate kadar artış gösterdi. Amilaz ve laktat, AMI'de erken tanısal belirteçler olarak kullanılabilir. Prokalsitonin artışı sadece 6. saatte anlamlıydı. Ferritin ise ilk 24 içinde hiç artış göstermediğinden AMI için erken faz belirteci olarak kabul edilmedii.

Anahtar sözcükler: *Akut mezenterik iskemi, Prokalsitonin, Laktat, Amilaz, Ferritin*

INTRODUCTION

Acute mesenteric ischemia (AMI) is a life-threatening acute abdominal disease that occurs as a result of sudden insufficiency in the blood flow of mesenteric vessels in the intestine [1-4]. Dramatically, AMI, appears as a frightening surgical problem [5]. The clinical signs and symptoms seen as a result of the sudden obstruction of the superior mesenteric artery constitute the most common clinical form, with the most common cause being the embolism of the superior mesenteric artery or its branches [6-10]. It presents with arterial spasm, insufficient collateral circulation, and decreased perfusion pressure ischemia. Venous mesenteric thrombosis is the cause of acute mesenteric ischemia in about 10-15% of all cases [1,2]. Superior mesenteric artery embolism is the most common known underlying cause of acute occlusive mesenteric ischemia, seen at a rate of 50% [11,12]. Luther et al.[13] retrospectively examined a total of 91 patients who had undergone endovascular treatment over a period of 11 years. A stent was applied in 78 (85.7%) patients, and angioplasty was performed in 13 (14.3%), principally of the superior

Correspondence
+90 312 5126000 Fax: +90 312 5529982
drbilgeaslan@hotmail.com

mesenteric artery (n=81/91, 89%). Despite favorable early results, the outcome of endovascular treatment deteriorates over time, reaching a one-year patency rate of 63%, as reported by a multicenter analysis. This leads to secondary procedures in 30% of cases. A surgical conversion carries a risk of high mortality and a very high rate of superior mesenteric artery embolism, such as 86% [13,14]. In another study, the overall perioperative mortality was found to be 59% (192 patients), and cumulative survival was reported as 30.8%, 26% and 23% for the first, third and fifth years, respectively [15]. Since the superior mesenteric artery diameter is wider and oblique exits from the aorta, emboli are frequently seen in this artery [13,16]. Many laboratory parameters, such as serum d-lactate, amylase, ferritin, and procalcitonin have been tested for this purpose, but no clinically useful result has been obtained [5,8-10]. Most available tests are not specific and require severe tissue damage to be detected in serum. This has to be translated into surgical practice, there is a delay in diagnosis until irreversible bowel damage occurs. Therefore, it is very important to identify a specific and rapidly rising marker to diagnose intestinal ischemia in the early stage of the disease to increase the chance of successful surgery and survival. In light of this finding, in this experimental, controlled study based on a rat model, we investigated the blood lactate, amylase, ferritin and procalcitonin levels in the diagnosis of acute mesenteric ischemia.

MATERIAL and METHODS

Ethical Approval

Experimental applications in rats were performed after obtaining the approval of the Local Ethics Committee of Kobay Experimental Animals Laboratory Company (Approval number: 2020-471). Sixteen, male Wistar-Albino rats weighing 250-300 grams were randomly divided into two groups of eight animals in each group. The rats that had been used in prior studies/experiments and that had signs/symptoms of illness, such as dehydration, decreased body weight, and abnormal posture were excluded from the study. The rats were housed individually at an environmental temperature of 20-25°C, with 40% relative humidity under a 12-h light/dark cycle and were provided free access to food and water. After the 12 h fasting period, general anesthesia was induced by applying 50 mg/kg ketamine and 5 mg/kg xylazine intramuscularly.

Surgical Procedure

The abdominal aorta has three main branches that provide vascularization of the abdominal organs. The first of these, truncus coeliacus, provides blood from the distal esophagus to the second continent of the duodenum. The main branches of this artery are splenic, left gastric and main hepatic arteries. The celiac artery, which is short and large, has widespread collateral circulation, and acute mesenteric artery at this level is the most important reason for ischemia to be rare. The second branch of the abdominal aorta is the superior mesenteric artery. This artery provides vascularization of the intestines starting from the second continent of the duodenum to the distal of the transverse colon and anastomoses with the inferior mesenteric artery through Drummond's marginal artery and the arc of Riolan. The superior mesenteric artery is separated from the abdominal aorta at an angle of about 45 degrees and creates a suitable path for thrombi. This explains, albeit partially, why many mesenteric emboli occur at this level. Although there is a decrease in the blood flow of up to 75%, due to the high adaptation rate to increase oxygen extraction, the intestines can reach 12 h without serious damage and can preserve their viability in this period. However, if ischemia lasts long and is severe, the protective properties of compensatory mechanisms are impaired.

In this study, all rats were given 3 mL intraperitoneal saline. The mesenteric ischemia was created by ligating the superior mesenteric arteries (SMAs) from the aortic outlet area in the experimental group (Group 2). Blood samples were taken at hours 0, 2, 6, 12 and 24. The rats in the control groups (Group 1) were subjected to laparotomy only, and blood samples were collected and examined at the same intervals. In order to easily obtain sufficient blood, the samples were taken from the abdominal aorta and placed in tubes containing citrate. The tubes were centrifuged at 1.000 rpm for 15 min, and the plasma procalcitonin, lactate, amylase and ferritin levels were measured in the separated serum. At the end of the study, all rats were killed by decapitation. Blood samples were studied in the central biochemistry and hormone laboratory of Ankara City Hospital. The Atellica Solution Chemistry 930 analyzer and Atellica Solution Immunassay 1600 modular system analyzer (Erlangen, Germany) were used to test the blood samples.

Statistical Analysis

The control laparotomy and ischemia groups were analyzed separately. SPSS v. 13 for Windows (SPSS Inc., Chicago, IL, USA) was used to analyze all data obtained in the study. The procalcitonin, lactate, amylase and ferritin levels of the groups were investigated using the Kruskal-Wallis analysis of variance (Bonferroni-corrected Mann-Whitney U test). $P<0.05$ was regarded as statistically significant.

RESULTS

The biochemical parameters were compared between the ischemia and control groups at the same evaluation times. The basal mean biochemical values of the groups are shown in *Table 1*. In Group 2 (experimental group), the amylase values significantly increased at 6 h, 12 h and 24 h ($P<0.001$ for all) *(Fig. 1)*, the lactate values showed a significant increase at 2 h, 6 h and 12 h ($P=0.001$, $P=0.001$, and $P<0.001$, respectively) *(Fig. 2)*, ferritin maintained

Table 1. Comparison of the baseline marker levels of the groups			
Parameters	**Group 1 n=8**	**Group 2 n=8**	**P-value**
Amylase (U/dL)	996.9±199.1 (846-1316)	1015.0±172.7 (865-1345)	0.382
Lactate (mg/dL)	30.7±3.2 (26.9-35.6)	30.9±4.6 (25.6-38.3)	0.878
Ferritin (ng/mL)	0.50±0.00 (0.50-0.50)	0.50±0.00 (0.50-0.50)	0.999
Procalcitonin (pg/L)	0.17±0.14 (0.03-0.30)	0.06±0.10 (0.03-0.30)	0.234
Data are given as mean ± standard deviation and (min-max)			

started to increase from the 6 h and continued significantly until the 12 h. Among these rats with mesenteric ischemia, the amylase values continuously and significantly increased from the 6 h to the 24 h while the procalcitonin value peaked at the 6 h *(Table 2)*. There was no significant increase in the level of blood ferritin within 24 h.

DISCUSSION

Acute mesenteric ischemia is a life-threatening condition, which occurs as a result of the sudden insufficiency of blood flow of mesenteric vessels in the intestine, causing damage to not only the intestines but also other vital

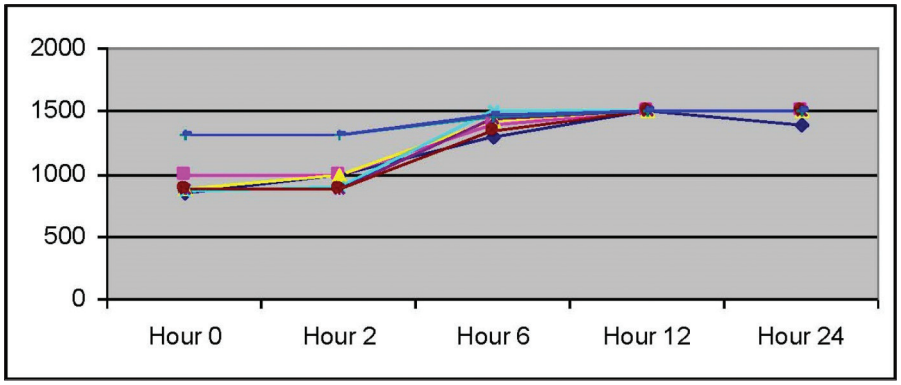
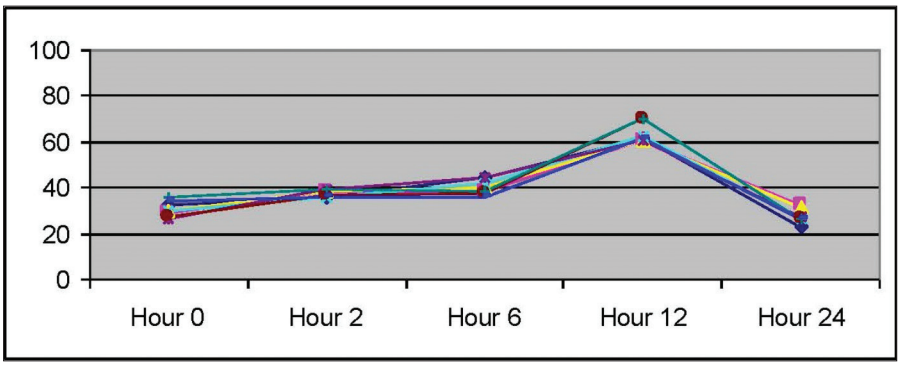


Fig 1. Changes in the amylase level of the AMI-induced rats over 24 h

Fig 2. Changes in the lactate level of the AMI-induced rats over 24 h



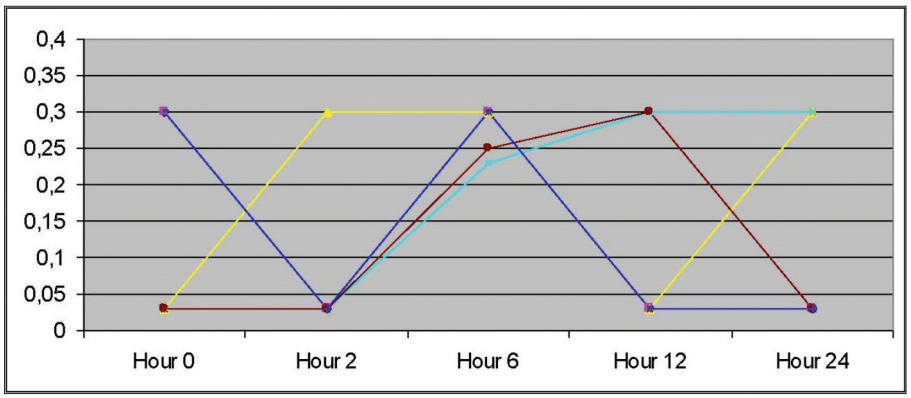


Fig 3. Changes in the procalcitonin level of the AMI-induced rats over 24 h

at the same level with no significant increase at any measurement time, and the serum procalcitonin level statistically significantly increased only at 6 h (P=0.035) *(Fig. 3)*. In addition, in Group 2, the mean of d-lactate levels

organs. AMI is an acute abdominal disease [10,16-18] remains to have a generally fatal outcome. The mortality rates of this disease are reported to be between 70 and 90% in recent years, which are very similar to those published

Table 2. *Changes in the marker levels of Group 2 compared to the baseline values*

Time Period	Amylase	Lactate	Ferritin	Procalcitonin
0 hour	996.9±199.1	30.7±3.2	0.50±0.00	0.17±0.14
2 hour	1.034.5±178.9 P= 0.121	37.5±1.5# p= 0.001	0.50±0.00	0.06±0.10 P= 0.197
6 hour	1.417.9±66.5* P<0.001	40.1±3.2# p= 0.001	0.50±0.00	0.29±0.03□ P= 0.035
12 hour	1.500.0±0.0* P<0.001	63.4±4.1# p<0.001	0.50±0.00	0.10±0.12 P= 0.451
24 hour	1.486.5±38.2* P<0.001	27.6±2.9 p= 0.111	0.50±0.00	0.10±0.12 P= 0.451
P value	0.034	0.001	0.999	0.033

Data are given as mean ± standard deviation. Values with different superscripts are significantly different at P<0.05 (, #, □)*

by Hibbert et al.[11] in 1933. The most important step in the management of AMI is, undoubtedly, to diagnose mesenteric ischemia before intestinal infarction develops. While the probability of life is 60% in patients diagnosed within 24 h, this rate drops to 30% in those diagnosed after this period. For this reason, studies on AMI and diagnostic investigations have increased in recent years [12]. The endovascular treatment of intestinal artery disease cannot be considered as the treatment of choice; it is rather an alternative method in patients with functional or local contraindications to surgery [13]. The common result of previous studies is that AMI lacks a sensitive and specific marker that can help achieve early diagnosis to increase survival. There are several different reasons why a sufficiently good diagnostic biochemical marker could not be detected. The first problem is due to the intestine consisting of mucous membranes, submucosa and smooth muscle layers. An ideal marker should be able to reflect this complex structure [5,10,14]. This marker should also ensure that damage to the mucosa is diagnosed before its progression into a full-thickness infarction. The second problem is that the vascular pathway that provides intestinal blood flow passes through the portal vein through the liver. Venous blood from the intestines can participate in systemic circulation after being exposed to the first transition effect in the liver. Lastly, the overlapping protein expression of the liver and intestine makes it difficult to identify an organ-specific marker [15,19,20].

Elevated serum lactate concentration also predicts mortality in mesenteric infarction cases [6,21]. Metabolic acidosis has been used as a biochemical parameter in the diagnosis of acute mesenteric ischemia for nearly three decades [7,16]. Accordingly, lactate levels were found to be high in approximately 90% of patients with AMI in the late period [8,19,20]. When the biochemical structure of lactate is evaluated, there are two isomers, D and L-lactate, according to the position of the alpha hydroxyl radical on the molecule, and both are products of anaerobic metabolism. Conflicting results have been reported in recent experimental studies regarding the use of lactate as a screening test in the early diagnosis of AMI, although pyruvate is reduced by specific lactate dehydrogenase [11,12,20]. Lactic dehydrogenase, amylase, alkaline phosphatase, creatinine phosphokinase, and ammonia were found to have no specificity for mesenteric ischemia. In mesenteric ischemia, the best marker evaluated to date remains serum lactate. Several clinical and experimental studies have shown that the serum lactate level increases following superior mesenteric artery occlusion [8,20]. Lange and Jackel have reported 100% sensitivity, but only 42% specificity for serum lactate as a marker for mesenteric ischemia. In another clinical study, the measurement of serum lactate was diagnostically helpful, although not confirmative [8,10,20]. Lactate is simply an indicator of an anaerobic state [8,9,21]. In our study, in the rats with mesenteric ischemia (Group 2-experimental group), the mean lactate level started to increase from the 6h, and this increase continued until 12 h *(Table 2)*.

In chronic experiments on dogs, soon after the ligation of the pancreatic duct, amylase increases in the peripheral blood, as well as the release of total amylase and lipase in the liquid and solid portions of the secretion from the isolated parts of the small intestine [9,12,20]. The α-amylase release was shown to depend on the level of blood amylolytic activity and the secretory activity of the small intestine. The α-amylase and lipase release is more increased upon mechanical rather than chemical stimulation of the small intestine. After the secretory function of the pancreas is excluded, two stages of compensatory adaptive changes occur in the secretory activity of the small intestine: consuming processes due to lower energy (secretion of blood enzymes) and involvement of the small intestine. In the diagnosis of AMI, many studies have been conducted to investigate the role of amylase for the last few decades. In a mesenteric ischemia model in dogs, Aydın et al.[21] reported that the amylase values significantly increased. Wilson et al.[22] evaluating 52 patients diagnosed with mesenteric ischemia, determined that 27 had higher amylase levels than normal. Aslan et al.[23] found that the amylase values of patients diagnosed with AMI started to increase at the third hour. The progresses in our understanding of the pathophysiology, diagnosis and treatment of the

acute occlusions of SMA or the superior mesenteric vein have made it possible to save lives and recover intestinal function in most patients with mesenteric ischemia [24]. In another previous study, amylase was found to be 42% specific and 68% sensitive in patients presenting with acute abdomen and diagnosed with mesenteric ischemia [25]. In our study, the amylase values in Group 2 increased steadily from 6 h to 24 h; thus, they exhibited an increasing trend. Based on these results, it can be commented that amylase rises in many diseases that play a role in the acute etiology of the abdomen, as well as AMI, as determined in our study. However, different studies have identified fluctuations in amylase levels and questioned whether this parameter is sufficiently specific to eliminate other causes of abdominal pain. Therefore, the use of amylase alone in the diagnosis of AMI remains controversial, and it should still be assessed together with other parameters due to its low sensitivity and specificity.

There are many laboratory parameters that are used in the diagnosis of inflammatory diseases based on their ability to show immune response. It is also known that inflammation-induced procalcitonin is released from neuroendocrine cells in the lung, liver, intestines, and pancreas. Bacterial endotoxins and TNF-α are the strongest procalcitonin inducers in experimental conditions [26,27]. The procalcitonin levels were previously found to be high in the early period in patients with acute myocardial infarction [28]. In recent years, procalcitonin has emerged a new parameter among infection markers in recent years [29]. It is a simple and practical marker that can be used in the early diagnosis of severe acute pancreatitis and monitoring of clinical prognosis [9,30]. PCT, as an inflammatory response parameter, it appears as an early and better marker in sepsis and serious infections [10,29,30]. In another study, the serum procalcitonin levels were evaluated in patients with acute stroke. In patients with acute stroke, the serum procalcitonin levels were found to be elevated starting from the first day, and it was observed to reach the highest levels on the seventh day [31]. Procalcitonin levels were also shown to increase in intestinal strangulation [32,33]. The procalcitonin levels were reported to be increased in the strangulated group compared to the controls. An increase in the procalcitonin levels was observed at the 30th and 60th min of the study, and a serious elevation was detected at the 120th min [32]. The high mortality seen in cases of mesenteric ischemia is also often caused by septic complications [2,32]. It is commonly agreed that in almost every situation, systemic inflammatory response syndrome and septic complications can develop. Plasma procalcitonin levels significantly increase in bacterial, fungal and parasitic infections, sepsis, and multi-organ failure syndrome [27,31,32]. Oruc et al.[33] found that the blood procalcitonin levels were higher in Crohn's disease, one of the inflammatory bowel diseases, compared to normal human blood levels. Procalcitonin can be used to monitor prognosis and response to treatment in this patient group [32,33]. In addition, increases in procalcitonin level does not change in autoimmune, viral and allergic diseases. Studies have shown that a small amount of bacterial endotoxin injection stimulates procalcitonin production in healthy individuals [34]. At two to three hours, procalcitonin rises to a level that can be measured, and then it increases rapidly from the sixth to eighth h, reaching its highest value at the 12th h. After this period, procalcitonin remains approximately the same for a further 12 h and returns to its normal level within the next two days. The half-life of procalcitonin varies from 20 to 24 h [27,34]. Procalcitonin measurement presents as an easy method and can be a marker for the diagnosis and follow-up of AMI and sepsis [29,34]. In our study, the PCT value in Group 2 was significantly increased only at 6 h. Since there is no study in the literature investigating procalcitonin in AMI, there is a need for research with larger series to confirm our results.

Ferritin has generally been considered to function as a "housekeeper" storage protein which can release iron required for cellular proliferation (e.g., ribonucleotide reductase) and metabolic renewal (e.g., cytochrome synthesis) [35]. The period of reperfusion after ischemia is thought to be a critical period of oxidant damage in many tissues, including the heart, brain, gut, and other organs [36]. The antioxidant properties of ferritin are likely mediated by the sequestration and storage of iron, thus preventing ferryl or hydroxyl radical generation [37,38], playing a key role in maintaining iron homeostasis. During the postischemic reoxygenation of the rat liver, early ferritin degradation was counteracted by enhanced ferritin transcription. It was suggested that this might act to re-establish ferritin levels and limit reperfusion damage [39]. Indeed, hypo-transferrinemic mice, which have high levels of ferritin and lactoferrin, are resistant to hyperoxia-induced lung injury [40]. Although serum ferritin levels usually correlate with total body iron stores, several physiologic conditions, such as inflammatory and infectious states can increase serum ferritin as one of the acute-phase proteins [41,42]. Rogers et al.[43] also reported that the inflammatory induction of ferritin synthesis was different from iron-dependent ferritin gene expression. Serum ferritin levels may increase solely as a consequence of cellular necrosis or damage [44]. This possibility is supported by previous clinical findings of an association between serum ferritin levels and the injury severity score [45]. Ferritin, a highly conserved iron-binding protein, plays a key role in the maintenance of cellular iron homeostasis and protection from oxidative stress. Ferritin mitigates oxidant stress by sequestering iron and preventing its participation in reactions that generate reactive oxygen species [46]. In our study, there was no statistically significant increase in the ferritin level of Group 2, which suggests that ferritin may be a late-phase reactant.

Çakır et al.[47] measured five parameters (White blood count,

Lactic dehydrogenase, Creatine Kinase, lactate, and D-dimer) in the blood of 92 AMI patients over 24 h. They detected cumulative percentages for amylase in 68 (73.9%) patients and lactate in 47 (51.1%). They also found that white blood cell count, creatine kinase, lactate dehydrogenase, lactate, and D-dimer levels increased in patients with acute mesenteric ischemia [47]. In our animal experiment, we simultaneously evaluated four early markers. We found similar blood values using different or same blood parameters in a more controlled rat model. The results of our animal study are consistent with clinical practice. We observed that the levels of blood amylase *(Fig. 1)* and d-lactate *(Fig. 2)* obtained from the SMA of the rats constantly increased from the 6 h to the 24 h. The amylase and lactate blood values reached their highest at the 12 h. On the other hand, we did not find a significant and stable increase in the procalcitonin and ferritin levels. Although the procalcitonin value peaked at the 6 h in the rats with mesenteric ischemia, we observed that the changes in the procalcitonin level were not consistent *(Fig. 3)*. Thus, we conclude that the lactate and amylase levels can be used as early diagnostic markers to achieve rapid and accurate results in patients with suspected SMA obstruction.

Author Contributions

FA designed the project, wrote the main paper. BA carried out data acquisition and statistical analyses. All authors contributed to the critical revision of the manuscript for important intellectual content and have read and approved the final version.

Conflict of Interest

All authors declare that they have no conflict of interest to disclose.

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Research Article

Optimisation of Indirect ELISA by Comparison of Different Antigen Preparations for Detection of Antibodies Against Schmallenberg Virus [1] [2]

Ahmet Kursat AZKUR [1,a] Emel AKSOY [1,b] Murat YILDIRIM [2,c] Kader YILDIZ [3,d]

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[2] *This study is presented in 2nd International Congress of Veterinary Microbiology, 16-19 October 2018, Antalya, Turkey*

[1] Department of Virology, Faculty of Veterinary Medicine, Kirikkale University, TR-71450 Kirikkale - TURKEY
[2] Department of Microbiology, Faculty of Veterinary Medicine, Kirikkale University, TR-71450 Kirikkale - TURKEY
[3] Department of Parasitology, Faculty of Veterinary Medicine, Kirikkale University, TR-71450 Kirikkale - TURKEY
ORCIDs: [a] 0000-0002-5597-8917; [b] 0000-0001-9536-4322; [c] 0000-0002-9576-2280; [d] 0000-0001-5802-6156

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Abstract

Schmallenberg virus (SBV) infection, discovered in 2011, was reported in Europe including Turkey, Africa and recently in some Asian countries. Commercial enzyme-linked immunosorbent assay (ELISA) kits were widely used by researchers in many epidemiological studies and SBV diagnosis. The aim of this study was to optimise indirect in-house ELISA that is based on different antigen preparations of cell-culture derived whole SBV particle. Antigen preparations were maintained with various methods: PEG precipitation, ultracentrifugation, dialysis, and antigen inactivation. Following antigen optimisation, steps of antigen coating, blocking, conjugate and stop solution were optimised and in-house ELISA was compared to commercial indirect SBV ELISA kit. The best result in ELISA antigen preparation for SBV was gained by 30% PEG purification method followed by formaldehyde inactivation. Although results of this study demonstrated that in-house ELISA for detection of SBV specific antibodies was equally sensitive and specific as commercial kit, purified SBV antigen based in-house ELISA development could increase S/P ratios.

Keywords: *Diagnosis, Dialysis, ELISA, PEG, Schmallenberg virus*

Schmallenberg Virüs Antikorlarının Belirlenmesi İçin İndirekt ELISA'nın Farklı Antijenler Karşılaştırılarak Optimizasyonu

Öz

Schmallenberg virüs (SBV) enfeksiyonu 2011 yılında keşfedilmiş ve enfeksiyon Türkiye dahil Avrupa kıtasında, Afrika'da ve bazı Asya ülkelerinde bildirilmiştir. Ticari ELISA kitleri çok sayıda epidemiyolojik çalışmada ve SBV tanısında sıklıkla kullanılmaktadır. Bu çalışmanın amacı, hücre kültüründen elde edilen tam SBV partikülünün farklı antijen hazırlama yöntemleri kullanılarak indirekt in-house ELISA optimizasyonudur. Antijen hazırlamak için; PEG presipitasyon, ultrasantrifüjleme, diyaliz ve antijen inaktivasyonu gibi farklı yöntemler kullanıldı. Antijen optimizasyonu sonrasında, antijen kaplama, bloklama, konjugat ve durdurma solüsyonları optimize edildi ve geliştirilen in-house ELISA ticari ELISA kiti ile kıyaslandı. ELISA antijen hazırlanmasında en iyi sonuçlar %30 PEG presipitasyon sonrasında formaldehit ile inaktivasyon sonucunda elde edildi. SBV özgül antikorların belirlenmesinde in-house ELISA ticari kit kadar duyarlı ve özgül olsa da saf SBV antijeni temelli in-house ELISA geliştirilmesinin S/P oranını yükseltebileceği düşünüldü.

Anahtar sözcükler: *Tanı, Diyaliz, ELISA, PEG, Schmallenberg virüs*

INTRODUCTION

Schmallenberg virus (SBV), first identified in 2011 in Germany, is classified in *Orthobunyavirus* genus of *Peribunyaviridae* family. SBV causes abortion, stillbirths, and congenital malformation in ruminants [1,2]. Since 2011, SBV infection in wild and domestic ruminants was reported in many countries in Europe including Turkey [2-6]. SBV infection was found in some African and Asian countries recently [7-9]. Seroepidemiological data of SBV infection in Turkey

Correspondence
+90 318 3574242/6487
kursatazkur@kku.edu.tr

revealed that SBV seropositivity in cattle, sheep, goats, and Anatolian water buffalo was 39.8%, 1.6%, 2.8%, and 1.5%, respectively [3]. SBV seropositivity in some European countries was estimated to be up to 98.5% in cattle, 89% in sheep, and 50.8% in goat [10]. SBV infection has been detected in both domestic and wild ruminants, including cattle, sheep, goat, buffalo, deer, and bison [3].

There are many assays developed for diagnosis of SBV infection to date. Reverse transcriptase PCR (RT-PCR), real-time RT-PCR assays, loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA) are developed for molecular diagnosis of SBV [11-13]. For detection of SBV specific antibodies, virus neutralisation, plaque reduction neutralisation, and enzyme-linked immuno-sorbent assay (ELISA) tests were used [14-17]. Indirect and competitive ELISA kits which are based on nucleoprotein (N) of SBV are commercially available for detection of SBV specific antibodies in serum, plasma and milk samples. The commercial kits are used by many researchers to determine SBV seropositivity for seroepidemiological surveys and diagnosis [3,18]. On the other hand, some researchers developed in-house ELISA to determine SBV specific antibody based on whole virus particle [16,17]. In this study it is aimed to optimise an indirect ELISA assay based on cell-culture derived whole viral particle for serological diagnosis of SBV infection by preparation and comparison of different SBV ELISA antigens.

MATERIAL and METHODS

Schmallenberg Virus

Schmallenberg virus isolate (strain F6; GenBank accessions: KC355457-KC355459) was kindly provided by Prof. Dr. Wim van der Poel (Wageningen Bioveterinary Research, Netherlands). SBV was propagated on Vero cells which were grown using Dulbecco modified Eagle medium (DMEM) with 10% foetal bovine serum (FBS) and incubated at 37°C with 5% CO_2. The viral titer was determined by plaque titration assay as described previously [19].

Serum Samples

Serum samples taken from 300 cattle in previous studies were tested with both commercial indirect ELISA kit (IDEXX, Westbrook, Maine, USA) and competitive ELISA kit (IDVet, Grabels, France) in order to determine SBV-seropositive and SBV-seronegative samples. Both commercial kits were used as following the instructions of the manufacturers. The positive and negative sera according to results of these commercial kits were accepted as SBV-seropositive and SBV-seronegative samples. In-house ELISA results were compared to commercial indirect ELISA results.

Preparation of ELISA Antigen

Vero cells were infected with SBV and cells were frozen after 80% or over of cells showed cytopathic effect. Freeze-thaw process was maintained for 2-3 times. Different methods which have implemented and/or not been implemented before such as, polyethylene glycol (PEG) precipitation, ultracentrifugation, dialysis, and inactivation for antigen preparation were carried out.

Precipitation with 50% PEG: 50% PEG 8000 (w/v) (Sigma, Missouri, USA) and 23% NaCl (w/v) (Merck, Darmstadt, Germany) were dissolved in distilled water. Supernatant of SBV infected cells was collected and centrifuged at 3000 rpm for 30 min at 4°C. The supernatant was mixed with 50% PEG and 23% NaCl solution was added to the mixture. Virus-PEG mixture was stirred at 100 rpm for 16 h at 4°C. The mixture was centrifuged at 12000 rpm for 30 min at 4°C. Pellet was suspended in 1× TNE buffer (pH 7.2) and centrifuged at 5500 rpm for 20 min at 4°C. After centrifugation supernatant was collected and centrifuged at 24000 rpm for 2 h at 4°C. Supernatant was discarded and the pellet was suspended in 1× TNE buffer and stored at -80°C until used [20].

Precipitation with 30% PEG: Viral supernatant was mixed at a ratio of 2:1 with 30% PEG 8000 (w/v) which was prepared in 0.4 M NaCl [21]. The mixture was incubated at 4°C overnight. The virus was recovered by centrifugation at 3200×g for 30 min at 4°C. Supernatant was discarded and the virus was suspended in PBS and stored at -80°C until used.

Ultracentrifugation: Supernatant of SBV infected cells was collected and centrifuged at 2000 rpm for 10 min at 4°C. After centrifugation, supernatant was collected and filtered with using 0.22 µm pore filter membranes. The filtrate was ultracentrifuged (in Department of Virology, Faculty of Veterinary Medicine, Firat University, Elazig, Turkey) using SW-28 rotor (Beckman Coulter, Brea, California, USA) at 25.000 rpm for 2 h at 4°C. DMEM containing 1% BSA was pour onto the viral pellet, incubated for 2 h at 4°C, and re-suspended.

Commercial PEG precipitation kit: SBV antigen was purified with PEG virus precipitation kit (Biovision, California, USA) according to manufacturer's instruction. Briefly, cells infected with SBV were centrifuged at 3200 ×g for 15 min at 4°C. Supernatant was collected, mixed with 5× PEG solution and incubated overnight at 4°C. The mixture was centrifuged the following day at 3200×g for 30 min at 4°C. The viral pellet was suspended in virus suspension buffer and stored at -80°C until used.

Dialysis: Dialysis was carried out with two different systems: 12-14 kDa cut-off dialysis tubes (Biovision, California, USA) and Spectra/Por2 dialysis membrane 12-14 kDa MWCO (Spectrum, Waltham, Massachusetts, USA). The dialysis tubes were soaked with distilled water and the tubes were dialyzed against water overnight at 4°C and against PBS for 4 h at 4°C [17]. After dialysis, virus collected and stored at -80°C until used. The dialysis membrane was initially

soaked in distilled water for 30 min. The supernatant was dialyzed against type I MilliQ water during day and overnight at 4°C and water was changed at least 4 times. Final dialysis was carried out against PBS overnight at 4°C [21]. The virus was collected and stored at -80°C until used.

Antigen inactivation: To inactivate SBV, the antigens were treated by Triton X-100 with final concentration of 1% [17] or formaldehyde with final concentration of 2% [22] and incubated in room temperature for 1 h.

SDS-PAGE and Western Blotting

SDS-PAGE and western blotting was carried out to confirm SBV isolate and antigen preparation method and to determine SBV-seropositive serum samples were reacting against viral proteins. For separation of protein suspensions, protein electrophoresis was carried out in 10% SDS-PAGE gel. The proteins were transferred onto a PVDF membrane (Thermo Scientific, Waltham, Massachusetts, USA) and the membrane was blocked overnight at 4°C with 5% skimmed milk powder in phosphate buffered saline with 0.05% Tween-20 (PBST). SBV-seropositive and -seronegative cattle sera were used as primary antibody and the membrane was incubated in sera diluted 1:100 in 0.01% PBST at room temperature for 2 h. Washing step was carried out with 0.1% PBST for three times. Rabbit anti-bovine IgG HRP secondary antibody (Life Technologies, Carlsbad, California, USA) diluted 1:1000 in 0.01% PBST was added onto the membrane and incubated at room temperature for 1 h. Following washing with 0.1% PBST, TMB substrate (Amresco, Solon, Ohio, USA) was poured onto the membrane, incubated for 5 min and evaluated with pre-stained protein marker (Thermo Scientific, Waltham, Massachusetts, USA).

In House Indirect ELISA Optimisation

Concentrations of differently prepared antigens were measured by Bradford protein assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). In-house indirect ELISA was optimised by performing checkerboard titrations of antigen and conjugate using positive and negative serum samples which were defined by commercial indirect and competitive ELISA kits. Further optimisations of SBV antigen, coating buffer, blocking, diluents, and stop solution were implemented for the in-house indirect ELISA. SBV antigen was diluted in both 0.05 M carbonate/bicarbonate buffer and PBS. Fifty µL of antigen solution at 10 µg/mL concentration was coated onto polystyrene ELISA plates by incubating at 4°C overnight. Undiluted sera, 1/2, 1/10 and 1/100 dilutions of sera are tested for sample step. The washing steps were carried out with 0.05% PBST. Two different blocking agents, 10% skimmed milk powder and 1:10 diluted foetal bovine serum (FBS), were tried for blocking step. The conjugate (Life Technologies, Carlsbad, California, USA) was diluted in different diluents, such as 5% skimmed milk powder (in 0.05% PBST) and 3% FBS (in 0.05% PBST). TMB substrate was used and the reaction was stopped by using 2 M sulphuric acid or 1% sodium dodecyl sulphate (SDS). Results were read at 450 nm using a spectrophotometer.

RESULTS

Three hundred cattle sera were tested by both commercial indirect and competitive ELISA kits and 22 out of 300 sera were positive for SBV specific antibody in both commercial kits. Positive and negative sera were used for development of in-house indirect ELISA. Differently prepared ELISA antigens were tested by SDS-PAGE and western blotting and SBV nucleoprotein (25 kDa) and Gc protein (110 kDa) were detected with SBV seropositive serum *(Fig. 1)*, whereas SBV seronegative serum cannot detect any SBV proteins by western blotting analysis (data not shown).

After checkerboard titrations of antigen and conjugate, optimisation steps of SBV antigen, coating buffer, blocking

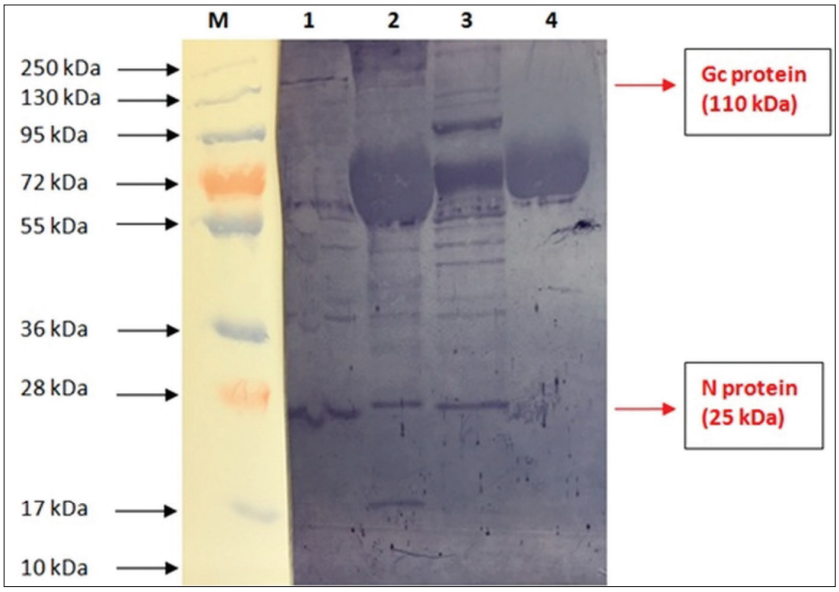

Fig 1. Western blotting results of different SBV antigen and bovine serum albumin (BSA). All differently prepared SBV antigens (Lines 1-3) showed Gc protein (110 kDa) and nucleoprotein (N) (25 kDa) bands in western blotting. M: Protein marker; 1: SBV antigen prepared by ultracentrifugation, 2: SBV antigen prepared by PEG precipitation; 3: SBV antigen gained from cell culture; 4: BSA (66.5 kDa) as control

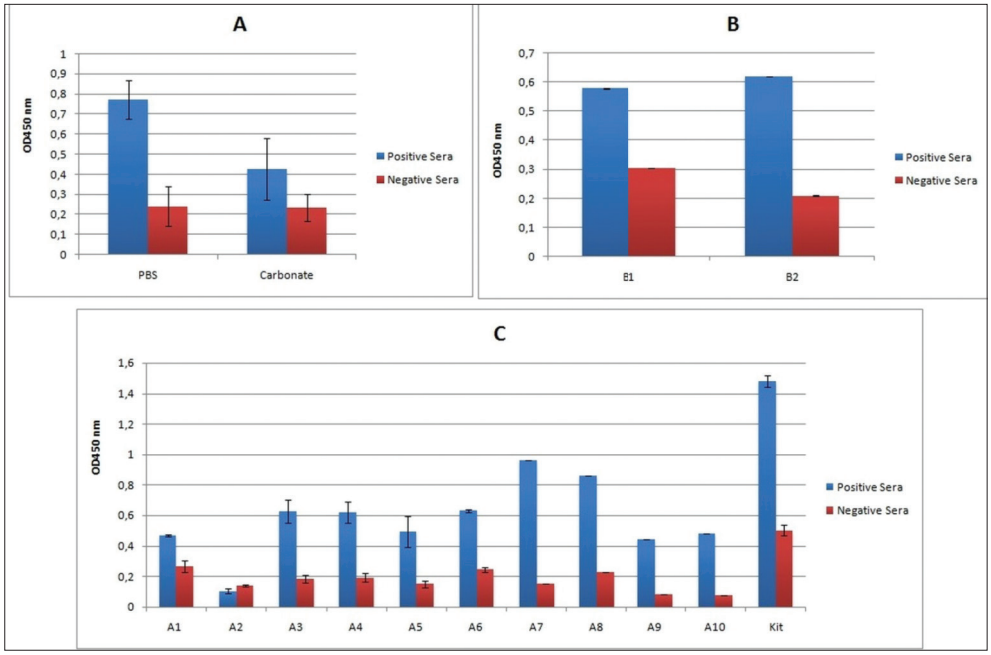

Fig 2. Comparison of optic density (OD) results of in-house ELISA for detection of SBV specific antibodies in cattle sera. **A:** Carbonate buffer and PBS were compared for antigen coating step, **B:** Blocking step was interpreted by 10% skimmed milk powder and 1:10 diluted FBS. B1: blocking with 10% skimmed milk powder, B2: blocking with 1:10 diluted FBS, **C:** Differently prepared ELISA antigens were compared to each other and indirect ELISA kit. SBV ELISA antigens were differently prepared; A1: 50% PEG, A2: ultra-centrifugation, A3: PEG kit, A4:30% PEG, A5: 30% PEG and dialysis, A6: dialysis and PEG, A7: 30% PEG and formaldehyde, A8: PEG kit and formaldehyde, A9: 30% PEG and triton X-100, A10: PEG kit and triton X-100. Kit: Indirect ELISA kit for SBV

Table 1. *The S/P% results of in-house indirect ELISA were calculated with formulation of indirect ELISA kit*

Antigens	**Positive Sera, S/P% (mean±SD)**	**Negative Sera, S/P% (mean±SD)**
30% PEG	53.0±2.181	9.0±0.371
30% PEG and Triton X-100 inactivation	21.3±0.876	-12.8±0.525
30% PEG and formaldehyde inactivation	69.9±2.877	-6.3±0.259
PEG kit	40.6±1.668	4.6±0.189
PEG kit and triton X-100 inactivation	24.8±1.019	-13.3±0.548
PEG kit and formaldehyde inactivation	60.4±2.483	1.0±0.042

The indirect ELISA kit results were calculated and interpreted as positive if S/P% ≥40%, as doubtful if 30% ≤S/P%<40%, and as negative if S/P%<30%

agent, and stop solution were implemented. SBV antigen was diluted at concentration of 10 µg/mL and coated successfully in PBS, not in 0.05 M carbonate/bicarbonate buffer. Antigen coating with PBS results in OD of 0.773±0.094 for positive sera and 0.241±0.098 for negative sera, whereas carbonate/bicarbonate buffer coating results in 0.4255±0.152 for positive sera and 0.234±0.067 for negative sera *(Fig. 2-A)*. Because coating with PBS resulted in a higher OD value for positive sera, PBS was preferred for the antigen coating step.

To optimise blocking step of in-house ELISA, skimmed milk powder and FBS were tested. The positive sera OD was 0.579±0.001 and negative sera OD was 0.3045±0.0015 in blocking with 10% skimmed milk powder. The OD values of blocking with 1:10 diluted FBS were 0.6205±0.0005 for positive sera and 0.209±0.001 for negative sera *(Fig. 2-B)*. For blocking step, 1:10 diluted FBS has the best OD results.

For sample step, undiluted sera, 1/2 and 1/10 dilutions of sera gave same O.D. results, whereas 1/100 dilution O.D. was undetectable. Therefore, optimisation of sample step was implemented with 1/10 dilutions of sera.

Skimmed milk powder and FBS were used as diluents of the conjugate to optimise conjugate step of in-house ELISA. The optimisation of conjugate step was maintained in commercial indirect ELISA kit (IDEXX, Westbrook, Maine, USA) with following the instructions of the manufacturer, except for conjugate step which was carried out with three different conjugates. The first one is the conjugate of commercial kit (IDEXX), the second one is the conjugate (Life Technologies) diluted 1:2000 in 5% skimmed milk powder, and the third one is the conjugate (Life Technologies) diluted 1:2000 in 3% FBS (in 0.05% PBST). When the full procedure of commercial ELISA kit was followed, the OD of positive sera and negative sera were 1.5905±0.0355 and 0.2545±0.0125, respectively. Once the conjugate (Life Technologies) was diluted 1:2000 in 5% skimmed milk powder (in 0.05% PBST), the OD values were 0.5105±0.0125 for positive sera and 0.2455±0.0035 for negative sera. The most optimised results gained by 1:2000 dilution of the conjugate (Life Technologies) in 3% FBS (in 0.05% PBST), with OD values of 2.2125±0.0235 and 0.3155±0.0205 for positive and negative sera, respectively. ELISA reactions were stopped by adding 2M sulphuric acid, but not with 1% SDS.

To find the best antigen preparation system, 50% PEG and 30% PEG precipitation, ultracentrifugation, commercial PEG precipitation kit, dialysis, and antigen inactivation (Triton X-100 or formaldehyde) methods were implemented and compared to each other. Comparison of OD values of the different antigens indicated that the ELISA antigen gained by 30% PEG precipitation with formaldehyde inactivation method has the highest efficiency as having 0.961 and 0.149 mean OD values for positive and negative sera, respectively *(Fig. 2-C)*. However, the result of commercial indirect ELISA kit has higher OD for positive sera (mean OD of 1.481). The S/P% values of different antigens-based in-house ELISA were given in *Table 1*.

Following optimisation of steps of in-house ELISA, results were compared with commercial indirect ELISA kit (IDEXX, Westbrook, Maine, USA) *(Fig. 2-C)*. All positive and negative sera which were already determined with two commercial kits were positive and negative, respectively, with in-house ELISA; however, the OD values were higher in commercial indirect ELISA kit in comparison to OD values of in-house ELISA *(Fig. 2-C)*. The sensitivity and specificity of in-house ELISA were determined as 100% when compared to commercial ELISA kits.

DISCUSSION

ELISA is one of the most commonly used serological techniques and the use of ELISA in serosurveys for viral diseases provides convenience to the scientists to assess the epidemiology, and rate of spread of the diseases. Since the first discovery of SBV infection in 2011, ELISA is widely used for SBV diagnosis and seroepidemiology. Commercial ELISA kits are available and in-house ELISAs are developed by some researchers. In present study, it is aimed to optimise indirect in-house ELISA for SBV antibody detection with assessing the results of different antigens and diluents which have not tested for SBV ELISA to date.

In the present study, different ELISA antigen preparation methods were implemented: precipitation with 50% PEG, precipitation with 30% PEG, commercial PEG precipitation kit, dialysis, and antigen inactivation with Triton X-100 or formaldehyde. Among these methods, the best ELISA antigen was prepared by 30% PEG purification following inactivation with formaldehyde in the present study. A previously used method for inactivation of SBV [17] with Triton X-100 has been resulted in low OD value when compared to formaldehyde inactivation method *(Fig. 2-C)*. Besides the methods used in the present study, sucrose gradient, caesium chloride density gradient, ultrafiltration could be applied for virus purification. Sucrose gradient method is found to be better for foot-and-mouth disease virus [23]. Some combination of purification methods could increase viral yield, such as sucrose gradient following dialysis and ultrafiltration method found to have the best yield for norovirus [24]. In the present study some combination of methods were tested and the antigen gained by 30% PEG purification method in combination with formaldehyde inactivation increased OD results *(Table 1)*. ELISA for detection of virus specific antibodies can be based on either whole virus [16,17] or recombinant protein of the virus [25,26]. In the present study, only whole SBV was implemented in indirect ELISA. Using the whole virus as antigen in ELISA may bring the risk of cross-reaction with other Orthobunyaviruses and this can be overcome by using pure SBV antigen.

ELISA antigen coating could be maintained by PBS and carbonate/bicarbonate buffer. It is known that using a coating buffer with pH of 1-2 units higher than the isoelectric point of the antigen could increase binding of the ELISA antigen [27]. Because isoelectric point of SBV still remains unknown, both PBS and carbonate/bicarbonate buffer were tested for in-house ELISA in the present study. Antigen coating step was successfully maintained in PBS in the present study, although other researchers used carbonate/bicarbonate buffer for coating of SBV antigen [16,17].

Foetal bovine serum, skimmed milk powder, normal goat serum, normal chicken serum, bovine serum albumin (BSA), and gelatine could be used as blocking agent in home-made ELISAs [27]. In this study blocking with 1:10 diluted FBS had the best results in comparison to 10% skimmed milk powder. Conjugate dilution buffer may differ among studies, e.g. PBS with 0.05% Tween 20, PBS with 0.05% Tween 80, 5% FBS, 1% BSA could be used for conjugate dilution [16,17,25,26]. Several diluents and concentrations were tried out for conjugate optimisation in the present study. Conjugate was diluted in 5% skimmed milk (in 0.05% PBST) and 3% FBS (in 0.05% PBST) with different concentrations. The most optimised result was gained by 1:2000 dilution of conjugate in 3% FBS (in 0.05% PBST).

In conclusion, an in-house ELISA for detection of SBV-specific antibodies was optimised with the antigen gained by 30% PEG purification following inactivation with formaldehyde. In further studies, different methods such as sucrose gradient, caesium chloride density gradient, and ultrafiltration can be tested for SBV ELISA antigen.

Conflict of Interest

The authors declare that they have no conflict of interest.

Statements Authors Contributions

AKA, EA designed the experiments, made serum samples, the preparation of ELISA antigen stages, and SDS-PAGE-western blotting, optimization works and wrote the manuscript. AKA, made a substantial contribution to interpretation of data. All authors discussed the results and contributed to the final manuscript.

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Research Article

The Protective Effect of Lactoferrin on Adenosine Deaminase, Nitric Oxide and Liver Enzymes in Lipopolysaccharide-Induced Experimental Endotoxemia Model in Rats [1]

Canan GULMEZ [1,a] Kezban YILDIZ DALGINLI [2,b] Emine ATAKISI [3,c] Onur ATAKISI [4,d]

[1] *This work was supported by Kafkas University Scientific Research Projects Coordinator (Grand number: 2017-FM-79), Turkey*

[1] Department of Pharmacy Services, Tuzluca Vocational High School, Igdir University, TR-76000 Igdir - TURKEY
[2] Department of Chemistry and Chemical Processing Technologies, Kars Vocational High School, Kafkas University, TR-36000 Kars - TURKEY
[3] Department of Biochemistry, Faculty of Veterinary Medicine, Kafkas University, TR-36000 Kars - TURKEY
[4] Department of Chemistry, Faculty Science and Letter, Kafkas University, TR-36000 Kars - TURKEY
ORCIDs: [a] 0000-0003-3253-140; [b] 0000-0002-1483-348; [c] 0000-0002-5685-1870; [d] 0000-0003-1183-6076

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Abstract

In this study, the effect of lactoferrin (LF) on adenosine deaminase (ADA) activity, nitric oxide (NO) and liver enzyme levels were investigated in lipopolysaccharide (LPS)-induced experimental endotoxemia model in rats. Forty Sprague Dawley female rats were divided into four groups as control, LF (20 mg/kg, i.p.), LPS (20 µg/kg, i.p.) *(E. coli* type 0111:B4) and LF+LPS (20 mg/kg LF+20 µg/kg LPS, i.p.). For a week, LF was given daily, while LPS was given a single dose. Liver adenosine deaminase, nitric oxide and liver enzymes (aspartate aminotransferase 'AST', alanine aminotransferase 'ALT' and gamma glutamyltranspeptidase 'GGT') levels of animals sacrificed six hours after LPS injection were determined. It was found that ADA activity, considered to be a marker of cellular immunity, and NO levels increased in LPS-induced endotoxemia and LF supplementation decreased these levels significantly ($P<0.001$). There was no statistically significant difference between liver ALT, AST and GGT activity levels of all groups, but serum AST and GGT activity levels were significantly higher ($P<0.001$ and $P<0.05$, respectively) in the LPS group than in the other groups, and LF supplementation significantly reduced these enzyme levels. As a result, it can be said that LPS increases ADA synthesis and NO release and LF acts as an anti-inflammatory and immunosuppressor in stimulating immune response.

Keywords: *Lactoferrin, Lipopolysaccharide, Endotoxemia, Adenosine deaminase, Nitric oxide*

Ratlarda Lipopolisakkarit İndüklü Deneysel Endotoksemi Modelinde Adenozin Deaminaz, Nitrik Oksit ve Karaciğer Enzimleri Üzerine Laktoferrinin Koruyucu Etkisi

Öz

Bu çalışmada, ratlarda lipopolisakkarit (LPS) ile indüklenen deneysel endotoksemi modelinde laktoferrin (LF)'in adenozin deaminaz (ADA) aktivitesi, nitrik oksit (NO) ve karaciğer enzim düzeyleri üzerine etkisi araştırılmıştır. Çalışmada 40 adet Sprague Dawley dişi rat kontrol, LF (20 mg/kg, ip), LPS (20 µg/kg, ip) *(E. coli* tip 0111: B4) ve LF+LPS (20 mg/kg LF+20 µg/kg LPS, ip) olmak üzere dört gruba ayrıldı. Bir hafta boyunca LF hergün verilirken, LPS tek doz uygulandı. LPS enjeksiyonundan 6 saat sonra sakrifiye edilen hayvanların karaciğer ADA aktivitesi, NO düzeyleri ve karaciğer enzim (aspartat aminotransferaz 'AST', alanin aminotransferaz 'ALT' ve gama glutamiltranspeptidaz 'GGT') aktiviteleri belirlendi. Hücresel immunitenin bir belirteci olan ADA aktivitesi ve NO düzeyleri LPS indüklü endotoksemide arttı ve LF takviyesi bu düzeyleri önemli şekilde azalttı ($P<0.001$). Tüm grupların karaciğer ALT, AST ve GGT aktivite düzeyleri arasında istatistiksel olarak önemli bir fark görülmezken, LPS grubunda serum AST ve GGT aktivite düzeyleri diğer gruplara kıyasla önemli şekilde daha yüksekti ve laktoferrin takviyesi bu düzeyleri önemli ölçüde azalttı (sırasıyla $P<0.001$ ve $P<0.05$). Sonuç olarak, LPS'nin ADA sentezini ve NO salımını arttırdığı ve LF'nin immün yanıtı uyarmada antienflamatuar ve immün baskılayıcı görev gördüğü söylenebilir.

Anahtar sözcükler: *Laktoferrin, Lipopolisakkarit, Endotoksemi, Adenozin deaminaz, Nitrik oksit*

Correspondence
+90 476 2230017/3426 Fax: +90 476 2230010
canan_glm@hotmail.com, canan.gulmez@igdir.edu.tr

INTRODUCTION

Lactoferrin (LF) is an iron-binding single-chain glycoprotein of the transferrin gene family, weighing 80-kDa [1]. Lactoferrin is a multifunctional protein that plays a role in many physiological processes such as regulation of iron metabolism, defense against a wide range of microbial infections, regulation of cell growth and differentiation, cancer prevention and metastasis [1,2]. Also, it is used in both health and industry because of its antiinflammatory, antifungal, antibacterial, antimicrobial, immunomodulatory, antioxidant and even antineoplastic properties.

Systemic status of bacterial infection is called sepsis and gram (-) bacterial endotoxin in circulation is called endotoxemia. As a systemic inflammatory response to infection, sepsis describes a complex clinical syndrome. The sepsis progressively results in septic shock, multiple organ failure, and ultimately death. Bacteria, viruses, fungi and parasites can cause septic shock and result in a mortality rate of around 20-80%. Lipopolysaccharide (LPS) is a group of substances in the glycolipid structure obtained from the cell wall of gram-negative bacteria, used in experimental animal septic shock models [3].

Lactoferrin binds to endothelial cells with specific affinity and crosses from the apical to the abluminal surface without being degraded, possibly via caveolae. Although the effect of LF on endothelial cells is not yet known, LF increases the production of nitric oxide, a vasodilating agent produced from endothelial cells, from macrophages. Adenosine deaminase (ADA), a key enzyme in purine metabolism, is considered to be a marker of cellular immunity. ADA activity has been demonstrated during the activation period of autoimmune diseases and has been reported to be associated with highly impaired cellular and humoral immunity, developmental retardation and serious infections [4,5]. LF can prevent the development of tissue damage caused by inflammation and the release of pro-inflammatory cytokines and reactive oxygen species, thanks to its antimicrobial activity and the ability to bind components of bacterial cell walls (LPS) or their receptors [6]. The mechanism of interaction between LF and LPS has not yet been understood. New studies are needed to better understand its activity and interactions to enable safe use of LF in clinical and veterinary medicine. In this study, it was aimed to investigate the effect of LF on adenosine deaminase, nitric oxide and liver enzymes in LPS-induced experimental endotoxemia model in rats.

MATERIAL and METHODS

Animal

The required permission for the study was obtained by the Local Ethics Committee of Kafkas University Animal Experiments (Approval No: KAU-HADYEK/2015-018). Housing, maintenance, and experimental procedures were carried out at Kafkas University Experimental Animals Production and Experimental Research Center. During the experiment, the subjects were placed in cages in a 12-h light and 12-h dark cycle and to reach food and water whenever they wanted. The rats were housed in plastic cages with chip shafts and five rats in each cage during the trial period.

Experimental Design

Forty Sprague Dawley female 6 months old rats were used in the study. Subjects were randomly distributed to four groups and rats were weighed for dose calculations. The rats were as follows: Control (n=10), LF (n=10), LPS (n=10), and LF + LPS (n=10) groups. During the experiment, saline was administered intraperitoneally to the control group daily for one week. Group II was given LF (20 mg/kg i.p.) once a day for one week. Group III received LPS (20 µg/kg i.p. *(E. coli* type 0111:B4)) single dose. Group IV was given LF (20 mg/kg i.p.) once a day for one week with lipopolysaccharide (20 µg/kg i.p.) single dose. Six hours after a single dose of lipopolysaccharide injection, animals were sacrificed under anesthesia and the intracardiac blood samples from animals were centrifuged at 3000 rpm and +4°C for 15 min to separate the serum. Liver tissues were stored to be used in subsequent analyzes.

Determination of Nitric Oxide Level

Nitric oxide concentrations in liver tissue were determined using a spectrophotometer (PowerWave XS, BioTek, Vermonts, USA) by the method of Miranda et al.[7]. The samples were de-proteinized with 10% zinc sulphate. Total NO (nitrate and nitrite) concentrations were measured colorimetrically by acidic Griess reaction via reaction involving reduction of nitrate to nitrite by vanadium (III) chloride.

Adenosine Deaminase Activity Assay

Adenosine deaminase activity in liver samples was performed according to the method of Giusti and Galanti [8]. Adenosine, used as a substrate, was incubated with the sample at 37°C for 30 min. The ammonia formed forms blue indophenol in the presence of sodium hypochlorite and phenol in alkaline medium. In this experiment where sodium nitroprusside has a catalyst effect, the ammonia concentration is directly proportional to the absorbance of indophenol. Briefly, 1 mL of phosphate buffer and 50 µL of distilled water into the reagent blind tube, 1 mL ammonium sulfate and 50 µL distilled water into the standard tube, 1 mL adenosine solution and 50 µL sample into the sample tube, 1 mL of adenosine solution was added to the sample blind tube. After the test tubes were closed, they were incubated in a 37°C incubator for 1 h and 3 mL of phenol/nitroprusside solution was added. After adding 0.05 mL of sample to the sample blind tube, 3 mL of alkaline hypochlorite solution was added to all tubes. The tubes were closed again and incubated at 37°C for 30 min. After incubation, the absorbance of the

tubes against the blind was read at 625 nm. One unit of ADA activity was defined as the amount of enzyme that releases 1 µmol of ammonia from adenosine per minute. ADA activities of the groups were expressed as U/mg protein, divided by the amount of protein.

Determination of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Gamma Glutamyltranspeptidase (GGT) Activities

The serum and liver aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyltranspeptidase (GGT) activities were determined colorimetrically using commercial kits (ERBA Diagnostics, Miami, Florida, ABD).

Determination of Total Protein

Liver total protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard at 595 nm.

Statistical Analysis

Statistical analysis of the data obtained from the study was done using SPSS Windows 16.0 package program (SPSS Inc.). Mean values between groups were determined by one-way analysis of variance (ANOVA) and differences between groups by Duncan test. Data (n=10) are expressed as mean±SD values.

RESULTS

Liver NO levels increased significantly in LPS group compared to other groups (P<0.001) *(Fig. 1)*. The NO levels in control, LF and LF+LPS groups were statistically similar. Liver ADA activity levels, considered to be a marker of cellular immunity, increased significantly in LPS group compared to other groups (P<0.001) *(Fig. 2)*. The ADA activity levels of other groups were statistically similar.

Serum AST and GGT activity levels were significantly higher in the LPS group than in the other groups, and LF supplementation significantly reduced these enzyme levels (P<0.001 and P<0.05, respectively). Serum ALT activity level was significantly higher (P<0.05) in the LPS group, but it was not statistically significant even though ALT activity decreased with lactoferrin supplementation. AST, ALT and GGT activities were significantly lower in the LF group compared to the LPS group *(Fig. 3-A)*. When liver ALT, AST

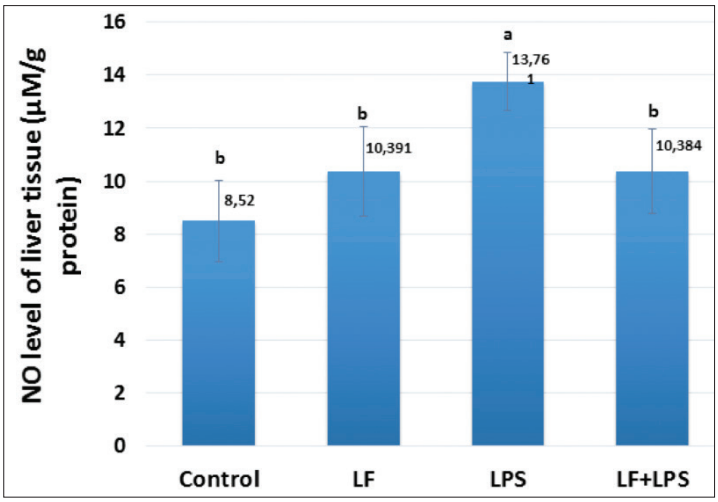

Fig 1. The effects of lactoferrin on liver nitric oxide levels in LPS-induced experimental endotoxemia model. Data (n=10) are expressed as mean±SD values. The difference between the groups indicated by different letters (a and b) is statistically significant (P<0.001)

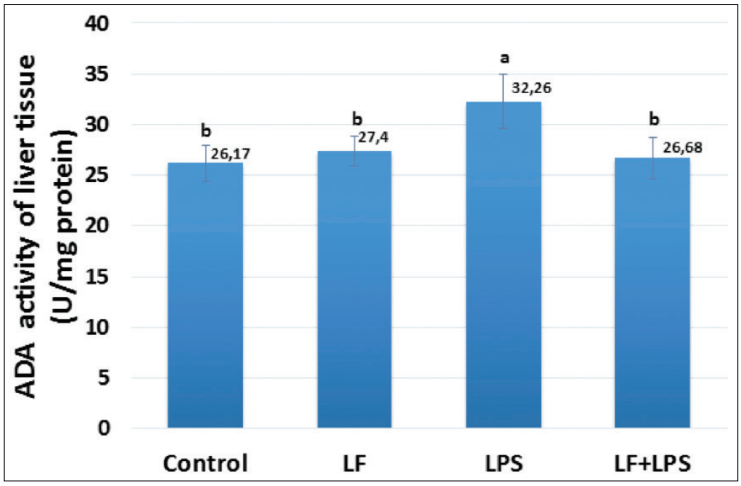

Fig 2. The effect of lactoferrin on liver adenosine deaminase activity levels in LPS-induced experimental endotoxemia model. Data (n=10) are expressed as mean±SD values. The difference between the groups indicated by different letters (a and b) is statistically significant (P<0.001)

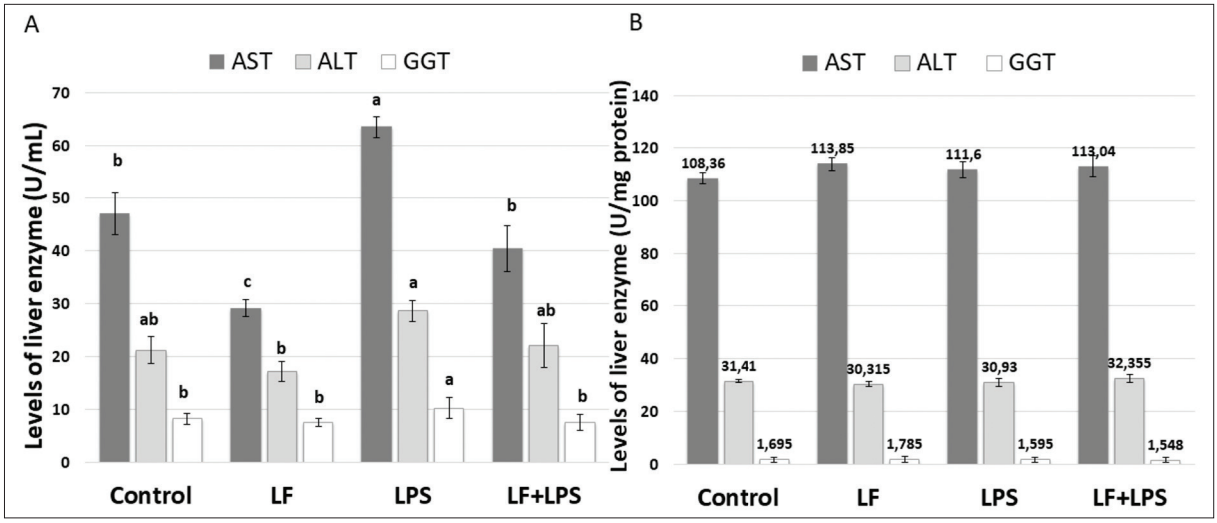

Fig 3. The effect of lactoferrin on AST, ALT and GGT activity levels in LPS-induced experimental endotoxemia model. **A:** Serum AST, ALT and GGT activity levels (P<0.05). **B:** Liver AST, ALT and GGT activity levels (Non-significant). Data (n=10) are expressed as mean±SD values. The diff erence between the groups indicated by different letters (a, b and c) is statistically significant

and GGT activity levels of all groups were compared, it was not statistically significant *(Fig. 3-B)*.

DISCUSSION

Lipopolysaccharide, the main outer membrane component of gram-negative bacteria, causes many pathophysiological processes such as fever, hypotension, disseminated intravascular coagulation, and multiple organ failure. When the history of sepsis is examined, there are many diagnostic approaches from microbiological evaluation of infection to the use of prognostic biomarkers. The main therapeutic approach to sepsis in recent years is intravenous immunoglobulin therapy and appropriate antibiotic use. LF binding to LPS causes bacterial surface damage and the binding to free LPS neutralizes its pro-inflammatory effects. Thus, LF is a potential adjunctive agent for inflammation and endotoxemia/sepsis [9]. LF, a major component produced from LF neutrophils, increases in bacterial infection sites [10,11]. Studies have reported that there is a direct binding between LF and LPS in LF-mediated LPS inhibition based on molecule-molecule interactions [12-15]. LF has been reported to interfere with intracellular events leading to nuclear factor kappa B (NF-κB) activation, inhibiting cytokine production and inhibiting LPS-induced cytokine gene expression within 2 h by inhibiting NF-κB binding to DNA [3]. LF from porcine-20 (LF-20), one of the anti-microbial peptides containing 20 amino acids, has been reported to inhibit the response to LPS-induced inflammation by inhibiting MyD88/NF-κB and MyD88/MAPK signaling pathways [16]. Although LF is a glycoprotein that plays an important role in immunomodulation, its mechanism of action has not been fully explained yet. In this study, the effect of LF on ADA activity, NO and liver enzyme levels were investigated in an experimental endotoxemia model in rats.

Adenosine deaminase, a key enzyme in purine metabolism, is an enzyme that catalyzes the conversion of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxynosine, respectively. ADA activity in humans is higher in thymus, spleen, lymph node, bone marrow and peripheral blood lymphocytes. ADA1, which has a low molecular weight of two isoforms, is found in the thymus, erythrocyte and heart, while ADA2 in high molecular weight is found in the liver, kidneys and intestines. ADA2 is also the dominant enzyme form in human blood plasma. ADA activities detected in T lymphocytes are higher than B lymphocytes, but there is a significant increase in ADA activity during T cell differentiation [17]. The ADA reaction is of great importance in controlling the intracellular concentration of adenosine. Since this reaction catalyzed by ADA is irreversible, it constitutes the control step in the destruction of adenosine. Increasing intracellular levels of adenosine and deoxyadenosine is toxic, and ADA plays an important role in regulating the intracellular levels of these nucleosides [18]. It is known that ADA activity increases during the mutagenic defense of the cell and is responsible for the proliferation and differentiation of lymphocytes and monocytes. ADA inhibition has been associated with lower plasma-mediated reactive oxygen species (ROS) production. In one study, incubation of neutrophils in plasma has been reported to increase total ADA activity 10 times from 1.3 U/mL to 12 U/mL [19]. ADA activity has been demonstrated in the autoimmune disease period such as typhoid, infectious mononucleosis, brucellosis, acute pneumonia, tuberculosis, sarcoidosis, liver diseases, acute leukemia, cancer, rheumatoid arthritis, systemic lupus erythematosus, psoriasis and Behçet disease [4]. ADA deficiency has been reported to be associated with lymphopenia, severely impaired cellular and humoral immunity, developmental retardation, and severe infections [5].

Nitric oxide is a signaling molecule that acts as an endocrine molecule in recent years [20]. During inflammation, NO

is increased by inducible nitric oxide species (iNOS) and activated by cytokines and reacts with superoxide anions leading to formation of peroxinitrite radical [21] and free radicals [22]. In a study conducted on CaCo-2 and RAW 246.7 cell lines, it was reported that recombinant LF (rLF) and its hydrolysates were effective in decreasing interleukin 8 and production of ROS in relieving the response to LPS-induced inflammation. Also, rLF did not affect the NO content in CaCo-2 monolayers [19]. The studies reported here indicate that recombinant human LF is effective in modulating the inflammatory responses of CD14+ and CD16+ macrophages during low and high stimulating conditions [23]. NO levels and ADA activity were increased in patients with *Entamoeba coli*, *Enterobius vermicularis*, *Giardia intestinalis*, *Demodex spp.*, hydatid cyst and *Toxoplasma gondii* serum positive compared to control [24].

Adenosine and its receptors have been reported to increase nitric oxide production in LPS -treated RAW 264.7 cells. Increased NO production by adenosine was inhibited by adenosine uptake inhibitors, such as dipyridamole, S(4-nitrobenzyl)-6-thioinosine and S(4-nitrobenzyl)-6-thio-guanosine [25]. In doxorubicin-induced cardio toxicity, ADA and NO levels were observed to be significantly higher than control. Erdosteine, which is given as a protector in cardiac toxicity, did not change the ADA activity, but significantly decreased the NO level in rats [26]. Akinyemi et al.[27] show that significantly increased adenosine deaminase activity with cadmium-induced renal toxicity in rats decreased curcumin. It was observed that cadmium toxicity decreased kidney nitric oxide level and this level increased with curcumin supplementation. The ADA activity and NO levels were examined by giving glutathione to rabbits at certain hours (0. 3. 6. and 12.) and no change was observed in plasma ADA levels. However, NO levels decreased significantly at 3. and 6. hours with significant glutathione supplementation [28]. In the study, it was found that ADA activity and NO levels increased in LPS-induced endotoxemia model and LF supplementation decreased these levels significantly ($P<0.001$). It can be said that LPS increases ADA synthesis and NO release and LF acts as an anti-inflammatory and immunosuppressor in stimulating immune response. Excessive amounts of neutrophils, macrophages, lymphocytes and eosinophils are produced as a response to microorganisms [20]. LPS stimulates immune responses and proinflammatory mediator secretion by monocytes, macrophages, and neutrophils, which are recruited into specific host tissues by LPS exposure [29,30]. The levels of TNF-α, IL-1b, IL-6, IL-8 and NO have been shown to increase during infection [20]. In a previous study, it was found that total oxidant capacity and NO was higher after the injection of LPS and total antioxidant capacity was demonstrated with a reduction in response to LPS in mice [31].

The alanine aminotransferase "ALT", alkaline phosphatase (ALP), and GGT enzymes are used to evaluate the functions of the liver and high levels of these enzymes increase risk of disease and all-cause mortality. In clinical practice, they are used as an indicator of damage in a spectrum ranging from inflammation to necrosis rather than indicating liver function [32]. In the presented study, there was no statistically significant difference between liver ALT, AST and GGT activity levels of all groups, but serum AST and GGT activity levels were significantly higher in the LPS group than in the other groups, and LF supplementation significantly reduced these enzyme levels. AST, ALT, and GGT activities were significantly lower in the LF group comparing to the LPS group.

As a result, LF showed a protective effect by reducing ADA synthesis and NO release in the LPS-induced experimental endotoxemia model. LPS increases ADA activity and NO levels and LF plays a complex role in modulating the inflammatory response. The results showed that ADA and NO may have the potential to become a marker in endotoxemia.

Author Contribution

O.A and E.A. conceived the original idea, designed and supervised the project. C.G. and K.Y.D performed experiments. All authors wrote the manuscript.

Conflict of Interest

All authors declare that there is no potential conflict of interest.

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Research Article

Effect of Resveratrol Supplemented to Japanese Quail *(Coturnix coturnix japonica)* Rations on Performance and Some Biochemical Parameters

Mükremin ÖLMEZ [1,a] Tarkan ŞAHİN [1,b] Mustafa MAKAV [2,c] Özlem KARADAĞOĞLU [1,d]

[1] Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Kafkas University, TR-36100 Kars - TURKEY
[2] Department of Physiology, Faculty of Veterinary Medicine, Kafkas University, TR-36100 Kars - TURKEY
ORCIDs: [a] 0000-0002-5003-3383; [b] 0000-0003-0155-2707; [c] 0000-0003-1879-8180; [d] 0000-0002-5917-9565

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Abstract

This experiment was conducted to evaluate the changes on growth performance, carcass and some biochemical parameters of Japanese quail fed supplemental Resveratrol (Res). A total of 200 quails were distributed at equal body weights into four experimental groups, each consisting of five replicate pens having ten birds/replicate pen: 1- basal diet without Res (Control), 2- basal diet + 100 mg/kg Res, 3- basal diet + 200 mg/kg Res, and 4- basal diet + 400 mg/kg Res. There were no significant effects of the supplementation of resveratrol on body weight gain and carcass traits (P>0.05). Inclusion of Res in quail rations improved feed consumption (P<0.05) and feed conversion ratio during the period from 3 to 5 weeks and the throughout (0-5 wks) of the treatment (P<0.05). Malondialdehyde decreased as compared with the control group (P<0.05), but glutathione and nitric oxide did not change by the effect of Res (P>0.05). Glucose, triglyceride, total protein was not influenced by the supplementation of Res (P>0.05). As a result, the findings of this study suggest that Res can be used safely as a growth promoter up to 400 mg/kg in quail rations.

Keywords: *Antioxidant, Japanese quail, Performance, Polyphenolic compounds, Resveratrol*

Japon Bıldırcın *(Coturnix coturnix japonica)* Rasyonlarına İlave Edilen Resveratrol'ün Performans ve Bazı Biyokimyasal Parametreler Üzerine Etkisi

Öz

Bu çalışmada Japon bıldırcın rasyonlarına ilave edilen resveratrolün (Res) büyüme aktivitesi ile ilişkisinin belirlenmesi amaçlandı. Çalışmada eşit canlı ağırlığa sahip 200 adet bıldırcın civcivi beşer tekrarlı (10 civciv) olacak şekilde ve her grupta 50 adet hayvan bulunan dört deneme grubuna ayrıldı. Resveratrolün Japon bıldırcınlarında performans, karkas özellikleri, serum antioksidan ve bazı biyokimyasal parametreleri üzerine etkisini belirlemek için 1- Res içermeyen bazal diyet (Kontrol), 2- bazal diyet + 100 mg/kg Res, 3- bazal diyet + 200 mg/kg Res ve 4- bazal diyet + 400 mg/kg Res gruplarına ayrıldı. Resveratrol ilavesinin canlı ağırlık artışı ve karkas parametrelerini etkilemediği belirlendi (P>0.05). Japon bıldırcın rasyonlarına Res ilavesi ile 3-5 haftalık periyotta ve çalışmanın tamamında (0-5 hafta) yem tüketimi ve yem dönüşüm oranı önemli derecede etkilendi (P<0.05). Resveratrolün etkisiyle malondialdehit kontrol grubuna göre azalırken (P<0.05), glutatyon ve nitrik oksit seviyeleri değişmedi (P>0.05). Resveratrol ilavesinin glikoz, trigliserit ve total protein üzerine etkisinin olmadığı tespit edildi (P>0.05). Sonuç olarak, elde edilen bulgulara göre Res'in bıldırcın rasyonlarında 400 mg/kg'a kadar büyüme destekleyici bir katkı maddesi olarak güvenle kullanılabileceği kanaati oluşmuştur.

Anahtar sözcükler: *Antioksidan, Fenolik bileşikler, Japon bıldırcın, Performans, Resveratrol*

INTRODUCTION

Antimicrobial feed additives have played an important role in the economic development of the poultry industry for many years by increasing the growth rate, improving the feed conversion ratio, and reducing the risk of disease [1]. However, the use of antibiotics in farm animals has caused significant concerns since they remain in meat and eggs

Correspondence
+90 474 2426807-5116
mukremin.olmez@hotmail.com

over time. Because of this, resistant bacterial populations have developed. As a result of these concerns, the use of antibiotics as a growth promoter for poultry has been banned in the European Union [2]. In addition to antibiotic prohibition in animal feeding, studies have focused on prebiotic, probiotic, medicinal and aromatic plants and phenolic compounds derived from them to minimize problems in poultry health and increase performance [3-7]. Resveratrol (Res) is a natural polyphenolic compound (3, 5, 4' - trihydroxytrans - stilbene) found in high concentrations in *Polygonum cuspidatum*, grape peel, hazelnut, Yucca schidigera and wine [8-11]. Resveratrol has antioxidant, anti-aging, and anti-inflammatory effects, and it plays a role in regulating energy metabolism [10,12-16]. Resveratrol or its derivatives exhibit antimicrobial properties and prevent cancer cell growth [12]. By protecting DNA from oxidative damage, malondialdehyde (MDA) and heat stress are reduced [12,17]. Resveratrol is considered an important monomeric bioactive compound that exhibits a strong antioxidant capacity to scavenging free oxygen and lipid radicals [18,19]. Resveratrol inhibits the formation of glutathione (GSH) disulfide and inhibits cellular damage produced by free radical reactions by maintaining GSH [18]. Resveratrol can prevent and treat cardiovascular diseases while preserving vascular endothelium, as well as cure micro-circulatory disorders. It can raise the level of cellular nitric oxide (NO) and also inhibits platelet aggregation [20,21]. Several animal and poultry experiments have been conducted to determine the dependence and functionality of Res and its supplementation in mammalian and poultry rations. Zhang et al.[9] found that Res (200, 400 or 800 mg/kg) supplementation in broiler chickens had a positive effect on their body weight compared to the control group. Sridhar et al.[22] reported that chicks given Res at 0.5% and 1.0% levels of ration help to increase antioxidative enzyme activities, at the same time contributing to the improvement of protein and total antioxidant capacity in plasma, and the degree of liver lesions in Res groups is much lower. It has been reported that the supplementation of grape seed extract (400 ppm) containing Res to broiler rations can improve serum biochemistry and lipid profile [23]. It is stated that dietary supplementation of Res, in addition to improving growth performance, gut morphology, and microbial balance, increased immunity in *E. coli* fighting broilers [24]. Therefore, the objective of this study was to determine the effects of dietary supplementation of Res on growth performance, antioxidant, and some serum biochemical parameters of Japanese quail.

MATERIAL and METHODS

Ethical Approval

The experimental protocol and animal care in this study were approved by animal experiments from the local ethics committee (KAÜ-HADYEK/2018-026) of Kafkas University.

Experimental Design

In this trial, the effect of 4 different levels of Res supplementation to Japanese quail rations on performance, carcass characteristics, serum antioxidant parameters and biochemical profile were investigated. The animal material of the study consisted of 200 Japanese quails *(Coturnix coturnix japonica)* of one day age in mixed gender, each consisting of five replicates having ten birds in each replicate. Quails were randomly divided into four treatment groups, 50 in each, and placed in cages. Research rations were prepared to take into account the criteria specified in the National Research Council (NRC) [25]. Ration containing 23.89% crude protein (CP) and 2929.28 kcal ME/kg was fed to all groups for 35 days in the trial. The raw material and nutrient contents of the ration used in the experiment are shown in *Table 1*. Quails were fed with ration containing 0 (control), 100, 200, and 400 mg/kg Res (50%, Resveratrol, Solgar, Istanbul, Turkey). The product contained naturally derived Res from 200 mg *Polygonum cuspidatum* (root). The experiment lasted for 35 days.

Growth Performance

Body weight gain (BWG) and feed consumption (FC) were recorded weekly as performance indicator parameters during the trial and the feed conversion ratio (FCR) was calculated. Feed and water were given as *ad libitum* and group feeding was applied to animals.

Table 1. *Nutrient and chemical composition of quail basal diet*

Ingredients	**Ratio, %**
Corn	48.45
Soybean meal (45% CP)	44.00
Wheat bran	1.50
Vegetable oil	2.80
Limestone	1.65
Dicalcium phosphate	0.80
Salt	0.25
DL-methionine	0.15
L-lysine sulfate	0.15
Vitamin mix[1]	0.15
Mineral mix[2]	0.10
Analysis of nutrient contents	
Crude protein (%)	23.89
Metabolizable energy (Kcal/kg)	2928.28
Calcium (%)	1.03
Available phosphorus (%)	0.58

[1] Vitamin mix makes per kilogram of ration: vit. D_3, 0.025 mg; menadione (menadione sodium bisulfate) 1.1 mg; vit. B_2, 4.4 mg; vit. B_1, 1.1 mg; vit. B_6, 2.2 mg; B_3, 35 mg; vit. B_{12}, 0.02 mg; folic acid, 0.55 mg; choline, 125.000 mg; d - biotin, 0.1 mg; calcium D-pantothenate, 10 mg; vit. A, 12.000 IU; vit. D_3, 2.400 IU; vit. E, 30 mg; vit. K_3, 5 mg
[2] Mineral mix makes per kilogram of ration: Se, 0.15 mg; Mn, 40 mg; Fe, 12.5 mg; Zn, 25 mg; Cu, 3.5 mg; I, 0.3 mg; Co, 0.1 mg

Carcass Parameters

At the end of the trial, 8 quails (a total of 32) were slaughtered from each group and their internal organs were exenterated. The weights of the carcasses and internal organs (heart, liver, and gizzard) were determined. The dressing percentage was calculated by proportioning the carcass weight to the body weight before slaughtering [26].

Blood Collection and Serum Separation

Blood samples were collected from each quail randomly selected for slaughtering (8/group, 32 in total). Blood samples taken from *Vena brachialis* during slaughtering were centrifuged for 10 minutes at 3000 rpm and stored at - 20°C a month until analysis.

Serum Antioxidant and Biochemical Parameters

Serum samples were dissolved at room temperature, antioxidant parameters as MDA, GSH, and NO and biochemical profile as glucose (GL), triglyceride (TG), and total protein (TP) concentrations were measured using commercial ELİSA kits (MyBioSource® Company, San Diego/USA) in the spectrophotometer device.

Statistical Analysis

For statistical analysis, the SPSS portable 18 (SPSS, Chicago, IL) statistical package program was used, and the data was analyzed with one-way analysis of variance (ANOVA). It was analyzed in terms of linear (L), quadratic (Q), and cubic (C) effects depending on the linear increase in the Res level (100, 200, and 400 mg/kg). Due to the significance is different, the P values for L, Q, and C effects are given in the tables. The mean separation among groups was performed utilizing Tukey's test. The means and standard errors of each group are indicated in the result tables. The significance level (P) was assessed as 0.05.

RESULTS

The effects of Res on BWG, FC and FCR in Japanese quails are presented in *Table 2*. It was determined that the differences between the groups in terms of BWG were not significant during the experiment (L=0.129). In the experiment, the average FC's belonging to the groups were significantly affected by increasing levels of dietary supplementation of Res.

In the trial, Res 100 group lower FC has occurred in comparison to control groups in 3-5. wks (Q=0.012; C=0.017) and 0-5. wks (Q=0.008). The effect of Res groups on FCR was significant (L=0.050; C=0.028). In the 3-5. wks of the trial, a cubic increase was observed in the FCR in Res 100 and 400 groups (C = 0.005). The carcass characteristics of the control and treatment groups thus obtained are presented in *Table 3*. There were no differences statistically among the treatment groups in terms of slaughter weight, dressing, heart, liver, and gizzard weights (L=0.119; L=0.630; L=0.814; L=0.968; L=0.816).

The effects of supplementation of Res on MDA, GSH, and NO are shown in *Table 4*. MDA level in serum was remarkably reduced by Res 200 and 400 groups (L=0.041 and L=0.047). Furthermore, no significant change was observed in the concentration of GSH (L=0.610) and NO (L=0.184) among the groups.

Supplementation with Res did not affect the levels of GL (L=0.723), TG (L=0.865) and TP (L=0.865) compared with the control group *(Table 5)*.

DISCUSSION

Based on the results of the present study, quail diets containing 100 mg/kg of Res were more effective in enhancing growth performance than the control group. In

Table 2. *Performance values of quails fed supplemental resveratrol (Mean±SEM[1])*

Parameters[2]	Weeks	Groups[3]				Contrasts[4]		
		Control	RES 100	RES 200	RES 400	L	Q	C
BWG (g)	0-3	20.04±0.09	19.88±0.05	19.98±0.16	19.95±0.01	0.716	0.482	0.129
	3-5	34.51±0.14	34.89±0.19	34.64±0.15	35.00±0.03	0.069	0.935	0.727
	0-5	27.27±0.09	27.39±0.11	27.31±0.04	27.48±0.02	0.129	0.062	0.213
FC (g)	0-3	51.09±0.34	50.82±0.13	50.52±0.17	50.78±0.30	0.276	0.306	0.607
	3-5	135.26±0.18^{a}	133.15±0.48^{b}	134.410.38^{ab}	134.46±0.45^{ab}	0.517	0.012	0.017
	0-5	93.18±0.20^{a}	91.98±0.21^{b}	92.47±0.21^{ab}	92.62±0.29^{ab}	0.258	0.008	0.064
FCR	0-3	2.55±0.01	2.56±0.01	2.53±0.02	2.54±0.01	0.457	0.811	0.247
	3-5	3.92±0.02^{a}	3.82±0.03^{b}	3.88±0.01^{ab}	3.84±0.01^{b}	0.058	0.102	0.005
	0-5	3.42±0.01^{a}	3.36±0.02^{b}	3.39±0.01^{ab}	3.37±0.01^{ab}	0.050	0.090	0.028

[1] SEM = Standard error mean; [2] BWG = Body weight gain; FC = Feed consumption; FCR = Feed conversion rate; [3] RES 100 = Resveratrol 100 mg/kg; RES 200 = Resveratrol 200 mg/kg; RES 400 = Resveratrol 400 mg/kg; [4] L = Linear; Q = Quadratic; C = Cubic; [a,b] Means within the same row with different superscript (P<0.05)

Table 3. *Carcass characteristics of quails fed supplemental resveratrol (Mean±SEM[1])*

Parameters[2]	Groups[3]				Contrasts[4]		
	Control	RES 100	RES 200	RES 400	*L*	*Q*	*C*
SW (g)	169.84±0.20	170.08±0.09	169.99±0.60	170.86±0.50	0.119	0.451	0.484
Dressing (%)	70.01±0.02	70.00±0.01	70.12±0.17	70.05±0.14	0.630	0.788	0.522
Heart (g)	1.03±0.03	1.02±0.03	1.03±0.00	1.02±0.03	0.814	0.895	0.639
Liver (g)	3.49±0.20	3.41±0.10	3.48±0.19	3.45±0.14	0.968	0.895	0.742
Gizzard (g)	3.61±0.09	3.42±0.11	3.76±0.11	3.45±0.21	0.816	0.671	0.078

[1] SEM = Standard error mean; [2] SW= Slaughter weight; [3] RES 100 = Resveratrol 100 mg/kg; RES 200 = Resveratrol 200 mg/kg; RES 400 = Resveratrol 400 mg/kg; [4] L = Linear; Q = Quadratic; C = Cubic

Table 4. *Serum antioxidant parameters of quails fed supplemental resveratrol (Mean±SEM[1])*

Items[2]	Groups[3]				Contrasts[4]		
	Control	RES 100	RES 200	RES 400	*L*	*Q*	*C*
MDA (mg/dL)	0.75±0.30[a]	0.31±0.08[a]	0.23±0.08[b]	0.23±0.10[b]	0.041	0.208	0.721
GSH (mg/dL)	0.07±0.00	0.06±0.00	0.06±0.00	0.06±0.00	0.610	0.275	0.785
NO (mg/dL)	10.63±2.49	13.28±2.59	23.70±4.95	23.44±7.48	0.184	0.780	0.736

[1] SEM = Standard error mean; [2] MDA = Malondialdehyde; GSH = Glutathione; NO = Nitric oxide; [3] RES 100 = Resveratrol 100 mg/kg; RES 200 = Resveratrol 200 mg/kg; RES 400 = Resveratrol 400 mg/kg; [4] L = Linear; Q = Quadratic; C = Cubic; [a,b] Means within the same row with different superscript (P<0.05)

Table 5. *Serum biochemical profile of quails fed supplemental resveratrol (Mean±SEM[1])*

Items[2]	Groups[3]				Contrasts[4]		
	Control	RES 100	RES 200	RES 400	*L*	*Q*	*C*
GL (mg/dL)	197.17±0.28	196.27±0.79	197.09±0.43	196.62±0.44	0.723	0.689	0.208
TG (mg/dL)	173.15±0.47	173.47±1.02	173.20±0.47	173.070.47	0.865	0.736	0.807
TP (g/dL)	3.20±0.08	3.20±0.12	3.20±0.11	3.22±0.10	0.865	0.942	0.982

[1] SEM = Standard error mean; [2] GL = Glucose; TG = Triglycerides; TP = Total protein; [3] RES 100 = Resveratrol 100 mg/kg; RES 200 = Resveratrol 200 mg/kg; RES 400 = Resveratrol 400 mg/kg; [4] L = Linear; Q = Quadratic; C = Cubic

the study, although BWG was not affected, the decrease of FC significantly improved FCR. He et al.[27] reported that the supplementation of Res at different levels (200, 350, 500 mg/kg) in broilers under heat stress does not affect the average daily feed consumption and improves body weight. Zhang et al.[28] reported that the average daily feed consumption, average body weight, and feed utilization rate improved with the supplementation of Res to broiler rations under heat stress. It has been stated that the Res supplementation of 0.5% and 1.0% to the ration of broiler chicks exposed to aflatoxin caused lower body weight and FC during five weeks period but did not affect FCR [22]. In a different trial, it was reported that the addition of different levels (200-400 and 800 mg/kg) of Res to broiler rations did not affect the performance values [9]. The observed decrease in feed intake in 100 and 400 mg/kg Res groups during the late period and whole experiment period may be due to the blandness of Res, leading to a direct reduction in FC [29]. The improved FCR in quails fed 100 mg/kg of Res could be attributed to the effects of resveratrol, which mediated reduction of pathogens and improved utilization of nutrients in the digestive system [30].

Kim et al.[31] stated that Res did not affect carcass efficiency and internal organ weights in a similar way to this study, but decreased liver weight. Abdel - Wahab et al.[32] revealed that grape seed does not affect carcass yield in quail. Sridhar et al.[22] reported that the supplementation of resveratrol decreased liver weight (P>0.05), but did not affect heart and liver weight in broilers.

It has been reported that the supplementation of Res to broiler rations helps chickens increase their antioxidant activities, and also contributes to the improvement of protein and total antioxidant capacity in plasma [22]. Also, Res could increase the expression of various antioxidant enzymes and reduce MDA content [28,33]. It has been determined that with the addition of Res, serum MDA level was decreased and serum GSH level in broilers was increased [27]. Sahin et al.[34] have noticed that serum MDA level was not affected in laying quail, differ from this study. Liu et al.[18] stated that adding 400 mg/kg Res to

chick rations increased antioxidant capacity and decreased MDA content. NO has a vital role as diverse physiological including, vasodilation and inflammation in cells [35]. Limited availability of studies was found about the effect of resveratrol on serum NO levels in poultry. In the current study, the effect of Res on serum NO can be considered as an initiator study investigating.

The inclusion of Res in quail diets did not affect GL, TG, and TP as compared to the control group. Zhang et al.[28] reported that with the addition of Res to broiler rations, triglyceride and total protein levels were affected and glucose levels were decreased compared to the control group. He et al.[27] reported that the supplementation of Res reduced serum glucose and total protein levels and increased the level of triglycerides. It has been reported that although the total protein level increased with the effect of Res, triglyceride and glucose levels did not change in broilers [22].

In conclusion, the study showed that Res has a positive effect on quail performance parameters as FC and FCR. Also, dietary res may reduce the serum MDA level that negatively affects health status of quails. So, Res can be supplemented to Japanese Quail rations as a feed additive (up to 400mg/kg). Besides, it is recommended to research using Res at different levels to reveal its antioxidant and biochemical effect in prospective poultry experiments.

Conflict of Interest

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

Author Contributions

Experimental Design; MÖ, TŞ and MM, collecting data for analysis; ÖK and MM, analysis of samples; MM, Software; MÖ and MM, Writing-original draft; MÖ, Writing-review and editing; TŞ.

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Short Communication

Anthelmintic Efficiency of Synthetic and herbal Compounds Against Gastrointestinal Nematodes in Naturally Infected Goats

Saqib REHMAN [1,a] Kiran AFSHAN [1,b] Abdul RAZZAQ [2,c] Munib HUSSAIN [3,d] Sabika FIRASAT [1,e]

[1] Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, 45320, PAKISTAN
[2] Animal Sciences Division, Pakistan Agricultural Research Council, Islamabad, 45320, PAKISTAN
[3] Animal Health Program, National Agriculture Research Centre, Islamabad, 45320, PAKISTAN
ORCIDs: [a] 0000-0003-3148-3554; [b] 0000-0002-3979-7606; [c] 0000-0001-8094-5335; [d] 0000-0001-9329-6303; [e] 0000-0003-4959-7832

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Abstract

The present study was aimed to assess the anthelmintic resistance against gastrointestinal (GI) nematode infections in goats. GI nematode infected goats (n=120) were randomly assigned to six treated groups, and untreated control group. Six treatments were given and faecal egg count reduction (FECR) was performed. Significant differences (P<0.05) were observed between the prevalence rate of different nematodes and their FEC levels. Ten nematodes species, *Haemonchus contortus, Ostertagia, Trichostrongylus, Strongyloides, Oesophagostomum, Nematodirus, Bunostomum, Chabertia ovina, Marshallagia* and *Cooperia* were identified. The FECR for the six anthelmintics showed significant (P<0.05) reduction in eggs per gram of feces of treated groups compared to control group on 3rd, 6th, 12th and 30th days post treatment. The highest efficacy was recorded of Oxyclozanide + Oxfendazole + Cobalt + Selenium treated group at 12th and 6th days of post treatment (99.3% and 98.6% respectively); whereas Levamisole + Oxyclozanide + Selenium + Cobalt treated group showed highest efficiency (97.7%) at 30th day. In herbal anthelmintics, lowest efficacy (87.4%) was with Deedani treated group and Atreefal Deedan treated group showed high efficacy of 91.2%. The most efficacious time in all groups treated with anthelmintics was 12th and 30th day of post treatment against GI nematodes in goats. In conclusion, imported anthelmintics need efficacy testing before use and novel combination of anthelmintics holds potential to reduce the burden and resistance in nematodes for control purposes in small ruminants.

Keywords: *Anthelmintics, Gastrointestinal nematod, Goats, Herbal medicines*

Doğal Olarak Enfekte Keçilerde Gastrointestinal Nematodlara Karşı Sentetik ve Bitkisel Bileşiklerin Anthelmintik Etkinliği

Öz

Bu çalışmanın amacı keçilerde gastrointestinal (GI) nematod enfeksiyonlarına karşı antelmintik direnci değerlendirmektir. GI nematodla enfekte keçiler (n = 120) rastgele olarak altı tedavi grubuna ve tedavi edilmemiş kontrol grubuna ayrıldı. Altı tedavi grubunda verilen ve dışkıda yumurta sayısının azalması (FECR) değerlendirildi. Farklı nematodların yaygınlık oranları ile FEC seviyeleri arasında önemli farklılıklar (P<0.05) gözlendi. On nematod türü, *Haemonchus contortus, Ostertagia, Trichostrongylus, Strongyloides, Oesophagostomum, Nematodirus, Bunostomum, Chabertia ovina, Marshallagia* ve *Cooperia* identifiye edildi. Altı antelmintik için FECR, kontrol grubuna oranla tedavi edilen grupların gram dışkı başına yumurtalarında tedavi sonrası 3., 6., 12. ve 30. günlerde önemli (P<0.05) azalma gösterdi. En yüksek etkinlik Oksiklozanid + Oksfendazol + Kobalt + Selenyum ile tedavi edilen grupta tedavi sonrası sırasıyla 12. ve 6. günlerde kaydedilmiştir (%99.3 ve %98.6); Levamisol + Oksiklozanid + Selenyum + Kobalt ile tedavi edilen grup ise 30. günde en yüksek etkinliği (%97.7) göstermiştir. Bitkisel antelmintiklerde en düşük etkinlik (%87.4) Deedani ile tedavi edilen grupta olmuştur ve Atreefal Deedan ile tedavi edilen grup %91.2 ile yüksek etkinlik saptanmıştır. Keçilerde antelmintiklerle tedavi edilen tüm gruplarda en etkili zaman, GI nematodlara karşı tedavinin 12. ve 30. günleri olduğu belirlenmiştir. Sonuç olarak, ithal edilen antelmintiklerin kullanımdan önce etkinlik testine ihtiyacı vardır ve antelmintiklerin yeni kombinasyonu, küçük ruminantlarda kontrol amaçlı nematodlardaki sayı ve direnci azaltma potansiyeline sahiptir.

Anahtar sözcükler: *Anthelmintik, Gastrointestinal nematod, Keçi, Bitkisel ilaç*

INTRODUCTION

Gastrointestinal (GI) nematode infections are one of the major problems to small ruminant industry and its sustainability worldwide. It causes immense monetary losses in livestock industry including reduction in weight, poor growth, infertility, poor wool quality, reduction in meat and milk yield [1]. The animal clinical symptoms

Correspondence
+92 51 90643252
kafshan@qau.edu.pk

associated with diarrhea, anorexia, anaemia and oedema [2] Around 90% of small ruminant population in Pakistan is at risk of parasitic infections [3]. Previous studies conducted on prevalence of GI nematodes in small ruminants have been reported from 26.5% to 91.7% in different areas of Pakistan [4]. The anthelmintics are used against GI nematode infections as prophylactic measures. The efficacy of anthelmintics has greatly reduced due to emergence of resistant in nematodes and extensive usage of anthelmintics for treatment and control purpose [5]. Resistivity of internal parasites to the commonly used anthelmintics has been a challenge for goat industry [6]. Current situation needs substitutes rather than relying on conventionally used compounds, alternative strategies for control of GI nematode infections to circumvent these resistance problems [7].

For helminth control many bioactive compounds extracted from medicinal plants are of growing interest in veterinary parasitology [8]. Moreover, most of the commercially available anthelmintic compounds are imported without proper test and registration, which may have effect on their efficacy [9]. Other than this increasing resistivity in nematodes have been contributed by different factors which includes low dose, use of drug with poor efficacy, prolong exposure to same drug, low protein diet and not isolating diseased animals [10]. Keeping in view the importance of these GI nematodes, current study was conducted to evaluate the anthelmintic efficacy of synthetic and herbal medicines against GI nematodes in flocks of dairy goats in Pakistan, in which data is lacking. Therefore, present study was designed to investigate these parameters for the wider benefits of livestock farming community.

MATERIAL and METHODS

The study was carried out between May 2018 to January 2019 at three livestock experimental stations, Islamabad i.e. National Agriculture Research Centre (NARC), Said-pur and Tramari. The study was approved by ethical committee of Quaid-i-Azam University, Islamabad Pakistan. The naturally infected goats (n=120) were selected which were not subjected to any anthelmintic drugs since last 3-4 months. The goats were reared on grazing and grazing pasture was shared by the multiple goats from farmer flocks. The fecal samples were collected in polythene bags from the rectum of each animals and examined with standard parasitological procedures i.e. flotation method and McMaster technique for counting of nematodes eggs [11]. The faecal samples were cultured to cultivate the L3 larvae for gastrointestinal nematodes identification according to the criteria of Coles [12]. The selected goats were equally divided into six treatment groups (n=20 each) and one control (untreated) group.

In certain time intervals (on day 3rd, 6th, 12th and day 30th), fecal samples of each group individuals were collected and the number of eggs per gram (EPG) of feces of each goat was evaluated. All goats were weighed, and randomly assigned to 6 treatments. Following treatments were given to each group orally:

Control: Untreated (no medication given)

Group A: Albendazole (Albasym; SYMANS Pharmaceuticals (PVT) Ltd.) at 1 mL/5 kg b.w.

Group B: Oxyclozanide + Oxfendazole + Cobalt + Selenium (Punch; Selmore Pharmaceuticals) at 1 mL/5 kg body weight (b.w.)

Group C: Oxfendazole (Systamex; ICI Pakistan Ltd.) at 1 mL/5 kg b.w.

Group D: Levamisole + Oxyclozanide + Selenium + Cobalt (Nilzan Plus; ICI Pakistan Ltd.) at 1 mL/5 kg b.w.

Group E: Deedani *(Mallolus philppinensis, Embelia ribes, Piper longum)* at 5 g/head/day for 3 days

Group F: Atreefal deedan *(Emblica officianalis, Terminalia bellerica, Terminalia chebula, Embelia robusta, Ipomoea turpethum, Saussurea lappa, Mallotus philippinensis, Lupinus albus, Artemisia absinthium, Darmina turki, Cascuta reflexa, Black salt, Brassica cernua, Citrullus colocynthis, Cyprus scariosus, Zingiber officinale,* liquid glucose and sugar*)* at 10 g/5 kg b.w. once

The Fecal eggs count percent reduction (FECR) was calculated by using the following formula and interpret according to efficiency assessment by WAAVP [12].

FECR (%) = EPG pre-treatment - EPG post-treatment/EGP pre-treatment x 100

The data was log transformed to confirm the normality and analysis of variance (ANOVA) was applied using Statistics 8.1 statistical package for Windows. The level of significance was set at P value <0.05. All values are expressed as mean EPG ± SD.

RESULTS

Significant differences (P<0.05) were observed between the prevalence rate of different nematodes and their FEC levels. Among these, *Haemonchus contortus* showed higher prevalence (54%) followed by O*stertagia* (15%), *Trichostrongylus* (9%) and *Strongyloides* (9%) during the study period *(Fig. 1)*.

All the treated groups of goats (A, B, C, D, E and F) showed statistically significant (P<0.05) difference of FEC reduction in post-treatment 3rd to 30th days *(Table 1)*. The goats treated with syntactic drugs i.e. Levamisole + Oxyclozanide + Selenium + Cobalt, showed higher reduction (97.6%) in nematode egg counts followed by Oxyclozanide + Oxfendazole + Cobalt + Selenium (95.5%), Oxfendazole (94.6%) and Albendazole (93.4%). These synthetic drugs were found effective against goat nematodes. Maximum

Table 1. *Mean EPG±SD of six treated groups and untreated control of experimental goats at livestock research stations, ICT, Islamabad*

Groups	Anthelmintics	Pre-Treatment	Post Treatments			
			3rd	6th	12th	30th
Control	Untreated	4134±426.5	4178±365.2	4029±419.7	3997±497.2	4012±456.8
A	Albendazole	4225±345.1^{bc}	1062.5±107.8^{c}	362.5±83.4^{b}	272.5±72.0^{b}	265±73.0^{a}
B	Oxyclozanide + Oxfendazole + Cobalt + Selenium	3977.5±432.3^{bc}	370±50.2^{d}	67.5±18.2^{b}	37.5±16.2^{b}	170±94.3^{a}
C	Oxfendazole	4895±464.2^{ab}	702.5±113.4^{cd}	200±36.4^{b}	277.5±46.5^{b}	220±36^{a}
D	Levamisole + Oxyclozanide + Selenium + Cobalt	5660±630.7^{a}	695±131.9^{cd}	385±84.4^{b}	327.5±93.0^{b}	197.5±136^{a}
E	Deedani	2955±355.9^{c}	2025±305^{b}	1685±309^{a}	1185±289^{a}	410±174^{a}
F	Atreefal Deedan	4205±558.6^{bc}	2966.5±456.7^{a}	1976.5±365.4^{a}	1246±234.5^{a}	355±124.9^{a}
P Value		0.004*	0.000*	0.000*	0.000*	0.6464^{NS}

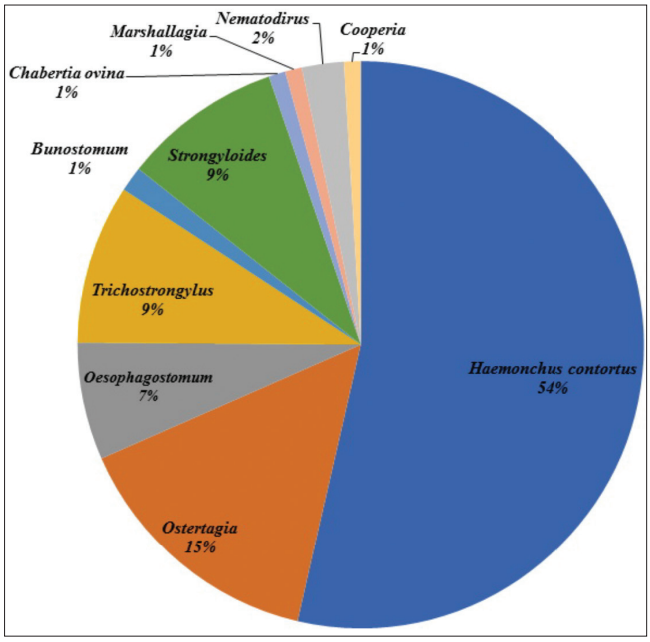

Fig 1. Prevalence (%) of gastrointestinal nematodes identified in six treated groups of goats

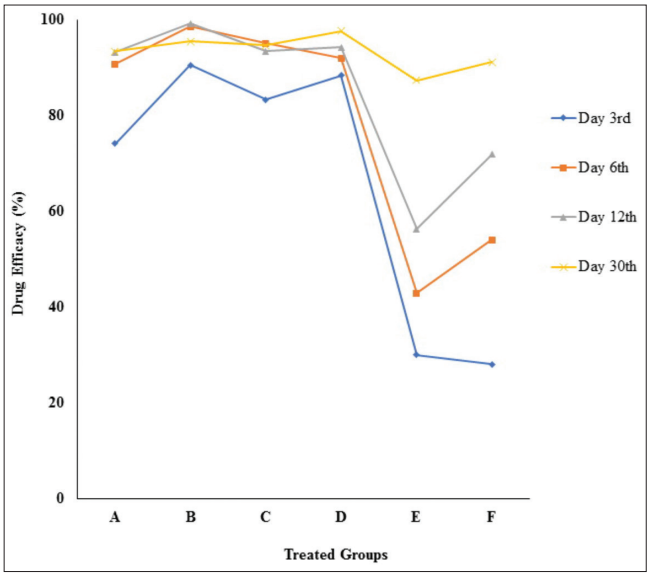

Fig 2. Anthelmintic efficacy (%) of six treated groups of goats

FECR percentage (99.27%) was recorded for group B in goats at 12th day and 6th (98.6%) after treatment. Two herbal products i.e., Atreefal deedan and Deedani also showed high percentage of egg counts reduction (91.2% and 87.4% respectively) and found effective against goat GI nematdes *(Fig. 2)*. The results indicate that all the medicines are effective and recommended as alternative to avoid problem of anthelmintics resistance in goats. However, in untreated control group regular increase in FEC % was observed during experiment.

DISCUSSION

To confirm synthetic and herbal medicines effects against GI nematodes in goats, a fully controlled experiment was performed. We used faecal egg output of goats as indicator of effect of medicines. Maximum value for FECR percentage remained 99.3% and 98.6% for Oxyclozanide + Oxfendazole + Cobalt + Selenium at day 12th and 6th respectively, which is similarly higher percentage recorded by Khan et al.[13]. Similarly, reduction in group of goats treated with Levamisole + Oxyclozanide + Selenium + Cobalt showed 97.7% reduction due to use of levamisole with combinations. Previous study recorded lower efficacy with levamisole up to 63.7% on day 14th of post-treatment [14] and up to 74.3% by Khan et al.[13]. The lower efficacy is thought due to development of resistance in parasites against levamisole [15].

The FEC reduction with albendazole observed in current study was 93.4%, which was lower percentage than 100% against GI helminthes of goats recorded in study by Bersissa et al.[16]. The lower efficay of albandaozle in current study may explained with development of resistance to this drug in goats reported in world (reference needed). The present result showed FECR with Oxfendazole was 94.7%, which is higher than 54-66% reported by Saddiqi et

al.[17]. This drug belongs to benzimidazoles group and is a broad spectrum anthelmintic, which shows higher efficacy against the nematode infection [13].

The treatment with two herbal medicines in current study showed high fecal egg count reduction in GI nematodes of goats. The efficacy of Atreefal Deedan treated group was recorded 91.2% against nematode infections in this study. Comparable results were recorded by Razzaq et al.[18] in sheep against GI helminths, which showed similarly high efficacy. The combinations of herbal products are commonly used to control parasitic infection and have significant contribution to reduce the development of resistance. In Deedani treated group, 87.4% efficacy was recorded at 30th day post treatment which is not compatible with the result of Razzaq et al.[18], who reported lower efficacy in sheep.

The combination therapy of the Oxyclozanide + Oxfendazole + Cobalt + Selenium and Levamisole + Oxyclozanide + Selenium + Cobalt and Atreefal Deedan resulted with lower resistance and effective control of nematode infections in the goats. The imported anthelmintics need efficacy testing before use to avoid resistance and overdosing of drugs to animals. Further studies on chemotherapeutic trials should be replicated for alternative drugs to avoid problem of anthelmintic resistance.

Declaration of Competing Interest

Authors declares that there is no conflict of interests

Author's Contribution

KA and AR designed the study. SR, AR and MH performed the experiment. KA, SF conducted the literature search, data analysis and manuscript preparation, advised on methods and interpretation of findings. KA reviewed the manuscript. All authors participated in the study and concur with the submission and subsequent revisions submitted by the corresponding author.

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Case Report

Treatment of Pathological Fractures in Two Lion Cubs (Panthera leo) with Nutritional Secondary Hyperparathyroidsm

Tuğba KURT [1,a] Yusuf ALTUNDAĞ [1,b] Serhat ÖZSOY [1,C]

[1] Istanbul University Cerrahpasa Faculty of Veterinary Medicine, Department of Surgery, TR- 34320 Avcilar, Istanbul - TURKEY
ORCIDs: [a] 0000-0002-1467-2145; [b] 0000-0001-6364-7512; [c] 0000-0003-0988-7691

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Abstract

Secondary nutritional hyperparathyroidism (NSH) is a metabolic disease characterized by the symptoms such as lameness, constipation, lordosis, and pathological fractures resulting from Ca and P imbalance. A five months old captive-bred two lion cubs were presented with pain in the whole body, reluctance to move, and growth retardation with no history of trauma. Radiographs revealed generalized osteopenia, multiple fractures of all limbs, and lordosis of the caudal vertebrae. Some of these fractures were nonunion. Based on radiographic findings and a history of an exclusive chicken liver diet and goat milk since weaning (after 1 month age), a diagnosis of nutritional secondary hyperparathyroidism was made. The diet was changed to a portion of commercial kitten food, vitamin D supplements, goat milk. Operative interventions were performed to repair the fractured bones and for those which were not suitable for operation, bandage was applied. Cubs were given cage rest for 8 weeks. At the end of these processes, patients were discharged walkable.

Keywords: *Lion cubs, NSH, Pathological fractures, Nutritional seconder hyperparathyroidsm, External fixation*

Nutrisyonel Sekonder Hiperparatiroidizmli İki Yavru Aslanda Patolojik Kırıkların Tedavisi

Öz

Nutrisyonel sekonder hiperparatiroidizm (NSH), Ca ve P dengesizliğine bağlı topallık, konstipasyon, lordoz ve patolojik kemik kırıkları gibi semptomlarla karakterize olan bir hastalıktır. Bu raporun materyalini oluşturan beş aylık iki aslan yavrusu, travma öyküsü olmaksızın tüm vücutta ağrı, hareket etme isteksizliği ve büyüme geriliği ile kliniğimize başvurmuştur. Radyografilerinde genel osteopeni, tüm uzuvlarda çok sayıda kırık ve kaudal omurlarda lordoz görülmüştür. Radyografik bulgulara ve sütten kesildikten (1 aylıktan sonra) bu yana tavuk karaciğeri ve keçi sütünden oluşan bir diyetle beslenme hikayesine dayanarak nutrisyonel sekonder hiperparatiroidizm teşhisi konulmuştur. Diyet; ticari bir yavru kedi maması, D vitamin takviyesi, keçi sütü olarak değiştirilmiştir. Hastaların kırıkları için operatif müdahaleler gerçekleştirilmiş, operasyona uygun olmayan kırıklar için bandaj uygulamaları yapılmıştır. Yavrulara 8 hafta boyunca kafes istirahati uygulanmıştır. Bu işlemlerin sonunda hastalar yürüyebilir şekilde taburcu edilmiştir.

Anahtar sözcükler: *Yavru aslan, NSH, Patolojik kırık, Nutrisyonel Sekonder Hiperparatiroidizm, Eksternal fiksasyon*

INTRODUCTION

Nutritional secondary hyperparathyroidism (NSH) is a metabolic disease associated with malnutrition. The affected animals were generally fed with diets such as meat and organ tissue, containing excess phosphate, insufficient calcium, or both [1,2]. Since balanced commercial foods can be easily reached today, nutrition-related diseases are less common. In this disease, bone production is normal but osteopenia results from excessive bone resorption [2,3]. Normal skeletal growth needs Ca:P of 1:1 to 1:2 but the red meat-based diet contains calcium and phosphorus in the ratio of 1:10 (horse meat) to 1:50 (chicken liver) [4]. Even if milk is added to the diet, the calcium content of the meat and milk diet is insufficient for bone development. The imbalance induces hypocalcemia, which increases the secretion of PTH. Increased parathyroid activity tends to normalize blood calcium and inorganic phosphate concentrations by promoting mineral resorption from bone. Also, hyperparathyroidism improves calcium absorption

Correspondence
+90 506 9258070 Fax: +90 212 4737240
tubikkurt@gmail.com

from the intestine and facilitates renal phosphate excretion and calcium retention. However, a long-term unbalanced diet sustains the hyperparathyroidism state and causes progressive skeletal demineralization and consequent clinical signs [2,3]. Clinical signs in young animals are more severe than in adult ones and include; lameness, reluctance to move, muscular or skeletal pain, generalized osteopenia, a predisposition to fractures, and rarely seizures that have been documented in some literatures [3-5]. Typical radiographical signs of osteopenia include generalized decreased bone opacity, thinned cortices, and trabeculation. Pathological fractures, spinal curvature changes, and pelvic deformation are often a sequel to longstanding osteopenia. A double cortical line is frequently seen in humans with various forms of osteopenia but is a rare finding in small animals [4,5]. In this case report, it was aimed to improve the health of 2 lion cubs with pathological fractures due to NSH by diet regulation, UV-B application and operative interventions together.

CASE HISTORY

This report; Republic of Turkey Ministry of Agriculture and Forestry General Directorate of Nature Protection and National Parks has been prepared with the permits dated 23.07.2020 and numbered 26137614. Five months old captive-bred male and female two lion cubs were presented with pain in the whole body, reluctance to move, and growth retardation with no history of trauma. These symptoms were noticed 45 days before they came to our hospital. In female patient, femoral and tibial fracture was detected in another clinic and operated with intramedullary grooved pin. In male patient, only femoral fracture was detected and operated with intramedullary Steinmann pins.

However, the symptoms worsened and they were finally admitted to our hospital. According to the anamnesis, the cubs were weaned at the age of 1 month and fed only with chicken liver and goat milk without supplementation of calcium and vitamins after weaning. On physical examination, lordosis, limb fractures, and dehydration were detected in both patients. During the musculoskeletal examination using palpation, pain reaction of the patients was observed. Therefore, it was noted that patients felt pain everytime they move and turned to their aching areas. Hematological and biochemical parameters of the patients were examined and evaluated normal at presentation *(Table 1, Table 2)*. Parathormon (PTH) and Vit D levels of the female patient were high at presentation while the levels were normal in male patient *(Table 3)*.

Multiple radiographs of the whole body and extremities were made under sedation using xylazine (Ksilazol 20 mg/mL, Provet, Turkey) at a dose of 2 mg/kg intramuscularly [9]. As a result of radiographic examinations, it was observed that in both patients had non-union problem in the

Table 1. *Hematological values of female and male lion cubs at presentation*

Parameters	Results		Reference Values
	Female	Male	
RBC M/µL	6.61	7.70	3.8-11.7 (M) [6] 3.8-10.0 (F) [6]
HCT %	28.6	36.4	24.8-54.0 [6]
HGB g/dL	9.9	11.6	4.9-23.0 [6]
MCV fL	43.3	47.3	29.9-76.0 (M) [6] 21.4-64.0 (F) [6]
MCH pg	15.0	15.1	11.2-27.2 (M) [6] 7.2-22.0 (F) [6]
MCHC g/dL	34.6	31.9	20.4-42.8 (M) [6] 20.5-49.7 (F) [6]
RDW %	25.6	25.5	
RETIC %	0.2	0.1	0-0.6
WBC K/µL	12.69	15.63	4.7-31.2 [6]
NEU%	50.9	70.5	45-64
LYM%	41.8	24.1	27-36
MONO%	6.8	4.4	0-5
EOS%	0.1	0.1	0-4
BASO%	0.4	0.9	0-1
NEU K/µL	6.46	11.02	2.30-10.29 [6]
LYM	5.31	3.77	0.92-6.88 [6]
MONO K/µL	0.86	0.69	0.05-0.67 [6]
EOS K/µL	0.01	0.01	0-0.8
BASO K/µL	0.05	0.14	0-0.2
PLT K/µL	213	266	300-800
MPV fL	14.0	14.0	12-18

Table 2. *Serum biochemical panel of female and male lion cubs at presentation*

Parameters	Results		Reference Values
	Female	Male	
GLU mg/dL	188	168	60-120 [7]
CREA mg/dL	0.3	0.4	53-327 [6]
BUN mg/dL	17	18	4.28-29.3 [6]
BUN/CREA	53	48	
PHOS mg/dL	5.1	5.3	3.0-6.1 [7]
CA mg/dL	8.6	9.7	8.7-11.7 [7]
TP g/dL	7.3	7.0	5.3-9.7 [6]
ALB g/dL	3.4	3.2	1.9-5.6 [6]
GLOB g/dL	3.9	3.8	1.9-5.7 [6]
ALB/GLOB	0.9	0.9	
ALT U/L	66	54	25-97 [7]
AST U/L	87	35	7-38 [7]
ALKP U/L	125	250	16-355 [6]
TBIL mg/dL	0.6	0.5	0-0.1 [7]
CHOL mg/dL	162	119	90.1-255.2 (M) [6] 91.2-293.9 (F) [6]

Table 3. *Presentation and after treatment results of blood serum values in lion cubs*

Parameters	Presentation Results		30 Days After Treatment Results		Reference Values
	Female	Male	Female	Male	
PTH	0.4 pm/mL	0.0 pm/mL	0.0 pm/mL	0.1 pm/mL	0-1.089 [8]
Vit D	111.0 ng/mL	70.0 ng/mL	12 ng/mL	12.4 ng/mL	26-68 [8]
Ca	9.4 mg/dL	8.5mg/dL	7.6 mg/dL	10.6 mg/dL	8.7-11.7 [7]
P	6.5 mg/dL	6.5mg/dL	4.9 mg/dL	5.6 mg/dL	3.0-6.1 [7]

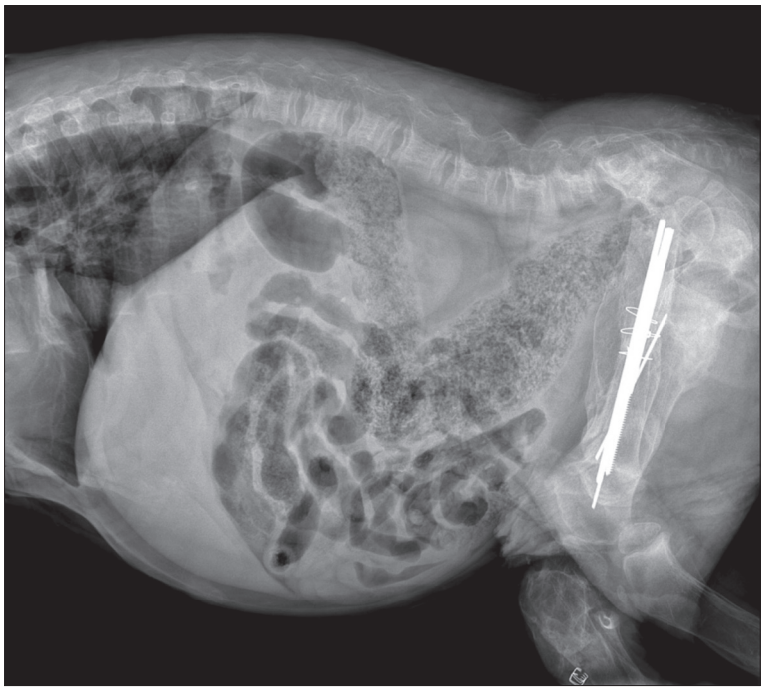
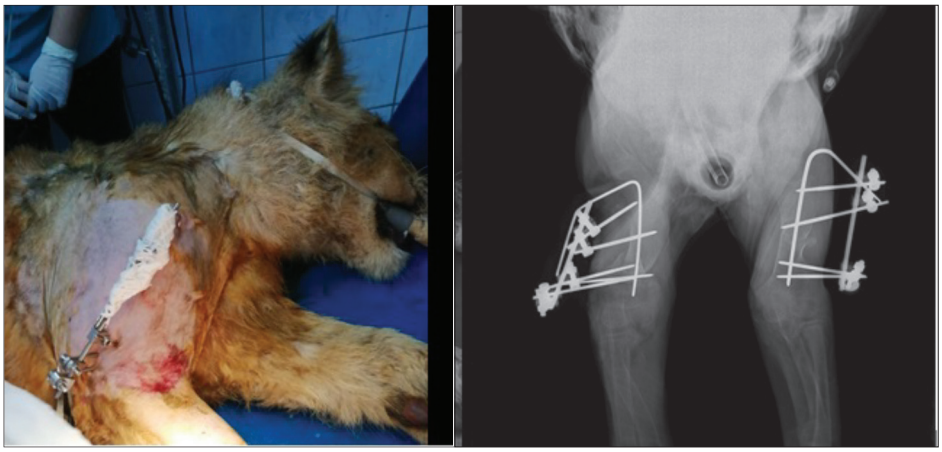


Fig 1. Generalized osteopenia and lumbar lordosis in male cub

Fig 2. After operation of humeral fracture with Kirschner- Ehmer apparatus in male lion cub



bones due to lack of stabilization, generalized osteopenia *(Fig.1)*. Bilateral humeral, femoral, and antebrachium fractures and lordosis in lumbal spines were detected in the male patient. Besides these, bilateral antebrachium fractures were detected in the female patient. On the basis of nutritional plan, clinical findings and radiographic examination, a diagnosis of NSH was deduced. First, the diet of patients was changed. Quality cat food, goat milk, vitamin D at the dose of 0.05 µg/kg PO [4], chicken neck, and egg were added to the diet. The area of lion cubs was restricted because the movement of patients was painful. Meloxicam 0.1 mg/kg (Meloxicam, BaVet, Turkey) were used for 5 days. Cage conditions were fixed and a UV-B lamp (red light 250w) was placed and used for 6 h per day. Vitamin D, helps calcium and phosphorus absorption from the intestines. It is obtained either by intestinal absorption after food intake or by the synthesis of Vit D3 by exposure to UV-B in the skin [10]. A thick layer of straw was used as

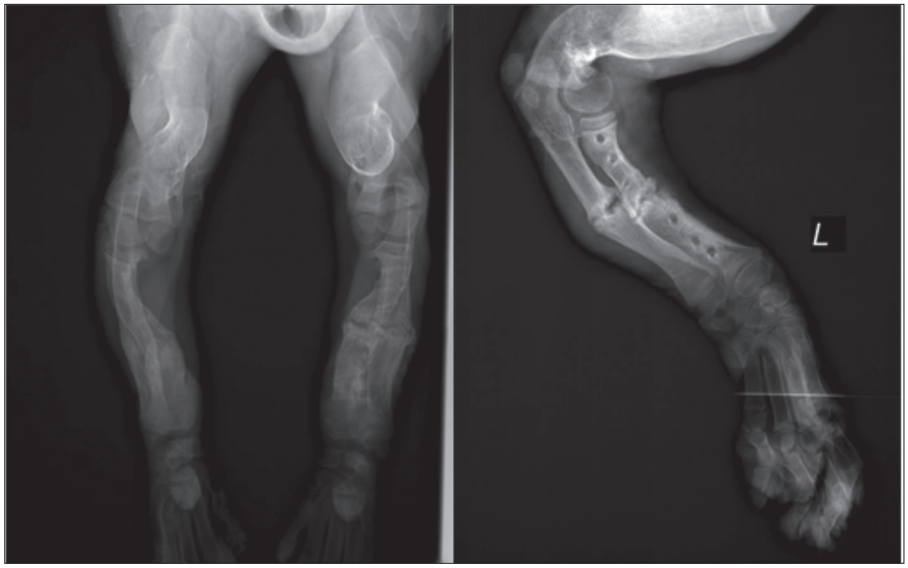


Fig 3. Bilateral forelimb radiograph of female lion cub. Patological fracture of both antebrachium was detected. Callus was identified. Medio-lateral radiograph of left antbrachium following removal of Kirschner-Ehmer fixator (female)

a base, because patients could not walk. As the fractures were old, only to the non-union ones were operated. Patients were sedated with xylazine then, anesthetized with propofol (Propofol-Lipuro 1%, B. Braun, Germany) at a dose of 2 mg/kg intravenously. The patient was intubated and followed by inhalation anesthesia (isofluorone 3%) [11]. The areas to be operated were shaved widely and disinfected with alcohol (70%), then with povidone-iodine (10% ADEKA, Turkey). Bilateral humeral closed technique of osteosynthesis with Kirschner- Ehmer apparatus was performed and the left tibia was bandaged for male patient. The same technique was performed for female patient's right antebrachium. The incision was not made in this technique *(Fig. 2, Fig. 3)*. After the operation, povidone-iodine was applied to the pin bottoms. Ceftriaxone sodium 25-50 mg/kg (Novosef, ZENTIVA, Czech Republic) and meloxicam 0.1 mg/kg were used postoperative for 5 days. Bandages were renewed every 7 days. External fixations materials and bandages were removed together after 45 days. In this process, they were given cage rest for 8 weeks. Last, of all patients were discharged walkable.

DISCUSSION

Despite the widespread use of commercially formulated pet diets, owners sometimes still prefer non-commercial diets, often without taking into account the specific dietary requirements of their pet, thus increasing the risks for health problems such as nutritional bone diseases. The authors believe that NSH is a common disease in captive species of lions associated with nutritional problems [4]. The cubs described in this report were weaned at 1 month and chicken livers and goat milk were added to their diet. The symptoms started at 3.5 months, whereas the cubs were completely dependent on the mother until 3 months of age, and should be weaned at 6-8 months [12]. Normal skeletal growth needs Ca:P of 1:1 to 1:2 while this diet contains calcium and phosphorus in the ratio of 1:50. Kittens in pioneering experiments fed only with red meat began to show locomotory disturbances after 41 days [5]. In this case symptoms developed within 2 months of weaning. NSH results from chronic hypocalcemia due to a continuous diet with low calcium. This causes excessive absorption of calcium from the bones, which makes the bones more fragile and disrupts the bone structure [13,14]. Persistently decreased calcium intake lead to chronic parathyroid stimulation, with increased parathyroid hormone secretion in these patients [4]. As a result of these compensatory mechanisms, blood Ca [2,15] and phosphorus levels are reported to be normal or decreased, blood PTH level and vitamin D level increased in patients with NSH [16]. In this case report, blood samples were collected from the patients on the first day, and on the 30th day after treatment and, Ca, PTH, vitamin D and P were measured *(Table 3)*. There is no specific blood result suggestive of NSH. Since the case was delayed, Ca levels were normal in the blood test before treatment. But with clinical examination results, radiographic findings and anamnesis, potentiated the diagnosis towards NSH. In the blood test 30 days after the treatment, Ca and P values were within normal limits in both patients. PTH and Ca levels were increased and P and Vit D levels were decreased. In the female lion cub, PTH, Ca, P and vit D levels decreased compared to the initial state. Normally enough Vit D synthesis occurs when exposed to sunlight for a sufficient period of time [17,18]. Hypovitaminosis D is formed as a result of deficiencies in diet and low exposure to sunlight of animals. In case of vitamin D deficiencies, endogenous vitamin D production is triggered by UV lamps. There is no information about the use of UV-B lamp in the studies conducted on lions. In our study, the bony development of animals was indirectly supported by the use of UV-B lamp for offspring.

Radiographically, reduced bone opacity, pathological fractures and thin cortices are sufficient to define osteopenia [4]. Our patients have multiple pathological fractures

without trauma history. Intramedullary pin application [19] external fixation [20] and plate osteosynthesis [21,22] are used for fracture treatment in wild animals [23]. In this case, external fixation with K-wires was preferred because of shortening anesthesia time, minimal tissue trauma, and easy postoperative care. Only pin-bottom infection was observed as postoperative complications and this situation was easily controlled. There was no study on the operative treatment of pathological fractures with NSH in cubs [4,5]. Pain control was achieved with intermittent use of meloxicam. With cage rest, the patient was followed for a long time. Despite spinal deformites, urination and defecation problems were not observed in patients, and at the end of the treatment, pain stress decreased and they became able to walk. Having a positive result even in such a chronic case may be a ray of hope for other patients in similar situations who are recommended euthanasia.

As a result, NSH is a preventable condition with a balanced diet and early diagnosis.

Conflict of Interest

The authors report no conflicts of interest.

Author Contributions

The authors alone are responsible for the content and writing of this case report.

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Case Report

Intervention of Atrial Septal Defect Embolization Using Three-Dimensional Transesophageal Echocardiography-Guided Thoracotomy in a Yorkshire Terrier

Akiko UEMURA [1,a] Tomohiko YOSHIDA [2,b] Katsuhiro MATSUURA [2,c] Ryou TANAKA [2,d]

[1] Department of Veterinary Surgery, Division of Veterinary Research, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inadacho Nishi, Obihiro-Shi, Hokkaido, 080-8555, JAPAN
[2] Department of Veterinary Surgery, Faculty of Veterinary Medicine, Tokyo University of Agriculture and Technology, 3-5-8 Saiwaicho, Fuchu, Tokyo 183-8509, JAPAN
ORCIDs: [a] 0000-0003-2671-5074; [b] 0000-0003-4311-9311; [c] 0000-0002-6047-2865; [d] 0000-0001-9948-6490

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Abstract

Atrial septal defect (ASD) is a relatively rare congenital heart disease in dogs. A radical treatment is defect closure under open heart or occlusion by intervention. Small dogs face challenges with either treatment. This case was an 8-month-old female Yorkshire Terrier, weighing 2.4 kg. She suffered from dyspnea due to deterioration of circulatory dynamics caused by ASD. An Amplatzer was positioned under thoracotomy, guided by three-dimensional transesophageal echocardiography. Postoperative recovery was quick and uneventful. Clinical symptoms disappeared and circulatory dynamics were stable after growth. This method is considered to be a useful treatment option for small dogs with ASD.

Keywords: *Atrial septal defect, Congenital heart defect, Small dog, Interventional cardiac procedures, 3D transesophageal echocardiography*

Bir Yorkshire Terrier'de Üç Boyutlu Transözofageal Ekokardiyografi Rehberli Torakotomi İle Atriyal Septal Defekt Embolizasyonuna Müdahale

Öz

Atriyal septal defekt (ASD) köpeklerde nispeten seyrek görülen bir doğmasal kalp hastalığıdır. Radikal tedavi, açık kalp altında defektin kapatılması veya girişimsel bir müdahale ile tıkanmasıdır. Küçük köpeklerde her iki tedavide de zorluklarla karşılaşılır. Bu olgu, 2.4 kg ağırlığında, 8 aylık dişi Yorkshire Terrier idi. Olgu, ASD'nin neden olduğu dolaşım dinamiklerinin bozulması nedeniyle nefes darlığı yaşamaktaydı. Torakotomi altına, üç boyutlu transözofageal ekokardiyografi rehberliğinde bir Amplatzer yerleştirildi. Postoperatif iyileşme hızlı ve sorunsuz oldu. Büyümeden sonra klinik semptomlar kayboldu ve dolaşım dinamikleri stabil hale geldi. Bu yöntem, ASD'li küçük köpekler için faydalı bir tedavi seçeneği olarak dikkate alınabilir.

Anahtar sözcükler: *Atrial septal defekt, Kongenital kalp defekti, Küçük köpek, Girişimsel kardiyak prosedür, 3D transözofagal ekokardiyografi*

INTRODUCTION

Atrial septal defect (ASD) is a malformation that occurs in about 1% of congenital heart diseases in dogs [1,2]. The prognosis for a small, isolated ASD is usually good, but dogs with defects larger than 12 mm can have difficulty breathing, exercise intolerance, and poor growth [3]; these dogs therefore require treatment. The treatment for ASD includes medical management with drugs or surgical closure of the defect [4]. In ASD that requires surgery, physical closure of the defect is necessary. Surgical approaches include direct visual closure of the obturator via open-heart surgery and the use of a cardiopulmonary bypass [4], and defect obstruction using a device, via percutaneous endovascular treatment [3]. In cases where percutaneous endovascular treatment is not feasible, for

Correspondence
+81-155-495376 (A. U.) +81-42-3675904 (R. T.) Fax: +81-155-495376 (A. U.) +81/42/3675904 (R. T.)
anco@vet.ne.jp (A. U.) ryo@vet.ne.jp (R. T.)

example if the diameter of the blood vessels is too small, a hybrid approach can be adopted, in which a device is inserted directly into the heart without opening, via a thoracotomy. The use of such a hybrid approach to treating heart defects was reported by Saunders and colleagues for a ventricular septal defect (VSD) in a small dog [5], and we used this approach for a small dog with ASD (a Norfolk Terrier weighing 6.9 kg) [6].

As is clear from the shape of heart-defect embolization devices currently on the market for use in humans, ASD occlusion plugs generally have a larger umbrella diameter compared with the umbrella diameter of VSD occlusion plugs. This is thought to be because the atrial wall is thinner than the ventricular fistula, and an obturator plug inserted into a defect cannot be fixed there unless it is firmly held in place with the large area associated with a large umbrella. However, using a large umbrella can partly inhibit intracardiac blood flow and valve movement. The authors have previously experienced the need to abandon the device, especially in the small hearts seen in small dogs because, even though the ASD may be occluded, the circulatory dynamics deteriorate when using a large device. It should therefore be taken into account that the same approach cannot be taken with VSD and ASD, even in small dogs of the same size.

CASE HISTORY

The case was a female Yorkshire Terrier, aged 8 months, and weighing 2.4 kg. She was admitted to the hospital for further examination and treatment because her home veterinarian suspected ASD. She had a breathing boost and persistent shortness of breath during exercise.

On day 1, physical examination revealed a heart murmur of Levine 1/6, blood pressure of 120/61 (85) (systolic/diastolic (mean)), and a heart rate of 95 bpm. A chest X-ray showed right ventricular enlargement. By transthoracic echocardiography (TTE), the ASD diameter was determined to be 5.7 mm, but the Qp/Qs was 2.97. Based on the location of the ASD, a diagnosis of secundum atrial septal defect was made. The clinical manifestations were suspected to be due to the deterioration of circulatory dynamics, therefore it was judged that physical closure of the defect was necessary. This case involved a small dog of low weight, and therefore at high risk of unsuccessful open-heart surgery, but percutaneous endovascular treatment was physically difficult because of the peripheral vessel diameter. Thus, the hybrid approach was planned, with the aim of inserting an occluder directly into the heart via thoracotomy, after gaining the owner's informed consent.

On day 25, anesthesia was induced with atropine (0.05 mg/kg, subcutaneous injection, sc), ampicillin (Ampicillin sodium injection 1 g, Kyoritsuseiyaku Corporation, Tokyo, Japan) (30 mg/kg, intravenous injection, iv), butorphanol (Vetorphale®, Meiji Seika Pharma Co Ltd., Tokyo, Japan) (0.3 mg/kg, iv), midazolam (Dormicum Injection 10 mg, Astellas Pharma Inc., Tokyo, Japan) (0.2 mg/kg, iv), meloxicam (Boehringer Ingelheim Animal Health Japan Co., Ltd., Tokyo, Japan) (0.2 mg/kg, sc), and propofol (Mylan Injection 1%, Mylan Inc., Tokyo, Japan) (to effect, iv). Anesthesia was maintained with isoflurane (Isoflurane for animals, Intervet K.K., Tokyo, Japan) (1.7%-2.2%).

After induction of anesthesia, ECG-synchronized CT was performed prior to surgery to confirm the position and size of the defect. The left atrial appendage was sutured to the left atrial appendage by the left fourth intercostal thoracotomy. After applying a tourniquet, the left atrial appendage was incised several millimeters. After insertion of a 9Fr sheath (Medikit Sheath Introducer, Medikit Co. Ltd., Tokyo, Japan) via the incision, the sheath tip was positioned near the defect under the guidance of three-dimensional (3D) transesophageal echocardiography (TEE). An occluder (waist diameter 9 mm, disk portion 14 mm) (ACDO, Infiniti Medical LLC, Malibu, CA, USA) was inserted via the sheath into the defect. After confirming that stable placement was possible, under 3D TEE guidance, the occluder was released *(Fig. 1)*. Once it was established that there was

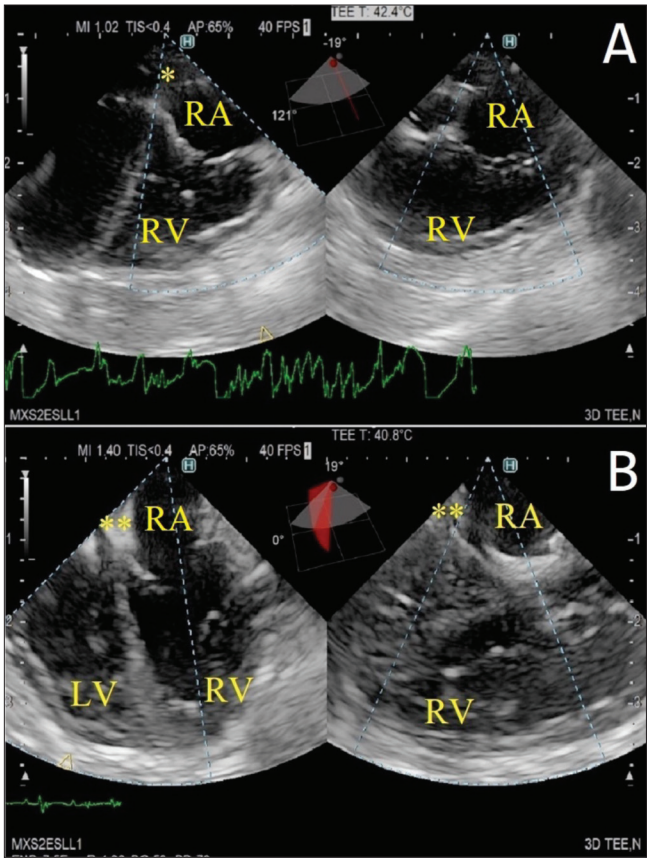


Fig 1. Imaging of three-dimensional (3D) transesophageal echocardiography (TEE) on day 25 By using 3D-TEE, a device inserted into the heart can be confirmed from two directions at once. Because the device status can be confirmed from two directions, it can be placed once the device is stable. RA, right atrium; RV, right ventricle; LV, left ventricle; *Amplatzer

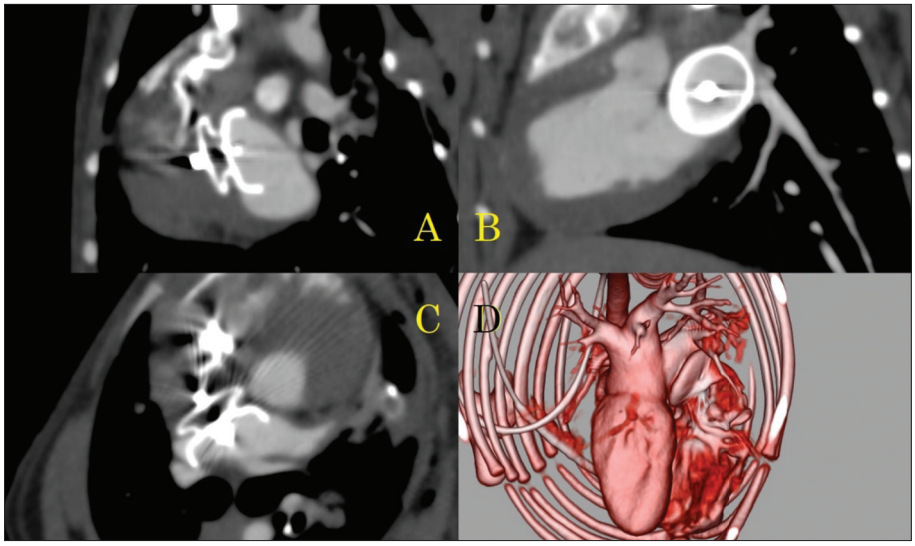
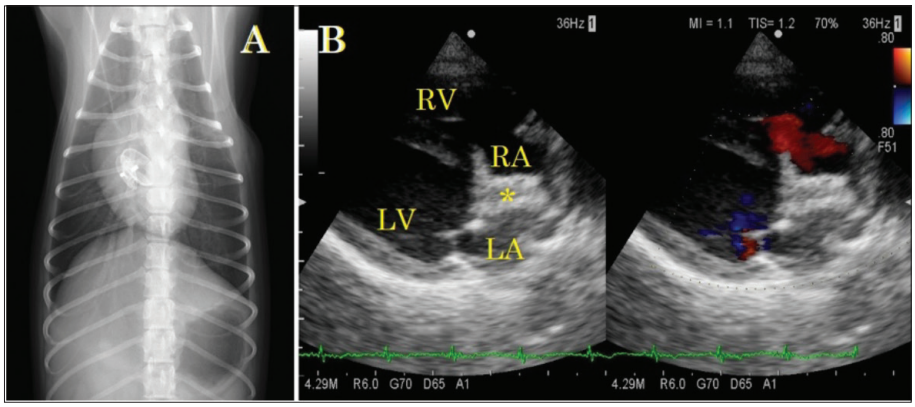


Fig 2. ECG-synchronized CT on day 25
Immediately after the operation of Amplatzer placed in the ASD. **(A)** Coronal plane; **(B)** sagittal plane; **(C)** axial plane; **(D)** 3D image

Fig 3. Imaging on day 97 (72 days post-operative)
There was no change in the position of the Amplatzer. The ASD was embolized by the Amplatzer and stable circulatory dynamics were achieved. **(A)** Chest X-ray; **(B)** transthoracic echocardio-graphic right parasternal long-axis four-chamber section; RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; *Amplatzer



no change in circulatory dynamics, the chest was closed according to the standard method.

After the operation, ECG-synchronized CT was again performed to confirm the position of the occluder *(Fig. 2)*. The dog's arousal from anesthesia was good. On the day following the surgery, TTE showed a Qp/Qs of 1.01, indicating a marked improvement in circulatory dynamics.

She was discharged on day 27 (the day following her surgery). No arrhythmia was observed from the post-operative period until discharge.

On day 97 (72 days postoperative), periodic postoperative examinations and OHE were performed. There was no medical treatment required, there were no problems with the circulatory dynamics, including during anesthesia or from the positioning of the obstructive plug, and no clinical symptoms were observed *(Fig. 3)*.

DISCUSSION

The medical treatment for ASD in dogs is a coping therapy. There are no clear guidelines for the surgical reduction of defects. Cases with a defect pore size of 12 mm or more have dyspnea [3], and surgical reduction is performed if there are signs of congestive heart failure [7,8].

The defect size in this case was 5.7 mm, as measured by TTE, but clinical symptoms thought to be caused by a deterioration in circulatory dynamics and congestive heart failure were observed. Medical management in this case may have improved the dog's clinical symptoms. However, even a small defect in the small heart found in a small dog can lead to a deterioration in circulatory dynamics, so it was determined that physical closure should be performed, based on the circulatory dynamics rather than the diameter of the defect. Previous study has described the usefulness of doppler echocardiography in confirming the pathogenesis of congenital heart disease [9]. In the present case, doppler echocardiography with TTE and TEE was utilized in this case as well.

By inserting an occluder directly into the heart during a thoracotomy, the invasiveness of thoracotomy is present, but the perioperative risk is reduced compared with the risk associated with open-heart surgery. Also, it is physically very difficult to insert a 9Fr sheath into the peripheral blood vessels of a small dog, but this limitation due to the diameter of the blood vessels can be eliminated by inserting the device directly into the heart.

This method can be performed under a surgical fluoroscope as well as via a conventional intervention. However, 3D TEE can provide improved stereoscopic images of intracardiac structures. By using 3D TEE as a guide instead of a fluoroscopic device, delicate movements can easily be reflected directly on the image, making release determination after placement much easier. Furthermore, it is beneficial to eliminate the risk of exposure for both healthcare professionals and patients.

In ASD, the defect size may be larger than the measurement of diameter made preoperatively, due to the thinness of the wall and its extensibility. Unlike with percutaneous intervention, it is much easier to insert and remove devices using this method, so it is also easy to replace devices of multiple sizes based on their stability and hemodynamics during device insertion.

The weight of a dog's heart is reported to be between 0.61% and 0.94% of its total body weight [10,11]. Based on this, the heart weight is 6.9 kg (42.1 g to 64.9 g heart) [6] and 2.4 kg (14.6 g to 22.6 g heart) in this case. There will be a difference of about 1.9- to 4.4-times. Heart weight and intraventricular volume are not simply proportional. However, larger dogs are less prone to circulatory dynamic deterioration due to intracardiac structure inhibition by an intracardiac defect occluder, while the weight difference can be disadvantageous for defect obstruction in smaller dogs.

In this study, we treated ASD in a Yorkshire Terrier, which weighed even less than the dog described in our previous report [6], and obtained good results. It was confirmed that there were no abnormalities in circulatory dynamics, even under anesthesia for a hysterectomy after growth.

We performed Amplatzer embolization using a 3D TEE-guided/thoracotomy approach for a case of ASD in a Yorkshire Terrier. The postoperative awakening was rapid, with no complications, and markedly improved postoperative circulatory dynamics and a disappearance of clinical symptoms. Based on the above, embolization using a 3D TEE-guided/thoracotomy approach for ASD is considered to be a useful radical treatment option, especially for ASD in small dogs.

Author Contributions

AU was a surgeon in this case, wrote the manuscript, and prepared all the figures. TY and KM were responsible for the patient's clinical examination. RT was a surgeon in this case and also the primary investigator.

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Case Report

Rhabdomyolysis Triggered by Septic Shock in a Dog: A Case Report

Kursad TURGUT [1,a] Amir NASERI [2,b] Havva SULEYMANOGLU [1,c] Merve ERTAN [1,d] Mehmet Ege INCE [1,e]

[1] Near East University, Faculty of Veterinary Medicine, Department of Internal Medicine, 99280, Nicosia, NORTH CYPRUS
[2] Selcuk University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-42003 Konya - TURKEY
ORCIDs: [a] 0000-0001-8725-8044; [b] 0000-0001-9140-5879; [c] 0000-0002-6681-0212; [d] 0000-0003-3436-481; [e] 0000-0002-1859-1449

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Abstract

The purpose of this report is to describe an unusual case of severe rhabdomyolysis associated with septic shock. A 13-year-old Pekingese male dog was admitted to our department with a history of respiratory distress, fatigue, severe myalgias, vomiting, and generalized severe asthenia. The creatine kinase (CK) level was very high (13.690 UI/L) on the 1st day of hospitalization and increased to 31.587 UI/L on the 2nd day. CK values during the 4th and 9th days were 15.796 UI/L and 1.064 UI/L, respectively. Despite aggressive shock treatment and adequate treatment of secondary infections and the complication of rhabdomyolysis (azotemia and liver failure), the patient developed progressive myalgia, progressive respiratory failure, and low compliance, resulting in death on the 9th day of hospitalization.

Keywords: *Rhabdomyolysis, Creatine kinase, Septic shock, Dog*

Bir Köpekte Septik Şoka Bağlı Gelişen Rabdomiyoliz Olgusu: Olgu Sunumu

Öz

Bu vaka raporunun amacı septik şokla birlikte ender görülen şiddetli rabdomiyoliz olgusunu bildirmektir. Onüç yaşlı, erkek, Pekinez ırkı köpek hastanemize solunum güçlüğü, halsizlik, şiddetli kas ağrısı, kusma ve güçsüzlük şikayetleri ile başvurdu. Serum biyokimyası analizi sonucu 1. gün CK seviyesi 13.690 UI/L olurken, 2. gün 31.587 UI/L değerine yükseldi. Dördüncü ve 9. günlerdeki CK değerleri sırasıyla 15.796 UI/L ve 1.064 UI/L olarak ölçüldü. Agresif şok tedavisi ve rabdomiyoliz sonucu gelişen komplikasyonlara yönelik yapılan tüm tedaviye rağmen hastada ileri miyalji ve şiddetli solunum yetmezliğine bağlı 9. günde ölüm gerçekleşti.

Anahtar sözcükler: *Rabdomiyoliz, Kreatin kinaz, Septik şok, Köpek*

INTRODUCTION

Rhabdomyolysis is an acute necrosis of striated muscle [1]. It ranges in severity from an asymptomatic elevation of creatine kinase (CK) level in the blood, to severe life-threatening cases associated with very high CK levels [2]. Muscle cell contents such as myoglobin are released into the circulatory system, causing acute tubular necrosis and resulting in acute renal failure. Presence of myalgias, significant muscle weakness, red-to-brown urine (myoglobinuria), and elevated CK levels are considered as clues to rhabdomyolysis [3]. There are many traumatic and non-traumatic causes of rhabdomyolysis in humans. In the first category, causes include: crush injuries, long-lasting muscle compressions such as that caused by prolonged immobilization, electrical shock injury, and venom from a snake or insect bite. Nontraumatic causes of rhabdomyolysis include extreme muscle strain (exertional rhabdomyolysis), the use of medications such as antipsychotics or statins, especially when given in high doses, elevated body temperature (hyperthermia) or heat stroke, seizures or delirium tremens, metabolic disorders such as diabetic ketoacidosis, viral infections such as the flu, HIV, or herpes simplex virus, bacterial infections leading to the presence of toxins in tissues or bloodstream (sepsis) [4].

In veterinary medicine, exertional rhabdomyolysis has been reported as a common cause of rhabdomyolysis in dogs and racehorses [5,6]. Holahan et al.[7] reported a case in a dog with presumptive hepatotoxicity and rhabdomyolysis secondary to phenazopyridine toxicity. Lechowski et al.[8] reported acute idiopathic rhabdomyolysis in a dog in Poland.

Correspondence
+90 533 8594430
kursad.turgut@neu.edu.tr

Septic shock-induced changes in the lung and kidneys have been studied extensively both clinically and modeled experimentally, but little is known of alterations in other organ systems. Skeletal muscle is a well-perfused and voluminous tissue and when in shock it may be assumed that its endothelium reacts similarly to the endothelium of the lung and kidneys. We present the clinical findings of severe rhabdomyolysis triggered by septic shock in dogs.

CASE HISTORY

A 13-year-old Pekingese male dog was admitted to our department with a history of respiratory distress, fatigue, severe myalgias, vomiting, and generalized severe asthenia. There was no history of trauma, seizures, surgery, snake or insect bite, contact with chemical agents. Additionally, the patient was not regularly receiving prescribed drugs.

Clinical Examination

On the initial examination, the dog was unconcise, and in a stupor state, with dehydration (8-10 percent). The patient was unable to stand, lying on his sternum, with pain in the abdominal cavity and muscles *(Fig. 1)*.

There were cyanotic mucosal membranes and prolonged capillary refill time ([CRT]: 4sec). The dog had reduced bilateral palpebral reflexes and decreased myotatic reflexes in all four limbs. The panniculus reflex was considered normal, and all other cranial nerve reflexes were intact. Pulse was very weak.

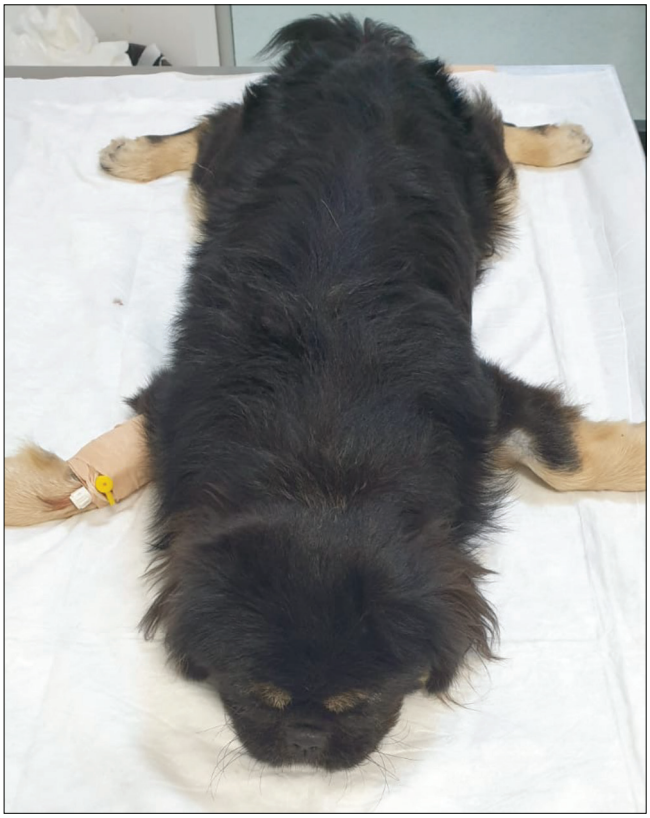

Fig 1. The patient was unable to stand, lying on his sternum, with pain in the abdominal cavity and muscles

After clinical examination, blood samples were collected from the cephalic vein. The diagnosis of severe sepsis was based on meeting the criteria of at least two variables, compatible with SIRS and the dysfunction of no less than one organ or have evidence of tissue hypoperfusion [9]. The considered SIRS variables were hypothermia (36°C) *(Table 1)*, tachypnoea (35/min [reference range: <25/min]), tachycardia (180 bpm [reference range: 60-160 bpm]) and leukocytosis (30.20×10^9/L) with granulocytosis (26.71×10^9/L) and a thrombocytopenia (85.00×109/L) *(Table 2)*. Haematocrit value was decreased (35.40%) *(Table 2)*. C-reactive protein (C-RP) value was elevated (47.13 mg/L) *(Table 2)* (Mindray BS120, Shenzen, China). Venous acid-base analysis (Epocal Inc., Ottawa, ON, Canada) showed that the dog had hypobasemia (pH: 7.37; bicarbonate [HCO_3]: 18 mmol/L; partial pressure of carbon dioxide [pCO_2]: 31.80 mmHg; base excess [BE]: -7 mmol/L) *(Table 3)*.

The variables associated with organ dysfunction included arterial hypotension (systolic blood pressure [SBP]: 83 mmHg, mean arterial pressure [MAP]: 63 mmHg), decreased oxygen saturation [SpO_2]: 74%) *(Table 1)* (Mindray BS120, Shenzen, China), decreased ionized calcium (0.69 mmol/L), decreased glucose (55 mg/dL) (Epocal Inc., Ottawa, ON, Canada), increased alanine aminotransferase (ALT) (141 UI/L), increased ALP (201 UI/L), decreased albumin (2.47 g/dL) (Mindray BS120, Shenzen, China), increased blood urea nitrogen (BUN) (53.85 mg/dL), increased creatinine (1.60 mg/dL) (Epocal Inc., Ottawa, ON, Canada), and increased phosphor (8.13 mg/dL), slightly increased LDH (297 UI/L) and extremely high creatine kinase (CK) level (13690 UI/L) *(Table 3)* (Mindray BS120, Shenzen, China). Plasma lactate

Table 1. *Body temperature, blood pressure and tissue oxygenation parameters in dog on day 1, 2, 4, 9*

Parameters	Reference Range	Day1	Day2	Day4	Day9
Body temp. (°C)	37.5-39.3	36	38.5	38.2	35.5
SBP (mmHg)	>90	83	110	115	78
MAP (mmHg)	>65	63	85	85	60
SpO_2 (%)	>92	74	91	94	71

Table 2. *Hematological parameters and C-reactive protein (C-RP) values in dog on day 1, 2, 4, 9*

Parameters	Reference Range	Day1	Day2	Day4	Day9
WBC ($\times10^9$/L)	6-17	30.20	21.00	30.20	16.20
Granulocyte ($\times10^9$/L)	4-12	26.71	18.11	26.71	18.11
Haematocrit (%)	39-56	35.40	30.50	35.40	19
Thrombocyte ($\times10^9$/L)	180-460	85	27	41	25
C-RP (mg/L)	0-10	47.13	45	37.03	30.33

Table 3. *Acid-base balance and biochemical analysis parameters in dog on day 1, 2, 4, 9*

Parameters	Reference Range	Day1	Day2	Day4	Day9
pH	7.35-7.45	7.37	7.40	7.45	7.45
PCO_2 (mmHg)	35-38	31.80	35.30	39.20	49.00
HCO_3 (mmol/L)	20-30	18	22	27.50	18.80
BE (mmol/L)	-5-0	-7	–2.7	3.50	-4.7
Lactate (mmol/L)	<2.3	1.59	1.70	1.63	7.37
Ionized calcium (mmol/L)	1.12–1.40	0.69	1.34	0.89	0.37
Glucose (mg/dL)	60-110	55	121	123	91
BUN (mg/dL)	10-28	53.85	7.45	21.49	15.14
Creatinine (mg/dL)	0.5-1.5	1.60	0.90	0.87	1.27
Phosphor (mg/dL)	2.60-6.20	8.13	3.25	4.24	3.24
ALT (UI/L)	21-102	141	142	140	340
ALP (UI/L)	20-156	201	195	178	274
Total bilirubin (mg/dL)		0.64	0.41	0.59	4.74
LDH (UI/L)	45-233	297	219	189	299
CK (UI/L)	48-261	13.690	31.587	15.796	1.064
Albumin (g/dL)	2.60-3.30	2.47	2.42	2.46	1.89

(1.59 mmol/L) *(Table 3)*, sodium (143 mmol/L [reference range: 139-150 mmol/L]) chlorine (116 mmol/L [reference range: 106-127 mmol/L]) and potassium (3.20 mmol/L [reference range: 3.40-4.90 mmol/L]) concentrations were in normal reference ranges (Epocal Inc., Ottawa, ON, Canada). Thyroid function tests were normal. Tests for toxoplasmosis and neosporosis were negative (Mindray BS120, Shenzen, China). Urine was normal in color with no proteinuria and myoglobinuria. Blood culture was performed, and *S. pseudintermedius* was the Gram-positive isolate.

Volume resuscitation was initiated using normal saline infusion (90 mL/kg, bolus) by peripheral venous access, to improve the clinical parameters and blood pressure (BP), followed by administration of diuretics (Furosemide (Lasix®, Sanofi Ltd.,Turkey) 4 mg/kg. IV, every 6 h). Maintenance fluid therapy was administrated with NaCl 0.9% (Polifarma, Turkey) at a dose of 40 mL/kg/day to maintain urine output.

After one bolus volume resuscitation, BP did not increase. Vasopressor (Norepinephrine) (1-2 mg norepinephrine (Cardenor®, Vem, Turkey) in 250 mL 0.9% NaCl (Polifarma, Turkey), IV) was applied. Systolic blood pressure (SBP) and mean arterial pressure (MAP) increased to 110 mmHg and 85 mmHg, respectively *(Table 1)*.

The dog received oxygen therapy, with a nasal oxygen mask (100 mL/kg/min) according to SpO_2. Concurrent administration of triple antimicrobial therapy with ceftriaxone (Eqiceft®, Tüm Ekip, Turkey) 30 mg/kg, IV, every 12 h, metronidazole (Polgyl®, Polifarma, Turkey) 10 mg/kg, IV, every 12 h, enrofloxacin (Dufafloxacin®, Holland) 5 mg/kg, IM, every 12 h and, theophylline (Biofleks Teosel®, Osel, Turkey) 10 mg/kg, IM, every 12 h, dexamethasone (Dexaveto-0.2®, Belgium) 0.2 mg/kg, IM, every 12 h and enoxaparin sodium (Oksapar®, Koçak Farma, Turkey) 1.5 mg/kg, IM, every 12 h were administrated. The diagnosis of septic shock was based on the presence of severe sepsis and hypotension that could not be reversed with fluid resuscitation [9]. Decreased ionized calcium and glucose concentrations were corrected by IV administration of calcium (Calcium Picken 10%®, Adeka, Turkey) 0.1 mL/kg, IV and dextrose (30% Dextrose® (Polifarma, Turkey) 0.5-1 mL/kg, IV. The chest radiograph and abdominal X-ray and ultrasound examinations were unremarkable.

The ECG was normal. Transthoracic echocardiography using right parasternal long and short-axis windows was performed to evaluate LV systolic function [10-12]. The echocardiographic findings included a normal LV systolic function with ejection fraction (EF) (82%) and stroke volume (SV) (12.74 mL).

The CK level was very high (13.690 UI/L) on the 1st day of hospitalizations and increased to 31.587 UI/L on the 2nd day. CK values during the 4th and 9th days were 15.796 UI/L and 1.064 UI/L, respectively *(Table 3)*.

Venous acid-base status and azotemia were normalized by the 2nd day of hospitalization *(Table 3)*.

On the fourth day of the treatment, the dog started to walk and receive food orally, however, liver enzymes (ALT, ALP) *(Table 3)* and C-RP values were still high *(Table 2)*. His urine was still normal.

On the ninth day of illness, the dog was in a sternal paralyzed position with a diffusely tender abdomen and depressed. Despite intensive therapy, the cyanotic mucosal membranes, prolonged CRT, low body temperature *(Table 1)*, increased granulocyte count *(Table 2)* and high lactate concentration persisted *(Table 3)*. There was no azotemia *(Table 3)*. However, liver enzymes (ALT, ALP) and total bilirubin concentration were high *(Table 3)*. pCO_2 increased to 49.00 mmHg in the venous acid-base analysis. Despite restoration to a normal hydration status (dehydration degree <5%), the BP was still hypotensive *(Table 1)*. The acute phase response never responded to treatment, with the C-RP 3 times upper limit of normal *(Table 2)*. He became acutely worse, with no oral intake, persistent severe abdominal pain, and severe myalgias that made movement difficult.

Despite aggressive shock treatment and adequate treatment of secondary infections and the complication of rhabdomyolysis (azotemia and liver failure), the patient developed progressive myalgia, progressive respiratory failure, and low compliance, resulting in death on the 9th day of hospitalization. Because of the emotional reason, the patient owner was reluctant to consider necropsy.

DISCUSSION

In humans, signs and symptoms of rhabdomyolysis may be hard to pinpoint. This is largely true because the course of rhabdomyolysis varies, depending on its cause. And, symptoms may occur in one area of the body or affect the whole body. Also, complications may occur in early and later stages [4]. The "classic triad" of rhabdomyolysis symptoms in humans are (1) muscle pain in the shoulders, thighs, or lower back; (2) muscle weakness or trouble moving arms and legs; and (3) dark red or brown urine or decreased urination. However, half of the people with the condition may have no muscle-related symptoms [4]. In this case, there was no history of trauma, seizures, surgery, snake or insect bite, contact with chemical agents. He did not take any drugs regularly. We agree that the course of rhabdomyolysis may vary because we did not observe dark red or brown urine and most prominent symptoms were muscle-related. Khan [2] informed that the definitive diagnosis of rhabdomyolysis should be made by laboratory tests including serum CK and urine myoglobin. In our case, there was no myoglobinuria, however significant increments in serum CK were determined *(Table 3)*. So, severe myalgia, unexplained muscle weakness, and elevated CK were the key to diagnosis.

Serum CK concentration, mainly the CK-MM subtype, is the most sensitive indicator of muscle damage. Serum CK begins to rise approximately 2 to 12 h after the onset of muscle injury, peaks within 24 to 72 h, and then decline at a relatively constant rate of 39% per day [13]. In this dog, the CK level was very high (13690 UI/L) on the 1st day of hospitalizations and increased to 31587 UI/L by the 2nd day of hospitalizations. CK values at the 4th and 9th days were 15796 UI/L and 1064 UI/L, respectively. In this case, the peak value of 31587 UI/L was during the 2nd day of hospitalizations and declined at the constant rate to 1064 UI/L on the 9th day of the treatment *(Table 3)*. This could be a result of intense volume repletion, followed by the administration of diuretics (Furosemide).

Although various values of CK have been postulated to define rhabdomyolysis, the magnitude of elevation is rather arbitrary; and there is no cut-off value that conclusively diagnoses rhabdomyolysis in humans. A serum CK activity greater than five times the normal value (in the absence of heart or brain diseases) was accepted as a criterion for the diagnosis of rhabdomyolysis [14]. However, the Clinical Advisory on Statins defined statin-induced rhabdomyolysis as muscle symptoms with marked CK elevation typically substantially greater than 10 times the upper normal limit, with a creatinine elevation consistent with pigment nephropathy and usually with brown urine with myoglobinuria [6,15]. In veterinary medicine, marked CK elevation in exertional rhabdomyolysis and toxication has been determined as high as 187380 U/L [8]. There is no data concerning septic shock. In this dog, serum CK activity was ten times greater than the normal value (13.690 UI/L) seen on the 1st day of admission *(Table 3)*.

Myoglobin is normally bound to plasma globulins and has a rapid renal clearance which maintains a low plasma level up to a certain serum concentration (0 to 0.003 mg/dL). After the occurrence of muscle damage, the circulating myoglobin levels exceed the plasma protein binding capacity, reach the glomeruli, and are eventually excreted in the urine [2]. We failed to detect myoglobinuria in the initial and following evaluations. This could be explained by studies in human medicine where Cervellin et al.[3] informed that myoglobinuria is detected in a varying proportion (28-70%) of patients with rhabdomyolysis. Khan [2] and Minnema et al.[16] also stated that serum myoglobin precedes the rise in CK and drops rapidly. Serum myoglobin usually increases before a rise in CK and drops more rapidly than the decline in CK concentration (in one to six hours). Moreover, myoglobinuria may not be visible or may resolve early in the course of rhabdomyolysis. These facts make this parameter less sensitive and therefore should not be relied upon to rule out the diagnosis of rhabdomyolysis. Thus, myoglobinuria may be undetectable in a patient presenting with muscle weakness and high CK. Our patient had a 2 days history of weakness and myalgias thus myoglobinuria may be undetectable at presentation [17]. Finally, we may say that myoglobinuria does not occur without rhabdomyolysis, but rhabdomyolysis does not necessarily lead to visible myoglobinuria (tea or cola-colored urine).

Once the diagnosis of rhabdomyolysis is established, a search must be instituted for a cause. In our case, there was no history of trauma, seizures, surgery, snake or insect bite, contact with chemical agents, and no medication regularly. Our dog had all the criteria for septic shock including *S. pseudintermedius* isolate in blood culture [9,18]. Rhabdomyolysis may occur as part of the septic syndrome in which hemodynamic instability and elaboration of bacterial toxins and other cytokines that may either selectively or collectively contribute to muscle necrosis. It is noteworthy in this regard that both the tumor necrosis factor-a (TNF-α) and interleukin-1β, elaborated by septic patients, are capable of causing acute proteolysis in the skeletal muscle cells [19]. Cytokines are known to activate branched-chain a- ketoacid dehydrogenase, the rate-limiting enzyme in branched-chain amino acid oxidation in the muscle, leading to a severe catabolic state. TNF-α is also known to cause an acute reduction in the cross skeletal muscle cell plasma membrane, implying direct injury to the muscle cell or an increase in Na permeability of the muscle cell. An increase in cytosolic Ca rapidly follows the increased Na permeability of the cell, resulting in swelling and eventual death of the muscle cell [10,19]. So, decrements in calcium and albumin concentrations in our case could be the result of muscle cell death. The accumulation of substantial amounts of fluid into the affected muscle cause

hypovolemia. At the same time, high intra-compartmental pressure provokes additional damage and necrosis [20]. This further muscle damage is manifested as the 'second wave phenomenon', with persistent elevation or rebound elevation in CK levels at 48 to 72 hours after the initial insult [21] *(Table 3)*.

High liver enzymes (ALT, ALP) and total bilirubin concentration, azotemia at admission, high lactate, high LDH hypothermia, hypobasemia, hypoglycemia, low blood pressure, decreased SpO_2 and thrombocyte count, and increased WBC, and C-RP could be explained by septic shock or/and rhabdomyolysis, and dysfunctional organ systems *(Table 3)*.

Blood gas analysis allows the interpretation of acid-base status as well as respiratory function, including both oxygenation and respiration. In our dog, there was hypobasemia (HCO_3: 18 mmol/L; BE: -7 mmol/L) and respiratory alkalosis due to decreased pCO_2 (31.80 mmHg) despite normal blood pH (7.37) on the day1 *(Table 3)*. The decreased pCO_2 can be assumed to be respiratory compensation of the hypobasemia. However, pCO_2 increased to 49.00 mmHg on the day9. A high pCO_2 is compatible with respiratory acidosis. This could be the result of sepsis-associated ARDS. Increased lactate concentration (7.37 mmol/L) on day9 supports this conclusion.

Our patient presented with severe rhabdomyolysis with a peak CK level of 31587 UI/L complicated by renal, respiratory, and hepatic failure. It should be emphasized that the risk of renal, respiratory, and hepatic failure could be decreased by early detection of rhabdomyolysis through routine measurement of CK level in patients with sepsis. Established shock and elevated CK level subsequently resulted in a cascade of renal failure, hepatic failure, secondary infections, and respiratory failure due to progressive ARDS with eventually a fatal course. Perhaps the clinical outcome would have been different if alarming signs had been recognized on time and shock could have been prevented.

In conclusion, we describe an unusual case of severe rhabdomyolysis associated with septic shock. This unusual case may further add to the understanding of rhabdomyolysis with sepsis.

Statement of Author Contributions

KT: Conceptualization, Methodology, Writing - review & editing; AN, HS, ME and MEI: Writing - review & editing..

Conflict of Interest

None

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Case Report

Occurrence and First Molecular Characterization of *Cryptosporidium felis* in a Cat in Turkey

Neslihan SURSAL [1,a] Emrah SIMSEK [2,b] Kader YILDIZ [3,c]

[1] Aksaray University, Faculty of Veterinary Medicine, Department of Parasitology, TR-68100 Aksaray - TURKEY
[2] Erciyes University, Faculty of Veterinary Medicine, Department of Preclinical Science, TR-38039 Kayseri - TURKEY
[3] Kirikkale University, Faculty of Veterinary Medicine, Department of Parasitology, TR-71450 Kirikkale - TURKEY
ORCIDs: [a] 0000-0002-4144-9520; [b] 0000-0002-0492-9840; [c] 0000-0001-5802-6156

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Abstract

Cryptosporidium species are highly prevalent and significant zoonotic protozoan parasites that cause severe diarrhea in many hosts. To date, cryptosporidiosis has been molecularly reported from humans and different animal hosts in Turkey. The aim of this case report is to reveal the molecular characterization based on small subunit (SSU) rRNA gene sequence of *Cryptosporidium* oocysts that were microscopically found in a three-month age female cat with severe diarrhea. SSU rRNA gene of *Cryptosporidium* was amplified for sequence and phylogenetic analyses. According to sequence analysis, *Cryptosporidium* spp. oocysts were characterized as *C. felis* (isolate name: ANK_1; accession number: MN394123). ANK_1 isolate showed a range from 99.1% to 100% identity with the most of *C. felis* sequences available in GenBank. However, it also exhibited high genetic distance (1.5-4.3%) with some of *C. felis* sequences. The intraspecific genetic differences among all SSU rRNA sequences of *C. felis* isolates included in the data set were determined ranged from 0.0% to 6.9%. As a conclusion, we provided the first microscopic and molecular evidence of the occurrence of *C. felis* in a house cat in Turkey. Although *C. felis* has a relatively low zoonotic risk to public health when compared with *C. parvum*, infected cats should not be neglected for zoonotic transmission due to close relationships between cats and humans.

Keywords: *Cryptosporidium felis, Cat, Molecular characterization, SSU rRNA gene, Turkey*

Türkiye'de Bir Kedide *Cryptosporidium felis*'in Varlığı ve İlk Moleküler Karakterizasyonu

Öz

Cryptosporidium türleri, birçok konakta ciddi ishale neden olan oldukça yaygın ve önemli zoonotik protozoan parazitlerdir. Bugüne kadar cryptosporidiosis, Türkiye'de insanlarda ve farklı hayvan konaklarında moleküler olarak rapor edilmiştir. Bu olgu sunumunun amacı, ağır ishalli üç aylık dişi bir kedide mikroskobik olarak saptanan *Cryptosporidium* ookistlerinin small subunit (SSU) rRNA gen sekansına dayanan moleküler karakterizasyonunu ortaya koymaktır. *Cryptosporidium*'un SSU rRNA geni, sekans ve filogenetik analizler için amplifiye edilmiştir. Sekans analizi sonucu, *Cryptosporidium* spp. ookistleri *C. felis* (izolat adı: ANK_1; erişim numarası: MN394123) olarak karakterize edilmiştir. ANK_1 izolatı, GenBank'ta mevcut *C. felis* sekanslarının birçoğuyla %99,1 ile %100 arasında benzerlik göstermiştir. Ancak, bazı *C. felis* sekanslarıyla ise yüksek düzeyde genetik farklılık (%1.5-4.3) göstermiştir. Veri setine dahil edilen tüm *C. felis* izolatlarının SSU rRNA sekansları arasındaki tür içi genetik farklılık %0.0 ile %6.9 arasında belirlenmiştir. Sonuç olarak, bu olgu sunumu ile Türkiye'de bir ev kedisinde *C. felis*'in varlığı mikroskobik ve moleküler olarak ilk kez gösterilmiştir. *Cryptosporidium felis, C. parvum*'a kıyasla halk sağlığı açısından nispeten daha az zoonotik risk taşımasına rağmen, insanlar ve kediler arasındaki yakın ilişkiler dikkate alındığında enfekte kedilerin zoonotik bulaşmada önemli olabileceği göz ardı edilmemelidir.

Anahtar sözcükler: *Cryptosporidium felis, Kedi, Moleküler karakterizasyon, SSU rRNA geni, Türkiye*

INTRODUCTION

Cryptosporidium species are significant apicomplexan parasites that infect the gastrointestinal system of numerous hosts (mammals, birds, reptiles, and fishes), including humans [1,2]. To date, 38 *Cryptosporidium* species have been recognized [3]. Cats are commonly infected with *C. felis* [4-6]. In addition, *C. parvum* [6,7], *C. muris* [4], *C. ryanae* [4], and *Cryptosporidium* rat genotype III-IV [4,8] species have also been reported from cats.

Correspondence
+90 382 2882854, Fax: +90 382 2882899
neslihansursal@hotmail.com, neslihansursal@aksaray.edu.tr

Most of the cats infected with *Cryptosporidium* spp. are usually asymptomatic. Diarrhea occurs more frequently in young and newborn kittens [9,10]. Feces are usually watery and do not contain mucus, blood, and melena. Other common clinical signs of cryptosporidiosis in cats are anorexia and weight loss [11].

Different diagnostic techniques including the microscopic examination (wet mount preparation, staining methods such as modified acid-fast stain or fluorescent stains), histological examination, immunological and various molecular methods are available for detection of cryptosporidiosis [12]. Molecular characterization of *Cryptosporidium* species is important and necessary for precise identification of organisms and to understand the zoonotic transmissions [13]. PCR-RFLP and DNA sequencing are the most frequently used assays for molecular identification and characterization of *Cryptosporidium* species. The small subunit (SSU) rRNA gene is commonly preferred for genotyping *Cryptosporidium* in many hosts and environmental samples [14].

Cryptosporidium infections have been reported from humans and different animal hosts including cats in Turkey [15-21]. It was aimed to reveal the first molecular characterization based on SSU rRNA gene sequence of *Cryptosporidium* isolate found in a cat in this case report.

CASE HISTORY

A fecal sample obtained from a mixed-breed, three-month old female cat with severe diarrhea was sent to Kırıkkale University, Faculty of Veterinary Medicine, Department of Parasitology Laboratory for parasitological examination by a veterinary clinic in June 2019. According to anamnesis, despite the long-term antibiotic treatment, no reduction in the severity of diarrhea was observed. It was also informed that the cat mostly lived in a house and had also access to the garden of the house. Written informed consent of the cat's owner was obtained for using the data in scientific publications.

Firstly, fecal consistency scores were evaluated according to the modified fecal scoring system [22] and determined as 6 (watery and no texture, occurs as puddles). After routine parasitological examination, the fecal sample was stained with carbol-fuchsin dye (CF) to detect *Cryptosporidium* oocysts [23]. *Cryptosporidium* spp. oocysts (~5 µm diameter) in the fecal sample stained with CF dye were visualized as bright white color on the red background using the light microscope (Olympus BX43 Tokyo, Japan equipped with Olympus DP73 digital camera) *(Fig. 1)*. No other parasites were found in the fecal sample.

For advanced molecular diagnosis, the genomic DNA (gDNA) was extracted from the fecal sample using a QIAamp Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Nested-PCR was performed to

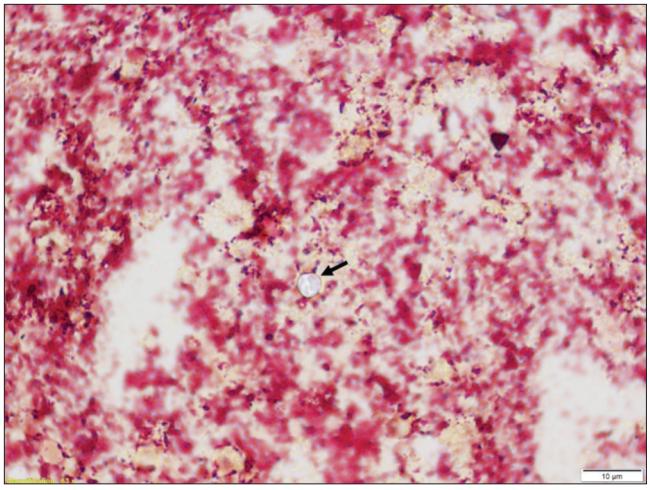

Fig 1. *Cryptosporidium* spp. oocyst that determined by the carbol-fuchsin staining method was shown with black arrow

amplify the SSU rRNA gene region of *Cryptosporidium*. 18SiCF2/18SiCR2 and 18SiCF1/18SiCR1 primer pairs were used for the first and second PCR amplification, respectively [24]. PCR reactions were performed in a total volume of 20 µL, consisted of a commercial master mix (Phusion High-Fidelity PCR Master Mix 5X, Thermo Scientific, Waltham, MA, USA), 0.5 µM each primer and 10-30 ng of gDNA. For the second PCR, 1 µL of the first PCR's product was used as template. An automated thermocycler (Applied Biosystems, Thermo Scientific, Waltham, MA, USA) were used in the PCR analyses and the amplification conditions (both first and second PCR amplifications) included a pre-denaturation at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 61.8°C for 20 s, and extension at 72°C for 45 s. The final extension step was carried out at 72°C for 10 min. Ultra-pure grade water and gDNA of *C. parvum* were used as the negative and positive controls in PCR assays, respectively. PCR amplicons (20 µL) were analyzed in 1.5% agarose gel, stained with SafeView™ (Applied Biological Materials, Richmond, BC, Canada), and visualized (Quantum CX5, Vilber Lourmat, France). Approximately 587 bp fragment of SSU rRNA gene region of *Cryptosporidium* isolate was amplified by nested PCR analysis and visualized on agarose gel *(Fig. 2-A)*. After that, the PCR product was purified (High Pure PCR Product Purification Kit, Roche, Germany) from gel to increase sequence quality. Purified product (5 µl) was re-analyzed in agarose gel to check the purification result *(Fig. 2-B)* and sequenced in both directions (Macrogen, Amsterdam, The Netherlands) using the nested PCR primers.

The obtained sequences were assembled and edited in Geneious Prime 2020.0.3 *(https://www.geneious.com)* by evaluating the quality value of sequence chromatograms. The forward and reverse sequence electropherograms were also visually checked for the presence of the double nucleotide peaks that might show possible mixed infections. The SSU rRNA gene consensus sequence (560 bp) was

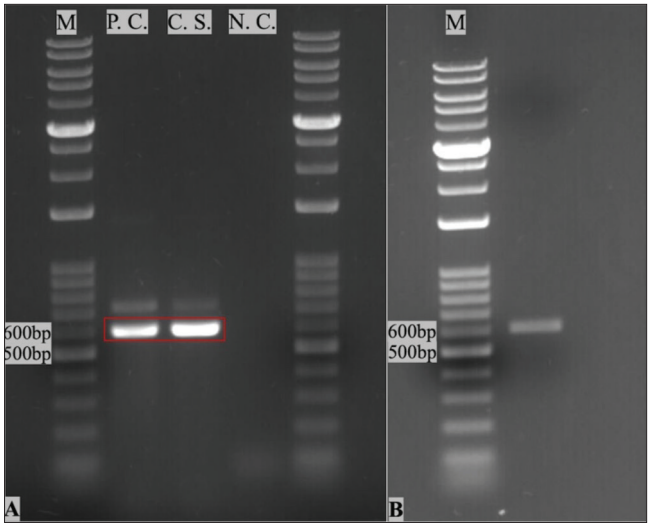

Fig 2. a) Gel electrophoresis image of nested PCR analyzes performed with primers 18SiCF1-18SiCR1 (SSU rRNA gene region). P.C.: Positive control, C.S.: Cat sample, N.C.: Negative control **b)** Gel electrophoresis image of purified amplicon for sequencing

successfully obtained from *Cryptosporidium* isolate. There was no double peak indicative of mixed infections. A BLASTn search was conducted using the final nucleotide sequences to make species-based identification and create a data set. The sequence and BLASTn analysis of the SSU rRNA gene region confirmed the microscopic examination result and *Cryptosporidium* isolate was molecularly assigned as *C. felis*. The nucleotide sequence of the SSU rRNA gene region of *C. felis* was deposited in GenBank with ANK_1 isolate name (accession number: MN394123).

For the phylogenetic analyses, the sequence data set was created by considering BLASTn results and using the available *Cryptosporidium* sequences (54 *C. felis* out of 61 *Cryptosporidium* isolates) from cats in GenBank. In created data set, trimmed alignment was 560 bp in length. The data set was tested with MEGA X model test to determine the most suitable DNA model according to the correct Akaike's Information Criterion (AIC). Phylogenetic analysis was conducted using the maximum-likelihood (ML) analysis based on GTR+G (the General Time Reversible + Gamma distributed) model in MEGA X [25] with 1000 bootstrap replicates. Genetic distances were determined using the Kimura two-parameter model [26] in MEGA X [25]. ANK_1 isolate showed a range from 99.1-100% identity with the most of *C. felis* sequences available in the GenBank *(Fig. 3)*. However, it exhibited 1.5%, 2%, 2.8%, 3.3%, and 4.3% high genetic distance with some of *C. felis* sequences with the accession numbers MG888051, KX174306, JQ312664, KX168415, and MG889862, respectively. The intraspecific genetic differences among all SSU rRNA sequences of *C. felis* isolates included in the data set were determined ranged from 0.0-6.9%. The ML tree based on the SSU rRNA gene region of *C. felis* revealed two main clades with the support of a 99% bootstrap value. Different *C. felis* haplotypes from various geographical regions were observed in the phylogenetic tree *(Fig. 3)*.

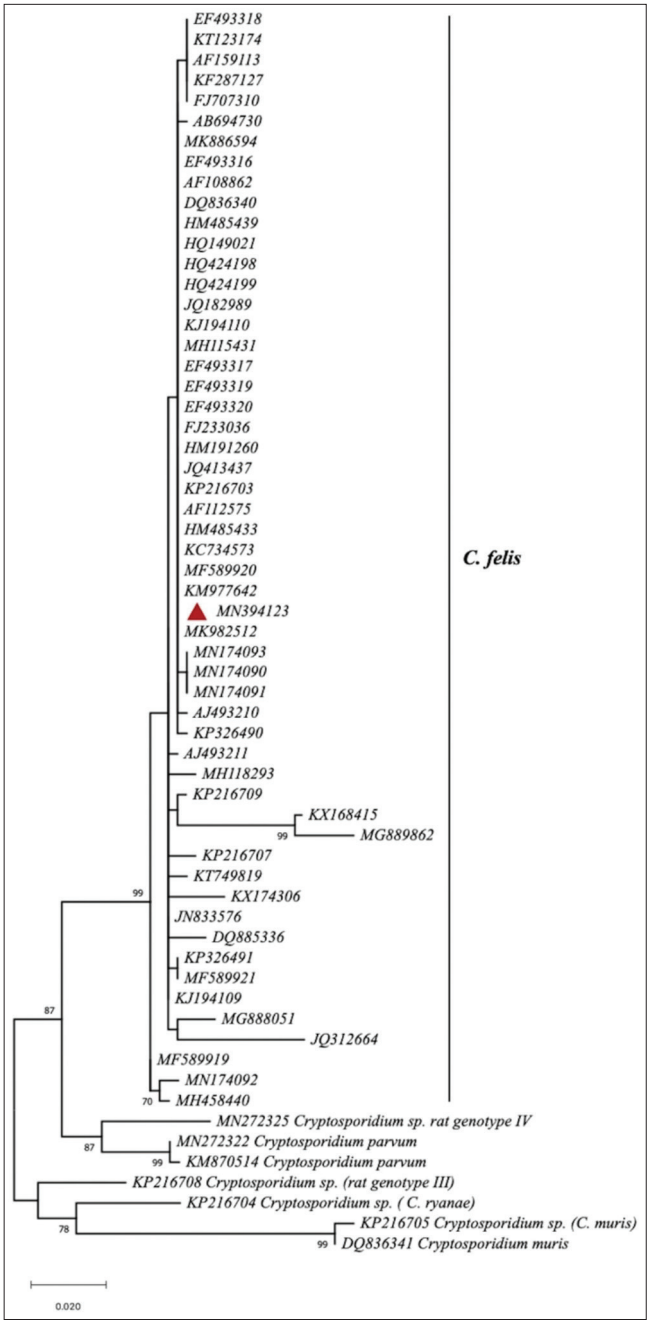

Fig 3. Phylogenetic relationships among *Cryptosporidium* isolates reported from cats in GenBank based on the SSU rRNA gene. Isolates that are included in data sets were given with GenBank accession numbers and species names. The isolate (ANK_1) found in this case report was shown with red character. Scale bar represents 0.020% substitutions per nucleotide position

DISCUSSION

Several risk factors such as breed, age, sex, and outdoor access have previously been analyzed for cryptosporidiosis in cats [27,28]. *Cryptosporidium* infection detected in this case might be related to the living area and age of the

cat. Because outdoor conditions could provide greater opportunity for young cats to exposure to infected hosts or contaminated soil/water. Some authors state that the infection risk with *Cryptosporidium* agents is higher in outdoor/stray cats than indoor/pet cats [27]. Besides cryptosporidiosis has been found more prevalent in young cats and this situation has been attributed with their immature immune system [28].

The prevalence of cryptosporidiosis in cats has been reported range from 0% to 29.4% in the world and these differences have been associated with diagnosis techniques used (reviewed by Lucio-Foster et al.[29]). The prevalence of *Cryptosporidium* spp. in cats has been reported from 1% to 13% in Turkey [19-21]. However, there is no data available regarding to molecular characterization of *Cryptosporidium* isolates in cats in Turkey. In this case, the SSU rRNA gene region of *Cryptosporidium* isolate found in a cat was successfully amplified and sequenced for the first time in Turkey. According to the BLASTn analysis, *Cryptosporidium* spp. isolate was molecularly assigned as *C. felis*, which is consistent with the reported dominance of this species in cats [4,6,30,31]. Similar to the findings of Ito et al.[31], ANK_1 isolate showed a range from 99.1% to 100% identity with most of *C. felis* sequences. However, high intraspecific genetic differences (0.0-6.9%) were determined among all SSU rRNA sequences of *C. felis* isolates included in the data set. Moreover, different *C. felis* haplotypes were observed in the phylogenetic tree. Considering high genetic variability, a suitable genetic marker may be needed to identify the subtypes family of the *C. felis* isolates.

Most of the human cases are associated with *C. hominis* and *C. parvum*. Besides these species, *C. felis, C. canis, C. meleagridis, C. cuniculus, C. ubiquitum,* and *C. viatorum* species are also responsible for human cryptosporidiosis [5,14]. Particularly, *C. felis* has been reported from many humans in different countries [32-35]. In a study, the identical *C. felis* SSU rRNA, HSP70, and COWP gene sequences were confirmed in both the cat and its immunocompetent owner in Sweden [33]. This result was an important step to molecularly confirm the zoonotic transmission of *C. felis* from cat to human. The authors highlighted that the cat could be the initial source of infection [33]. In another study on human cryptosporidiosis in the UK, contact with cats has been found as a significant risk factor for *C. felis* cases determined in humans [36].

In conclusion, we provided the first microscopic and molecular evidence of the occurrence of *C. felis* in a cat in Turkey. Considering the studies mentioned above, although *C. felis* has a relatively low zoonotic risk to public health compared with *C. parvum* [5,29], infected cats should not be neglected for zoonotic transmission due to close relationships between cats and humans. Especially, immunocompromised humans and children should be avoided from cats infected with *Cryptosporidium* spp. New studies with a large-scale sampling in a wide geographic area are necessary to determine prevalence, species, and genetic diversity of *Cryptosporidium* species in cats and to reveal potential risk factors for public health.

Statement of Author Contributions

NS designed the study. Parasitological examinations and molecular analyses were conducted by NS and ES. The manuscript was written by NS, and reviewed by ES and KY. All authors contributed to the improvement of discussion and reviewed the final manuscript not only for spelling and grammar but also for its intellectual content.

Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Letter to the Editor

Dystocia Caused by Spinal Paraplegia in a Cat with Superfetation

(Süperfetasyonlu Bir Kedide Spinal Paraplejinin Neden Olduğu Distoşya)

Emsal Sinem ÖZDEMİR SALCI [1,a] Barış GÜNER [2,b] Volkan İPEK [3,c]

[1] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Bursa Uludag University, 16059 - Bursa - TURKEY
[2] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Balıkesir University, 10145 - Balıkesir - TURKEY
[3] Department Pathology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, 15030 - Burdur - TURKEY

ORCIDs: [a] 0000-0003-0154-9938; [b] 0000-0001-6414-6752; [c] 0000-0001-5874-7797

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Dear Editor,

Dystocia is commonly encountered gynecological problem in veterinary medicine. The cause of dystocia is usually maternally fractured pelvis, inadequate uterine contractions, etc.; fetally it is caused by oversized fetus, fetal mal-presentation, malformations and deaths [1]. Although superfetation is generally not common, it describes the mating and fertilization if a pregnant female shows an estrus again [1,2]. En-block overiohystectomy is a safe and effective alternative technique that allows cesarean and sterilization in cats and dogs with dystocia [1]. As reported in humans, the spinal paraplegia may lead to in the motor activity of abdominal muscles and in the autonomic nervous system, which prevents the normal birth process [3]. In this presented case, it has been emphasized that dystocia due to spinal paraplegia can be observed in cats, and superfetation is possible in paralyzed cats mated at different times.

A mix breed, 4-year-old pregnant cat with spinal paralysis was brought to our clinic with the complaint that the birth started but the cat could not give birth. In the anamnesis, it was reported that the cat was paralyzed in the thoraco-lumbar spinal region due to a traffic accident about 2.5 years ago, the cat spontaneously delivered once before paralysis, and the kitten born at that time were normal. It was reported that the cat lived with different male cats at home, was not sterilized by the idea that it could not be mating because it was paralyzed, but pregnancy was diagnosed with an ultrasound examination performed by a veterinarian as a result of abdominal enlargement.

In the clinical examination, the vital parameters of the cat were determined to be normal and in the ultrasonographic examination, 1 live kitten was found. Apart from the live kitten, 2 other kittens, smaller in size but not beating the heart, were identified. Due to the paralysis of the cat and the occurrence of dystocia, the patient owner asked for the live kitten to be removed and the cat spayed.

For premedication and induction, xylazine HCl (1 mg/kg, im) and ketamine HCl (10 mg/kg, im) was applied respectively. Vascular cannulation was achieved with 24 no angiocath and 0.9% isotonic NaCl was infused. General anesthesia and maintenance were provided with 2% isoflurane.

After surgical preparation and antisepsis of the ventral abdominal region, the caudal abdominal area was limited to sterile covers. The abdominal cavity was reached with a median postumbilical laparotomy incision. Right and left uterine horn differed in diameter, there was local 3x4 cm enlargement in the cranial part of the left uterine horn and thinning in the uterine wall *(Fig. 1-A)*. Routine en-bloc ovariohysterectomy operation was performed. In the right uterine horn, which was taken out explicitly, 1 live mature kitten and 2 immature different size fetuses were observed in the left uterine corn, which was determined as superfetation *(Fig. 1-B)*. Abdominal incision was routinely closed. The two immature puppies are 5 and 7 cm in size, and were thought to be at the age of 4 and 6 weeks, respectively. While no abnormality was observed in the examination of the large immature fetus, arthrogryposis was detected in the hind limbs of the smallest fetus. There was no abnormality of the internal organs in the necropsy of these fetuses. Postoperative cefazolin Na (20 mg/kg, im., bid) and tolfenamic acid (4 mg/kg, oral, qd) were recommended for 5 days. In the 1st week and after the other controls, the newborn kitten was reported to have

Correspondence
+90 224 2940828 GSM: +90 531 3651173 Fax: +90 224 294 08 78
ssalci@uludag.edu.tr

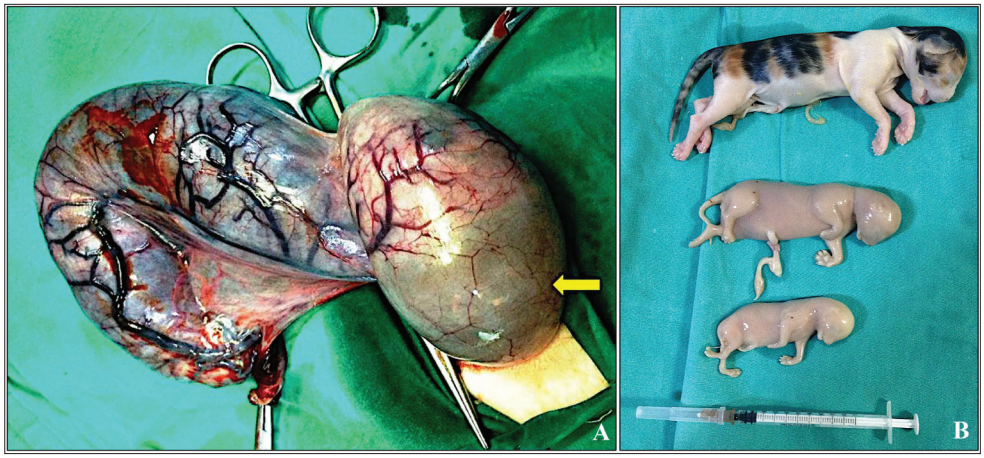

Fig 1. A: Intraoperative view points out the local 3x4 cm enlargement in the cranial part of the left uterine horn and thinning in the uterine wall *(arrow)*, **B:** Appearance of a live mature kitten and 2 immature different size fetuses resulted from superfetation

survived and the paralyzed cat was in good condition.

Female cats with spinal paralysis are also sexually active and it is even possible to encounter mating and pregnancies with superfetation. Autonomic dysfunction and motor nerve damage caused by spinal paralysis in cats causes dystocia by creating impaired activity in organs and muscles that help birth. For this reason, ovariohysterectomy may be recommended by veterinary clinical practitioner to owners have female cat with spinal paraplegia.

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Review

An Overlooked Entities in Small Animal Surgery: Splenic Disorders

Kürşat ÖZER [1,a] Burak GÜMÜRÇİNLER [2,b] Murat KARABAĞLI [1,c]

[1] Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Surgery Department, TR-34320 Avcillar/ Istanbul - TURKEY
[2] Istanbul Municipality Kısırkaya Temporary Animal Shelter, TR-34450 Sarıyer/Istanbul - TURKEY
ORCID's: [a] 0000-0002-3314-7581; [b] 0000-0003-2922-7279; [c] 0000-0002-3936-1730

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Abstract

The spleen, which has a vitally important function in the body, is located in the left part of the abdomen. The spleen's primary tasks are hematopoiesis and immunity, such as the reservoir of blood cells and the production of the immune system's defense cells. On most occasions -due to the lack of clinical manifestation- splenic disorders are coincidental findings at surgery despite their prevalence. Splenosis is a heterotopic autotransplantation of splenic tissue in other body compartments, which may be interpreted as a pathological entity through imaging techniques. Foreign substance accumulation, such as iron deposition, namely siderosis, may be monitored in the spleen due to the aging process. The pathologies of the spleen are mostly characterized by the enlargement of the organ (splenomegaly). Hemangiosarcoma is the most frequently encountered neoplasia in the spleen, usually metastasizing to the heart's right atrium. Ultrasonography is the most commonly utilized diagnostic tool in splenic disorders. Besides, the magnetic resonance imaging (MRI) and computed tomography (CT) techniques may be applied. Whole blood, plasma, or both are prerequisites that should be kept at disposal in the preoperative approach to the splenic disorders. Surgical interventions of splenic pathologies include splenorrhaphy, partial splenectomy, and total splenectomy. Splenic injuries usually result from blunt trauma. Surgery is not an indication unless there is a life-threatening amount of blood loss. Splenic torsion, which is an acute disorder of the spleen usually encountered in deep-chested dog breeds due to gastric dilatation and volvulus presents a diagnostic challenge. Total splenectomy is the favored treatment of choice in the approach of the relevant conditions rather than splenic derotation. Early phase complications include hemorrhage, cardiac arrhythmia, and ischemia in the pancreas and gastric wall, and metastasis and gastric dilatation and volvulus appear as the complications of the postoperative course. This review has aimed to open a new gate to better understanding the importance and functions of the spleen in normal physiology and also aimed to share information about the diagnostic tools and guide through to apply appropriate surgical approach in term of disorders.

Keywords: *Splenosis, Hemangiosarcoma, Siderosis, Total splenectomy, Splenic torsion*

Küçük Hayvan Cerrahisinde Gözardı Edilen Bir Başlık: Dalak Hastalıkları

Öz

Vücutta hayati öneme sahip bir işlevi olan dalak, karnın sol kısmında yer alır. Dalağın birincil görevleri, kan hücrelerinin rezervuarı ve bağışıklık sisteminin savunma hücrelerinin üretilmesi gibi hematopoez ve bağışıklık olaylarıdır. Çoğu zaman -klinik belirtilerin patognomik olmaması nedeniyle- dalak hastalıkları, operasyon esnasında rastlantısal olarak tespit edilmektedir. Splenoz dalak dokusunun diğer vücut kompartmanlarındaki heterotopik bir ototransplantasyonudur ve görüntüleme esnasında patolojik bir durum olarak yorumlanabilir. Yaşlanma sürecine bağlı olarak dalakta demir birikimi yani sideroz gibi yabancı madde birikimleri izlenebilir. Dalağın patolojileri çoğunlukla organın genişlemesi (splenomegali) ile karakterizedir. Hemanjiyosarkom, dalakta en sık karşılaşılan neoplazidir ve genellikle kalbin sağ atriyumuna metastaz yapar. Ultrasonografi, dalak bozukluklarının tanısında en sık kullanılan tanı aracıdır. Ayrıca manyetik rezonans görüntüleme (MRG) ve bilgisayarlı tomografi (BT) teknikleri de kullanılabilir. Preoperatif dönemde tam kan, plazma veya her ikisi birden hazır edilmelidir. Dalak patolojilerinin cerrahi müdahaleleri arasında splenorafi, parsiyel splenektomi ve total splenektomi sayılabilir. Dalak yaralanmaları genellikle künt travmadan kaynaklanır. Yaşamı tehdit eden miktarda kan kaybı olmadıkça cerrahi müdehale endikasyonu doğmaz. Genellikle derin göğüslü köpek ırklarında mide dilatasyonu ve volvulusa bağlı olarak karşılaşılan ve akut bir dalak hastalığı olan dalak torsiyonun tanısı oldukça zordur. Bu durumda total splenektomi, dalak derotasyonundan ziyade tercih edilen tedavi seçeneğidir. Postoperatif erken dönem komplikasyonları, pankreas ve mide duvarında kanama, kardiyak aritmi ve iskemiyi içerir; postoperatif seyrin komplikasyonları olarak metastaz ve mide dilatasyonu ve volvulus görülür. Bu derleme, bir organ olarak dalağın fonksiyonel önemini ve işlevlerini daha iyi anlamak için yeni bir kapı açmayı, tanı araçları hakkında bilgiler paylaşmayı ve hastalıklarında uygun cerrahi yaklaşımın seçilmesine rehberlik etmeyi amaçlamıştır.

Anahtar sözcükler: *Splenoz, Hemanjiyosarkoma, Siderozis, Total splenektomi, Dalak torsiyonu*

Correspondence
+90 532 2538253 (Cell Phone)
ozer_kursat@yahoo.com

INTRODUCTION

Although the spleen is not an essential organ for survival, it cannot be considered unimportant. Accurate recognition of this organ, which has many tasks related to erythrocytes, platelets, and the immune system, is vital in small animal surgery, especially when evaluating traumatic and oncological disease. This review has aimed to open a new gate to better understanding the importance and functions of the spleen in normal physiology and also aimed to share information about the diagnostic tools and guide through to apply appropriate surgical approach in term of disorders.

ANATOMY AND PHYSIOLOGY OF THE SPLEEN

The spleen is a hook-shaped organ predominantly located in the left cranial part of the abdomen. It is situated parallelly in the left hypogastric region adjacent to the *curvature major* of the stomach. Particularly having been shrunk, it is a consistent organ, with a trabecular framework. Roughly, it has a tongue-like shape. Once the spleen has enlarged, it gets extended and the trunk of the organ contracts. It has a triangular shape on the sections [1]. The organ's color was defined as purple-red, red-brown, amaranth, mahogany, and grey-brown [2,3]. Its shape, size, and location vary depending on several parameters such as the species, breed, medication, and the pathologic translocation of the organ. The spleen's portion occupying the thorax varies depending on its contraction capacity and gastric fullness [4].

When contracted, the spleen might thoroughly translocate into the thoracal cavity and appears pale bluish-purple. On the contrary, when congested, the caudal portion of the spleen might advance to the urinary bladder and appear dark brownish-red. A cat spleen is narrower and smaller compared to the dog spleen [4]. The *celiac* artery, which is originated from the abdominal aorta and branches into three segments such as the hepatic, left gastric and splenic artery, nurtures the spleen [5]. Blood enters the spleen through the splenic artery as a branch of the *celiac* artery into the gastrosplenic vessel and exists through the splenic vein that finally opens into the portal vein. Short gastric arteries arising from the splenic artery anastomose with the branches of the left gastric artery [1]. The splenic artery supplies blood from several hilar vessels and connects with the gastrosplenic vessel before entering the portal vein. Splenic lymph nodes are situated adjacent to the artery merely a few centimeters away from the spleen and the hilus [2]. A normal spleen is soft and consistent, yet not friable as the liver. The spleen constitutes approximately 0.2% of the body weight in small animals [5].

The spleen is supported by the portion of the *omentum major*, which leaves the left *cupola* of the diaphragm located between *esophageal hiatus* and *celiac* artery and *frenicosplenic* ligament. This portion of the omentum is enlarged in the caudal direction and having to cover the splenic hilus initially, and then the *curvature major* of the stomach emerges as a broad *gastrosplenic* ligament [1]. The spleen is composed of a capsule, rich in elastic and smooth muscle fibers, trabeculae, and parenchyma of red and white pulp. Trabeculae form a spider net-like tangled structure. Some trabeculae join and reinforce the blood vessels, while other blood vessels are self-contained [6]. Larger intrasplenic arteries are mainly situated within the trabeculae. Collagenous fibers of trabeculae directly proceed to reticular fibers in the white pulp [1]. The white pulp of the dog is composed of widespread nodular lymphoid tissue. The nodules are usually smaller than 1 mm in diameter and are not visible to bare eyes. Their germinal centers appear paler than the peripheral pulp. The red pulp is composed of splenic cords, which form a spongy network filled by venous sinuses. The cellular component of splenic cords includes abundant lymphocytes, megakaryocytes, mobile and resident macrophages, and circulating blood cells, the majority of which comprise agranulocytes [7].

Intrauterine malformation or malfunctioning of the spleen is called "asplenia" [8]. Accessory splenic nodules widely found on the omentum of small animals represent revascularized tiny splenic tissue portions that possibly occurred secondarily to trauma [9]. Ectopic splenic tissue embedded alongside the pancreas' surface as nodules were found only in cats; however, its cause was not conclusively linked with trauma. Siderotic and siderofibrotic plaques may be found on a geriatric dog spleen as amorphous calcium and iron deposits [2]. Splenosis is a benign condition that emerges as a result of trauma or rupture occurred during a surgical intervention. It is usually a coincidental finding and is not considered an indication for treatment unless symptomatic. Moreover, it may radiographically mimic a malignancy, which should be thoroughly examined. The diagnostic method of choice is nuclear scintigraphy in relevant cases. Splenosis is usually detected in the abdominal and pelvic cavity, yet intrathoracic, subcutaneous, intrahepatic, and intracranial lesions were defined in the patients [4].

The spleen's size and weight in cats and dogs vastly vary since it serves as a significant blood reservoir in the body. The contracted spleen after exercise, due to the smooth muscle fibers found throughout the organ rhythmically relax in the resting position. These smooth muscle fibers relax with anesthetic administration, which results in prominent splenomegaly. Catecholamines, stress, and injections lead to the removal of mobile blood cells and the plasma from the red pulp, and thus the spleen shrinks. No parasympathetic nerve fibers are found in the spleen. Splenic rupture may be detected due to a traumatic impact that occurred during traffic accidents, yet it is highly likely possible to remove the organ with no life-critical complication surgically. The respectively loose bond of

the spleen with the stomach facilitates splenectomy on a vascular basis [10].

SPLEEN DISORDERS

Splenomegaly

A preliminary clinical examination includes spleen's size, whether it is enlarged (splenomegaly) or shrunk. Splenomegaly may result from congestion and some other infiltrative diseases. The cut surface of a congested spleen readily emits the blood, yet an infiltrated spleen is more consistent restraining the blood [11].

Splenitis and Inflammation

Splenitis refers to an inflammatory accumulation, which may further be categorized according to the predominant cell component. Bacteria, viruses, fungi, and protozoal agents may cause splenitis [12]. The most frequent causes of uniform splenomegaly in the dog include bacteriemia, hypothermic sepsis, and contagious chronic diseases, in which necrotic residues are filtrated through the spleen. On certain occasions, neutrophilic infiltration that might have advanced with a localized abscess predominates an infectious splenitis. Eosinophilic splenitis may accompany the course of the disease in cats with hypereosinophilic syndrome and dogs with eosinophilic gastritis along with other clinical signs. Pyogranulomatous splenitis was demonstrated in feline infectious peritonitis [13]. Lymphoplasmacytic infiltrative splenomegaly may be monitored as chronic or subacute forms accompanying mycotic and mycobacterial infections. Necrotizing splenitis results from gas-producing bacteria. Splenic vascular thrombosis is simultaneously detected with hypercoagulative pathologies resulting from the *Clostridia spp* growth in the hypoxic areas. Blood flow and thus, oxygen transportation is abundant in both the red and white pulp hampering the growth of coagulative infectious agents; therefore, necrotizing splenitis is rare in cats and dogs [4].

Congestion

Splenic torsion is commonly encountered in the pig and dog, including the concurrent translocation of the spleen and stomach, particularly in deep-chested breeds. Unlike ruminants, the spleen of pig and dog is loosely bound with the stomach through a gastrosplenic ligament. Splenic torsion leads to splenic infarction by an initial clogging of the blood vessels and then blocking blood flow in the spleen and finally, the occlusion of the main splenic artery. The spleen turns on a bluish-black color with a prominent, yet regular enlargement, usually rendering a "C" shape overlapping the medial surfaces, which is considered an indication for surgery. The majority of the surgeons recommend a direct removal of the spleen without derotation to prevent thrombosis and the release of endotoxins and free radicals [14].

Intravenous injection of barbiturates causes acute congestion in the spleen. Drug-induced splenomegaly is detected due to the administration of phenothiazines such as acepromazine, barbiturates, and short-acting barbiturates such as thiopental. It was shown in a study that splenomegaly was more likely induced by thiopental and ketamine-diazepam combination than propofol-based anesthesia protocols. Causes of congestive splenomegaly include portal hypertension, right heart failure, and splenic torsion [10].

Immune-mediated Reactions and Hyperplasia

Generalized splenomegaly may physiologically arise from the overproduction of the spleen's cellular component while functioning normally. Red pulp and white pulp hyperplasia or both may occur in animals suffering from chronic diseases. Subacute and chronic disorders, such as immune-mediated hemolytic anemia and thrombocytopenia, usually lead to red and white pulp hyperplasia. Splenomegaly is widespread in dogs with immune-mediated hemolytic anemia, which might have resulted from extramedullary hematopoiesis and reticuloendothelial hyperplasia associated with the destruction of IgG coated erythrocytes. Macrophage hyperplasia induced by increased phagocytic activity initially affects the red pulp and is usually detected in histoplasmosis and leishmaniasis. Acute hemolytic anemia, babesiosis and immune-mediated hemolytic anemia may cause prominent congestion in the spleen. A large number of circulating phagocytized erythrocytes and their accumulation lead to the congestion of the spleen [11,15-19].

Infection Associated Disorders

Splenic infiltrations may occur due to bizarre cell accumulation or substance depositions during neoplastic processes (primary and metastasizing) and splenic amyloidosis, which is a rare condition. Neoplasia-associated splenomegaly usually results from the neoplastic proliferation of the resident cells such as lymphocytes, macrophages, fibroblasts, smooth muscle, and endothelial cells, which emerges as primary neoplastic lesions. The most common cause of generalized splenomegaly is myeloproliferative neoplasias, such as lymphosarcoma and mastocytosis [4]. Metastasis to the spleen is an uncommon finding and usually appears as focal solitary or multifocal lesions. Lymphoma is the most frequently detected metastasizing lesion in the spleen [19]. Rarely, certain lysosomal storage diseases and splenic amyloidosis may lead to generalized splenomegaly. The spleen appears as a pale beige firm and waxy tissue in dogs with splenic amyloidosis [4].

Localized Splenomegaly

Localized splenomegaly may result from neoplastic and non-neoplastic entities. The most common causes of localized splenomegaly are addressed as nodular hyperplasia, splenic hematoma, and hemangiosarcoma [4].

Splenic Hematoma

Hemorrhage in the red pulp is a prerequisite for splenic hematoma, restricted within the splenic capsule of an approximate diameter of 12-15 cm, red to dark red with a soft consistency. Coagulation mechanisms, transforming blood into dark reddish-brown pulpy material, macrophage infiltration to phagocytize erythrocytes, and degraded hemoglobin are the constituents of splenic hematoma, which takes days to weeks to develop. The indeed repairing process elicits hematoidin and hemosiderin, and thus fibrosis in the spleen. On certain occasions, the splenic capsule that covers the hematoma may rupture, damaging the visceral peritoneum, resulting in hemoperitoneum, hypovolemic shock, and death. The causes of hematoma are not precisely recognized. Some may develop due to trauma, while some others result from splenic nodular hyperplasia. Splenic hematomas were reported to have occurred due to the rupture of hemangiosarcomas located within the spleen [11].

Hamartoma

Canine splenic hamartomas are rare benign lesions resulting from the mature splenic residential cells and tissues' overgrowth as in nodular hyperplasia [20]. Furthermore, hamartomas may be differentiated by not mimicking the surrounding tissue. Despite the lack of specific diagnostic data, these entities are highly likely differentiated from the other forms of localized splenomegaly merely on ultrasonography. Computed tomography (CT) and magnetic resonance imaging (MRI) techniques, widely applied in human medical practice, are more reliable diagnostic tools to differentiate hamartomas from other miscellaneous benign proliferation [4].

Nodular Hyperplasia

Nodular hyperplasia, formerly referred to as "splenoma" is a prevalent, yet coincidental splenic pathology in geriatric dogs. The nodules, which are composed of clusters of lymphoid, erythroid, and myeloid cells, and megakaryocytes, are known to be benign lesions. On the other hand, some authors reported that nodular hyperplasia might have advanced to a hematoma due to the peripheral circulation's failure that caused accumulation of abundant blood within the nodules. Splenic hematomas and hyperplastic nodules, which histologically differ, might be permanent lesions in dogs. Non-sinusoidal nature of the feline spleen is not prone to venous congestion hampering the occurrence of nodular hyperplasia, a rare entity in the cat [21].

Acute Splenic Infarctions

Splenic infarctions are triangular-shaped hemorrhagic lesions that initially occur at the periphery of the organ. Splenic infarctions usually result from hypercoagulative diseases, such as liver and kidney disorders, Cushing syndrome, as well as neoplastic lesions and cardiovascular disease in the dog. It may be highly unlikely to demonstrate the splenic infarction precisely in the early phases, yet it may develop into a wedge-shaped lesion with its baseline situated toward the capsule, which appears as a separate dark-red swollen tissue. The lesion turns into a grey-white focus in the chronic lesions and shrinks due to fibrosis [11].

Pseudotumors

Inflammatory pseudotumors are rare entities both in dogs and humans; therefore, they should be separately assessed in the differential diagnosis of nodular hyperplasia and some malignant tumors, such as lymphoma and sarcoma. The prognosis is favorable in humans after splenectomy [22].

Hemangiosarcoma

Hemangiosarcoma is a malignant neoplasm, which is a widespread primary tumor of the spleen in dogs. On the other hand, benign splenic hemangiomas are quite rare. Hemangiosarcomas may appear as solitary, multifocal, dark reddish-purple tumors that are not easily differentiated from hematomas. The cut surface may appear as sanguineous pulpy red tissue, while the tumor may be firm and pale in the solid areas. Metastasis may develop in the early phases. Multiple reddish-black masses on the omentum, abdominal organs, and the serosa manifest metastasis to the abdomen. Metastasis to the liver and the lungs is prevalent through the hematogenous route. In dogs, hemangiosarcomas were previously defined in the heart's right atrium, retroperitoneal adipose tissue, and the skin, while hemangiosarcomas with multiple organ involvement were identified in horses, cats, and cattle. Usually, since hemangiosarcomas have already metastasized on the initial diagnosis, the primary tumor is unlikely to be defined [11].

Hemangioma

Benign hemangiomas are demonstrated in the vast majority of dogs with localized splenomegaly. The relevant entities are solitary masses composed of well-differentiated endothelial cells [11]. The defined tissue structure enables hemangiomas to be easily differentiated from hemangiosarcomas, forming irregular, amorphous masses of neoplastic endothelial cells and vascular spaces. Nevertheless, hemangiomas, hematomas, and hemangiosarcomas reveal similar images in the dog [4].

Parasites

Cystic parasitic nodules may be encountered on the spleen, usually intermediate forms of *Echinococcus granulosus* and *Cysticercus tenuicollis*, prevalent in wild animals [11].

Splenic Abscess

Splenic abscess, which usually arises from pathologies

impairing the vascular network and lymph drainage of the spleen, is rare in small animals [23]. Certain microorganisms cause chronic suppurative splenitis, which typically ends up in splenomegaly [4].

Plaques

Siderocytic plaques commonly seen on the spleen's surface as golden and black spots are non-malignant lesions, which is an outcome of a hemorrhage resulting from erythrophagocytosis subsequently occurring with hemoglobin breakdown. Siderocalcific plaques, which appear as yellowish-white crusty depositions at the edges or within the parenchyma of the spleen, are considered a natural outcome of the aging process in geriatric animals, yet on certain occasions; it may be linked with trauma [11].

Exceptional Conditions: Splenic Torsion

Splenic torsion has never been identified in the cat, yet is a rare disorder of the dog's spleen. Deep-chested dog breeds and males are more prone to develop splenic torsion [20,24-26]. Splenic torsion initially obstructs the venous circulation, which leads to splenic congestion, followed by arterial occlusion in further phases, which ends up in splenic infarction. Even though pathogenesis is unclear, splenic torsion is assumed to have concurrently occurred with gastric dilatation and volvulus. Clinically, chronic splenic torsion is manifested by lethargy, anorexia, vomiting, diarrhea, polyuria, polydipsia, and weight loss, which intervene with those of other disorders causing abdominal pain, hampering a differential diagnosis. Dogs with acute splenic torsion may show clinical signs of evidence of hypovolemic and toxic shock. Hematological findings are interpreted as leukocytosis, anemia, and thrombocytopenia on hemogram. Biochemical changes comprise elevated hepatic and pancreatic enzymes lacking peculiarity. Treatment of choice in the patients with splenic torsion includes palliative care and splenectomy. Derotation of the spleen is not considered an option since it might result in the intravascular release of sequestered blood components, thrombus and microthrombus particles, free radicals, and cytokines such as tumor necrosing factor and vasoactive amines into the systemic circulation. Emergency splenectomy should be performed in acute cases [4].

DIAGNOSIS

Diagnostic Radiography

It is an effortless process to image the spleen in dogs and cats radiographically, yet its location may differ by the movable caudal portion of the organ. A standard ventrodorsal shooting reveals a transversal triangular-shaped section of the spleen's cranial extremity extending from the left cranial region to gastric fundus caudolaterally, and the left kidney craniolaterally. The canine spleen is more visible with the right lateral imaging [27,28]. Large splenic masses are usually visualized on the abdominal midline and lateral regions. Generalized splenomegaly is characterized by round or blunt splenic edges. Hemoabdomen that emerge from splenic hemorrhage and rupture may fade in visceral radiographical details hampering the imaging of splenic and hepatic silhouettes [4].

Ultrasonography

Ultrasonography is the most preferred diagnostic tool for assessing the spleen and the metastasized masses in the abdominal cavity [25]. Contours of the spleen should be carefully evaluated during ultrasonography in terms of potential traumatic alterations, hematoma, and different types of focal expansions and irregularities due to neoplastic lesions. The spleen's size should be assessed, yet there are no standard maximum and minimum values established concerning the spleen's size in dogs and cats. Therefore, an experienced eye plays a crucial role in interpreting the ultrasonographic findings. It is highly unlikely to monitor a physiological enlargement in the cat spleen due to the feline spleen's non-nervous nature [26]. Hypoechoic nodules within the spleen are mostly associated with infiltrations, infarction, and necrosis, while widespread hypoechoic parenchyma is usually observed with splenic congestion and torsion.

Hematomas, neoplasms, abscesses, and cysts may appear as cavitary lesions by ultrasonography. Doppler ultrasonography is used to assess the characteristics of the focal and multifocal splenic lesions in cats and dogs [29,30]. While ultrasonography is utilized as a diagnostic tool for splenic disorders, a throughout abdominal examination concerning potential effusions, lymphadenopathy, hepatic abnormalities, and masses is of great importance. Right atrial hemangiosarcoma may concurrently be found in 25% of the dogs with splenic hemangiosarcomas, which points out the necessity of cardiac Doppler ultrasonography before surgical intervention [4].

Computed Tomography (CT) and Magnetic Resonance Imaging (MRI)

Computed tomography and MRI have become vastly utilized techniques also in veterinary medicine to image splenic disorders. Both techniques have long been routinely applied in human medicine, particularly to assess a concurrent underlying disease [31-33]. A recent prospective study investigating the splenic lesions by contrast CT revealed a reduced density for hemangiosarcomas than nodular hyperplasia and before and after contrast scans of hematomas [34].

SURGERY

Preoperative Management

Generalized and focal splenomegaly or both might be

monitored in the animals with splenic disorders requiring surgical intervention. Widespread (symmetrical) splenomegaly may result from congestion (i.e., splenic torsion, right cardiac failure, gastric dilatation/volvulus [GDV], and drugs), infiltrations due to infection (i.e., mycotic, bacterial, and rickettsial), foreign bodies, immune-mediated diseases (i.e., immune-mediated thrombocytopenia and immune-mediated hemolytic anemia), and neoplasms (i.e., lymphosarcoma, histiocytic sarcoma, and feline mastocytosis). Benign lesions (i.e., nodular regeneration, hematoma, and trauma) and neoplastic entities (i.e., hemangiosarcoma and lymphoma) may cause focal splenomegaly. Neoplasia-associated infiltrative splenomegaly is the most common cause of spontaneous splenomegaly in dogs and cats. Anemia, splenic trauma, rupture of a hematoma, and underlying diseases (i.e., chronic infections and disseminated coagulopathy [DIC]) might have induced a hemorrhage. Coagulation factors should be measured in the animals with non-traumatic splenic hemorrhage. Whole blood and plasma or both should be at disposal before splenectomy. In case DIC is suspected, treatment protocols should include heparin. Dehydrated animals should be parenterally treated before the surgical procedure, and preoxygenation should be applied for anemic animals, which should be maintained even in the recovery period. The choice of perioperative prophylactic antibiotics is crucial in the dog to be performed splenectomy. Perioperative antibiotic administration is unnecessary in healthy animals, yet it might be applied during the induction of anesthesia and should be ceased in 24 h. Immunosuppressed and cachectic animals may require a more extended antibiotic application. It was reported that splenectomy performed along with dental detartrage and extraction might have resulted in multiple abscesses, septicemia, and even death in the dog [4].

Surgical Techniques

Splenorrhaphy

Small iatrogenic lacerations or perforations are indications for splenorrhaphy, yet maintaining hemostasis is vitally important. If there is hemorrhage, a capsular repair might be considered at appropriate pressure by appropriate technique [4]. Trauma-associated splenic rupture or laceration may spontaneously heal without the requirement of splenorrhaphy or splenectomy [25].

Partial Splenectomy

Partial splenectomy is a more challenging technique than total splenectomy, yet it allows the spleen's physiological function to be maintained. The procedure is an indication for focal splenic abscess or injuries; however, it is out of the question in neoplastic lesions. Partial splenectomy is most commonly performed on animals with blunt or penetrated abdominal trauma that has impaired the vascular structure of the spleen [35-37].

Total Splenectomy

Total splenectomy is an indication for animals with definitive or suspected neoplastic lesions, splenic torsion, severe trauma, widespread infiltrative diseases, and with some immune-mediated diseases [4]. Total splenectomy is most commonly performed on animals with a history of splenic neoplasms, gastric dilatation/volvulus, and severe trauma manifested by a life-threatening hemorrhage. Splenectomy was previously applied for non-responsive hematologic disorders (i.e., thrombocytopenia and hemolytic anemia); however, immunosuppressive drugs and corticosteroids extenuated the requirement for the procedure. Nevertheless, it might be considered if the medication has failed or generated intolerable side effects. Even though sepsis has been reported after splenectomy in humans, there is no known case in dogs, yet -if possible- partial splenectomy should be preferred rather than total splenectomy. Splenectomy is contraindicated in patients with bone marrow hypoplasia since it is the leading site for hematopoiesis [38,39]. It is not uncommon for veterinary practitioners to recommend prophylactic gastropexy for large breed dogs that underwent splenectomy to prevent potential gastric dilatation and volvulus [4].

POTENTIAL COMPLICATION OF SURGICAL INTERVENTIONS

Hemorrhage

Acute blood loss may be detected after splenectomy, which might have been resulted from unsuccessful surgical intervention and hemostatic anomalies. Incomplete ligation of omentum vessels, loosely inserted surgical clips and inaccurate electrocautherization may cause hemorrhage. Potential causes that may result in hemorrhage after splenectomy may be listed as autoimmune disorders that lead to endothelial injury, thrombocytic activation or breakdown, splenic masses, infection, and trauma [31-34]. It has been reported that a thrombocyte count of 25.000/µL allows a safe splenectomy [33]. According to a published study, DIC is more likely to have developed in dogs with malignant tumoral lesions when the activated partial thromboplastin time (aPTT) has prolonged [34].

Cardiac Arrhythmia

Arrhythmia causes reduced cardiac output. It usually occurs as ventricular tachycardia or multifocal ventricular arrhythmia, which is monitored in dogs with splenomegaly due to malignant tumors, torsion, and benign tumoral masses after splenectomy [35-37].

Ischemia on the Left Lobe of the Pancreas and Gastric Wall

Left pancreatic ischemia results from the occlusion of the pancreatic and splenic blood vessels, which might

emerge as a life-threatening condition. Potential risks include splenic torsion, splenic tumors causing secondary DIC or coagulation disorders, and defective surgical manipulations [35-37].

Metastasis

Hemangiosarcoma is a commonly encountered entity in the spleen [31-33]. Recent studies have been aimed at the differentiation of benign entities from malignant splenic lesions. However, the studies turned out to be inconclusive. Multifocal lesions may be attributed to metastasis. Some neoplasms (i.e., lymphoma, mast cell tumor) may be primary or secondary lesions on the spleen [38-42].

Gastric Dilatation and Volvulus

Gastric dilatation and volvulus (GDV) is a risky condition with a potentially dangerous outcome, in which the stomach rotates around its axis and, most frequently, in a clockwise fashion. A recent study demonstrated that splenectomized patients with GDV had a poorer prognosis than those that underwent only a derotation procedure [42-45]. The relevant findings favor considering a prophylactic gastropexy in splenectomized dogs, particularly bearing other risk factors for developing GDV [45-49].

CONCLUSION

Splenic disorders and their surgical treatment are currently becoming more prominent in veterinary medicine as in their human counterparts. The developing technology enables the widespread utilization of advanced imaging and surgical techniques in assessing and treating splenic disorders. Since splenic pathologies do not reveal prominent clinical signs, they are prone to be overlooked by veterinary practitioners. Instant intervention in acute cases like splenic torsion might have a life-saving outcome.

Statements of Authors Contributions

All authors contributed to the content and main topics of the manuscript. Introductions, anatomy and physiology prepared by MK, spleen disorders prepared by BK and diagnosis, treatment and complications prepared by KÖ. Final controls made by KÖ.

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Review

The Role of Oxidant and Antioxidant Parameters in the Infectious Diseases: A Systematic Literature Review

Sena ÇENESİZ [1,a]

[1] Ondokuz Mayis University, Faculty of Veterinary Medicine, Department of Biochemistry, TR-55139 Samsun - TURKEY
ORCID: [a] 0000-0002-3544-503X

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Abstract

The formation of reactive oxygen species is a physiological event in aerobic life. In case of infection, if the increased oxidant substances cannot be cleaned sufficiently by antioxidants, oxidative stress occurs. As a result, a number of pathological problems occur by damaging DNA, protein, carbohydrate and lipids. In biological systems, the balance between oxidants and antioxidants is important. In oxidative stress situations where the balance cannot be achieved sufficiently, support can be provided with the use of exogenous antioxidants. However, the molecular structure, route of administration and concentrations of these exogenous antioxidants are important. Otherwise, they may show a pro-oxidative effect. The present rewiev study makes a general overview of oxidative-nitrosative stress markers commonly used in infective clinical studies, antioxidant enzymes and parameters and antioxidant supplements.

Keywords: *Infectious diseases, Oxidant, Antioxidant, Oxidative stress*

Enfektif Hastalıklarda Oksidan ve Antioksidan Parametrelerin Rolü: Sistematik Bir Literatür Değerlendirmesi

Öz

Reaktif oksijen türlerinin oluşumu arerobik yaşamda fizyolojik bir olaydır. Bu serbest radikal türlerinin daha fazla üretilmesine neden olan paraziter, bakteriyel ve viral enfeksiyonlarda antioksidan sistemin kapasitesi bu reaktifleri yeterince temizleyemez ise oksidatif stres gelişir. Sonuçta DNA, protein, karbonhidrat ve lipidlerde hasar oluşarak bir takım patolojik problemler meydana gelir. Biyolojik sistemlerde oksidan ve antioksidanlar arasındaki denge önemlidir. Dengenin yeterince sağlanamadığı oksidatif stres durumlarında bazan eksojen antioksidan kullanımı ile destek sağlanabilir. Ancak bu eksojen antioksidanların molekül yapısı, veriliş yolu ve konsantrasyonları önemlidir. Aksi takdirde pro-oksidatif etki gösterebilirler. Bu çalışma ile yaygın olarak görülen enfektif klinik çalışmalarda kullanılan oksidatif-nitrozatif stres belirteçlerinin, antioksidan enzim ve parametreleri ile ileriye yönelik olarak yapılacak bazı antioksidan supplementlerinin genel bir durumu değerlendirilmiştir.

Anahtar sözcükler: *Enfeksiyöz hastalıklar, Oksidanlar, Antioksidanlar, Oksidatif stres*

INTRODUCTION

Free radicals are high energy atoms or molecules that contain one or more unpaired electrons in their outer orbitals. Nitric oxide (·NO), nitrogen dioxide (NO_2·), superoxide (O_2·$^-$), hydroxyl (·OH), lipid peroxy (LOO·), peroxyl (ROO·) and alkoxyl (RO·) radicals can be given as examples [1,2]. Of these free radicals, those originating from oxygen area called reactive oxygen species (ROS) and those originating from nitrogen are called reactive nitrogen species (RNS) [2]. Under physiological conditions, the most important source of intracellular reactive products is mitochondria. These reactive products are produced in large quantities in neutrophils, macrophages and monocytes. In addition to being produced with mitochondrial electron transfer system (ETS) chain and phagocyte activation, endogenously produced free radicals are also released as a result of the activity of many enzymes such as xanthine oxidase (XOD), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, neutrophil myeloperoxidase (MPO), cyclooxygenase, lipoxygenase [3-6]. Sources of exogenous free radicals (ROS) are UV, X-ray, gamma and microwave rays, air pollutants such as asbestos, benzene, carbon monoxide, formaldehyde, ozone and toluene, chemicals such as cleaning products, glue, paint, thinner, perfumes and pesticides, sudden and excessive oxygen entry, medical hyperbaric oxygen exposure, increase in catecholamines, increased lactic acid in muscles and blood, elevation in lytic enzyme activities such as lactate

Correspondence
+90 362 3121919-3893 Cell Phone: +90 505 5914096
scenesiz@omu.edu.tr

dehydrogenase, creatine phosphokinase, different and difficult physiological conditions such as exercise, pregnancy and old age, intense stress, cigarette and alcohol use, large amounts of and long-term consumption of diets containing polyunsaturated and easily peroxidable fats. Antioxidant system deficiencies or exceeding the defensive wall disrupts the oxidant-antioxidant balance in favour of oxidants and oxidative stress occurs [7-10]. Because reactive species have very short shelf life and difficult measurement, NO, lipid peroxidation marker malondialdehyde (MDA), total oxidant capacity (TOC), which is easy to measure with kits and 8-hydroxydeoxyguanosine as the marker of ROS in DNA oxidation are the most used parameters [11-14].

A large number of studies have been published on oxidative stress markers in the field of veterinary science. This review makes a general overview of oxidative-nitrosative stress markers commonly used in infective clinical studies, antioxidant enzymes and parameters and antioxidant supplements to be made prospectively.

PHYSIOLOGICAL STATES OF RADICALS

In addition to being harmful, free radicals also fulfil some physiological functions depending on their concentrations. For example, ·NO is an important mediator in the relaxation of smooth muscles and regulation of microcirculation as an intracellular messenger; additionally in addition, free radicals are used in vascular tone and signal transmission, protein phosphorylation, transcription factor activation, cell differentiation, apoptosis, oocyte maturation, embryonic development, steroidogenesis, spermatogenesis, pregnancy and immune defence system [15-20]. NO leukocyte adhesion, which is used by endothelial cells, is necessary for leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis and for vascular smooth muscles to regulate blood pressure. In addition to these, NO produced by neurons is an important transmitter substance and has a key in role for neural plasticity. On the other hand, NO produced by macrophages is an important mediator to create an immune response. Superoxide and H_2O_2 can act like second messengers. NO is a molecule that has significant cytotoxic effect in defence against tumour cells, parasite, fungi, protozoa, helminths and mycobacteria, but it is not effective against extracellular pathogens. $O_2^{\cdot-}$ can stimulate collagen synthesis through fibroblast proliferation, H_2O_2 has a role in NFkB activation. They can be said to have important roles on cellular signals such as killing cancer cells with cytotoxic lymphocytes and macrophages during phagocytosis in viral, parasitic and microbial infections; detoxification of xenobiotics with p450; activation of ROS and RNS nuclear transcription factors, Ca release from intracellular stores, tyrosine phosphorylation amino acid; activation of non-receptor tyrosine kinase and activation of some cytokines and growth factor signals. In addition to being produced with phagocyte activation, mitochondrial ETS chain also occurs as a result of the activation of many enzymes such as XOD, NADPH oxidase, MPO, cyclooxygenase, lipoxygenase. Apart from these, ROS participates in the biosynthesis of molecules such as prostaglandin and thyroxin and stimulates the development of these processes. In luteal phase in the oestrus cycle and in follicular (oestrus) period, oxidation level is high. Reactive oxygen species are dissolved in cell and are used in the regulation of guanylate cyclase activity and in vital activities such as gene transcription [2,21-30].

DISEASES AND SYNDROMES REPORTED TO BE CAUSED BY OXIDATIVE DAMAGE

There are a large number of studies reporting that oxidative stress markers are the basis of many diseases. MDA, hydroxy-2-nonenal (HNE), 2-propenal (acrolein), isoprostanes, oxide glutathione (GSSG), NO, total oxidant capacity (TOC), 8-hydroxy-2-deoxyguanosine (8-OHdG) are among the most studied oxidative markers clinically for this purpose. Although oxidative stress has been studied with too many diseases and syndromes, it has been reported to be effective in the development of cancer, cardiovascular disease, neural disorders, Alzheimer's disease, mild cognitive disorder, Parkinson's disease, alcohol-related liver disease, ulcerative colitis, aging and atherosclerosis, lead poisoning, liver damage due to carbon tetrachloride, aminoglycoside, reactions from drugs and toxins, such as heavy metal toxicity, chronic and degenerative diseases such as glomerulo-nephritis, emphysema, porphyria, bronchopulmonary dysplasia, atherosclerosis, pancreatitis, rheumatoid arthritis, aging, neurodegenerative disorders, hemolytic anemia, cardiovascular diseases, pneumonia, sepsis, mastitis, metritis, retentio secundinarum, genital tract inflammation, acidosis, tongue-playing, sepsis, mastitis, ketosis, enteritis, respiratory, joint diseases and autoimmune disorders [14,26,28,31-43].

ROS FORMATION IN INFECTIOUS CONDITIONS

In many animal studies, the common picture in the clinical biochemistry of parasitic, microbial or viral diseases is the increase in oxidant parameters such as ROS and NO in the cell in order to fight the infectious agent. An increase in MDA level has been reported as a result of lipid peroxidation with the increase in cell damage. Although ROS production is useful in removing invasive pathogens, its excessive and prolonged production can cause permanent damage to host and non-infected cells. ROS production can affect the pro-inflammatory response of inflammatory cell significantly. Intracellular ROS formation by NADPH oxidase triggers pro-inflammatory cytokine production in macrophages, neutrophils and microglia. Free radicals are particularly known to be effective in the last step of phagocytosis, that is, in the step of killing microorganisms. The rapid production of free radicals by a mechanism induced by

inflammation and known as Respiratory Burst causes oxidative stress and cell damage. During this event, O_2 consumption in phagocytic cells increases 4 to 100 times. Activated phagocytic cells (neutrophil, eosinophil and all types of macrophages) produce $O_2^{\cdot-}$ with NADPH oxidase. This function is important in cleaning phagocytosed bacteria. There are five mechanisms under the control of the oxidative explosion;

Endogenous GTPase limits NADPH oxidase activation,

Lactoferrin in phagocyte granules binds free iron,

Phagocytes have self-protective antioxidant systems such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase; dense taurine substance is thought to neutralize hypochlorous acid (HOCl),

Some cells sacrifice themselves apoptosis,

Liquids and cells surrounding the target have protective systems [30,44].

When cells are activated, as a result of the activation of macrophages against the agent, chemotactic chemokine such as tumour necrosis factor- alpha (TNF-α), interleukin-6 (IL-6), interferon gamma (IFN-γ), interleukin-8 (IL-8), monocyte chemo attractant protein-1 (MCP-1), macrophage inflammatory protein 2 (MIP-2) are released. As an early response to inflammation, they can cause an abnormal persistent cascade known as cytokine storm as a result of excessive release of inflammatory cytokines. $O_2^{-}\bullet$ is released as a result of the reaction of NADPH oxidase enzyme with respiratory burst in order to eliminate the pathogen in the pathogenesis of the infection. $O_2^{-}\bullet$ radical formed as a result of reaction is converted into H_2O_2, a cytotoxic molecule catalysed by SOD. The most toxic radical, hydroxyl radical ($HO^{\cdot}$) is produced as a result of H_2O_2 and O_2^{-} reaction (Heber-Weiss reaction) or H_2O_2 and Fe^{2+} reaction (Fenton reaction), while hypochlorous acid is produced as a result of the reaction of H_2O_2 and chlorine (Cl^{-}) catalysed by the enzyme myeloperoxidase. This acid produced forms a part of the antimicrobial defence by destroying bacterial DNA and causing DNA replication to stop. The events that characterize leukocyte activation are rapid increase in oxygen use, increase in glycogenolysis and glucose oxidation and rapid formation of ROS. Especially neutrophils and monocytes form highly reactive oxidants by using the H_2O_2-MPO-Halid system. Chloride, bromide, iodide and thiocyanate can be used as substrate in this system; however, due to its *in vivo* concentrations, chloride (Cl^{-}) appears to be the most preferred halide. Highly reactive hypochlorous porphyrins react rapidly with a large number of molecules such as proteins and amines and kill microorganisms with halogenation, protein, lipid peroxidation reactions [30,45-51].

Oxidative Stress in Parasitic Infestations

Parasites cause tissue and cell damage in the host as they increase the amount of free radical and lipid peroxidation in the tissues, organs and cells they settle. It has been reported that following *Nippostrontrongylus brasilliensis*, *Trichinella spiralis* and *Dictyocaulus viviparus* infestation, MDA level increases significantly in the lung tissue of the host and that this increase may have been caused by the damage of free radicals to various cell components during infection. Increased lipid peroxidation has been associated with lung lesions due to parasite and oxidative stress caused by respiratory distress that occurs as a result of these lesions [52-54].

It is known that cellular and humoral immune response occurs against cystic echinococcosis, T-lymphocytes play an important role in the immunological control of parasite and macrophage and neutrophils fight metasestodes [55]. NO produced by macrophages is an important mediator to create immune response. In addition, superoxide and hydrogen peroxide increase in macrophage and neutrophils due to XO, MPO and NADPH oxidase activity, which causes an increase in oxidant production [56]. Lipid peroxidation caused by ROS causes cell membrane disruption and ultimately necrotic deaths [57]. In studies conducted on patients infected with liver cystic echinococcosis, an increase was found in MDA levels, while a decrease was found in antioxidant levels; thus, oxidative stress was reported [55-61]. In addition, in the study conducted by Heidarpour et al.[59], the decrease in albumin level was associated with its being used as free radical scavenger in oxidative process. In lung infections, an increase occurs in TOC level, while a decrease occurs in TAC level and has in OSI index [60,62].

Similarly, oxidative stress increase was found in *Toxocara vitulorum*, *Eimeria spp and* coccidiosis, *Nematodiasis*, *Leishmaniasis, Hypoderma spp., Toxoplasma gondii, Eimeria*, *Anaplasmosis, Theileriosis, Babesiosis, Cryptosporidium parvum* infections in other parasitic diseases involving intestines, kidneys and blood, etc. [12,13,63-72]. In Helminth infestations, these are fought thanks to oxidizing enzymes of increased eosinophil [73]. When studies conducted were examined, the common point found was increased oxidation in parasitic infestations, and increased oxidative stress as a result of decreased antioxidative defence. Specifically, the use of oxidant-antioxidant parameters in differential diagnosis is difficult because oxidative stress is similarly increased in the pathogenesis of many parasitic infestations. On the contrary, these parameters can be used to get information about the healing success or clinical course of the disease.

Oxidative Stress in Bacterial Infections

Although there are different types of microorganisms causing respiratory diseases, studies conducted on beef cattle with microorganisms such as *Actinomyces spp., Staph. epidermidis, Corynebacterium spp., Pasteurella multocida, Manheimia haemolytica, S. aureus and Escherichia coli* have reported increased oxidative stress [74]. In studies conducted with different types and biological materials, similar to

bacterial pneumonia, increase was fond in serum, broncho-alveolar fluid, while decrease was found in antioxidant system. M. bovis increases neutrophil apoptosis and ROS production, while it decreases NO production [30,75-77]. High oxidative stress index in brucella can be used in showing the severity of inflammation [67,78]. Paratuberculosis, one of the inflammatory bowel diseases of the digestive system, *Mycobacterium avium* subtype paratuberculosis (MAP) infections in the aetiology of Johne and Crohn disease are frequently encountered [79]. In the pathophysiology of such inflammatory bowel diseases, high production of ROS and RNS species is closely associated with decreased antioxidant activity and oxidative stress shaped with increased glutathione peroxidase (GPx) activity. In addition, in nonsteroidal anti-inflammatory drugs, it signals apoptosis with oxidative stress through mitochondrial pathway. As a result of increased oxidative stress, the functions of fatty acids and proteins in cell membrane deteriorate, even causing DNA damage and mutations. Another factor contributing to these inflammatory diseases is T-helper cells releasing high amount of interferon (Th-1, Th-2). With the deterioration of epithelial barrier, intestinal permeability increases and inflammation becomes un-controlled [1,80-82]. While an increased picture is seen in studies conducted in general on infective diseases due to an increase in oxidative parameters and a decrease in antioxidant activity, Johne and Crohn reported increased oxidation and increased GPx activity unlike other studies [83,84]. In Crohn's aetiology, there is no change in GPx activity in case of absence of MAP, while typical finding in those with MAP is on increase in GPx activity. It has been reported that the consistent correlation between MAP infection and GPx activity can potentially be used to find out the MAP infection status.

In a study conducted on calves with septicaemia caused by *E. coli*, while no change was reported in MDA and albumin levels, increase was reported in SOD and GPx activity. The absence of increase in MDA was attributed to the important increase in bilirubin level [85]. Negative correlation was reported between MDA and hyperbilirubinemia [86].

Oxidative Stress in Viral Infections

Parvovirus infection in dogs has been stated to be significantly associated with oxidative stress and reactive oxygen/nitrogen species, lipid peroxidation and poor antioxidant reserve. In a study conducted by Aydoğdu et al.[87] TOS was increased, while no change was seen in TAS and naturally OSI index was high. There are also situations in which increase was detected in SOD and GPx activity with oxidants such as MDA, H_2O_2 and decrease in CAT activity [88]. In sheep infected with sheeppox virus, an increase was found in MDA, while a decrease was found in GSH and albumin [89]. This decrease in albumin results from suppression of the antioxidative system and negative acute phase reactant due to infection.

In studies conducted on bulls, cattle and sheep with Foot-and-mouth disease (FMD), the common point is the increase in MDA, NO, TOC level and decrease in GSH and TAC level [90-94]. Researchers have reported that oxidant stress is strong in such a viral disease. In viral infectious diseas, even if the disease changes, results are similar in oxidative stress markers. In malignant catarrhal fever (MCF) disease seen in cattle, while MDA and NO levels were increased GSH was decreased [95,96]. In the pneumonia table in goats, MDA was increased, while SOD, GPx, GSH were decreased [97]. Low level of GSH causes higher ROS production, resulting in unbalanced immune response, inflammation and susceptibility to infection.

Increased RNS production in viral infections is increased through induction of inducible NO synthase enzyme. Cytokines related to Th1 trigger ROS/ RNS production in tissues in infected host tissues. Imbalance in ROS/RNS production and its removal results in oxidative/nitrosative stress that can increase virus replication and the mutation rate of viral RNA, resulting in increased damage to host tissues [98-101]. In viral hepatitis, the main mechanism that triggers cell death is reactive oxygen species formed against pathogen in neutrophil and kupffer cells. ROS increase causes an increase in collagen production by stimulating profibrinogenic cytokines such as TGF-β, PDGF and the regulation of collagen gene transcription in fibroblasts, which is involved in the pathogenesis of the formation of fibrosis. Increased 8-OHdG with infection can be used as a marker in hepatocarcinogenesis [102-104]. While a decrease has been reported in GPx and SOD activity in Herpes virus infections, increase in GPx activity and suppression of SOD activity has been reported in BHV-1 infections [96,102-106]. Since an increase in MDA and NO and decrease is seen in GSH in zoonotic viral diseases such as ecthyma contagiosum, oxidative stress can be mentioned in the pathogenesis [107].

We have stated that the weapon used by the body to phagocytize the pathogen as a defence system to prevent parasitic, bacterial and viral infections from entering the cell and infecting the host is free radicals. The common point in various diseases mentioned above was the increase of oxidants in biological fluids such as serum, tissue, etc. and increase in oxidative stress as a result of the deficiency or suppression in the antioxidative defence system. Oxidative stress (OS) occurs when the antioxidant defence system is insufficient against free radical products. If this stress continues excessively and for a long time, lipid, protein and DNA modifications are seen. Oxidative damage to DNA can form base or sugar lesions of DNA, single and double-strand breaks, abasic regions, DNA-protein or cross-linking between strains. Damaged nucleosides accumulate in both nuclear and mitochondrial DNA. Disruption of this redox balance triggers cell signal change, causing loss of basic cellular functions, tissue inflammation, ageing, apoptosis and ultimately tissue damage [2,6,108-113].

ANTIOXIDANTS

High doses of oxidants used clinically to clarify the pathogenesis of the disease cause pathophysiological changes, especially in parasitic, bacterial and viral infective conditions. The defence system developed by the organism to decrease oxidative stress in the face of this increased oxidation is antioxidants. While endogenous antioxidant enzymes form the superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (GR) enzymatic defence line; endogenous protective antioxidants are non-enzymatic antioxidant defence systems such as glutathione (GSH), uric acid, melatonin, bilirubin, coenzyme Q10, albumin, α-lipoic acid, lactoferrin, ferritin, haptoglobulin, ceruloplasmin, transferrin. Cleansing or chain breaking antioxidants taken exogenously are lipid or water soluble materials such as tocopherol, carotenoids, ascorbate, urate. Besides, zinc, selenium, lycopene, lutein, zeaxanthin, chlorogenic acids, gallic acid, caffeic acid, quercetin, kaempferol, myricetin, naringenin, eriodictyol, hesperetin, luteolin and apigenin are also antioxidants that can be taken in the organism exogenously. These react with free radicals before living structures, take this burden on them and form a product that cannot sustain the harmful reaction. These antioxidant defence systems are parameters used in determining oxidative stress index in serum, plasma, tissue and urine. CAT, GSH, GPx, GR activities and total antioxidant capacity (TAC), ceruloplasmin, albumin, bilirubin parameters are frequently measured in clinical studies as antioxidant markers. Exogenous antioxidants and endogenous antioxidants work together to maintain redox balance [2,6,114,115]. Deficiencies in advanced age caused by insufficient intake or excessive consumption of antioxidant vitamins disrupt immune regulation [116]. As with inhibition by Vit-C or regeneration of Vit-E by GSH, endogenous and exogenous antioxidants work together to maintain redox balance. XO activity, which is accepted as the primary source of ROS, is inhibited by some flavonoids such as quercetin, silibin and luteolin. The most studied polyphenols in clinical infective animal studies due to both their anti-inflammatory and antioxidant effects are resveratrol, quercetin, rutin, tangeretin, homoplantaginin, ellagic acid, catechin, apigenin [117-119]. While flavonoids decrease peroxidase activity, they inhibit the release of free radicals by neutrophils and the activation of these cells by *α* 1-antitrypsin [120]. Anti-inflammatory activities of polyphenols such as quercetin, rutin, morin, hesperetin and hesperidin have been reported in acute and chronic inflammation of animal models. For example, rutin is only effective in chronic inflammatory processes, especially in arthritis, while flavones are effective in the neurogenic inflammation caused by xylene. It has been reported that inflammatory reaction induced by LPS injection can be modulated with daidzin, glycidyl, genistein and their glycosides. Also, exogenous antioxidants such as polyphenols can affect enzymatic activity such as protein kinase and signalling systems during inflammation process, and these enzymes play a role in cell activation processes such as cell proliferation, B lymphocyte activation or cytokine production by stimulated monocytes. They also significantly inhibit the release of arachidonic acid from the cell membrane [117,121,122]. *In vivo* and *in vitro* studies conducted show that plant derived polyphenol molecules have anti-genotoxic and anti-cytotoxic effect on cells exposed to oxidative stress [123]. Again, the ability of quercetin and naringenin to inhibit cytochrome P450 enzymes which include bioactivation of chemical carcinogens constitutes the other chemopreventive mechanism of polyphenols against cancer development, including lung cancer [124]. Exogenous antioxidants also show pro-oxidant activity in high concentrations and especially in the presence of metal ions such as iron and copper. Pro-oxidative effects of polyphenol compounds such as quercetin, catechin and gallic acids, the antioxidant properties of which are known and emphasized in previous studies, have also come to the fore in recent studies. It has been shown that prooxidant activity in flavonoids is associated with the number of hydroxyls in the molecule and flavonoids containing more than three OH in B group increase the production of hydroxyl radicals [2,119,125-128]. For this reason, molecular structures and concentrations of antioxidant substances to be used in studies should be well determined.

ANTIOXIDANTS IN INFECTIOUS ANIMAL DISEASES

If considered in a broad sense, it can be thought that antioxidant stress resulting from the excessive or inappropriate inflammatory response in severe-fulminant infections and complications of chronic infections may be at the centre of events that harm the patient or even lead to death to overcome the disease. In which infections, duration and doses can supplemental antioxidant treatment be used for the benefit of the host? There are many unclear issues about how these treatments will affect the immune response. Therefore, it may be recommended to study the effects of antioxidant supplements first *in vitro* and then start *in vivo* studies to clarify the situation in the whole system.

Antioxidant Use in Parasitic Infestations

Studies on the use of antioxidant supplements in parasitic infestations are more limited compared to viral and bacterial infections. The nephrotoxic effect occurs as a side effect during the application of first generation platinum containing cisplatin, which is mostly used as an antineoplastic for the treatment of Leishmaniasis. The application of antioxidant complex (Vit-C, Vit-E, silibinin) to reduce nephrotoxicity has been described as a promising study because of the decrease in the parasite load and toxic effect [129]. Ram et al. recommend the use of copper, manganese, selenium and zinc injections in new born calves with theileriosis since they help improve immunological

imbalance [130]. Similarly, it is stated that N-acetyl-L-cystein, which is known to have antioxidant activity, can be used as a drug in the treatment of babesiosis [131]. Vit-E and selenium application can be used as an adjunct therapeutic agent to regulate intravascular hemolysis caused by oxidative stress in babesiosis in cattle [132].

Antioxidant Use in Bacterial Infections

In the samples of the infectious studies that we found in our review, oxidative stress has been reported in the pathogenesis of patients with sepsis and it has been suggested mitochondrial dysfunction may be a causal factor in the development of multiple organ failure. This is because ROS, which increases as a result of oxidative stress in the cell, simultaneously causes the collapse of mitochondrial membrane potential and pathological ROS burst due to ETZ. These may cause ROS release in neighbouring mitochondria by being released into the cytosol; that is, ROS-induced ROS release occurs [133-137]. It has been shown that mitochondrial functions are significantly impaired in the created liver sepsis model and the impairment is strongly associated with the extent of mitochondrial ultra-structural abnormalities [138]. Oxidative stress is one of the main pathogenic factors causing mitochondrial dysfunction in acute kidney injury. Since the kidney suffers from oxidative stress during sepsis, one of the most promising approaches to mitigate such damaging results has been proposed as the use of antioxidants. For this purpose, the results of the study in which the mitochondria targeted antioxidant, plastoquinol decylrhodamine 19 (SkQR1) is applied, showed that antioxidant use is beneficial against renal tissue damage. In order not to disturb the redox balance, it has been deemed appropriate to use mitochondria-specific oxidative explosion-extinguishing supplements instead of high-dose traditional antioxidant application [139]. Mitochondria are one of the key organelles involved in the development of pathogenic cascades under septic conditions, acting both as a source and as a target for ROS. For this reason, antioxidant use has been recommended to prevent the development of oxidative stress by stopping or decreasing pathological ROS production in mitochondria. Besides, antioxidants such as multi-antioxidant Ceria-Zirconia nanoparticles developed to remove ROS for sepsis treatment have been studied and recommended for the treatment of inflammatory diseases [140]. It has been stated that selenium, which is used for antioxidant purposes in animal models of bacterial infections such as *Escherichia coli, Listeria monocytogenes, Dichelobacter nodosus, Staphylococcus aureus, has positive effects and can be used in healing* [141-144]. In the experimental study of *Microcystis aeruginosa* against Microcystin LR toxin, melatonin, Vit-E and Vit-C, which are used as a supplement to reduce the cell damage and increased 8-OH-dG, have a very high protective effect. Among these antioxidants, the effect of melatonin was 60 times higher against Vit-C and 70 times higher against Vit E [11]. Melatonin protects the DNA against oxidative damage by activating antioxidant enzymes and inhibiting prooxidative enzymes. Additionally, it is unique as an antioxidant in its ability to cross biological barriers and multiple action pathways. It is also known to have minimum toxicity even in high doses and thus to be within a wide range of dosage [145-147].

Antioxidant Use in Viral Diseases

It has been reported that the application of Vit-E, Vit-C and Zn as antioxidants provides antioxidant protection during the treatment process against oxidative damage caused by both viral infection and antiviral therapy in common viral hepatitis [148]. In studies conducted with selenium, it has been reported that selenium not only enhances Th1 type host immunity against viral infections, but also inhibits the evolution of more virulent strains of viral pathogens in RNA viruses [149-151]. Resveratrol has been reported to be effective as an inhibitor against viral replication and viral-induced inflammation in diseases caused by various pathogenic viruses, including respiratory viruses such as RSV, HCoV and HRV, influenza virus [152]. Anti-inflammatory and antioxidant activities of resveratrol may contribute to alleviating the symptoms of the virus associated with pathological signs [153]. Although it has been reported that the application of N-acetylcysteine (NAC) in infection with influenza virus clinically decreases the incidence of the disease, there are also some researchers who think that it is not healthy to recommend NAC without conducting further studies since it may decrease GSH while increasing the amount of GSSG as a result of showing prooxidant effect when the dose gets higher [154-156]. It has been reported that high doses of ascorbic acid in viral infections clears superoxide anion, inhibits virus proliferation and decreases expression of viral antigens and cellular viral load. It has also been reported to have immunomodulatory characteristics, concentrate in leukocytes, lymphocytes and macrophages, heal chemotaxis, increase neutrophil phagocytic capacity and oxidative killing, support lymphocyte proliferation and function, significantly restore decreased mitochondrial membrane potential and decrease gene expression of pro-inflammatory cytokines [157,158]. It has been reported that ROS production increases for viral replication in BHV-1 infection and causes mitochondrial dysfunction in the cell [159]. SOD activation decreases in cells infected with this virus, quercetin application increases SOD activation and ceruloplasmin level and number of apoptotic cells [105]. In a study conducted on poultry, it was suggested that oxidative stress with duodenal and jejunal mucosa in Newcastle disease virus infection causes pathological damage, while this damage can be eliminated with Vit-E given as supplement and can be used in the treatment of Newcastle disease [160]. It has also been reported that resveratrol application in poultry is useful both as antiviral and as an antioxidant [161]. It has been reported that the application of antioxidant preparates (zinc, methionine,

Vit-E, selenium) to sheep with foot-and-mouth disease improves the general health conditions and performance of animals since it increases TAC and GPx activity and decreases DNA damage [94]. It has been stated that in viral hepatitis, antioxidants can be used against viruses because they reduce virus replication and oxidant damage due to virus and increase in response to oxidative damage. Antioxidants may be effective in weakening replication and making antiviral interferon therapy more effective [162].

CONCLUSION

There is a great deal of research in animal studies on the mechanism of oxidative stress in the pathogenesis of the disease. Basically, the common point in infectious diseases is the increase in oxidative stress. When the organism encounters an infective pathogen, it increases the amount of ROS in neutrophils to protect the immune defence system. With the high and prolonged continuation of ROS which increases initially to remove the pathogen agent, oxidation and DNA modifications start in lipids and proteins. Disruption of this redox balance triggers cell signal change, causing loss of basic cellular functions, change in tissue structure, apoptosis and eventually tissue damage. It is difficult to use increased oxidative parameters in the differential diagnosis of diseases; however, it will be useful to determine oxidative stress index in determining the pathogenesis and severity of the infection and following the treatment process. On the other hand, antioxidant parameters vary in infective disease studies conducted. While GPx activity and ceruloplasmin increased in some of the studies conducted, there are also studies reporting a decrease. Since SOD activity and TAC are generally suppressed, redox balance cannot be maintained and oxidative stress occurs in the organism. Studies to supplement with antioxidants exogenously are carried out so as not to cause to give more damage with the increasing OSI. In some of these studies, prooxidative effects are seen in the presence of especially metal ions such as iron and copper depending on the molecular structure and concentrations of antioxidants, contrary to what is expected. Studies investigating the preventive effects of rations or supplements rich in antioxidants on the development, progression and treatment in studies on infectiously ill patients or in experimental studies are limited or in dose-determination stages. As the pro-oxidative effects of polyphenol compounds such as quercetin, catechin and gallic acid have come to the fore in recently conducted studies, the need for *in vitro* and *in vivo* studies on the dose, solubility, reliability and administration route of antioxidative substances has been increasing. Therefore, epidemiological studies on the effects of antioxidant molecules in healthy and sick animals will play a key role in research.

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AUTHOR INDEX - 2020

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