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Research Article

Pernicious Anemia Due to Cobalamin Deficiency in Dogs with *Helicobacter* Gastritis

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Abstract

Pernicious anemia due to cobalamin deficiency is a predominant finding in human *Helicobacter* infections, but is generally considered in exocrine pancreatic insufficiency and intestinal disease in veterinary medicine. The aim of this study is to investigate the cobalamin levels in dogs infected with *Helicobacter* spp. Material of the study were selected from 81 dogs referred to our clinics with probable gastrointestinal system origined clinical complaints including vomiting, neusea, epigastric pain and anorexia, and consisted of 36 female and 22 male (n:58) dogs in which *Helicobacter* spp. gastritis was diagnosed with Urea Breath test (^{14}C-UBT). To these dogs, gastroscopy, complete blood count, serum biochemistry including blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alkalen phosphatase (ALP), lipase, amylase, total protein and albumin levels were performed. In 38 dogs (65.51%) microcytic anemia was observed in complete blood count. All animals were treated with metronidazole, amoxycillin and famotidine and dietary management was scheduled. ^{14}C-UBT test, complete blood count and cobalamine levels were reperformed after ceasion of the therapy. Cobalamin levels were analysed in 38 dogs with anemia. Low cobalamin levels were determined in dogs with gastric *Helicobacter* infection and anemia and after therapy-without any B_{12} supplementation- cobalamin levels and mean corpuscular volume (MCV) and Haemoglobin (Hgb) values elevated to normal reference limits were observed. This suggests that cobalamin level must be determined in dogs with *Helicobacter* infections and in deficiencies which are still present after therapy, essential supplementation would be clinically beneficial. In conclusion, this is the first study demonstrating that pernicious anemia must be a considered factor in *Helicobacter* gastritis with microcytic anemia in dogs and further investigation will be beneficial to demonstrate the cobalamin levels in dogs with or without microcytic anemia in *Helicobacter* gastritis.

Keywords: *Cobalamin, Dog, Helicobacter, Pernicious anemia, Gastritis*

Helicobacter Gastritisli Köpeklerde Kobalamin Eksikliğine Bağlı Pernisiyöz Anemi

Özet

Kobalamin eksikliğine bağlı pernisiyöz anemi insan *Helicobacter* enfeksyonlarında baskın bir bulgu olmakla birlikte, bu anemi veteriner hekimlikte genellikle ekzokrin pankreatik yetmezlik ve intestinal hastalıklarda göz önüne alınmaktadır. Bu çalışmanın amacı, köpeklerde *Helicobacter* spp. enfeksiyonlarında kobalamin seviyelerinin araştırılmasıdır. Çalışmanın materyalini kliniklerimize kusma, iştahsızlık, bulantı ve anoreksi gibi muhtemel gastrointestinal sistem şikayetleriyle getirilen 81 köpekten üre nefes testi (^{14}C-UBT) ile helikobakter enfeksiyonu tanısı konulan 58 (36 dişi ve 22 erkek) köpek oluşturmuştur. Bu köpeklere gastroskopi, tam kan sayımı, kan üre nitrojen (BUN), alanin aminotransferaz (ALT), aspartat aminotransferaz (AST), gama glutamil transferaz (GGT), alkalen fosfataz (ALP), lipaz, amilaz, total protein ve albumin seviyelerini kapsayan serum biyokimyasal tetkikler uygulanmıştır. Bu köpeklerden 38'inde (%65.51) mikrositik tipte anemi gözlenmiştir. Tüm hayvanlar metronidazol, amoksisilin, famotidin ve diyetsel düzenleme ile tedavi altına alınmış ve tedavinin tamamlanmasından sonra. ^{14}C-UBT test, tam kan sayımı ve kobalamin seviyesi ölçümü tekrarlanmıştır. Anemili 38 köpekte kobalamin seviyesi ölçülmüş ve gastrik *Helicobacter* enfeksiyonlu ve mikrositik anemi gözlenen bu köpeklerde kobalamin seviyelerinin düşük olduğu görülmüştür. Anemili köpeklerde tedaviden sonra (B_{12} desteği verilmediği halde) kobalamin seviyesi ile ortalama hücresel hacim (MCV) ve hemoglobin (Hgb) değerlerinin normal referans aralıklara döndüğü gözlenmiştir. Bu durumda, klinik pratikte *Helicobacter* enfeksiyonlu köpeklerde kobalamin seviyesi belirlenmesi ve tedavi sonrası normal değerlere ulaşmadığı durumlarda takviye yapılması gerekebilir. Sonuç olarak, bu çalışmada *Helicobacter* gastritisli ve kan tablosunda mikrositik anemi gözlenen köpeklerde kobalamin eksikliği saptanması bu alandaki ilk çalışma olup, köpeklerde mikrositik anemi olan ve/veya olmayan *Helicobacter* gastritis olgularında kobalamin seviyesini ortaya koyan ileri çalışmalar faydalı olacaktır.

Anahtar sözcükler: *Kobalamin, Köpek, Helicobacter, Pernisiyöz anemi, Gastritis*

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INTRODUCTION

Helicobacter spp. are gram-negative, microaerophilic, motile and curved-spiral bacteria found predominantly in the stomach and also intestines and liver of many species [1]. In dogs the isolated Helicobacter spp. from the stomach are *H. felis, H. bizzozeronii, H. salomonis, Flexispira rappini, H. bilis,* and *H. heilmannii* [2]. In humans, *H. pylori*, injures the gastric mucosal barrier, diminishes the parietal cell responsiveness and alters the gastric secretory procedure and results with gastric inflammation, atrophic gastritis, peptic ulcers, and also predisposes humans to the development of gastric cancer [3,4].

In dogs, gastric infection with *Helicobacter* has a prevalence of 67 to 100% in healthy pet dogs, and 74 to 90% in dogs presented with vomiting [5,6] and is characterized by the predominant mucosal cellular infiltrate as lymphocytic, lymphoplasmacytic, eosinophilic, or granulomatous glandular degeneration, enlarged canaliculi and pyknotic parietal cells resulting with gastritis, ulcerations [2,6] nearby development of canine inflammatory bowel disease due to enterohepatic Helicobacter spp. [7].

Cobalamin and iron deficiency anemias are predominant findings in human *Helicobacter* infections [8-10]. There are few suggestions on *Helicobacter* infections cause cobalamine deficiency; *H. pylori* is usually located on the surface of the gastric antral epithelial cells and directly effects the gastric mucosa by either preventing iron uptake and B_{12} or causing an increased loss of iron in the stomach [11]. Another way of pernicious anemia development is by loss of gastric parietal cells, either by autoimmune destruction which are responsible, in part, for the secretion of intrinsic factor, a protein essential for subsequent absorption of vitamin B_{12} in the ileum [9,11]. Severe food cobalamin malabsorbtion and low acid-pepsin secretion are other factors considered [8,12].

Cobalamin is essential for many metabolic functions. It has a major role in DNA replication, in the synthesis of red blood, and in maintaining the myelin sheath that surrounds nerve cells and deficiency of cobalamin leads primarily to central and peripheral neuropathies, immunodeficiency and gastrointestinal abnormalities [13].

Pernicious anemia due to cobalamin deficiency is generally considered in exocrine pancreatic insufficiency and intestinal disease in veterinary medicine. Pernicious anemia often goes undetected and there is a weak correlation between vitamin B_{12} deficiency and mean corpuscular volume (MCV), therefore subjects with low serum vitamin B_{12} level should not be absolutely anemic [14]. According to the literature, there is no research performed on cobalamine levels in *Helicobacter* gastritis in dogs. The aim of this study is to investigate the cobalamin levels in dogs infected with *Helicobacter* spp..

MATERIAL and METHODS

Material of the study were selected from 81 dogs referred to our clinics with probable gastrointestinal system origined clinical complaints including vomiting, neusea, epigastric pain and anorexia, and consisted of 36 female and 22 male (n:58) dogs weighing 15-25 kg, in which *Helicobacter* spp. gastritis was diagnosed with Urea Breath test (^{14}C-UBT). Breed distribution of the subjects was as follows; 33 mixedbreeds, 5 Golden Retrievers, 3 Labradors, 12 Terriers, 4 Turkish shepherd dogs and 1 German shepherd. The mean age of dogs was 4.5 years with a range of 3 to 6 years.

Each of 81 dogs was performed a detailed physical examination and ^{14}C-UBT test. To 58 dogs with grade 2 ^{14}C-UBT test *(Helicobacter* infection positive) gastroscopy, complete blood count, serum biochemistry including blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alkalen phosphatase (ALP), lipase, amylase, total protein and albumin levels were performed. Complete blood counts were studied with Abacus Vet Junior Hematology Analyzer (Diatron, Austria). Serum biochemistry analyses were performed with Idexx Vet 8008 Analyser. All data were recorded into their individual protocols.

^{14}C-UBT test was applied to all subjects. Dogs were starved for 6 h, free from antibiotics and active acid inhibitors for at least a month. Before the test, the animals were made to swallow a ^{14}C- Urea capsule (HELICAP®)-with plenty of water. Following the ingestion of the capsule, 2 mg/kg of 2% Xylazine HCl was administered to the patient for sedation. Within 10 min breath collector was connected to the dry cartridge. Suitable endotracheal tube was applied to sedated patient. The breath is collected with the attached system until the membrane's color of the cartridge changed from orange to yellow within 20 min (approximately this period longs for 20 min). Then, dry cartridge system was scanned at an analyser (Heliprobe Analyser Nosterkibion System 2223- A™), and the results were received in 250 sec. The results evaluated as GRADE 0 meaning negative infection; GRADE 1 meaning suspicious; GRADE 2 meaning infected. Evaluation of the Heliprobe Analyzer Results are presented in *Table 1*. Fifty eight dogs with grade 2 results were included in the study.

Gastroscopy was performed to 58 dogs with grade 2 ^{14}C-UBT test results before and after the therapy, with

Table 1. *Evaluation of the heliprobe analyzer results*
Tablo 1. *Heliprob analizer ile alınan sonuçların değerlendirilmesi*

Grading	Infection Status	d (cpm)
0	Infection negative	d ≤ 25 cpm
1	Suspicious	25 cpm < d < 50 cpm
2	Infection positive	d ≥50 cpm

Olympus XQ20 model endoscopy equipment. Starting from the pharynx, in addition to the systematic examinations of oesophagus and antrum, the examination of the stomach was completed with the scrutinization along the angulus antrum and pyloric canal as a whole.

Serum cobalamin levels were investigated in 38 dogs with microcytic anemia and determined with Immulite 2000 Vitamin B_{12} Macro ELISA kit, according to the manufacturers instructions.

All animals were treated with metronidazole (10 mg/kg three times a day; po) for three weeks, amoxycillin (20 mg/kg three times a day; po) and famotidine (1 mg/kg twice a day; po) and dietary management was scheduled [15]. ^{14}C-UBT test, complete blood count and cobalamine levels were reperformed after ceasion of the therapy.

The assessment of the results has been done using T-test in statistical package program (SPSS, 12.0). The findings have been presented as average values and standard error. For this study, the ethical committee report numbered 2009-38 has been taken from OMU Animal Ethical Committee.

RESULTS

Serum biochemistry including BUN, ALT, AST, GGT, ALP, lipase, amylase, total protein and albumin levels of the dogs were within reference ranges. Complete blood count revealed microcytic anemia in 38 subjects (65.51%). The distributions of "d" values obtained via ^{14}C- UBT method used in the study are presented in *Table 2* [16]. Serum cobalamine levels and MCV and Hgb values of the anemic dogs are presented in *Table 3*. Endoscopy revealed ulcerative-atrophic gastritis in 11 dogs and antral nodules, red patches, erythematous, scabraus mucosal view in the rest of the Helicobacter positive subjects. Clinical and gastroscopic evaluations revealed complete recovery with the disappearance of vomiting, neusea, epigastric pain and anorexia and healthy gastric endoscopic observation.

DISCUSSION

In dogs, various reports had been published on the prevalance of gastric infection with *Helicobacter* spp. in a range of 67 to 100% in healthy pet dogs, and 74 to 90% in dogs presented with vomiting [5,6,17]. In the present study, from the 81 dogs presented with vomiting, epigastric pain, anorexia and neusea to our clinics, ^{14}C-UBT test referred 58 (71.6%) gastric infection with Helicobacter spp.

Urea breath test used in the present study, demonstrates the actual *Helicobacter* colonization, it is the preferred noninvasive method to document a successful eradication in humans and animals [18]. Sensitivity rate of 96.55% was reported for ^{14}C-UBT in dogs in the detection of the existence of spiral bacterium before the designation of species. Wong et al.[19] reported a 94.5%, where Ricci et al.[20] reported a 95-97% sensitivity. Urea test was found 65 times more reliable than Helicobacter staining method, twice as reliable as PCR analysis method, that it is a non-invasive method increases its suitability among other methods used for diagnosis [21]. Similarly, in the study of Kopanski et al.[22] it was reported that urea breath test as a non-invasive choice for detection of Helicobacter infections had higher sensitivity.

Table 2. *The distributions of "d" values obtained via ^{14}C- UBT method used in the study*

Tablo 2. Çalışmada uygulanan ^{14}C- UBT yöntemi ile elde edilen "d" değer dağılımları

^{14}C Isotope Values (cpm)	Group I Before Treatment (n=38) (cpm) (mean±SE)	Group II After Treatment (n=38) (cpm) (mean±SE)
d	97±36 [a]	11±4.1 [b]

** Groups with different letters are significant among themselves (P≤0.001)*

Table 3. *Serum cobalamine levels and mean corpuscular volume and haemoglobin values of the anemic dogs*

Tablo 3. *Anemik köpeklerde serum kobalamin değerleri ile MCV ve Hgb değerleri*

Parameter/Unit	Before Therapy (n=38) (mean±SE)	After Therapy (n=38) (mean±SE)	Range
Cobalamin (ng/L)	117±19[a]	231±41[b]	150-1000
MCV (fl)**	57.66±3.2[a]	63.17±2.1[b]	60-77
Hgb (g/dL)***	10.89±2.6[a]	12.12±0.8[b]	12.00-18.00

** Groups that are assigned a different letter have been found to be statistically significant at the level of P≤0.05; ** Mean corpuscular volume; *** Haemoglobin*

Cobalamin is an essential micronutrient that plays an important role in the differentiation, proliferation and metabolic stability of cells. Cobalamin has fundamental roles in CNS function at all ages, especially the methionine-synthase mediated conversion of homocysteine to methionine, which is essential for nucleotide synthesis and genomic and non-genomic methylation [23]. It has a major role in DNA replication, in the synthesis of red blood, and in maintaining the myelin sheath that surrounds nerve cells and deficiency of cobalamin leads primarily to central and peripheral neuropathies, immunodeficiency and gastrointestinal abnormalities [13,23].

Pernicious anemia is an important disorder caused by cobalamin deficiency and is usually underdiagnosed [24]. Megaloblastic anemia occurs because of impaired DNA synthesis that results from deficiencies of vit B_{12}, folic acid or both [23]. In complete blood counts of the subjects, anemia observed in 65.51% of the dogs was in microcytic character resembling an iron deficiency etiology. This is not unexpected, regarding the reports suggesting a weak correlation between vitamin B_{12} deficiency and MCV and that subjects with low serum vitamin B_{12} level are not

absolutely anemic [14,25]. A likely explanation for the microcytic anemia in the present study may be iron deficiency accompanying gastritis [13,15,26]. Since gastritis both impair dietary iron and cobalamin absorbtion, the factors determining its clinical presentation in the form of microcytic or macrocytic megaloblastic anemia are suggested as age, gender, duration, severity of the disease as the cobalamin stores last longer than iron [26].

In the present study, low cobalamin levels were determined in dogs with gastric *Helicobacter* infection and anemia and following therapy-without any B_{12} supplementation- cobalamin levels and MCV and Hgb values elevated to normal reference limits were observed similar to the literatüre [9]. In human medicine *H. pylori* is determined as the causative agent of vitamin B_{12} deficiency for the last two decades [3,8,9,24].

In the present study, low cobalamin level determined in dogs with gastric Helicobacter infection and anemia is unexpected, since stomach is not the major source of the intrinsic factor in dogs. This cobalamin deficiency may be due to the following factors;

Pepsinogen and gastric acid liberates the dietary cobalamin ingested from the dietary proteins in the stomach and cobalamin immediately bounds to gastric and salivary R-protein (haptocorrin) which transfers cobalamin to duodenum where pancreatic proteases breake down this complex and intrinsic factor bounds to cobalamin, and finally this cobalamin-intrinsic factor complex is readily absorbed from ileal mucosa enterocytes [13,27].

In human medicine one reason of pernicious anemia observed in Helicobacter infections is suggested as the decreased secretion of intrinsic factor by parietal cells [8,28]. This is not valid for the dog, as pancreas is the major source of intrinsic factor and only a minor intrinsic factor secretion is from the stomach [13,29].

Intrinsic factor theory do not explain the cobalamin deficiency in *Helicobacter* infected dogs and we guess this is why B_{12} deficiency is considered only in intestinal and exocrine pancreatic disorders in the dog. However intrinsic factor deficiency is not the only reason for cobalamin decrease in Helicobacter gastritis and especially in the last decade few other predominant factors are stated.

Low acid-pepsin secretion in gastritis results in decreased release of free vitamin B_{12} from food proteins [8]. This maldigestion decreases the cobalamine consumption. Another factor of maldisgetion is that *Helicobacter* antigenically cross react with antral mucosa and parietal cells in dogs [6] leading to food cobalamine malabsorbtion. Also decrease in pepsin secretion and hypochlorhydria due to decreased parietal cell function promotes overgrowth of bacteria that bind vitamin B_{12} for their own use in the hypochlorhydric stomach [30].

As mentioned, R-protein (haptocorrin) has a crucial role in cobalamin metabolism. Haptocorrin location is found in parietal cells in the stomach of rat, human and dog. Haptocorrin in gastric juice comes from both salivary glands and gastric mucosa [27]. The parietal cells located in the upper third of the fundic gland secrete hydrochloric acid, R-protein and intrinsic factor in dog [13] and therefore impairment of parietal cell function due to gastritis effects cobalamin metabolism.

Return to normal cobalamin levels after therapy without and cobalamine supplementation may be explained by the remission of the above factors.

This is the first study demonstrating cobalamin deficiency in *Helicobacter* infections in dogs to the authors' knowledge. Up to date, cobalamine deficiency is only considered in exocrine pancreatic insufficiency and intestinal disorders, perhaps due to pancreatic secretion of intrinsic factor in dogs. However there are other contributing factors as mentioned above, in the etiology of cobalamine decrease in *Helicobacter* gastritis and the predominant factor in dogs playing role in this deficiency needs further investigation. Cobalamin level must be determined in dogs with *Helicobacter* infections and in deficiencies which are still present after therapy, essential supplementation would be clinically beneficial. In conclusion, this study demonstrates that pernicious anemia must be a considered factor in *Helicobacter* gastritis with microcytic anemia in dogs and further investigation will be beneficial to demonstrate the cobalamin levels in dogs with or without microcytic anemia in *Helicobacter* gastritis.

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Research Article

Comparison of the Common Immunogenic Protein Components of *Pasteurella multocida* Serotypes B:2 and B: 3,4

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Abstract

Serotype B of *Pasteurella multocida* is a major agent of haemorrhagic septicaemia (HS), a form of blood poisoning widespread in cattle and buffalo from Southern Asia. The vaccines currently used against the disease are not potent enough, being effective only for a limited duration only. In an attempt to identify immunogenic components of *P. multocida* for use as live/subunit vaccines, the outer membrane proteins (OMPs) of B:2 and B:3,4 serotypes were isolated and their profiles compared. Three common immunogenic components; polypeptides of molecular weight (MW) 39.0, 33.5, 31.0 kDa, were identified by immunoblotting. The immunogenicity of identified OMPs was also confirmed using antisera generated against them. The antigenic OMPs identified in current research could be explored in the formulation of new, safe and effective vaccines for indigenous buffalo and cattle breeds with a prolonged efficacy.

Keywords: *Cattle, OMP, Vaccine, Haemorrhagic septicemia*

Pasteurella multocida B:2 ve B:3,4 Serotiplerinin Ortak İmmunojenik Protein Komponentlerinin Karşılaştırılması

Özet

Pasteurella multocida B serotipi Güney Asya'daki sığır ve mandalarda yaygın olarak görülen ve kanda septisemi ile seyreden hemorajik septisemi (HS)'nin en önemli nedenidir. Hastalık için günümüzde kullanılan aşılar yeteri kadar etkili değildir ve sadece sınırlı bir sürede etkisini göstermektedir. Canlı/subunit aşılarda kullanılmak üzere *Pasteurella multocida*'nın immunojenik komponentlerinin belirlenmesi için yapılan çalışmamızda, B:2 ve B:3,4 serotiplerinin dış membran proteinleri (OMP) izole edilmiş ve bunların profilleri karşılaştırılmıştır. İmmunbloting sonucunda 39.0, 33.5 ve 31.0 kDa moleküler ağırlıklarında 3 adet ortak immunojenik polipeptit belirlenmiştir. İdentifiye edilen dış membran proteinlerinin immunojenitesi ayrıca bunlardan elde edilen antiserumlar ile doğrulanmıştır. Bu çalışmada identifiye edilen antijenik OMP'ler uzun süre etki gösteren yeni, güvenli ve etkin aşıların formülasyonlarının geliştirilmesine katkı sağlayacaktır.

Anahtar sözcükler: *Sığır, OMP, Aşı, Hemorajik septisemi*

INTRODUCTION

Haemorrhagic Septiceamia (HS), a widely distributed animal disease of tropical countries, is mainly associated with Asian and African countries. Prevalence of HS is mostly observed with moist conditions with most probably disease spread during winter season resulting in high morbidity and mortality rate. Several serotypes of *Pasteurella multocida* (Gram negative bacilli) are responsible for disease pathogenicity. Clinical signs included high temperature, respiratory distress with nasal discharge and frothing from the mouth, leading to death.

Cattle and buffalo are the most susceptible host for HS, buffalos as compared to other animals [1]. The major *P. multocida* pathogenicity factors include polysaccharide capsule, endotoxins or lipopolysaccharide (LPS) and outer membrane proteins (OMPs) playing an important role for conferring protective immunity in a wide range of hosts [1-4]. There is a clear correlation between capsular type and the disease; however, contribution to the disease specificity, of a particular type of lipopolysaccharide or the type and amount of bacterial surface proteins is still unclear [4-7]. Molecular techniques have been employed to understand pathogenicity and epidemiology of *P. multocida* antigenic

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components [7-11]. The common serological methods, based on capsular and somatic antigen, are; indirect haem-agglutination (IHA) test for capsular serotypes detection and agglutination/agar gel immunodiffusion (AGID) tests for somatic serotyping. Capsular type B belongs to Asian countries and serotype B:2 is the main disease causing agent. Serotypes B:3, B:4 and B:3,4 cause HS in cattle and wild ruminants producing signs and lesions similar to those by serotypes B:2 in water buffaloes and cattle [12].

An effective treatment of sulphur drugs with antibiotics was reported, but some strains of *P. multocida* show resistance against antibiotic [13,14]. Immune serum therapy was considered to be ineffective. Commonly, immunity against HS are provided by using three common vaccines namely, (i) bacterins (ii) alum-precipitated vaccine (APV) and (iii) oil-adjuvanted vaccine (OAV). *P. multocida* serotype B:3, 4 isolated from a fallow deer in the United Kingdom was used in manufacturing live heterotypic vaccine. The vaccine protected cattle vaccinated subcutaneously against a serotype B:2 challenge and conferred immunity against HS for a year [15]. Cross-protection by *P. multocida* serotype B:3,4 in a live intranasal vaccine against a subcutaneous challenge with serotype B:2 has been confirmed in Myanmar [16]. This study revealed outer membrane proteins (OMPs) as common immunogenic components (MW 39.0, 33.5, 31.0 kDa) of *P. multocida* serotypes B:3,4 and B:2, rendering B:3,4 a successful candidate for use as a live vaccine against haemorhhagic septicaemia. Moreover the polypeptides identified could be used in future for the development of sub unit vaccine

In Pakistan, cattle and buffalo populations are at high risk of HS infection owing to high animal losses. In an attempt to provide animals protection against disease, a comparative study was conducted to identify local pathogenic strain specific immunogenic components use as vaccine. The identified immunogenic polypeptides could be explored for subunit vaccine production in future.

MATERIAL and METHODS

For culturing purposes, *P. multocida* strains; B:2 and B:3,4, were obtained from Bacteriology Laboratory, Animal Sciences Institute (ASI), National Agricultural Research Centre (NARC) Islamabad, Pakistan. After reconstitution in normal saline, 0.5 mL of each strain was injected into mice subcutaneously (2 mice for each isolate) maintained in animal house for 24 h. Blood samples were collected from treated animals through cardiac puncture under anaesthesia. Blood was streaked on Trypton Soya Agar medium plates (Oxoid Ltd., England) and incubated at 37°C for 24 h. Positive colonies were initially selected by Gram staining and, for further confirmation, API 20NE identification kit (Biomerix 20NE System) was used. Mass cultivation of pathogenic organisms was performed in 250 mL flasks containing 100 mL brain heart infusion (BHI) broth kept in shaker incubator at 37°C for 24 h. Liquid cultures were centrifuged at 10.000 rpm for 30 min at 4°C; pellet was re-suspended in normal saline overnight at 4°C followed by centrifugation at 10.000 rpm for 20 min at 4°C. The resulting pellet was again suspended in normal saline, centrifuged and suspended in 10 mM N-(2-hydroxyethyl) piperazine-N-2 ethane sulfonic acid (HEPES) buffer, pH 7.4 (a ratio of 0.1 g pellet wet weight/mL of extraction buffer). The suspended cellular pellet was sonicated (Sonicator G-055 UP 400S) at 100% amplitude for 2 min with 30 sec intervals for cell disruption. The sonicated supernatant was then centrifuged at 1.700×g for 20 min at 4°C and supernatant containing whole cell proteins was divided into aliquots for storage. The study was ethically approved.

Preparation of OMP Enriched Fraction

The OMPs were extracted by the method of Choi *et al*.[17]. The supernatant was ultra-centrifuged at 100.000×g /2.8500 rpm at Beckman Coulter L-100XP ultracentrifuge using SW41 Ti-Rotor for 1 h at 4°C. The pellet was collected and re-suspended in 2% sodium lauryl sarcosinate detergent and then incubated at 22°C for 1 h. The solution was ultracentrifuged at 100.000×g for 1 h at 4°C. The pellet was kept in distilled water overnight and ultra-centrifuged followed by another washing step and dissolved in Nano-pure water. Next the pellet was dialyzed against distilled water for 8 h using dialysis membrane of 6.000-8.000 Dalton. The final protein concentrations of 3.05 mg/mL for B: 2 strain and 1.1 mg/mL of B:3,4 strain were obtained. The protein rich extract was then separated by SDS-PAGE [18] using 4% stacking and 12% separating gels visualised using Coomassie brilliant blue stain. Molecular weights of proteins were determined through Rf values (Rf = band distance/distance covered by dye front). Molecular weights obtained through plots were then log transformed.

Hyperimmune Sera Production

Antisera were raised in two groups of rabbits (3 animals per group, two for each strain and one as a control) while two groups of animals were used for raising antisera in calves [19]. The blood of infected animals was collected and serum separated by centrifugation at 3.000 rpm for 10 min.

Immunoblotting of Anti-Sera Proteins

Effectiveness of raised antisera was checked using dot blot method with standard rabbit antisera (Chinchilla breed). Three experiments were performed: one with B:2 and B:3,4 antigens (OMPs and LPSs) and B:2 antisera raised in rabbits; other with B:2 and B:3,4 antigens with B:3,4 antiserum raised in rabbits; third with B:2 and B:3,4 antigens (OMPs and LPSs) and B:2 antisera raised in calves. The primary antisera (raised in rabbits) and secondary anti-body (goat anti-rabbit IgG Alkaline Phosphatase conjugated) dilutions were prepared in TEN buffer. The substrate contained nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphate buffer (APS).

Immunoblotting was performed using standard protocol and transblot apparatus by the protocol given on the manufacturer's website [20] followed by SDS-PAGE using pre-stained markers (SM 0671, Fermentas). Blotting was performed using E844 CONSORT Electrophoretic power supply at 4°C. Two dilutions (1:50 and 1:100) of B:3,4 antiserum were used as the primary antibody following the same conditions as mentioned above. The 1:100 dilution of primary antibody (B:2 antiserum in calf) was prepared using 3% bovine serum albumin (BSA), incubated for 2 h at room temperature with constant shaking. The BSA was removed and antiserum washed thrice with 15 mL of 0.1% phosphate buffered saline with tween (PBST). The horse-radish peroxidase conjugated secondary antibovine IgG antibody (1:1000 dilution) and primary antibody (1:50 dilution) were added followed by three washings with 0.1% PBST. The filter was placed in Diaminobenzidine (DAB) substrate solution and reaction was stopped by rinsing with water. The images were scanned and stored for further analysis. In another experiment, the 1:50 and 1:100 dilutions of B:3,4 antiserum were used as the primary antibody following the same conditions as mentioned above.

LPS Profiling

For LPS extraction from B: 3,4 and B:2 strains was performed using O.I. E Manual (2004) and enzymatic method Bastuji *et al.*[21] respectively. Bacterial strains were mass cultivation using BHI broth and cells were harvested. The extracted LPS were analysed on 4% stacking gel and 12.5% separating gel, along with L4130 as standard LPS marker and then silver stained [21]. The gels were then transferred to nitrocellulose membranes following the method described by Towbin *et al.*[22].

RESULTS

The B:2 and B:3,4 strains of *P. multocida* were found to be Gram negative coccobacilli as they gave pink colour after Gram staining under microscope. The data gathered from kit-based tests was transformed with a numerical value of 3.000,004 and was confirmed in the Analytical Profile Index.

The OMPs concentration was found to be 3.05 mg/mL for B:2 strain and 1.1 mg/mL of B:3,4 strain. The SDS-PAGE results showed five polypeptides of 94.8, 56.5, 33.5, 31.0 and17.06 kDa for strain B:3,4 whereas five polypeptides of 71.61, 33.5, 31.0, 23.44 and 17.06 kDa *(Table 1)* for strain B:2. Among these, three polypeptides (33.5, 31.0, and 17.0 kDa) were common in both of the strains *(Fig. 1)*. As far as the intensity of bands was concerned, the polypeptide having MWof 33.5 kDa showed the most intense band among bacterial strains.

The dot blot results were positive for all the dilutions (1:100, 1:200 and 1:400) of primary antisera (raised against

Table 1.*Calculation of OMPs molecular weights using Rf values and log of molecular weight plot*

Tablo 1. *OMP'lerin molekül ağırlıklarının Rf değerleri ve moleküler ağırlık plot logaritması kullanılarak hesaplanması*

Specimen ID	Distance Covered by Bands (cm)	Rf Values	MW (kDa)	Log MW
SM0431	0.6	0.6/8 = 0.075	116.0	2.064
	1.2	1.2/8 = 0.15	66.2	1.820
	2.2	2.2/8 = 0.275	45.0	1.653
	2.9	2.9/8 = 0.362	35.0	1.544
	4.1	4.1/8 = 0.512	25.0	1.397
	4.9	4.9/8 = 0.61	18.4	1.264
	5.3	5.3/8 = 0.662	14.4	1.158
B:3,4	0.6	0.6/8 = 0.075	94.83	1.976
	1.9	1.9/8 = 0.237	56.5	1.752
	3.2	3.2/8 = 0.4	33.5	1.52
	3.4	3.4/8 = 0.425	31.0	1.491
	4.9	4.9/8 = 0.612	17.06	1.232
B:2	1.3	1.3/8 = 0.1625	71.61	1.855
	3.2	3.2/8 = 0.4	33.5	1.52
	3.4	3.4/8 = 0.425	31.0	1.491
	4.1	4.1/8 = 0.512	23.44	1.370
	4.9	4.9/8 = 0.612	17.06	1.232

Distance covered by dye front= 8.0 cm

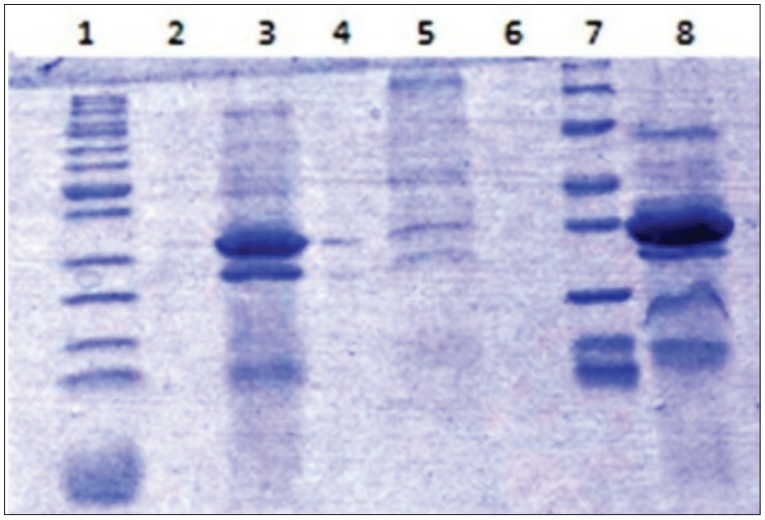

Fig 1. Outer membrane protein profiles of *Pasteurella multocida* strains B:2 and B:3,4

Lane 1: Protein marker (SM 0661), Lane 3 and 5: B:3,4 (1:2 dilution), Lane 7: Protein marker (SM 0431), Lane 8: B:2(1:2 dilution)

Şekil 1. *Pasteurella multocida* B:2ve B:3, 4 suşlarının dış membran protein profilleri

Kolon 1: Protein markeri (SM 0661), Kolon 3 ve 5: B:3,4 (1:2 sulandırma), Kolon 7: Protein markeri (SM 0431), Kolon 8: B:2(1:2 sulandırma)

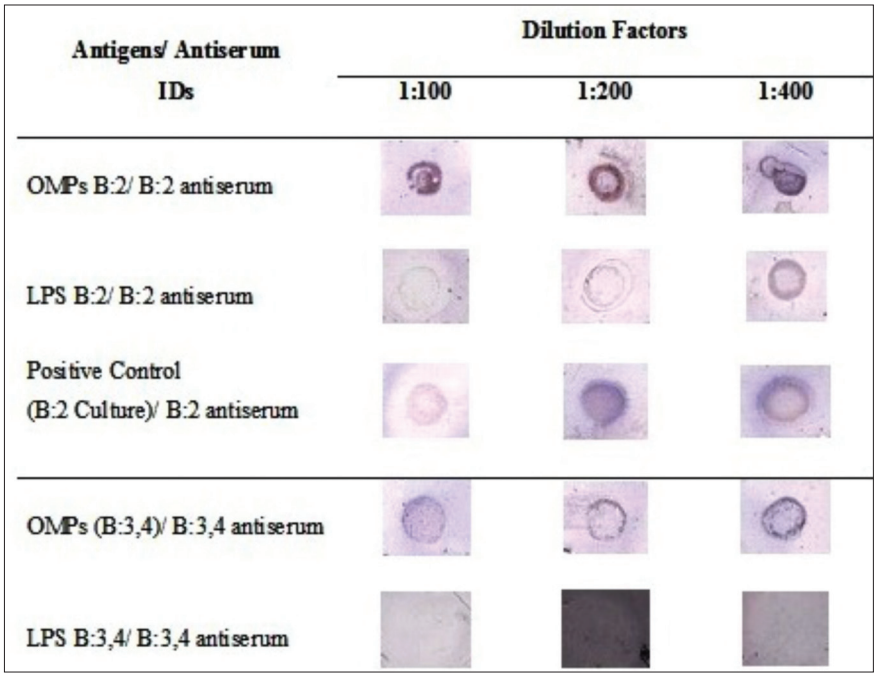

Fig 2. Dot blot results of B:2 and B:3,4 antigens against B:2 antiserum raised in rabbits

Şekil 2. Tavşanlardan elde edilen B:2 antiserumuna karşı B:2 ve B:3,4 antijenlerinin Dot-Blot sonuçları

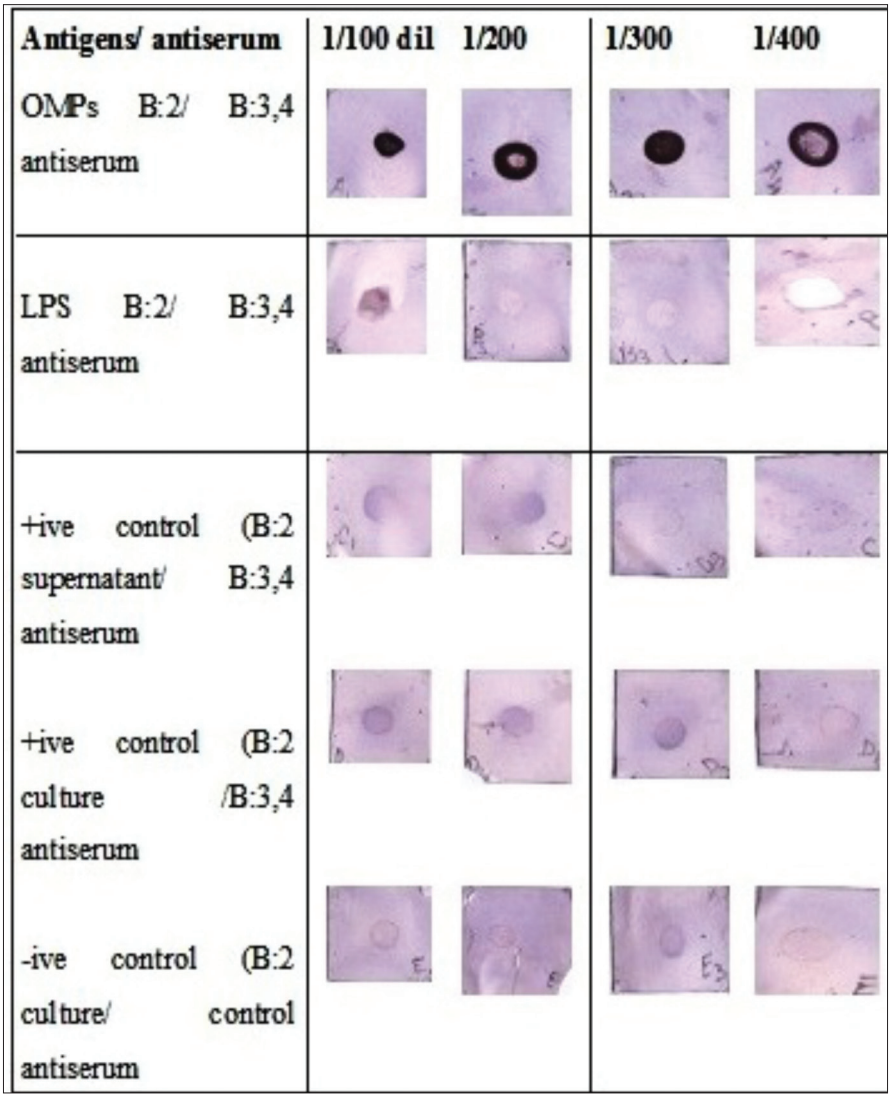

Fig 3. Cross reactivity of B:2 antigens against B:3,4 antiserum raised in rabbits

Şekil 3. Tavşanlardan elde edilen B:3,4 antiserumuna karşı B:2 antijenlerinin çapraz reaktivitesi

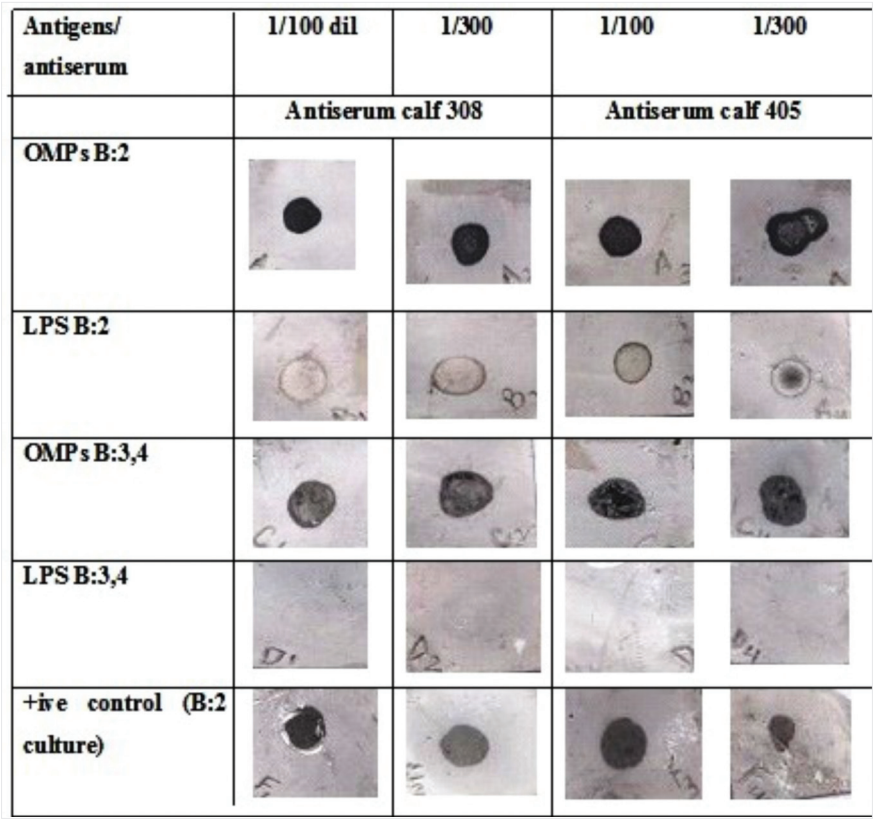

Fig 4. Dot blot results of B:2 and B:3,4 antigens against B:2 antiserum raised in calves

Şekil 4. Buzağılardan elde edilen B:2 antiserumuna karşı B:2 ve B:3,4 antijenlerinin Dot-Blot sonuçları

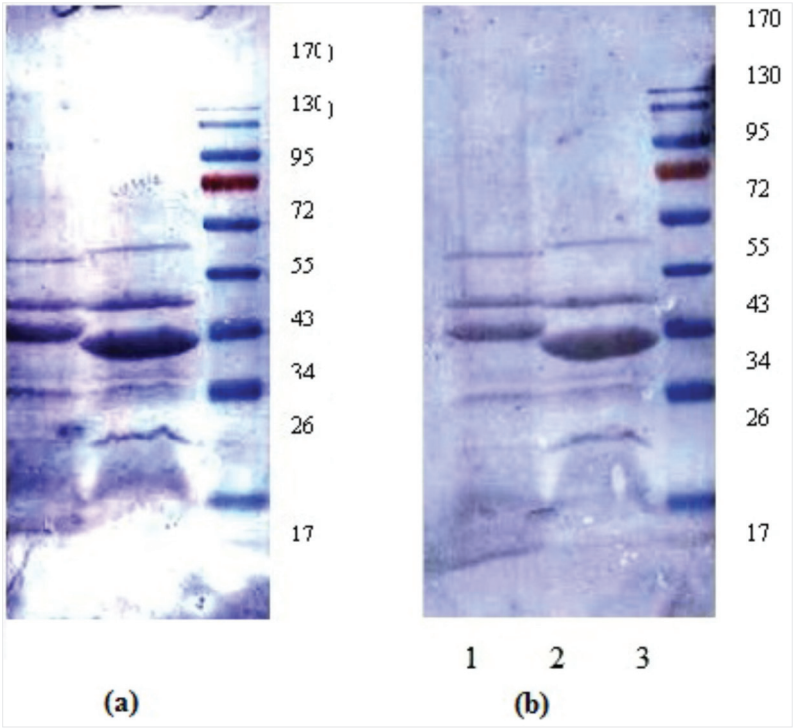

Fig 5. Immunoblot result of *P. multocida* B:2 and B:3,4 OMPs against B:2 antiserum raised in calves; **a-** 1:50 dilution, **b-** 1:100 dilution

Lane 1: B:3,4 OMPs, Lane 2: B:2 OMPs, Lane 3: Pre-stained protein marker (SM0671)

Şekil 5. Buzağılardan elde edilen B:2 antiserumuna karşı *P.multocida* B:2 ve B:3,4 dış membran proteinlerinin immunoblot sonucu; **a-** 1:50 sulandırma, **b-** 1:100 sulandırma

Kolon 1: B:3,4 OMPs, Kolon 2: B:2 OMPs, Kolon 3: Ön-boyamalı protein markeri (SM0671)

P. multocida B:2 and B:3,4 in rabbits). The OMPs of B:2 and B:3,4 strains reacted with all three dilutions of their respective antiserum (self reactivity) whereas 1/400 dilution was positive for B:2 LPS only *(Fig. 2, Fig. 3)*. The antisera raised in buffalo calves and B:2 antiserum dilutions (1/100 and 1/300) from two groups of animals was also checked for its efficacy against its own antigens (B:2 OMPs and LPS i.e., self reactivity) and antigens of the other strain, B3,4. (cross reactivity). The results were positive for OMPs with both the dilutions whereas B:2 LPS gave positive result only with 1/300 dilution only *(Fig. 4)*.

The immunoblot of OMPs confirmed common bands 33.5, 31.0 and 17.0 kDa among B:2 and B:3,4 strains of *P. multocida* as revealed by SDS-PAGE. The polypeptides having molecular weight of 39.0, 33.5 and 31.0 kDa were found immunogenic in B:2 and B:3,4 strains as revealed by western blotting. The band of molecular weight of 39.0 kDa was not obvious in the gel but was visualised in Western blotting thus proving that this method is more sensitive in detecting immunogenic proteins than Coomassie blue staining *(Fig. 5)*.

As in case of LPS, dot blot results were found positive for 1/100 and 1/300 dilution of primary antisera with 1/1000 dilution of secondary antibody though immunoblots with LPS were unsuccessful.

DISCUSSION

Asian and African cattle and buffalo are highly susceptible to HS infection resulting from *P. multocida*. The disease prevalence, morbidity and mortality rate is high in tropical environments including certain regions of Pakistan. Several serotypes of *P. multocida* exist ranging in disease pathogenicity. Serotype B:2 is the common disease causing agent in Asian water buffaloes while B:3, B:4 and B:3,4 serotypes cause HS in cattle and wild ruminants [4]. Disease pathogenicity arises from polysaccharide capsule, endotoxins or LPS whereas *P. multocida* OMPs especially B:2 serotype are reported for conferring immunity against disease [2,4,23]. Therefore, detection of immunogenic proteins common to B:2 and B:3,4 serotypes of *P. multocida* are being explored as future targets for subunit vaccine production against HS infection.

The OMPs of B:2 and B:3,4 serotypes of *P. multocida* were studied for their immunogenicity. The SDS-PAGE profiling of OMPs from B:2 and B:3,4 serotypes identified five proteins with three common bands; 33.5, 31.0, and 17.06 kDa. The polypeptide with 33.5 kDa showed the most intense band between both bacterial strains. No research has been reported regarding the comparative analysis of B:2 and B:3,4 strains. As far as the band intensity is concerned, Jain *et al.*[24] reported 31.7 and 34.9 kDa as the major OMPs among nine protein bands with MW ranging from 21.1-89. 2kDa. Kedrak and Opacka [25] found protein bands of 22 to 86kDa in the OMP profile of serotype B:2 while Pati *et al.*[23] reported ten protein bands ranges between MW of 25 to 88 kDa. In another study, Johnson *et al.*[26] demonstrated polypeptides of 32 kDa as the major OMP. With a little difference from our findings, Tomer *et al*.[27] concluded 31, 33 and 37kDa as the major OMPs while comparing the outer membrane protein profiles of *P. multocida* B:2 isolates.

The immunogenic and protective role of OMPs against HS infection has been elucidated previously [22,25,28]. With regards to the immunogenicity of OMPs of *P. multocida* B:2 and B:3,4, both strains reacted with respective antiserum raised in rabbits, thus confirming cross-reactivity. Western blotting analyses revealed three polypeptides from B:2 and B:3,4 strains; 39.0, 33.5 and 31.0kDa possessing immunogenicity against infection *(Fig. 5)*. Based on quantitative analysis of gel bands, Tomer *et al*.[16] also reported three polypeptides MW of 31, 33 and 37 kDa, with 37 kDa fraction being highly antigenic. Whereas other study reported polypeptides of 44, 37 and 30 kDa being major immunogens among ten polypeptide bands of 25 to 88 kDa in a study on outer membrane protein from *P. multocida* serotype B:2 [24].

The efficacy of antisera raised in both groups of animals (rabbits and calves) is indicative of the immunogenic nature of both the antigens (OMPs and LPS). Experiments showing cross reactivity of antisera with antigens reveal the common nature of the mentioned immunogenic components also evident from SDS-PAGE analysis of OMPs where three common bands (MW of 33.5, 31.0 and 17.0 kDa) were found among B:2 and B:3,4 strains. Therefore, present study clearly revealed outer membrane proteins (OMPs), as common immunogenic components of *P. multocida* serotypes B:2 and B:3,4 for use as a live vaccine against haemorrhagic septicaemia. Moreover, the polypeptides identified could be employed for the development of subunit vaccine(s).

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Research Article

The Importance of Ejaculate Volume for the Physical Parameters of Ejaculates and Sperm Morphology of Hypor Boars

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Abstract

The study aimed at analysing the effects of ejaculate volume on the physical parameters of ejaculates and the sperm morphology of Hypor boars. The analyses involved 114 ejaculates collected from 12 Hypor insemination boars. The ejaculates were classified according to the criterion of ejaculate volume. Three groups were specified: ejaculates with a volume of 251 ml or lower (Group I), ejaculates with a volume of 252-310 ml (Group II), and ejaculates with a volume of 311 ml or higher (Group III). The ejaculates were assessed to identify the basic physical traits and determine the incidence of morphological abnormalities in the spermatozoa, specifying major and minor abnormalities. Furthermore, the morphological structure indices for the spermatozoa were also calculated. Rising ejaculate volume accompanied with a rise in the total number and motility of spermatozoa, and a simultaneous slight fall in sperm concentration in the ejaculates. The ejaculates with the highest volumes turned out to contain more morphologically well-formed spermatozoa. We also determined that rising ejaculate volume is accompanied with increasing sperm dimensions, especially those of the head. The increased parameters were the length and the width of sperm heads, as well as their perimeters and areas. Ejaculate volume has an impact on the shape of Hypor boar spermatozoa. As the ejaculate volume increases, the shape of sperm heads becomes increasingly more oval. Additionally, spermatozoa in ejaculates with greater volumes have larger heads in relation to flagellum length.

Keywords: *Boar, Ejaculate volume, Morphometric traits, Semen, Spermatozoa*

Hypor Domuzlarında Sperm Morfolojisi ve Ejakülatların Fiziksel Özellikleri Yönünden Ejakülat Hacminin Önemi

Özet

Çalışmada Hypor erkek domuzların sperm morfolojisi ve ejakülatın fiziksel özellikleri üzerine ejakülat hacminin etkilerini incelemek amaçlandı. Bu amaçla, 12 adet Hypor ırkı suni tohumlama domuzundan alınan 114 ejakülat incelendi. Ejakülatlar hacme göre gruplandırıldı: Numuneler, 251 ml ve altında (Grup 1), 251-310 ml (Grup 2) ve 310 ml ve üstünde (Grup 3) olarak ayrıldı. Sperma, major ve minor morfolojik sperm anomalilerinin sıklığı ve temel fiziksel özellikleri yönünden değerlendirildi. Ayrıca, spermatozoa'nın morfolojik yapısına ait değerler saptandı. Ejakülat hacmi artış ve ejakülasyondaki sperm konsantrasyonunda az miktarda düşüş ile, motilite ve toplam sperm sayısında bir artışla beraber bulundu. En yüksek hacimli ejakülatlarda düzgün yapıda morfolojiye sahip daha fazla sayıda spermatozoa saptandı. Ayrıca, daha yüksek ejakülat hacmiyle birlikte spermatozoonların özellikle baş kısmında olmak üzere ebatlarının büyüdüğü saptandı. Spermatozoonun baş uzunluğu ve genişliği ile çevre uzunluğu ve yüzey alanı büyüdü. Ejakülat hacmi Hypor domuz spermatozoonlarının şekli üzerinde bir etkiye sahipti. Ejakülat hacminin artmasıyla spermatozoa başı daha oval bir şekil aldı. Ek olarak, hacmin artmasıyla ejakülattaki spermatozoa kuyruk uzunluğu ile daha uzun başa sahip olma arasında bir ilişki saptandı.

Anahtar sözcükler: *Erkek domuz, Ejakülasyon hacmi, Morfolojik özellikler, Sperma, Sperm*

INTRODUCTION

The efficacy of insemination largely depends on the quality of spermatozoa inferred from analyses of sperm morphology [1,2]. Sperm quality can be also determined on the basis of an analysis of the damage to the sperm membrane [3], the state of chromatin structure, the anti-oxidative potential of the spermatozoa, apoptotic changes or on the basis of sperm ATP level assays [4,5].

Morphological abnormalities in spermatozoa reduce male fertility [6]. Particular individuals exhibit differences in the quality of spermatozoa they produce. This includes difference in the incidence of spermatozoa with morpho-

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logical abnormalities [7]. The shape and dimensions of spermatozoa are also important. Their dimensions and shapes determine their motility and capacity to penetrate the egg [8]. This issue has been dealt with by numerous researchers who concentrated on the variation in morphometric parameters of spermatozoa, depending on the physical traits of the ejaculates produced by the sires [9,10]. It is believed that the presence of spermatozoa with head abnormalities in the semen can be the reason for reduced embryo quality [11] and miscarriages in the initial period of pregnancy [12].

The size and shape of the sperm head is a species-related trait. However, differences have been identified between males representing different breeds of the same species, or even between particular individual animals [13]. Some scientists believe that motor parameters of spermatozoa depend on their sizes and shapes [14-16]. This has impact on the competitiveness of spermatozoa in the reproductive organs of the female. Faster and viable spermatozoa undergo spontaneous selection following capacitation. Spermatozoa negotiate a long way in the reproductive tract of the female, surmounting the immunological barrier, disadvantageous pH, complex oviduct topography and the untoward conditions prevalent there [17].

The volume of ejaculated semen varies among animals depending on nutrition, genetics, breed and management [18]. High-volume ejaculates are considered to be particularly useful for insemination because they make it possible to prepare numerous insemination doses containing the required numbers of spermatozoa. Ejaculate volume is also important for fertilization efficacy and embryo survival [19]. Semen volume affects the distribution of spermatozoa [20]. Increased ejaculate volume positively influences the transport of semen by stimulating the central layer of the uterine accelerate its contractions and inducing the pituitary body to release hormones that stimulate contractions of the smooth muscles of the matrix [19]. Ejaculate volume may also affect the quality dimensions and shape of sperms, as well as their motor parameters that determine their fertilizing ability. Currently, there are studies that indicate a connection between the sperm morphometric parameters and concentration in the ejaculates of boars [9].

The present study was aimed at analysing the relationship between ejaculate parameters and sperm morphology and the volume of ejaculates produced by Hypor boars, on the basis of physical traits of the ejaculates, morphometric measurements of the spermatozoa, and an evaluation of the incidence of morphological sperm abnormalities.

MATERIAL and METHODS

The study concerned 114 ejaculates collected from 12 Hypor boars used at three insemination centres. The boars (aged 8 to 18 months), were managed in accordance with the rules of animal welfare [21]. The individual pen area was 10 m^2/boar, and the pen had a concrete slatted floor. The boars were fed according to Swine Nutrition Requirements [22], with *ad libitum* access to water. Temperature, relative air humidity, and atmospheric pressure were measured during semen collections. Temperature was measured with a precision of one degree Celsius. Humidity, expressed as a percentage, was measured with a precision of one percentage point. Temperature and humidity was recorded using a thermo-hygrometer TERMIK PLUS (1000209, Termo-produkt, PL). Atmospheric pressure was measured using an ADLER barometer (Bar 003, Demus, PL) with a resolution one hPa. Relative humidity was close to 70%. The air temperature in the boar pens was 16ºC (average minimum 13ºC and maximum 21ºC). Air pressure inside the buildings averaged 1005 hPa (min. 987 hPa, max. 1016 hPa). Ejaculates were collected using the manual method [23] in one-month intervals over a period of nine months. A total of 114 ejaculates were collected from July 2013 to April 2014. Each boar provided at least 10 ejaculates for the analysis. The ejaculates were grouped according by volume as follows:

Group I : ejaculates with a volume below 251 ml - 32 ejaculates,

Group II : ejaculates with a volume between 251 ml and 310 ml - 38 ejaculates,

Group III : ejaculates with a volume above 310 ml - 44 ejaculates.

The following physical parameters were determined in the freshly collected ejaculates: ejaculate volume (ml), sperm concentration (x10^6/ml), sperm motility (%), total number of spermatozoa (x10^9), and number of insemination doses per ejaculate (n). Ejaculate volumes were determined by weight, without the gelatinous fraction, using electronic scales. Sperm concentration in the ejaculates was determined with a photometric method, using a spectrophotometer (IMV Technologies, France). Sperm motility was evaluated with a Nikon Eclipse 50i light microscope equipped with a heated stage. A sample of 5 µl of sperm suspension was placed on a pre-warmed slide and sealed with a coverslip at 37ºC. Under 200x magnification, the percentage of normally motile spermatozoa was determined in the overall number of sperms present in the field of vision of the microscope. The total number of motile spermatozoa and the number of insemination doses per ejaculate were calculated using SYSTEM SUL (v. 6.35; Gogosystem, Poland) software package.

Semen samples from the collections were used to prepare microscopic slides. The slides for morphological analyses were stained using eosin and gentian violet, according Kondracki et al.[24]. Microscopic analyses of the smears were performed under 100x magnification with immersion lenses, using the Nikon Eclipse 50i light microscope. The morphology of 500 spermatozoa was assessed per slide, identifying the number of well-formed and malformed spermatozoa and differentiating those

with primary and secondary changes, according to Blom's classification [25].

Also, sperm morphometric measurements were carried out on 15 randomly selected normal spermatozoa in each slide. The measurements were performed using a suite for computer image analysis (Screen Measurement v. 4.1), according to methodology proposed by Kondracki et al.[26]. The following sperm measurements were taken: head length (µm), head width (µm), head area (µm^2), head perimeter (µm), flagellum length (µm), and total length (µm). The following morphological indices were calculated on the basis of the measurements:

head width/head length,
head length/total length,
head length/flagellum length,
flagellum length/total length,
head perimeter/total length,
head area/total length,
head length x width/total length.

Experimental data were analysed using a program STATISTICA® 10 PL (StatSoft, Tulsa, USA) [27]. All results are expressed as mean (X) ± standard deviation (Sx). The obtained material was statistically analysed according to the following mathematical model:

$$Y_{ij} = \mu + a_i + e_{ij}$$

Where: Y_{ij} is the value of the analysed parameter, µ is the population mean, a_i is the effect of ejaculate volume, e_{ij} is the error. The significance of the differences between the groups was assessed with the Tukey test at $P \leq 0.05$ and $P \leq 0.01$.

RESULTS

Table 1 contains data on the physical parameters of the Hypor boar ejaculates in relation to ejaculate volume. The data reveal that ejaculate physical parameters are correlated with ejaculate volume. An increase in ejaculate volume was accompanied by a decrease in sperm concentration and an increase in sperm motility. With an increase in volume, the total number of sperm and the number of insemination doses per ejaculate significantly increased too. Group I, which comprised the lowest-volume ejaculates, showed the highest sperm concentrations, which averaged 430.66 x 10^6/ml and were by more than 18 x 10^6/ml higher than in Group II, and by nearly 29 x 10^6/ml higher than in Group III, the one with the highest ejaculate volumes. These differences were not, however, statistically confirmed.

The data in *Table 1* show that spermatozoa in the higher-volume ejaculates have greater progressive motility. Group III, comprising ejaculates with the highest volume, was found to contain spermatozoa with the highest motility. The percentage of motile spermatozoa in the ejaculates from this group was more than 3% higher than in Group II ($P \leq 0.01$) and almost 5.5% higher than in Group I ($P \leq 0.01$). The highest sperm counts were found in Group III. The ejaculates in this group contained over 114 billion spermatozoa with progressive motility, over 26 billion more than those in Group II ($P \leq 0.01$) and approximately 40 billion more than those in Group I, with the lowest volumes ($P \leq 0.01$). The number of spermatozoa in the ejaculate also determines the number of insemination doses that can be prepared out of the ejaculate. The most numerous doses were prepared from the ejaculates in Group III, those with the highest volumes. Each ejaculate in this group provided more than 37 insemination doses, approximately 8.4 doses more than the ejaculates in Group II and over 13 doses more than the ejaculates in Group I ($P \leq 0.01$). *Table 2* contains the results of the analysis of morphological abnormalities in the spermatozoa.

The mean percentage of normally formed spermatozoa remained within the range from 94.72% to 96.90%. The fewest spermatozoa with normal morphology were found in the ejaculates in Group III. The data presented in *Table 2* show Hypor boar semen quality to be very high. The mean percentage of spermatozoa with major morphological abnormalities did not exceed 1.71%. The differences

Table 1. *Physical traits of ejaculates in relation to ejaculate volume*
Tablo 1. *Ejakülat hacmine ilişkin ejekülatların fiziksel özellikleri*

Specification		Groups & Ejaculate Volume (ml)		
		I (< 251)	II (251-310)	III (> 310)
Number of ejaculates (n)		32	38	44
Ejaculate volume (ml)	X±Sx	217.19±33.14^A	284.47±15.54^B	364.09±46.37^C
Spermatozoa concentration (x 10^6/ml)	X±Sx	430.66±131.65	412.50±129.98	401.86±103.20
Percentage of spermatozoa with progressive motility (%)	X±Sx	73.44±4.82^a	75.79±5.00^b	78.86±3.21^c
Total number of spermatozoa (x 10^9)	X±Sx	75.21±38.57^a	88.39±25.15^b	114.58±18.54^c
Number of insemination doses per ejaculate (n)	X±Sx	24.22±8.49^A	29.37±8.78^B	37.75±8.00^C

^{a,b} Differences between average values, represented by different letters in the same row, are important ($P \leq 0.05$); ^{A,B} Differences between average values, represented by different letters in the same row, are important ($P \leq 0.01$)

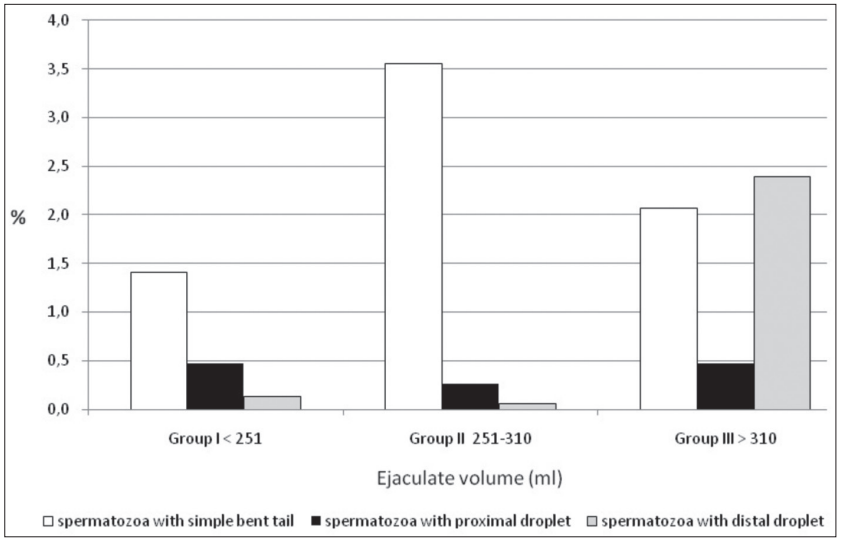

Fig 1. Frequency of occurrence of chosen anomaly of morphological spermatozoa depending on the ejaculate volume

Şekil 1. Ejakülat hacmine ilişkin seçilmiş morfolojik sperm anomalilerinin görülme sıklığı

Table 2. *Frequency of spermatozoa with morphological changes in relation to ejaculate volume*
Tablo 2. *Ejakülat hacmine ilişkin morfolojik değişikliklere sahip spermatozoa sıklığı*

Specification		Groups & Ejaculate Volume (ml)		
		I (< 251)	II (251-310)	III (> 310)
Number of ejaculates (n)		32	38	44
Ejaculate volume (ml)	X±Sx	217.19±33.14^{A}	284.47±15.54^{B}	364.09±46.37^{C}
Percentage of normal spermatozoa (%)	X±Sx	96.08±3.20^{B}	96.90±2.63^{B}	94.72±3.72^{A}
Spermatozoa with major abnormalities (%)	X±Sx	1.09±1.08	0.91±1.80	1.71±2.05
Spermatozoa with minor abnormalities (%)	X±Sx	2.88±2.69	2.18±1.81	3.60±2.93

^{A,B} Differences between average values, represented by different letters in the same row, are important (P≤0.01)

Table 3. *Morphometric traits of spermatozoa with regard to ejaculate volume*
Tablo 3. *Ejakülat hacmine ilişkin morfometrik spermatozoa özellikleri*

Specification		Groups & Ejaculate Volume (ml)		
		I (< 251)	II (251-310)	III (> 310)
Number of ejaculates (n)		32	38	44
Ejaculate volume (ml)	X±Sx	217.19±33.14^{A}	284.47±15.54^{B}	364.09±46.37^{C}
Head length (µm)	X±Sx	8.99±0.55	9.10±0.56	9.19±0.46
Head width (µm)	X±Sx	4.73±0.32	4.85±0.30	5.02±0.34
Head perimeter (µm)	X±Sx	23.24±1.02	23.59±1.14	23.75±1.32
Head area (µm^2)	X±Sx	37.88±4.39^{A}	39.16±4.57^{AB}	40.53±4.28^{B}
Flagellum length (µm)	X±Sx	43.19±1.12^{a}	43.84±1.16^{b}	43.59±1.37^{ab}
Total length (µm)	X±Sx	52.18±1.00^{A}	53.02±1.15^{B}	52.80±1.62^{AB}

^{a,b} Differences between average values, represented by different letters in the same row, are important (P≤0.05); ^{A,B} Differences between average values, represented by different letters in the same row, are important (P≤0.01)

between the groups were slight and statistically unconfirmed. The highest percentage of spermatozoa with major abnormalities was found in the semen of the boars with the highest ejaculate volumes. Among the major morphological abnormalities, cytoplasmic droplets in the proximal position in the spermatozoa were most frequent. The mean percentage of spermatozoa with this defect was low and did not exceed 0.5% *(Fig. 1)*. The highest numbers of sperm with minor morphological abnormalities were found in the semen of Group III (3.60%). The differences between the groups were, however, low and statistically non-significant.

The data in *Table 2* suggest that the volume of ejaculates collected from Hypor boars insignificantly affected the frequency of morphological abnormalities in the spermato-

Table 4. *Morphometric indices of spermatozoa in relation to ejaculate volume*
Tablo 4. *Ejakülat hacmine ilişkin morfometrik spermatozoa endeksleri*

Specification		Groups & Ejaculate Volume (ml)		
		I (< 251)	II (251-310)	III (> 310)
Number of ejaculates (n)		32	38	44
Ejaculate volume (ml)	X±Sx	217.19±33.14^{A}	284.47±15.54^{B}	364.09±46.37^{C}
Head width/head length	X±Sx	52.71±3.08^{A}	53.42±2.55^{AB}	54.61±2.88^{B}
Head length/total length	X±Sx	17.23±1.09	17.15±0.96	17.40±0.66
Head length/flagellum length	X±Sx	20.84±1.59	20.76±1.40	21.09±0.99
Head area/total length	X±Sx	82.76±1.09	82.69±1.31	82.54±0.79
Head length x width/total length	X±Sx	44.54±2.09	44.48±1.94	44.96±1.69
Perimeter of the head/total length	X±Sx	72.61±8.50^{a}	73.79±7.94^{ab}	76.66±6.82^{b}
Flagellum length/total length	X±Sx	81.79±9.54^{a}	83.46±8.94^{a}	87.37±7.88^{b}

^{a,b} Differences between average values, represented by different letters in the same row, are important (P≤0.05); ^{A,B} Differences between average values, represented by different letters in the same row, are important (P≤0.01)

zoa. The results of the morphometric measurements of the spermatozoa are presented in *Table 3*.

The data in *Table 3* show that the spermatozoa in the ejaculates with the highest volumes (Group III) have larger head dimensions than those in the ejaculates with intermediate and low volume (Group II and I). The head lengths and widths increase with ejaculate volume. The spermatozoa in the ejaculates with the highest volumes (Group III) had 0.17 µm wider heads than those in the ejaculates in Group II (P≤0.05) and 0.29 µm wider heads than the spermatozoa in Group I (P≤0.01). The spermatozoa in the ejaculates of the highest volumes were also characterized by the largest head areas. The head area exhibits a clear rising trend in line with the increase in ejaculate volume (P≤0.01).

Sperm flagella in the ejaculates in Group II were on average 0.65 µm longer than those in in Group I (P≤0.05), and 0.25 µm longer compared to Group III. The spermatozoa total length was also the greatest in the ejaculates in Group II, principally due to the longer flagella. *Table 4* contains data on the structural indices defining the sperm shape.

The data in *Table 4* suggest that the effect of ejaculate volume on the shape of Hypor boar spermatozoa is non-significant. Most of the structural sperm morphology indices assumed similar values in all groups, and the observed differences largely remained within the range of statistical error. It was recorded that the spermatozoa in the ejaculates with the lowest volume (Group I) had the most elongated heads, and as the ejaculate volume grew, the shape of the sperm heads turned increasingly more oval. This has been confirmed in the head width/head length index, the highest in the ejaculates in Group III - 1.90 times higher than in Group I (P≤0.01). The data in *Table 4* also show that as the ejaculate volume rises, the proportions between the spermatozoa head and the flagellum change as well. With an increase in ejaculate volume, the head area/total length and head length x width/total length ratios also increased. Both indices were higher in Group III, compared to those in Groups II and I (P≤0.05). This suggests that spermatozoa in ejaculates with higher volumes have larger heads in relation to the flagellum length.

DISCUSSION

The ejaculate volume has a physiological basis and is associated with the secretory function of the accessory sexual glands, which produce seminal plasma forming environment for development, and existence of sperm. Functionality of the accessory sexual glands depends on many factors including genetic and non-genetic factors. Important for the physiology of plasma secretion of semen components is sexual development of pig males. The sexual development of pig males is not over at 8-9 months of age, when boars start to be used for insemination, but proceeds until a much more advanced age. Some authors have reported that boar ejaculate volume and sperm count of the boars grows until the age of around 27-28 months [28,29]. The further development of sexual glands in sexually mature and active breeding boars is confirmed by testicular morphology analysis. It was shown that boar testes increase in size until the age of 20 months [30]. Oestrogens play a crucial role in the control of testicular development and functionality [31,32]. Dynamically rising weight of testes during pubescence as well as the number of reproductive and somatic cells within the parenchyma of testes may be determined by oestrogen levels.

An essential parameter in the qualitative assessment of boar semen is the percentage of sperm with superior motility. The reason is that motility is a symptom of viability and indirectly reveals the fertilization capability of spermatozoa. Acceptable, fertilisation-capable boar semen should contain at least 70% of spermatozoa with progressive rectilinear motion [33]. The data of the present study showed

the spermatozoa in all the analysed groups having a good motility, much above the values reported by Shipley [34]. It was essential to identify the positive effect of ejaculate volume on sperm motility. Raising ejaculate volume was accompanied with a significant increase in sperm motility. Numerous factors affecting sperm motility have been reported. Some authors have reported the negative impact of morphological defects on sperm motility [35], while others have pointed out the considerable impact of the hyperosmotic environment of spermatozoa on their motility [36]. Frequent causes of reduced sperm motility include spermatogenetic disorders, anomalies in the functioning of the epididymal epithelium and debilitated functioning of the additional sexual glands [7]. The correlation between sperm motility and the physical parameters of the ejaculate has not been clearly confirmed yet. Publications on the subject provide inconclusive observations. The study of Pietrain boars by Kondracki et al.[9] showed the highest motility in spermatozoa in ejaculates with the lowest volumes. The progressively motile spermatozoa identified in the previous study ranged from 75 to 79%, and was slightly higher in ejaculates with the lowest volumes, i.e. contrarily to the correlation observed in this study. The observed changes were, however, non-significant, and the percentage of spermatozoa with progressive motility was not too much. Studies of the importance of sperm concentration have revealed that sperm motility is not in significant correlation with sperm concentration in the ejaculate [37,38]. There was no correlation between sperm motility and the total number of spermatozoa in the ejaculate has been identified either [17]. The results of the present study showed that sperm concentration was slightly higher in ejaculates with lower volumes. This confirms the existence of an inversely proportional correlation between ejaculate volume and sperm concentration in boar ejaculates, as identified in previous studies [24,39].

The total number of sperm increased with ejaculate volume. The differences were significant and very pronounced. This is consistent with the expectations, since the content of motile spermatozoa in the ejaculate depends on ejaculate volume and sperm concentration. A directly proportional correlation between the number of spermatozoa and ejaculate volume has also been observed in other studies [9].

The results of the present study justify the conclusion that ejaculate volume affects sperm morphology. The spermatozoa in ejaculates with different volumes differ in their sizes and shapes, as well as in the incidence of morphological abnormalities. The spermatozoa in the ejaculates with the highest volumes (above 310 ml) were larger in size than those in the ejaculates in Group I - with the lowest volumes (below 251 ml). Sperm size affects the motility and fertilization capacity [14,30]. According to Noorafshan & Karbalay-Doust [16], sperm length is positively correlated with the speed of sperm motion. Spermatozoa with longer mid-pieces and flagella have stronger tails [40]. The correlation between flagellum length, and primarily mid-piece length, and sperm motility has also been revealed [14,15,41].

It is probable that, sperm mid-piece length can be associated with the level of energy originating in mitochondria [42]. Spermatozoa with longer flagella are more competitive since they might reach the egg faster. The present authors found that spermatozoa with the longest flagella were present in ejaculates with intermediate volumes (251-310 ml). A study by Marmor and Grob-Menendez [43] revealed that spermatozoa with low motility could have flagella that are shorter by as much as 50%. The results of the above study were confirmed by Noorafshan and Karbalay-Dust [16]. The data presented in this work also validate this correlation, because the lower flagellum length in ejaculates containing spermatozoa with low motility was statistically confirmed. The interrelation between flagellum length and ejaculate parameters has already been identified in several studies [44,45]. It has been revealed that ejaculates with a high sperm concentration contain spermatozoa with shorter flagella [37,46]. However, no clear-cut correlation was identified between flagellum length and the total number of spermatozoa in the ejaculate [17].

The rising ejaculate volume was accompanied with increasing sperm head dimensions, including the length, width, perimeter and area *(Table 3)*. The sperm head contains the cellular nucleus, which is the primary carrier of genetic information transferred during fertilization. The variation in the dimensions of sperm heads can stem from differences in chromatin structure [47]. Some reports informed that even slight modifications in the sperm head shape can be associated with changes in chromatin structure in the cellular nucleus [48], leading to reduced fertility [49]. A correlation has been found between sperm head dimensions and male fertility. It was observed that the spermatozoa of males with higher fertility had narrower and shorter heads [50,51]. The studies by Villalobos et al.[52] demonstrated a positive correlation between fertility and spermatozoa head morphometry in swine. It was concluded that males with high fertility showed the values of 8.9 µm in length and 4.5 µm in width. The data of the present work revealed that the spermatozoa in the ejaculates with the lowest volumes had the lowest head dimensions. Their heads were shorter and narrower than the heads of spermatozoa in the ejaculates with higher volumes. The association of sperm head dimensions with the physical parameters of ejaculates has also been identified in other studies [9]. Sperm head dimensions have been observed to be dependent on the sperm concentration in bull [24] and boar ejaculates [37,38].

The head shape can be significant in the context of sperm motility. Spermatozoa with an elongated head shape move faster than those with rounded heads [53]. The current data showed that the spermatozoa in the ejaculates

with the lowest volumes (Group I) had the most elongated heads, and as the ejaculate volume increases, the shape of the sperm heads turned increasingly more oval *(Table 4)*. Helfenstein et al.[54] have reported the existence of a correlation between the length of the head and flagellum and the speed of sperm motion. Spermatozoa with a lower ratio of head length to tail length move faster. Considering the results of the experiment, this refers to the spermatozoa from the ejaculates classified in Group II in terms of the volume.

Sperm head dimensions can be affected by the manner of storage and preservation of semen [55,56]. A study by Hidalgo et al.[56] revealed that buck sperm heads in refrigerated were smaller than those in fresh semen. This was explained with the damage to or loss of the acrosome, or a possible change in sperm chromatin structure as a result of refrigeration. The detection of abnormalities in sperm heads makes it possible to recognize fertile animals and those with reduced fertility [57]. Morphometric analyses of ram spermatozoa have revealed that sires with reduced fertility have larger sperm heads than fertile males [58]. The reason for the increase in head size can be disordered spermatogenesis, or changes in chromatin structure during the maturation and transport of spermatozoa. Sperm head defects often cause deterioration in the quality of embryos and lead to miscarriages in the first period of pregnancy [12,59].

The data presented in this work indicate a moderate correlation between the incidences of sperm morphological abnormalities and ejaculate volume. However, ejaculates with the highest volumes had the lowest proportions of spermatozoa with correct morphology. The presence of morphologically abnormal spermatozoa reduces male fertility and was an index of a reduced performance of the seminiferous epithelium. The incidence of morphological abnormalities in spermatozoa can result from the influence of seasonal factors [60], genetic conditions [12,61], and individual predispositions [62]. The incidences of morphologically abnormal spermatozoa also depend on feeding factors [63]. Large differences in the frequency of morphological abnormalities in spermatozoa have also been identified in relation to the age of sires [28,64-66].

Boars with normal fertility always have a certain percentage of morphologically abnormal spermatozoa [63]. A maximum of 15% spermatozoa with major and 10-15% with minor abnormalities is acceptable. The presence of spermatozoa with major abnormalities that have appeared during spermatogenesis is particularly disadvantageous. A high percentage of spermatozoa with major modifications, especially acrosome defects, substantially reduces the chances for insemination. The data presented in this study showed that the samples of spermatozoa with major abnormalities was low and did not exceed 1.71% in any of the groups. Among the major morphological abnormalities, the proximal cytoplasmic droplet in the spermatozoa was the most frequent defect. Such defects result from anomalies in sperm maturation. The reason for the appearance of the abnormalities can be a short a time of sperm maturation in the epididymal duct [67]. It should be noted that the incidence of the tail defects could be a consequence of an osmotic difference between the spermatozoa and the solution in which the sample is immersed [68]. According to Martin-Rillo et al.[69], a maximum of 20% spermatozoa with a proximal droplet is acceptable in collected semen. Any amount in excess of this level leads to a considerable reduction of male fertility [70].

Ejaculate parameters depend on the volume of produced ejaculates. The rise in ejaculate volume was accompanied with an increase in the total number and motility spermatozoa, as well as with a concomitant slight fall in sperm concentration. Ejaculates with the highest volume were highly usable for preparation of more insemination doses. Ejaculates with the highest volumes had a larger proportion of spermatozoa with normal morphology. However, ejaculate volume does not substantially affect the frequency of morphological sperm abnormalities in Hypor boar ejaculates. Ejaculate volume influences morphometric parameters of Hypor boar spermatozoa. The rise in ejaculate volume is accompanied with an increase in sperm dimensions, especially with regard to the sperm head. The increased parameters were the length and the width of sperm heads as well as their perimeters and areas. Ejaculate volume had an impact on the shape of Hypor boar spermatozoa. As the ejaculate volume increases, the shape of the sperm heads changes from elongated to increasingly more oval. Spermatozoa in ejaculates with higher volume had a larger heads in relation to the flagellum length. When using Hypor boars for insemination purposes, it is preferred to choose sires with a high ejaculatory efficacy and producing ejaculates of high volume. Such ejaculates allow not only for generating more insemination doses, but also doses including spermatozoa of higher mobility and quality.

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Research Article

Development and Preliminary Application of an Indirect ELISA to Detect Infectious Bovine Rhinotracheitis Virus Using Recombinant Glycoprotein D of IBRV Strain SD [1]

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Abstract

Glycoprotein D (gD) is the major structural protein of infectious bovine rhinotracheitis virus (IBRV). It can induce both humoral and cellular immunity thus it is a preferable protein for IBR diagnostic reagent. Regarding to DNAstar analysis, the major antigenic region of the gD fragment was amplified by PCR using the IBRV genomic DNA as a template, and subsequenly constructed into the recombinant plasmid pET32a-gD. The fusion protein was expressed upon IPTG induction. The fusion protein was purified by immobilized Ni ion affinity chromatography with a Ni-NTA Kit after verification by SDS-PAGE and Western-blotting analysis, then was utilized as a coating antigen to detect antibodies to infectious bovine rhinotracheitis virus (IBRV) in an indirect ELISA method. Cross-reactivity examinations have showed that the recombinant antigen had no cross reaction with positive sera of other common viral diseases (bovine ephemeral fever, bovine viral diarrhea-mucosal disease, calf diarrhea, bovine intestinal virus infection, bovine coronavirus disease) which indicating a strong specificity. Application of this diagnostic method in 1315 clinical serum samples displayed an antibody positive rate of 23.7% (311/1315), with respect to a coincidence rate of 96.8% as compared to a commercialized IBRV whole virus ELISA. Our method is stable and sensitive hence provides a quick and convenient serological diagnosis favoring epidemiology and disease identification of domestic IBR.

Keywords: *gD, Indirect ELISA, Infectious bovine rhinotracheitis virus, Serology*

Enfeksiyöz Bovine Rhinotracheitis Virusun Tespitinde IBRV Suş SD Rekombinant Glikoprotein D Kullanılarak İndirekt ELISA Yönteminin Geliştirilmesi ve Uygulanması

Özet

Glikoprotein D (gD) sığır enfeksiyöz rhinotracheitis virus (IBRV)'ün majör yapısal proteinidir. Glikoprotein D hem humoral hem de hücresel bağışıklığı uyarması nedeniyle IBR'nin tespitinde tercih edilen bir proteindir. DNAstar analizi ile ilgili olarak gD fragmanının majör antijenik bölgesi BRV genomik DNA'sı şablon olarak kullanılmak suretiyle PCR ile amplifiye edildi; sonrasında rekombinant plazmid pET32a-gD içinde yapılandırıldı. Füzyon proteini IPTG oluşturulmak suretiyle ifade edildi. Füzyon proteini; SDS-PAGE ve Western Blotting analizleriyle onaylandıktan sonra Ni-NTA Kit kullanılarak immobilize Ni iyon affinite kromotografi ile saflaştırıldı. Sonrasında, saflaştırılan protein indirekt ELISA metodunda enfeksiyöz bovine rhinotracheitis virüs (IBRV) antikorlarını belirlemek amacıyla örtü antijeni olarak kullanıldı. Çapraz reaksiyon incelemeleri ise diğer yaygın viral hastalıkların (bovine ephemeral fever, bovine viral diare-mukozal hastalık, buzağı dizanterisi, bovine intestinal virüs enfeksiyonu, bovine coronavirus hastalığı) pozitif serumları ile çapraz reaksiyon oluşturmayarak güçlü bir spesifiteye sahip olduğunu gösterdi. Tanı amaçlı olarak metodun 1315 serum örneğinde uygulanması neticesinde antikor pozitif oranı %23.7 (311/1315) olarak belirlendi. Bu sonuçla güvenli kullanım oranı ticari IBRV tüm virüs ELISA ile karşılaştırıldığında %96.8 olarak tespit edildi. Çabuk ve kullanışlı serolojik tanı sağlaması nedeniyle mevcut yöntem IBR'nin hastalık tanısı ve epidemiyolojisinde istikrarlı ve duyarlı bir test olarak kullanılabilir.

Anahtar sözcükler: *gD, İndirekt ELISA, Enfeksiyöz Bovine Rhinotracheitis Virus, Seroloji*

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INTRODUCTION

Infectious bovine rhinotracheitis (IBR) is an acute, feverish and contagious disease caused by infectious bovine rhinotracheitis virus (IBRV) [1-3]. IBRV is one of critical pathogens causing bovine respiratory diseases (also known as shipping fever). Apart from direct pathogenicity, IBRV also induces immune suppression resulting in secondary bacterial infection, which subsequently weakens cattle reproductivity and causes mortality in severe cases. This virus has a typical pantropism enabling it to attack a variety of tissues and organs with diverse symptoms. The cattle are characterized with latent and persistent infections after virus invasion and can be a long-term, or even life-long carrier [4,5]. The potential carriers intermittently release viruses during a period of up to one and half years which rapidly spread around in a high density stock causing exclusive infections. Meanwhile, certain amounts of IBRV in the semen also lead to a widespread of viruses making it extremely difficult to control and eliminate the disease.

From the 1850s when the disease was identified for the first time in the US, so far there have been IBR infections reported worldwide and thus it became a prominent global disease. Since IBR was isolated in China in 1980 from the cattle imported from New Zealand, the presence of this disease in most Chinese provinces is rising up and impacting greatly on the rates of cattle fattening, milk production and reproductivity [6,7]. IBR is classified as a class B infectious disease by the World Organization for Animal Health (OIE), and is a key quarantine by the Law of the People's Republic of China on the entry and exit animal [8]. Therefore, further development of diagnostic method for IBR is of great importance for prevention and control of the disease.

IBRV also called bovine herpesvirus type I (BHV-1). The BHV-1 genome is an approximately 138 kb linear double-stranded DNA [3]. It encodes about 30 to 40 structural proteins including 11 glycoproteins among which, the gD is a major one located at the surface of virus particle associated with virus infection [9]. It is mainly involved in virus entry and can induce humoral and cellular immunity in host cells [10]. The whole virus ELISA method experiences a risk of virus contamination although with a better antigenicity. The neutralization antibody against gD largely produced in the host has the best performance to antagonize IBRV, hence is favored as the top choice for manufacturing diagnostic reagents and subunit vaccines [11]. This study utilizes a prokaryotic system to express recombinant gD. Preliminary data showed that the recombinant product of the whole gD gene mainly existed as an inactive form in inclusion body and needed further complicated re-activation after purification. Hence in this study, a fragment of the gD gene centralized with antigen clusters was truncated depending on the outcome of biological software analysis. The majority product of this truncated gD gene was in a soluble form. In addition, Western-blot analysis of purified protein indicated that this shortened gD protein exhibited a relative high reactivity to be used as the antigen in ELISA [12].

This study aims to establish an indirect ELISA method to detect IBRV using the recombinant gD protein expressed in prokaryotic cells as the coating antigen. It provides a quick and convenient technique for IBRV serological and epidemiological investigation.

MATERIAL and METHODS

Virus, Bacterial Strains, Plasmids and Serum Samples

The IBRV-SD strain was isolated from a dairy farm in Jinan, Shandong, China. The cloning vector pEASY-T3, and DH5a, BL21(DE3) competent cells were purchased from Transgene Biotechnology Co., Ltd, Beijing, China. The pET32a (+) expression plasmid, IBRV positive/negative sera and 1315 blood samples collected from dairy cattle in some Shandong farms were stored in the Dairy Cattle Research Center, Shandong Provincial Academy of Agricultural Sciences, Jinan, China.

Enzymes and Reagents

The enxymes and reagents were purchased from different companies *(Table 1)*.

Extraction of Virus Genomic DNA

Monolayer MDBK cells were inoculated with 100$TCID_{50}$ IBRV ($TCID_{50}$ = $10^{-7.62}$/0.1 mL) and underwent 3 freeze-thaw cycles once 80% of lesion achieved. The IBRV genomic DNA was purified with the virus genomic DNA extraction kit (Takara Biotechnology Co., Ltd (Dalian, China) following manufacturer's instructions.

Construction and Verification of the gD Vector for Prokaryotic Expression

The gD ORF was amplified by PCR from the IBRV SD strain genome. The primer sequences for the gD gene were gD-F and gD-R. The primers were designed specifically using a primer design software *(Table 2)* and synthesized by Sangon Biotechnology Co., Limited (Shanghai, China). The truncated gD fragment was amplified by PCR and digested with BamH I/Hind III after purification. Afterwards it was cloned into the pET32a (+) backbone to generate the recombinant plasmid pET32a-gD which was transformed into DH5α competent cells. The plasmids were purified from a bulk culture and verified by restriction analysis and sequencing.

Expressionand Activity Evaluation of Recombinant gD Protein

BL21 (DE3) cells were transformed with positive recombinant pET32a-gD plasmids and a single colony was picked

Table 1. *Enzymes and reagents used in this study*
Table 1. *Çalışmada kullanılan enzim ve maddeler*

Enzymes and Reagents	Sellers
LA Taq enzyme	Takara Biotechnology Co., Ltd (Dalian, China)
T4 DNA ligase	Takara Biotechnology Co., Ltd (Dalian, China)
restriction endonucleases	Takara Biotechnology Co., Ltd (Dalian, China)
viral genomic DNA extraction kit	Takara Biotechnology Co., Ltd (Dalian, China)
protein loading buffer and marker	Sangon Biotech Company (Shanghai, China)
Ni-NTA spin kit	QIAGEN (Hilden, Germany)
IPTG	Sangong Biotechnology Co.,Ltd (Shanghai, China)
X-Gal	Sangong Biotechnology Co.,Ltd (Shanghai, China)
plasmid extraction and gel extraction kits	Tiangen Bioech (Beijing) Co.,Ltd (Beijing, China)
mouse anti-6-histidine (His) monoclonal antibody	Sigma-Aldrich Co. LLC (Saint Louis, USA)
horseradish peroxidase-conjugated goat anti-mouse IgG antibody	Sigma-Aldrich Co. LLC (Saint Louis, USA)
96-well plates	Sigma-Aldrich Co. LLC (Saint Louis, USA)

Table 2. *Primers for prokaryotic expression*
Table 2. *Prokaryotik ifade tespitinde kullanılan primerler*

Primer Name	Primer Sequences (5′-3′)
gD-F	ATGGATCCTTCGCCTACCCCACGGAC
gD-R	GCAAGCTTGTTGACGTTGCCAAAGGCC

and inoculated into 5 mL of LB containing 0.1% ampicillin followed by overnight growth at 37°C, 200 r/min in a shaking incubator. The next morning the overnight culture was inoculated into 5 mL of LB containing 0.1% ampicillin at 1 in 100 dilution and shake at 200 r/min, 37°C until OD600 to be 0.6 to 0.8. Then IPTG was added to a final concentration of 1 mmol/L and the bacteria were induced at 30°C for 6 h before harvesting. The pellet from 1 mL of broth was vortexed and boiled in 80 μL 1% SDS plus 20 μL of loading buffer for 5 min, after short spin, 10 μL of the supernatant was loaded on a 12% SDS-PAGE gel for analysis.

The induced bacteria were harvested and washed for three times with ice-cold PBS. The cells were disrupted by sonication and centrifuged at 1200 r/min for 15 min. Then the target protein expression in the supernatant and cell pellet were separately analyzed by SDS-PAGE electrophoresis. The target protein was purified with the Ni-NTA protein purification kit according to manufacturer's instructions.

The proteins separated by 12% SDS-PAGE gel were transferred onto a PVDF membrane. The membrane was blocked in PBST containing 5% of skimmed milk, 4°C for overnight, followed by sequential staining with primary mouse anti-6-Histidine (His) monoclonal antibody (1:5000 dilution) and secondary HRP-conjugated goat anti-mouse IgG antibody (1:5000 dilution). The immunoactivity of recombinant gD protein was detected through X-ray spectroscopy.

Development of Indirect ELISA (iELISA) to Detect Recombinant gD

- Procedure of Indirect ELISA

The purified protein was appropriately diluted in carbonate buffer (pH 9.6) and coated onto a 96-well ELISA plate at 4°C, overnight. The plate was washed for 3 times with 0.5% PBST buffer for 3 min each, and blocked at 37°C followed by repeated washes as above. The diluted serum samples were added into the reaction plate and incubated at 37°C. Then the plate was washed again as above. The secondary HRP-conjugated antibody was added into the reaction plate after appropriate dilution. After washing, the TMB chromogenic substrate solution was added and reacted for certain time before stopped by 2 mol/L of H_2SO_4. The absorbance of degraded substrate was measured at 450 nm in a microplate reader.

- Optimization for Indirect ELISA

The main factors impacting on the outcome of indirect ELISA include the concentration of coating antigen, the blocking buffer and time, serum dilution, serum-antigen reaction time, the secondary antibody dilution and incubation time. The optimized conditions were determined by the square matrix titrimetry. Under the same reaction conditions, the plate was coated with different concentrations of antigen (0.5 μg/well, 1.0 μg/well, 1.5 μg/well, 2.0 μg/well, 2.5 μg/well, 5.0 μg/well, 7.5 μg/well, 10.0 μg/well, 12.5 μg/well, and 15 μg/well), added in serially diluted serum samples (1:20, 1:40, 1:80, 1:100, 1:120, 1:140, 1:160, and 1:320), blocked with different blocking solution (5% horse serum, 10% horse serum, 20% horse serum in PBST and the PBST control) for various duration (30 min, 60 min, 90 min, 120 min, and 150 min), incubated with serially diluted secondary antibody solution (1:1000, 1:2000, 1:3000, 1:4000, 1:5000, 1:6000, 1:7000, and 1:8000) with a range of reaction time (30 min, 60 min, 90 min, 120 min,

and 150 min). All these conditions made up of a square with which the negative and positive OD values at 450 nm were measured via a plate reader, and subsequently the maximum P/N value was selected as the best combination.

- Determination of Positive and Negative Thresholds of iELISA

Indirect ELISA was performed with 40 bovine serum samples in duplicate (all samples were negative in serum neutralization test and IBRV whole virus analysis). The final results of OD450nm were calculated as mean (x) and standard deviation (S). In accordance with statistical principles, the thresholds were set up based on the criteria as follows: a sample OD450nm value ≥ x + 3S was considered as positive; a sample OD450nm value ≤ x + 2S was considered as negative; and the values between x + 3S and x + 2S were regarded as suspicious. The suspicious samples were double examined and considered as positive if the value still questionable. In this circumstance, clinical symptom was taken into account for diagnosis if applicable, and follow-up monitoring was carried out [13].

- Specificity Analysis

Under the same conditions, the bovine sera positive of other viral diseases (bovine ephemeral fever, bovine viral diarrhea-mucosal disease, calf diarrhea, bovine intestinal virus infection, and bovine coronavirus disease) were examined by indirect ELISA in parallel with standard IBRV positive and negative serum samples as reference. Cross-reactivity is determined according to the standard.

- Experiment Reproducibility

Intra-batch experiment reproducibility: under the same experimental conditions, 6 serum samples were chosen randomly with each examined for 6 times. The average coefficient of variation was calculated.

Inter-batch experiment reproducibility: under the same experimental conditions, 6 serum samples were chosen randomly with each examined by ELISA for twice using 4 batches of proteins purified at different time. The average coefficient of variation was calculated (C.V %).

$$C.V\ \% = S/x \times 100\%$$

- Evaluation of Clinical Samples

In total 1315 bovine serum samples were examined simultaneously using the indirect ELISA method with IBRV recombinant gD developed in this study and commercialized IBRV whole virus ELISA diagnostic kit. The results from whole virus ELISA were used as reference to evaluate the coincidence rate, sensitivity, specificity of gD-ELISA.

Coincidence rate = sample number with the same result/total number of samples × 100 %

Sensitivity = sample number with positive results by gD ELISA/sample number with positive results by whole virus ELISA kit × 100%

Specificity = sample number with negative results by gD ELISA/sample number with positive results by whole virus ELISA kit × 100%

RESULTS

Expression and Purification of Recombinant Protein

The recombinant plasmid pET32a-gD transformed into *E. coli* BL21 (DE3) displayed efficient expression under IPTG induction. There were a large amount of recombinant proteins in expected size in the induced group over the non-induced one as verified by 12% SDS-PAGE electrophoresis *(Fig. 1)*. The majority of expressed target proteins were present as inclusion bodies in the precipitates, whereas some were in a soluble form in the supernatant. The concentration of purified protein was measured by the BCA assay with an average of 0.210 mg/mL.

Identification of Recombinant Protein by Western-blot

Western-blot analysis was carried out to evaluate recombinant gD. As a result, there was a specific band presented at the desired location for the target protein only in the positive serum sample instead of the negative one *(Fig. 2)*. This demonstrates that the expressed recombinant protein could react specifically with the standard IBRV positive serum with a promising reactogenicity.

Establishment of Indirect ELISA using Recombinant gD

- Confirmation of ELISA Conditions

The final optimal reaction conditions were confirmed after the square titration experiments as follows: protein coating concentration: 2.5 μg/mL; blocking solution: 20% (v/v) horse serum, blocking time: 37°C for 2 h; serum sample dilution: 1:100; incubation time: 37°C for 1 h; optimal secondary antibody dilution: 1:5000; incubation time: 37°C for 1 h; chromogenic reaction time: 37°C for 5 min.

- Establishment of Positive and Negative Thresholds

The range of OD450nm values was between 0.138~0.338 with an average of 0.198 and a standard deviation of 0.055, as measured from 40 negative serum samples. The threshold between negative and positive samples was 0.308 which indicates that, a serum sample can be regarded as positive at OD450nm ≥0.363, or negative at OD450nm ≤0.308, or as suspicious with a value between 0.308-0.363. The suspicious sample was measured again and considered as positive with a questionable value.

- Specificity Evaluation

According to established iELISA conditions, the positive sera of other common viral diseases (bovine ephemeral

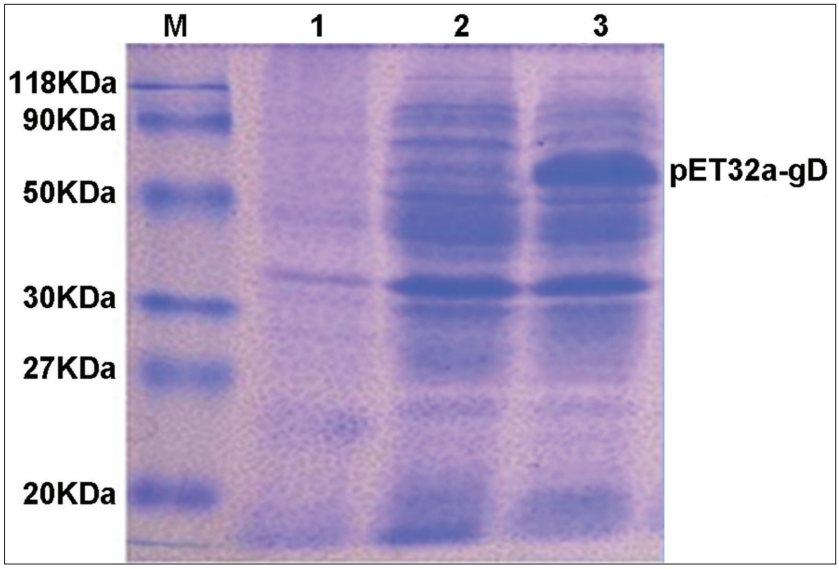

Fig 1. Induced expression of pET32a-gD. M. Protein marker; 1. Non-induced lysates from BL21 transformed with pET32a (+); 2. Non-induced lysate from BL21 transformed with pET32a-gD; 3. Lysates from BL21 transformed with pET32a-gD and induced with IPTG

Şekil 1. pET32a-gD ekspresyonu. M. Protein markırı; 1. pET32a (+) ile oluşturulan BL21'in uyarılmamış lizatları; 2. pET32a-gD ile oluşturulan BL21'in uyarılmamış lizatı; 3. pET32a-gD ile oluşturulan ve IPTG ile uyarılmış BL21'in lizatları

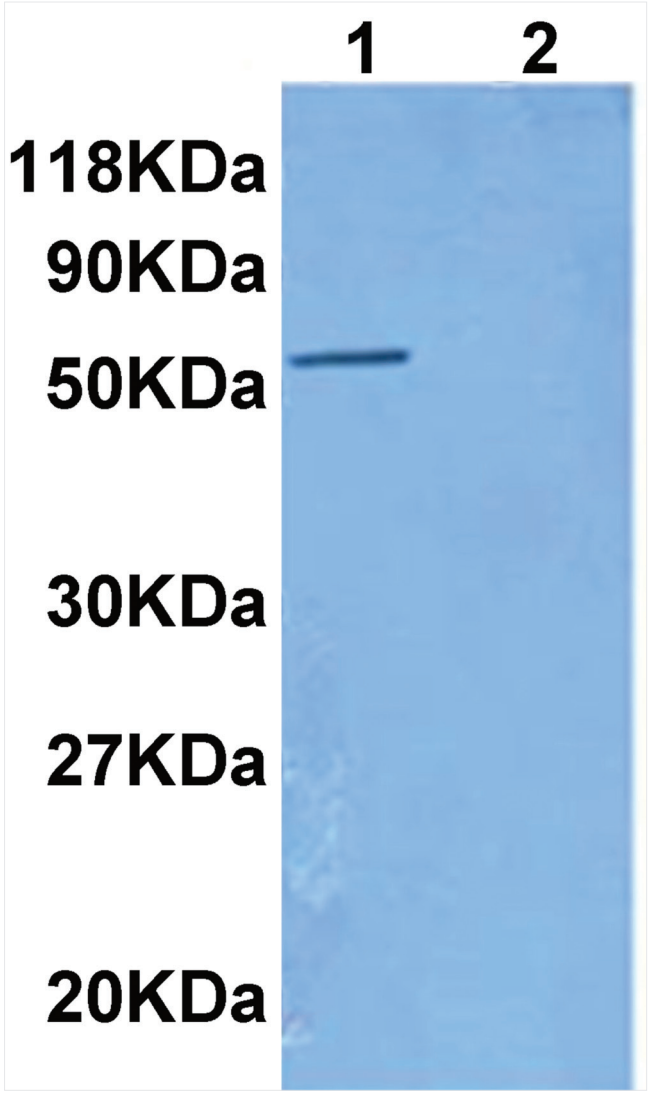

Fig 2. Western blot analysis of pET32a-gD. 1. Positive serum of IBRV; 2. Negative serum of IBRV

Şekil 2. pET32a-gD'nin Western blot analizi. 1. IBRV'nin pozitif serumu; 2. IBRV'nin negatif serumu

fever, bovine viral diarrhea-mucosal disease, calf diarrhea, bovine intestinal virus infection, and bovine coronavirus disease) all showed negative OD450nm values, indicating a negative cross-reactivity of this antigen with above sera.

Experiment Reproducibility

The variation coefficient is less than 7% within intra-batch experiments, whereas less than 11% with inter-batch experiments using different batches of purified recombinant antigens. This result suggests a good reproducibility of this study.

Clinical Sample Evaluation

Examination of serum samples from 1315 cows in Shangdong Province using the indirect ELISA method and commercialized IBRV whole virus ELISA kit (Laboratorios HIPRA, Girona, Spain) showed that, there were 311 samples with positive IBRV antibodies and a 23.7% of seropositivity rate by indirect ELISA. The coincidence rate is 96.8% in comparison to the results using commercialized whole virus ELISA kit *(Table 3)*.

DISCUSSION

Infectious bovine rhinotracheitis is prevalent worldwide as a globally important disease. Almost all countries had seropositive cattle (even including a small number of countries in South American yet without isolated viruses). World Organization for Animal Health (OIE) included infectious bovine rhinotracheitis virus in the statutory reporting list of animal diseases, whilst China also obliged IBRV assessment as an obligation for all imported and exported cattle. Since the disease was detected and reported in China for the first time from cows imported from New Zealand in 1980, its prevalence all over the country has been rising up. A seroprevalence survey in 2010 revealed 66.7% of positive serological antibodies in

Table 3. *Comparison of the results obtained from gD-ELISA and the commercialized IBRV whole virus ELISA*
Table 3. *gD-ELISA ve ticari IBRV tüm virus ELİSA'dan elde edilen sonuçların krşılaştırması*

Detection Method and Results		gD-ELISA Detection				
		Positive	Negative	Specificity/%	Sensitivity/%	Coincidence Rate/%
IBRV whole virus ELISA	Positive	294	25	98.3%	92.2%	96.8%
	Negative	17	979			
Total		319	996			

cows distributing in five northern regions (Beijing, Liaoning, Jilin, Inner Mongolia, and Shanxi). There were also 1505 out of 5000 breeding bulls imported from Australia exhibiting positive reactions to IBRV antibodies during quarantine in 2005, with 2 of them having isolated viruses. Taken together, the epidemic situation is not optimistic on domestic herds under double pressure from both imported carries with hidden infection and also local cattle with high infectious bovine rhinotracheitis. Thus, it is of top importance to establish a fast and accurate method for diagnosis of the disease.

Serological detection of antibodies is an essential immunological technology for diagnosis of the disease. Serum neutralization assay and ELISA are two major serological methods and also commonly used techniques worldwide. Serum neutralization assay has high accuracy, yet requires a long period and complicated procedure to perform therefore is not suitable for a large number of samples with limited use. ELISA becomes a widely used serological diagnostic technique, as also recommended by OIE International Trade, in animals quarantine for infectious diseases and epidemical investigation due to several advantages including strong specificity, sensitivity and objective criteria, etc.

The specificity of ELISA can be affected by many factors, notably the antigen coating concentration and serum sample dilution factors. High antigen coating concentration leads to multi-layer formation of proteins so that some are detached from the support surface to be easily washed off during the wash process causing a non-specific effect. On the other hand, low concentration would leave some un-occupied spaces by antigen on the surface of the ELISA plate also contributing to high non-specificity. There are also some extra effects from various blocking buffer and time, reaction time with serum samples and secondary antibody, as well as chromogenic reaction time. In this study all optimal experimental conditions were probed by the square titration and the thresholds were established via statistical analysis. Evaluation of clinical samples suggested that, as compared to the commercialized IBRV whole virus ELISA kit, our method has 96.8% coincidence, 92.2% sensitivity and 98.3% specificity. Meanwhile, the variation coefficient of intra-batch experiments is less than 7%, and the one of inter-batch experiments is less than 11%, indicating that this methodology has decent reproducibility and stability. One of the important characteristics of BoHV-1 is establishment of life long latency in sensory neurons of the peripheral nervous system after replication in mucosal epithelium. Reactivation from latency can occur after natural stimulus exposure [14,15] or corticosteroid treatment [16] culminating in recurrent virus transmission to uninfected animals generally without clinical signs. Currently the most developed and widely used technique for IBRV detection includes virus isolation, virus neutralization test, ELISA, and PCR, etc.[17,18]. Rational use of these detection technologies in combination with regulatory procedures can greatly improve the efficiency of IBRV detection and consequently reduce the possibility of hidden IBRV. It applies particularly on the imported cattle in quarantine in order to reassure the quality of animals and keep carriers out of the country. Our study establishes an indirect ELISA method for detection of IBRV antibody which provides a promise for developing IBRV ELISA kit in the future and also technical support for quarantine, diagnosis, antibody surveillance, prevention and control of IBR.

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Research Article

The Protective Effects of L-Carnitine Against Lead (II) Acetate Toxicity in *Capoeta capoeta* (Guldensteadt 1773) [1]

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Abstract

In this study, the protective effects of L-carnitine (LC) against Lead (II) acetate ($Pb(C_2H_3O_2)_2$) toxicity in *Capoeta capoeta* were investigated by means of histopathologically, via electrophoretic and biochemical methods. Fish caught from Kars creek were divided into four groups, which include 10 fish each in 500 L tanks. Lead and liquid LC were added to water. Fish in the first group were adjusted as control. Fish in the II. group were applied 1 mg/L lead (as Lead (II) acetate) for 10 days. Fish in the III. group were administered 100 mg/L LC. Fish in the IV. group were administered 1 mg/L lead and 100 mg/L LC. Degenerations in liver, gill, intestine and kidney tissues were observed to reduce LC administration against the toxic effects of lead acetate. Electrophoretically, inhibitions of some protein bands in the group, which was applied lead acetate, were caused, and increases in protein expression in the group, which was applied L-carnitine, have occurred. While high a level of total protein in the group that was administered lead was found; in the group that was treated lead + LC, it was found to be lower ($P<0.05$). Levels of globulin in the group that was administered LC + lead were observed to be significantly lower ($P<0.05$). Total oxidant capacity (TOC) in lead treatment group were higher than the control group, TOC levels in lead + LC treatment group were determined to be between the control and lead group. LC was concluded to show a protective effect on *Capoeta capoeta* that were exposed to lead.

Keywords: *Lead (II) acetate, L-carnitine, Capoeta capoeta, Electrophoresis, TAC, TOC, Histopathology*

Capoeta capoeta (Guldensteadt 1773)'larda Kurşun (II) Asetat Toksisitesine Karşı L-Karnitinin Koruyucu Etkileri

Özet

Çalışmada, *Capoeta capoeta*'da Kurşun (II) asetat ($Pb(C_2H_3O_2)_2$) toksisitesine karşı L-karnitinin (LK) koruyucu etkileri histopatolojik, elektroforetik ve biyokimyasal yöntemlerle incelendi. Kars Çayı'ndan yakalanan balıklar 500 L'lik tanklarda her grupta 10'ar adet balık bulunan 4 gruba ayrıldı. Kurşun ve sıvı LK suya ilave edildi. I. gruptaki balıklar kontrol grubu olarak belirlendi. II. gruptaki balıklara 10 gün süreyle 1 mg/L kurşun asetat uygulandı. III. gruptaki balıklara 100 mg/L LK uygulandı. IV. gruptaki balıklar ise 1 mg/L kurşun ve 100 mg/L dozunda LK uygulandı. Karaciğer, solungaç, bağırsak ve böbrek dokularında kurşun asetatın toksik etki gösterdiği ve buna karşı L-karnitin uygulamasının koruyucu etki göstererek oluşan bu dejenerasyonların şiddetini azalttığı gözlemlendi. Elektroforetik incelemede, kurşun asetatın birçok protein bandında inhibisyona neden olduğu, LK uygulaması sonucunda da protein ekspresyonlarında artış meydana geldiği saptandı. Kurşun uygulanan grupta total protein düzeyinin yüksek, kurşun + LK verilen grubun ise düşük olduğu belirlendi ($P<0.05$). Globulin düzeylerinin, LK + kurşun verilen grupta istatistiksel olarak düşük olduğu saptandı ($P<0.05$). Kurşun uygulanan grubun Total Oksidan Kapasitesinin (TOK) kontrol grubuna göre yüksek, kurşun + LK verilen grubun TOK düzeyinin ise kontrol ile kurşun verilen grubun arasında olduğu belirlendi. Sonuç olarak; LK'in *Capoeta capoeta*'da kurşun toksisitesine karşı koruyucu özellik gösterdiği kanısına varıldı.

Anahtar sözcükler: *Kurşun (II) asetat, L-karnitin, Capoeta capoeta, Elektroforez, TAK, TOS, Histopatoloji*

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INTRODUCTION

Sources of lead in the environment are either natural or unnatural (anthropogenic) sources. While geological and volcanic events form natural resources of lead; anthropogenic sources include mining and resources in the industrial area (batteries, cables, pigments, oil, solder and steel) [1,2]. Lead in living organisms are not necessary for normal physiological events [3]. Lead exposure generally occurs through occupational or environmental contamination [4]. The main contamination form of lead in the environment is by air, and airborne. Lead can accumulate in soil and aquatic environments. Later, it can be transported to people through the food chain by living organisms in the aquatic environment and plants. Lead shows toxic effects on many organs and organ systems of living organism (blood, brain, kidney, heart and immune system) [1,2]. L-carnitine, being a vitamin-like compound, is endogenously synthesized from lysine and methionine that are essential amino acids mainly in the liver, kidney and brain [5]. For this synthesis, lysine, methionine, iron, vitamin C, vitamin B6 and niacin are needed [6]. The most important function in the organism of L-carnitine is to facilitate the entry into the mitochondria of fatty acids to be used in energy production [7,8]. In addition, L-carnitine plays an important role in reducing toxic effects of various drugs and chemical substances in the organism [6]. Nevertheless, carnitine is known to increase the blood antioxidant level [9]. Deficiency of L-carnitine emerges from immune function defects, such as systemic sclerosis and chronic fatigue syndrome [10].

In this study, investigation of productive effects of LC on *Capoeta capoeta* that was exposed to Lead (II) acetate was aimed.

MATERIAL and METHODS

Experimental Design

This study has been conducted under the approval (KAÜ-HADYEK/2015-043) of Kafkas University Animal Experiments Local Ethics Committee. *Capoeta capoeta* (200-270 g) caught by electro shock from Kars creek was divided into four groups, which included 10 fish each in 500 L tanks. Fish were fed with ad libitum until experimental studies. Feeding was stopped during experimental study. Fish in the first group were adjusted as control. Fish in the II. group were applied 1.00 mg/L lead for 10 days. Fish in the III. group were administered 100 mg/L L-carnitine. Fish in the IV. group were administered 1.00 mg/L lead and 100 mg/L L-carnitine.

The water temperature and oxygen concentration was adjusted to 18.0±0.20ºC by thermostat thermometer and 5.00±0.40 mg/L, respectively. At the end of the experimental period, blood samples from dorsal vein of fish for biochemical and/or electrophoretic analysis and, liver, kidney, intestine and gill tissue samples for histopathological analysis were taken.

In tanks, Kars creek water was used and water was changed daily. The quality of the water in the Kars creek was identified as pH: 7.80-8.40, O_2: 5.00-8.50, conductivity 213 mS/cm^2, NH_3: 408 mg/L, PO_4: 53.7 mg /L, NO_3: 0.25 mg/L and temperature: 16.3-19.0°C.

Histopathology

Tissues taken from the samples were passed through graded alcohol and xylene series after being fixed in 10.0% phosphate buffer formaldehyde solution. Gills were decalcified with Osteotec (Bio-Optica, Italy). Paraffin blocks from tissue samples were prepared by routine methods and slices from paraffin blocks were taken in 3.00 to 5.00 microns thickness. Slices were stained according to hematoxylin-eosin method (HE) and evaluated under a light microscope (Olympus BX51, JAPAN).

Sodium Dodecyl sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Blood samples were centrifuged at 4.00°C for 10 min at 3.000 rpm and serums were separated. Serums were stored at -20.0°C until analysis. The protein concentrations of the samples were measured by the biuret method [11]. SDS-PAGE was performed by the methods modified from Laemmli and O'Farrell [12,13]. Bovine albumin (66 kDa), egg albumin (45 kDa) and trypsinogen (24 kDa) were used as protein standards in electrophoresis. Molecular weight of the protein was performed according to the method of Weber et al.[14].

Biochemical Analysis

Antioxidant and oxidant levels of Serum were measured by Total Antioxidant Status and Total Oxidant Status Assay kit (Assay Rel Diagnostics, Clinical Chemistry Solutions, Gaziantep, Turkey) [15]. Blood glucose, total protein, albumin and globulin levels were measured using a commercial kit (ERBA DDS, Turkey).

Statistical Analysis

One-way analysis of variance for the statistical analysis of the differences between the groups (ANOVA), Tukey's test was used to determine differences between groups. as SPSS Statistics 18.0 software package was used. Values are expressed as mean ± standard deviation ($P<0.05$).

RESULTS

Histopathology

The livers of the fish in the control group did not exhibit any histopathological findings *(Fig. 1A)*. Structure of livers of fish, which were applied 100 mg/L L- carnitine, was

similar to the livers of fish in the control group *(Fig. 1B)*. The fish livers that was applied 1.00 mg/L lead acetate were observed to deteriorate in the remark cords with severe degenerative and necrotic changes. Large and small sharp-edged vacuolar degeneration in the cytoplasm of hepatocytes was detected. Hydropic degeneration were observed in a small number of cells. Due to changes in the cytoplasm of hepatocytes, cells demonstrated quite growth and loss of intercellular sinusoidal space. Focal necrotic areas were detected by the death of a few cells *(Fig. 1C)*. In the group, which was applied 1.00 mg/L of lead acetate +100 mg/L L-carnitine, the prevalence and severity of the degeneration of the liver were observed to decrease significantly compared to group, which was applied only lead acetate. Hepatocytes in this group have often been found small and sharp edged vacuoles in the few cells with hydropic degeneration. Necrosis was also detected in a few cells. Although the general structure of the remark cord was protected; particularly, the remark cord was found to be disrupted in necrotic areas. Light activation was formed in Kupffer's Star cells *(Fig. 1D)*.

There were no histopathological findings in the control group and LC of kidney tissue *(Fig. 2A-B)*. Kidney tissues of fish, which were applied 1.00 mg/L lead acetate, especially drew attention to leave the basal layer with hydropic degeneration in the proximal tubule epithelium and segmental necrosis. Integrity in the basal layer of the tubules was slightly lost. Tubule lumen were observed in a small number of pink hyaline cylinders. These degenerative changes were observed less in the distal tubule. Increased mesangial cells in glomeruli and thickening in bowman capsule were determined *(Fig. 2C)*. In kidney tissues of fish that were applied Lead acetate + L-carnitine, although reductions in the severity and prevalence of degeneration and necrosis occurring in tubules were observed compared to the group that was applied only lead; degenerations still continue. While glomerulus appear to be robust in some areas in the experimentals in this group; in some areas, especially in cytoplasms of the endothelial and epithelial cells close to the Bowman space, increase in eosinophilic staining and formation of pyknosis in nucleus attracted attention *(Fig. 2D)*.

In tissue samples taken from intestine, the control group and LC did not show any histopathological findings like in kidney tissues *(Fig. 3A-B)*. In the lead acetate applied group, the inner layer of the epithelium was observed to increase in goblet cells filled with secretory granules. Lamina propria was seen in mononuclear cell infiltration, edema and light hyperemia *(Fig. 3C)*. In the group, which was administered Lead acetate + L-carnitine, a decrease in degenerative and necrotic changes and a significant increase in goblet cells were seen, compared to the control and LC groups. However, compared to the group that was applied lead, it was quite a small number of increase in goblet cells. Mononuclear cell infiltration in the lamina propria was similar to control group and the one that was applied LC *(Fig. 3D)*.

The fish gill tissues in control and LC groups had normal histological appearance *(Fig. 4-A-B)*. In gill tissues of fish, which were applied 1.00 mg /L lead acetate, degeneration in the secondary lamellae epithelium, loss in necrosis, swelling of the chloride cells, and hydropic degeneration in the lamellar epithelium were observed *(Fig. 4C)*. In the

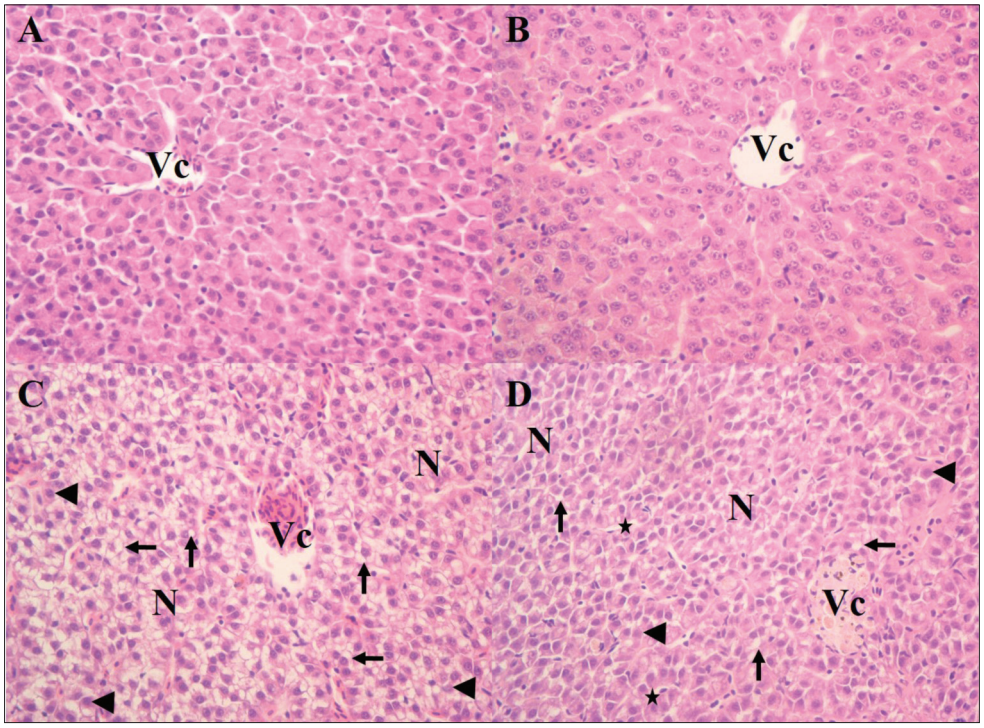

Fig 1. Liver tissue of fish in the control and experimental groups (Hematoxylin and eosin, x40). **A-** Control and **B-** liver tissues of fish in LC group, **C-** Liver tissue of fish applied 1.00 mg/L lead acetate, vacuolar *(arrows)* and hydropic *(arrowhead)* degeneration, focal necrosis (N), **D-** Liver tissue of fish that were administered 1.00 mg/L lead acetate +100 mg/L LC. Vacuolar *(arrows)* and hydropic *(arrowhead)* degeneration, focal necrosis (N)

Şekil 1. Kontrol ve deney gruplarındaki balıklara ait karaciğer dokusu (Hematoksilen ve eozin, x40). **A-** Kontrol ve **B-** LC grubundaki balıklara ait karaciğer dokusu, **C-** 1.00 mg/L kurşun asetat içeren tankta 10 gün süreyle tutulan balıklara ait karaciğer dokusu vakuoler *(oklar)* ve hidropik *(ok başı)* dejenerasyon, fokal nekroz alanları (N), **D-** 1.00 mg/L kurşun asetat ile birlikte 100 mg/L dozunda LK verilen balıklara ait karaciğer dokusu, vakuoler *(oklar)* ve hidropik *(ok başı)* dejenerasyon, fokal nekroz alanları (N)

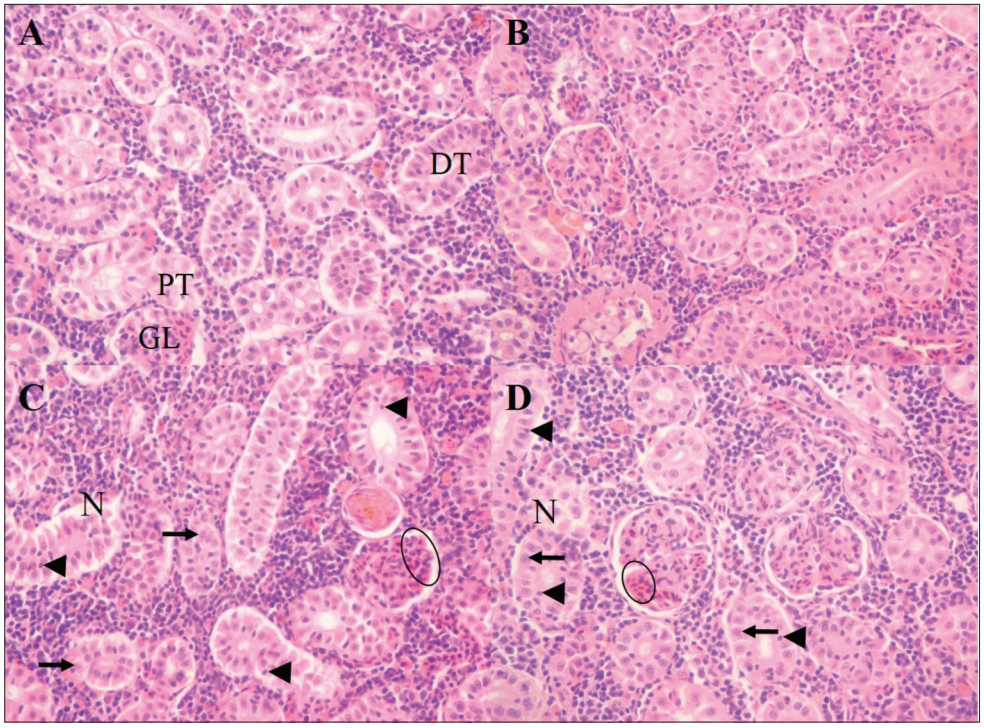

Fig 2. Kidney tissue of the fish in the control and experimental groups (Hematoxylin and eosin, x40). **A-** Control and **B-** kidney tissues of fish in group LC, **C-** Kidney tissue of fish,which were applied 1.00 mg/L lead acetate. Hydropic degeneration in the proximal tubule epithelium *(arrows),* pyknosis *(arrowhead),* segmental necrosis (N), **D-** Kidney tissue of fish that were applied 1.00 mg/L lead acetate + 100 mg/L LC. Hydropic degeneration in the tubule epithelium *(arrows),* pyknosis *(arrowhead),* segmental necrosis (N).

Şekil 2. Kontrol ve deney gruplarındaki balıklara ait böbrek dokusu (Hematoksilen ve eozin, X40). **A-** Kontrol ve **B-** LC grubundaki balıklara ait böbrek dokusu, **C-** 1.00 mg/L kurşun asetat içeren tankta 10 gün süreyle tutulan balıklara ait böbrek dokusu. Proksimal tubul epitellerinde hidropik dejenerasyon *(oklar),* piknoz *(ok başı),* segmental nekroz (N), **D-** 1.00 mg/L kurşun asetat ile birlikte 100 mg/L dozunda LK verilen balıklara ait böbrek dokusu. Tubul epitellerinde hidropik dejenerasyon *(oklar),* piknoz *(ok başı),* segmental nekroz (N)

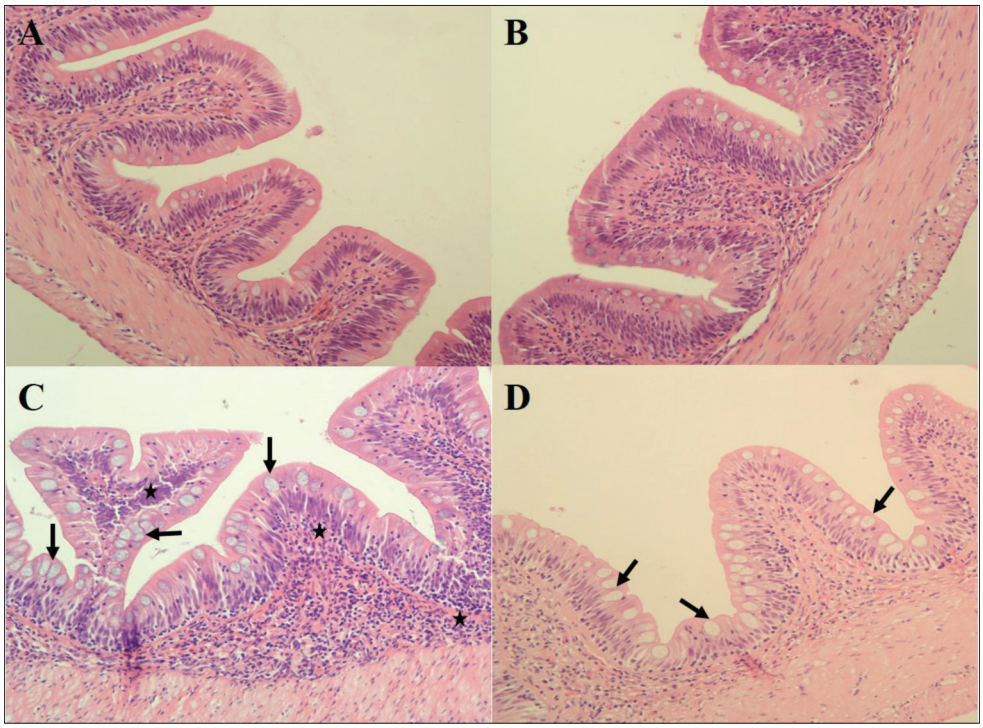

Fig 3. Intestinal tissues of fish in the control and experimental groups (Hematoxylin and eosin, x40). **A-** Control and **B-** Intestinal tissue of fish in the group, which was applied LC, **C-** Intestinal tissue of fish that were applied 1.00 mg/L of lead acetate. The increase in goblet cells *(arrows),* mononuclear cell infiltrate in lamina propria and edema, **D-** Intestinal tissue of fish, which were applied 1.00 mg/L of lead acetate +100 mg/L LC. Increase in goblet cells *(arrows)*

Şekil 3. Kontrol ve deney gruplarındaki balıklara ait bağırsak dokusu (Hematoksilen ve eozin, x40). **A-** Kontrol ve **B-** LK grubundaki balıklara ait bağırsak dokusu, **C-** 1.00 mg/L kurşun asetat içeren tankta 10 gün süreyle tutulan balıklara ait bağırsak dokusu. Goblet hücrelerindeki artış *(oklar),* Lamina propriyada mononükleer hücre infiltrasyonu, ve ödem, **D-** 1.00 mg/L kurşun asetat ile birlikte 100 mg/L dozunda LK verilen balıklara ait bağırsak dokusu. Goblet hücrelerinde artış *(oklar)*

gill tissues of lead acetate + LC applied fish, degenerations and necrosis in the secondary lamellae were significantly reduced and degeneration were only seen at the starting of secondary lamellae *(Fig. 4D)*.

SDS-PAGE

The electrophoretic examination, thinning in expressions of high molecular weight albumin bands and thickening in expressions of low molecular weight globulin bands were observed in lead applied fish. In the group, which was administered Lead + L-carnitine, compared to the control group, proteins with 98 kD and 43 kD molecular weights were over-expressed *(Fig. 5)*.

Biochemical Analysis

Glucose levels of groups treated with lead, LC and lead + LC were significantly lower than the control group ($P<0.001$). Total protein levels in the group of fish that

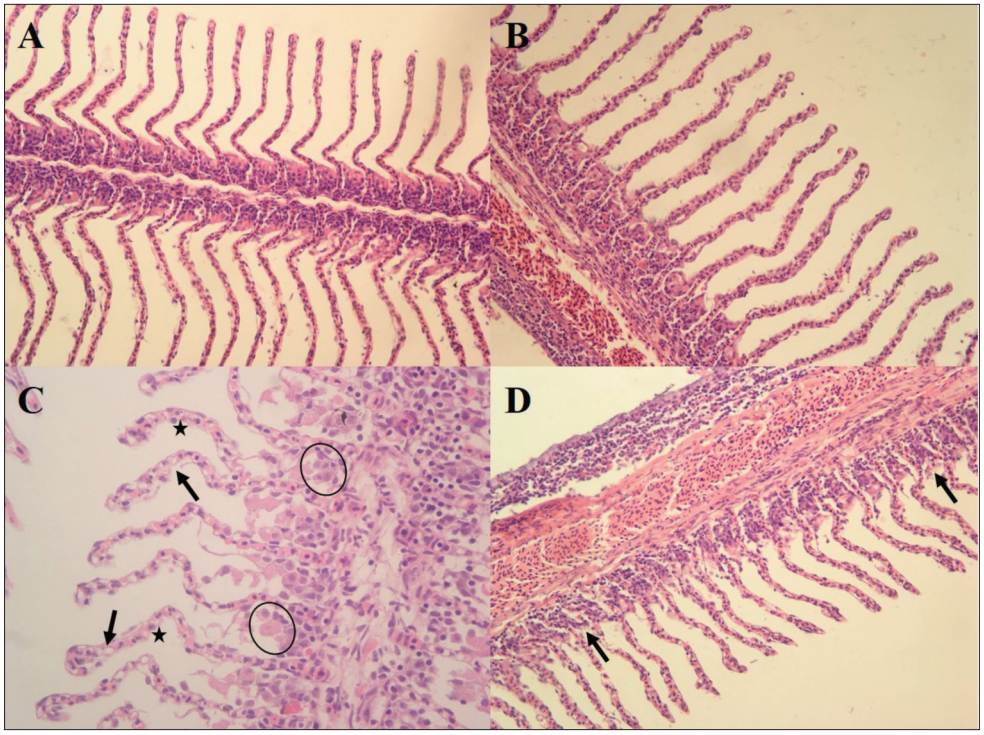

Fig 4. Gill tissue of fish in the control and experimental groups (Hematoxylin and eosin, x20). **A-** Control and **B-** Gill tissue of fish in the LC applied group, **C-** Gill tissue of fish that were applied 1.00 mg/L lead acetate. Degeneration in epithelial of secondary lamellae and necrosis, loss *(arrows),* swelling in the chloride cells (circle), **D-** Gill tissue of fish that were administered 1.00 mg/L of lead acetate + 100 mg/L LC. Degeneration in epithelial of secondary lamellae and necrosis *(arrows)*

Şekil 4. Kontrol ve deney gruplarındaki balıklara ait solungaç dokusu (Hematoksilen ve eozin, x20). **A-** Kontrol ve **B-** LC grubundaki balıklara ait solungaç dokusu, **C-** 1.00 mg/L kurşun asetat içeren tankta 10 gün süreyle tutulan balıklara ait solungaç dokusu. Sekonder lamel epitellerinde dejenerasyon ve nekroz, dökülme *(oklar),* Klorid hücrelerde şişme (daire), **D-** 1.00 mg/L kurşun asetat ile birlikte 100 mg/L dozunda LK verilen balıklara ait solungaç dokusu. Sekonder lamel epitellerindeki dejenerasyon ve nekroz *(oklar)*

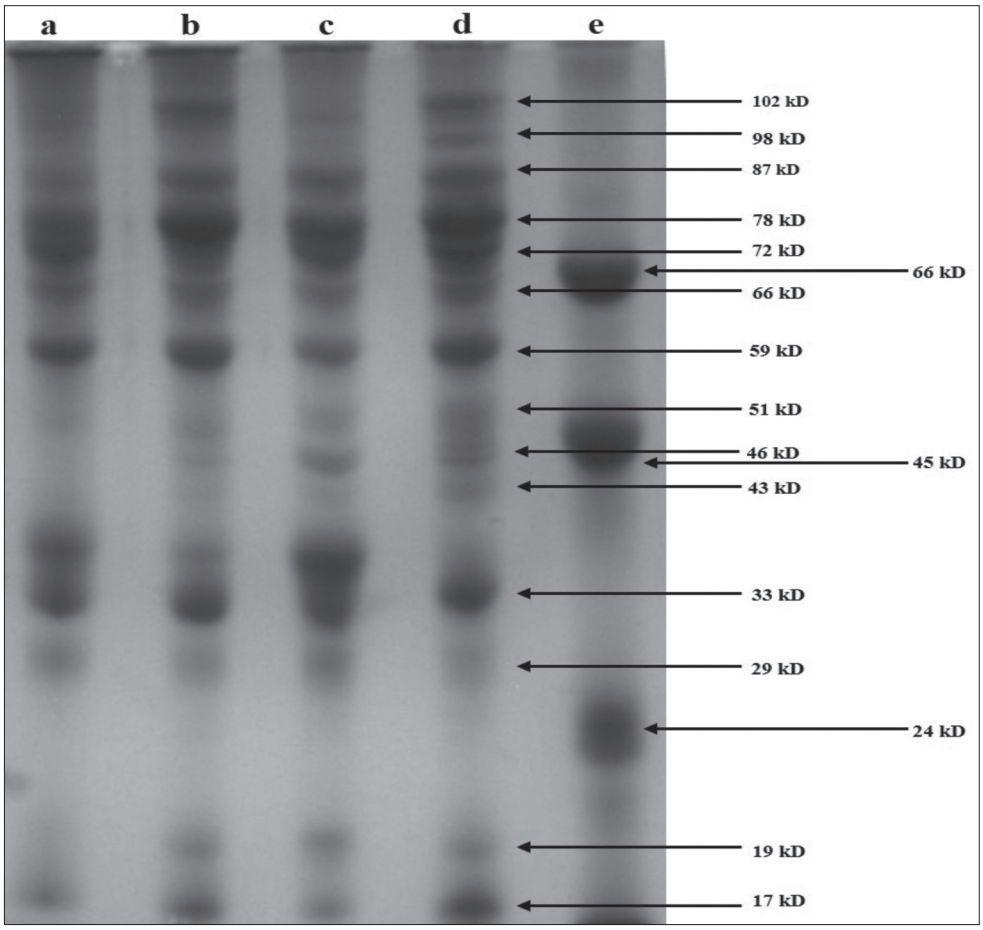

Fig 5. SDS-PAGE electropherogram obtained from fish, which were administered LC with Lead. **a-** Control group, **b-** Lead applied group, **c-** LC applied group, **d-** Lead+LC applied group, **e-** Standard proteins

Şekil 5. Kurşun ile birlikte LC verilen balıkların SDS-PAGE'den elde edilen elektroferogramı. **a-** Kontrol grubu, **b-** kurşun uygulanan grup, **c-** LC uygulanan grup, **d-** Kurşunla birlikte LC uygulanan grup, **e-** Standart proteinler

was applied LC were not observed to have a statistically significant difference; while total protein levels of the group that was applied lead were found to be high, and total protein level of group that was administered Lead + LC was determined to be low ($P<0.05$). Albumin level of the group that was applied LC was observed to have a

Table 1. *The biochemical analysis of fish that were applied LC and lead*
Tablo 1. *Kurşun ve LC uygulanan balıkların biyokimyasal analizleri*

Parameter	Control	Lead	LC	Lead + LC	P Value
Glucose (mg/dL)	73.19±3.03^{a}	57.06±3.16^{c}	49.74±3.67^{bc}	47.89±1.16^{b}	0.000
Total Protein (g/dL)	4.06±0.33^{ab}	4.26±0.07^{a}	4.02±0.15^{ab}	3.55±0.12^{b}	0.044
Albumin (g/dL)	1.95±0.11^{b}	2.18±0.04^{ab}	2.23±0.10^{a}	2.08±0.05^{ab}	0.117
Globulin (g/dL)	2.11±0.31^{a}	2.08±0.09^{a}	1.78±0.09^{ab}	1.46±0.08^{b}	0.018
Albumin/Globulin	1.11±0.19^{b}	1.07±0.07^{b}	1.27±0.07^{ab}	1.45±0.07^{a}	0.055
TAC (mmol Trolox Equiv./L)	0.47±0.06^{a}	0.46±0.03^{a}	0.45±0.01^{a}	0.51±0.02^{a}	0.658
TOC (μmolH_2O_2 Equiv./L)	9.68±1.62^{b}	17.02±1.37^{a}	10.91±2.21^{b}	13.20±1.79^{ab}	0.061

** Means with different superscript letters are statistically different in line (P<0.05)*

statistically insignificant increase (P>0.05), compared to the control group. Globulin level of the group that was applied lead +LC was significantly lower than the control group (P<0.05). Globulin levels of the LC applied group fish were found to be between total globulin levels of groups that were applied lead and lead+L-carnitine. Albumin/globulin ratio of group treated with Lead + LC statistically insignificant (P>0.05) compared to the control group, but was found to be high. TAC levels in the experimental groups and the control group were not observed to have a statistically significant difference. Total oxidant capacity (TOC) did not occur as a statistical difference between the groups (P>0.05). TOC levels in the group treated with lead were higher than the control group. TOC levels in group treated with lead + LC were determined to be between the control and lead group *(Table 1)*.

DISCUSSION

Although the literature about the effects of metals on aquatic organisms is quite wide, studies on fish are limited with lead. The use of LC in the aquatic environment is emphasized, which will be important in many ways. In particular, to accelerate the growth, to provide protection against xenobiotics and toxic levels of ammonia, to facilitate adaptation to environments of the fish in the changing temperature in water, the reduction of stress and increasing of reproductive performance of LC are some factors that make it important for fish [6]. In particular, the research relating to lead toxicity was found to have adverse effects on the antioxidant system of the lead. In studies conducted on the effects of different tissues and organs of fish exposed to lead, lead has been reported to cause oxidative damage in hepatocytes of liver [16], and kidney tissue [17]. In a histopathological study, formation of mononuclear cell infiltration and necrosis, expansion in sinusoidal space of in liver tissue depending on the lead toxicity have been indicated [18]. In another study, necrosis and cytoplasmic vacuolization in liver tissue of rat that were applied lead have been observed, whereas different degeneration and necrosis in kidney tissue have been reported [19]. Mutlu et al.[20] reported that degenerative and necrotic deterioration in liver tissue of lead acetate applied rats have occured. In the present study, while degenerations in the liver, kidney and intestines gill tissue of fish that were applied lead were formed, LC administration was observed to reduce the severity of degenerations.

There were no studies in the literature about the protective role of LC against lead toxicity on fish, electrophoretically. Changes in increase and decrease occurring in protein expression according to applied substance and the animal species have clearly been demonstrated in the toxicity studies [21,22]. In the present study, changes in protein expression due to lead the application have occurred, and the administration of LC showed an increase in expression of some proteins. The reason for increases in expression of these proteins was thought to be formed due to reduction of the toxic effects of lead exposure.

Proteins show the excessive sensitivity to free oxygen radicals [23]. In a study conducted in rats induced by acetaminophen, comparing to control group, globulin levels were significantly decreased, however, the application of LC did not change the level of globulin [24]. Another study, dietary protein and glucose levels have increased in the application of LC on *Oreochromis niloticus* [25]. In the present study, while lead application increased levels of the total proteins; Lead + LC administration was determined to decrease levels of the total proteins. However, globulin levels of the group treated with L-carnitine + lead were statically lowered more than the control group. Furthermore, glucose levels of the group treated with with L-carnitine + lead were statically lowered more than the control group.

Reactive oxygen species have quite increased in physiological and pathological conditions [26]. LC shows features similar to antioxidants and the harmful effects reactive oxygen species caused by various toxic substances reduces [27]. In a study, it was found that LC showed to be protective against oxidative damage induced by ethanol [28]. In another study, researchers reported that LC reduces the peroxidation of lipids and increases activities of antioxidant enzyme in rats fed with fish oil [9]. Protective effect of LC

against oxidative damage generated in Tilapia *(Oreochromis niloticus)* that were applied cylindrospermopsin has been determined [29]. In another study, oral administration of LC increased the level of antioxidant enzymes, whereas reduced the level of oxidative enzymes against lead stress in rats [30]. In the present study, in terms of TAC levels between treatment groups and the control group, a statistically significant difference was not observed. In terms of TOC levels, while there were increases in levels of TOC in the group that was administered lead, there were decreases in levels of TOC in the group treated with lead + LC. According to these data, LC reduces the oxidative damage and shows an antioxidant feature.

In conclusion, while there were histopathologically degenerations in livers, gills, intestines and kidney tissues of fish exposed to lead, LC caused a reduction in the severity of the toxicity of lead.

Increases and decreases in the expression of some proteins in lead toxicity occurred, electrophoretically, increase in expressions of proteins with 98 kD and 43 kD molecular weights in the group that was administered lead + LC were observed.

In the biochemical analysis, while levels of total protein in the group which applied lead were found to be high, levels of total protein in the group that was applied lead + LC were found to be low.

LC increases the albumin, insignificantly.

Compared to control group, globulin levels in the group that was applied LC + lead were found to be lower ($P<0.05$).

TAC levels against the lead toxicity were found to show a protective effect of L-carnitine.

While depending on the dose and duration lead acetate administered revealed various toxic effects on *Capoeta capoeta*, LC showed protective effect on fish that were exposed to lead.

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Research Article

Hidrazon Yapısındaki On Farklı Bileşiğin Antileishmanial Aktivitesinin Araştırılması [1]

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Özet

Leishmaniasis Türkiye ve dünyada önemli paraziter bir hastalıktır. Leishmaniasis tedavisinde sodyum stibogluconate, miltefosin, paramomisin, amfoterisin B ve pentamidin gibi anti-leishmanial ilaçlar kullanılmaktadır. Fakat leishmaniasisin kemoterapisinde kullanılan bu ilaçların nefrotoksik, hepatotoksik ve teratojenik yan etkileri görülebilmektedir. Ayrıca antimon bileşiklerine karşı direnç gelişmesi nedeniyle de yeni terapötik ajanların keşfedilmesi ve geliştirilmesine öncelik verilmesi gerektiği düşünülmüştür. Bu çalışmanın amacı, sentezlenmiş hidrazon yapısındaki on farklı bileşiğin (5a-5j) *Leishmania infantum* promastigotlarına karşı anti-leishmanial aktivitesinin mikrodilüsyon yöntemiyle belirlenmesidir. Hazırlanan hidrazon bileşikleri, konsantrasyonu 6 µg/ml olacak şekilde RPMI-1640 besiyerlerine eklenmiş ve bileşiklerin mikroplakta konsantrasyon aralığı 3 - 0.003 µg/ml olacak şekilde seri dilüsyonları yapılmıştır. Mikroplaklarda mikrodilüsyon besiyeri yöntemi uygulanarak, üzerine hemositometrede hücre sayısı $2.5x10^7$ hücre/ml olacak şekilde ayarlanmış standart *Leishmania infantum* promastigotları eklenerek 27°C'de inkübe edilmiştir. Yirmi saat sonra mikroplakların üzerine alamar mavisi eklenerek 4 saat inkübe edilmiştir. Promastigotların üremesi 24, 48 ve 72. saatlerde değerlendirilmiştir. Kuyucuklarda rengin maviden pembeye dönmesi parazitin ürediği, rengin değişmeden kalması ise parazitin üremediği şeklinde değerlendirilmiştir. Bu çalışmada, *Leishmania infantum* promastigotlarına karşı en etkili maddelerin 5e, 5g ve 5h bileşikleri (MİK 0.187 µg/ml) olduğu, en etkisiz bileşiğin ise 5i bileşiği (MİK 3 µg/ml) olduğu saptanmıştır. Sentezlenen bileşiklerin ilaç olarak kullanılabilmesi için; gerekli olduğu düşünülen *in vitro* makrofaj kültüründe *Leishmania* amastigotlarına karşı etkinliği ve *in vivo* olarak deneysel hayvan modellerinde kontrol çalışmalarına ihtiyaç vardır.

Anahtar sözcükler: *Leishmania infantum, Hidrazon, Anti-leismanial aktivite, Alamar mavisi*

Investigation of Anti-leishmanial Activity of the Ten Different Hydrazone Derivatives

Abstract

Leishmaniasis is an important parasitic disease in Turkey and the world. Anti-leishmanial drugs such as sodium stibogluconate, miltefosine, paramomycin, amphotericin B, and pentamidine are used for the treatment of leishmaniasis. However, the drugs, used for the chemotheraphy of leishmaniasis, have some side effects such as nephrotoxicity, hepatotoxicity, and teratogenicity. In addition, it is deemed that the discovery and development of the new therapeutic agents must be given priority due to the development of resistance against antimony compounds. The purpose of this study is detecting the anti-leishmanial activity of ten different synthesized hydrazone compounds against *Leishmania infantum* promastigotes via the microdilution method. The prepared hydrazone compounds, having the concentration of 6 µg/ml, were added to RPMI-1640 media and the dilution of the hydrazone derivates was performed in the wells of microplates in the range of concentrations of 3 - 0.003 µg/ml. The microdilution broth method in the microplates was prepared, than the adjusted standard *Leishmania infantum* promastigotes, $2.5x10^7$cells/mL, were added, into the mentioned microplates which was incubated in 27°C. Twenty h later, the alamar blue were added on the microplates and they were incubated for 4 h. The proliferation of promastigotes was evaluated in 24, 48, and 72 h. It was considered that changing the color from blue to pink in the wells were exhibiting the growth of parasites, while the unchanged color was not. The present study has revealed that the most effective substances against *Leishmania infantum* promastigotes were 5e, 5g, and 5h compounds (MIC 0.187 µg/ml) while the least effective compound was 5i (MIC 3 µg/ml). There is a need for further studies on *in vitro* activity against the *Leishmania* amastigotes in macrophages cultures and *in vivo* experimental animal models for the synthesized compounds showing anti-leishmanial effect in the present study.

Keywords: *Leishmania infantum, Hydrazone, Anti-leishmanial activity, Alamar blue*

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GİRİŞ

Dünya Sağlık Örgütü (DSÖ) verilerine göre; Afrika, Asya, Avrupa, Güney ve Kuzey Amerika'da toplam 98 ülke ve yerleşim yerinde yaklaşık 350 milyon insan leishmaniasis açısından risk altındadır [1]. Hastalık, Türkiye'nin de içerisinde yer aldığı Akdeniz havzasına kıyısı olan ülkelerde, Ortadoğu ülkeleri, Hindistan ve Güney Amerika ülkelerinde ciddi bir sağlık problemi oluşturmaktadır [2,3]. Türkiye'de 2000-2014 yılları arasında toplamda 413 vaka bildirimi yapılmış olan visseral leishmaniasis (VL) enfeksiyonunun en önemli etkeni *Leishmania infantum*'dur [4]. En önemli etkeni *Leishmania tropica* olan kutanöz leishmaniasis (KL) vakaları 2000-2014 yılları arasında toplamda 29845 olarak rapor edilmiştir [4].

Parazit memeli vücuduna inoküle olurken, vektörün tükürüğündeki hyalüronidaz-maksadilan salgısı ile vazodilatatör etki oluşturularak enjeksiyon bölgesinde temel immün engellerin hızla aşılması ve enfeksiyonun başlamasına yardımcı olur [5]. Promastigotların fagositozunu takiben, tutunma ve bağlanmada önemli rol oynayan parazit ürünleri ve virülans faktörleri, tekrar devreye girerek parazitin zarar görmeksizin makrofaj içinde yaşamasını ve çoğalmasını sağlarlar [5-8].

Leishmaniasis tedavisinde, sodyum stiboglukonat, miltefosin, paramomisin, amfoterisin B ve pentamidin gibi anti-leishmanial ilaçlar kullanılmaktadır [9]. Fakat leishmaniasisin kemoterapisinde kullanılan bu ilaçların etkileri oldukça sınırlıdır. Ayrıca bu ilaçların çoğunun nefrotoksik, hepatotoksik ve teratojenik etkiler başta olmak üzere ciddi yan etkileri bulunmaktadır [3,9,10]. Dünyada ve Türkiye'de KL ve VL'in tedavisinde birincil ilaç olarak kullanılan beş değerli antimon türevleri, sodyum stiboglukonat (Pentostam®) ve megluminantimoniat (Glucantime®) 1940 yılında geliştirilmiş, ancak bu ilaçlara karşı direnç geliştiği, araştırıcılar tarafından bildirilmiştir [6,11-14].

Hastalığa karşı etkili bir profilaktik aşının bulunmaması, mevcut kullanılan ilaçların toksik etkileri ve bu ilaçlara karşı direncin giderek artması nedeniyle yeni terapötik ajanların keşfedilmesi ve geliştirilmesine öncelik verilmesi gerektiği bildirilmiştir [10,15].

Antiparaziter tedavilerde kullanılan hidrazon türevi bileşikler; aldehit veya ketonların, hidrazin veya alkil-hidrazinler ile kondansasyon reaksiyonunun sonucunda sentez edilen ve kimyasal yapısı (-C=N-NH-) grubu olan bileşik sınıfının genel adıdır. Hidrazon grubunun farmakolojik ve biyolojik olarak aktif bileşiklerin merkezinde yer alması, pek çok araştırmaya ve yeni ilaç geliştirme sürecine kaynak oluşturmuştur. Hidrazonların anti-mikrobiyal, anti-tüberküler, anti-konvülzan, analjezik, anti-enflamatuar, pıhtılaşmayı önleyici ve tümörler, virüsler ve malaryaya karşı etkinliklere sahip olduğu rapor edilmiştir [16,17].

Bu araştırmada alamar mavisi mikrodilüsyon yöntemi ile aksenik kültürde VL etkeni olan *L. infantum* promastigot izolatları kullanılarak, sentezlenmiş 6-sübstitüe-3(2*H*)-pridazinon-2-asetil-2-(sübstitüe/nonsübstitüeasetofenon) hidrazon yapısındaki on adet farklı bileşiğin (5a-5j) anti-leishmanial aktivitesinin belirlenmesi amaçlanmıştır.

MATERYAL ve METOT

Araştırmada standart aksenik *L.infantum* şuşları, hidrazon yapısındaki on farklı bileşik ve standart ilaç olarak amfoterisin B kullanılmıştır.

Leishmania Promastigotlarının Hazırlanması

Çalışmada aksenik standart *L. infantum* (MHOM/TN/19-80/IPT1) suşu kullanılmıştır. Temin edilen suşlar, içerisine %10 Fetal Bovin Serum (FBS F4135 Sigma-Aldrich USA), %1 Penisilin (P3032 Sigma-Aldrich USA) ve Streptomisin (S9137 Sigma-Aldrich USA) (100.000 ünite penisilin ve 10 mg streptomisin) eklenmiş RPMI-1640 (R8758 Sigma-Aldrich USA) besiyerinde çoğaltılmış ve pasajlanarak devamlılığı sağlanmıştır.

Promastigotlar, çoğaltıldıkları besiyerinden 20 ml alınarak steril falkon tüplerine aktarılmış ve 10 dak. 1.000 g'de santrifüj edilmiştir. Daha sonra tüplerdeki süpernatant atılarak çökeltide bulunan promastigotların üzerine 10 ml steril PBS eklenmiş ve düşük devirde vortekslenmiştir. Bu hazırlanan çözelti 10 dak. 1.000 g'de santrifüj edilmiş ve işlem üç kez tekrarlanarak promastigotların yıkanması sağlanmıştır.

Hidrazon Bileşikleri

İn vitro anti-leishmanial aktivite testinde kullanılan hidrazon türevi bileşikler (5a-5j) Utku ve ark.[18] tarafından daha önce sentezlenmiştir. Beş basmakta elde edilen sonuç ürünlerinin sentezini kısaca özetlenecek olursa; 3,6-dikloropiridazin ve 1-fenilpiperazin etanol içerisinde ısıtılmış ve ardından asetik asit ilave edilerek 6-sübstitüe-3(2H)-piridazinon türevi bileşikler sentez edilmiştir. Etil bromoasetat kullanarak 6-sübstitüe-3(2H)-piridazinon-2-il asetat türevinin sentezi yapıldıktan sonra hidrazin kullanarak hidrazon türevleri sentezi yapılmıştır. Farklı sübstitüent taşıyan aldehit türevleri kullanılarak sonuç ürünleri olan (5a-5j) türevlerinin sentezi tamamlanmıştır. Sentez edilen bileşiklerin yapıları ^{1}H-NMR (Varian Mercury 400 MHz FT-NMR spektrometre) ve IR (Bruker Vector 22 IR/Opus Spectroscopic Software Version 2.0) ve elemental analiz verileri (LECO 932 CHNS analizör) ile aydınlatılmıştır. Bu on farklı hidrazon bileşiği *Tablo 1*'de verilmiştir.

İn Vitro Anti-Leishmanial Aktivite testi

Sentezlenmiş olan 6-sübstitüe-3(2*H*)-pridazinon-2-asetil-2-(sübstitüe/nonsübstitüe asetofenon) hidrazon yapısındaki on adet bileşik DMSO/H_2O (%10) içerisinde çözülerek homojen hale getirilmiş ve 0.20-μm membran filtre (Millipore, USA) ile steril edilmiştir. Üzerine konsantrasyonu 6 µg/ml

Tablo 1. *Anti-leishmanial aktivitesi araştırılan hidrazon yapısındaki on farklı bileşik*
Table 1. *Anti-leishmanial activity investigated the hydrazone structure ten different compounds*

Sıra	Sentezlenen Bileşiğin Adı	Açıklama
1	**Bileşik 5a** 6-(4-fenilpiperazin)-3(2*H*)-piridazinon -2-asetil-2-asetofenonhidrazon	Verim%61, Erime noktası 234-235 °C,^{1}H-NMR (DMSO-d_6): δ (ppm) 11.65 (s, 1H, N-H),7.70-7.67 (d, 1H, piridazinon H5), 7.66-6.94 (m, 10H, aromatik protonlar), 6.84-6.81 (d, 1H, piridazinon H4),5.04 ve 4.66 (s, s, 2H, CH2), 3.39-3.37 (t, 4H, piperazina+a' protonlar), 3.29-3.27 (t, 4H, piperazinb+b'protonlar), 2.23 (s, 3H, CH3). FT-IR (KBr): υ 1705 (C=O, hidrazon), 1662 (C=O, 3(2*H*)- piridazinon), 3216(N-H). Analitik Hesaplanan. C24H26N6O2:C, 66.96; H, 6.09; N, 19.52. Bulunan: C, 67.13; H, 6.30; N, 19.37
2	**Bileşik 5b** 6-(4-fenilpiperazin)-3(2*H*)-piridazinon-2- asetil-2-(4 bromoasetofenon) hidrazon	Verim%73, Erime noktası 243-244 °C. ^{1}H-NMR (DMSO-d_6): δ (ppm) 11.67 (s, 1H, NH), 7.72-7.69 (d, 1H, piridazinon H5), 7.67-6.88 (m, 9H, aromatikprotonlar), 6.80-6.77 (d, 1H, piridazinon H4), 5.02 ve 4.63 (s, s,2H, CH2), 3.36-3.34 (t, 4H, piperazina+a' protonlar), 3.28-3.25 (t, 4H, piperazinb+b' protonlar), 2.22 (s, 3H,CH3). FT-IR (KBr): υ 1703 (C=O, hidrazon), 1666 (C=O, 3(2*H*)- piridazinon), 3217 (N-H). Analitik Hesaplanan. C24H25BrN6O2:C, 56.59; H, 4.95; N, 16.50. Bulunan: C, 56.81; H, 5.05; N, 16.69
3	**Bileşik 5c** 6-(4-fenilpiperazin)-3(2*H*)-piridazinon-2- asetil-2-(4-kloroasetofenon) hidrazon	Verim%67, Erime noktası 252-253 °C,^{1}H-NMR (DMSO-d_6): δ (ppm) 11.69 (s, 1H, NH), 7.73-7.70 (d, 1H, piridazinon H5), 7.68-6.91 (m, 9H, aromatik protonlar), 6.83-6.79 (d, 1H, piridazinon H4), 5.06 ve 4.68 (s, s,2H, CH2), 3.36-3.34 (t, 4H, piperazina+a' protonlar), 3.28-3.24 (t, 4H, piperazinb+b' protonlar), 2.23 (s, 3H,CH3). FT-IR (KBr): υ 1706 (C=O, hidrazon), 1665 (C=O, 3(2*H*)- piridazinon), 3214 (N-H). Analitik Hesaplanan. C24H25ClN6O2:C, 62.00; H, 5.42; N, 18.08. Bulunan: C, 62.07; H, 5.54; N, 17.96
4	**Bileşik 5d** 6-(4-fenilpiperazin)-3(2*H*)-piridazinon-2- asetil-2-(4-floroasetofenon) hidrazon	Verim%81, Erime noktası 262-263 °C,^{1}H-NMR (DMSO-d_6): δ (ppm) 11.70 (s, 1H, NH), 7.76-7.74 (d, 1H, piridazinon H5), 7.67-6.89 (m, 9H, aromatik protonlar), 6.82-6.80 (d, 1H, piridazinon H4), 5.05 ve 4.66 (s, s,2H, CH2), 3.39-3.37 (t, 4H, piperazinea+a' protonlar), 3.29-3.27 (t, 4H, piperazineb+b' protonlar), 2.24 (s, 3H,CH3). FT-IR (KBr): υ 1704 (C=O, hidrazon), 1668 (C=O, 3(2*H*)- piridazinon), 3215 (N-H). Analitik Hesaplanan. C24H25FN6O2:C, 64.27; H, 5.62; N, 18.74. Bulunan: C, 64.14; H, 5.74; N, 18.77
5	**Bileşik 5e** 6-[4-(4-florofenil) piperazin]-3(2*H*)- piridazinon -2- asetil -2-asetofenonhidrazon	Verim%80, Erime noktası 271-272 °C,^{1}H-NMR (DMSO-d_6): δ (ppm) 11.73 (s, 1H, NH), 7.77-7.75 (d, 1H, piridazinon H5), 7.72-6.96 (m, 9H, aromatikprotonlar), 6.85-6.83 (d, 1H, piridazinon H4), 5.07 ve 4.70 (s, s,2H, CH2), 3.39-3.37 (t, 4H, piperazina+a' protonlar), 3.30-3.28 (t, 4H, piperazinb+b' protonlar), 2.21 (s, 3H,CH3). FT-IR (KBr): υ 1705 (C=O, hidrazon), 1666 (C=O, 3(2*H*)- piridazinon), 3213 (N-H). Analitik Hesaplanan. C24H25FN6O2:C, 64.27; H, 5.62; N, 18.74. Bulunan: C, 64.04; H, 5.33; N, 18.81
6	**Bileşik 5f** 6-[4-(4-florofenil) piperazin]-3(2*H*)- piridazinon -2- asetil -2-(4-bromoasetofenon) hidrazon	Verim%62, Erime noktası 248-249 °C. ^{1}H-NMR (DMSO-d_6): δ (ppm) 11.80 (s, 1H, NH), 7.79-7.76 (d, 1H, piridazinon H5), 7.70-6.95 (m, 8H, aromatik protonlar), 6.86-6.84 (d, 1H, piridazinon H4), 5.07 ve 4.70 (s, s,2H, CH2), 3.40-3.38 (t, 4H, piperazina+a' protonlar), 3.30-3.28 (t, 4H, piperazinb+b' protonlar), 2.23 (s, 3H,CH3). FT-IR (KBr): υ 1705 (C=O, hidrazon), 1663 (C=O, 3(2*H*)- piridazinon), 3216 (N-H). Analitik Hesaplanan. C24H24BrFN6O2:C, 54.66; H, 4.59; N, 15.94. Bulunan: C, 54.82; H, 4.48; N, 16.12
7	**Bileşik 5g** 6-[4-(4-florofenil) piperazin]-3(2*H*)- piridazinon -2- asetil -2-(4-kloroasetofenon) hidrazon	Verim%50, Erime noktası 229-230 °C,^{1}H-NMR (DMSO-d_6): δ (ppm) 11.82 (s, 1H, NH), 7.77-7.75 (d, 1H, piridazinon H5), 7.79-6.72 (m, 8H, aromatik protonlar), 6.99-6.97 (d, 1H, piridazinon H4), 5.10 ve 4.80 (s, s,2H, CH2), 3.42-3.39 (t, 4H, piperazina+a' protonlar), 3.31-3.29 (t, 4H, piperazinb+b' protonlar), 2.24 (s, 3H,CH3). FT-IR (KBr): υ 1704 (C=O, hidrazon), 1665 (C=O, 3(2*H*)- piridazinon), 3214 (N-H). Analitik Hesaplanan. C24H24ClFN6O2:C, 59.69; H, 5.01; N, 17.40. Bulunan: C, 59.81; H, 5.16; N, 17.26
8	**Bileşik 5h** 6-[4-(4-florofenil) piperazin]-3(2*H*)- piridazinon -2- asetil -2-(4-floroasetofenon) hidrazon	Verim%40, Erime noktası 241-242 °C,^{1}H-NMR (DMSO-d_6): δ (ppm) 11.79 (s, 1H, NH), 7.75-7.72 (d, 1H, piridazinon H5), 7.68-6.90 (m, 8H, aromatik protonlar), 6.85-6.83 (d, 1H, piridazinon H4), 5.07 ve 4.70 (s, s,2H, CH2), 3.39-3.37 (t, 4H, piperazina+a' protonlar), 3.29-3.27 (t, 4H, piperazinb+b' protonlar), 2.23 (s, 3H,CH3). FT-IR (KBr): υ 1706 (C=O, hidrazon), 1662 (C=O, 3(2*H*)- piridazinon), 3216 (N-H). AnalitikHesaplanan. C24H24F2N6O2:C, 61.79; H, 5.19; N, 18.02. Bulunan: C, 61.51; H, 5.36; N, 17.93
9	**Bileşik 5i** 6-[4-(4-klorofenil) piperazin]-3(2*H*)- piridazinon -2- asetil -2-asetofenon hidrazon	Verim%56, Erime noktası 280-281 °C,^{1}H-NMR (DMSO-d_6): δ (ppm) 11.72 (s, 1H, NH), 7.73-7.70 (d, 1H, piridazinon H4), 7.67-6.90 (m, 9H, aromatik protonlar), 6.82-6.80 (d, 1H, piridazinon H4), 5.05 ve 4.70 (s, s,2H, CH2), 3.37-3.35 (t, 4H, piperazina+a' protonlar), 3.27-3.25 (t, 4H, piperazinb+b' protonlar), 2.22 (s, 3H,CH3). FT-IR (KBr): υ 1706 (C=O, hidrazon), 1664 (C=O, 3(2*H*)- piridazinon), 3218 (N-H). Analitik Hesaplanan. C24H25ClN6O2:C, 62.00; H, 5.42; N, 18.08. Bulunan: C, 62.17; H, 5.56; N, 17.98
10	**Bileşik 5j** 6-[4-(4-klorofenil) piperazin]-3(2*H*)- piridazinon -2- asetil -2-(4-bromoasetofenon) hidrazon	Verim%48, Erime noktası 231-232 °C,^{1}H-NMR (DMSO-d_6): δ (ppm) 11.70 (s, 1H, NH), 7.77-7.75 (d, 1H, piridazinon H4), 7.65-6.90 (m, 8H, aromatik protonlar), 6.80-6.79 (d, 1H, piridazinon H4), 5.07 ve 4.70 (s, s,2H, CH2), 3.38-3.36 (t, 4H, piperazina+a' protonlar), 3.28-3.26 (t, 4H, piperazinb+b' protonlar), 2.24 (s, 3H,CH3). FT-IR (KBr): υ 1704 (C=O, hidrazon), 1663 (C=O, 3(2*H*)- piridazinon), 3212 (N-H). Analitik Hesaplanan. C24H24BrClN6O2:C, 53.00; H, 4.45; N, 15.45. Bulunan: C, 53.08; H, 4.40; N, 15.35

olacak şekilde fenol red içermeyen RPMI-1640 besiyeri eklenmiştir. Besiyerine ayrıca ısı ile inaktive edilmiş %10 Fetal Bovine Serum (FBS) eklenmiştir. Hazırlanan her bir karışım sırayla 96 kuyucuklu mikroplakların ilk kuyucuklarına 225 µl dağıtılmıştır. İçerisine 112.5 µl besiyeri eklenmiş 2. kuyucuğa ilk kuyucuktan 112.5 µl eklenerek ½ oranında dilüe edilmiştir. Aynı şekilde seri dilüsyonla 10 kuyucukta işlem tekrarlanarak son kuyucuktan alınan karışım dışarı atılmıştır.

Kuyucukların üzerine, hemositometre ile hücre sayısı $2.5x10^7$ hücre/ml olacak şekilde ayarlanmış 112.5 µl *L.infantum* promastigotları eklenerek son konsantrasyonları 3 - 0.003 µg/ml olacak şekilde bileşikler sulandırılmıştır.

Pozitif kontrol olarak 11. sıradaki kuyucuklara sadece 225 µl promastigot, negatif kontrol olarak da 12. sıradaki kuyucuklara sadece 225 µl sentezlenmiş bileşik eklenmiştir. Mikroplaklar 27ºC'ye ayarlanmış soğutmalı etüvde 20 saat süreyle inkübasyona bırakılmıştır.

Hazırlanmış olan tüm kuyucuklara bu sürenin sonunda 25 µl alamar mavisi (Resazurin sodium salt R7017 Sigma-Aldrich USA) (0.1 mg/ml) eklenmiş ve tekrar 27°C'de 4 saat daha inkübasyona bırakılmıştır. Mikroplaklar 24, 48 ve 72 saat sonra değerlendirilerek sonuçlar kaydedilmiştir. Çalışmada kontrol ilacı olarak da amfoterisin B kullanılmıştır. Tüm testler iki kez tekrarlanmıştır.

BULGULAR

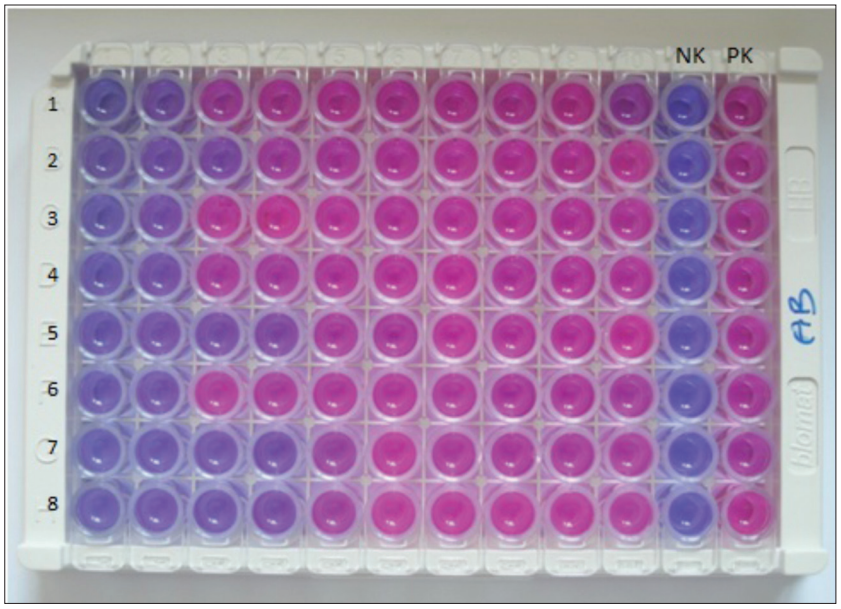
Çalışmada sentezlenmiş hidrazon yapısındaki 10 farklı bileşiğin anti-leishmanial aktivitesi 96 kuyucuklu mikroplaklarda mikrodilüsyon alamar mavisi yöntemi kullanılarak araştırılmıştır. Kuyucuklarda rengin maviden pembeye dönmesi parazitin ürediği, rengin değişmeden kalması ise parazitin üremediği şeklinde değerlendirilmiştir *(Şekil 1)*.

Araştırmada beklenildiği gibi pozitif kontrol kuyucuğunda renk maviden pembeye değişirken, negatif kontrol kuyucuğunda herhangi bir renk değişikliği gözlenmemiştir.

Çalışmada hidrazon yapısındaki on adet bileşiğin Minimal İnhibitör Konsantrasyon (MİK) değerleri *Tablo 2*'de verilmiştir.

TARTIŞMA ve SONUÇ

Dünya genelinde yaygın olan beş paraziter hastalık arasında yer alan leishmaniasis önemli bir halk sağlığı sorundur. DSÖ'ne göre her yıl çoğu gelişmekte olan ülkelerden yaklaşık 2 milyon yeni vaka bildirilmekte olup12 milyon insanı etkilemektedir [10].



Şekil 1. İlk 8 bileşiğe ait mikroplağın 24 saatlik inkübasyon sonrasındaki görüntüsü (11.sütun negatif kontroller 12. Sütun pozitif kontroller)

Fig 1. Image of microplate belong the first eight compounds in at the 24 h incubation (11. column negative controls, 12. column positive controls)

Tablo 2. *Anti-leishmanial aktivitesi araştırılan hidrazon yapısındaki on farklı bileşiğin MİK değerleri*

Table 2. *MIC values of ten different hydrazone derivatives investigated anti-leishmanial activity*

Sıra	Sentezlenmiş Bileşik	MİK (µg/ml)
1	5a	1.5
2	5b	0.75
3	5c	1.5
4	5d	1.5
5	5e	0.187
6	5f	1.5
7	5g	0.187
8	5h	0.187
9	5i	3
10	5j	1.5
Konrtol	Amfoterisin B	0.003

Leishmaniasis tedavisinde beş değerli antimon bileşikleri, amfoterisin B deoksikolat, miltefosin, paromomisin, sitamaquin, azoller ve pentamidin gibi farklı anti-leishmanial ajanların yanında termoterapi ve kriyoterapi gibi uygulamalar da kullanılmaktadır [19-21].

Tedavisinde kullanılan beş değerli antimon bileşiklerinin etkinliğinin %90'ın üzerinde olduğu bildirilirken, miyalji, artralji, karın ağrısı, hepatit, pankreatit ve çeşitli laboratuvar değerlerinde yükseklik gibi nadiren lokal ve sistemik yan etkilere neden olabileceği de rapor edilmiştir [21]. Amfoterisin B ise vücutta geniş bir yayılım göstermesi nedeniyle, infüzyon reaksiyonları, nefrotoksisite, hipokalemi ve miyokardit gibi yan etkilere sahiptir ve hasta 4-5 hafta yatarak takip edilmektedir. Amfoterisin B'nin yan etkilerini azaltmak için dalak ve karaciğer gibi organların makrofajlarında hızla konsantrasyonları yükselerek, uzun süre bu organlarda kalabilen çeşitli lipid formülasyonları (lipozomal amfoterisin B) geliştirilmiştir [19]. Antimon bileşiklerine direnç geliştiğinde, VL tedavisinde intravenöz

veya intramüsküler olarak amfoterisin B kullanılmaktadır. Ancak bu ilaçlara karşı da direnç geliştiği bildirilmiştir [20]. Sunulan çalışmada da hidrozon bileşiklerinin tedavide etkili olabileceği gözlemlenmiştir. Bu durum hastalığın etkili ve kısa sürede tedavisi ile ilgili olumlu olarak değerlendirilmiştir. Ayrıca tedavi sürecinde yan etkisi az olan ve etkili olan bir ilacın kullanılması hastanın yaşam kalitesi açısından da önemlidir.

Çalışmada parazitin canlılığını belirlemek için kullanılan alamar mavisi, oksidasyon-redüksiyon indikatörü olarak hücre canlılığı ve toksisite, hücresel büyüme ölçümleri için çeşitli deneylerde kullanılan ve uzun inkübasyon süresi boyunca canlı hücreler için toksik etki göstermeyen bir maddedir [22,23]. Bu özellikleri nedeniyle *Leishmania* parazitlerine karşı bileşiklerin sitotoksisitesini ölçmeye yönelik farklı yöntemlerde ve kantitatif kolorimetrik testlerde kullanılmıştır. Kültür ortamında canlı *Leishmania* promastigotlarının alamar mavisini indirgeyerek rengini maviden kırmızıya dönüştürdüğünü belirten çalışmalar mevcuttur [24].

Tedavide kullanılan anti-leishmanial ilaçların toksik yan etkileri ve bu ilaçlara karşı parazitin direnç geliştirmesi nedeniyle günümüze kadar yeni moleküllerin tanımlanması ve formülasyonları ile ilgili çalışmalar yapılmıştır [3]. Sudan'da yöresel olarak KL'nin tedavisinde kullanılan *Allium sativa* (sarımsak) ve *Azadirachta indica* (Neem)'dan elde edilen etanol ekstratlarının anti-leishmanial aktivitelerinin pentostamla kıyaslandığında anlamlı bir farkı olmadığını belirten çalışmalardan [14], makrolid grubunda yer alan iki antibiyotikten azitromisinin ve klaritromisine göre *L. topica* promastigot ve amastigotları üzerinen anti-leishmanial etkisinin daha yüksek olduğu vurgulayan [25] çalışmalara kadar farklı çalışmalar yapılmıştır [9,16,25-29].

Ulaşılan kaynak bilgilerde Coa ve ark.[26] on farklı kinolan-hidrazonhibridini, Alptüzün ve ark.[30] pyridinium hydrazone derivelerini, Al-Kahraman ve ark.[31] *N*-unsubstituted hidrazon yapıdaki 3 (benzilmonohydrazones) ve 5 (benzophenone-hydrazones) bileşiklerini ve Coimbra ve ark.[32] tarafından 7-chloro-4-quinolinyl hydrazone derivelerini kullanarak anti-leishmanial aktivitelerini denemişler ve olumlu sonuçlar elde ettiklerini bildirmişlerdir. Sunulan çalışmada kullanılmış olan hidrazonlar diğer araştırmalardan faklı yapıdadır *(Tablo 1)*.

Bu çalışmada kullanılan hidrazonların anti-leishmanial aktivitesi ile ilgili şimdiye kadar yapılan herhangi bir araştırmaya rastlanılmamıştır. Çalışmamızda elde edilen sonuçlara göre en etkili maddelerin 5e, 5g ve 5h bileşikleri (MİK=0.187 µg/ml) olduğu, en etkisiz bileşiğin ise 5i (MİK=3 µg/ml) olduğu görülmüştür *(Tablo 2)*. Standart ilaç olarak kullanılan amfoterisin B'nin ise 0.003 µg/ml konsantrasyonda etkili olduğu belirlenmiştir. Bu durum 5e, 5g ve 5h maddelerinin parazitin tedavisi için ilaç adayı olabilecekleri şeklinde açıklanabilir.

Leishmania türlerinin promastigot şekillerinin canlılığı ve çoğalmasının saptanmasında, enzimatik belirleyiciler, H^3 timin eklenmesi ve kolorimetrik metodların kullanılabileceği bildirilmiştir. Kolorimetrik metodlar arasında da alamar mavisinin tek aşamalı, uygulaması basit, düşük maliyetli, çevre dostu ve kolay transfer edilebiliyor oluşu ve ayrıca redoks indikatörünün hücre toksisitesinin olmaması ve uzun inkübasyon periyotlarında kullanılabiliyor olması gibi çeşitli avantajları rapor edilmiştir [3]. Mikus ve ark.[22] da, *L. major* promastigotlarıyla yapılan ilaç tarama testinde alamar mavisinin uygulanabilir kolorimetrik indikatör olduğunu göstermişlerdir. Çalışmamızda da indikatör boya olarak alamar mavisi kullanılmıştır.

Sonuç olarak; çalışmada sentezlenen 5e, 5g ve 5h (MİK = 0.187 µg/ml) bileşikleri antiparaziter olarak etkili bulunmuş olup bu hidrazonların ilaç adayı olarak ileri çalışmalarına devam edilebileceği kanısına varılmıştır. Sentezlenen bileşiklerin ilaç olarak kullanılabilmesi için; gerekli olduğu düşünülen *in vitro* makrofaj kültüründe *Leishmania* amastigotlarına karşı etkinliği ve *in vivo* olarak deneysel hayvan modellerinde kontrol çalışmalarına ihtiyaç vardır. Ayrıca alamar mavisi mikrodilüsyon yönteminin, anti-leishmanial ilaç aktivitesi araştırılmasında basit, güvenilir ve tekrarlanabilirliği yüksek bir yöntem olduğu saptanmıştır.

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Research Article

The Prevalence of Clinical Coccidiosis and the Estimation of the Costs of Disease Control and Treatment in Broiler Production [1]

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Abstract

This study aimed to determine the prevalence of clinical coccidiosis and to estimate the costs of disease control and treatment in broiler production. The study was performed with randomly selected poultry farms in Akşehir district of Konya Province. Each farm was visited for once at the day of 21 and over of the production period. Necropsy samples (5/10000) were collected from each farm for parasitological and histopathological examinations. The prevalence of clinical coccidiosis was found as 7.72%. A significant relationship was determined between the prevalence of clinical coccidiosis and age groups (P<0.05). Four Eimeria species: *E. brunetti* (35.48%), *E. necatrix* (35.48%), *E. maxima* (19.35%) and *E. tenella* (9.68%) were identified. Mixed infection was observed in 45.83% of the chicks. Disease control and treatment costs were estimated as 4604.17 $ and 2683.25 $ respectively. In conclusion, this multidisciplinary study showed that coccidiosis was still a serious disease for broiler production in the region. The costs of the control and treatment of coccidiosis may vary depending on its prevalence. Therefore, disease control strategies maintain its importance.

Keywords: *Clinical coccidiosis, Broiler production, Disease control cost, Treatment cost*

Broiler Üretiminde Klinik Koksidiyozisin Prevalansı ve Hastalık Kontrol ve Tedavi Maliyetlerinin Tahmini

Özet

Bu araştırma, broiler üretiminde klinik koksidiyozisin prevalansını belirlemek, hastalığın kontrol ve tedavi maliyetlerini tahmin etmek amacıyla yapılmıştır. Çalışma, Konya İli Akşehir İlçesinde rastgele seçilen işletmelerde yürütülmüştür. Her işletme üretim sürecinin 21 ve üzerindeki günlerde bir kez ziyaret edilmiştir. Bu işletmelerden, parazitolojik ve histopatolojik incelemeler için nekropsi numuneleri (5/10.000) toplanmıştır. Klinik koksidiyozisin prevalansı %7.72 bulunmuştur. Klinik koksidiyozisin prevalansı ile yaş grupları arasında anlamlı bir ilişki belirlenmiştir (P<0.05). Dört adet Eimeria türü tespit edilmiştir: *E. brunetti* (%35.48), *E. necatrix* (%35.48), *E. maxima* (%19.35) ve *E. tenella* (9.68%). Piliçlerin %45.83'ünde miks enfeksiyon gözlenmiştir. Hastalığın kontrol ve tedavi maliyetleri sırasıyla 4604.17 $ ve 2683.25 $ tahmin edilmiştir. Sonuç olarak, multidisipliner özelliğe sahip bu araştırma koksidiyozisin broiler üretimi için bölgede halen ciddi bir hastalık olduğunu göstermiştir. Hastalık kontrol ve tedavi maliyetleri koksidiyozisin prevalansına bağlı olarak değişebilir. Dolayısıyla, hastalık kontrol stratejileri önemini korumaktadır.

Anahtar sözcükler: *Klinik koksidiyozis, broiler üretimi, hastalık kontrol maliyeti, tedavi maliyeti*

INTRODUCTION

Coccidiosis caused by protozoan parasites of the genus *Eimeria*, is the most important parasitic disease of domestic poultry with substantial economic losses in the world. Commercial broiler production is mostly affected from coccidiosis. Production losses (mortality, decreased liveweight gain and increased feed conversion ratio-FCR)

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were reported up to 81-96% while disease control and treatment expenditures have responsibility up to 4-18% in total production costs [1,2].

The clinical form of the disease manifests through prominent signs of mortality, morbidity, diarrhoea or bloody faeces [2]. The attempts to control and treatment the disease can cause an increase in production costs [3,4].

Turkey, with an annual poultry meat production of 1.7 million tons has a 2% share in the world poultry meat market and has moved to 8th rank. In the last 12 years, production capacity of sector increased about 158%, its annual turnover is 4.5 billion US$, and export of poultry meat is about 567 million US$. As a growing sector, 2.4 million people work for it [5].

The aim of this study was to determine the prevalence of clinical coccidiosis and to estimate the costs of disease control and treatment in broiler production in Akşehir district of Konya province in Turkey.

MATERIAL and METHODS

Study Area

This study was conducted from July to November in 2011 in Akşehir district of Konya province where has a significant potential in terms of broiler production in Turkey. It is located some 135 km south-west of the Konya province, at an altitude of 1050 m above sea level with latitude 38° 22′N and 31° 26′E. The district has a continental climate: hot and dry summers, mild and rainy springs, cold and snowy winters. Broilers are reared in an intensive deep-litter system in this region.

Sampling

The material consisted of the farms that were allowed to give necropsy materials (approved by Afyon Kocatepe University, the Local Ethics Committee on Animal Experiments, 03/08/2011, B.30.2.AKÜ.0.8Z.00.00/76). Five necropsy sample per 10.000's capacity were collected from each farm [6]. Each farm was visited only one time from starting the 21st day of the production period (average slaughter age of chickens is 42 days in this area). Randomly selected necropsy samples were subjected to the routine post-mortem examination according to the method described by Conway and Mckenzie [7]. Charecteristics of lesions and sites were recorded. Mucosal scrapings were made from segments of intestine with gross lesions. Segments of intestines about 1-3 cm in length were taken for histopathology from parts of the intestine with gross lesions.

Parasitological Techniques

Mucosal scrapings were microscopically examined for developmental stages of coccidia. In positive samples, faeces were mixed thoroughly with a 2.5% potassium dichromate solution. The mixture was poured in thin layers into petri dishes and left to sporulate at 29°C for morphological identification of oocysts.

Histopathological Examination

The tissue samples taken from parts of the intestine with gross lesions were fixed with buffered formalin solution for microscopic examination and were blocked with paraffin after routine laboratory follow-up. Sections taken from paraffin blocks with 5 µ thickness were stained with hematoxylin-eosin (HE). The prepared sections were examined at light microscope (Olympus CX41) mounted with a digital camera and analysis system (Kameram®).

Species İdentification

Coccidial species were identified according to the site of infection, the sporulation time, morphological features of sporulated oocysts, nature of gross lesions and histological changes in tissues [7,8]. Sporulated oocysts from each sample were measured using a Nikon Eclipse i-Series 80i trinocular research microscope with 100x magnification and DS-5M-L1 digital camera system.

Estimation of the Costs of Disease Control and Treatment

The costs of disease control and treatment were calculated according to Williams [2] and Bera *et al.*[1]. General information of farms such as farmer's name, farm location, flock age, flock size, production period and feed consumption were collected through questionnaires at the time of sampling for estimating the costs of disease control and treatment. Otherwise, it was interviewed with the veterinarians who was working in the research area for disease control (the use and the price of coccidiostats) and treatment (medicine usage and the price of medicine).

Statistical Analysis

Chi-square (X^2) test was used for comparisons between infection and age groups.

RESULTS

In this study, clinical coccidiosis was diagnosed in 24 chicks (7.72%) out of 311 necropsied chicks. Results showed that the prevalence of clinical coccidiosis has statistically significant effects on age groups ($P<0.05$). Chicks with 36-42. days of age showed the highest prevalence of infection *(Table 1)*.

Four *Eimeria* species: *E. brunetti* (35.48%), *E. necatrix* (35.48%), *E. maxima* (19.35%), *E. tenella* (9.68%) were identified *(Table 2)*. Mixed infections with two species were found in 45.83% of the chickens *(Table 3)*.

The cost of disease control and treatment were calculated as 4604.,17 $ and 2683.25 $ respectively *(Table 4)*.

Table 1. *Prevalence of clinical coccidiosis by age groups*
Tablo 1. *Yaş gruplarına göre klinik koksidiyozisin prevalansı*

Necropsy samples	Age Groups (days)			Total
	21-28	29-35	36-42	
No. of examined	102	153	56	311
No. of positive	5	2	17	24
Prevalence (%)	4.90	1.31	30.36	7.72
x^2: 37.57, $P<0.05$				

Table 2. *Eimeria species by age groups*
Tablo 2. *Yaş gruplarına göre Eimeria türleri*

Eimeria species	Age Groups (days)			Total	%
	21-28	29-35	36-42		
E. brunetti	3	0	8	11	35.48
E. necatrix	2	1	8	11	35.48
E. maxima	1	0	5	6	19.35
E. tenella	0	0	3	3	9.68
Total	6	1	24	31	100.00

Table 3. *Frequency of Eimeria species according to single and mixed infections*
Tablo 3. *Tek ve miks enfeksiyonlara göre Eimeria türlerinin dağılımı*

Single and Mixed İnfections	Frequency	%
E. brunetti	7	29.16
E. necatrix	6	25.00
E. brunetti + *E. maxima*	2	8.33
E. brunetti + *E. tenella*	1	4.17
E. brunetti + *E. necatrix*	1	4.17
E. necatrix + *E. maxima*	3	12.50
E. necatrix + *E. tenella*	1	4.17
E. maxima + *E. tenella*	1	4.17
Eimeria spp.	2	8.33
Total	24	100.00

Table 4. *The costs of disease control and treatment*
Tablo 4. *Hastalık kontrol ve tedavi maliyetleri*

Cost parameters	Code	Value	Explanation
No. of chicks population	N	622000	Project data
No. of sampled	Ns	311	(N/10000)*5
No. of chicks in clinical form	Nc	48018	(N*7.72)/100
Cumulative feed consumption for 28 days (tonne/chick)	Cf	0.00219	Project data
Coccidiostat for control (kg/tonne)	Ac	0.5	Project data
Price of coccidiostat ($/kg)	Pa	6.76	Project data
Disease control cost (N x Cf x Ac x Pa) ($)	I	4604.17	
Water consumption for 21 day old chick (L/day)	Wc	0.25	Project data
Treatment period (days)	D	2	Project data
Medicine for treatment (ml/L)	Mt	0.001	Project data
Price of medicine ($/L)	Pm	111.76	Project data
Treatment cost (Nc x Wc x D x Mt x Pm) ($)	II	2683.25	
Total costs (I + II) ($)		7287.42	
1 US$: 1.7 Turkish Lira			

DISCUSSION

A diagnosis of clinical coccidiosis is warranted if oocysts, merozoites, or schizonts are seen microscopically and if lesions are severe [9]. In this study, the prevalence of clinical coccidiosis among examined broiler chicks was 7.72% (24/311). In the previous studies in different countries, the prevalence of coccidial infection in broiler chicks ranged between 21% and 92% [10-14]. In Turkey, there are limited studies on poultry coccidiosis [4,15,16]. Karaer *et al.*[15] suggested that prevalence of subclinical coccidiosis in broiler farms is 54.3% in different regions of Turkey.

In this study, the prevalence of clinical coccidiosis (7.72%) was very low compared to investigations in Turkey and other countries. Mismanagement, such as wet litter that encourage oocysts sporulation, contaminated drinkers and feeders, poor ventilation, and high stocking density can exacerbate the clinical signs [17]. Coccidiosis can be controlled by proper stocking density, the housing and handling of the chicks, the proper use of anticoccidial drugs [12,16]. In current investigation, this might be due to good management practices, and the proper use of anticoccidial drugs (questionnaires).

It was noted that the highest rate of clinical coccidiosis (30.36%) was determined in the 36-42 days age group in the present study. This is in agreement with the findings reported by Lobago *et al.*[12], Karaer *et al.*[15] and Amare *et al.*[18]. Hygienic measurements are not enough alone for coccidiosis control. In order to prevent infection usually supplemental anticoccidial feeds and for the treatment of disease drugs including active ingredients of sulphonamide, amprolium and toltrazuril are used [3].

In this study, it has been found that anticoccidial feed additives were used except the last two weeks (between 28^{th}-42^{nd} days) of the production period. It was determined that these drugs were mixed into the drinking water. We found pathogenic *Eimeria* species responsible for clinical coccidiosis: *E.brunetti*, *E. necatrix*, *E.maxima*, *E.tenella*. Previous studies reported that *E.tenella* was the most prevalent coccidial species in broiler chickens [13,17,19,20], where as the findings of the present study showed that *E. brunetti* and *E.necatrix* were the most common. This inconsistency may be arised from which the anticoccidial drugs in general use are developed specifically to control this pathogenic species.

Bera *et al.*[1] suggested that the major economic losses considered in relation to clinical coccidiosis in commercial broiler were expenses on control, therapy and mortality. Death in poultry is known to occur more often in situations where more than one pathogenic agent is combined. It has been reported that coccidiosis is seen with salmonella or gumboro [21]. However, lethal coccidiosis is becoming rare, because of widely implemented prevention and control strategies [2,22].

According to Williams [2], the anticoccidial drugs used constitute an extremely variable cost of chick production, both from drug to drug and from country to country. In this study, disease control was the most important parameter in broiler industry in Akşehir district of Konya province. This result was in consistent with the finding of Bera *et al.*[1] who reported prevention of coccidiosis in India mainly depends on control. Prophylactic use of anticoccidial feed additives are primary means of controlling coccidiosis in the broiler industry. However, this leads to a further problem of drug resistance and drug residue in consumable meat [1].

In conclusion, the costs of disease control and treatment may vary due to the prevalence of coccidiosis which is affected by several factors such as geography and climate. This study that carried out in multidisciplinary manner showed that coccidiosis is still a serious disease for broiler production in the region. Therefore, disease control strategies are going to be the main issue about coccidiosis.

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Research Article

First Molecular Characterization of *Raphidascaris acus* Bloch, 1779 (Nematoda: Anisakidae) from European eels *(Anguilla anguilla* Linnaeus, 1758) Caught off the Aegean Region Streams, Turkey [1]

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Abstract

In this study, the presence of anisakid nematodes in the European eels *(Anguilla anguilla* L.) was investigated. A total of 30 specimens of eels were caught by local fishermen from the Buyuk Menderes River, Turkey. Nematoda species of *Raphidascaris acus* was found from eels. This is the first record of *R. acus* from *A. anguilla* from Turkish waters. The ribosomal DNA (rDNA) internal transcribed spacer regions (ITS-1and ITS-2) and 5.8S of this parasitic species was amplified and sequenced. Also, all the nematodes were identified as *R. acus* based on nucleotide sequence comparisons. Pairwise comparison between the entire ITS regions and 5.8S of the *R. acus* isolates of *A. anguilla* (GenBank accession number: KT633862) and other *R. acus* isolates from Caspian Sea (KM047505), and Vistula Lagoon, Poland (AY603537) showed differences ranging from 0.0 to 1.9% intraspecific nucleotide differences, respectively. With the present study, *R. acus* infecting *A. anguilla* caught off the Aegean Region streams were characterized for the first time by sequencing of the rDNA ITS regions and 5.8S.

Keywords: *Raphidascaris acus, Anguilla anguilla, ITS gene regions, 5.8S, Aegean Region streams, Molecular characterization*

Ege Bölgesi Akarsularında Yakalanan Avrupa Yılan Balıklarında *(Anguilla anguilla* Linnaeus, 1758) *Raphidascaris acus* Bloch, 1779 (Nematoda: Anisakidae)'un İlk Moleküler Karakterizasyonu

Özet

Bu çalışmada balıkçılar tarafından Büyük Menderes Nehri'nden yakalanan 30 adet Avrupa yılan balığında *(Anguilla anguilla* L.) anisakid nematodların varlığı araştırılmıştır. Yılan balıklarında *Raphidascaris acus* türü nematoda rastlanmıştır. *Raphidascaris acus* Türkiye sularında yakalanan yılan balıklarında ilk kez bildirilmiştir. İdentifiye edilen parazitlerin internal transcribed spacer gen bölgeleri (ITS-1 ve ITS-2) ve 5.8S rDNA'sı amplifiye edilerek sekans analizleri gerçekleştirilmiştir. Nükleotid sekans karşılaştırmaları sonucu bu nematodların *R. acus* olduğu doğrulanmıştır. Yılan balığında tespit edilen *R. acus* izolatının (GenBank erişim numarası: KT633862) ITS gen bölgeleri (ITS-1 ve ITS-2) ve 5.8S bölgesinin ikili hizalamaları sonucunda Hazar Denizi (KM047505) ve Polonya, Vistula Lagünü'nden (AY603537) izole edilmiş *R. acus* izolatları ile arasında sırasıyla %0.0 ve 1.9 oranlarında tür içi nükleotit farklılığı saptanmıştır. Bu çalışmada Ege Bölgesi akarsularında sularında yakalanan yılan balıklarında *(A. anguilla)* saptanan *R. acus*'un ITS gen bölgeleri (ITS-1 ve ITS-2) ve 5.8S rDNA'sı sekanslanarak ilk kez moleküler karakterizasyonu yapılmıştır.

Anahtar sözcükler: *Raphidascaris acus, Anguilla anguilla, ITS gen bölgeleri, 5.8S, Ege Bölgesi akarsuları, Moleküler karakterizasyon*

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INTRODUCTION

Anisakid nematodes of the genus *Raphidascaris* (Railliet & Henry, 1915) are parasites of the digestive tract of a range of marine, brackish and freshwater fishes in world-wide. *Raphidascaris acus* is a cosmopolitan species reported from different host species [1].

Raphidascaris acus has three well developed lips that mouth surrounded. However, the interlabia are rudimentary or absent. Nerve ring is encircling approximately at border of first and second thirds of oesophagus. Excretory pore is located at behind level of nerve ring. The intestinal caecum is absent. *Raphidascaris acus* has various genital papillae. The tail of both sexes is short and conical [2].

Raphidascaris acus has been morphologically identified from Turkish waters [3,4]. However, to date there has not been any study regarding molecular characterization of *R. acus* from fish caught off the Turkish waters. Recently, molecular techniques, using DNA sequencing of the nuclear ribosomal DNA spacers, have been proven to be particularly useful for the accurate identification of ascaridoid nematodes at the species level for eggs, larvae, and adults [5-12]. Nevertheless, before the present study, there had been no reports of characterizing the *R. acus* from the Turkish waters using well-defined internal transcribed spacers (ITS1 and ITS2) and 5.8S region sequence.

Therefore, in the present study, *R. acus* from Aegean Region streams were genetically characterized for the first time by sequencing of ITS regions and 5.8S subunit markers.

MATERIAL and METHODS

Sampling and Parasitological Examination

A total of 30 specimens of *Anguilla anguilla* were caught by local fishermen from the Buyuk Menderes River in Aydın vicinity of Turkey. Fishes were dissected carefully and examined for nematodes in the stomach, intestine, abdominal cavity, and muscles. Nematodes were only found from the intestine and washed in physiological saline. For each nematode, a small piece of the mid-body was cut and stored in 70% ethanol for molecular analyses and anterior-posterior ends of specimens were cleared in lactophenol for morphological studies. The parasites were identified by using the morphology of the labia, the position of the excretory pore, ventricular appendix and the tail [1].

DNA Extraction, PCR Amplification, and Sequencing

Three nematodes (i.e., three individuals were randomly selected among the samples) were subjected to the molecular analysis. Genomic DNA (gDNA) was extracted from the nematodes using the DNA purification kit (Wizard Genomic, Promega) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed to amplify the ITS regions (ITS-1and ITS-2) and 5.8S. PCR reaction (50 µl) was contained 10-50 ng of extracted DNA, 1× TaqBuffer with KCl (Thermo Scientific), 3mM of $MgCl_2$ (Thermo Scientific), 0.3 mM dNTPs (Thermo Scientific), 2 pmol of each primer, 2.5 U of Taq DNA polymerase (Thermo Scientific), and DEPC-treated water. The ITS regions and 5.8S were amplified using the primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTA GTTTCTTTTCCTCCGCT-3′) [5]. The PCR was performed in an automated thermocycler (Applied Biosystems) and the conditions were modified as follows: 15 min at 95°C, then 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C followed by a final extension step at 5 min at 72°C. PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and visualized by UV illumination. Then the positive samples were purified by the commercial kit (High Pure PCR product purification kit, Roche) from the agarose gel. The purified products were commercially sequenced by Macrogen (Netherlands) in both directions, using NC5 and NC2 primers.

Data Analysis and Phylogenetic Tree Construction

The forward and reverse nucleotide sequences were assembled and edited with using Contig Express (Vector Nti® Advance 11.5, Invitrogen, Carlsbad, California, USA). A standard nucleotide Basic Local Alignment Search Tool (BLAST [blastn]) search was conducted [13]. Nucleotide sequences were aligned with the software CLUSTAL W in Mega 6.0 multiple sequence alignments [14]. Genetic distances were calculated using the Kimura two-parameter model with pairwise deletion in Mega 6.0 [15]. Phylogenetic analysis with other known *Raphidascaris* species was conducted using Maximum-Likelihood (ML) analysis in Mega 6.0 [15]. The aligned sequences were tested with Mega 6.0 model test to find the best DNA model to infer the phylogenetic trees [15]. The general-time reversible model (GTR+G) was selected using Akaine Information Criterion (AIC). The evolutionary history was inferred using the ML method based on the GTR+G model for ITS sequences with *Eustrongylides* sp. as an out group. Confidence in the ML trees was determined by analyzing 1.000 bootstrap replicates [16] using the Mega 6.0 program. The sequences of ITS regions and 5.8S of *R. acus* has been deposited in GenBank databases under accession number KT633862.

RESULTS

These species were analyzed in the present paper clearly belongs to the genus *Raphidascaris*. The morphology of our specimens agrees well with the description of *R. acus* [1]. The amplification of the ITS regions and 5.8S was produced a fragment of approximately 1.000 bp from each nematodes. No intraspecific differences were found

in the sequences of *R. acus,* which represented a single genotype. Therefore, only one sequence was submitted to GenBank. The length of ITS sequences of *R. acus* was 866 bp. *Raphidascaris acus* isolates from the Menderes River, Turkey (KT633862) showed 98.1 to 100% identity with various geographical isolates of *R. acus* from the Vistula Lagoon, Poland (AY603537) and Caspian Sea (KM047505) from GenBank, respectively. Pairwise comparison between the present data and other *R. acus* isolates from the Caspian waters (KM047505) and the Poland (AY603537) displayed only 0.0 to 1.9% intraspecific nucleotide differences *(Table 1)*, respectively. Phylogenetic relationships among *R. acus* isolates from *A. anguilla* of the Menderes River, Turkey and the other *Raphidascaris* species isolates as inferred by ML analysis of the ITS sequence are presented in *Fig. 1*.

DISCUSSION

The genus *Raphidascaris* Railliet & Henry, 1915 consists of three subgenera namely *Ichthyascaris* Wu, 1949, *Sprentascaris* Petter & Cassone, 1984, and *Raphidascaris* Railliet & Henry, 1915 [17-19]. The present material is assigned to *Raphidascaris* due to the lips well-developed, excretory pore slightly behind the nerve-ring, posterior directed ventricular appendix, and intestinal caecum absent [1]. *Raphidascaris acus* is found in different freshwater fishes (Esocidae, Salmonidae, Anguillidae, Gadidae) in Europe, Asia and North America [1]. Until now, only one species, *R. acus* has been morphologically identified from Esocidae *(Esox lucius* L.) from Turkish waters [3,4]. To the best of our knowledge, this is the first record of *R. acus* from Anguillidae *(A. anguilla*) from Turkish waters.

Accurate identification of a parasite at any stage of its development has important implications for studying parasite epidemiology and resolving taxonomic problems [20]. Different studies have demonstrated that the ITS regions and 5.8S provide useful genetic markers for the accurate identification of sibling species and morphospecies within ascaridoid species [5,10-12,21].

There are three *Raphidascaris* species [*R. lophii* (Wu, 1949), *R. trichiuri* (Yin & Zhang, 1983), and *R. longispicula* (Li et al., 2012)] belongs to the subgenus *Ichthyascaris* Wu, 1949 [22-24] and two species [*R. acus* (Bloch, 1779) Railliet

Table 1. Pairwise comparison of nucleotide sequence differences (%) in the ITS gene regions and 5.8S among *Raphidascaris acus* (Turkey) isolates and various geographical isolates

Tablo 1. *Raphidascaris acus* izolatı (Türkiye) ile değişik coğrafik bölgelere ait izolatların ITS gen bölgeleri ve 5.8S nükleotit sekans farklılıklarının (%) ikili karşılaştırmaları

Isolate No	GenBank Accession Numbers and Locations	Nucleotide Sequence Differences (%)		
		1	2	3
1	KT633862 Menderes River, Turkey			
2	KM047505 Caspian Sea	0.0		
3	AY603537 Vistula Lagoon, Poland	1.9	1.9	

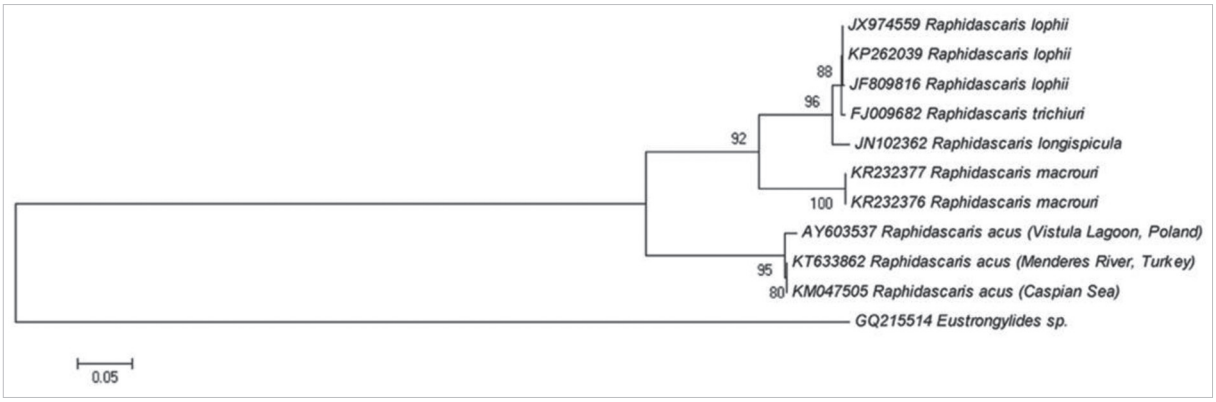

Fig 1. Phylogenetic tree reconstructed using Maximum-Likelihood (ML) analysis of ITS regions and 5.8S. The evolutionary history was inferred using the ML method based on the GTR+G model for ITS sequences with *Eustrongylides* sp. as an out group. The accession numbers of individual sequences determined in the present study are shown in each tree. A scale bar indicates estimated distance. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1.000 replicates) are shown at the internal nodes (>70% only)

Şekil 1. ITS gen bölgeleri ve 5.8S'in filogenetik ağacı Maximum-Likelihood (ML) analiz yöntemi kullanılarak oluşturulmuştur. ITS dizilimlerinin evrimsel sürecine *Eustrongylides* sp.'nin dış grup olarak kullanılması ile GTR+G modelini temel alan ML analiz yöntemi kullanılarak varılmıştır. Bu çalışmada belirlenen sekansların erişim numaraları ağaçta gösterilmiştir. Ölçek çubuğu tahmini uzaklıkları göstermektedir. Bootstrap testinde birbirleri ile ilişkili taksonların filogenetik ağaçtaki tekrar yüzdeleri ağacın iç düğümlerinde gösterilmiştir (>%70)

& Henry, 1915, *R. macrouri* (Pérez-i-García et al., 2015)] belongs to the subgenus *Raphidascaris* Railliet & Henry, 1915 studied of the ITS gene region [10,19].

This study provides the first molecular characterization of *R. acus* from the Turkish waters. *Raphidascaris acus* isolate of Turkey (KT633862) showed 100% identity with isolate of Caspian Sea (KM047505) from GenBank. Nonetheless, the ITS sequence variation between the Turkish and Polish populations was 1.9%. A significant ITS sequence variation between the two populations is evidence on the lacking gene flow between the Turkish waters and Vistula Lagoon, Poland. In this study, bootstrapping of the sequences with ML revealed significant support for one clade containing *R. acus* isolates from the Turkey (KT633862), Caspian waters (KM047505) and the Vistula Lagoon, Poland (AY603537), revealing a close relationship between these isolates *(Fig. 1)*.

As conclusions, the ITS gene sequences of *R. acus* from Turkish waters have been obtained for the first time in the present study, and further researches using more polymorphic genetic markers are required to examine the genetic variability and population genetic structure within *R. acus* from different freshwater and marine fish species and geographical locations in Turkey.

Conflict of Interest

The authors do not have any potential conflicts of interest to declare.

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Research Article

The Effect of β-carotene on Acute Phase Response in Diethylnitrosamine Given Rabbits [1]

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Abstract

The aim of this study was to investigate the effect of β-carotene in acute phase response (APR) in rabbits which were administered high doses of toxic diethylnitrosamine (DEN). Twenty-one New Zealand race rabbits at 5-7 months of age were divided into 3 groups each having 7 ones. Control group received single dose of 0.9% NaCl solution intraperitoneally (IP); Group 1 received single dose of 100 mg/kg DEN (IP); Group 2 received both 100 mg/kg DEN (IP) and 2 mg/kg/day β-carotene orally for 7 days. After applying administrations, blood samples were obtained on 1st, 4th, and 7th days. The levels of ceruloplasmin was determined by the Colombo and Richterich technique; serum amyloid A (SAA) and haptoglobin using ELISA; iron (Fe) and unsaturated iron binding capacity (UIBC), AST and ALT using a commercial colorimetric kit; total iron binding capacity (TIBC) and transferrin saturation (TS) were determined using a formula based on Fe and UIBC. We determined that the levels of haptoglobin and SAA on 1st, 4th, and 7th days ($P<0.01$) and the level of ceruloplasmin on 1st and 4th days significantly increased ($P<0.01$) relative to the controls. On the other hand, the levels of TS decreased on 4th and 7th days relative to control ($P<0.05$). These results suggest that β-carotene has a protective role on APR resulted from the toxic effect of DEN.

Keywords: *Acute phase protein, β-carotene, Diethylnitrosamine (DEN), Rabbit*

Dietilnitrozamin Verilen Tavşanlarda β-karotenin Akut Faz Yanıta Etkisi

Özet

Bu çalışmada, tavşanlara yüksek dozda verilen dietilnitrozaminin (DEN) toksik etkisine karşı oluşabilecek akut faz yanıt (AFY) üzerine β-karotenin etkisinin araştırılması amaçlandı. Materyal olarak kullanılan 21 adet, 5-7 aylık Yeni Zelanda ırkı tavşan, her birinde 7 adet olacak şekilde 3 gruba ayrıldı. Kontrol: Tek doz %0.9'luk NaCl solüsyonu intraperitoneal (İP), Grup I: Tek doz 100 mg/kg DEN (İP), Grup II: Tek doz 100 mg/kg DEN (İP) + 2 mg/kg/gün β-karoten 7 gün boyunca günlük oral yolla verildi. Tavşanlardan enjeksiyondan sonraki 1., 4. ve 7. günlerde kan örnekleri alındı. Seruloplazmin düzeyi Colombo ve Richterich yöntemiyle, serum amiloid A (SAA) ve haptoglobin ELISA kit ile albümin, demir (Fe), doymamış demir bağlama kapasitesi (DDBK), AST, ALT ticari kit kullanılarak kolorimetrik yöntem ile, total demir bağlama kapasitesi (TDBK) ve transferin doyumu (TD) ise Fe ve DDBK üzerinden formülle hesaplanarak saptandı. Haptoglobin ve SAA düzeylerinin 1., 4. ve 7. günlerde ($P<0.01$), seruloplazmin düzeyinin 1. ve 4. günde kontrol grubuna göre arttığı ($P<0.01$), TD düzeyinin ise 4. ve 7. günlerde kontrol grubuna göre azaldığı ($P<0.05$) gözlenmiştir. Sonuç olarak, DEN'in toksik etkisine bağlı olarak meydana gelen AFY'ye β-karotenin koruyucu etkisinin olabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Akut faz protein, β-karoten, Dietilnitrozamin (DEN), Tavşan*

INTRODUCTION

Nitrosamines which are found in different types of foodstuff including, meat, salted fish, alcoholic beverages, agricultural drugs, insecticides, cigarette and several vegetables are known to have carcinogenic effects [1-4]. Nitrosamines cause tissue damage and inflammation because they increase the levels of free radicals, and ultimately leads to acute phase response (APR) [5]. To evaluate the damage in the liver, the levels of AST and ALT are routinely measured [6].

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The precursor of vitamin A, β-carotene, is taken via diet because it cannot be synthesized in the body [7]. Due to conjugated double bonds in their chemical structures, carotenoids have an inhibitory action for superoxide [8-11]. Carotenoids not only remove already existed superoxide and peroxide radicals but also prevent the formation of them [12,13].

Acute phase response is an unspecific reaction in an organism in response to inflammation, tissue damage, neoplastic formations, or immunologic disorders. It actually delineate changes in the concentration of numerous plasma proteins which are produced by the liver in response to above listed events [14,15]. Assessing APR is important for determining diagnosis, prognosis, and treatment strategies [16]. There-fore, it is imperative to better understand the mechanism of APR in various pathological conditions in various animal species.

It is believed that free radicals are one of the main players causing inflammation and tissue damage. Hence, in this study, the toxic effects of high DEN exposure on liver damage at the tissue level, and the role of β-carotene on APR were investigated.

MATERIAL and METHODS

In this study, 21 New Zealand race rabbits at 5-7 months of age were utilized. Before the experimental procedure, the permission for the use of laboratory animals was obtained from Kafkas University Animal Experimentation Ethics Board (Decision no: KAU-HADYEK: 2012/9).

Animals were adapted in an environment with temperature (25±2°C), and light (12 h light/dark cycles), and ventilation. All animals allowed to access *ad libitum* nourishing. According to their weight, 3 groups each having 7 rabbits were formed. The weight and total feed consumption of each group was recorded weekly basis throughout the experiment. Control group received single dose of 0.9% NaCl solution intraperitoneally (IP); Group 1 received single dose of 100 mg/kg DEN (IP); Group 2 received both 100 mg/kg DEN (IP) and 2 mg/kg/day β-carotene (Sigma) daily by oral gavage for 7 days. For biochemical analyses, blood samples obtained from *vena auricularis* at 1st, 4th, and 7th days post-injections, and serum was separated and stored at -20°C until further analysis.

The levels of ceruloplasmin was determined by the Colombo and Richterich [17] technique; SAA and hapto-globin using ELISA (Cusabio Biotech, China); albumin, Fe, and UIBC, AST and ALT using a commercial colorimetric kit (DDS, Turkey and Epoch, Biotech, USA); TIBC and TS were determined using a formula based on Fe and UIBC (TS (%) = Fe/TIBC x 100) [18].

Statistical Analysis

The data were analyzed using SPSS [19] for Windows 16.0.2 software. The difference among groups using ANOVA and Tukey multiple comparison test; and the difference among days was determined via variants analysis of repeated measures. Values were presented as mean +/- standard error.

RESULTS

When comparing groups relative to the control, the levels of haptoglobin and SAA on 1st, 4th, and 7th days in group I and II significantly increased relative to control ($P<0.01$), and the level of haptoglobin peaked on day 1, the levels of SAA peaked on day 4. The level of ceruloplasmin increased relative to control on 1st and 4th days ($P<0.01$) with the highest level on day 4th. The concentration of albumin and Fe in groups increased relative to the control but not significant. The level of TS (%) significantly decreased ($P<0.05$) on day 4 and 7 relative to the control group.

When comparing days, haptoglobin levels in both DEN and DEN + β-carotene were significantly higher on day 1, and significantly lower on days 4 and 7 ($P<0.01$). The level of ceruloplasmin in DEN given group was highest on 4th day ($P<0.01$), and it was lowest on 7th day in DEN + β-carotene group ($P<0.05$). The concentration of albumin significantly lower on 4th and 7th days in DEN and DEN + β-carotene relative to 1st day. The increase in TIBC in those days were not significant relative to day 1. The levels of AST and ALT in response to DEN was highest on 7th day relative to other days ($P<0.05$), and they was significantly higher in DEN and DEN + β-carotene group on 4th and 7th days relative to 1st day ($P<0.05$).

When comparing groups, the levels of AST and ALT in all groups significantly increased in all three days relative to control ($P<0.05$), and reached the highest on 7th day. In addition, the levels of AST and ALT were significantly lower in DEN + β-carotene relative to only DEN given group ($P<0.05$) *(Table 1)*.

DISCUSSION

Nutrients meet animals' nutritional needs and may play roles in preventing diseases; however, they may also cause diseases. N-nitrosamines in the environment and in nutrients are one of the risks [1]. Although the mechanism is not fully understood, nitrosamines which are used as preservative in meat and meat products are proposed to be carcinogenic for numerous tissues [4,20]. DEN metabolism is catalyzed by the enzymes in monooxygenase system which belongs to cytochrome P-450. As a consequence of its metabolic activation, it displays its toxic effects. The intermediate products stemmed from its bioactivation have low binding affinity to the binding sites of various enzymes, and therefore, instead of excretion with urine, they may form covalent bonds with important components

Table 1. *The levels of acute phase protein and the activities of AST and ALT in DEN given rabbits*
Tablo 1. *DEN verilen tavşanlarda bazı akut faz protein düzeyleri ile AST ve ALT aktiviteleri*

Parameters	Groups	Time (day)			P Values
		1	4	7	
Haptoglobin (g/L)	Control	0.108±0.003^{x}	0.105±0.003^{x}	0.104±0.005^{x}	NS
	DEN	1.003±0.021^{y,a}	0.894±0.032^{y,b}	0.873±0.032^{y,b}	P<0.01
	DEN+β-Carotene	0.756±0.021^{z,a}	0.637±0.019^{z,b}	0.594±0.014^{z,b}	P<0.01
	P Values	P<0.01	P<0.01	P<0.01	
SAA (µg/mL)	Control	14.36±0.90^{x}	13.76±0.62^{x}	14.96±0.25^{x}	NS
	DEN	24.88±0.33^{y,a}	25.82±0.15^{y,b}	23.84±1.50^{y,ab}	P<0.01
	DEN+β-Carotene	23.52±0.67^{y,a}	24.34±0.70^{y,a}	21.68±0.76^{y,ab}	P<0.01
	P Values	P<0.01	P<0.01	P<0.01	
Ceruloplasmin (mg/dL)	Control	16.33±1.49^{x}	17.11±2.42^{x}	16.47±2.34	NS
	DEN	27.26±1.44^{ya}	28.87±1.43^{ya}	22.81±1.72^{b}	P<0.01
	DEN+β-Carotene	24.58±2.17^{ya}	25.15±2.25^{ya}	19.20±1.04^{b}	P<0.05
	P Values	P<0.01	P<0.01	NS	
Albumin (g/dL)	Control	3.40±0.11	3.44±0.13	3.41±0.13	NS
	DEN	3.23±0.13	3.06±0.17	3.05±0.09	NS
	DEN+β-Carotene	3.31±0.19	3.12±0.10	3.08±0.12	NS
Fe (µg/dL)	Control	184.70±5.36	181.90±3.25	185.23±5.09	NS
	DEN	176.02±4.92^{a}	171.53±3.45^{b}	170.62±4.60^{b}	P<0.05
	DEN+β-Carotene	180.26±6.16	174.28±4.80	178.58±4.83	NS
TIBC (µg/dL)	Control	264.80±2.49	262.57±2.95	268.89±5.82	NS
	DEN	278.48±4.94	279.46±4.91	282.77±5.93	NS
	DEN+β-Carotene	274.93±3.54	276.77±6.26	277.84±4.07	NS
TS (%)	Control	69.85±2.45^{x}	69.28±1.08^{x}	68.97±1.79^{x}	NS
	DEN	63.33±2.11^{x,a}	61.58±2.13^{y,b}	60.57±2.37^{y,b}	P<0.05
	DEN+β-Carotene	65.72±2.84^{x}	63.13±2.06^{y}	64.29±1.62^{y}	NS
	P Values	NS	P<0.05	P<0.05	
AST (U/L)	Control	100.70±6.05^{x}	99.86±3.20^{x}	99.33±4.44^{x}	NS
	DEN	122.11±6.33^{y,a}	130.90±6.73^{y,a}	148.03±7.85^{y,b}	P<0.05
	DEN+β-Carotene	108.43±3.35^{z,a}	116.79±5.23^{z,a}	121.43±8.30^{z,b}	P<0.05
	P Values	P<0.05	P<0.05	P<0.05	
ALT (U/L)	Control	86.93±5.75^{x}	85.41±6.92^{x}	86.61±5.72^{x}	NS
	DEN	118.17±5.78^{y,a}	122.73±12.76^{y,a}	128.57±6.41^{y,b}	P<0.05
	DEN+β-Carotene	98.50±6.56^{z,a}	99.39±8.95^{z,a}	103.21±6.49^{z,b}	P<0.05
	P Values	P<0.05	P<0.05	P<0.05	

^{a,b} The groups in the same line labeled different letters are statistically significant (P<0.05, P<0.01)
^{x,y,z} The groups in the same column labeled different letters are statistically significant (P<0.05, P<0.01), ***NS:*** *Non Significant*

of the cells, and ultimately cause mutations, necrosis and cancer [5,21]. One of the intermediate products formed by the bioactivation of DEN is superoxide anion free radical that may cause inflammation and tissue damage [22]. Inflammation and tissue damage triggers APR, and as a result, acute phase proteins (APP) are produced by the liver [16].

Atakisi and Ozcan [3], Chiarello et al.[23], Bansal et al.[24] and Pradeep et al.[25] have reported that single dose of 150 mg/kg or 200 mg/kg DEN (IP), respectively caused significant liver damage. In addition to these studies, Sahin et al.[26] reported that exposure of 100 and 200 mg/kg DEN (IP) increased serum concentrations of AST, ALT and GGT in rats. In this study, we believe that the increase in AST and ALT resulted from the hepatotoxicity due to DEN exposure. In order to decrease the damage caused by DEN, Atakisi and Ozcan [3], Atakisi et al.[27] reported that

omega-3 rich fish oils might decrease the toxic effects of DEN. Similarly, in other studies in rats, when Karaca and Baysu Sozbilir [28] administered α-lipoic acid; when Sadik et al.[29] administered blueberries, and when Liu et al.[30] administered barley enriched with selenium, the activities of AST, ALT, ALP, and GGT were decreased relative to only DEN given group, and accordingly they decreased the toxic effects of DEN. Parallel to these studies, in our study, β-carotene decreased the serum levels of AST and ALT on 7th day suggesting the protective effect of β-carotene.

Free oxygen radicals play important roles in stress induced tissue damage and pathogenesis of inflammation [31,32]. The imbalance between protective and damaging mechanisms results in acute inflammation. Cytokines, such as IL-1 and TNF-a, are released from damaged tissues due to inflammation. Because of the effects of these cytokines, APPs are synthesized in the liver [33]. The levels of these proteins are important for diagnosis and prognosis of inflammation, tissue damage, and formation of tumors [34]. There are a very limited number of studies on the effect of DEN on APR up-to-date. Sadik et al.[29] and Sukata et al.[35] have reported that the concentrations of α-fetoprotein and α-macroglobulin positive APPs are increased in response to DEN. Consistently, in our study, a positive APP, haptoglobin, on 1st day, and SAA and ceruloplasmin reached their highest levels on 4th day, and subsequently their concentration significantly dropped on 7th day. The reduction in APP on day 7 might be due to the inhibiting effects of β-carotene on superoxide anions.

Active cytokines during acute phase reaction released from the damaged tissues also affect other organs, such as the brain, liver, and other tissues and cause a reduction in the levels of various minerals, such as Ca, Zn, and Fe [15]. In this study, we found that the level of Fe dropped possibly due to APR. The level of TS is also reduced proportionally to the level of Fe.

In conclusion, our study revealed that toxic effect of DEN led to APR; and as a consequent of this, the synthesis APP and the hepatotoxicity were induced. Our results also support the idea that exogenous β-carotene supplementation has beneficial roles against APR resulted from the toxic effects of DEN, and this should be further scrutinized for both medical and veterinary clinical pathology.

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Research Article

Effects of Borax on Inflammation, Haematological Parameters and Total Oxidant-Antioxidant Status in Rats Applied 3–Methylcholanthrene [1]

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Abstract

In this study was investigated effects of borax (BX) on inflammation markers, haematolojical parameters and total oxidant (TOS)-antioxidant status (TAS) in rats applied 3–methylcholanthrene (3-MC). In this research a total of 24 Wistar Albino rats were used. They were divided into 4 groups each containing 6 rats. 1st group was separated as a control group. 3-MC was applied twice a week first 2 weeks 25 mg/kg dose to the 2nd group with i.p. way. BX was given to 3rd group 300mg/L/day dose with drinking water during 150 days. 3-MC was applied twice a week first 2 weeks 25 mg/kg dose with i.p. way and BX were given with drinking water during 150 days to 4th group. At the end of the study blood analysis, tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) levels in 3-MC group; TOS and oxidative stress index (OSI), platelet (PLT) levels in 3-MC and 3-MC+BX groups showed significantly increases when compared to other groups. It was determined that lymphocytes % (LY%) of ever 3 groups were significantly higher; however, neutrophil % (NEU%) were significantly fewer according to control group. Haemoglobin (HGB) and hematocrit (HCT) values of 3-MC+BX groups showed significantly decrease according to other groups (P≤0.05). Mean corpusculer volume (MCV) in 3-MC and 3-MC+BX groups showed significantly decrease when compared to other groups (P≤0.05). As a result, in case of exposure to 3-MC, long-term use of BX with oral ways may not decrease oxidative stress, may changes haematolojical parameters such as, WBC, LY%, NEU%, PLT, HGB, HCT, MCV. However, these changes remain within physiological limits. Even so, in the use of BX should be considered use of iron. Furthermore, BX with the abovementioned dosage may be used to reduce the levels of TNF-α, IL-1β, IL-6 being inflammation and cancer markers.

Keywords: *Borax, Haematology, Inflammation, Interleukin, Rat, Tas, Tos, Tnf-α, 3-MC*

3-Metilkolatren Uygulanan Sıçanlarda Boraksın İnflamasyon, Hematolojik Parametreler ve Total Oksidan-Antioksidan Durumlar Üzerine Etkileri

Özet

Bu çalışmada, 3-metilkolatren (3-MC) uygulanan sıçanlarda boraksın (BX) inflamasyon göstergeleri, hematolojik parametreler ve total oksidan (TOS)-antioksidan durumlar (TAS) üzerine etkileri araştırıldı. Çalışmada toplam 24 Wistar Albino sıçan kullanıldı. Sıçanlar her grupta 6'şar adet olacak şekilde 4 gruba ayrıldı. Birinci Grup kontrol grubu olarak ayrıldı. İkinci gruba 25 mg/kg dozunda haftada iki kez ilk 2 hafta 3-MC i.p. yolla uygulandı. Üçüncü gruba BX 300 mg/L/gün dozunda içme suları ile 150 gün boyunca verildi. Dördüncü gruba 3-MC 25 mg/kg dozunda haftada iki kez ilk 2 hafta i.p. yolla uygulandı ve BX 300 mg/L/gün dozunda içme suları ile 150 gün boyunca verildi. Çalışma sonunda kan analizlerinde, diğer gruplarla karşılaştırıldığında 3-MC grubunda tümör nekrozis faktör alfa (TNF-α) ve interlökin 1 beta (IL-1β); 3-MC ve 3-MC+BX gruplarında ise, TOS, oksidatif stres indeksi (OSI) ve trombosit (PLT) seviyeleri istatistiksel önemde artış gösterdi. Kontrol grubuna göre her 3 gruptaki % lenfosit (%LY) seviyeleri yüksek; fakat % nötrofil (%NEU) seviyeleri önemli düzeyde düşük olduğu belirlendi. 3-MC+BX grubunda hemoglobin (HGB) ve hematokrit (HCT) değerleri diğer gruplara gore önemli bir azalma gösterdi (P≤0.05). 3-MC ve 3-MC+BX gruplarındaki ortalama alyuvar hacmi (MCV) diğer gruplarla karşılaştırıldığında önemli bir azalma gösterdi (P≤0.05). Sonuç olarak, 3-MC'ye maruziyet durumunda BX'ın uzun sureli oral kullanımı oksidatif stresi azaltamayabilir, WBC, %LY, %NEU, PLT, HGB, HCT, MCV gibi hematolojik parametreleri değiştirebilir. Fakat bu değişimler fizyolojik sınırlar içersinde kalır. Yinede BX'ın kullanımında demir kullanımına dikkat edilmelidir. Ayrıca, bu dozda BX'ın kullanımı inflamasyon ve kanser göstergeleri olan TNF-α, IL-1β, IL-6 seviyelerini azaltabilir.

Anahtar sözcükler: *Boraks, Hematoloji, İnflamasyon, İnterlökin, Sıçan, Tas, Tos, Tnf-α, 3-MC*

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INTRODUCTION

Boron (B) is an essential element being responsible in metabolic reactions, which affect physiological systems of organism. Borax (BX) mineral being a component of Boron on the other hand, is a boric acid salt [1]. Used especially in pharmaceutical industry, Borax is a necessary mineral as a trace element for human, animals and plants [2].

Borax affects activity [3], mineral metabolism (Ca and P) [4], hormones [5] and lipid metabolism [6], free radicals of many enzymes [7,8].

Being one of the unsaturated aromatic hydrocarbons, 3-methylcholanthrene (3-MC) is a chemical carcinogenic which is used in experimental studies. Therefore, it can be applied through hypodermic, peritoneal spread and oral ways to test animals [9].

Tumor necrosis alpha-factor (TNF-α), interleukin-6 (IL-6) and interleukin-1 beta (IL-1β) are released by adipositis. Adipositis can initiate tumor formation in angiogenesis and cancer cells [10]. It is reported that these formations occur by means of cytokines such as TNF-α, IL-6, ve IL-1-receptor agonist [10,11].

Several pro-inflammatory cytokines released by innate and adaptive immune cells have been shown to regulate cancer cell growth and thereby contribute to tumor promotion and progression [12].

There is a remarkable balance between antioxidants and oxidants in a healthy body. Health problems occur due to a rise of free radicals or fall of antioxidants. Total antioxidant status (TAS) and total oxidant status (TOS) measurement is the most practical, economic and rapid practice that detects oxidant [13] and antioxidant [14] amounts.

There is limited information about whether any effects of BX on inflammation, TAS- TOS and haemataological parameters in rats could be applied 3-MC. Therefore, in the present study, we have investigated the effect of BX on TNF-α, IL-1β, IL-6, TAS, TOS, WBC, RBC, PLT values in rats applied 3-MC.

MATERIAL and METHODS

Study Groups

In this study were used total of 24 Wistar albino rats weighing between 200 and 250 g in a climate-controlled animal care facility, with a 12 h light/dark cycle. The animals were given with standard rat chow and water, *ad libitum*.

They were divided into 4 groups each containing 6 rats. Group 1 (Control) was separated as a control group and saline (1 mL of 0.9% NaCl) was injected twice a week first 2 weeks of study with i.p. way. Group 2 (3-MC) was applied 25 mg/kg dosage of 3-MC (Sigma- Aldrich Code: 213942) 2 twice a week first 2 weeks of study with i.p. way and was given normal drinking water during 150 days. Group 3 (BX) was given BX (Aldrich, Code: 2 21732) 300 mg/L/day dose with drinking water, during 150 days. Group 4 (3-MC+BX) was applied 25 mg/kg dosage of 3-MC twice a week first 2 weeks with i.p. way and BX was given 300mg/L/day dose with drinking water, during 150 days. All applications were simultaneously begun first day of study.

This study was approved by the local ethics committee of Yuzuncu Yil University (YUHADEK-Approval No: 2014/5).

Blood Collection

At the end of 150 days, blood samples were collected from all the rats and sacrificed by anesthetizing with a i.p. injection of 70 mg/kg of ketamine HCl (Ketalar, Pfizer) and xylasine HCl 10 mg/kg i. p. xylazine (Rompun, Bayer)

Blood samples were taken from hearts with sterile injector and placed into tubes with EDTA and coagulated tubes. Then bloods were separated into serum by centrifugation at 1.800 g (3.000 RPM) for 10 min. Serum was stored (-20°C) until the analysis.

Assay

The TNF-α, IL-1β, IL-6 levels were analysis by ELISA kits (eBioscience, Austria); TAS, TOS values using a novel automated measurement method developed by Erel [13,14] by colorimetric kits (Rel Assay, Türkiye) in serum. The oxidative stress index (OSI) was calculated with the ratio of TOS to TAS.

Hematology parameters, WBC, % leukocyte, RBC, HGB, HCT, PLT, were determined using rat mode of veterinary the blood cell counter (Abocus Junior Vet-5, Austria) in whole blood.

Statistical Analysis

All data were analyzed using the Kruskal-Wallis test. Dunn test was performed to determine the different groups. Statistics Calculator taken as 5% level of significance and SPSS statistical software 16.0 for Windows was used for the calculations. The data was given as means±stantard deviation (X±SD)

RESULTS

The serum levels of TNF-α, IL-1β and IL-6 are shown in *Table 1*. According to the *Table 1* the levels of TNF-α ($P \leq 0.05$), IL-1β ($P \leq 0.05$) and IL-6 in group 2 increased compared to other groups. But increase of IL-6 was not statistically significant.

The hematolojical parameters are shown in *Table 2*. According to the *Table 2* the increase of WBC levels in group 2 were not significantly compared to other groups. LY% levels of every 3 groups were significantly higher ($P \leq 0.05$);

however, its NEU% (P≤0.01) and MO% were lower than control group. But, MO% was not statistically significant. The HGB and HCT values of group 4, MCV in group 2 and group 4 were obtained significantly decrease (P≤0.05) compared to other groups. The PLT counts in groups 2 and 4 were determined significantly higher than others groups (P≤0.05) *(Table 2)*.

The serum levels of TAS, TOS and OSI are shown in *Table 3*. According to the table 3 serum TOS and OSI levels in groups 2 and group 4 were determined significantly higher (P≤0.01) than other groups. There was no difference for TAS among the groups.

DISCUSSION

Being of great importance for environmental health, 3-MC changes metabolism and toxicity of physiological substances and drugs and leads to mutation after being taken into body. As a result of genotoxic effects of 3-MC, teratogenicity, leucemia, especially lung and cervix cancer types occur.

Cytokines are multi-functional polypeptides which are synthesized by various cells in body and have significant roles in the development of cellular, humoral immune and

Table 1. *Serum TNF-α, IL-1β and IL-6 levels in all the groups (mean±SD)*
Tablo1. *Tüm gruplardaki serum TNF-α, IL-1β and IL-6 seviyeleri*

Inflammation Markers	Control Group n:6	3-MC Group n:6	BX Group n:6	3-MC+BX Group n:6	P Value
TNF-α (pg/mL)	309.76±24.83^{b}	379.70±44.37^{a}	312.92±34.11^{b}	305.31±67.45^{b}	≤0.05
IL-1β (pg/mL)	301.72±95.76^{b}	481.85±79.06^{a}	365.94±80.08^{b}	339.70±89.88^{b}	≤0.05
IL-6 (pg/mL)	162.88±41.12	184.46±47.37	163.22±39.91	157.28±22.73	≥0.05

^{a,b} in the same line values with different letters show statistically significant differences

Table 2. *The haematolojical parameters in all the groups (mean±SD)*
Tablo 2. *Tüm gruplardaki hematolojik parametreler*

Haematolojical Parameters	Control Group n:6	3-MC Group n:6	BX Group n:6	3-MC+ BX Group n:6	P Value
WBC (10^9/L)	6.81±1.93	7.74±1.13	6.53±1.78	6.02±1.88	≥0.05
LY (%)	64.87±9.84^{b}	74.82±4.05^{a}	78.40±2.48^{a}	77.57±2.17^{a}	≤0.05
MO (%)	6.98±3.98	2.96±2.17	3.03±2.05	4.83±4.46	≥0.05
NEU (%)	28.13±6.29^{a}	22.18±2.79^{b}	18.57±2.69^{b}	17.58±3.88^{b}	≤0.01
RBC (10^{12}/L)	7.70±0.19	7.63±0.47	7.52±0.90	7.13±0.76	≥0.05
HGB (g/dL)	13.93±0.37^{a}	13.58±0.53^{a}	13.98±0.66^{a}	12.72±0.79^{b}	≤0.05
HCT (%)	45.74±1.34^{a}	43.80±2.45^{a}	44.43±5.55^{a}	40.71±4.18^{b}	≤0.05
MCV (fl)	59.50±1.52^{a}	57.60±1.49^{b}	59.17±2.23^{a}	57.33±1.37^{b}	≤0.05
MCH (pg)	18.10±0.30	17.88±0.49	18.88±3.47	17.95±1.66	≥0.05
MCHC (g/dL)	30.52±0.82	31.10±0.63	31.98±5.71	31.42±2.83	≥0.05
RDWc (%)	14.83±0.37	14.94±0.71	14.22±0.48	14.97±0.74	≥0.05
PLT (10^9/L)	607.00±69.57^{c}	715.83±71.37^{b}	605.50±75.63^{c}	779.17±67.81^{a}	≤0.05
PCT (%)	0.47±0.22	0.51±0.08	0.44±0.05	0.59±0.14	≥0.05
MPV (fl)	7.85±0.74	7.72±25.14	7.27±0.12	7.53±0.38	≥0.05
PDWc (%)	34.85±1.23	35.05±0.96	34.63±0.44	35.27±0.86	≥0.05

^{a,b,c} in the same line values with different letters show statistically significant differences

Table 3. *Serum TAS-TOS and OSI values in all the groups (mean±SD)*
Tablo 3. *Tüm gruplardaki Serum TAS-TOS and OSI değerleri*

Oxidant-Antioxidant Parameters	Control Group n:6	3-MC Group n:6	BX Group n:6	3-MC+BX Group n:6	P Value
TAS (mmol Trolox Equiv/L)	0.54±0.09	0.52±0.06	0.47±0.7	0.56±0.03	≥0.05
TOS (μmol H_2O_2 Equiv/L)	4.83±1.03^{c}	9.17±1.56^{b}	4.57±1.35^{c}	16.16±2.08^{a}	≤0.01
OSI (Arbitrary Unit)	0.89±0.13^{c}	1.76±0.36^{b}	0.96±0.28^{c}	2.89±0.42^{a}	≤0.01

^{a,b,c} in the same line values with different letters show statistically significant differences

inflamatuar responses; supervising the cell growth and differentiation and initiating cicatrisation processes [1,15]. Main cytokines being responsible for chronic inflammation are TNF-α, IL-6 and inflammasome-activated IL-1β; and TNF-αs and IL-6 play a significant role in cell growth and differentiation [16].

It has been emphasized that IL-6 being a significant cytokine that plays role in inflamatuar response and pathogenesis of cancer [17] is a remarkable marker of experimental cancer, IL-6 levels rise in some cancer patients [18] and anti-apoptotic effects are observed in tumor cells [12].

In a study [9], serum IL-6 and TNF-α levels were investigated in fibrosarcoma induced by 3-MC (0.2 mg). The experiment took about 150-210 days until the appearance of tumor tissue in mause. IL-6 and Tnf-α was higher than controls. In another study, 1 mg of 3-MC was injected into rats with i.p. way; it was determined that it leads to tumor with 66.6% rate and it was reported that 3-MC plays role in cancer biology by means of mutation directly or immune system depression indirectly [19]. In this study, levels of serum TNF-α and IL-1β (P≤0.05), IL-6 in 3 MC group was higher than other groups; these values in BX+3MC group were smiliar to control group.

3-MC injected into rats with 30 mg/kg dosage leads to the synthesis of oncogenic proteins that can be used for the diagnosis [20,21]. In a study [22] detected that 3-MC injected with 200 mg/kg dosage leads to atrophy in thymus glan, T and B lymphositopeny and cancer. However, in these study, ever 3 groups were demonstrated nötropenia (P≤0.01) and lenfositosis (P≤0.05) according to control group. This finding could result from a chronic inflammation which was a result of 3-MC effect. As a matter of fact, it was reported that TNF-α has a toxic effect on β cells of pancreas, ensures vein adhesion of inflamatuar cells, matures monocyte and macrophages and B and T lymphocytes [23,24]. In addition, IL-1 increases expression of surface molecules which help the aggregation of leucocytes; does not directly activate inflammatuar leucocytes as neutrophile does, but affects mononuclear and endothelium cells instead; thus leads to the synthesis of chemokines that activate leucocystes [15,25].

IL-1 has also many inflammatuar characteristics of TNF. For example, it was reported that IL-1 affects endothelium cells and increases coagulation [15,25]. In these study, the PLT counts in 3-MC and 3MC+BX groups were determined to be significantly higher (P≤0.05) while the MCV values significantly lower (P≤0.05) than others groups. At the same time, HGB ve HCT values in 3MC+BX group was significantly decrease according to others groups (P≤0.05). However, these decreases were found to be within physiological limits. Furthermore, decrease of HGB and HCT and increase of PLT may be caused by iron deficiency. Although iron is an essential element for hemoglobin the free iron is moved binds to the transferin, stored as proteins such as ferritin or hemosiderin complexes, it is used holding in the hemoglobin and myoglobin. Because free iron is toxic for cells [26].

Oxidative stress is the imbalance between free radicals and antioxidant defense systems and associated with the etiology and progression of aging and many diseases [27-29]. Having genotoxic effects, 3-MC increases oxidative stress as well [30]. It is asserted that antioxidants taken through nutrition may decrease tumor incidents of antioxidants [20]. Anti-mutagens and antioxidants decrease oxidative stress and lead to decrease in genotoxicity and cancer risk [31].

Studies have demonstrated that B compounds are effective in maintaining the balance of prooxidants and antioxidants by reducing tissue damage resulting from oxidative stress [3,7,32]. Pawa and Ali [7] demonstrated that B limits oxidative damage by enhancing the glutathione store or inducing other free radical elimination.

In our study, TOS values were analyzed to assess the total effect of oxidants. Likewise, we measured the TAS level instead of evaluating antioxidant molecules separately.

In a study [32], B compounds supplementation in diet (100 mg/kg) significantly decreases the lipid peroxidation (LPO) and malondialdehit (MDA) concentration, and enhances the antioxidant defense mechanism such as GSH in blood. However, in this study, serum TOS ve OSI levels in 3MC and 3MC+BX (300 mg/L) groups were determined significant increase according to others groups (P≤0.01). This increase may be from iron deficiency in HGB and increase free iron in blood plasma. In this case free radicals and oxidative stress is increase [33]. Also this stuation may be due to the difference in dose. Turkez et al.[8] reported that B did not alter MDA concentration at low doses (5-50 mg/L) but increased it at high doses (500 mg/L) in human peripheral blood. However, in this study this level of B is nontoxic. Because B compounds are given orally to animals for a short term, the LD50 values for borax in laboratory animals are in the range of approximately 400-700 mg B/kg of body weight [34,35]. Furthermore, the maximum tolerable level of B is 150 mg/kg; diet B deficiency may occur in animals when their diet contains B at 0.3 mg/kg [36].

Antioxidant capacity is an important factor in all physiological standards, and for the performance of humans and all animals [37,38]. Turkez et al.[8] observed that at low doses (15 mg/L) B compounds increased both SOD and CAT activities, while at high doses decresed (500 mg/L) in erythrocytes. Koç et al.[39], were demonstrated that B compounds (100 mg/kg), increases antioxidan capacity in spinal cord ıschemia/reperfusion injury. However, in the present this study, serum TAS levels did not alter in between groups. This result is consistent with literature [32]. Ince et al.[32] showed that dietary B supplementation did not alter the plasma antioxidant capacity when compared to control. Turkez et al.[8] determined TAA in erythrocytes under *in vitro* conditions while we measured it in plasma,

which contains many nonspecific antioxidants such as urea, uric acid, and proteins [32].

According to these studies, the use of BX different doses and time has been reported that its different effects are on oxidative stress and the antioxidant status, but it has not revealed their impact on inflammation markers and the haematolojical parameters. Therefore these effects of BX were evaluated in this study.

In summary in the present study, TNF-α and IL-1β ($P \leq 0.05$), IL-6 ($P \geq 0.05$), WBC ($P \geq 0.05$) levels in 3-MC group, TOS and OSI ($P \leq 0.01$), PLT ($P \leq 0.05$) levels in 3-MC and 3-MC+BX groups were detected increases compared with other groups. It was determined that LY% levels of ever 3 groups were increased ($P \leq 0.05$); however, NEU% ($P \leq 0.01$) and MO% ($P \geq 0.05$) levels were decreased according to control group. Also, MCV in 3-MC and 3-MC+BX groups ($P \leq 0.05$), HGB and HCT values in 3-MC+BX group were decrease a physiology limited compared to other groups ($P \leq 0.05$).

As a result, this experimental study has demonstrated that 3MC may increase the level of inflammation and cancer markers, oxidative stress and some haematolojical parameters. In case of exposure to 3-MC, use alone of BX with 300 mg/L/day dosage with drinking water during 150 days does not decrease oxidative stress, may changes haematolojical parameters such as, WBC, LY%, NEU%, PLT, HGB, HCT, MCV. However, these changes remain within physiological limits. Even so, iron metabolism should be considered in the use of BX. Furtermore, BX with the abovementioned dosage may be used to reduce the levels of TNF-α, IL-1β, IL-6 being inflammation and cancer markers.

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Research Article

Serum Concentrations of Anti-Müllerian Hormone and its Expression in the Remnant Ovarian Tissue of Rats with Experimentally Induced Ovarian Remnant Syndrome

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Abstract

Anti-Müllerian hormone (AMH) is synthesised in the Sertoli cells of the testes and granulosa cells of the ovary. As the ovaries seem to be the primary source of AMH, it may be used for determination of the presence or absence of ovaries or ovarian remnants in mammalians. The purpose of the present study was to compare the serum AMH concentration of rats with experimentally induced ovarian remnant syndrome and the expression of AMH in the ovarian tissue removed during ovariohysterectomy and remnant ovarian tissue. A total of eighteen Sprague Dawley rats were used in the study. Group I consisted of 6 rats that were gone through ovarian remnant syndrome (ORS) experimentally, group II consisted of 6 rats in which both ovaries were removed and group III consisted of 6 rats that were sham-operated. Median laparotomy was performed in the all groups under general anaesthesia. AMH mRNA expression was determined using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). AMH mRNA expression levels in group I were decreased on day 30 after surgery when compared to day 0 ($P>0.05$). Mean concentration of serum AMH on day 10 after surgery in group I, II and III were found 2.27±0.52 ng/ml, <0.312 ng/ml and 3.96±0.53 ng/ml, respectively ($P<0.05$). In conclusion, this finding suggests that evaluation of serum AMH concentration could be an useful method to determine the presence or absence of ovaries or ovarian remnants in the rat.

Keywords: *Anti-Müllerian hormone, Ovarian remnant syndrome, Rat*

Deneysel Olarak Ovaryum Kalıntısı Sendromu Oluşturulan Ratlarda Serum Anti-Müllerian Hormon Konsantrasyonu ve Kalıntı Ovaryum Dokusunda AMH mRNA Ekspresyonu

Özet

Anti-Müllerian hormon (AMH), testislerde Sertoli hücrelerinden ve ovaryumlarda granuloza hücrelerinden sentezlenmektedir. Anti-Müllerian hormonun ovaryum kökenli olmasından dolayı, memelilerde ovaryum varlığının veya kalan ovaryum dokusunun tespit edilmesinde kullanılabilmektedir. Bu çalışmanın amacı, deneysel olarak ovaryum kalıntısı sendromu oluşturulan ratlarda serum AMH konsantrasyonları ve ovaryohisterektomi ile uzaklaştırılan ovaryum dokusunda ve kalan ovaryum dokusundaki AMH gen ekspresyonlarını karşılaştırmaktı. Çalışmada toplam 18 Sprague Dawley rat kullanıldı. Deneysel olarak ovaryum kalıntısı sendromu oluşturulan 6 rat grup I'i, her iki ovaryumu da uzaklaştırılan 6 rat grup II'yi ve sham operayonu yapılan 6 rat ise grup III'ü oluşturdu. Bütün gruplarda genel anestezi altında median laparotomi gerçekleştirildi. AMH mRNA ekspresyonu kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR) ile ölçüldü. Operasyon sonrası 30. günde, 0. güne kıyasla AMH mRNA ekspresyon düzeylerinde azalma oldu ($P>0.05$). Operasyondan 10 gün sonra ortalama AMH değeri grup I, II ve III'de sırasıyla 2.27±0.52 ng/ml, <0.312 ng/ml ve 3.96±0.53 ng/ml olarak bulundu ($P<0.05$). Sonuç olarak, çalışmanın bulgularına göre serum AMH konsantrasyonunun, ratlarda ovaryum varlığının veya kalıntı ovaryum dokusunun tespit edilmesinde kullanılabilir bir yöntem olduğu belirlendi.

Anahtar sözcükler: *Anti-Müllerian hormon, Ovaryum kalıntısı sendromu, Rat*

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INTRODUCTION

Anti-Müllerian hormone (AMH), named additionally Müllerian inhibiting substance is a member of the transforming growth factor-β (TGF-β) superfamily and is synthesised in the gonads of mammalians [1-3]. AMH is produced from the Sertoli cells of the testes and granulosa cells of preantral and small antral follicles of the ovary particularly in the layer nearest to the oocyte whereas its expression is absent when follicles become atretic [4-8]. Durlinger [1] showed that AMH inhibits the initiation of primordial follicle growth in anti-Müllerian hormone-deficient female mice. Thus, it is thought that serum AMH concentration represents the size of the small antral follicles, the number of residual primordial follicles or the ovarian reserve in both women [9,10] and rodents [4,5,8,11].

In rats, no alterations was detected during the estrus cycle in the expression of AMH and its type II receptor (AMHRII), however some heterogeneity has been determined in AMH mRNA expression in preantral and small antral follicles at estrus and diestrus [4,5,7,12]. In addition, it has been reported that ovariectomy in regularly cycling women causes undetectable AMH concentrations [13]. Hence, the ovaries seem to be the primary source of AMH; therefore, it may be used for determination of the presence or absence of ovaries or ovarian remnants in dogs and cats [14,15].

Several researches emphasized the evaluation of AMH concentrations in various ovarian pathological conditions including polycystic ovary syndrome, granulosa cell tumors and premature ovarian failure in the women and rat and also ovarian remnant syndrome in the bitch and queen [9,16,17]. The purpose of the present study was to compare the serum concentration of AMH and the expression of AMH gene in the ovarian tissue removed during ovariohysterectomy and in remnant ovarian tissue in rats with experimentally induced ovarian remnant syndrome.

MATERIAL and METHODS

Animals and Study Design

A total of eighteen adult female Sprague Dawley rats weighing 200-250 g were obtained from Harlan Laboratories B.V. (The Netherlands). Animals were maintained with a 12-h light/dark schedule and supplied standard rat chow (Korkuteli Food Industry, Turkey) and water *ad libitum*. The rats were divided into three groups. Group I consisted of 6 rats gone through ovarian remnant syndrome experimentally, group II consisted of 6 rats performed total ovariohysterectomy and group III consisted of 6 rats sham operated. Median laparotomy was performed in all groups under xylazine (5 mg/kg) and ketamine (45 mg/kg) anaesthesia. In group I; uterus, left ovary and half of the right ovary were removed (total hysterectomy and unilateral hemiovariectomy) [18]. Remnant ovarian tissue was removed 30 days later with the same technique. In group II; uterus and both ovaries were removed at the same time (total ovariohysterectomy). All the ovarian tissue samples were stored in liquid nitrogen at -196°C until mRNA isolation. Intravenous blood samples from tail vein were obtained into plain tubes on day 0, 1, 5, 10 and also day 30 from group I and were immediately centrifuged at 1550 g for 10 min. Sera were removed and stored at -80°C until analyzed for AMH concentration. In order to compare the serum AMH concentrations of different stages, we performed vaginal cytologic examination to determine the stages of the estrus cycle in all rats. Vaginal secretion was collected with a plastic pipette filled with 10 IU of normal saline (NaCl 0.9%) by embedding the tip into the vagina. Vaginal fluid was dropped on glass slides. Unstained material was evaluated under the light microscope (Leica CME Microscope, 1349522X, NY, USA, 40x objective lenses) according to Marcondes et al.[19].

All procedures involving the use of animals were approved by the Gazi University Animal Experiments Local Ethics Committee (Approval no:15.31; Turkey) and were performed at Laboratory Animal Breeding and Experimental Researches Center of the same university.

Serum AMH Assays

The serum AMH concentration was determined using an enzyme-linked immunosorbent assay kit (MBS701712, MyBioSource, Inc. San Diego, CA 92195-3308, USA), according to the manufacturer's instructions. All serum assays were performed in duplicate. The minimum detectable concentration of the assay was 0.375 ng/ml. The lower and upper limits of detection were 0.375 ng/ml and 150 ng/ml, respectively. The intra-assay coefficient of variation (CV) was < 8% and the inter-assay CV was <10%.

Quantitative Real-Time PCR Analysis

Total RNA was extracted from each tissue using the TriReagent (peqGOLD TriFastTM, Peqlab, Erlangen, Germany) and treated with DNase I, RNase-Free (Thermo scientific, Fermentas) according to the recommendations of the supplier. The RNA concentration of each sample was measured at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, USA). The reverse transcription reaction of RNA was performed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Samples were analyzed on a LightCycler® 480 instrument (Roche Diagnostics, Mannheim, Germany) and relative mRNA expression of the AMH was normalized to the expression levels of beta actin (ACTB) expression. The sequences of the gene-specific primers and probes for AMH and ACTB transcripts were designed using the online Universal Probe Library (UPL) Assay Design Center (https://www.universalprobe library.com). The sequences of the primers used for QRT-PCR experiments were as

follows: rat ACTB forward (5′-CCCGCGAGTACAACCTTCT-3′) and reverse (5´-CGTCATCCATGGCGAACT-3´), probe no 17; rat AMH forward (5′-CTGGACACCGTGCCTTTC-3′) and reverse (5′-CACTGTGTGGCAGGTCCTC-3′), probe no 26. The QRT-PCR reactions with the following program: after initial denaturation at 95ºC for 15 min, followed by 45 cycles consisting of 95ºC for 15 sec, and 60ºC for 20 sec and cooled down to 40ºC. The concentration of the expression level for each sample was determined from the threshold cycle (Ct), which is the cycle where an increase in PCR product is first detected at a statistically significant level.

Statistical Analysis

The statistical significance of the differences in the AMH concentration between groups was analyzed by One-Way ANOVA analysis of variance and Duncan's multiple range test using SigmaStat (Jandel Scientific Software Inc.; San Jose, CA, USA). Data were expressed as mean± standard deviation (SD). A P value of <0.05 was considered statistically significant. The relative expression for each gene is obtained by comparing the CT values for each gene using the equation 2−ΔΔCT following the Pfaffl-based method with the Relative Expression Software Tool 2008 (REST©) [20]. Each experiment was carried out three times.

RESULTS

Vaginal Cytologic Examination

Estrus cycle stages of the rats in all groups were determined as six proestrus, four estrus, four metestrus and four diestrus before surgery. There was no significant difference in serum AMH concentrations among the stages of the estrus cycle in all groups (P>0.05) *(Fig. 1)*.

Serum AMH Concentrations

The pre-operative serum AMH concentrations in group I, II and III were 4.61±1.13 ng/ml, 4.32±0.71 ng/ml and 4.15±0.71 ng/ml, respectively (P>0.05). As shown in *Table 1*, the pre-operative serum AMH concentrations in group I and II were significantly different compared to post-operative day 1 and 5 (P<0.05). In group III, there was no significant difference between pre-operative and post-operative stage in serum AMH concentrations (P>0.05). In

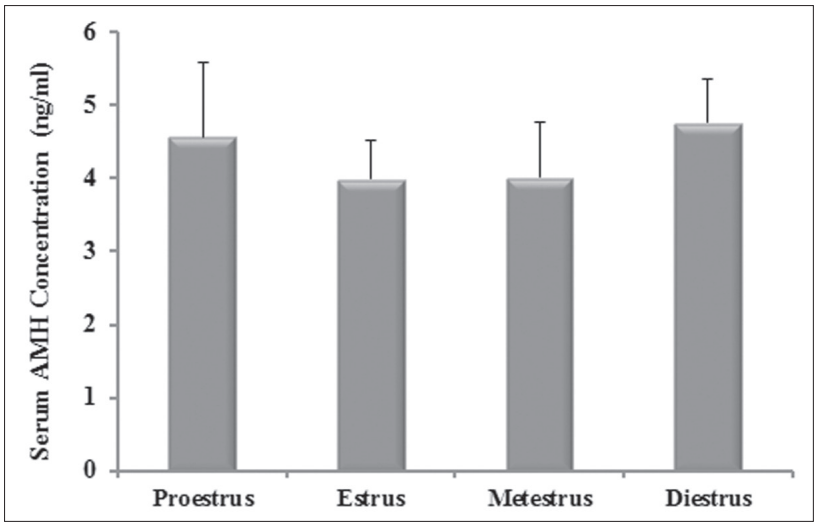

Fig 1. Mean serum AMH concentrations (ng/ml) at different stages of the estrus cycle in all groups at day 0, P>0.05

Şekil 1. Tüm gruplarda 0.günde farklı östrus siklus evrelerindeki serum AMH konsantrasyonları (ng/ml), P>0.05

Fig 2. Relative mRNA expression of AMH in group I at day 0 and 30. mRNA: messenger RNA; PCR: polymerase chain reaction, P>0.05

Şekil 2. Grup I'de 0. ve 30. günlerdeki göreceli AMH ekspresyon düzeyleri. mRNA: messenger RNA; PCR: polymerase chain reaction, P>0.05

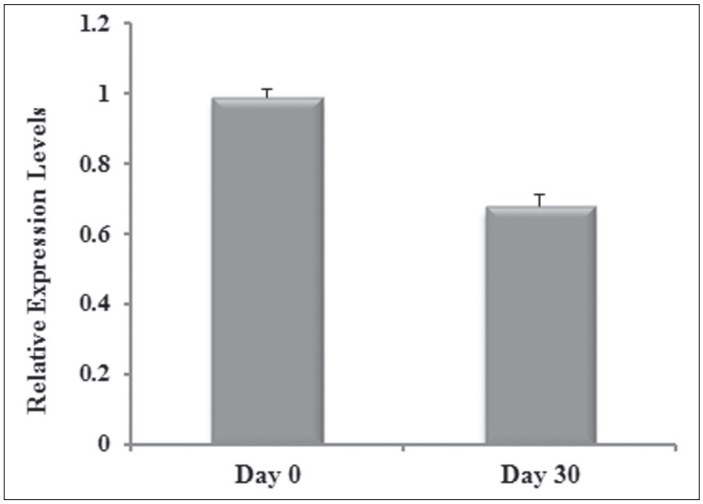

Table 1. *Mean serum AMH concentrations (ng/ml) of the groups at day 0, 1, 5 and 10*
Tablo 1. *Gruplardaki 0., 1., 5. ve 10.* günlerdeki ortalama serum *AMH konsantrasyonları (ng/ml)*

Groups	Day 0	Day 1	Day 5	Day 10
Group I (ORS)	4.61±1.13^{a}	2.72±0.71^{A,b}	1.35±0.24^{A,c}	2.27±0.52^{B,b}
Group II (OHE)	4.32±0.71^{a}	1.78±0.24^{B,b}	0.52±0.13^{B,c}	ND
Group III (Sham)	4.15±0.71	3.98±0.42^{C}	4.09±0.50^{C}	3.96±0.53^{C}

ND: *non-detectable. Different superscript capital letters indicate significant difference between the groups in the same column, Different superscript small letters indicate significant difference between the days in the same row (Duncan's test; P<0.05)*

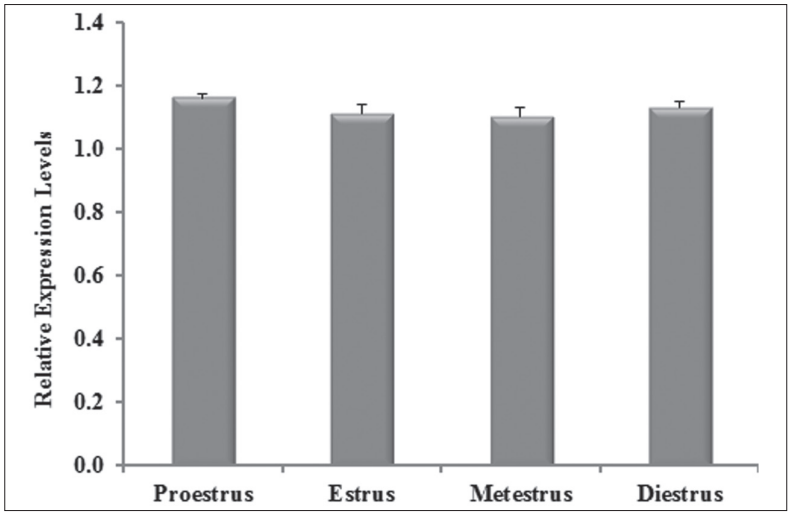

Fig 3. Relative mRNA expression of AMH at different stages of the estrus cycle in all groups at day 0. mRNA: messenger RNA; PCR: polymerase chain reaction, P>0.05

Şekil 3. Tüm gruplarda 0. günde farklı östrus siklus evrelerindeki göreceli AMH ekspresyon düzeyleri. mRNA: messenger RNA; PCR: polymerase chain reaction, P>0.05

group II, serum AMH concentrations were below the non-detectable concentration (0.312 ng/ml) on day 10 after the surgery *(Table 1)*. In addition, serum AMH concentration in group I was determined to be 2.03±0.71 ng/ml on day 30.

Relative Expression Levels of AMH

AMH mRNA expression levels in group I showed no significant difference in comparison with the group II on day 0 (P>0.05). In addition, AMH mRNA expression levels in group I were decreased on day 30 after surgery when compared to day 0, but this reduction was not statistically significant (P>0.05) *(Fig. 2)*. There was no significant difference between the stages of the estrus cycle in AMH mRNA expression levels in all groups (P>0.05) *(Fig. 3)*.

DISCUSSION

Anti-Müllerian hormone (AMH) is only produced by granulosa cells of the ovary [6,7,8,21]. AMH may be used for determination of the presence or absence of ovaries or ovarian remnants in mammalians since the ovaries appear to be the primary source of AMH. It has been determined that serum AMH concentration was undetectable on day 3-5 after bilateral ovariectomy in women [13]. Similarly, the present study showed that serum AMH concentrations gradually decreased from day 1 to 5 and were undetectable on day 10 after ovariohysterectomy and this finding made us thought that serum AMH concentration might be affected by the absence of the ovaries.

Recent studies revealed that serum AMH concentrations were not significantly different during the menstrual cycle in normocycling women [22,23]. However, a comparison of serum AMH concentrations between the stages of the estrus cycle in the rat has not been investigated before. In our study, we demonstrated that serum AMH concentrations before surgery were not statistically different amongst all groups considering the stages of the estrus cycle (P>0.05).

AMH and AMHRII mRNA expression in preantral follicles did not vary during the estrus cycle, despite the heterogeneous decrease at estrus in rats [12]. Hirobe et al.[7] also presented that AMH mRNA expression are wide and uniform in recruited growing antral follicles on the morning of estrus but become heterogeneous on diestrus, when selection for atresia occurs. However, expression of AMH gene in the ovarian tissues at all stages of the estrus cycle in rats has been identically strong and homogenous in our study.

AMH mRNA expression initiates at the peri-natal period, declines along reproductive life and becomes undetectable at the postmenopausal period in women [1,24]. In rodents, AMH expression in the ovary has been observed by postnatal day 3 in granulosa cells of growing primordial follicles [5,25]. Kevenaar et al.[8] found that serum AMH concentrations decrease in mice with increasing age and this reduction in serum AMH is not represented by a similar change in AMH mRNA expression level. Immunostaining intensity of AMH expression in granulosa cells of growing follicles does not show an alteration with age in mice [8]. The

rats used in the study were particularly chosen from the young adults already reached puberty and aged 2.5 to 3.0 months to exclude the diversity of results due to different ages. Therefore, both the measurement of serum AMH concentrations and gene expressions were performed when the rats were active reproductively.

AMH mRNA expression levels in group I declined on day 30 after surgery when compared to day 0, but this reduction was not statistically significant. This is because the amount of ovarian tissues taken at day 0 and 30 in order for measurements of AMH mRNA expression level were same. Therefore, it was thought that the statistically insignificant difference of AMH mRNA expression was not dependent upon the amount of tissue. The reason for the measurement of AMH mRNA expression of remnant ovarian tissue at day 30 was to wait the tissue to complete its repairment. One major drawback of our study is lack of comparison of AMH mRNA expression in remnant ovarian tissue on day 1, 5 and 10 after surgery.

In a study by Minke et al.[26], seventy-five percent of the devascularized ovarian tissue revascularized and forty-three percent of them demonstrated follicullar growth. One step ahead, our present study indicated that devascularized ovarian tissue remained functional and this was confirmed by increased serum AMH concentration between 5th and 10th postoperative days in group I, which was attributed to the revascularization of remnant ovarian tissue.

In conclusion, the present study revealed that serum AMH concentrations were below the non-detectable concentration on day 10 after ovariohysterectomy. This finding suggests that evaluation of serum AMH concentration could be an useful method to determine the presence or absence of ovaries or ovarian remnants in the rat. This result may be considered for future studies to clarify serum AMH concentrations and AMH mRNA expression levels in the stages of the estrus cycle in rats.

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Research Article

Anticoccidal Efficacy of Usnic Acid in Broilers [1]

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Abstract

This experiment was conducted to investigate if usnic acid, a lichen metabolite, exerts therapeutic action against coccidiosis. A total of 160 one-day-old male Ross 308 broiler chicks were divided into 5 experimental groups (A-E), each replicated in 4 pens of 8 chicks. At 16 days of age Groups B-E were infected orally with a mixture of purified oocysts including 30 x 10^4 sporulated oocysts from field isolates of *E. acervulina, E. brunetti, E. maxima, E. mitis, E. praecox* and *E. tenella*, whereas Group A was remained uninfected. Seven days after the coccidiosis induction, infected birds were divided into 4 groups to receive orally with 20 mg usnic acid (Group C), 100 mg usnic acid (Group D) and 7 mg toltrazuril (Group E) per kg body weight. The birds in Group B were untreated and served as infected control. The experiment was terminated at 29 days of age. As a result of the study it was evaluated that infected birds had lower feed intake and body weight and worse feed conversion, higher intestinal lesion score, longer small intestine and cecum, and higher fecal oocyst count than healthy birds. The anticoccidial effect of usnic acid at 100 mg/kg application dose was comparable to toltrazuril as reflected by alleviations in performance and pathology findings. In conclusion, it is demonstrated that usnic acid possesses some anticoccidial effects, but not nearly as good as toltrazuril.

Keywords: *Lichen, Usnic acid, Toltrazuril, Broiler, Coccidiosis*

Usnik Asitin Broylerlerdeki Anticoccidial Etkinliği

Özet

Bu çalışmada, bir liken metaboliti olan usnik asitin coccidiosise karşı terapötik etkinliğinin araştırılması amaçlanmıştır. Toplam 160 adet, 1 günlük yaştaki erkek Ross 308 civciv, her bir grup 4 tekerrürlü olacak şekilde 5 deneysel gruba (A-E) ayrılmıştır. Hayvanlar 16 günlük yaşa geldiğinde Grup B-E'dekiler *E. acervulina, E. brunetti, E. maxima, E. mitis, E. praecox* ve *E. tenella*'nın saha izolatlarından elde edilmiş inokulumdan 30 x 10^4 sporlanmış oocyst ile enfekte edilmiştir. Grup A'da yer alanlar civcivler ise enfekte edilmeden kontrol olarak bırakılmıştır. Hastalık oluşturulduktan 7 gün sonra, enfekte hayvanlara ağız yolu ile 20 mg/kg usnik asit (Grup C), 100 mg/kg usnik asit (Grup D) ve 7 mg/kg toltrazuril (Grup E) uygulanmış; B grubundaki enfekte hayvanlara tedavi uygulanmadan enfekte kontrol olarak bırakılmıştır. Broylerler 29 günlük olduğunda çalışma sona erdirilmiştir. Enfekte hayvanların sağlıklı hayvanlara kıyasla daha düşük yem tüketimi, vücut ağırlığı ve yemden yararlanma oranına; daha yüksek lezyon skoruna, daha uzun bağırsak uzunluğuna ve daha yüksek oocyst sayısına sahip olduğu tespit edilmiştir. Performans ve patolojik bulgulardaki değişimler göz önünde bulundurulduğunda 100 mg/kg dozda uygulanan usnik asitin toltrazuril ile karşılaştırılabilecek derecede anticoccidal etkinliğe sahip olduğu belirlenmiştir. Sonuç olarak; usnik asitin bazı anticoccidial etkinliklere sahip olduğu ancak bu etkilerin toltrazuril kadar güçlü olmadığı kanısına varılmıştır.

Anahtar sözcükler: *Liken, Usnik asit, Toltrazuril, Broyler, Coccidiosis*

INTRODUCTION

Coccidiosis is a widespread poultry disease caused by *Eimeria* parasites. Primarily, seven species of *Eimeria (E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella)* cause coccidiosis in chickens [1]. The disease is associated with reduced growth rate, impaired feed conversion leading to poor performance, increased susceptibility to other diseases and increased mortality [2].

Traditionally, coccidiosis control is largely dependent on anticoccidial drug usage and on live vaccines on a limited scale at intensive poultry production systems. However, problems with drug resistance in *Eimeria* strains

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in the field, withdrawal period for drugs prior to slaughter, consumer pressure for poultry products free of drug residues, restriction of using antibiotics as growth promoters by European Union and the cost of expensive vaccines urged researchers to explore cheap and safe alternative agents [3,4]. There are a number of research articles proving anticoccidial activity of natural products including plants [5], pre- and probiotics [4,5] and fungi [6,7]. Natural products are expected to be alternative in the coccidiosis control promisingly by including new therapeutic molecules to which *Eimeria* strains have not yet developed resistance [8-10].

Lichens are symbiotic associations between an exhabitant fungus and one or more inhabitant photosynthetic partners (algae or cyanobacteria) and they synthesize more than 800 types of metabolites [11,12]. Numerous biological investigations have showed that secondary lichen metabolites have a broad range of biological activities including antibiotic [13], antiviral [14], analgesic and antipyretic [15], antifungal [16,17], anti-inflammatory [18], cytotoxic and antimicrobial [19], antiulcerogenic and antioxidant [20] and immunologic modulator [21].

Usnic acid is one of the most extensively studied lichen metabolites and present in the lichen genera *Alectoria*, *Cladonia*, *Evernia*, *Lecanora*, *Ramalina* and *Usnea* [22]. It is shown to exert a number of biological activities including anti-inflammatory, analgesic and antipyretic, antibiotic, antiviral, antimicrobial, antiproliferative, gastroprotective, antitumor, antioxidant, antimycotic, antigrowth and anti-insect properties [22,23]. Although few studies [9,24-27] dealing with antiprotozoal effect of usnic acid are available, its anticoccidial effect is largely unknown. The present experiment was set out to investigate therapeutic action of usnic acid against coccidiosis in broilers.

MATERIAL and METHODS

Animals and Management

A total of 160 one-day-old male Ross 308 broiler chicks were purchased from a commercial hatchery and housed in an experimental house from 1 to 29 days of age. They were reared altogether in a large pen from d 1 to 7 for adaptation. Chicks were then divided randomly into the final 5 groups (A, B, C, D and E) in 4 replicate subgroups containing of 8 chicks per subgroup. Each subgroup was housed in a separate floor pen (1.5 m x 2.5 m) equipped with one hanging bell drinker, two tube-type feeders and electrical heater. Wood shavings were used as bedding material with a depth of 5 cm. The room temperature was gradually decreased from 33ºC on d 1 to 22ºC on d 21, and then remained unchanged. The chicks were vaccinated against infectious bronchitis and Newcastle disease with Nobilis MA5+Clone30 (Intervet, Boxmeer, Netherlands) at 1 d of age via drinking water. The feed contained no anticoccidials or growth enhancers. Birds were offered feed and water *ad libitum*. The experiments were conducted according to the ethical norms approved by the Atatürk University Ethic Committee of Experimental Animal Teaching and Researcher Center (No: 2012-49).

Parasites and Usnic Acid

The reference parasite stock was provided by the Department of Parasitology at the Veterinary Medicine Faculty of Ataturk University, Turkey. The stock, containing 30 x 10^4 sporulated oocysts from field *Eimeria* isolates *(E. acervulina*, *E. brunette*, *E. maxima*, *E. mitis*, *E. praecox* and *E. tenella)*, was passaged in *Eimeria*-free chickens to keep them infective.

(+)- Usnic acid (Sigma-Aldrich, Steinheim, Germany) was suspended in 1% carboxymethyl cellulose (CMC) water solution and then had been filtered through a 0.2-µm-pore-size filter.

Acute Toxicity Test

An acute toxicity test was conducted on 10-day-old broiler chicks. The birds were divided into 5 groups (CMC and 10, 100, 500 or 1.000 mg (+)-usnic acid extract per kilogram diet, respectively), each consisting 5 birds. The birds were observed for 24 h for signs of toxicity or death.

Experimental Design

After one-week adaptation period, chicks in Group A were not infected and served as uninfected control, whereas those in Group B-E were infected orally with a mixture of purified oocysts including 30 x 10^4 sporulated oocysts from field isolates of *E. acervulina, E. brunetti*, *E. maxima, E. mitis, E. praecox* and *E. tenella* on 16 days of age. The oocyst inoculum was washed several times with tap water and then a 2 ml suspension of 30 x 10^4 sporulated oocysts administered directly into the crop via oral gavage.

After observation of typical lesions of coccidiosis at d 7 post-infection (PI), one chick from each subgroup was chosen randomly and euthanized to inspect lesion scores in order to confirm success of the infection. Group B was untreated and served as infected control. Starting from d 8 PI the birds in Group C, D and E were dosed orally with 20 mg usnic acid, 100 mg usnic acid and 7 mg toltrazuril (Baycox 2.5%, Bayer, Leverkusen, Germany) per kg BW for 5, 5, and 2 days, respectively. The experiment was terminated on d 13 PI.

Fecal oocysts were enumerated a day before infection and performed daily between 20-29 days of age. For this purpose, approximately 200-300 g fecal samples were collected daily from each replicate pen in several spots. Representative fecal samples for each pen were placed in screw cap containers and stored at +4ºC until oocyst counts were performed (oocyst per gram of feces, OPG) using the McMaster counting technique [28].

At the end of the experiment, all birds were slaughtered

for scoring intestinal lesions caused by *Eimeria* species according to the method of Johnson and Reid [29], 0 indicating normal and 1, 2, 3 or 4 indicating the degree of severity of infection. The upper, middle and cecal sections of the chick intestine were examined for lesions. Because the mixed infection was induced, the sectional data were averaged by the group prior to statistical analysis.

Performance Measurements

Feed intake (FI) and body weight (BW) were measured and performance variables (BW gain, BWG and feed conversion ratio, FCR) were calculated at d 1, 16, 23, and 29 on a pen basis.

Statistical Analysis

Considering 5% reduction in oocyst count upon treatment with usnic acid to be significant, sample size was calculated to be 4 replicates. Data were analyzed by one-way ANOVA in a completely randomized design. The oocyst count data were log transformed prior to statistical analysis. The model to test effect of treatments included treatment effect as fixed effect and treatment within group as random effect. Time and group by time interactions were also fixed factor for FI and oocyst data. Statistical significance was considered at $P<0.05$.

RESULTS

No acute toxic effect or mortality was detected in the toxicity test.

Typical clinical signs of coccidiosis including inappetence, wing drooping, distorted feathers, huddling and bloody droppings were observed in all the infected groups of chickens at day 7 PI which were inferred the success of the experimental infection. Reduction in severity of the clinical signs was observed in groups C-E, conspicuously in group E, after the treatment. On the contrary, severity of clinical signs in group B was increased progressively and resulted with a mortality of 10% by day 13 PI.

Table 1 summarizes performance parameters in response to treatment effects in broilers subjected to the coccidiosis induction. After oocyst inoculation, the coccidiosis-induced birds had depressed FI (by 12.1%) and BWG (by 12.2%) and elevated FCR (by 9.9%). Toltrazuril treatment alleviated FCR as compared to the healthy control group. The high level of usnic acid treatment was as effective as toltrazuril treatment *(Table 1, Fig.1)*.

Responses of changes in intestinal pathology and fecal oocyts count to the treatments were ambiguous. Comparing with the healthy control group, the oocyst inoculation caused an increase in lesion scores and elongation of intestine sections as well as presence of oocyst in feces *(Table 2)*. Toltrazuril and usnic acid treatments considerably reduced intestinal lesion score at a similar extent as compared to the untreated groups. However, usnic acid was not as effective as toltrazuril to recover intestinal length. Both usnic acid and toltrazuril caused reduction in fecal oocyst count at a similar level as compared to the untreated group *(Fig. 2)*.

DISCUSSION

In acute toxicity test, neither mortality nor toxicity signs were recorded. This result confirms the previous report [20], which indicated that usnic acid is well tolerated up to 1000 mg/kg BW

Table 1. *Performance parameters in response to treatments in broilers subjected to coccidiosis induction*
Tablo 1. *Coccidiosis oluşturulan broylerlerde tedavilere karşı gelişen performans parametreleri*

Parameter/Stage	Groups[1]					P<
	Group A	Group B	Group C	Group D	Group E	
Body weight (BW), g						
Before trial (d 7)	----------189.4±1.3----------					
Before infection (d 16)	----------320.4±2.5----------					
End of infection (d 23)	661.2±3.1^{a}	----------581.4±10.2^{b}----------				0.0005
End of treatment (d 28)	1399.2±29.4^{a}	1190.0±13.4^{c}	1159.7±39.5^{c}	1331.7±17.0^{ab}	1293.3±39.2^{b}	0.0002
Feed intake (FI), g						
Before trial	----------59.0±0.8----------					
During infection period cumulative	520.3±6.9^{a}	----------456.7±10.0^{b}----------				0.002
During treatment period cumulative	1127.9±31.4^{a}	1056.2±23.2^{ab}	994.6±33.2^{b}	1110.4±37.6^{a}	1070.8±16.8^{ab}	0.05
Feed conversion ratio (FCR, Feed: BW Gain)						
Before trial	----------0.31±0.004----------					
During infection	1.58±0.02^{a}	----------1.74±0.03^{b}----------				0.01
During treatment	1.53±0.02^{a}	1.68±0.04^{b}	1.65±0.07^{ab}	1.51±0.05^{a}	1.59±0.04^{ab}	0.10

Superscripts among columns indicate group differences at P<0.05

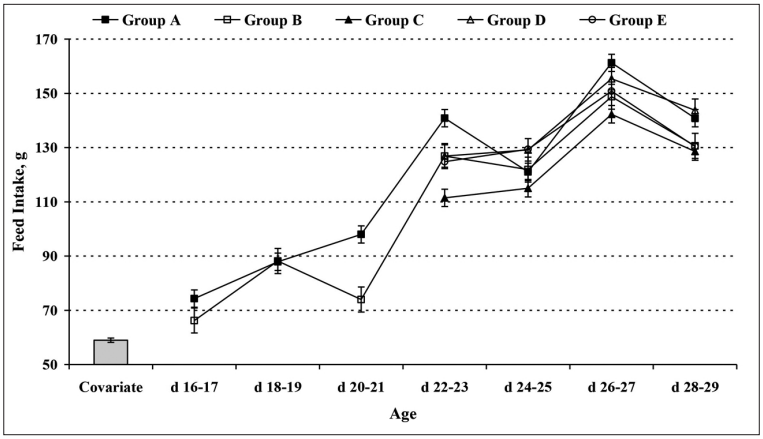


Fig 1. Effect of treatment on feed intake in broilers subjected to coccidiosis induction. Covariate represents feed intake on d 7. The birds in Group A were not infected and served as positive control, the birds in Groups B-E were infected and then subdivided into untreated and served as negative control (Group B) or treated with 20 mg usnic acid (Group C), 100 mg usnic acid (Group D) and 7 mg toltrazuril. Pooled SE was 3.79

Şekil 1. Coccidiosis oluşturulan broylerlerde tedavinin yem tüketimine etkisi. Eşdeğişken 7. gündeki yem tüketimini gösteriyor. A grubundaki hayvanlar enfekte edilmeyerek pozitif kontrol olarak ayrılırken B-E gruplarındakiler enfekte edilip daha sonra tedavi uygulan-mayan negatif kontrol (Grup B), 20 mg/kg usnik asit ile tedavi edilen (Grup C), 100 mg/kg usnik asitle tedavi edilen (Grup D) ve 7 mg/kg toltrazuril uygulanan alt gruplara ayrılmıştır. Ortalama standart hata 3.79

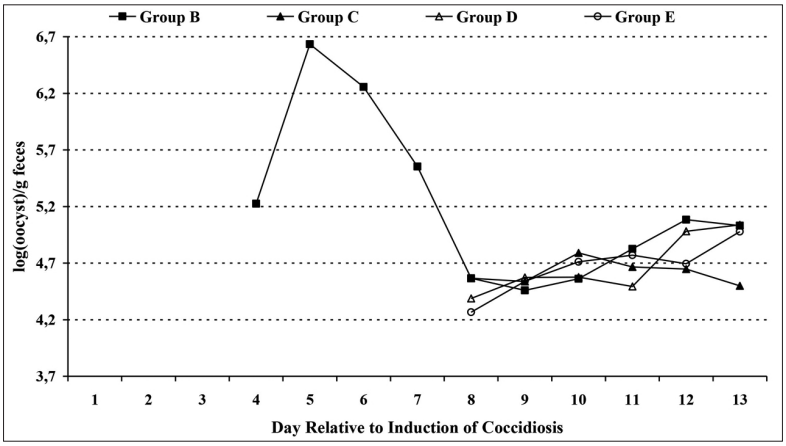
Table 2. *Intestinal lesion score and fecal oocyst count in response to treatments in broilers subjected to coccidiosis induction*
Tablo 2. *Coccidiosis oluşturulan broylerlerde tedaviler sonrası bağırsak lezyon skorları ve oocyst sayıları*

Parameter	Part/Period	Groups[1]					P<
		Group A	Group B	Group C	Group D	Group E	
Lesion score		0.00±0.00^{a}	2.96±0.12^{c}	0.9±0.04^{b}	0.6±0.03^{b}	0.5±0.13^{b}	0.0001
Length (mm)	Small Intestine	156.5±1.8^{b}	177.2±5.6^{ab}	186.2±9.3^{a}	187.2±11.6^{ab}	158.5±7^{b}	0.03
	Cecum	16.2±0.7^{b}	19.12±0.5^{a}	18.0±0.5^{ab}	18.1±0.9^{ab}	16.9±0.6^{b}	0.05
Oocyst count[2]	Infection Period	0.00±0.00^{a}	----------5.92±0.07^{b}----------				0.0000
	Treatment Period	0.00±0.00^{a}	5.27±0.12^{c}	5.13±0.12^{b}	5.17±0.12^{b}	5.12±0.13^{b}	0.0001

[1] The birds in Group A were not infected and served as uninfected control, the birds in Groups B-E were infected and then subdivided into untreated control (Group B) or treated with 20 mg usnic acid (Group C), 100 mg usnic acid (Group D) and 7 mg toltrazuril (Group E) per kg body weight. Superscripts among columns indicate group differences at P<0.05; [2] Time effect, P<0.0001. Group x Time effect, P<0.0001

Fig 2. Effect of treatment on fecal oocyst count in broilers subjected to coccidiosis induction. The birds in Groups B-E were infected and then subdivided into untreated control (Group B) or treated with 20 mg usnic acid (Group C), 100 mg usnic acid (Group D) and 7 mg toltrazuril (Group E) per kg body weight. Time effect, P<0.0001. Group x Time effect, P<0.0001. Pooled SE was 0.12

Şekil 2. Coccidiosis oluşturulan broylerlerde tedavinin fekal oocyst sayısına etkisi. B-E gruplarındakiler enfekte edilip daha sonra tedavi uygulanmayan negatif kontrol (Grup B), 20 mg/kg usnik asit ile tedavi edilen (Grup C), 100 mg/kg usnik asitle tedavi edilen (Grup D) ve 7 mg/kg toltrazuril uygulanan alt gruplara ayrılmıştır. Zaman etkisi, P<0.0001. Grup x Zaman etkisi, P<0.0001. Ortalama standart hata 0.12



In the present experiment, poor performance *(Table 1, Fig. 1)* as well as intestinal lesions, prolonged intestine and OPG count *(Table 2, Fig. 2)* confirm success of the coccidiosis induction [30-33]. These are related to significant damage to the intestinal mucosa and enterocytes during the progression of *Eimeria* lifecycle after the *Eimeria* challenge [34]. It appears that body responds to the challenge through elongation of the intestine.

As an alternative to antibiotics, anticoccidial effect of various herbal extracts has been reported [31,34-39]. Their effects were related to protection and/or relieve of intestinal mucosa. To our knowledge, no data on anticoccidial effect of usnic acid are available. However, few investigations have been performed on its antiprotozoal activity. Wu et al.[27] stated that (K) usnic acid exhibited a strong effect against *Trichomonas vaginalis in vitro*. Intralesional administration of (+) usnic acid in mice infected with *Leishmania* promastigotes produced a significant reduction of cutaneous lesions and parasite loads [40]. Luz et al.[40] also determined the antileishmanial activity of usnic acid on *L. infantum chagasi* promastigotes and suggested that usnic acid as a possible phytotherapic agent in the treatment of visceral leishmaniasis. It appears that the

antileishmanial action mode is linked to a complete lysis of promastigotes of the *Leishmania* species [17]. Lichen constituents (thallus, methyl evernate, tenuiorin and three hopane triterpenoids) exerted a weak trypanocidial effect in comparison with the conventional drug in use against epimastigotes of *Trypanosoma cruzi* [20]. De Carvalho et al.[24] investigated the effects of usnic acid against *Trypanosoma cruzi* epimastigotes and trypomastigotes, and reported that usnic acid treatment resulted in growth inhibition in a dose-dependent manner. Lauinger et al.[26] reported antiplasmodial effect of some lichen compounds (*e.g.*, evernic acid, vulpic acid, psoromic acid and (+)-usnic acid) against liver stages of *Plasmodium berghei*.

Anticoccidial effect of usnic acid was comparable to toltrazuril that is a well-known anticoccidial agent [32,41]. In this study, usnic acid increased FI and BWG and alleviated FCR *(Table 1, Fig. 1)*. These could be consequence of its relieve effects on intestinal mucosa *(Table 2)*, which was associated with decreased OPG *(Fig. 2)* and partially shortened cecal length *(Table 2)*.

In summary, data showed that usnic acid (100 mg/kg) was effective in the treatment of coccidiosis as reflected by performance and pathology parameters but regrettably not as good as toltrazuril. Further studies are needed to substantiate our findings and elucidate its action in detail to suggest usnic acid as an alternative anticoccidial agent.

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Research Article

PCR Detection of Soy Protein in Ready to Eat Meat Doners [1]

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Abstract

Addition of soy protein sources in food products is widely used because of their functional properties such as water binding, fat binding, beneficial effects on texture and emulsification capability and providing improved economy with increasing yield. However, the use of soy protein in food products causes economical disadvantages because of replacement of an expensive ingredient like meat with a cheaper ingredient like soy and health risks for the consumers as well. Soy is an important allergy source for sensitive consumers. Because of these reasons, the most recent meat products regulation of Turkish Food Codex has banned the addition of soy in doners since 2012, like several other countries. Detection of soy in food products is performed by detection of soy protein or soy DNA. Because DNA is more stable to processing, PCR methods are shown to be more reliable when used in processed foods. In our study, 50 doner samples were collected from various retail sales points. Twenty-five doner samples were collected before and 25 after the regulation was enacted. DNA was isolated from doner samples and PCR testing of these DNA extracts were performed. The detection results of the doner samples showed that any of the 25 samples collected after the regulation enacted did not contain soy ingredient while 3 of 25 sample (12%) collected before the regulation came into force contained soy.

Keywords: *Soy, Doner, PCR, lectin, GM soy*

Tüketime Hazır Dönerlerde PCR ile Soya Proteininin Aranması

Özet

Soya proteinleri, gıda endüstrisinde, su bağlama, yağ bağlama, tekstür ve emülsifikasyon yeteneği, verim arttırma gibi fonksiyonel özelliklerinden ve verimin artışına bağlı olarak ekonomik karlılığı arttırmasından dolayı geniş kullanım alanı bulmaktadır. Ancak gıda ürünlerinde soya kullanımı, tüketiciler için ekonomik kayıplar ve sağlık riskleri gibi olumsuzluklara sebep olmaktadır. Soya, hassas tüketiciler için önemli bir alerji kaynağıdır. Bu gibi nedenlerden ülkemizde Aralık 2012'de çıkan Türk Gıda Kodeksi Et ve Et Ürünleri Tebliği ile dönerlerde soya kullanımı birçok diğer ülkelerde de olduğu gibi yasaklanmıştır. Gıdalarda soyanın tespit edilmesi soya proteinin ya da soya DNA'sının tespit edilmesi ile gerçekleştirilir. Ancak, DNA gıda işleme şartlara daha dayanıklı olması sebebiyle işlenmiş gıdalarda çok daha güvenilirdir. Çalışmamızda, 25 adedi Et ve Et Ürünleri Tebliğinin yürürlüğe girmesinden önce, diğer 25 adedinin ise tebliğin yürürlüğe girmesinden sonra olacak şekilde 50 adet döner örneği çeşitli perakende satış noktalarından toplanmıştır. Döner örneklerinden DNA izole edilmiş ve bu DNA'ların PZR testleri gerçekleştirilmiştir. Elde edilen sonuçlara göre Et ve Et Ürünleri Tebliğinin yürürlüğe girmesinden sonra toplanan hiçbir örnekte soya tespit edilemezken, tebliğin yayınlanmasından önce toplanan 25 örnekten 3'ünde (%12) soya varlığı saptanmıştır.

Anahtar sözcükler: *Soya, döner, PCR, lektin, GD soya*

INTRODUCTION

Addition of non-meat protein sources in food products, is widely used because of it is capability of improving the product properties and reducing the production cost. Soy protein fractions are preferred because of their higher protein content and functional properties [1,2]. The use of soy protein fractions in meat products is also widely applied for their properties such as water binding, fat binding, texture and emulsification capability and providing improved economy with increasing yield [1-3]. Soy protein fractions are available in various forms such as; flour, grits, concentrates, isolates and textured [3]. However, the use of soy in food products, causes health and economic risks for the consumers as well. It brings economical disadvantages because of replacement of an expensive

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ingredient like meat with a cheaper ingredient like soy. Besides soy is an important allergy source for sensitive consumers [3-5]. Because of these reasons, use of soy in food products are limited or banned in various countries [4,5]. In our country presence of soy in a food product has to be declared in the label. Additionally, meat products regulation of Turkish Food Codex banns the addition of soy in doner [6]. Despite, addition of soy in ready to eat meat products like doner, meat patties is not uncommon for reducing the cost.

Several methods have been used for detection of soy in food products so far [3,4,7]. However, most reliable methods are based on detection of soy protein or DNA. Protein based methods includes, electrophoretic or serologic (ELISA) methods [7-9] while PCR is most widely used as DNA based methods [4,10]. Because DNA is more stable to processing conditions than protein, PCR methods are shown to be more reliable when used in processed foods [10].

In this study, the presence of soy in doner kebabs sold in local sales points were investigated. The doner samples examined were purchased before and after the regulation enacted, to evaluate the effect of regulatory enforcement. For this, PCR detection of soy specific *lectin* gene with PCR was performed. The positive samples were further analyzed to detect whether they are Genetically Modified (GM) or not.

MATERIALS and METHODS

Doner Samples

For the study, 50 meat and poultry doner samples were collected from various retail sales points. Twenty five of these doner samples were collected before the regulation was released, while 25 were collected after the regulation. Additionally, soybean powder (IRMM, Geel, Belgium) and beef were used as positive and negative controls respectively. All the meat samples were stored in -20°C freezer till they were used.

DNA Extraction and Purification

For DNA isolation from doner samples and positive and negative control samples, the Promega Wizard™ DNA isolation kit (Promega, Madison, USA) was used according to the manufacturer's instructions. Two hundred to three hundred milligrams of food material taken from a previously homogenized sample was mixed with 860 µl of extraction buffer (10 mM Tris-OH, 150 mM NaCl, 2 mM EDTA and 1% w/v sodium dodecyl sulfate), 100 µl of guanidine hydrochloride (5M) and 40 µl of proteinase K (20 mg/ml), then incubated at 65°C overnight. The samples were then centrifuged at 13.500 g for 10 min. After centrifugation, 500 µl of the supernatant was mixed with 1 ml of Wizard™ resin (Promega, Madison, USA) and pushed through a Wizard™ minicolumn (Promega, Madison, USA). The column was further washed with 2 ml of isopropanol. Following centrifugation of the column at 12,000 g for 5 min, the DNA was eluted with 50 µl of pre-warmed (65°C) elution buffer (10 mM Tris-OH). The columns were incubated at room temperature for 1 min and centrifuged at 10.000 g for 2 min. The collected DNA was stored at -20°C until used.

DNA quantification was achieved by measuring the UV absorption at 260 nm using a T80 UV/VIS spectrometer (PG Ins. Ltd., UK).

PCR Primers and PCR Conditions

The primers GMO3 (5'-GCC CTC TAC TCC ACC CCC ATC C-3') and GMO (5'-GCC CAT CTG CAA GCC TTT TTG TG-3') were used for the amplification of soy-specific *lectin* sequence and yielded a longer PCR product (118 bp) [10,11]. The primers 35s-f2 (5'-TGATGTGATATCTCCACTGACG-3') and petu-r1 (5'-TGTATCCCTTGAGCCATGTTGT-3') were used for the amplification of GM soy-specific Round Up Ready (RUR) soy sequence and yielded a longer PCR product (172 bp) [11]. All PCR reactions were performed with a CG Palm-Cycler (CG 1-96 Genetix Biotech, Australia & Asia).

Amplification reactions for *lectin* contained; 5 µl of genomic DNA and 20 µl of the appropriate PCR reaction mixture. PCR reaction mixture consisted of 1X buffer (Fermentas), 1.5 mM $MgCl_2$ (Fermentas), 0.2 µM of each primers, 0.8 mM of each dNTP (Fermentas) and 0.5 IU of Maxima™ Hot Start *Taq* polymerase (Fermentas). The amplification profile used for this mixture was as follow: denaturation for 10 min at 95°C; amplification for 30 s at 95°C, for 30 s at 60°C, for 60 s at 72°C; number of cycles 35; final extension for 3 min at 72°C.

For detection of GM soy, amplification reactions which consisted of; 1X buffer (Fermentas), 1.5 mM $MgCl_2$ (Fermentas), 0.2 µM of each primer for RUR soy amplifications, 0.8 mM of each dNTP (Fermentas) and 0.5 U of Maxima™ Hot Start *Taq* polymerase (Fermentas) were used. The amplification profile used for this mixture was as follow: denaturation for 10 min at 95°C; amplification for 30 s at 95°C, for 30 s at 60°C, for 25 s at 72°C; number of cycles 40; final extension for 3 min at 72°C.

PCR products were electrophoresed through a 2% agarose gel containing ethidium bromide. As a size reference, a 50 bp DNA ladder (Fermentas) was used. Visualization of the gels was performed with a UV trans-illuminator, and the gels were captured with the Dolphin-DOC system and Dolphin 1D Gel analyzing software (Wealtec, Nevada,USA).

RESULTS

Total of fifty commercially sold ready to eat doner samples which 25 of them were collected before the related regulation came in to force while the rest 25 were collected after the enforcement were detected for

presence of soy protein with PCR in the present study.

For ensuring the reliability of the detection tests appropriate quality control studies were performed throughout the whole study.

For confirmation of the specificity of the primers PCR tests were performed with DNA extracts obtained from soybean powder and beef. The results showed that the primers were specific to soy and did not give any false result with the other main ingredient of doner like beef *(Fig. 1)*.

False positive results related to carry over contamination during DNA sampling and extraction were avoided by processing sterile milli Q water in parallel with the samples at each step of extraction and PCR [12].

For elimination of false negative results related to PCR inhibitors that might be present in the sample, DNA extracts of each sample were run in triplicate for each PCR reaction which one of the extracts were spiked with soybean powder DNA while the other were run without spiking. The results showed that any of the 50 samples did not contain any inhibitor.

The detection results of the doner samples showed that 3 of all 50 samples (6%) we analyzed were positive for soy. Any of the 25 sample collected after the regulation released, did not contain soy ingredient while 3 of 25 sample (12%) collected before the regulation came into force contained soy. The gel electrophoresis results of the positive samples are given at *Fig. 2*.

The *lectin* positive samples were further analyzed for presence of GM soya. The results of this detection proved that any of these samples contained soy from a GM source.

DISCUSSION

There is not much study performed on detection

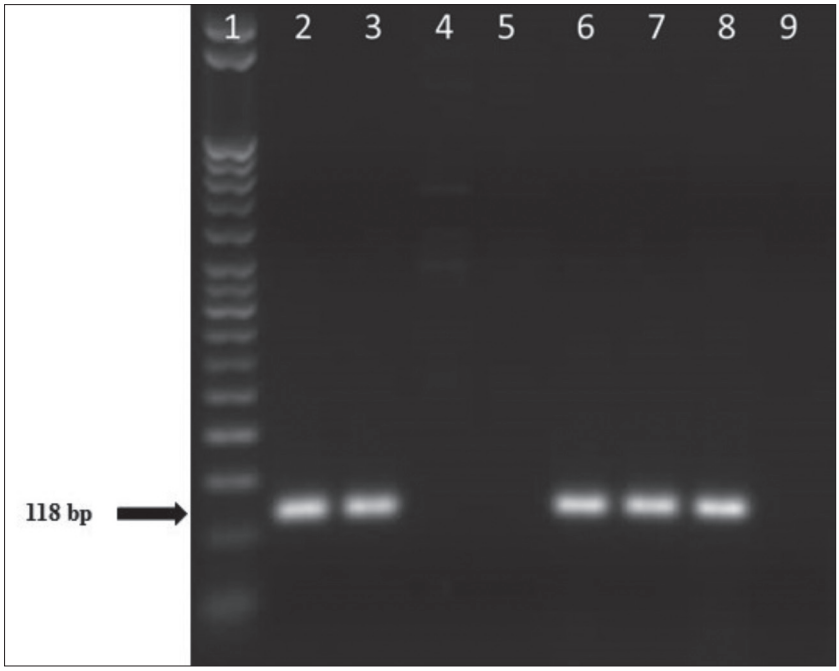


Fig 1. Specificity of primers

Lane 1: DNA ladder, Lane 2-3: soy flour, Lane 4-5: Beef, Lane 6-8: soybean powder, Lane 9: PCR mili q water

Şekil 1. Primerların özgünlüğü

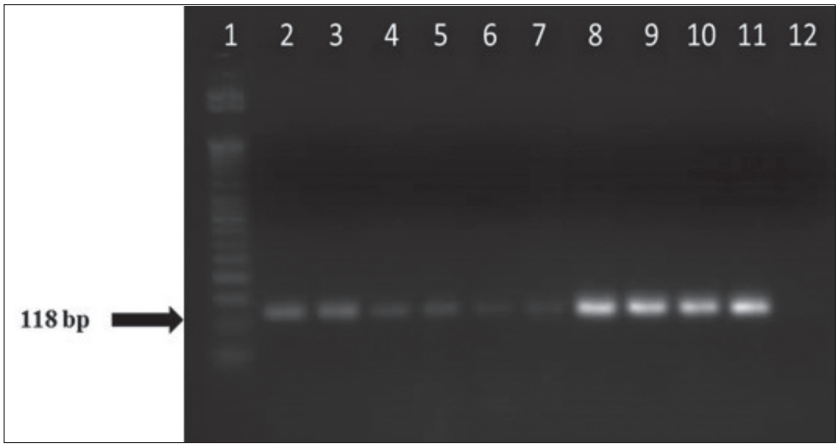
1. Sıra: DNA marker, 2-3. Sıra: Soya unu, 4-5. Sıra: Sığır eti, 6-8. Sıra: Soya fasulyesi tozu, 9. Sıra: PCR mili q su

Fig 2. Agar gel electrophoresis of positive doner samples

Lane-1: DNA ladder, Lane-2-3: doner sample-12, Lane-4-5: doner sample-13, Lane 6-7: doner sample-19, Lane 8-11: soybean powder Lane 12: PCR milli q water

Şekil 2. Pozitif döner örneklerin agar jel elektroforezi

1. Sıra: DNA marker, 2-3. Sıra: 12. Döner örneği, 4-5. Sıra: 13 Döner örneği, 6-7. Sıra: 19. Döner örneği, 8-11. Sıra: Soya unu, 12. Sıra: PCR milli q su



on soy in meat products. The main reason of this issue is that, it is not banned in several countries and it has been banned in our country only in 2012. For this purpose, few studies performed on detection of soy is mainly focused on detection of GMOs [13,14]. Ulca et al.[13] detected the presence of GM soy in various type of meat products and the samples of this study were collected recently after the regulation was released. According to these results, 32 out of 38 total samples contained soy and 2 of these positive samples were GM. In our previous study on detection of GM soya in food products which we performed before the regulation was enacted, we detected several meat products containing soy ingredients. Because it was legal, it was declared on the label as well [14]. However, different than our results (6% in our study) all of the doner samples Ulca et al.[13] analysed were positive for soy. According to the *Ct* results of this study, the soy level of these doner samples are quite low and most probably reflects trace amounts of presence. Thus, this difference might be related to the difference in the limit of detection of the two methods. The possible disadvantage of too low detection limit (below 0.1%) in authenticity testing is discussed by several authors because of its effect on discriminating technical unavoidable contamination and intentional addition [15,16]. For this purpose, it is not evaluated as a weakness of the method used in our study.

Based on the results of our study, we can conclude that intentional addition of soy was not commonly used in the case of doner even before the regulation. The level of usage has decreased after the regulation came in to force which showed that the producers comply with the regulation requirements. However, it is strongly recommended to further monitor the other type of processed meat products which are more commonly contained soy before the regulation.

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Research Article

Prevalence of Cartilage Erosion in Canine Patellar Luxation and Gene Expression in Affected Joints

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Abstract

The objectives of this study were to assess the prevalence of cartilage erosion in small dogs with patellar luxation (PL), and related osteoarthritis (OA)-related gene expression. In Study 1, 71 dogs were examined to determine risk factors associated with PL, including breed, age, weight, sex, and affected joint. In Study 2, a total of 39 dogs were divided into four groups: normal articular cartilage in the stifle joint (G1; n=5); PL without cartilage erosion (G2; n=11); PL with cartilage erosion (G3; n=14); and OA in the stifle (G4; n=9). Articular cartilage and synovial membranes were collected during surgical operations to correct PL. Real-time PCR was used to quantify the expression levels of 11 OA-related genes, including *AGG, COL2A1, HAS-1, HAS-2, TIMP-1, MMP-3, IL-1β, TNF-α, IFN-γ, COX-1,* and *COX-2*, with *GAPDH* used as a reference gene. From Study 1, it was found that the risk factors related with cartilage erosion lesion were age, sex, and PL grade (all variables showed $P<0.05$). From Study 2, it was demonstrated that PL with or without cartilage erosion expressed pro-inflammatory cytokines and enzymes; some biomolecules were up regulated *(IL-1β, MMP-3, AGG, TIMP-1)* but some were down regulated *(COL2A1, HAS-2, COX-1, COX-2)*. This expression was the difference between the articular cartilage and the synovial membrane; however, the expression of genes from PL with cartilage erosion was observed to be similar to that of OA. From our results, it can be concluded that PL can develop into secondary OA due to an increase of *IL-1β* in cartilage and synovial membrane.

Keywords: *Cartilage erosion, Dog, Gene expression, Patellar luxation*

Köpeklerde Patellar Luksasyonda Kıkırdak Erozyonunun Prevalansı ve Etkilenmiş Eklemlerdeki Gen Ekspresyonu

Özet

Bu çalışmanın amacı patellar luksasyonlu (PL) küçük cüsseli köpeklerde kartilaj erozyonunun prevalansını ve osteoartritis (OA)-alakalı gen ekspresyonunu belirlemektedir. Birinci araştırmada; cins, yaş, cinsiyet ve infekte eklemi içeren PL ile ilgili risk faktörlerini belirlemek amacıyla 71 köpek incelendi. İkinci araştırmada toplam 39 köpek dört gruba ayrıldı; diz ekleminde normal artikular kartilaj (G1; n=5), kartilaj erozyon olmayan PL (G2; n=11), kartilaj erozyonlu PL (G3; n=14) ve dizde OA (G4; n=9). Cerrahi operasyon sırasında PL'u düzeltmek amacıyla artiküler kartilaj ve sinoviyal zarlar alındı. Toplam 11 adet OA ile ilgili genin *(AGG, COL2A1, HAS-1, HAS-2, TIMP*-1, *MMP-3, IL-1β, TNF-α, IFN-γ, COX-1* ve *COX-2*; referans gen olarak GAPDH kullanıldı) ekspresyon düzeylerini belirlemek amacıyla PCR tekniği uygulandı. Birinci araştırmanın sonucunda yaş, cinsiyet ve PL derecesi kartilaj erozyonu ile ilgili risk faktörleri olarak belirlendi ($P<0.05$). İkinci araştırmada kartilaj erozyonlu veya erozyon bulunmayan PL'lu köpeklerde pro-inflamatuar sitokinleri eksprese ettikleri, bazı biyomoleküllerin ekspresyonunu artırdıkları *(IL-1β, MMP-3, AGG, TIMP-1)* bazılarını ise azalttıkları (IL-1β, MMP-3, AGG, TIMP-1) gözlemlendi. Bu ekspresyon artiküler kartilaj ve sinoviyal zar için farklıydı. Ancak kartilaj erozyonlu PL'da genlerin ekspresyonu OA ile benzerlik göstermekteydi. Çalışma bulguları doğrultusunda kıkırdak ve sinoviyal zarlarda artan *IL-1β*'ya bağlı olarak PL'un sekonder OA'e yol açabileceği sonucuna varıldı.

Anahtar sözcükler: *Kartilaj erozyonu, Köpek, Gen ekspresyonu, Patella çıkığı*

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INTRODUCTION

Patellar luxation (PL) is one of the most common joint diseases in small breed dogs [1-3], the prevalence of which has been studied worldwide. In Chiang Mai, Thailand, for example, 128 out of 317 dogs (40.3%) were reported to be affected with PL, predominantly in poodles (34.4%), Pomeranians (28.9%), and Chihuahuas (12.5%). Even in the United States, a study found that 43% of Pomeranians and 23.6% of Dutch flat-coated retrievers had PL [4]. Originally, clinicians focused on finding an effective surgical technique for treating this condition [5-8]. So far, targeted gene studies have detected loci on chromosomes 7 and 31 that are involved in PL [2,9,10]. Patellar luxations can be medial or lateral and are graded based on severity [11]. A higher grade of PL is associated with certain joint diseases, such as cranial cruciate ligament rupture [1,12].

Osteoarthritis (OA) is one of the most common joint diseases in animals, and in humans as well. Many joint diseases in dogs have been proven to be the cause of OA, such as cranial cruciate ligament rupture [13-15], meniscus injury [14,15], elbow dysplasia [16], and hip dysplasia [17]. Although a relationship between PL and OA has not been well established in dogs or in humans, three reports have indicated that PL is a possible cause. The other study also reported significant potential in treating PL without surgery [18]. Patellar luxation causes joint instability from the lateral or medial movement of the patella on the femoral groove. The movement of the patella in and out of the femoral groove in PL can cause cartilage erosion [19-21], which may then develop into OA [18,22,23].

As yet, PL has not been reported to be associated with expression of OA-related genes in dogs or in humans. This work aims to study the expression of some OA-related genes from articular cartilage and synovial membrane in canine PL. The objectives of this study were to determine the prevalence of cartilage erosion in PL and to compare the expression of genes in PL, with or without cartilage erosion, to OA gene expression. The hypothesis is, if PL is related to development of OA, then the expression of some OA-related genes will differ from normal and be similar to OA joint. Additionally, we study the incidence of cartilage erosion in PL.

MATERIAL and METHODS

This research consisted of two independent studies. The first is retrospective data showing the prevalence of cartilage erosion in canine PL. The second study investigated the expression level of some OA-related genes from the articular cartilage and the synovial membrane. The Ethics Committee of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand, approved this study in 2014.

Study 1: Incidence of Cartilage Erosion in Canine

Animals: The data *-including breed, age, weight, sex, and affected stifle joint- were recorded from 71 PL dogs (Table 1)* that had visited the Animal Hospital for stifle surgery from 2010 to 2014.

Patellar Luxation Grading: The degrees of PL were classified into four grades, as determined by manipulation [1,24,25]. *Grade I:* The patella can be luxated from the femoral groove when the stifle was fully extended and the patella can return into the femoral groove immediately. *Grade II:* The patella moves out of the femoral groove for sometime, but it can return to the normal position spontaneously. *Grade III:* The patella is normally luxated from the femoral groove but can be returned to a normal position by manipulation. *Grade IV:* The patella is permanently luxated from the femoral groove and cannot returned to this normal position.

Cartilage Erosion Lesion: During exploratory stifle arthrotomy, the lesions on six anatomical sub-regions of articular cartilage on the patella and femoral trochlea, including the central patella, medial patella, lateral patella, medial trochlea, lateral trochlea, femoral groove, and osteophyte formation were examined and evaluated as positive or negative lesions.

Statistical Analysis: Age and weight were reported as mean±SD, sex was reported in terms of number of male and female, and affected joints were reported in terms of percentage. The prevalence of cartilage erosion lesions in canine PL cases was reported with 95% confidence interval (95% CI). The relationship between degrees of PL and positive/negative cartilage erosion was analyzed using Fisher's exact test. The R statistical program was used to analyze the risk factors of cartilage erosion lesion finding. Univariable analysis was performed using Fisher's exact test. A thres-hold value of $P<0.05$ was used to screen variables for the multivariable model. A multivariable logistic regression model was used to assess the risk factors of this outcome. The Akaike information criterion was used to select the best-constructed model. The receiver operating characteristic curve was tested to evaluate model accuracy.

Table 1. *Information on patients included in study 1*
Tablo 1. *Birinci araştırmadaki hasta bilgileri*

Breed	Number	Age (months) min-max (mean)	Weight (kg) mean±SD	Sex	
				Male	Female
Pomeranian	33	6-120 (37)	3.7±1.6	15	18
Chihuahua	22	8-96 (25)	2.7±1.5	7	15
Shih Tzu	6	36-84 (49)	5.8±1.2	3	3
Yorkshire Terrier	5	18-96 (40)	2.4±0.8	4	1
Poodle	5	24-120 (64)	5.1±2.3	2	3

Study 2: Articular Cartilage and Synovial Membrane Gene Expression in Canine Patellar Luxation

Animals: A total of 39 dogs were divided into four groups *(Table 2)*: G1=dogs without gross evidence of pathology of articular cartilage from the stifle joint (n=5); G2=PL without cartilage erosion (n=11); G3=PL with cartilage erosion (n=14); and G4=dogs with stifle OA (n=9), when OA lesions were present in the joint, based on the following criteria: cartilage fibrillation, erosion, and osteophytes [26].

Inclusion/Exclusion Criteria for Samples: The dogs belonging to G2 and G3 were small-breed dogs less than 5 years old with clinical signs of medial PL. Animals that were pregnant, were with neurological disease, or had undergone musculoskeletal surgery were excluded. Dogs with lameness due to cranial cruciate ligament rupture or meniscal injury, and those with nerve injury, lumbosacral instability, infection, immune diseases, and fractures were also excluded. Dogs belonging to G1 had visited the hospital for hind limb amputation (from a traffic accident). Gross pathology reports of previously collected cartilage and synovial membrane were evaluated, and it was confirmed that the reports did not show cartilage and synovial membrane lesions. Moreover, these dogs had no documented history of stifle disorder. Dogs in G4 were diagnosed with cranial cruciate ligament rupture or meniscus injury at least 1 month prior to surgery; moreover, this group was free from PL. Samples of articular cartilage and synovial membrane were collected during the operation.

Patellar Luxation and Articular Cartilage Erosion: The degrees of medial PL were classified into four grades by manipulation, as mentioned previously. Dogs with medial PL had been recommended to undergo surgical correction of the condition by a veterinarian. Articular cartilage erosion was evaluated during the operation. The criterion of cartilage erosion was applied from macroscopic scoring of femoral condyle and patella as mention in Cook et al.[27]. Cartilage erosion was classified as either positive or negative.

Collected Cartilage and Synovial Membrane: During stifle the operation, the cartilage was collected at the lateral site of the femoral trochlea using a scalpel blade, while the synovial membrane was collected at the incision site immediately following arthrotomy *(Fig. 1)*. For dogs in G1 and G4, samples were collected at the same site as for G2 and G3, to avoid the location of articular cartilage being a factor in the analysis. The size of each sample of the cartilage and the synovial membrane was approximately 0.5 cm in length. The samples were ground and homogenized with TRIzol® reagent for RNA isolation [28,29]. Due to ethical considerations, sample collection was performed so that the procedure itself would not cause OA or other joint disease. Cartilage samples could not be collected at cartilage lesions because of the possibility that this could lead to progressive OA [30]. A previous study found that collection of cartilage at the lateral site of the femoral trochlea would not cause OA [31].

Gene Expression Analysis: The genes involved in OA were investigated for their expression levels in the cartilage, and the synovial membrane tissues collected from dogs with OA and PL, using the quantitative real-time PCR method. The tissues were evaluated for the expression level of 11 genes, as follows: 5 anabolic-related genes: aggrecan *(AGG)*, collagen type II alpha 1 *(COL2A1)*, hyaluronan synthase 1 *(HAS-1)*, hyaluronan synthase 2 *(HAS-2)*, and the tissue inhibitor of metalloproteinase 1 *(TIMP-1)*; 1 catabolism-relatedgene:matrix metalloproteinase 3 *(MMP-3)*; 3 pro-inflammatory cytokine genes: interleukin 1 beta *(IL-1β)*, tumor necrosis factor alpha *(TNF-α)*, and interferon gamma *(IFN-γ)*; and 2 inflammatory enzyme genes: cyclooxygenase 1 *(COX-1)* and cyclooxygenase 2 *(COX-2)*. Glycer-

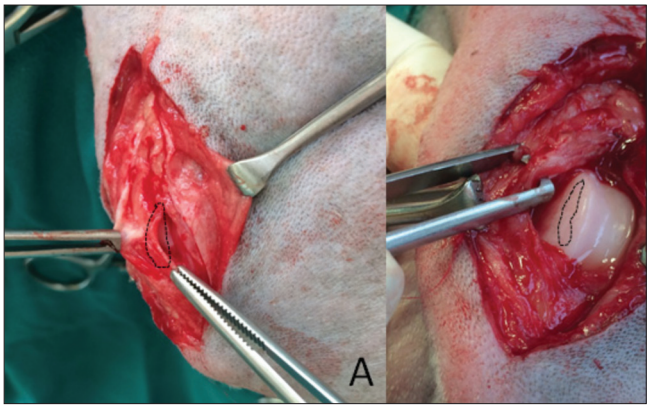

Fig 1. The excision sites of (A) synovial membrane and (B) articular cartilage

Şekil 1. Sinoviyal zar (A) ve artikular kartilajdaki (B) kesit alanları

Table 2. *Information on patients included in study 2*
Tablo 2. *İkinci araştırmadaki hasta bilgileri*

Groups	Articular Cartilage	Number Total Number (male:female)	Age (months) min-max (mean)	Weight (kg) mean±SD
G1	Normal	5 (2:3)	12-58 (34)	1.81±4.67
G2	Patellar luxation without cartilage erosion	11 (7:4)	6-60 (20)	3.76±2.37
G3	Patellar luxation with cartilage erosion	14 (3:11)	6-60 (20)	3.76±2.37
G4	Osteoarthritis	9 (4:5)	24-120 (67)	7.33±5.32

aldehyde-3-phosphate dehydrogenase *(GAPDH)* was used as the endogenous control gene (reference gene) [28].

RNA Isolation, cDNA Synthesis and Quantitative Real-Time PCR: The total RNA of the cartilage and the synovial membrane were isolated by using aninnuPREP DNA/RNA Mini Kit (Analytik Jena AG, Germany), as described in the manufacturer's protocol. -Reverse transcription of the total RNA from the cartilage and the synovial membrane was carried out to synthesize first-strand cDNA. The Expression of the genes related to OA was measured by quantitative real-time PCR by using an Eco™ Real-Time PCR System (Illumina, USA). The PCR reaction was incubated according to the following protocol: 95°C for 10 min, 45 cycles of denaturation at 95°C for 20 s, annealing at different annealing temperatures *(Table 3)* for 15 s, and extension at 72°C for 15 s. The relative expressions were calculated using threshold cycles (C_T) with normalization to the reference gene (*GAPDH*) [28].

Statistical Analysis: The amplification efficiency of genes reported to the GAPDH expression as the internal control. The mRNA level, expressed as Ct, ΔCt (Ct gene - Ct GAPDH), was used to calculate the relative quantification (Rq), using $2^{-\Delta\Delta Ct}$ methods. The expression of the control group (G1) served as a reference (Rq=1). The data were presented as box plots and statistically analyzed using the SPSS 17 software. The expression level difference groups were determined using ANOVA and multiple comparison tests. $P<0.05$was considered to be statistically significant.

RESULTS

Prevalence of Cartilage Erosion in Canine Patellar Luxation: Out of a total of 71 dogs surveyed for cartilage erosion, 39% (28/71) demonstrated cartilage lesions predominantly on the femoral trochlea and patella. The majority of these animals (24/28) had one lesion, whereas three animals had two, and one had three lesions on the articular surfaces and bone *(Table 4)*. Out of 33 lesions observed, the majority were observed on the medial patella (30% of total lesions, 10/33), while 27% (9/33), 21% (7/33), 18% (6/33), and 3% (1/33) were observed on the lateral patella, the center patella, the medial trochlea, and lateral trochlea osteophytes, respectively *(Fig. 2)*.

Cartilage erosion was not found on the femoral groove or the lateral trochlea. On five stifles (18%) were found two lesions; four stifles showed lesion on the medial and the lateral patella, one stifle revealed lesion on the center of the patella and osteophyte on the lateral trochlea. Three lesions were found on one stifle (3%) on the medial and the lateral patella as well as the medial trochlea. Risk factors that were related to cartilage erosion were age, sex, and PL grade ($P<0.05$). The prevalence of cartilage erosion was higher (OR = 7.05, $P<0.05$) in female dogs compared with male dogs and increased with age (OR = 1.04, $P<0.05$).

Gene Expression: The quantity and quality of the cDNA samples synthesized from the RNA was evaluated using a spectrophotometer (Biodrop Ltd., Cambridge, UK). It was

Table 3. *Sequences of primers used in quantitative real-time PCR*
Tablo 3. *Kantitatif gerçek zamanlı PCR'da kullanılan primer sekansları*

Gene	Primer Sequence (5'→3')	Length (bp)	Accession Number	Annealing Temperature (°C)
GAPDH	Fw: AGTATGATTCTACCCACGGC Rw: CGAAGTGGTCATGGATGACT	362	DQ403060	55
MMP-3	Fw: CTCACCCAGCAATACCTAGA Rw: CAGAGCTTTCTCAATGGCAG	318	AY183143_1	57
TIMP-1	Fw: ATCCTGCTGTTGCTGTGG Rw: GTCGGTCTGGTTGACTTCTGC	138	NM_001003182	57
AGG	Fw: GCCACCATCAGAAACCTAC Rw: AGACACCTCGGAAGCAGA	350	NM_001113455	57
COL2A1	Fw: CAGCGAGCGTTCCCAAGA Rw: CAGGCGGAGGAAGGTCAT	158	NM_001006951	60
HAS-1	Fw: CAGACACGCTGGTCCAAATC Rw: GCATAGAAGAGCCGCAACAC	149	XM_849398	55
HAS-2	Fw: GGTCATAGATGGGAACTCG Rw: GACTCATCCGTCTCACCAG	135	XM_539153	51
TNF-α	Fw: AGTGCCGTCAGATGGGTTG Rw: CCAGGTAGATGGGCTCGTA	215	NM_001003244	58
IFN-γ	Fw: AGGTCCAGCGCAAGGCGATA Rw: TCGATGCTCTGCGGCCTCGAA	117	NM_001003174	60
IL-1β	Fw: CACAGGTTCTCTGGTAGATGAGG Rw: TGGCTTATGTCCTGTAACTTGC	264	Z70047.1	50
COX-1	Fw: GGATGGAGAGATGTACCCGC Rw: CCCAATGAGGATGAGTCGGG	244	NM_001003023.2	60
COX-2	Fw: GGGAACTCCGCCGCGA Rw: CCGTAGAATCCTGTTCGGGT	167	NM_001003354.1	55

found that cDNA concentrations ranged from 0.6 to 2.3 µg/µl. The purities of the OD 260/280 and 260/230 ratios were 1.6-1.7 and 1.7-2.8, respectively.

Cartilage Gene Expression: The expressions of all 11 genes in cartilage are shown in *Fig. 3*. In comparing the OA group (G4) to controls (G1), seven transcripts were expressed to a lower degree in G4 ($P<0.05$), which included *HAS-1, HAS-2, COL2A1, AGG, IFN-γ, COX-1,* and *COX-2*, while the other four transcripts had higher expression in G4. Only *MMP-3* and *IL-1β* had higher ($P<0.05$) expression in G1 than in G4. The expressions of *COL2A1* and *IFN-γ* were highly expressed ($P<0.05$) in G2 compared with G3. In G2, the expressions of *HAS-1, HAS-2 AGG, TIMP-1, MMP-3,* and *TNF-α* did not differ ($P>0.05$); however, expressions of *IL-1β* and *IFN-γ* was higher ($P<0.05$) compared with G1. Between G2 and G4, expression of *HAS-1, HAS2, COL2A1, AGG, IFN-γ, COX-1,* and *COX-2* were higher ($P<0.05$) in G2, while in G3, the expression of *TIMP-1, MMP-3, IL-1*β, COX-2, and *TNF-α* were not different ($P>0.05$), nor were did they differ compared to G1. The expressions of *AGG, IFN-γ,* and *COX-1* were higher ($P<0.05$) in G3 compared with G4. Last, expression of *MMP-3* was lower ($P<0.05$) in G3 compared with G4.

Synovial Membrane Gene Expression: The expressions of genes from the synovial membrane are shown in *Fig. 4*. Comparing G1 and G4 groups, two genes, namely *HAS-1* and *HAS-2,* were expressed to a lower extent ($P<0.05$) in G4, whereas *IL-1β* and *COX-1* were similar ($P>0.05$). Three genes, which included *TNF-α*, *IFN-γ,* and *COX-2,* had higher expression ($P<0.05$) in G4. Between G2 and G3, *TIMP-1* expression was higher ($P<0.05$) in G3, whereas *COL2A1, MMP-3, COX-2, IFN-γ* and *TNF-α* was not ($P>0.05$). *IL-1β* expression was lower ($P<0.05$) in G3, but *HAS-1, HAS-2, AGG,* and *COX-1* expression was similar ($P>0.05$). *TIMP-1* in G2 was expressed more ($P<0.05$) than that in G1, while *HAS-1, COL2A1, AGG, MMP-3, IL-1β, TNF-α,* and *IFN-γ* had similar expression levels ($P>0.05$). Notably, *HAS-2* showed lower ($P<0.05$) expression compared with G1. The expression of *HAS-2, AGG,* and *IL-1β* in G2 was higher ($P<0.05$), while *TIMP-1, TNF-α, IFN-γ* and *COX-2* was lower ($P<0.05$) in comparison with G4. *MMP-3* in G3 showed higher ($P<0.05$) expression in comparison with G1, while *HAS-1, COL2A1, AGG, TIMP-1, IL-1β, COX-2,* and *TNF-α* did not show a difference ($P>0.05$) inexpression. Lower epression of *HAS-2* ($P<0.05$), but not *COX-1* or *COX-2* ($P>0.05$), was observed in G3 as compared to G1. *AGG* and *TIMP-1* in G3 showed higher ($P<0.05$) expression, while *COX-2* showed no difference ($P<0.05$) in expression in comparison with G4.

DISCUSSION

The relationship between cartilage erosion and PL in humans has been widely reported [19,20,32], and a few studies have been performed in dogs [21,23]. In the reports on humans, a high prevalence of cartilage lesions, of 40-97%, has been reported [19,20,32], while in dogs, prevalence reported has been 39.5% [21]; in addition, our study reports the percentage of prevalence in dogs to be 39%.

Our study also found that the grade of PL had an effect on cartilage erosion ($P<0.05$). However, the odds ratio (OR) cannot be reported because of the low number of dogs in each group of patellar grade, making the OR number of this factor extremely high. A larger number of dogs is needed in each group for finding the OR number of the patellar grade and the cartilage erosion. A total of 79% of the cartilage erosion in PL was found in a single location, 18% was observed in two locations, and 3% was found in three locations. These findings are in accordance with a previous report [21] which demonstrated that very high percentages of cartilage erosion were found in 21-60% of the examination areas. The reason that we did not conduct the evaluation in terms of areas of cartilage erosions because during the operation it is not possible to do a measurement of the actual area of all the articular cartilage, precise measurement of the area was not possible at the time of surgery. In our study, low grades of PL (1-2) were

Table 4. *Percentage of positive and negative cartilage lesions as well as location of cartilage lesions in 71 cases of patellar luxation*
Tablo 4. *Patella çıkıklı 71 olgudaki lezyonlar*

Patella Grade	Cartilage Lesions (total stifle joints = 71)			Cartilage Lesions in Different Sub-regions (total lesions = 33)						
	Total	Pos.	Neg.	A	B	C	D	E	F	G
1	14% (10/71)	0% (0/28)	23% (10/43)	0% (0/33)	0% (0/33)	0% (0/33)	0% (0/33)	0% (0/33)	0% (0/33)	0% (0/33)
2	13% (9/71)	0% (0/28)	21% (9/43)	0% (0/33)	0% (0/33)	0% (0/33)	0% (0/33)	0% (0/33)	0% (0/33)	0% (0/33)
3	37% (26/71)	29% (8/28)	42% (18/43)	12% (4/33)	0% (0/33)	6% (2/33)	6% (2/33)	0% (0/33)	0% (0/33)	0% (0/33)
4	37% (26/71)	71% (20/28)	14% (6/43)	18% (6/33)	27% (9/33)	15% (5/33)	12% (4/33)	0% (0/33)	0% (0/33)	3% (1/33)
Total	71	39% (28/71)	61% (43/71)	30% (10/33)	27% (9/33)	21% (7/33)	18% (6/33)	0% (0/33)	0% (0/33)	3% (1/33)

Pos. = positive, Neg. = negative, A = medial patella, B = lateral patella, C = center patella, D = medial trochlea, E = lateral trochlea, F = femoral groove, G = osteophyte

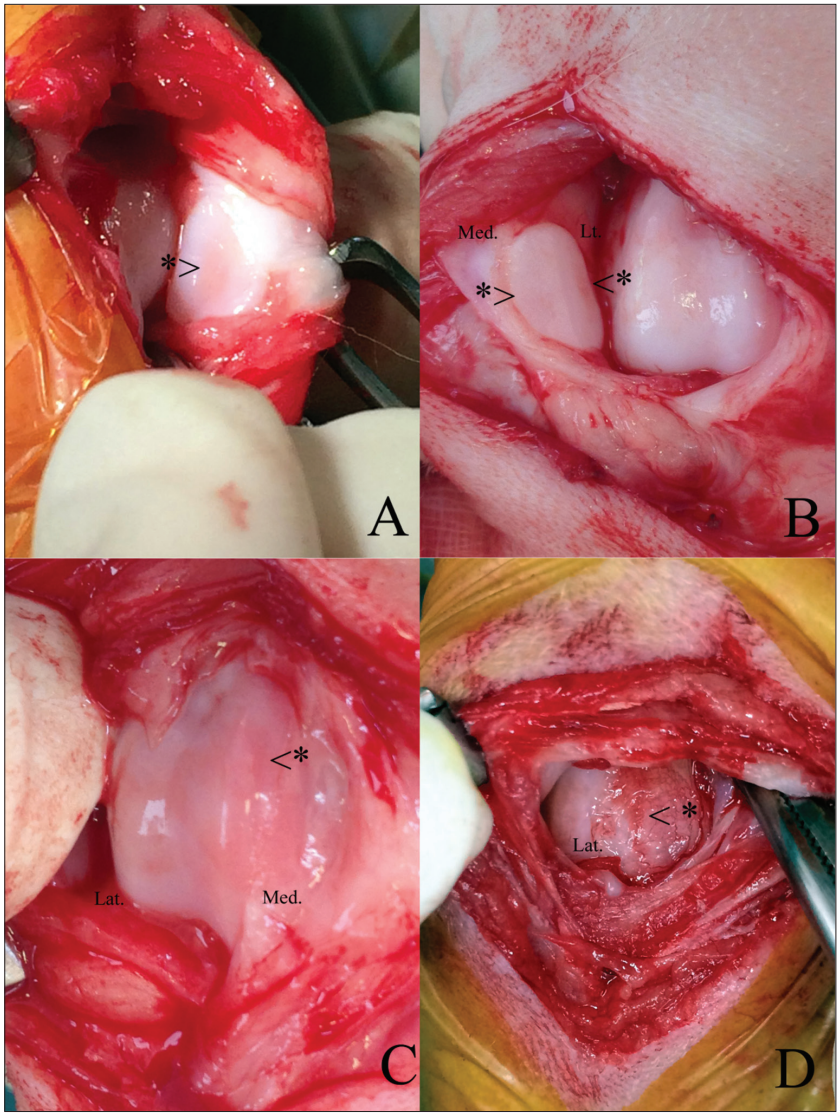

Fig 2. A representative photo of cartilage erosion on the (A) center of patella, (B) medial and lateral patella, (C) medial trochlea, and (D) osteophyte (Lat. = lateral; Med. = medial)

Şekil 2. Patellanın merkezinde (A), medial ve lateralinde (B), medial trocleada (C) ve osteohytede (D) kartilaj erozyonlarının göstüntüsü (Lat. = lateral; Med. = medial)

not found to be affected with cartilage erosion, while a report from Daems et al.[21] demonstrated 55% and 42% from grade 1 and grade 2 as being affected with cartilage erosion; the contrasting reports may be due the difference in the sizes of dogs in the two studies. The study conducted by Daems et al.[21] included all breed sizes (small to giant breeds) with weights in the range of 1-42 kg (the median weight being 8.8 kg), while our study included only small breeds with weights in the range of 1.2-9.0 kg (the median weight being 3.5 kg). The conclusion arrived at by Daems et al.[21] suggests a weak significant correlation between cartilage erosion and body weight, but taken together with the findings in this study, it may be possible that weight does have an effect on cartilage erosion in PL. Moreover, our first study found the prevalence of cartilage erosion to be significantly higher in female dogs compared with males. Additionally, with increasing age, the prevalence of cartilage erosion was also found to increase significantly. It has been well documented in many publications that aging has an effect on cartilage morphology and biology, which can cause OA [33,34].

In the process of harvesting articular cartilage, we collected at the lateral aspect of the femoral trochlea in all four groups. From a previous study on chondrocyte transplant, including research done by our team [31], this location is the best for harvesting articular cartilage without causing OA. Moreover, this is in accordance with ethical standards, whereby any clinical research method must not be the cause of disease or illness. Indeed, the experimental design was for samples to be collected at the same location in all joints: normal, PL with or without cartilage erosion, and OA groups. This is because if samples were collected at different locations (weight-/non-weight-bearing sites or lesion/normal sites) it would affect the comparison of gene expression among the four groups. In the PL with cartilage erosion group, the expression of genes in cartilage taken from a lesion was similar to that of OA [35]. But our findings have shown than not only do chondrocytes from a lesion demonstrate a similarity to chondrocytes from OA, they also have a marked effect on normal cartilage tissue in the same bone.

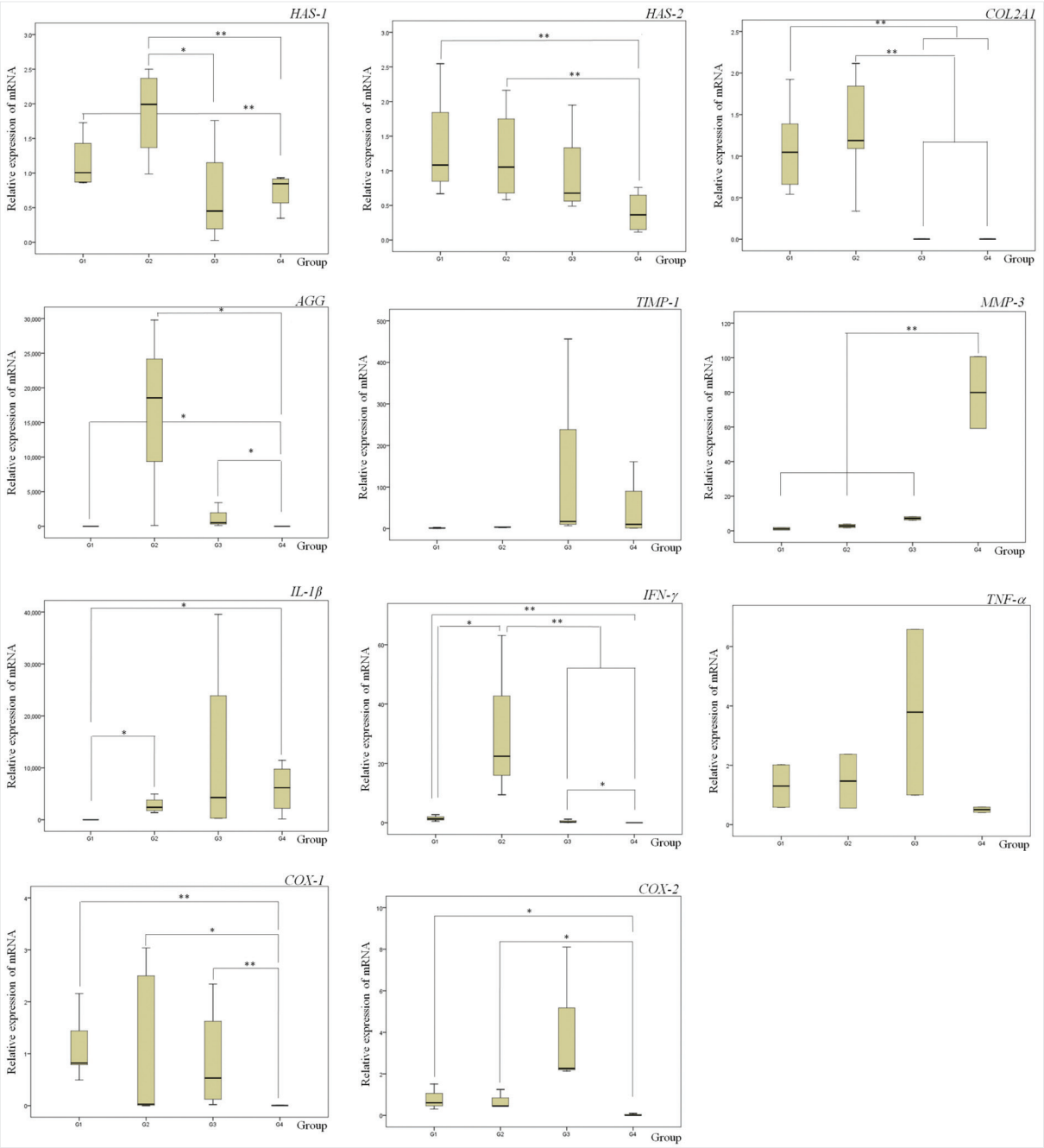

Fig 3. The relative expressions of 11 genes from the articular cartilage, as considered in the four study groups: G1 = normal, G2 = patellar luxation without cartilage erosion, G3 = patellar luxation with cartilage erosion, and G4 = osteoarthritis (*P<0.05; **P<0.01)

Şekil 3. Artikular kartilajda 11 genin orantısal ekspresyonları G1 = normal, G2 = kartilaj erozyonu olmayan patella çıkığı, G3 = kartilaj erozyonlu patella çıkığı ve G4 = osteoartritis (*P<0.05; **P<0.01)

To the best of our knowledge, this research is the first to demonstrate the expression of genes in PL with and without cartilage lesion as having the potential to develop into OA. This study has shown that the expression levels of *AGG* (8,302-fold) and *IFN-γ* (18-fold) from the articular cartilage and *HAS-1* (1,404-fold), *AGG* (19,814-fold), and *IL-1β* (365-fold) from the synovial membrane of PL without cartilage erosion are the highest among all the four groups. The expression of *IL-1β* (9,872-fold) and *COX-2* (48-fold) from the articular cartilage and *TIMP-1* (332-fold) and *MMP-3* (44-fold) from the synovial membrane of PL with cartilage erosion was found to be the highest among all the four groups. In PL with or without cartilage erosion, it was found that the *AGG* and the *IL-1β* expression levels from the articular cartilage and the *HAS-1* expression from the synovial membrane had up-regulated in comparison with the normal and the OA groups.

Among the cytokines involved in the OA process, *IL-1β* and *TNF-α* are found to play important roles as major

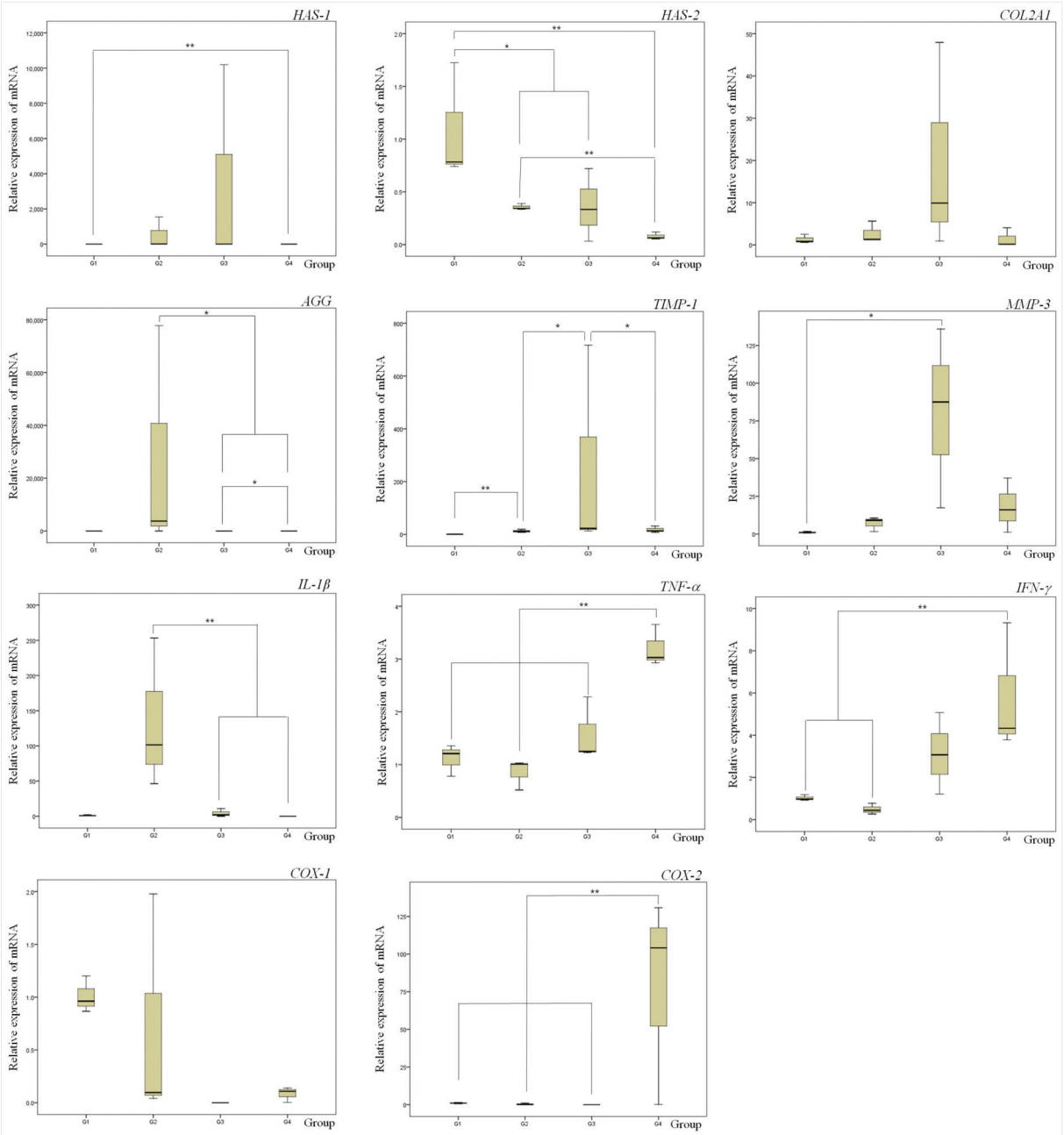

Fig 4. The relative expression of 11 genes from the synovial membrane, as considered in the four study groups: G1 = normal, G2 = patellar luxation without cartilage erosion, G3 = patellar luxation with cartilage erosion, and G4 = osteoarthritis (*P<0.05; **P<0.01)

Şekil 4. Sinoviyal zarda 11 genin orantısal ekspresyonları G1 = normal, G2 = kartilaj erozyonu olmayan patella çıkığı, G3 = kartilaj erozyonlu patella çıkığı ve G4 = osteoartritis (*P<0.05; **P<0.01)

OA-induced cytokines. Our study found that the cartilage and synovial cells of PL expressed *IL-1β* in levels higher than normal and OA. In the articular cartilage of PL with and without cartilage erosion, *IL-1β* was observed to have the highest level of expression in comparison with other genes. But the expression levels from the synovial membrane of both the groups were observed to have slightly up-regulated. From this result, it is possible to conclude that PL with or without cartilage erosion can increase the expression levels of *IL-1β* and *TNF-α*, which can lead to the development of a catabolic pathway to OA. Moreover, *MMP-3* is an enzyme that responds to the catabolic pathway, and the expression of this gene was found to be significantly high in the articular cartilage. The modulated expression of *MMP-3* was found in the articular cartilage and the synovial membrane of PL with cartilage erosion, while the expression from PL without cartilage erosion was observed to be mild. It is possible that cartilage erosion is one cause of *MMP-3* expression, which increases the degradation process in articular joints affected with PL. The enzyme *MMP-3* can cleave collagen, aggrecan, and link protein, while *TIMP*s inhibit the activity

of *MMP*s. This study evaluates the expression of *MMP-3* and *TIMP-1* because these two genes are related, as previously described [36,37]. An increase in the level of the enzyme *MMP-3* in comparison with *TIMP-1* in the cartilage and the synovial membrane would explain the decrease in proteoglycan. In this study, the expression levels of *MMP-3* and *TIMP-1* from the articular cartilage of PL with and without cartilage erosion and OA are together up regulated.

As is well known, *IFN-γ* is a pro-inflammatory cytokine that plays a key role in maintaining immune homeostasis in patients with rheumatoid arthritis (RA) and joint inflammation [8]. This study found that the expression of *IFN-γ* in the articular cartilage from patella luxation without cartilage erosion was up-regulated, but that the expression of *IFN-γ* in the articular cartilage from PL with cartilage erosion and OA were down-regulated; however, they were not found to be very significantly different. This finding is similar to the finding reported by Tsuchida et al.[18], which is that the level of the synovial fluid of the cartilage defect joint is higher than the level of the cartilage of OA. Whereas in the synovial membrane of PL with and without cartilage erosion, it was found that *IFN-γ* showed mild up regulation, significant ($P<0.05$) up regulation was found in the synovial membrane of OA.

One of the multifunctional enzymes involved in the normal and the pathologic pathways is COX, of which two isoforms have been characterized. *COX-1* is expressed constitutively in many organs/tissues in body, while *COX-2* up-regulates in the inflammation pathway. Our study found a low expression of *COX-1* in the cartilage and the synovial membrane of PL with cartilage erosion and OA groups, while *COX-2* expression from the cartilage and the synovial membrane was observed to be the highest in PL with cartilage erosion and OA group. Increasing amounts of *IL-1β* and *TNF*-α were detected in cartilage and synovial membrane samples taken from both the OA and PL with cartilage erosion groups. Both of these cytokines have the ability to upregulate *COX-2* gene expression. *TNF* activates not only the degradation pathway in OA, but also the sensory neurons, which is what induces neuropathic pain [38]; an increase in *TNF-α* in PL and OA can be a cause of pain.

The HAS enzymes are secreted by chondrocyte and synoviocyte, and the three related synthase isoenzymes are *HAS-1*, *HAS-2*, and *HAS-3*. The predominant enzymes are *HAS-1* and *HAS-2*; *HAS-1* is a major HAS isoform produced from synoviocyte, while *HAS-2* is a major isoform produced from cartilage [39]. Our study found the gene expression of *HAS-1* and *HAS-2*, but not *HAS-3* because *HAS-1* and *HAS-2* are active during the process of tissue damage and repair and produce high molecular weight HA, whereas*HAS-3* produces low molecular weight HA [40]. This study found the *HAS-1* expression from the synovial membrane to be extremely high in PL, both with and without cartilage erosion groups, while it was downregulation the OA group in comparison with the control group. *HAS-2* was observed to be upregulated in PL with or without cartilage erosion but down regulated in the OA group.

Both the synovial membrane and the articular cartilage play important roles in controlling OA. But the difference between the cartilage and the synovial membrane lies in the expression of the genes; even for the same gene, there exists differences in the expression between two tissues in normal or OA joint [18]. All the cytokines that are produced from these two tissues influence the OA mechanism. In the early stages of OA, the expression of cytokine from the synovial membrane is found to be associated with the presence of synovial inflammation [41,42]. Our study found down-regulation of the *COX-2* gene from the synovial membrane of patella luxation with and without cartilage erosion, but the highest occurrence of up-regulation was from the synovial membrane of the OA group. Moreover, *COX-2* in the articular cartilage was observed to be up regulated in PL with cartilage erosion, but down-regulated in PL without cartilage erosion and OA.

This study has demonstrated that PL with or without cartilage erosion expresses pro-inflammatory cytokines and enzymes, and that some anabolic biomolecules are up regulated but some are down regulated. The expression was different between articular cartilage and synovial membrane. The expression of genes from PL with cartilage erosion is similar to that of OA. In conclusion, PL with or without presentation of articular cartilage erosion can lead to OA, based on increasing levels of *IL-1β* observed.

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Research Article

Identification of Genetic Variation of Melatonin Receptor 1A (MTNR1A) Gene in Kıvırcık Breed Ewes by *Mnl*I and *Rsa*I Restriction Enzymes [1] [2]

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Abstract

Melatonin receptor 1A (MTNR1A) gene encodes melatonin hormone which regulates the function of seasonal reproductive activity in sheep. The aim of this study was to make the genetic characterization and identify the variant alleles of MTNR1A gene in Kıvırcık breed. Blood samples of 110 Kıvırcık sheep were collected from five different farms located in Kırklareli and Istanbul. DNA extraction was performed from blood samples. Exon 2, the polymorphic region of Melatonin receptor 1A gene, was amplified and PCR products were genotyped by using *Mnl*I and *Rsa*I enzymes. The observed alleles and genotypes for *Mnl*I enzyme were; M (0.891), m (0.109) and MM (0.782), Mm (0.218) respectively. Kıvırcık sheep was null from mm genotype. Also identified alleles were C (0.682), T (0.318) and genotypes were CC (0.582), CT (0.200), TT (0.218) for *Rsa*I enzyme. The most frequent genotypes were MM (78%) and CC (58%) in Kıvırcık ewes. Since MM and CC genotypes were known with their positive effect on out of season reproductive activities, Kıvırcık ewes with these genotypes might suggested to be used in out of season lambing when demanded.

Keywords: *Kıvırcık, Sheep, Melatonin, Receptor, Genetic variation*

Kıvırcık Irkı Koyunlarda Melatonin Reseptör 1A (MTNR1A) Geninin *Mnl*I ve *Rsa*I Restriksiyon Enzimleri ile Genetik Varyasyonunun Belirlenmesi

Özet

Melatonin reseptör 1A (MTNR1A) geni koyunlarda mevsime bağlı üreme fonksiyonlarını düzenleyen bir hormon olan melatonini kodlamaktadır. Bu çalışmanın amacı Kıvırcık ırkı koyunda MTNR1A geninin genetik varyasyonunun ve allel çeşitliliğini belirlemektir. Kırklareli ve İstanbul illerindeki 5 farklı çiftlikten olmak üzere toplam 110 adet Kıvırcık ırkı koyununa ait kan örnekleri toplanarak DNA izolasyonu yapılmıştır. MTNR1A geninde polimorfik olan ekzon 2 bölgesi PZR ile yükseltgenmiş olup *Mnl*I ve *Rsa*I enzimleri kullanılarak allel ve genotip tespitleri yapılmıştır. Gözlenen alleller ve genotipler *Mnl*I için M (0.891) ve m (0.109) alleleri ile MM (0.782) ve Mm (0.218) genotipleri, *Rsa*I için C (0.682) ve T (0.318) alleleri ile CC (0.582), CT (0.200) ve TT (0.218) genotipleri olmuştur. Kıvırcık koyunlarında mm genotipi gözlenmemiş olup, en yüksek oranda gözlenen genotipler, koyunlarda mevsim dışı üreme faaliyetlerini pozitif olarak etkilediği bilinen MM (%78) ve CC (%58) olarak tespit edilmiştir. Büyük bir çoğunluğu MM ve CC genotiplerine sahip olan Kıvırcık ırkı koyunların mevsim dışı kuzulatmada yaygın olarak kullanılması yetiştiricilere önerilebilir.

Anahtar sözcükler: *Kıvırcık, Koyun, Melatonin, Reseptör, Genetik varyasyon*

INTRODUCTION

Kıvırcık is an important red meat source in Turkey and a native sheep breed known with its good meat quality [1]. Kıvırcık breed is raised in Thrace region, southern and eastern provinces in Marmara region and in some Aegean provinces of Turkey [2]. Age, body weight and photoperiod are the most significant factors that effect of puberty in ewes [3]. Small ruminant reproductive activity increases during decreasing photoperiods. Related process

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depends on melatonin hormone which plays an essential role in controlling seasonal reproduction by photoperiodic information. Melatonin is secreted from pineal gland in proportion to the period of darkness [4] and its production is controlled by day/night alteration. The peak level of melatonin secretion is positively correlated with the length of the dark hours [5]. Short photoperiods influence positively on melatonin level, enhance secretion of gonadotropic releasing hormone (GnRH) and correspondingly luteinizing hormone (LH). Melatonin is link with two specific high affinity receptors, melatonin receptor 1A and 1B that are located in hypophyseal pars tuberalis [6]. However Melatonin receptor 1A (MTNR1A) is the main receptor mediating melatonin action to modulate GnRH pulsatile activity [6], therefor it is involved in the regulation of reproductive activity [7]. Futhermore melatonin has a protective effect against aluminum accumulation [8]. Exogenous applications of this hormone during the summer encourage the onset of puberty [3]. To activate out of season reproduction hormonal treatments are used in sheep breeding. Variations in MTNR1A gene have significant effect on melatonin binding sites to pars tuberalis of hypothalamus [7]. Therefor these variations also effect the respond to melatonin treatment [3]. However demands for hormone free products directs to a search for alternative methods [9]. Knowledge of genes and genetic markers that influence on out of season lambing would allow more efficient and intensive selection programs for reproduction [10]. The use of genetic markers for reproduction, especially photoperiod sensitivity, is a promising method in sheep [9]. The variation among animals can be determined at the DNA level with various molecular techniques. Utilizing this information in selection program is a growing interest, especially for the traits that are difficult to improve with conventional methods [10].

MTNR1A gene located on chromosome 26 of sheep genome. Its genomic structure consist of two exons divided by a large intron [11]. Exon 1 encodes the first transmembrane domain and the first intracellular loop and exon 2 codifies for the remaining part of the receptor. Various studies in different sheep breeds were reported two single nucleotide polymorphisms (SNPs) at position 606 (C>T) and 612 (G>A) in exon 2 region which are also identified as silent mutations. Related SNPs can be identified by *Rsa*I and *Mnl*I enzymes respectively. Polymorphic regions in both *Rsa*I and *Mnl*I recognition sites were also reported about their association with the seasonal ovulation and reproductive activity in ewes [7,12]. Related polymorphic sites were studied various sheep breeds such as Columbia [13], Merino d'arles [7,14], small tailed Han sheep [15], Ile de France sheep [16], Prolific Olkuska, Polish Mountain sheep, Suffolk, Merino-Romanov sheep [17], Karakul [18], Awasi [19-21], Mouflon wild sheep [12], Sarda [3,22], crossbred of 50% Dorset, 25% Rambouillet, and 25% Finnsheep ewes [23], Akkaraman, Chios [20,21], Rasa Aragonesa [9], Local Starozagorska, Local Karnobatska, Breznishka and Sofiiska [24], Dağlıç, Gökçeada, Karacabey Merino, Karayaka, Kıvırcık [21], Zandi sheep [25], Dorset [10], Zel, Naeini [26], Indian Chokla [27], Marwari and Magna [28]. Trechel *et al.*[29] provide evidence of a modification in the melatonin signaling pathway by comparing two polymorphic variants which makes MTNR1A gene a potential DNA marker for out of season breeding.

The aim of this study was identify the genetic variation of MTNR1A gene in Kıvırcık which is a noted and desirable native sheep breed with its meat quality in Turkey.

MATERIAL and METHODS

This study was approved by Ethic Committee of the Istanbul University Veterinary Faculty (Approval number: 2010/184).

Animals

Animal samples of this study come from five purebred Kıvırcık flocks. The four of the flocks were located in Kırklareli province. Twenty ewes were selected randomly from each flock. The fifth flock was belong to Research and Education Farm of Istanbul University Faculty of Veterinary Medicine in which thirty ewes were selected randomly. Blood samples of Kıvırcık (n=110) ewes were collected from Vena jugullaris into steril vacuumed EDTA tubes from Kırklareli (n=80) and Istanbul (n=30) provinces.

Genotyping

DNA isolation was performed from blood samples by using DNA Pure Kit (Geneaid Biotech™, Taiwan). The region of the MTNR1A gene in sheep was amplified by using PCR with the forward 5'TGTGTTTGTGGTGAGCCTGG3' and reverse 5'ATGGAGAGGGTTTGCGTTTA3'primers [30], which captured a fragment that has a length of 824bp from exon 2 (HQ658144.1). PCR amplification was performed in total volume of 25µl consist from 5 µl Taq PCR Master Mix (200 U/ml Ultra-Pure Taq DNA Polymerase, 1.25 mM dNTPs, 10 mM $MgCl_2$; Geneaid Biotech™, Taiwan), 0.5 µl 20 pmol each primer, 3 µl genomic DNA (100 ng) and 16 µl dH_2O (AccuGENE™, Lonza, Belgium). PCR was performed with the following conditions; denaturing at 94°C in 5 min, 34 cycles of 94°C in 1 min, 62°C in 1 min, 72°C in 1 min and final extension at 72°C in 10 min (Bio-Rad T100, Bio-Rad Laboratories Inc., CA, USA).

PCR products were digested with both *Mnl*I and *Rsa*I enzymes (MBI Fermentas). Incubation was performed at 37°C by overnight for both *Mnl*I and *Rsa*I cleavage. After performing the digestions, band patterns were visualized on 4% agarose gel stained with ethidium bromide.

The ovine MTNR1A nucleotide data HQ658145.1 and HQ658147.1 which include C606T and G612A SNPs respectively, was aligned with HQ658144.1 nucleotide which includes wild type alleles (C and M). Alignment was performed with nucleotide BLAST tool (http://blast.ncbi.

nlm.nih.gov/Blast.cgi) in order to compare and confirm restriction sites among related nucleotides.

Statistical Analysis

Allele and genotype frequencies, observed and expected heterozygosity values and chi square (X^2) for Hardy-Wienberg equilibrium (HWE) was estimated with PopGene32 program [31].

RESULTS

Two alleles were identified for *Mnl*I (M and m) and *Rsa*I (C and T) digestions of ovine MTNR1A locus. Observed genotypes with *Mnl*I enzyme restriction were MM (78%) and Mm (22%), no mm genotype was determined. With *Rsa*I enzyme restriction observed genotypes were CC (58%), CT (20%) and TT (22%). MTNR1A locus had seven restriction sites for *Mnl*I and four for *Rsa*I enzyme. Band pattern sizes for M allele were; 220bp, 218bp, 135bp, 83bp, 82bp, 36bp, 28bp, 22bp and for C alleles were 411bp, 267bp, 70bp, 53bp, 23bp. However existence of G>A transition in *Mnl*I recognition site (GAGG-AAGG) was result to divergence in the band patterns (303bp, 218bp, 135bp, 82bp, 36bp, 28bp, 22bp) thus it causes to m allele. Also existence of C>T transition in *Rsa*I recognition site (GTAC-GTAT) results to T allele (411, 290, 70, 53 bp) *(Fig. 1)*.

Band patterns for *Mnl*I (M and m) and *Rsa*I (C and T) were visualized on 4% agarose gel *(Fig. 2 A,B)*. However all DNA fragments resulted after *Mnl*I and *Rsa*I digestions could not been observed on agarose gel. Observable DNA fragments for M allele were 303bp, 218bp, 135bp and for m allele were 220bp, 218bp, 135bp. Also visualized band patterns for C allele were 411bp, 267bp and for T allele were 411bp, 290bp.

Allele and genotype frequencies, observed and expected heterozygosity and chi square (X^2) values resulted from both *Mnl*I and *Rsa*I enzyme digestions of ovine MTNR1A locus were given in *Table 1*. Kıvırcık breed ewes were found in HWE at *Mnl*I locus. However deviation from HWE was found significant at *Rsa*I locus (P<0.01).

DISCUSSION

Through conventional breeding program, genetic improvement in out of season fertility trait is challenging. For reproductive traits using genetic markers in selection programs will be useful since the trait has low heritability, furthermore it is expressed late in life; observed in one gender; exhibited only in some environmental conditions or management systems [10,23]. The unproductive time period that passes between birth and first lambing is one of the biggest problems in management of sheep breeding [3]. Sezenler *et al.*[32] performed a study to determine some reproductive characteristics of Kıvırcık, Chios and Imroz indigenous sheep breeds of Turkey. Mating season duration

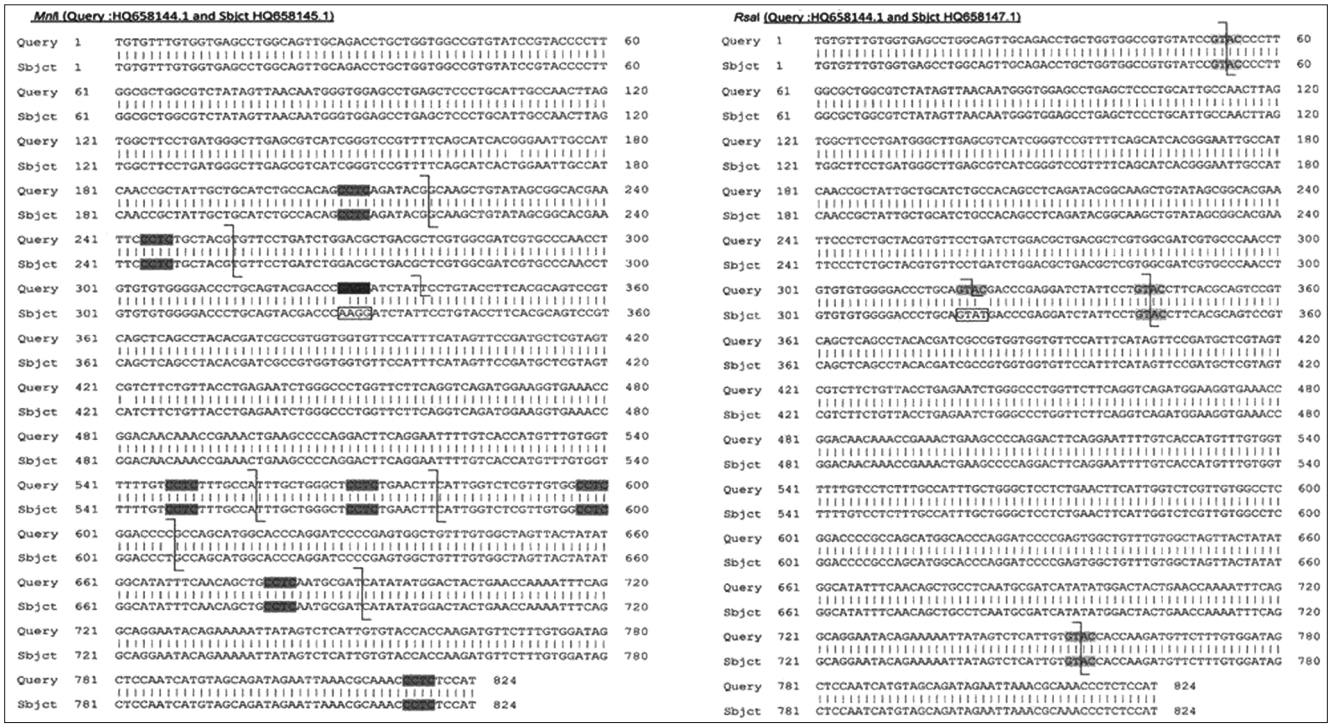

Fig 1. Restriction sites of *Mnl*I (Query; HQ658144.1; M allele and Sbjct; HQ658145.1; m allele) and *Rsa*I (Query; HQ658144.1; C allele and Sbjct; HQ658147.1; T allele) enzymes within ovine MTNR1A gene

Şekil 1. Koyun MTNR1A geninde *Mnl*I (Query; HQ658144.1; M alleli ve Sbjct; HQ658145.1; m alleli) ve *Rsa*I (Query; HQ658144.1; C alleli ve Sbjct; HQ658147.1; T alleli) enzimleri için kesim bölgeleri

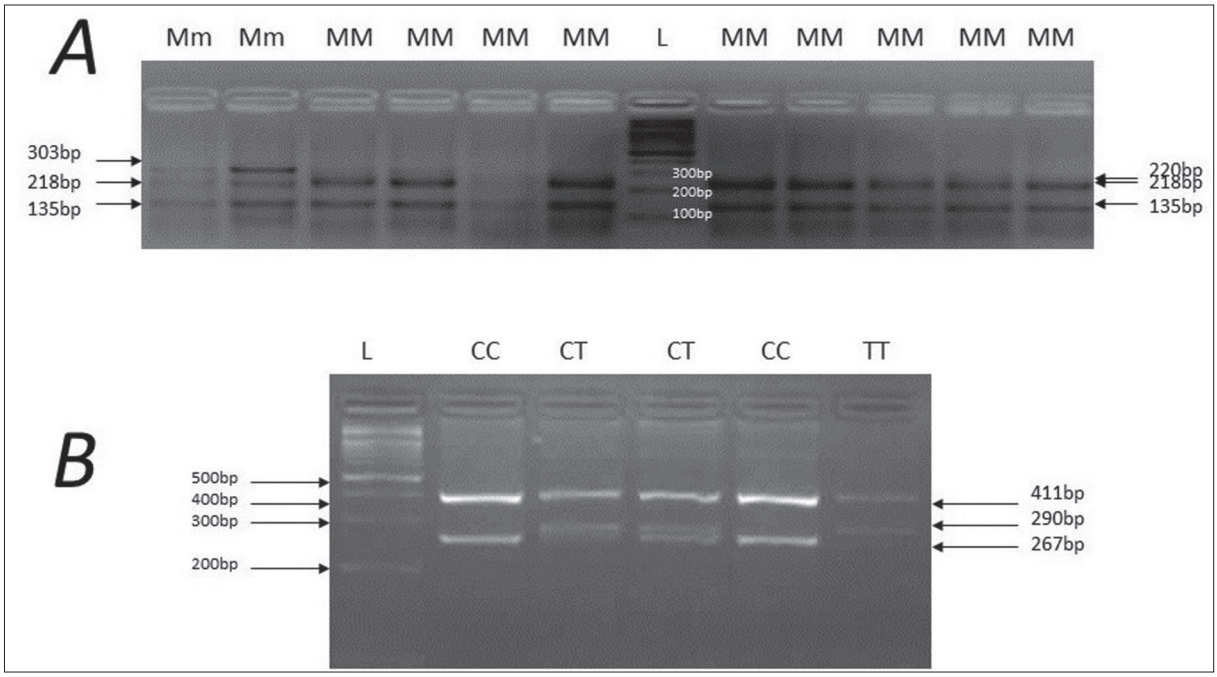

Fig 2. The observed genotypes in Kıvırcık sheep after *Mnl*I (A. Mm; 303bp, 218bp, 135bp in lanes 1, 2 and MM; 218bp, 135 bp in lanes 3, 4, 5, 6, 8, 9, 10, 11, 12) and *Rsa*I (B. CC; 411bp, 267bp in lanes 2, 5, CT; 411bp, 290bp, 267bp in lanes 3, 4, TT; 411bp, 290bp in lane 6) enzyme digestions of MTNR1A gene on 4% agarose gel (L= 100bp ladder)

Şekil 2. Kıvırcık koyununda %4'lük agaroz jelde MTNR1A geninin *Mnl*I (A. 1, 2 nolu kuyucuklarda Mm: 303bç, 218bç, 135bç; 3, 4, 5, 6, 8, 9, 10, 11, 12 nolu kuyucuklarda MM: 218bç, 135 bç) ve *Rsa*I (B. 2, 5 nolu kuyucuklarda CC: 411bç, 267bç; 3, 4 nolu kuyucuklarda CT: 411bç, 290bç, 267bç; 6 nolu kuyucukta TT: 411bç, 290bç) enzim kesimlerini takiben gözlenen genotipler

Table 1. *Allele and genotype frequencies, observed and expected heterozygosity, chi square (X^2) values of MTNR1A gene in Kıvırcık sheep breed for both MnlI and RsaI enzymes*

Tablo 1. *Kıvırcık koyununda MnlI ve RsaI enzimleri için MTNR1A genine ait allel ve genotip frekansları, gözlenen ve beklenen heterozigotluk ve Ki kare (X^2) değerleri*

Enzyme	Alleles	Allele Frequency	Genotypes	Genotype Frequency	Heterozygosity		X^2
					Ho	He	
*Mnl*I	M	0.891	MM	0.782	0.218	0.195	1.57^{ns}
	m	0.109	Mm	0.218			
			mm	0.000			
*Rsa*I	C	0.682	CC	0.582	0.200	0.436	32.9^{*}
	T	0.318	CT	0.200			
			TT	0.218			

ns: *nonsignificant, *P<0.01*

(225.03, 222.58 and 167.67 days resp.) and anestrus period (139.97, 142.59 and 197.33 days resp.) were reported for Kıvırcık, Chios and Imroz respectively. Kıvırcık had the longest mating duration and the shortest anestrus period among three native breeds. Duration of reproductive season of Kıvırcık was reported approximately up to 8 months. When estrus distribution analysed for months, Sezenler *et al.*[32] found that Kıvırcık show estrus mostly in October. Distribution of reproductive season among the months of a year would be the early summer (June) to winter (January) for Kıvırcık breed.

Pelletier *et al.*[7] reported that M allele has an effect of ovulatory cycling during out of season (in spring) in Merinos d'Arles ewes. Furthermore the homozygous genotype for the absence of a polymorphic *Mnl*I sites (mm) at position 612 of exon 2 was found associated with seasonal anovulatory activity in Merino d'Arles [7]. Moreover M allele was reported with its positive influence on autumn lambing success in Columbia ewes [13]. The mm genotype was more frequent (50%) in wild Mouflon [12] ewes and its reproductive activity was reported as seasonal. Martinez-Royo *et al.*[9] found significant differences in estrous cyclicity among months and genotypes for SNP C606T. The most significant differences between TT and CC genotypes in the percentage of estrous cyclic ewes were reached in May (27.8%, P<0.1), June (29.4%, P<0.05) and July (28.9%, P<0.05). Therefore T allele was reported associated with

a greater percentage of nonseasonal estrous cyclic ewes of Rasa Aragonesa breed. During the anestrus season Rasa Aragonesa ewes with TT genotype showed more estrus activity. C allel is related with a greater percentage of seasonal estrus cyclic ewes in Rasa Aragonesa breed [9]. Sarda sheep that carry one of MM and CC genotypes showed estrus in spring. As a consequence they lambed in autumn (September-December), therefore reproductive activity of Sarda ewes was reported as non-seasonal. Lambs that were born in autumn can be reach puberty by the early summer of the following year. However ewes that were born in spring do not reach puberty until the next autumn, later than those which were born in autumn. Lambs which were born in autumn are being chosen by breeders as replacement ewe lambs and these ewes were probably MM and CC genotype [22]. Small Tail Han [15] and Awassi [19] ewes which were identified to have MM, CC genotypes were reported that they show non-seasonal estrus and ewes with mm, TT genotypes were showed seasonal estrus. However Teyssier *et al.*[14] reported that *Mnl*I site of the MTNR1A gene cannot be used alone as a genetic selection marker for spring (out-of-season) breeding in Merino d'Arles ewes. Furthermore M allele was not found to be related with seasonal reproduction trait in Rasa Aragonesa sheep [9]. Kaczor *et al.*[17] reported that prolific Olkuska ewes with different genotypes did not show significantly different average melatonin concentration during the dark phase (December); an association had not been found between MTNR1A polymorphism and blood melatonin concentration. The effect of related polymorphisms might be determined by the breed and /or environmental conditions.

In present study we found that MTNR1A gene had two alleles; M and m, and two genotypes; MM and Mm for *Mnl*I enzyme; C and T alleles, CC, CT and TT genotypes for *Rsa*I restriction site in Kıvırcık breed. We observed that M allele (89%) was much more frequent than m allele (11%) in Kıvırcık breed similar with Magna (95%), Chokla (92%), Zandhi (92%) [25], Marwari (90%) [27], Chios (90%), Awasi (84%), White Karaman (80%) [20], Hu (80%), Karakul (79%) [18], Sarda (78%) [22], Small Tail Han (75%) [15] and Naeini (71%) [28] breeds. However Elmacı *et al.*[21] reported that M allele was less frequent (26%) than m allele (74%) in Kıvırcık sheep. Genotype frequencies of MM and Mm genotypes (78%; 22%) in Kıvırcık breed were resemble with the frequencies reported in Chokla (77%; 21%), Marwari (80%; 19%) [27], Zandhi (82%; 18%) [25], Chios (80%; 20%) and Karakul (70%; 30%) [18] sheep breeds. Similar to our results mm genotype was not observed in Zandhi [25], Awasi, White Karaman, Chios [20] and Karakul [18] breeds. Observed heterozygosity for Mm genotype (0.22) in Kıvırcık breed was found similar with Naeini (0.22) and Zel (0.25) breeds [28]. Observed heterozygosity that Elmaci *et al*.[21] reported for Mm genotype in Kıvırcık breed was higher than our results (0.31). Similar to our findings, C allele (68%) was more frequent than T allele (32%) in Magna (95%; 5%), Chokla (87%; 13%), Marwari (89%, 11%) [27], Gokceada (79%; 21%), Awasi (73%; 26%) [20], Local Karnobatska (73%; 27%) [24] and Small Tail Han (71%, 29%) [15] sheep breeds. Elmaci *et al*.[21] found frequency of C allele (53%) closer to T allele (47%). After RsaI digestion genotypes frequencies from the most frequent to less were; CC (58%), CT (20%) and TT (22%) respectively, which were found similar with Sarda (53%; 26%; 21%) [22] sheep breed. In current study observed heterozygosity (0.2) for CT genotype was found similar with Karayaka (0.24) [21] and Local Karnobatska (0.23) [24] breeds. However Elmaci *et al*.[21] reported observed heterozygosity in Kıvırcık breed for CT genotype was much higher than our result (0.54). We found that Kıvırcık sheep was not in HWE for *Rsa*I site of MTNR1A gene, similarly as reported in Zel and Kıvırcık breeds [21,28]. Differences between findings of Elmaci *et al*.[21] in MTNR1A variation in Kıvırcık breed (n=39) and ours may result from sampling size and inbreeding levels of sampled animals.

In conclusion the current study showed that MTNR1A gene varies for both *Mnl*I and *Rsa*I enzymes in Kıvırcık ewes. Since mm genotype was known to be related with seasonal estrus and anovulatory activity in ewes, it can be assumed that selection process may occurred negatively for this genotype in Kıvırcık breed. The desired alleles for out of season cycling; MM (78%) and CC (58%) were found more frequent than Mm (22%), CT (20%) and TT (22%) genotypes. Kıvırcık ewes, that shows MM and CC genotype, can be suggested to use for autumn lambing when demanded. Further studies are needed to clarify the characterization and genotype variation of MTNR1A gene and its impact on out of season reproductive activities. Our next aim is to investigate the association of non-seasonal (autumn) lambing with MM and CC genotypes in Kıvırcık ewes that may help to develop new suggestions in sheep breeding.

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Research Article

Effects of Milk Thistle *(Silybum marianum)* Seed Supplementation to High-Calorie Basal Diets of Quails on Egg Production, Egg Quality Traits, Hatchability and Oxidative Stress Parameters

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Abstract

The purpose of this study was to examine the changes to occur in egg production, egg quality traits, hatchability, and oxidative stress parameters of laying quails fed with high calorie diets and effect of milk thistle (*Silybum marianum*) seed on these changes. A total of 75 45-day-old quails including 60 females and 15 males were used in the study. The quails were divided into 5 groups with three repetitions. 4 females and 1 male were used in each repetition. The groups of the study were arranged as following; control group (C) consuming corn-soybean based basal diet, oil group (SFO) in which 5% sun flower oil was added into basal diet, oil + milk thistle group (SFO+MT) in which 5% sun flower oil + 1% milk thistle seed was added into basal diet, syrup group (CS) in which 10% corn syrup was added into basal diet, and syrup + milk thistle group (CS+MT) in which 10% corn syrup + 1% milk thistle was added into basal diet. Total egg production was found to be significantly higher in SFO and SFO+MT groups of present study ($P<0.05$). Feed intake, total egg weight and feed conversion were found similar among the groups ($P>0.05$). The differences among the groups in albumen rate, yolk rate, dried shell rate and shape index were not statistically significant ($P>0.05$). The best yolk colour was obtained in SFO+MT and CS groups ($P<0.01$). There were no statistically differences among the groups in hatchability, hatchability of fertile and embrionic mortality ($P>0.05$), but the difference in examined fertility were statistically significant ($P<0.05$). Malonyl dialdehyde (MDA) values of blood, liver and heart tissues were similar among the groups of study ($P>0.05$). MDA level of kidney significantly increased in CS group, however addition of milk thistle seed into diet was reduced MDA level of kidney in CS+MT group ($P<0.01$). High calorie diets caused changes in antioxidant system in blood and tissues. Consequently, high-calorie basal diets especially addition of SFO improved only egg production and fertility rate. However feeding quails with corn syrup had significantly higher lipid peroxidation in kidney. Supplementation of milk thistle seed into CS group may protect kidney against free radical damage.

Keywords: *Quail, Egg yield, Hatchability, Milk thistle, High calorie diets, Oxidative stress*

Yüksek Kalorili Bıldırcın Karma Yemlerine Deve Dikeni *(Silybum marianum)* Tohumu İlavesinin Yumurta Verimi, Yumurta Kalite Özellikleri, Kuluçka Randımanı ve Oksidatif Stres Parametreleri Üzerine Etkisi

Özet

Bu araştırmada yüksek kalorili yemlerle beslenen yumurtacı bıldırcınlarda yumurta verimi, yumurta kalite özellikleri, kuluçka performansı ve oksidatif stres parametrelerinde meydana gelebilecek değişimler ve bu değişimler üzerine deve dikeni (*Silybum marianum*) tohumunun etkisinin incelenmesi amaçlanmıştır. Araştırmada, 45 günlük yaşta 60 adet dişi, 15 adet erkek olmak üzere toplam 75 adet bıldırcın kullanılmıştır. Araştırma 5 grup ve her grupta 3 tekerrürden oluşturulmuştur. Her bir tekerrürde 4 dişi ve 1 erkek bıldırcın kullanılmıştır. Deneme grupları; mısır-soya esasına dayalı temel karma yemi tüketen kontrol grubu (C), temel diyete % 5 ay çiçeği yağı ilave edilen yağ grubu (SFO), temel diyete %5 ay çiçeği yağı + %1 deve dikeni ilave edilen yağ + deve dikeni grubu (SFO+MT), temel diyete %10 mısır şurubu ilave edilen şurup grubu (CS) ve temel diyete %10 mısır şurubu + %1 deve dikeni ilave edilen şurup + deve dikeni grubu (CS+MT) şeklinde oluşturulmuştur. Çalışmada, toplam yumurta verimi SFO ve SFO+MT gruplarında önemli ($P<0.05$) derecede yüksek bulunmuştur. Yem tüketimi, toplam yumurta ağırlığı ve yemden yararlanma yönünden gruplar birbirine benzer bulunmuştur ($P>0.05$). Yumurta akı ve sarı oranı, kurutulmuş kabuk oranı ve şekil indeksi yönünden gruplar arasında fark bulunamamıştır ($P>0.05$). En iyi sarı rengi SFO+MT ve CS gruplarında belirlenmiştir ($P<0.01$). Kuluçka randımanı, çıkış gücü ve embriyonik ölüm açısından gruplar arasında fark saptanmazken ($P>0.05$), döllülük açısından gruplar arasında önemli ($P<0.05$) fark tespit edilmiştir. Kan, karaciğer ve kalp dokularının Malonil dialdehid (MDA) değerleri benzer bulunmuştur ($P>0.05$). Böbrek MDA değeri CS grubunda önemli olarak artarken, diyete deve dikeni tohumu ilavesi CS+ MT grubunda böbreğin MDA değerini azaltmıştır ($P<0.01$). Yüksek kalorili diyetler kan ve dokularda antioksidan sistemde değişikliğe sebep olmuştur. Sonuç olarak, yüksek kalorili diyetler özellikle SFO ilavesi sadece yumurta üretimini ve döllülük oranını iyileştirmiştir. Diğer taraftan mısır şurubu tüketimi böbrekte lipit peroksidasyonunu artırmıştır. CS gruplarında deve dikeni tohumu ilavesi böbreği serbest radikal hasarına karşı koruyabilir.

Anahtar sözcükler: *Bıldırcın, Yumurta verimi, Kuluçka, Deve dikeni, Yüksek kalorili diet, Oksidatif stres*

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INTRODUCTION

Poultry products constitute a significant source in order to meet protein deficit of increasing population in the world. Quail takes an important place in poultry farming because it has high reproduction rate and fertility, short reproduction period, highfarming per unit area and high feed conversion ratio, easy farming, products especially rich in protein and minerals, and also is fondly consumedby people [1].

Natural and reliable alternative sources have attracted attention of researchers after growth factors like antibiotics, hormones etc. and productive additive substances were prohibited in the animal feeding area. In this context, alternative supplements such as organic acids, probiotics, prebiotics, bioenzymes, aromatic plants, and essential oils have acquired currency. Aromatic plants and essential oils are used in alternative medicine for many centuries and made a research subject by scientists in all fields because they are natural and contain several active components. Aromatic plants and essential oils have been conveniently used as alternatives to these synthetic growth factors in farm animal diet [2,3].

Milk thistle *(Silybum marianum* L. Gaertn.) is a plant belonging to Asteraceae family. Its seeds have been used for protecting liver against diseases of liver and gallbladder, and intoxication; and also for treatment of cases such as mushroom poisoning, snake bite, bug bites since 2000. Extracts acquired from milk thistle seeds contain plenty of silymarin. Silymarin is chemically composed of isomer flavanolignans named as silybin (silybinin), isosilybin, silychristin, silydianin, and dehydrosilybinin [4]. Basic component of silymarin which is thought to be responsible for its biological activity is silybin; however, it is also thought that other flavanolignans found in silymarin can have a role in this biological activity [5].

In the studies conductedup to the present, positive results have been obtained from feed supplements with various antioxidant and immune system promoting characteristics added into diets. Some of these supplements were banned because they are synthetic and there are also difficulties in supplying some of them; therefore researchers direct to alternative supplements [6]. In this regard; aromatic plants and essential oils have been used by humans for centuries and become a research subject for scientists in every field because they are natural and contain numerous active components [2,3].

The aim of this study was to examine the changes to occur in egg production, egg quality traits, hatchability, and oxidative stress parameters of laying quails fed with high calorie diets and effect of milk thistle seed on these changes.

MATERIAL and METHODS

Management

The study was conducted atthe Poultry Unit, Faculty of Veterinary Science, Fırat University. A total of 75 45-day-old quails including 60 females and 15 males were used in the study. The quails were divided into 5 groups with three repetitions. 4 females and 1 male were used in each repetitions.The groups of the study were arranged as following; control group (C) consuming corn-soybean based basal diet, oil group (SFO) in which 5% sun flower oil was added into basal diet, oil + milk thistle group (SFO+MT) in which 5% sun flower oil + 1% milk thistle seed was added into basal diet, syrup group (CS) in which 10% corn syrup was added into basal diet, and syrup + milk thistle group (CS+MT) in which 10% corn syrup + 1% milk thistle was added into basal diet. The experiment was conducted under the protocol which was approved by Firat University Animal Use Local Ethical Committee (No: 2015/101). All groups were given diet and water as *ad libitum*. Rations used in the study were arranged according to standards of National Research Council [7] and given in *Table 1*. The milk thistle (*Silybum marianum*) was provided from a commercial company (Naturoil Food and Chemical Industry Company Limited). The chemical composition of milk thistle was given in *Table 2*. Quails were kept in special laying cages and in a room with temperature between 15 and 25°C. A lighting program including daylight of 16 hours and dark program of 8 h was applied during the laying period. Records of egg production in quails were taken as beginning from the period of 5% egg production quails-days. For this purpose, eggs were collected in the same hour of every day by counting them, daily egg production (%) was determined by dividing number of the obtained eggs into number of quails in that day. Diets were weighed and given daily, remaining diet in feeders were weighed weekly and daily feed consumption was determined with the difference between them. Feed conversion ratio was calculated by using values of egg production, egg weigh, and feed consumption. As from the 3rd week of the study, a total of 250 eggs including 50 eggs from each group (including sub-groups) were cracked in order to determine internal quality traits after external quality traits were determined. Fertility rate, hatchability, and hatchability of fertile eggs, were determined by incubating eggs collected for 3 weeks as from the 3rd week of the study. Blood, liver, kidney, and heart samples were taken during 6 animals from each experimental group were slaughtered by using decapitation method at the end of the study.

Chemical Analysis

After blood samples were taken into tubes containing heparin and whole blood was separated for determination of glutathione (GSH) and glutathione peroxidase (GSH-Px), remaining blood samples were centrifuged at 3.000 rpm

Table 1. *Ingredients and nutrient composition of experimental diets (g/kg)*
Tablo 1. *Araştırma diyetinin bileşimi ve besin madde içeriği (g/kg)*

Experimental Groups	C	SFO	SFO+MT	CS	CN+MT
Ingredients, g/kg					
Corn	530.0	444.5	440.4	453.1	450.0
Wheat bran	85.0	112.0	105.0	29.0	21.9
Soybean meal (48% CP)	192.0	188.4	190.0	224.8	225.0
Corn gluten (43% CP)	90.0	113.0	112.5	90.0	90.0
Vegetable oil	25.0	65.	65.0	25.0	25.0
Milk thistle seed	-	-	10.0	-	10.0
Corn syrup	-	-	-	100.0	100.0
DL-Methionine	1.7	1.7	1.7	1.9	1.8
Dicalcium phosphate	18.0	17.1	17.1	18.0	18.1
Ground limestone	51.7	51.7	51.7	51.6	51.6
$NaHCO_3$	1.0	1.0	1.0	1.0	1.0
Salt	2.6	2.6	2.6	2.6	2.6
Vitamin-Mineral Mix*	3.0	3.0	3.0	3.0	3.0
Nutritional Composition, g/kg					
Dry matter	904.0	908.0	908.0	906.0	906.0
Crude protein	170.0	170.0	170.0	170.0	170.0
Crude fiber	525.0	525.0	525.0	525.0	525.0
Ether extract	630.0	630.0	630.0	630.0	630.0
Calcium	25.1	25.0	25.0	25.0	25.1
Available phosphorus	4.1	4.0	4.1	4.0	4.1
Sodium	1.8	1.8	1.8	1.8	1.8
Meth+Sist	7.6	7.6	7.6	7.6	7.6
Lysine	8.1	8.1	8.2	8.6	8.6
Threonine	6.4	6.4	6.4	6.5	6.5
Tryptophan	2.0	2.0	2.0	2.1	2.1
ME, kcal/kg**	2750	2900	2900	2900	2900

** Provided per kg of diet: retinol, 2.64 mg; cholecalciferol, 0.04 mg; dl-a-tocopherol-acetate, 11 mg; riboflavin, 9.0 mg; pantothenic acid, 11.0 mg; vitamin B12, 0.013 mg; niacin, 26 mg; choline, 900 mg; vitamin K, 1.5 mg; folic acid, 1.5 mg; biotin, 0.25 mg; iron, 30 mg; zinc, 40 mg; manganese, 60 mg; copper, 8 mg; selenium, 0.2 mg; ** Calculated, ME (kcal/kg) = 53+38 B used formula. B = (% Crude protein) + (2.25) (%Ether extract) + (1.1)(% Starch) + (% Sugar); Group control: C, Group sun flower oil: SFO, Group sun flower oil + milk thistle: SFO+MT, Group corn syrup: CS, Group corn syrup + milk thistle: CN+MT*

Table 2. *The chemical composition of milk thistle seed (%)*
Tablo 2. *Deve dikeni tohumunun kimyasal bileşimi (%)*

Chemical Composition	(%)
Dry Matter	94.29
Moisture	5.71
Crude protein	14.96
Crude fiber	31.3
Ether extract	25.48
Ash	5.63
Carbohydrates	16.92

for ten minutes and their plasma was used for Malonyl dialdehyde (MDA) determination. Remaining erythrocyte packages were washed with 0.9% NaCl 3 times and used for catalase (CAT) assay. MDA and GSH levels and CAT and GSH-Px activities were determined in liver, kidney, and heart tissues. While MDA level was determined based on the method of Placer et al.[8], GSH level was determined based on method of Chavan et al.[9]. While the method of Matkovics et al.[10] was used for measurement of GSH-Px activity, Aebi [11] method was used to measure CAT activity.

Statistical Analysis

After test of normality, all data including performance, egg quality, hatchability and oxidative stress parameters were subjected to one-way analysis of variance (anova). Significant differences were then subjected to Tukey HSD test. All analyses were performed by using Statistical Packages for the Social Sciences for Windows [12]. The results were considered as significant when P values were less than 0.05.

RESULTS

The hen-day egg production, feed intake, total egg weight, feed conversion and egg quality parameters of treatment groups are shown in *Table 3*. Total egg production was found to be significantly higher in SFO+MT and SFO groups of present study (P<0.05). There was not any significant difference in total egg production of the other control, CS and CS+ MT groups of the study. Feed intake, total egg weight and feed conversion were found similar among the groups (P>0.05). Some egg quality parameters in laying quails fedhigh-calorie diet are also shown in *Table 3*. The differences among the groups in albumen rate, yolk rate, dried shell rate and shape index were not statistically significant (P>0.05). The best yolk colour was obtained in SFO+MT and CS groups (P<0.01). C group had lower value in this parameter and SFO and CS+MT groups were similar to other groups (P>0.05).

The effect of dietary milk thistle seed on some hatchability traits in laying quails fedhigh-calorie diet are shown in *Table 4*. There were no statistically differences among the groups in hatchability, hatchabilitiy of fertile and embrionic mortality (P>0.05), but the difference in examined fertility were statistically significant (P<0.05). When fertility rate was examined, it was found that higher fertility rate was obtained in CS and CS+MT group. Addition of silybum marianum in to diet did not affect neither fertility rate nor other hatchability traits.

The effect of dietary milk thistle seed on oxidative stress parameters of different tissues in laying quails fed high-calorie diet are shown in *Table 5*. According to *Table 5*, MDA values of blood, liver and heart tissues were similar among the groups of study (P>0.05). MDA level of kidney significantly increased in CS group, however addition of milk thistle seed into diet was reduced MDA level of kidney

Table 3. *Effects on some performance and egg quality parameters of milk thistle (Silybum marianum) seed supplementation to high-calorie basal diets of quails*
Tablo 3. *Yüksek kalorili bıldırcın karma yemlerine deve dikeni tohumu (Silybum marianum) ilavesinin bazı performans ve yumurta kalite parametrelerine etkisi*

Performance Parameters	C	SFO	SFO+MT	CS	CS+MT	P-Significance
Hen-Day Egg Production (egg production/100 female birds/day)						
0-14	51.19±1.19	50.59±5.18	63.09±10.73	52.97±9.90	46.42±8.24	NS
15-28	55.95±6.76	81.54±8.05	77.38±10.40	70.83±9.83	64.88±12.45	NS
29-42	69.64±6.76	91.07±1.03	92.87±4.49	83.33±6.29	74.40±11.86	NS
43-56	78.57±9.16	89.28±1.03	89.23±2.72	85.11±4.16	85.71±7.14	NS
Total	63.83±3.58^{b}	78.12±2.60^{a}	80.65±5.51^{a}	73.06±4.39^{ab}	67.85±1.03^{b}	*
Feed Intake(g/bird/day)						
0-14	25.34±0.72	27.44±1.77	28.00±1.39	28.64±2.30	27.35±0.47	NS
15-28	29.93±1.40	30.83±0.65	32.00±0.38	32.04±0.45	30.85±0.28	NS
29-42	29.78±1.80	32.71±1.59	31.29±1.15	31.77±1.09	31.05±0.70	NS
43-56	30.11±2052	30.33±1.39	29.65±0.54	29.60±0.75	30.78±0.31	NS
Total	28.79±1.27	30.32±1.33	30.28±0.72	30.51±0.14	30.01±0.31	NS
Total Egg Weight (g)						
0-14	11.78±0.28	11.90±0.46	11.28±0.20	11.80±0.24	12.08±0.20	NS
15-28	11.65±0.67	12.20±0.13	11.47±0.16	11.92±0.21	12.20±0.25	NS
29-42	11.92±0.62	12.71±0.30	12.24±0.20	12.65±0.24	12.41±0.40	NS
43-56	11.92±0.40	12.71±0.62	12.24±0.30	12.65±0.20	12.41±0.24	NS
Total	12.16±0.09	12.99±0.62	12.38±0.29	12.75±0.05	12.50±0.27	NS
Feed Conversion (g feed intake x female number/egg production x egg weight)						
0-14	4.20±0.13	4.55±0.53	3.93±0.80	4.58±0.63	4.87±0.98	NS
15-28	4.59±0.30	3.09±0.07	3.60±0.42	3.79±0.34	3.89±0.81	NS
29-42	3.58±0.47	2.82±0.11	2.75±0.13	3.01±0.32	3.36±0.49	NS
43-56	3.21±0.06	2.67±0.10	2.71±0.05	2.73±0.22	2.89±0.14	NS
Total	3.70±0.16	2.98±0.13	3.03±0.30	3.27±0.28	3.53±0.26	NS
Egg Parameters						
Albumen rate (AR), %	52.52±0.85	51.91±0.32	51.98±1.28	53.03±0.49	52.13±0.29	NS
Yolk rate (YR), %	31.29±0.21	31.41±0.10	32.29±0.85	30.64±0.21	30.74±0.31	NS
Dried shell rate(DSR), %	8.08±0.06	7.93±0.03	8.10±0.16	8.21±0.06	7.86±0.07	NS
Shape index, (SI)	77.15±1.38	76.67±0.46	77.67±0.42	78.65±0.39	78.45±0.41	NS
Yolk colour	8.09±0.18^{b}	8.39±0.10^{ab}	8.78±0.16^{a}	8.75±0.11^{a}	8.65±0.15^{ab}	**

Data were given as Mean ± SEM, ***NS:*** *Not statistically significant; * P≤0.05, ** P≤0.01; ^{a,b} Mean values wirth different superscripts within a row differ significantly. AR = (Albumen weight/Egg weight)*100, YR = (YYolk weight/Egg weight)*100, DSR = (Shell weight/Egg weight)*100, SI = (Egg with/Egg length)*100*

Table 4. *Effects on some hatchability traits of milk thistle (Silybum marianum) seed supplementation to high-calorie basal diets of quails*
Tablo 4. *Yüksek kalorili bıldırcın karma yemlerine deve dikeni (Silybum marianum) tohumu ilavesinin bazı kuluçka özellikleri üzerine etkisi*

Hatchability Parameters	C	SFO	SFO+MT	CS	CS+MT	P-Significance
Fertility*(n/100 eggs set)*	73.95±3.91^{b}	78.32±3.42^{ab}	71.67±1.51^{b}	91.64±1.17^{a}	82.57±4.92^{a}	*
Hatchability*(commercial chicks/100 eggs set)*	57.30±4.61	62.19±3.87	58.21±6.64	77.27±6.74	64.25±3.28	NS
Hatchability of fertile*(chicks/100 fertile egg)*	78.48±9.71	79.97±7.56	80.91±7.76	84.18±6.51	78.74±8.09	NS
Embrionic mortality *(n/100 fertile eggs)*	21.50±7.72	20.01±5.56	19.08±5.62	15.80±4.51	21.24±6.15	NS

Data were given as Mean ± SEM, ***NS:*** *Not statistically significant; * P≤0.05, ^{a,b} Mean values wirth different superscripts within a row differ significantly*

Table 5. *Effects on oxidative stress parameters of milk thistle (Silybum marianum) seed supplementation to high-calorie basal diets of quails*
Tablo 5. *Yüksek kalorili bıldırcın karma yemlerine deve dikeni tohumu (Silybum marianum) ilavesinin oksidatif stres parametrelerine etkisi*

Stress Parameters	C	SFO	SFO+MT	CS	CS+MT	P-Significance
Blood						
MDA nmol/ml	1.70±0.15	1.84±0.14	1.68±0.07	1.85±0.10	1.46±0.07	NS
GSH mmol/g protein	39.96±1.07	43.69±3.31	37.10±1.52	35.46±2.98	40.01±2.31	NS
GSH-Px U/g Hb	72.68±5.46^{a}	73.81±4.06^{a}	60.69±2.50^{b}	59.18±3.32^{b}	61.75±2.65^{b}	**
CAT k/g Hb	16.66±1.30^{b}	22.42±1.18^{ab}	27.37±1.81^{a}	25.00±3.44^{ab}	26.65±2.15^{a}	*
Liver						
MDA nmol/g tissue	19.71±1.26	17.50±0.68	18.60±1.74	21.35±1.39	17.58±0.68	NS
GSH mmol/g protein	3.90±0.09^{b}	3.22±0.35^{b}	5.92±0.64^{a}	4.17±0.25^{b}	3.25±0.24^{b}	***
GSH-Px U/g protein	4.66±0.25	4.98±0.31	5.76±0.81	5.88±0.53	5.21±0.28	NS
CAT k/g protein	118.38±7.05^{b}	157.44±17.02^{ab}	151.42±6.86^{ab}	134.92±9.43^{b}	189.25±8.77^{a}	**
Kidney						
MDA nmol/g tissue	24.73±2.57^{b}	30.02±1.56^{b}	32.84±3.68^{ab}	43.40±4.05^{a}	24.96±1.20^{b}	**
GSH mmol/g protein	4.45±0.32^{a}	3.18±0.15^{bc}	2.65±0.06^{c}	4.09±0.40^{ab}	3.11±0.22^{bc}	***
GSH-Px U/g protein	4.86±0.24^{a}	3.94±0.20^{ab}	3.65±0.25^{ab}	3.93±0.51^{ab}	2.98±0.19^{b}	**
CAT k/g protein	68.77±2.40^{a}	50.89±3.37^{b}	53.51±3.51^{b}	56.05±3.56^{b}	53.42±1.35^{b}	**
Heart						
MDA nmol/g tissue	12.95±1.03	14.35±0.39	13.19±0.68	11.95±0.81	12.77±0.89	NS
GSH mmol/g protein	21.41±1.15^{a}	17.69±0.94^{ab}	20.05±1.00^{ab}	17.40±1.35^{ab}	16.26±1.31^{b}	*
GSH-Px U/g protein	34.96±2.27	32.97±3.48	37.15±3.84	30.96±4.05	26.08±2.12	NS
CAT k/g protein	46.51±2.07^{a}	45.61±4.28^{ab}	42.99±2.89^{ab}	32.95±2.90^{b}	38.89±2.77^{ab}	*

Data were given as Mean ± SEM, ***NS:*** *Not statistically significant; * P≤0.05; ** P≤.0.01; *** P≤0.001;* ***MDA:*** *Malondialdehyde,* ***GSH:*** *Glutathione,* ***GSH-Px:*** *Glutathione peroxidase,* ***CAT:*** *Catalase;* ^{a,b,c} *Mean values wirth different superscripts within a row differ significantly*

in CS+MT group (P<0.01). MDA levels of SFO groups slightly increased but not significant (P>0.05). The differences in GSH levels of blood, GSH-Px activities of liver and heart in the examined groups were not significant (P>0.05). GSH-Px activities of blood in control and SFO groups were higher than the other treatment groups (P<0.01), CAT activity of blood was found lower in control group (P<0.05). GSH level of liver in SFO+MT (P<0.001) and also CAT activity in CS+MT group were significantly higher (P<0.01). GSH levels and GSH-Px, CAT activities of kidney (P<0.01), in addition, CAT activities of heart were higher in control group (P<0.05). Addition of milk thistle seed into diet significantly reduced GSH level (P<0.001) and GSH-Px (P<0.01) activity of kidney and also GSH level of heart (P<0.05).

DISCUSSION

Table 3 also shows the effect of MT supplementationto diet on some performance and egg quality traits in laying quails fed with high calorie diets. When total hen-day egg production was examined, it was determined that egg production significantly increased in SFO+MT and SFO groups. Even though high calorie diets increased egg production compared to C group, this increase was found to reach significant levels in oil groups. While acquired egg production values were similar to those reported by Kaplan et al.[13] and Silici et al.[14], they were lower than values reported by Yörük et al.[15], Kaplan et al.[16], and Erişir et al.[17]. It was determined that addition of MT did not make any contribution to egg production. The fact that egg production was higher in oil added groups compared to CS group was associated with extra-caloric and extra-metabolic effect of oils [18]. Different from this study, in the study of Midilli et al.[19] it was reported that addition of 1.5% and 3% SFO into basal diet did not affect egg production. Daily feed consumption in study groups varied between 28.78 g and 30.51 g, and the difference between the groups was statistically insignificant *(Table 3)*. While feed consumption was expected to decrease depending on energy content in groups consuming high calorie diet Griffiths et al.[20] similar results were achieved in all groups of this study and thus it was determined that quails use the extra energy they consumed for egg production and accordingly egg production increased in parallel with energy consumption. Furthermore, the fact that they used extra energy, they produced due to extra-caloric and extra-metabolic effects of oils, for egg production did not caused a significant difference in feed consumption also in these groups. While the obtained feed consumption values were similar to those reported by Silici et al.[14] and Erişir

et al.[17], they were higher than those reported by Kaplan et al.[13] and Kaplan et al.[16] and lower than values reported by Yörük et al.[15]. In their study, Midilli et al.[19] did not found any difference between control group and groups with addition of 1.5% and 3% SFO in terms of daily feed consumption. Some related studies have revealed that addition of essential oils into basal diets increased feed consumption [21,22]. Egg weigh varied between 12.16 and 12.99 g in the study. There was no difference between the groups in terms of egg weight. While the studies Whitehead et al.[23] and Lelis et al.[24] have emphasized that essential oils added into diet increase egg weight due to essential fatty acids they contain, it was thought that the increase in number of eggs in these groups is a factor inhibiting the increase of egg weight. Egg weight values found in this study were similar to egg weight values reported by Silici et al.[14] Yörük et al.[15] and Karabayır et al.[25] and were higher than those reported by Kaplan et al.[13], Kaplan et al.[16] and Erişir et al.[17]. Feed conversion values in the study varied between 2.98 and 3.70. Despite a significant difference in egg production, no difference was found between the groups in terms of feed conversion. This result is thought to be caused by small differences in parameters of egg weight and feed consumption which are effective in the calculation of feed conversion ratio. While acquired feed conversion ratios were found similar to values reported by Kaplan et al.[13] Silici et al.[14] Yörük et al.[15] and Erişir et al.[17] they were higher than those reported by Kaplan et al.[16]. When some parameters belonging to egg quality traits in the study were examined, it was determined that the groups were similar in terms of albumen, yolk, and shell rates, and shape index and there was a significant improvement in all study groups regarding color of yolk compared to C group. It could be asserted that addition of MT into diet increased color of yolk. This situation can vary depending on xanthophyll or carotene contents of supplements used [26].

Table 4 illustrates the effect of milk thistle supplementation to diet on some hatchability traits in laying quails fed with high calorie diet in the study. As is seen in *Table 4*, a significant difference was determined between the groups in terms of fertility. In CS and CS+MT groups, fertility was found to be the highest. It was seen that addition of MT into ration decreased the fertility rate from 78.32% determined in SFO group to 71.67% in SFO+MT group and the fertility rate from 91.64% determined in CS group to 82.57% in CS+MT group. The fertility rates determined in the study was lower than fertility rates reported by İpek et al.[27] and Silici et al.[14]. Similarity was found between the groups in terms of hatchability, hatchability of fertile eggs, and embryonic mortality in the study. Hatchability and hatchability of fertile eggs found in the study was lower than values reported by Silici et al.[14] and İpek et al.[27]. Embryonic mortality rate was determined to be quite higher than embryonic mortality rate reported by İpek et al.[27]. In this study, fertility increased in groups fed with high calorie diets and especially in CS group. This result supports the result that high energy has a challenging effect on fertility [28]. It was thought that while sharing of energy in oil groups was towards egg production more, it promoted mating and sperm quality in sugar groups. Additionally, it was observed that MT added into diet did not have a positive effect on fertility and hatchability traits. Being different from this study, Şimşek et al.[28] emphasized that oils of cinnamon and rosemary added into ration increased fertility depending on their bioactive characteristics, some active components or mineral and vitamin contents.

Oxidative stress may be caused by excess Reactive oxygen species (ROS) production and/or deficient antioxidant capacity [29,30]. ROS produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation, and DNA damage. As a result of the degradation of lipid peroxides, MDA forms and is used as an indicator for lipid peroxidation [31]. In this study, CS caused oxidative stress with statistically significant increase of MDA and decrease of antioxidants (GSH, GSHPx, CAT) in kidney tissue of quails. On the other hand, SFO caused a statistically insignificant increase in MDA and decreases in antioxidants in kidney. Liu et al.[32] reported that high concentration of poly-unsaturated fatty acid in the diet made membrane susceptible to peroxidative degradation and increased oxidative stress. In a study conducted similar to our results, insignificant differences was found in serum MDA of ducks fed with SFO diet [32]. It is known that *Slybum marianum* extracts and active substances like *silymarin* show antioxidant characteristics and inhibit oxidative stress in various tissues such as liver, kidney, brain of rats [5,33-35]. It was determined that it increased in kidneys of diabetic rats; while the use of milk thistle extracts and *silymarin* decreased Thiobarbituric acid reactive substances (TBARS) values in a statistically significant way, the decreased SOD, CAT, and GSHPx activities increased [34,36]. In this study, the use of both CS and MT enabled MDA levels to become normal in kidney. The fact that MDA level return to normal with addition of MT indicated that the addition of MT is beneficial for kidney. However, since antioxidants levels were lower in CS+MT and SFO+MT groups, antioxidants could remain insufficient in case of using MT longer than 60 days. Silybin and silymarin are effective in returning the increased TBARS and lipid hydroperoxide levels, caused by Arsenic, Gentamicin, and Aflatoxin in kidney, and the decreased nonenzymatic and enzymatic antioxidants to normal, and are suggested for decreasing nephrotoxicity [29,31,33]. According to results of this study, we can suggest that MT is nephroprotective in poultry as well due to its antioxidant characteristics.

Consequently, high-calorie diets could be used to obtain higher egg production and fertitility rate in quail production. However these diets caused changes in anti-

oxidants in blood and tissues in the birds. Particularly, in quails feeding with corn syrup significantly increased lipid peroxidation in kidney. Supplementation of milk thistle seed into corn syrup groups may protect kidney against free radical damage, on the contrary the additive was not able to any success on performance and hatchability parameters.

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Research Article

The Relationship Between von Willebrand Factor Gene and von Willebrand Factor Antigen Levels in Dogs [1]

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Abstract

In this study, the relationship between plasma vWF antigen level and polymorphism occurring due to the leucine (L) to proline (P) substitution at position 2380 in exon 42 in von Willebrand factor gene (*VWF*) in dogs was aimed to investigate. The present study was performed on 161 dogs of various breeds and ages (95 male, 66 female) referred to Faculty of Veterinary Clinics at Erciyes University between January 2014 and 2015. Blood samples were collected in EDTA and Na citrate tubes after clinical examination of the dogs. PT, APTT, TT, fibrinogen, D-dimer, thrombocyte, vWF antigen (vWF: Ag) levels and *VWF* were determined from the blood samples. Genotypes were examined using PCR and restriction endonucleas enzymes. In the laboratory examination, 34 (21.1%) of the cases were positive and 127 (78.9%) of the cases were negative with concern to von Willebrand. Prevalence of the disease in different dog breeds that included to the present study were Golden Retreiver (n=35) 34.3%, Kangal (n=45) 11.1%, German Shepherd (n=11) 36.4%, Labrador Retriever (n=7) 42.9%, English Cocker Spaniel (n=2) 100%, English Pointer (n=8) 25.0%, mix breed (n=8) 33.3%, Husky (n=7) 12.5%, Malinois (n=1) 100%, Dogo Argentino (n=2) 50.0%, Samoyed (n=1) 100% and some other breeds were 0%. The genotype of *VWF* was not statistically significant in both positive and negative dogs with concern to vWF: Ag values (P=0.675). When comparison were made in terms of proportional distribution in positive and negative dogs, statistical importance were not observed between genotype and the disease ratio (P=0.969). In conclusion, relationship between *VWF* and vWF antigen level were not determined in dogs. According to our knowledge; this study is the first repot on *VWF* and vWF: Ag relationship therefore believed to be important.

Keywords: *Coagulation, Dog, Haemostasis, Mutation, von Willebrand factor*

Köpeklerde von Willebrand Faktör Antijen Seviyesi ve von Willebrand Faktör Geni Arasındaki İlişkinin Belirlenmesi

Özet

Bu çalışmada, köpeklerde von Willebrand faktör geninin 42. ekzonunun 2380. pozisyonunda meydana gelen lösin (L) - prolin (P)'nin yer değiştirmesine neden olan mutasyon sonucu oluşan polimorfizm ile plazma vWF antijen seviyeleri arasındaki ilişkinin araştırılması amaçlandı. Çalışmaya, Ocak 2014 ve 2015 yılları arasında, Erciyes Üniversitesi Veteriner Fakültesi Kliniklerine getirilen farklı ırk ve yaşta (95 erkek, 66 dişi) 161 köpek dahil edildi. Köpeklerin klinik muayeneleri yapılarak EDTA ve Na sitratlı tüplere kan örnekleri toplandı. Kan örneklerinde PT, APTT, TT, fibrinojen, D-dimer, trombosit, vWF antigen (vWF: Ag) seviyeleri belirlendi ve *VWF* elde edildi. Genotipler, yapılan PCR sonucunda elde edilen ürünlerin *Msp*I endonükleaz enzimi ile kesilerek belirlendi. Laboratuar muayenesinde 34 köpek (%21.1) von Willebrand açısından pozitif olarak belirlenirken, 127 köpeğin (%78.9) negatif olduğu belirlendi. Çalışmaya dahil edilen hayvanlarda ırklara göre hastalığın görülme oranları Golden Retreiver (n=35) %34.3, Kangal (n=45) %11.1, German Shepherd (n=11) %36.4, Labrador Retriever (n=7) %42.9, English Cocker Spaniel (n=2) %100, English Pointer (n=8) %25.0, melez (n=8) %33.3, Husky (n=7) %12.5, Malinois (n=1) %100, Dogo Argentino (n=2) %50.0, Samoyed (n=1) %100 ve diğer ırklar %0 olarak belirlendi. Hem pozitif hem de negatif köpeklerde *VWF* genotipi ile vWF: Ag değerleri arasında istatistiksel olarak bir ilişkili bulunamadı (P=0.675). vWD pozitif ve negatif köpeklerde oransal dağılım açısından karşılaştırıldığında hastalık oranı ve genotip arasında istatistik bir önem gözlenmedi (P=0.969). Sonuç olarak, köpeklerde vWF antigen seviyesi ve *VWF* arasında ilişki bulunmadı. Bilgilerimize göre bu çalışma, *VWF* and vWF: Ag arasındaki ilişkinin araştırıldığı ilk çalışma olması nedeniyle önemlidir.

Anahtar sözcükler: *Pıhtılaşma, Köpek, Hemostaz, Mutasyon, von Willebrand faktör*

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INTRODUCTION

Von Willebrand's disease (vWD) is the commonest an acquired or inherited bleeding disorders of dogs and humans being [1-3].

The most inherited haemostatic disorder of dog is von Willebrand's disease. Von Willebrand syndrome is transferred as autosomal recessive character in dogs [4]. The quantitative deficiencies or qualitative abnormalities of the von Willebrand's factor (vWF) or both causes bleeding tendency at the patients suffers from vWD [1,3]. The vWD phenotype can be classified as type I, II, and III on the basis of the plasma vWF concentration and the vWF multimer structure [1,2]. The measurement of plasma vWF antigen (vWF: Ag) concentration is the current gold standard for diagnosis of the vWD and have been used as screening test for carrier detection in dogs [2]. It is reported that vWD is negative in dogs with vWF: Ag concentration >70% and is positive in dogs with vWF: Ag concentration <50% [2,5]. At the nucleotide sequences of von Willebrand factor gene (*VWF*) is determined to be 85% identical of human and canine and can be used as cross-species modelling of their vWDs [3]. The human *VWF* is located near the telomere of the short arm of chromosome 12 and split into 52 exons, spanning 180 kb of deoxyribonucleic acid [3].

In dogs; *VWF* has 178 kb size, and is in dog caryotype on number 12 chromosome, which has multiple repeated regions [6]. Two widespread mutations about inherited presence of von Willebrand syndrome have been determined. One of the mutations is on the number 4 exon and the other is on the number 42 exon [7].

Venta and Vidal [8] found a polymorphism of the canine *VWF* which can be characterized using the *Msp*I restriction enzyme. This polymorphism occurs due to the leucine (L) to proline (P) substitution at position 2380 in exon 42 in the canine *VWF* [8].

We aimed to investigate the relationship between plasma vWF antigen level in different breed dogs and polymorphism occurring due to the L to P substitution at position 2380 in exon 42 in *VWF* in dogs. Furthermore, we proposed to determine whether the different simultaneous distribution with the vWD phenotype and vWF: Ag concentration as a criteria of elimination in the future for selecting vWD free dogs.

MATERIAL and METHODS

In the present study, 161 dogs which vaccinated, not pregnant, out of eustrus cycles and have no hypothyroidism signs of various breeds and ages (95 male, 66 female) referred to Faculty of Veterinary Clinics at Erciyes University between January 2014 and 2015 were used as animal material. Blood samples were collected into EDTA and Na citrate tubes after clinical examination of the dogs. The citrated blood was centrifuged at 1.500 x g for 15 min at room temperature, and then plasma was separated. Complete blood counts were examined by electronic cell counter (Mindray BC-2800 Vet®, China). All plasma and blood samples with EDTA were stored at -80°C until needed.

Von Willebrand's factor antigen were performed on a CS-5100 analyser (Sysmex UK Ltd, Milton Keynes, UK), all reagents were from Siemens Healthcare Diagnostics. Prothrombin time (PT), Activated thromboplastin time (APTT), thrombin time (TT), fibrinogen, D-dimer were analysed on the Sysmex CA-7000 (Siemens Healthcare Diagnostics) at the Central Laboratory of Erciyes University.

The DNAs were extracted using phenol-chloroform method from leukocytes. PCR analysis for the gene was performed in a 25 µl reaction mixture, containing 1.5 mM $MgCl_2$, 50 µM of dNTP mix, 0.2 µM of each primers, 1 X PCR buffer, 1U Taq polymerase and 50 ng of genomic DNA template. The *VWF* was amplified using the primers: primer 1 5'-TCTTGTCCCCCGCACTGGA-3'; primer 2 5'-TGGTTGTGG TGCAGCCACAGTC-3'. Primers were designed to the canine *VWF* sequence (Genbank accession numbers L76227, U66246, AF099154) by Venta and Vidal [8]. Thermal cycling conditions were followed by 50 cycles of 94°C 30 sec, 62°C 1 min and 72°C 1 min and PCR products were digested at least 60 min at 37°C with the 10U *Msp*I restriction endonuclease (MBI Fermentase®, Lithuania).

The Chi-Square Test ($\chi 2$) was used to check whether the populations were in Hardy-Weinberg equilibrium. Genotypic data were compared to vWD prevalence by using Chi-Square Test in MİNİTAB 16 software.

Erciyes University Local Board of Ethics Committee for Animal Experiments has approved the study protocol of this research (Decision no: 2013/09).

RESULTS

Dogs included to the study were 95 male (59%), 66 female (41%). A comparison of vWF: Ag concentration between males and females were found no significant difference (P>0.05). Out of 161 animals 75 (46.6%) were < 1 years old and 86 (53.4%) were >1 years old. There were no statistically significant effects of age on vWF: Ag levels (P>0.05). According to vWF: Ag concentration; higher than 50% of this concentration (127, which was about 78.9%) considered negative and lower than 50% of vWF: Ag level (34, which was about 21.1%) considered positive [2]. In positive breeds according to vWF: Ag concentration; allele frequencies the *VWF* and prevalence of vWD given in *Table 1*.

The PCR amplification was yielded a 147 bp product. The PCR products were digested with 10U of *Msp*I restriction endonuclease. Restriction digestion of 147 bp PCR products with *Msp*I enzymes revealed three genotypes *(Fig. 1)* of LL

Table 1. *The allele frequencies and prevalence the VWF in vWD positive dog breeds according to vWF: Ag concentration*
Tablo 1. *vWF: Ag seviyelerine göre vWD pozitif olan köpek ırkların VWF genotip frekansları ve vWD görülme oranları*

Breed	N (%)		Genotypes		
	vWD Negative	vWD Positive	LL	LP	PP
Golden Retriever	23 (65.7%)	12 (34.3%)	5	6	1
Kangal	40 (88.9%)	5 (11.1%)	4	1	0
German Shepherd	7 (63.6%)	4 (36.4%)	1	1	2
Labrador Retriever	4 (57.1%)	3 (42.9%)	1	1	1
English Cocker Spaniel	0 (0%)	2 (100%)	1	0	1
English Pointer	6 (75.0%)	2 (25.0%)	1	1	0
Mixed breed	4 (66.7%)	2 (33.3%)	1	0	1
Husky	7 (87.5%)	1 (12.5%)	0	1	0
Malinois	0 (0%)	1 (100%)	1	0	0
Dogo Argentino	1 (50.0%)	1 (50.0%)	0	0	1
Samoyed	0 (0%)	1 (100%)	0	0	1
Other Breeds	35 (100%)	0 (0%)			

N: *Sample Size;* ***vWD:*** *von Willebrand Disease*

Table 2. *The allele, genotype frequencies and Hardy-Weinberg chi-square analysis of the VWF*
Tablo 2. *VWF'nin allel, genotip frekansları ve Hardy-Weinberg Ki-kare analizi*

Groups	Genotypes Frequency			Allele Frequency		Statistical Significant (Chi-squared HWE)
	LL	LP	PP	L	P	
vWD Negative	53 (41.73%)	43 (33.86%)	31 (24.41%)	58.7	41.3	$X^2 = 11.57$, $P < 0.001$ (df = 1)
vWD Positive	15 (44.12%)	11 (32.35%)	8 (23.53%)	60.3	39.7	$X^2 = 3.576$, $P = 0.059$ (df = 1)

HWE: *Hardy-Weinberg Equilibrium;* ***X^2:*** *Chi-Square value; df: Degree of freedom;* ***vWD :*** *von Willebrand Disease;* ***VWF:*** *von Willebrand factor gene*

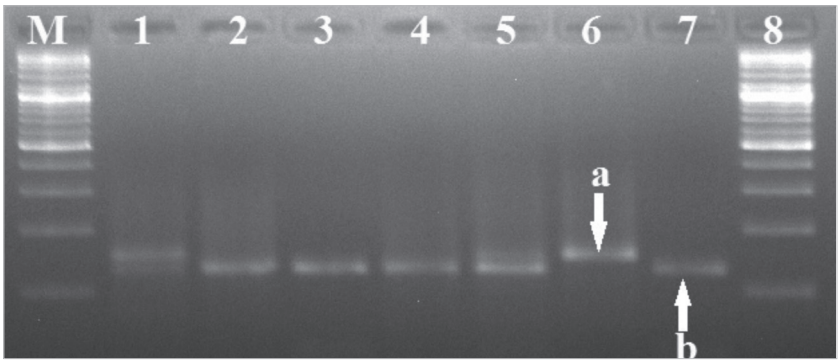

Fig 1. Electrophoresis of RFLP of canine *VWF* after digestion by *Msp*I. Lanes 2-4 and 7; PP (-/-) genotypes; Lanes 1 and 5: LP (+/-) genotypes; lane 6: LL (+/+) genotype; lane M: DNA ladder (100 bp); a: 147 bp and b: 124 bp

Şekil 1. Köpek *VWF* geninin *Msp*I enzim kesim ürünlerinin elektroforez görüntüsü. 2-5 ve 7 nolu kuyucuklar: PP (-/-) genotipli bireyler; 1 ve 5 nolu kuyucuklar: LP(+/-) genotipli bireyler; 6 nolu kuyucuk: LL(+/+) genotipli birey. M: 100 bç'lik DNA merdiveni; a: 147 bç ve b: 124 bç

(+/+) (147 bp), LP (+/-) (147 and 124 bp) and PP (-/-) (124 and 23 bp).

The allelic, genotypic frequencies and heterozygosity of the *VWF*, polymorphisms for the dog are given in *Table 2*.

There was no significant difference between *VWF* genotypes and vWF: Ag values in vWD positive and vWD negative dogs (P=0.675) *(Table 3)*.

In addition, no statistical significance was observed for the prevalence between vWD and genotype (P=0.969), compared the proportional distributions according to genotypes of the *VWF* in vWD positive and vWD negative dogs *(Table 4)*.

The differences between vWD positive and vWD negative dogs in levels of D-dimer, fibrinogen, leucocytes, erythrocyte, haemoglobin and PCV were significant (P<0.05) *(Table 5)*.

DISCUSSION

Von Willebrand's disease is the most common bleeding disease in humans and dogs, resulting various clinical

Table 3. *The proportional distributions of vWF: Ag concentration according to genotypes*
Tablo 3. *Genotipe göre vWF: Ag konsantrasyonunun oransal dağılımı*

Genotype	N	Mean ±SEM	Statistical Significance (ANOVA)
LL	68	66.16 ± 2.51	F: 0.394 P = 0.675
LP	54	68.67 ± 2.95	
PP	39	70.10 ± 4.61	

***vWD:** von Willebrand Disease; **vWF:** Ag: von Willebrand factor antigen; **N:** Sample size; **SEM:** Standard error of the mean; **F:** F statistics value; **P:** P value*

Table 4. *The proportional distributions according to genotypes of the VWF in vWD positive and vWD negative dogs*
Tablo 4. *vWD pozitif ve negatif köpeklerde VWF'nin genotipine göre oransal dağılımı*

Genotype		vWF: Ag		Total
		-	+	
LL	Count	53	15	68
	%	77.9%	22.1%	100.0%
LP	Count	43	11	54
	%	79.6%	20.4%	100.0%
PP	Count	31	8	39
	%	79.5%	20.5%	100.0%
Total	Count	127	34	161
	%	78.9%	21.1%	100.0%

***vWF:** Ag: von Willebrand factor antigen; **vWD :** von Willebrand Disease*

Table 5. *Some hematological parameters in vWD positive (1) and vWD negative (0) dogs*
Tablo 5. *vWD pozitif (1) ve negatif (0) köpeklerde bazı hematolojik parametreler*

Parameters	vWD	N	Mean± SEM	Statistical Significance (Student T test)
D-dimer (µg/l)	0	127	2914.96±611.49	T = 2.91 P = 0.04
	1	34	1063.82±174.69	
Prothrombin time (sec)	0	127	7.30±0.08	T = 0.380 P = 0.780
	1	34	7.24±0.10	
Activated thromboplastin time (sec)	0	127	17.09±0.19	T = 0.887 P = 0.376
	1	34	16.74±0.25	
Thrombin time (sec)	0	127	17.75±0.19	T = 0.732 P = 0.466
	1	34	17.44±0.42	
Fibrinogen (mg/dl)	0	127	294.06±12.90	T =0 .491 P < 0.001
	1	34	193.03±16.06	
Thrombocyte (10^3/µl)	0	127	313.42±12.18	T = 1.788 P = 0.077
	1	34	281.97±12.69	
Leucocyte (10^3/µl)	0	127	20.72±1.06	T = 2.138 P = 0.035
	1	34	17.32±1.19	
Erythrocyte (10^6/µl)	0	127	6.28±0.14	T = -2.544 P = 0.012
	1	34	7.03±0.22	
Haemoglobin (g/dl)	0	127	15.54±0.30	T = -2.578 P = 0.011
	1	34	17.18±0.53	
Haematocrit (%)	0	126	44.55±0.87	T = -2.582 P = 0.011
	1	34	49.47±1.75	

***vWD:** von Willebrand Disease; **N:** Sample size; **SEM:** Standard error of the mean; **T:** T statistics value; **P:** P value*

conditions from none to death causing severe hemorrhagic diathesis [9,10]. In vWD; to determine diagnosis and carriers, vWF: Ag levels have been used. According to Brooks et al.[11] and Kayar et al.[12] vWF: Ag concentrations classified as non-carrier if it is 70-179%, borderline if it is 51-59%, carrier if it is 2-49% and effected if it is 0.1-0.3% in individuals. On the other hand, Raymond et al.[13] defined over 60% vWF: Ag concentration as non-carrier, between 50-59% as border-line, and between 0-49% as carriers. In addition, Pathak [14] evaluated 70-180% as normal range, 50-69% as borderline and 1-49% as carrier. In the present study; vWF: Ag concentration <50% individuals were considered to be vWD positive as reported by Burges et al.[2] and Thomas [15].

Von Willebrand's disease is an acquired or inherited bleeding disorder common in humans and dogs [3]. In human beings estimated prevalence of vWD has been reported between 0.01-0.02% [9,16]. Castaman et al.[17] point out that with a prevalence of 1% in general population; the ratio of visible clinical symptoms is only 0.01%.

The prevalence of vWD in Scottish Terrier dogs has been reported to change between 18% to 30% [18-21]. In German Wirehaired Pointers; carrier ratio reported to be 12% and disease developing ratio is reported to be 1% [11]. On the other hand, Dutch Kookier dogs this ratio determined to be 5.2% (in 717 dogs; 38 dogs had type III vWD) [22]. According to Brooks et al.[19] when lower than 50% vWF: Ag concentration taken into consideration; 73% of dobermans, 30% of scotties, and 28% of shelties had abnormal vWF: Ag concentration. Furthermore; Kayar et al.[12] found this ratio in German Shepherd dogs as 26%. In our study, when lower than 50% vWF: Ag concentration evaluated; vWD ratio was determined to be 21.1%. Prevalence of the disease in different dog breeds that included to the present study were Golden Retreiver (n=35) 34.3%, Kangal (n=45) 11.1%, German Shepherd (n=11) 36.4%, Labrador Retriever (n=7) 42.9%, English Cocker Spaniel (n=2) 100%, English Pointer (n=8) 25.0%, mixed breed (n=8) 33.3%, Husky (n=7) 12.5%, Malinois (n=1) 100%, Dogo Argentino (n=2) 50.0%, Samoyed (n=1) 100% and some other breeds were 0%. The difference observed in the prevalence; believed to be occurred due to diagnostic method, animal breed and number of animals used in studies.

Brooks et al.[1] examined German Wirehaired Pointer dogs and found no relation between vWD, age and sex. Similary, Kayar et al.[12] also found no relation between vWD and age. In the present study no correlation between the occurrence of the vWD, sex or age in dogs also determined.

In vWD dogs; number of platelet, TT, PT, APTT times were reported to be in generally reference values or APTT time were slightly a bit longer than normal [2,12,14,22]. Similarly, in the present study, platelet number, TT, PT and APTT times were in reference borders and differences between two groups were not significant.

In the present study, serum fibrinogen level in dogs with vWD were lower statistically (P<0.001) compared to the level obtained from the dogs without vWD but the determined concentrations in both groups were within the reference values. Obtaining normal fibrinogen levels in vWD dogs was in agreement with the previous studies [15]. D-dimer is a product of cross-linked of fibrin primer degradation [23]. Determining low D-dimer level in vWF: Ag level low dogs can be explained by the insufficient formation of clot which found in the present study.

It is important that the affected dogs and asymptomatic carriers of vWD should be excluded from breeding programs. Quantitative assays of plasma vWF antigen concentration have been used for definitive diagnosis of vWD and have been used to detect vWD carrier dogs [1,19,24,25]. Additionally, Brooks et al.[11] reported that a single vWF: Ag levels can be applied breeding dogs selection without vWD in German Wirehaired Pointers. However, Slappendel et al.[22] speculated that plasma vWF: Ag levels ranged between 30 to 89% in 39 Dutch kookier dogs are obligate carriers of Type III vWD. In addition, in people for the estimates of the heritability is used as plasma vWF: Ag levels in between 35-75% [16]. Because of the uncertainty in defining carrier status by ELISA method measuring vWF: Ag concentration in plasma, the location and character of the mutation causing vWD should be determined [3]. Identification of the affected gene and its action mechanism is also important in developing treatment for vWD. The encode of *VWF* is located on short arm of chromosome 12 and the 52 exons of the gene in humans [9,26]. The single-gene associations between plasma vWF: Ag concentrations and variants at the angiotensin-coverting enzyme, the FUR2 locus, lipoproteinreceptor-related protein, and arginine vasopressin 2 receptor have been reported in previous studies in humans [16]. Kramer et al.[3] investigated German Shorthaired Pointers having type II vWD and they found statistical importance between vWF variant nucleotide in exon 28 and vWF multimer deficiencies. Furthermore, in Scottish Terriers single base deletion in fourth exon were determined [18].

Venta and Vidal [8] reported that in exon 42 of the canine *VWF,* a polymorphic region cutting by an *Msp*I is present. In parallel with Venta and Vidal [8] findings; in the present study at 11 different dog breeds, *VWF* had polymorphic regions when the gene cut by an *Msp*I. Furthermore, breed specific difference in different dog breeds on the examined cutting regions were not determined in the present study as reported by Venta and Vidal [8].

In the present study, 11 different dog breeds raised in Turkey were investigated. In this study, apart from Venta and Vidal's [8] study, the relationship between *Msp*I restriction site polymorphism and vWF: Ag levels in plasma were examined. As a result of the present study; relationship between *Msp*I cutting region and vWF: Ag levels could not be determined. The reason could be; investigated each breed a vWD; the levels of vWF: Ag were different levels as reported by Slappendel et al.[22], or it was thought that the number of animals that diagnosed as vWD were too low. Furthermore, in the present study it was believed that, *Msp*I region that used genotyping of dog breeds could not be effective in determining individuals having mutant allele. To test these possibilities; dog breeds that the disease seen more often, studies having more animal materials should be performed in the future.

As a result of the present study; the relationship between plasma vWF antigen level and *Msp*I polymorphism at position 2380 in exon 42 in *VWF* in dogs could not be determined. In the present study; it was thought that the detection of *Msp*I polymorphism at position 2380 in exon 42 in *VWF* is not sufficient for the definitive diagnosis of vWD. In addition to this, to own best knowledge, the present study is the first report on *VWF* and vWF: Ag relationship, thus this study will contribute to the literature. Furthermore, the other importance of the present study may also come from, its' one of rare study with concern to clinical syndrome and their molecular relationship. Besides; vWF: Ag levels and vWD reported first time in Kangal breed dogs. Additionally; presence of *Msp*I polymorphism on the *VWF* determined first time in the Kangal breed dogs that raised in Turkey.

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Research Article

Detection of Extended-spectrum β-lactamase and AmpC β-lactamase Producing *Escherichia coli* Isolates from Chickens [1]

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Abstract

The aim of this study was to investigate the presence of Extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase producing *Escherichia coli* strains isolated from meat and faecal samples of chicken. Faecal samples (n: 384) were collected from broiler and egg-type healthy chickens in 43 flocks and 384 fresh chicken meat samples from local markets, supermarkets and slaughterhouses. *E. coli* isolates were tested phenotypically according to the guidelines of CLSI (2013) for ESBL production. Phenotypic detection of AmpC production was carried out by determination of resistance to cefoxitin and susceptibility to cefepime. Also, presence of genes encoding different types of β-lactamases (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and the mutation in the promoter of AmpC) in all phenotypically ESBL or AmpC producing *E. coli* was investigated by PCR assays. In this study, *bla*$_{CTX-M}$ (95.4%), *bla*$_{CTX-M-1}$ (81.8%), *bla*$_{CTX-M-8}$ (4.5%), *bla*$_{TEM}$ (45.4%) and *bla*$_{SHV}$ (2.2%) genes were detected from faecal samples and *bla*$_{CTX-M}$ (100%), *bla*$_{CTX-M-1}$ (28.5%) and *bla*$_{TEM}$ (7.1%) from meat samples. The only plasmidic AmpC β-lactamase found was the CIT type.

Keywords: *Escherichia coli, ESBL, AmpC, Chicken, PCR*

Tavuklarda Genişlemiş Spektrumlu β-laktamaz ve AmpC β-laktamaz Üreten *Escherichia coli* İzolatlarının Saptanması

Özet

Bu çalışmanın amacı, tavuk eti ve dışkı örneklerinden izole edilen Genişlemiş Spektrumlu β-laktamaz (GSBL) ve AmpC β-laktamaz üreten *Escherichia coli* izolatlarının varlığını ortaya koymaktır. Bu çalışmada, 43 kümesten sağlıklı tavuklara ait 384 dışkı örneği ve yerel marketlerden, süpermarketlerden ve kesimhanelerden 384 taze tavuk eti örneği toplandı. *E. coli* izolatları fenotipik olarak CLSI (2013) standartları kullanılarak GSBL üretimi yönünden incelendi. AmpC üretiminin fenotipik olarak saptanması sefoksitine direnç ve sefepime duyarlılık kriterleri ile belirlendi. Fenotipik olarak GSBL ya da AmpC üreten *E. coli* olarak saptanan izolatlarının hepsi PCR ile değişik tipteki β-laktamaz genlerinin (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, AmpC) varlığını ortaya koymak için incelendi. Bu çalışmada, dışkı örneklerinde *bla*$_{CTX-M}$ (%95.4), *bla*$_{CTX-M-1}$ (%81.8), *bla*$_{CTX-M-8}$ (%4.5), *bla*$_{TEM}$ (%45.4), *bla*$_{SHV}$ (%2.2) genleri ve tavuk eti örneklerinde ise *bla*$_{CTX-M}$ (%100), *bla*$_{CTX-M-1}$ (%28.5), *bla*$_{TEM}$ (%7.1) genleri saptandı. AmpC β-laktamaz grubunda sadece CIT geni belirlendi.

Anahtar sözcükler: *Escherichia coli, GSBL, AmpC, Tavuk, PCR*

INTRODUCTION

During the past decade, drug resistance has increased worldwide and also extended-spectrum β-lactamases (ESBL) is the most common mechanism of resistance to broad-spectrum cephalosporins in members of Enterobacteriaceae (mostly *Escherichia coli*) [1-3]. ESBL genes are located on plasmids those can easily harbour in bacterial species. Some ESBL genes are mutant derivatives of established plasmid-mediated β-lactamases (e.g., *bla*$_{TEM/SHV}$), and others are mobilized from environmental bacteria (e.g., *bla*$_{CTX-M}$). The epidemiology of ESBL genes is rather complex, changing quickly and shows marked geographic differences in distribution of genotypes of *bla*$_{CTX-M}$ β-lactamases [3]. Another large group of broad-spectrum β-lactamases are the AmpC enzymes, which are typically

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encoded on the chromosome of many Gram-negative bacteria. Also, AmpC-type β-lactamases may be carried on plasmids of bacterial species lacking the chromosomal AmpC gene [4].

The use of antimicrobial agents in poultry husbandry is a common problem. The authors emphasize that the antimicrobial-resistant poultry faecal *E. coli* strains can be transmitted to humans both directly and via the food chain [5]. The source of the colonization of ESBL/AmpC producing bacteria in humans is not completely understood, circumstantial evidence points also to a food-borne source [6]. Poultry meat can be contaminated with antimicrobial-resistant *E. coli* at slaughter and can also act as a reservoir of drug resistant bacteria. The use of cephalosporins in food-producing animals and in veterinary medicine could be a selective factor for the appearance of ESBL producing bacteria in animals [7].

Recent studies in Spain [8], France [9], Tunisia [10], Belgium [11], China [12], Great Britain [13], Netherlands [6], and England [14] show that CTX-M *E. coli* isolates are likely to be present in chickens globally. In Turkey, the prevalence has not been extensively investigated in poultry.

The present study was undertaken to determine the presence and prevalence of ESBL and AmpC β-lactamase producing *E. coli* strains in chicken in Marmara Region of Turkey.

MATERIAL and METHODS

The present study was approved by the Animal Care Committee of Istanbul University, Faculty of Veterinary Medicine, Approval no: 2012/17.

A total of 384 faecal samples (10 faeces pooled in a sterile faeces container and counted as one sample) were collected from healthy chickens in 43 flocks on 14 farms located in different areas of the Marmara Region in Turkey (Kırklareli, Edirne, Tekirdağ, Istanbul, Kocaeli, Yalova, Sakarya, Bursa, Balıkesir, Çanakkale), at intervals between November 2012 and October 2013. Additionally, a total of 384 retail chicken meat samples (breast, leg quarter) were collected from local markets, supermarkets and slaughterhouses located in the same region, between January-October 2013.

Five gram of the faecal and meat samples were inoculated into 15 ml of tryptic soy broth/TSB (Oxoid, USA) and were incubated for 16-18 h at 37°C. Subsequently, 10 µL of TSB was transferred onto MacConkey agar plate (Becton Dickinson, USA) supplemented with 1 mg/L cefotaxime (Sigma-Aldrich, United Kingdom) and incubated aerobically overnight at 37°C. One presumptive *E. coli* colony was randomly selected on MacConkey agar and subcultured onto blood agar plate and identified by biochemical tests [15]. *E. coli* isolates were subjected for antibiotic susceptibility testing by using, cefotaxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefpodoxime (10 µg), aztreonam (30 µg), cefoxitin (30 µg), cefepime (30 µg) discs. Clinical Laboratory Standards Institute (CLSI) guidelines were followed for inoculum standardization, medium and incubation conditions, and internal quality control organisms *(E. coli* ATCC 25922) were used. The diameter of the zone of inhibition was measured and interpreted according to the guidelines of CLSI (2013). The isolates were tested for ESBL production by combination of disc diffusion test including cefotaxime and ceftazidime with and without clavulanic acid. An increase in the zone diameter of 5 mm or more, when either of the antimicrobial agents was combined with clavulanic acid, was considered evidence of ESBL production [16]. Phenotypic detection of AmpC production was carried out by determination of resistance to cefoxitin and susceptibility to cefepime [16,17].

DNA was extracted by Roche High Pure PCR Template Preparation Kit (Roche, France), according to the manufacturer's instructions. The presence of genes encoding different types of β-lactamases (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and the mutation in the promoter of *AmpC*) in all phenotypically ESBL or AmpC producing *E. coli* was studied.

For specific detection of the *bla*$_{CTX-M}$ genes (CTX-M-1, CTX-M-2, CTX-M-9, CTX-M-14, CTX-M-20 and CTX-M-21), consensus primers were chosen from regions with high levels of sequence homology to the *bla*$_{CTX-M}$ genes [18].

bla$_{CTX-M}$ genes groups 1, 2 and 9 were detected by multiplex PCR. Primer pairs and predicted amplicon sizes were: group 1, 5′-AAA AAT CAC TGC GCC AGT TC and 5′-AGC TTA TTC ATC GCC ACG TT (415 bp); group 2, 5′-CGA CGC TAC CCC TGC TAT T and 5′-CCA GCG TCA GAT TTT TCA GG (552 bp); group 9, 5′-CAA AGA GAG TGC AAC GGA TG and 5′-ATT GGA AAG CGT TCA TCA CC (205 bp). Amplification conditions were: initial denaturation at 94ºC for 5 min; 30 cycles of 94ºC for 25 s, 52ºC for 40 s and 72ºC for 50 s; and a final extension at 72ºC for 6 min [19].

The primer sequences, product sizes and cycling conditions used to amplify different β-lactamase genes by PCR are listed in *Table 1*.

Multiplex PCR for the purpose of identifying family-specific *AmpC* β-lactamase genes was performed as previously described [21]. The targets, *primers sequences used for PCR amplification and product sizes are summarized in Table 2*. The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 25 cycles of DNA denaturation at 94°C for 30s, primer annealing at 64°C for 30s, and primer extension at 72°C for 1 min. After the last cycle, a final extension step at 72°C for 7 min was added.

PCR product were analysed by gel electrophoresis with 2% agarose (Sigma-Aldrich, United Kingdom) and visualizations were performed.

Table 1. *Primer sequences, product sizes, cycling conditions and references*
Tablo 1. *Gen dizilimleri, amplikon büyüklüğü, amplifikasyon koşulları ve kaynaklar*

Target(s)	Primers (5' to 3', as synthesized)	Size (bp)	Cycling Conditions					References
bla_{CTX-M}	SCS ATG TGC AGY ACC AGT AA	550	94°C 3 min.	35 cycles			72°C 5 min.	[18]
	CCG CRA TAT GRT TGG TGG TG			95°C 30 s.	57°C 30 s.	72°C 45 s.		
CTX-M Group 8	TCG CGT TAA GCG GAT GAT GC	666	94°C 5 min.	25 cycles			72°C 6 min.	[19]
	AAC CCA CGA TGT GGG TAG C			94°C 25 s.	52°C 40 s.	72°C 50 s.		
CTX-M Group 25	GCA CGA TGA CAT TCG GG	327	94°C 5 min.	25 cycles			72°C 6 min.	[19]
	AAC CCA CGA TGT GGG TAG C			94°C 25 s.	52°C 40 s.	72°C 50 s.		
bla_{TEM}	ATG AGT ATT CAA CAT TTC CG	858	94°C 3 min.	35 cycles			72°C 5 min.	[20]
	CCA ATG CTT AAT CAG TGA GC			95°C 30 s.	55°C 30 s.	72°C 45 s.		
bla_{SHV}	CTT TAC TCG CTT TAT CG	475	95°C 5 min.	30 cycles			72°C 5 min.	[20]
	TCC CGC AGA TAA ATC ACC A			95°C15 s.	52°C 30 s.	72°C 90 s.		

Table 2. *Targets, primer sequences and product sizes*
Tablo 2. *Hedefler, gen dizilimleri ve amplikon büyüklükleri*

Target (s)	Primer	Primers (5' to 3', as synthesized)	Size (bp)
MOX-1, MOX-2, CMY-1, CMY-8-9-10-11	MOXM-F MOXM-R	GCT GCT CAA GGA GCA CAG GAT CAC ATT GAC ATA GGT GTG GTG C	520
LAT-1-2-3-4, CMY-2-3-4-5-6-7, BIL-1	CITM-F CITM-R	TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC	462
DHA-1, DHA-2	DHAM-F DHAM-R	AAC TTT CAC AGG TGT GCT GGG T CCG TAC GCA TAC TGG CTT TGC	405
ACC	ACCM-F ACCM-R	AAC AGC CTC AGC AGC CGG TTA TTC GCC GCA ATC ATC CCT AGC	346
MIR-1T, ACT-1	EBCM-F EBCM-R	TCG GTA AAG CCG ATG TTG CGC CTT CCA CTG CGG CTG CCA GTT	302
FOX-1-2-3-4-5b	FOXM-F FOXM-R	AAC ATG GGG TAT CAG GGA GAT G CAA AGC GCG TAA CCG GAT TGG	190

RESULTS

Phenotypically Monitoring and Confirmation Tests

From faecal samples, 76 (19.7%) isolates on MacConkey agar (supplemented with cefotaxime) were positive for cefotaxime-resistant *E. coli*.

Distribution of β-lactam resistance phenotypes among cefotaxime-resistant faecal *E. coli* isolates from broilers on each farm is shown at *Table 3*.

From meat samples, 14 (3.6%) isolates on MacConkey agar (supplemented with cefotaxime) were positive for cefotaxime resistant *E. coli*. Out of those isolates only 2 (40%) isolates were ESBL producers, and 3 (60%) were AmpC β-lactamase producers.

Genetic Confirmation by PCR

In 44 phenotypically confirmed ESBL and/or AmpC β-lactamase producing *E. coli* isolates from faecal samples, 42 (95.4%) harboured at least one of the ESBL-gene: bla_{CTX-M} (95.4%), $bla_{CTX-M-1}$ (81.8%), $bla_{CTX-M-8}$ (4.5%), bla_{TEM} (45.4%) and for bla_{SHV} (2.2%). The only plasmid encoded AmpC β-lactamase found was the CIT type (n=29). $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, $bla_{CTX-M-25}$ or AmpC FOX, EBC, ACC, DHA and MOX groups were not detected.

Five phenotypically confirmed ESBL and AmpC β-lactamase producing *E. coli* isolates obtained from meat samples, all of them (%100) was harbouring at least one of ESBL-gene: bla_{CTX-M} (100%), $bla_{CTX-M-1}$ (28.5%) and/or bla_{TEM} (7.1%). The only plasmidic AmpC β-lactamase found was the CIT type (n=3). $bla_{CTX-M-2}$, $bla_{CTX-M-8}$, $bla_{CTX-M-9}$, $bla_{CTX-M-25}$, bla_{SHV} or AmpC FOX, EBC, ACC, DHA and MOX groups were not found. Distribution of β-lactamase genes is given in *Table 4*.

DISCUSSION

Antimicrobial resistance is recognized as one of the greatest threat and the most important global health challenge. In recent years, the prevalence of ESBL producing *E. coli* has been increasing in both human and veterinary medicine [3,22].

In this study, it has been demonstrated that ESBL producing *E. coli* strains are frequently present (39.4%) in faecal samples of broilers studied. The prevalence of ESBL producing *E. coli* has been reported between 6.7% and

Table 3. *Distribution of β-lactam resistance phenotypes among faecal E. coli isolates from broilers on each farm*
Tablo 3. *Fenotipik olarak β-laktam dirençli fekal E. coli izolatlarının broyler çiftliklerine göre dağılımı*

Farm Number	Number of Faecal Samples Analysed	Number of Cefotaxime -resistant *E. coli* isolates	Number of ESBL Producers	Number of AmpC Producers
1	31	3	1	-
2	38	8	-	5
3	33	9	4	-
4	22	11	2	4
5	35	7	3	1
6	19	6	3	-
7	28	7	2	1
8	25	-	-	-
9	18	-	-	-
10	25	14	8	3
11	28	-	-	-
12	32	10	7	-
13	32	-	-	-
14	18	1	-	-
TOTAL (%)	384	76 (19.7%)	30 (39.4%)	14 (18.4%)
			44 (57.8%)	

Table 4. *Distribution of β-lactamase genes*
Tablo 4. *β-laktamaz genlerinin dağılımı*

Samples	Genetic confirmation by PCR						
	bla_{CTX-M}	CIT Type	bla_{CTX-M} + CIT Type	$bla_{CTX-M-1}$	$bla_{CTX-M-8}$	bla_{TEM}	bla_{SHV}
Faecal (n=44)	42 (95.4%)	29 (65.9%)	27 (61.3%)	36 (81.8%)	2 (4.5%)	20 (45.4%)	1 (2.2%)
Meat (n=5)	5 (100%)	3 (60%)	3 (60%)	4 (80%)	-	1 (20%)	-

60.7%. Our results were relatively low according to some studies [9,13] while showed similarities to others [11,12].

ESBL producing *E. coli* was detected 40% of the isolates obtained from meat samples in this study. In European countries, the prevalence of ESBL genes in chicken meat have been detected in various studies; in the Netherlands, 79.8% [23], in Spain 67% [24] and in the United Kingdom 37% [25]. The different prevalence rates may be originated from detection methods, sampling procedures, regions, geographical conditions and the antibiotic policies.

In this study, 14 (18.4%) *E. coli* isolates from faecal samples were identified as AmpC (plasmidic or chromosomally) β-lactamase producers. The prevalence was lower in comparison to those detected by Smet et al.[11] 43% and Kolar et al.[20] 25.9%. On the other hand, Blanc et al.[23] reported the prevalence rate of *E. coli* isolates as AmpC β-lactamase producers was 6.7%. The comparison of our results with the mentioned studies shows that *E. coli* strains producing AmpC enzymes are mostly less prevalent.

According to our results, the CTX-M group β-lactamases were the predominant ESBL type. Similar results have been reported by Randall et al.[13], Overdevest et al.[3], Morris et al.[26], and Leverstein-van Hall et al.[27]. Also, the majority of those ESBL genes were identified as $bla_{CTX-M-1}$ (81.8%) in this study. Recent studies identified CTX-M-1 as the most prevalent ESBL type shared by human patients, healthy carriers, poultry, and retail chicken meat, suggesting recent cross-transmission between human and avian hosts [22]. In Turkey, Zarakolu et al.[28] and Gülamber et al.[29] reported that CTX-M-1 members were the most prevalent gene types in human isolates.

In this study, ESBL-producing *E. coli* isolates were detected in both faecal and food samples and $bla_{CTX-M-8}$ gene were detected from only two faecal isolates. On the contrary of our results, Jouini et al.[10] indicated that ESBL-producing *E. coli* isolates were detected in 10 (26%) of 38 food samples analyzed and in none of the tested animal faecal samples. Also, these authors reported that $bla_{CTX-M-8}$ gene was not detected from any of the tested animal faecal samples and pointed that the first time that the unusual CTX-M-8 β-lactamase has been detected in bacteria of non-human origin.

Phenotypically confirmed *E. coli* isolates obtained from faecal samples carried an ESBL-gene: bla_{TEM} (45.4%) and

from meat samples carried an ESBL-gene: bla_{TEM} (7.1%) Also, bla_{TEM} was always detected present in combination with other β-lactamase genes ($bla_{CTX-M-1}$, bla_{SHV}, bla_{CIT}). These findings were similar with previous studies reporting that TEM β-lactamases were the most frequent mechanism in *E. coli* isolates from food-producing animals [6,30].

bla_{SHV} was detected only in one isolate from faecal samples and was not detected in any of the isolates from meat samples. On the contrary to these results, Kolar et al.[20] described the most frequent ESBL types were SHV. In Spain, SHV-12-producing *E. coli* strains were then sporadically recovered in faecal samples from healthy chickens [24]. Carattoli [31] emphasize that this gene variant has been described in *E. coli* isolated from poultry in different countries; thus, it would be of particular interest to monitor its future global diffusion.

In the current study, of the plasmidic class C β-lactamases, only the bla_{CIT} type was identified. Various authors indicated that the most prevalent AmpC gene family was CIT including CMY-2, CMY-4, and two CMY-2 variants. Plasmid-encoded AmpC genes belonging to the CIT family have already been reported in food-producing animals and humans worldwide. These findings were consistent with previous studies as a major factor contributing to AmpC resistance [8,19,32].

Multiple β-lactamases within the same organism (e.g., multiple ESBLs or ESBL-AmpC combinations) can make phenotypic identification of the β-lactamases difficult. Unfortunately, for this reason, plasmid-mediated AmpC β-lactamase resistance goes undetected in most clinical laboratories [20]. Also, some researchers stated that two or more of β-lactamase resistance genes can be found in the frequently isolated Gram-negative bacteria [33]. In the current study, 27 faecal (61.3%) and 3 (60%) meat *E.coli* strains were observed as they possess the various ESBL and AmpC genes combinations.

Our results clearly show that ESBL and/or AmpC producing *E. coli* are present in most of the farms (71.4%) studied. The differences could be originated from antibiotic policies or increasing usage of cephalosporins in worldwide. Moreover, there are various methods for detection of ESBL and/or AmpC producing *E. coli*, making the results difficult to compare. Both the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have revised and updated breakpoints for Enterobacteriaceae. The microbiology laboratories in Turkey, like many European laboratories, have implemented the new EUCAST guidelines in 2015. In the present study, the screening and confirmation tests of ESBL were applied and interpreted according to the guidelines of CLSI. In EUCAST guidelines, the formation of the zone of inhibition around cefpodoxime (10 mg) disk <21 mm in diameter (in CLSI this value is ≤17 mm) was accepted ESBL positive screening test. So that, if EUCAST standard had been used as a method, more isolates would be positive screening test, and confirmation test should be applied. Consequently, the prevalence is thought to be higher according to ESBL screening test breakpoints in guidelines issued by EUCAST [16,33].

To conclude, ESBL and/or AmpC producing *E. coli* strains are frequently present in this study and CTX-M type enzymes are the predominant ESBL type. CTX-M-1 is the most prevalent ESBL type and of the plasmidic class C β-lactamases, only the bla_{CIT} type is present. Further multi-disciplinary studies, parallel monitoring and surveillance programmes, and novel strategies in the spirit of 'One Health' are required since such data are currently missing.

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Research Article

The Effect of Decompression on The Treatment of Chronic Constriction Injury in Peripheral Nerve [1]

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Abstract

Chronic constriction injury (CCI) is a common clinical entity and characterized by allodynia or spontaneous neuropathic pain. Treatment of neuropathic pain is difficult, because a lack of knowledge about the underlying mechanisms and limited effectiveness of the existing drugs. Surgical decompression enables a more radical treatment by releasing the compressed nerve. Beside the pain behaviour morphological changes occur in CCI. Ultrastructural morphological changes at the injury site of the sciatic nerve and in the dorsal root ganglia (DRG) are believed to play role in the pathogenesis of CCI and in the development of neuropathic pain behaviour in individuals. However, the effects of surgical decompression on the ultrastructure of constricted nerve site as well as in the dorsal root ganglia have not been studied in details. We investigated the effect of nerve decompression on ultrastructure of rat sciatic nerve and DRG by light and transmission electron microscopic methods. For this aim, CCI was established on the rat sciatic nerve with four loose ligatures. Surgical decompression was held at 1st, 3rd and 5th the weeks after CCI by removing the ligatures. Our results suggest that the efficacy of decompression was superior when applied one week after compression. The results of the study verify the need for early surgical decompression to prevent irreversible damage of the peripheral nerve and DRG.

Keywords: *Decompression, Sciatic nerve, Chronic constriction injury, TEM*

Periferik Sinirde Kronik Konstrüksiyon Hasarı Tedavisi Üzerine Dekompresyonun Etkisi

Özet

Kronik konstrüksiyon hasarı (CCI), yaygın klinik bir oluşumdur ve allodini ya da spontan nöropatik ağrı ile karakterize edilir. Nöropatik ağrının tedavisi, altta yatan mekanizmaların yeterince bilinmemesi ve mevcut ilaçların sınırlı etkisinden dolayı oldukça zordur. Cerrahi dekompresyon uygulaması, sıkışmış sinirin serbestleştirilmesi suretiyle oldukça radikal bir tedavi imkanı sağlar. CCI'da ağrının yanısıra morfolojik değişimler de olur. Siyatik sinirin yaralı bölgesi ve dorsal kök gangliyonundaki (DRG) ultrayapısal morfolojik değişimlerin, CCI patojenezinde ve bireylerdeki nöropatik ağrı davranışı gelişiminde rol oynadığına inanılır. Ancak, sıkışmış sinir bölgesinde ve dorsal kök gangliyonlarındaki ultrayapı üzerine cerrahi dekompresyonun etkileri henüz detaylı olarak çalışılmamıştır. Çalışmamızda rat siyatik siniri ve DRG'nun ultrayapısı üzerine sinir dekompresyonunun etkisini ışık ve geçirimli elektron mikroskobik metodlarla inceledik. Bu amaçla CCI rat siyatik siniri üzerine yapılan dört gevşek bağ ile oluşturulmuştur. Cerrahi dekompresyon, CCI sonrası 1. 3. ve 5. haftalarda bağların uzaklaştırılmasıyla sağlanmıştır. Verilerimiz dekompresyon etkisinin sinir sıkıştırılmasından bir hafta sonra uygulandığında çok daha iyi sonuç verdiğini desteklemektedir. Erken cerrahi dekompresyonun, periferik sinir ve dorsal kök ganglionunun geri dönüşümsüz hasarını önlemeye etkileri üzerine ilave çalışmalara ihtiyaç bulunmaktadır.

Anahtar sözcükler: *Dekompresyon, Siyatik sinir, Kronik konstüksiyon hasarı, TEM*

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INTRODUCTION

Neuropathic pain arises as a consequence of nerve injury either of the peripheral or central nervous system. It tends to be chronic, and less responsive to the administration of analgesic drugs and other conventional medical management [1,2]. Following peripheral nerve injury, a cascade of events in the primary afferents leads to peripheral sensitization, resulting in spontaneous nociceptor activity, decreased threshold, and increased response to suprathreshold stimuli [3]. However, the pathophysiological mechanisms underlying neuropathic pain are poorly understood and the available treatments are unsatisfactory [4].

Animal models of neuropathic pain are available that help to clarify the underlying mechanisms [5]. Especially, compression-related nerve injury is the main model for neuropathic pain, and it includes chronic construction injury (CCI), partial sciatic nerve ligation, and spinal nerve ligation [6]. CCI is relatively simple to perform, and produces robust and stable pain hypersensitivity for at least one month after injury. It is also commonly used to investigate both the pathophysiology, and potential therapeutic agents for treatment of neuropathic pain [7].

In clinical practice, surgical decompression is frequently used to relieve symptoms of neuropathic pain, e.g. carpal tunel syndrome, spinal root compression, and trigeminal neuralgia due to vascular compression [8]. Few studies have investigated the molecular mechanisms of CCI and decompression [9] whereas some studies evaluated morphological changes [10-12]. However experimental evidence on neuropathic pain and CCI is still lacking. Further research is required to correlate the effects of decompression on neuropathic pain mechanisms and morphological changes in constricted nerves.

The aim of this study is to investigate changes in the ultrastructural morphology of constricted peripheral nerve and its associated DRGs and whether surgical decompresion at given times after established CCI can reverse these changes.

MATERIAL and METHODS

All the experiments were approved by the Local Ethical Committee of Osmangazi University (Protocol No: 376/2014) in Eskisehir (Turkey) and conducted according to the healthcare guidelines for the laboratory animals and Universal Declaration on Animal Welfare. The animals used for the experiments were provided by the Center for Medical and Surgical Research (TICAM). The animals were housed in seperate cages with day and night cycle. Access to free standard rodent food and water *ad libitum* were allowed for all animals in the course of experiments.

Thirty adult female Sprague-Dawley rats, weighing 250-300 g, were used in this study. Rats were divided randomly in five groups (n=6). The first group comprised of SHAM operated rats (SHAM). CCI was established in the other groups which were entitled as control (C) and chronic constriction release groups at first, third and fifth weeks after CCI (CCR1w, CCR3w, CCR5w) respectively.

Surgical procedures were performed under Thiopenthal Sodium (40-50 mg /kg) anesthesia via intraperitoneal injection. After depilation of the right hindlimb the right sciatic nerve was exposed at the mid-thigh level. CCI was induced by four silk ligatures loosely tied around the nerve at 1 mm intervals proximal to the trifurcation. Thereafter the wound was closed by muscle and skin layers and animals were left to recover. The constricting ligatures were remained in control animals for 8 weeks. CCR groups were operated for surgical decompression at given times under the same anesthesia procedure. All four ligatures were carefully removed at first, third and fifth weeks after CCI. All experiments were ended for CCR groups at the end of the 8th week from the CCI operation.

Animals were euthenized by blood exsanguination and intracardiac perfusion of 2.5% gluteralaldehyde in 0.1M pH 7.4 Sodium phosphate buffer (PBS). Right Sciatic nerve segments (site of the constriction and its 5mm proximal and distal parts) and associated DRGs were dissected out carefully and stored in the same fixative solution.

Light and Transmission Electron Microscopy

Tissue samples were prepared by fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 24 h at 4°C and then rinsed with phosphate buffer. Specimens postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h at room temperature. All specimens were then dehydrated in graded solutions of ethyl alcohol (30%, 50%, 70%, 90%, 96% and 100%) and embedded in epon-araldite resin. Semithin sections (700 nm) were collected at a variety of depths of the sample and stained with toluidine blue. The sections were examined by light microscope (Olympus BX50) to select appropriate areas for TEM analysis. Degenerated axons were determined by two criteria involving myelin debris formation and finer degeneration in axons. In addition, the number of normal and degenerated nerve fibres was counted and Degenerated/Normal Axons Ratio (Deg/Nor) were calculated. The measurements were done blindly by two investigators (O.O and I.D) to minimize subjectively affected differences in results. Later, ultrathin sections (60 nm) were taken on an ultramicrotome (Leica Ultracut RM, Wetzlar, Germany) and counterstained with uranyl acetate-lead citrate [13,14]. They were examined and photographed using a TEM (JEOL JEM 1220) with digital imaging capabilities.

Data Analysis

All data were obtained and originally analyzed uzing SPSS v2.2 for Windows (IBM Inc. Istanbul, Turkey). Shapiro-Wilk test was used to assess the normal distribution of

data. Independent samples Kruskal -Wallis test was used for comparing differences between groups. Data were presented both as mean ±SD and median (25%, 75%) percentiles. *P* <0,05 was accepted as statistically significant.

RESULTS

Light Microscopic Results

Light microscopic findings showed the normal structural features in the SHAM animals. Myelinated axons of sciatic nerve was normal in appearance and a few unmyelinated axons were observed *(Fig. 1a)*. After CCI, axonal damage and thinly myelin sheaths were increased and common decrease was seen in the number of myelinated axon. Some areas with a depletion of nerve fibers were also shown *(Fig. 1b)*. One week after decompression, significant increase in the number of myelinated axon was observed and only a few degenerated fibres were determined. Remyelinised axons were also abundant *(Fig. 1c)*. After the third week, several degrees of axonal injury were seen and its progression to myelinated fiber degeneration are shown. Degenerated myelin fibres increased from the third to fifth week decompression. Morphological signs of axonal regeneration were reduced remarkably *(Fig. 1d-e)*. DRGs associated both in the control and CCR1w animals showed compact and regularly arranged structures *(Fig. 2a-c)*. After CCI, the major pathological change was that of microvacuolisation of the DRG cells *(Fig. 2b)*. In addition, cell membrane lines were lost and their sizes were larger. These changes were seen in CCR3w and CCR5w groups less frequently *(Fig. 2d-e)*.

In the present study compared to the the SHAM group (0.07±0.01), CCI group (1.98±0.20) demonstrated a significant increase in Deg/Nor ($P<0.05$). A significant decrease was found in Deg/Nor of CCR1w (0.20±0.03) compared to CCI ($P<0.05$). However there were no significant differences between CCI, CCR3w (0.50±0.07) and CCR5w group (1.00±0.22) ($P>0.05$). In addition, no significant differences were found in Deg/Nor among CCR1w, CCR3w and CCR5w ($P>0.05$). The data were summarized in *Table 1* and *Fig. 3*.

Electron Microscopic Results

Changes in the ultrastructure of nerve fibres became increasingly noticeable in TEM studies. Axons in SHAM groups exhibited an organized, dark stained filament network. The cytoplasm of the axon appears normal ultrastructural morphology. In CCI group, the most remarkable morphological changes that occurred in myelinated axons were vacuolisation, myelin sheath degeneration loss of axoplasm, and lamellar separation. Also, blebbing lipid-like materials on axonal shrinkages were determined *(Fig. 4a-c)*. In addition, increased numbers of mitochondria and dense neurofilament network were observed in myelinated axon. There were no significant changes in the ultrastructure of unmyelinated fibers in all groups compared to SHAM. Decompression at one week after CCI, no axonal shrinkage or myelin sheath degeneration were observed *(Fig. 5a)*. In particular areas, uncompleted remyelinisation was also observed *(Fig. 5b)*. In general, a few large vacuoles and several mitochondria were observed in axoplasm. Decompression at third and fifth week, myelin sheath degeneration accompanied with less marked regeneration of related structures. Myelinated axons exhibited axon-myelin separations and numerous intracellular organelles.

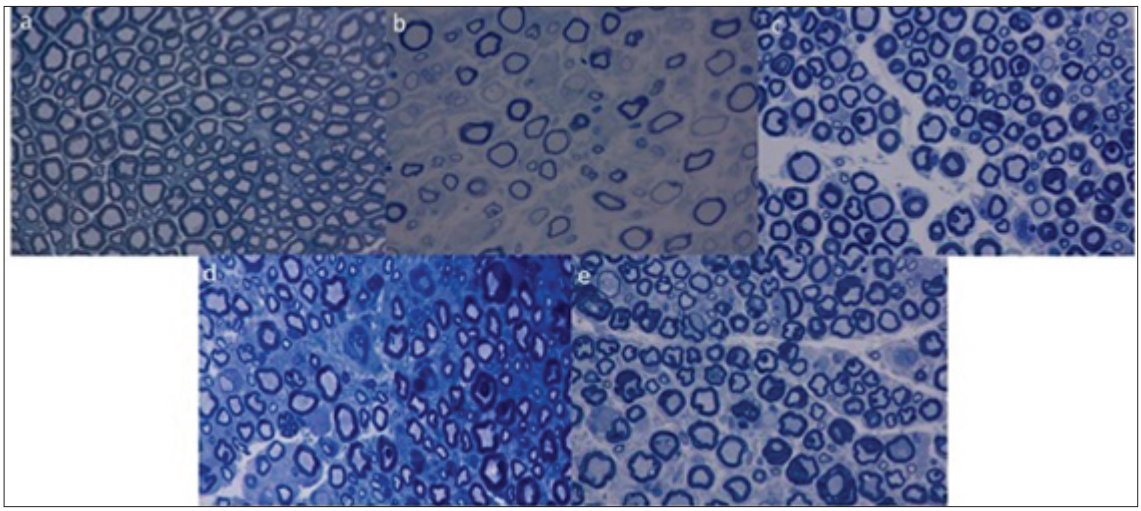

Fig 1. Semithin sections stained with toluidine blue of the sciatic nerve. a- Normal neural morphology in SHAM group, b- Note that the swollen appearance of the axons and the decrease in number of axons with intact myelin sheath in CCI group, c- Marked regeneration of axons of CCR1w group, d,e- Less marked recovery of the neural structures in CCR3w and CCR5w group, respectively (Bar: 10 µm)

Şekil 1. Siyatik sinirin toluidin mavisi ile boyanmış yarı ince kesitleri. a- SHAM grubunda normal noral morfoloji, b- CCI grupta aksonların şişmiş görünümü ve intakt miyelin kılıflı aksonların sayısında azalma, c- CCR1w grubunda aksonların belirgin rejenerasyonu, d,e- Sırasıyla CCR3w ve CCR5w gruplarında noral yapının daha az belirgin düzelmesi (Bar: 10 µm)

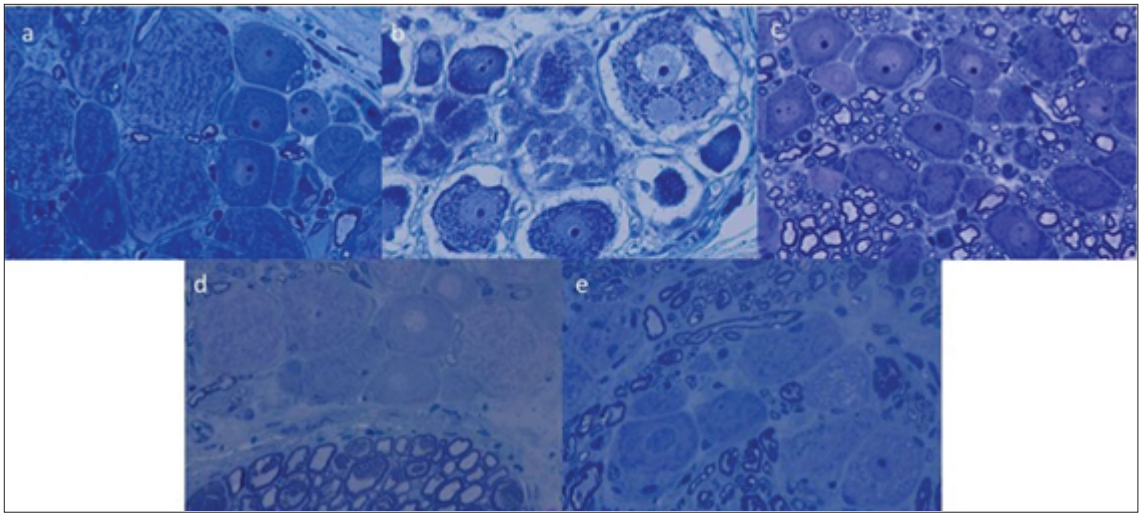

Fig 2. Semi-thin sections of the dorsal root ganglions of same samples. Figures show parallel data with sciatic nerve. a- SHAM, b- CCI group, c- CCR1w, d- CCR3w, e- CCR5w, note that the microvacuolisation of DRG cells in CCI and CCR5w groups (Bar: 10 µm)

Şekil 2. Aynı örneklerin dorsal root ganglionlarının yarı ince kesitleri. Şekiller siyatik sinir ile benzer verileri gösterir. a- SHAM, b- CCI grup, c- CCR1w, d- CCR3w, e- CCR5w, CCI ve CCR5w gruplarında DRG hücrelerinin mikrovakuolizasyonu görülmektedir

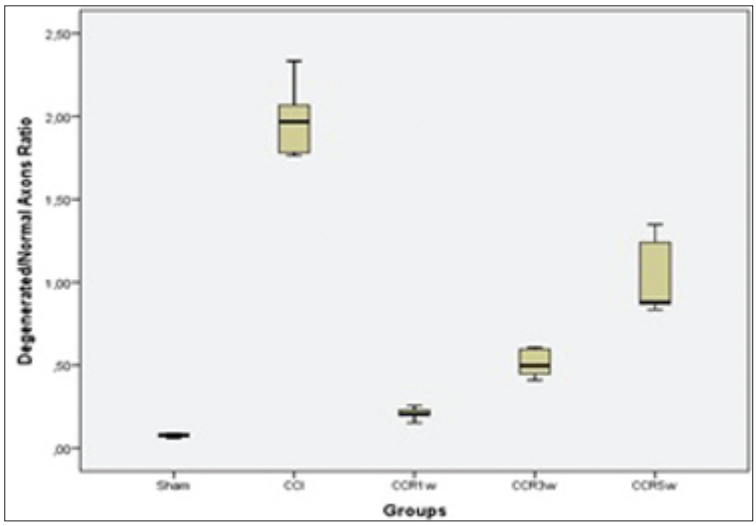

Fig 3. Graphic presentation of the mean numbers of degenerated/normal axon ratios

Şekil 3. Dejenere/normal akson oranlarının ortalama sayılarının grafik gösterimi

Table 1. *The number of degenerated/normal axon ratios counted in semithin sections obtained from each study group*

Tablo 1. *Her çalışma grubunda yarı ince kesitlerin sayımından elde edilen dejenere/normal akson oranları*

Groups	Degenerated/Normal Axons Ratio	
	Mean±STD	Median (25%-75%)
SHAM	0.07±0.01	0.07 (0.06- 0.08)
CCI	1.98±0.20	1.96 (1.77-2.13)
CCR1w	0.20±0.03	0.20 (0.18-0.23)
CCR3w	0.50±0.07	0.49 (0.43-0.59)
CCR5w	1.00±0.22	0.88 (0.86-1.26)

In some areas, axonal shrinkage occurred *(Fig. 6a)*. Loss of axoplasm was abundantly. Separations between the axoplasm and myelin sheath were prominent. Widened interface was observed between the Schwann cell and the axoplasm *(Fig. 6b)*. In addition, unmyelinated nerve fibres showed no important morphological changes except a few vacuol formation.

The DRG of SHAM showed normal histologic features. Neurons are surrounded satellite glial cells (SGCs) and nuclei of SGC is visible *(Fig. 7a)*. The DRG morphology was found similar in both CCR1w and CCR3w groups as in SHAM. No degenerative changes were observed in DRGc and Surrounding SGCs. Massive degenerations such as loss of cell membrane and swelling were determined in CCI and CCR5w. However the most striking alteration in these latter groups was vacuolisation in DRGc. These vacuoles appeared as variably sized, but generally they were in clear content. Some dark inclusions were also determined within the cytoplasm *(Fig. 7b)*. Loss of cell membranes of DRGc and cytoplasmic vacuolation were the typical characteristics of apoptosis in some sections of both CCI and CCR5w groups *(Fig. 7c)*. Electron microscopic results were summarized in *Table 2*.

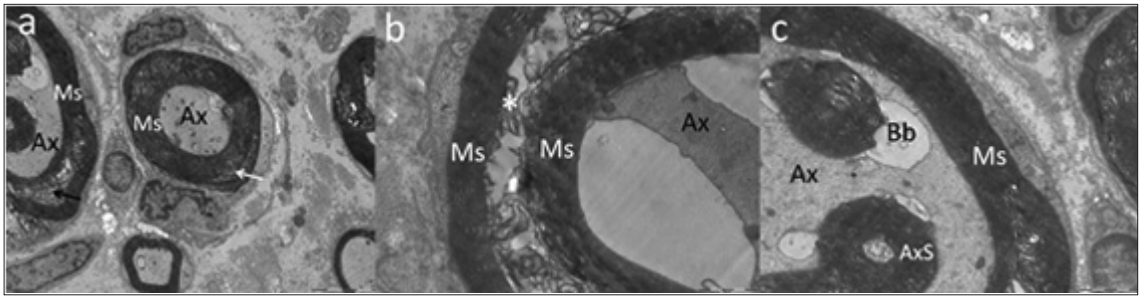

Fig 4. Transmission electron microscopic (TEM) analysis results of rat sciatic nerve after chronic construction damage (CCI). a- There are some vacuoles *(white arrow*) and myelin separations *(black arrow)* (Ms: myelin sheath; Ax: axon), (Bar: 5 µm), b- Axon showed the high degree of axoplasm loss. White asterisks indicates the lamellar separation of myelin sheath (Bar: 1 µm), c- Axonal shrinkage (AxS) is seen in axon. Bb indicates blebbing lipid-like materials on axonal shrinkages (Bar: 2 µm)

Şekil 4. Rat siyatik sinirine uygulanan Kronik Konstriksiyon hasarından (CCI) sonra elde edilen transmisyon elektron mikroskobik (TEM) sonuçların analizi. a- myelin seperasyonlar *(siyah ok)* ve vakuoller *(beyaz ok)* (Ms: myelin kılıf; Ax: akson), (Bar: 5 µm), b- aksoplazma kaybının çok olduğu aksonlar görülmektedir. Beyaz yıldızlar myelin kılıftaki lameller seperasyonu göstermektedir (Bar: 1 µm), c- aksonda aksonal şişme (AxS) görülmektedir. Bb aksonal büzüşmede blebbing lipid benzeri materyali göstermektedir (Bar: 2 µm)

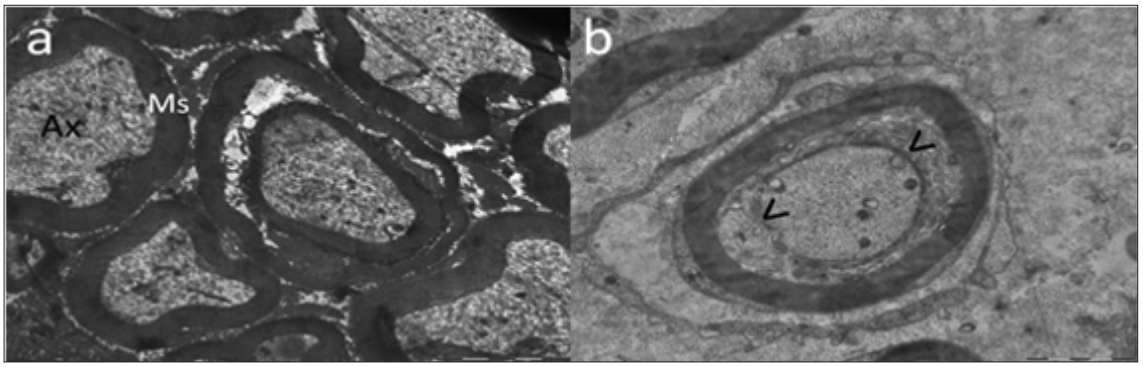

Fig 5. a- Transmission electron microscopic (TEM) analysis results of sciatic nerve from decompression at one week after CCI (CCR1w). Myelinated sheaths (Ms) show normal ultrastructural features. The cytoplasm of the axon (Ax) demonstrates no morphological abnormality (no axonal shrinkage or myelin sheath separation) (Bar: 5 µm), b- *black arrowheads* indicate uncompleted remyelinisated areas (Bar: 2 µm)

Şekil 5. CCI (CCR1w) den bir hafta sonra serbestleştirilen siyatik sinirin transmisyon elektron mikroskobik (TEM) analiz sonuçları. Miyelin kılıflar normal ultrastrüktürel yapı göstermektedir. Aksonların sitoplazması morfolojik olarak normal görünümdedir (aksonal büzüşme veya miyelin kılıf seperasyonu yok) (Bar: 5 µm), b- *siyah okbaşları* inkomplet remyelinize alanları göstermektedir (Bar: 2 µm)

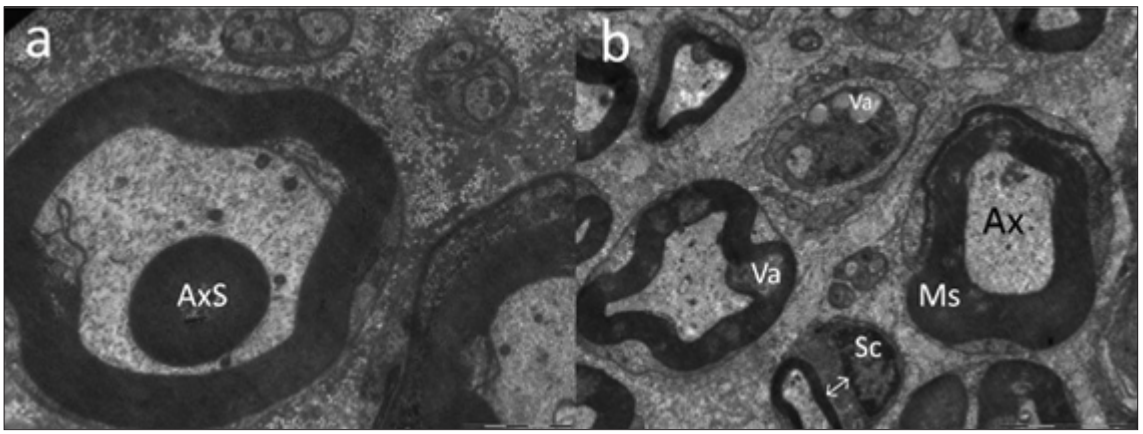

Fig 6. Transmission electron microscopic (TEM) analysis results obtained from decompression at five week after chronic construction injury (CCR5w). a- on left image, there is an axonal shrinkage (AxS) (Bar: 2 µm), b- The nerve fibers included in figure on right are vacuolisation areas (Va); *two headed arrow* demonstrate the widened interface was observed between the schwann cell and the axoplasm (Bar: 5 µm)

Şekil 6. Kronik konstriksiyon hasarı sonrası beşinci haftada serbestleştirilen gruptan (CCR5w) elde edilen Transmisyon elektron mikroskobik (TEM) görüntüler. a- soldaki görüntüde bir axonal büzüşme var (AxS) (Bar: 2 µm), b- sağdaki şekilde sinir lifinde vakuolizasyon alanı (Va); *iki başlı ok* schwann hücresi ve aksoplazma arasındaki genişlemiş arayüzü göstermektedir (Bar: 5 µm)

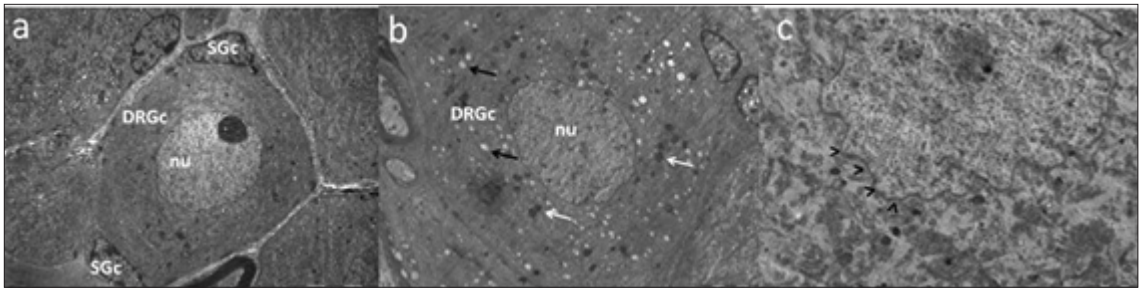

Fig 7. Transmission electron microscopic (TEM) analysis results of normal and damaged DRGc. a- Normal ultrastructural morphology in SHAM. Similar findings were found in CCR1w and CCR3w groups (Bar: 10 µm), b- loss of cell membrane and swelling in CCI. Clear vacuoles *(black arrow)* and dark inclusions *(white arrow)* in the cytoplasm (Bar: 5 µm), c- Apoptotic changes in cell membranes *(arrowhead)*, DRGc: Dorsal Root Ganglion cells, SGc: Satellite Glial cell, Nu: Nucleus (Bar: 2 µm)

Şekil 7. Normal ve hasarlı DRG hücrelerinin transmisyon elektron mikroskobik (TEM) analiz sonuçları. a- SHAM grubunda normal ultrastrüktürel yapı. CCR1w ve CCR3w gruplarında benzer bulgular elde edildi (Bar: 10 µm), b- CCI grubunda hücre membran kaybı ve şişme. Sitoplazmada vakuoller *(siyah ok)* ve siyah inklüzyonlar *(beyaz ok)* (Bar: 5 µm), c- hücre membranında apopitotik değişiklikler *(okbaşı)*, DRGc: Dorsal Root Gangliyon hücreleri, SGc: Satellit Glial hücreler, Nu: Nucleus (Bar: 2 µm)

Table 2. *Summary of the ultrastructural changes in sciatic nerve and its associated DRG*
Tablo 2. *Siyatik sinir ve ilişkili DRG nin ultrastrüktürel değişikliklerinin özeti*

Groups	Sciatic Nerve		Dorsal Root Ganglion	
	LM*	TEM*	LM	TEM
SHAM	Normal myelinated and unmyelinated fibers	Normal myelinated and unmyelinated fibers, typical appearance axoplasm and myelin sheath	Centrally placed nuclei, neurons are completely covered by satellite cells, most of the cell have an angular outline	Satellite glial cells around the neurons, quite evenly distributed organelles throughout the cytoplasm
CCI	Axonal damage, thin myelin sheaths,common decrease in the number of myelinated axon	Vacuolisation, loss of axoplasm, myelin sheath, degeneration lamellar separation. increased numbers of mitochondria, blebbing lipid-like materials on axonal shrinkages	Microvacuolisation of the DRG cells, weakness in cell membrane lines, swollen cells	Microvacuolisation of the DRG cells, weakness in cell membrane lines, swollen cells
CCR1w	Marked increase in the number of myelinated axon, only a few degenerated fibres, Remyelinised axons	No loss of axoplasm, axonal shrinkage or myelin sheath degeneration, uncompleted remyelinisation areas. a few large vacuoles several mitochondria	Normal morphological findings	SHAM like, normal morphological findings, a few axon-myelin separation
CCR3w	Myelinated fiber degeneration	Less marked regeneration	A few microvacuolisation in DRG cells, weakness in cell membrane lines, swollen cells	Axon-myelin separation, Loss of axoplasm, Increased mitochondria, Lamellar separation, Normal nuclear membrane morphology, Axonal shrinkage
CCR5w	Marked increase in degenerated myelin fibres counts	Axon-myelin separations Increasing electron dense in some unmyelinated axons, axonal shrinkage, Lost of axoplasm	Increasing microvacuolisation in DRG cells, loss of cell membrane	Loss of cell membranes, Many vacuolisation in cytoplasm, increased mitochondria, dark inclusions, axon-myelin separation, neuronal membrane damage

DISCUSSION

CCI of the rat sciatic nerve is an injury model established to investigate neuropathic pain. The injury is characterised behaviorally as mechanical allodynia and thermal hyperalgesia after a few day of the lesion. While some neurotransmitters such Substance P,CGRP and pERK play role in development of neuropathic pain [9,15] morphological changes may occur at the injury site of sciatic nerve [10,16-18], endorgan [8] or dorsal horn [9,15] which is central terminals of for nociceptive information and DRG [11]. Neuropathic pain is treated with drugs symptomatically in clinical practice [19,20] and also various agents were tested experimentally after CCI established in animals [21,22]. However conservative treatment of neuropathic pain is still limited [19]. Unique radical intervention is decompression which is performed by removing of the ligatures on the sciatic nerve and works for treatment of underlyig cause. It is shown that decompression not only relieves neuropathic pain; in addition, it also promotes the regeneration in associated central and peripheral nerves [23].

In the present study the effect of CCI and CCR on morphology of constricted site of nerve and associated DRG have been investigated in an conventional animal

model. CCI caused marked decrease in the number of myelinated axon, loss of axoplasm and vacuolisation at the constricted site of the nerve. deg/reg axon ratio was high in CCI group. In TEM analysis these pathological features were more evident. Axon-myelin separations, vacuolisation and loss of axoplasm were observed exhaustively. Large myelinated axons were found more degenerated compared to other structures. Surprisingly, unmyelinated fibers were not affected from CCI. This findings are somewhat consistent with previous studies. Similar microscopic findings have been reported with CCI injury in the rat, with massive degeneration of large myelinated fibers and less severe changes in small myelinated fibers and unmyelinated fibers [24-26]. Later Prinz et al.[10], have reported the ultrastructural changes of peripheral nerve lesions induced by chronic compression using an experimental model by light and electron microscopy myelinated axons at the compression sites displayed a remarkable increase in the number of small axons up to 60% in comparison with the normal axons. Additionally, remarkable axonal degeneration was obswerved both in central and in marginal regions of the distal to the constriction. In contrast, we observed significant decrease in the number of both small and large myelinated axons. However, distal and proximal nerve sections were found less damaged, and they were limited with a few lamellar separations in our study (data not shown).

The current scientific information suggests that the DRG is an active participant in the development of neuropathic pain [27]. Although Changes in gene expression of inflammatory cytokines, neuropeptides and ion current of ion channels in response to CCI or other types of nerve injuries have been reported previously [27-29] morphologic changes within the DRG have been less documented [11]. In addition to constricted peripheral nerve segment histopathologic changes were observed within the DRG in the present study. The major morphologic change was formation of microvacuoles within the DRG cells of the CCI group which appeared both in in light and TEM sections. As known, neuronal vacuolisation may be seen in neurons undergoing degeneration and it is a particular concern for neuropathologic significance of DRG. Besides loss of cell mebrane and swelling of the DRG cells was also characteristic.

The morphologic changes in CCI injury were clearly demonstrated in the present work and previous studies. decompression can prevent or reverse these pathological conditions, in particular if performed early. In CCR1w group deg/normal axon ratio was found lower than CCI, CCR3w and CCR5w groups. Myelin structures and axoplasms thereby myelin-axon integrity were preserved in CCR1w. Normal schwann cell appearance and less lamellar separation was observed in TEM. Similar to our findings Jancalek and Dupovy [11] showed that decompression after 1-week compression caused a rapid increase in the number of both small and large myelinated axons within the spinal root including the site of compression. In contrast the degenerative processes with a reduction of the MA number were very distinct when spinal roots were compressed for 5 weeks with subsequently decompressed for 3 weeks similar to the findings of our CCR3w and CCR5w groups.

Decompresssion also reversed the changes in the sensorial terminals of the peripheral nerves. Attenuation of neuropathic pain by surgical decompression caused normalization of dorsal horn activities [9,15]. The structure of DRG cells were preserved after decompression at first and third weeks whereas degeneration of DRG cells in CCR5w group persisted. However the recovery of peripheral nerve and its central terminals do not guarantee recovery at the endorgan *etc* skin. Although nerve decompression was accompanied with the disappearance of neuropathic pain behaviors after CCI, morphological studies have shown the evidence of an incomplete skin reinnervation [8,17].

The present study posesses two limiting factors. First the scope was restricted to investigate only morphologic changes of constricted nerve and its associated DRGs during CCI and after decompression. Thus behavioral tests to evaluate the signs of neuropathic pain such as allodynia and hyperalgesia or biochemichal analysis to determine neuroinflammatory response were not conducted. Second the pathologic condition is limited to eight weeks period intervened at first, third and fifth week to determine the appropriate timing of decompression for a given disease period. Therefore time course after decompression was not equal between the groups. One can argue that recovery period was shorther for late decompressed groups. Thus degeneration is more apparent in these groups. On the contrary, El-Barrany et al.[30] reported that degenerative changes have been reversed as time course after decompression prolonged. According to their findings, severe degenerative changes in schwann cells and myelinated axons were observed one week after decompression. After the second week of decompression, the endoneurium showed extentive edema with an increase in the regenerating myelinated and unmyelinated nerve fibers. Three weeks after decompression, edema decreased and six weeks after decompression, the endoneurium appeared nearly normal. Nevertheless a further study in our laboratory was planned to investigate how decompression at first, third and fifth weeks affect on morphologic recovery of peripheral nerve and DRG and whether these changes are reversible after a constant follow-up period (8 week after decompression).

Results of our light and electron microscopy studies showed that early decompression preserve the histopathological features of nerve at the constriction site and DRG cells. In the late decompression groups, ultrastructural damages were observed as more intensely similar to the CCI group *(Table 2)*.

Our study data support previous reports of the importance of early surgical intervention in providing optimal treatment for nerve compression conditions. Future quantitative histological studies on regeneration of constricted sciatic nerve will enable us to have better understanding of the these mechanisms.

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Research Article

Protective Effect of *Morinda citrifolia* (Noni) on 3-methyl-4-nitrophenol-induced Injury in Rat Testes [1]

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Abstract

In this study, the protective effect of *Morinda citrifolia* (Noni) against 3-methyl-4-nitrophenol (PNMC) toxicity in rat testes was investigated with an experimental period of five days. Fifty-six adult male Sprague-Dawley rats were allocated into seven experimental groups and a control (n:7). One group received only Noni. Testicular tissue injury of six experimental groups was induced by subcutaneous injection of PNMC at three different doses (1, 10 and 100 mg/kg) and three received Noni treatment (2 ml per rat by gavage). On day six all rats were sacrificed and then blood samples and testis tissues were collected. Serum testosterone, FSH and LH levels were assessed. Testicular tissues were evaluated histomorphometrically in terms of tubular diameter, seminiferous epithelium density, luminal space and interstitial tissue and immunohistochemically labelled with inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) markers to assess oxidative damage. Histomorphometrically, most severe tissue injury was observed in the group of 10 mg/kg PNMC. Tissue injury improved significantly in its corresponding treatment group (with Noni). iNOS and eNOS levels increased in all PNMC groups and decreased with Noni treatments. Noni was most effective in the group of 100mg/kg PNMC in terms of oxidative damage. Serum hormone levels revealed no significant results. In conclusion, Noni reduced PNMC-induced tissue injury in rat testes.

Keywords: *3-methyl-4-nitrophenol, Rat, Noni, Testes, iNOS, eNOS*

Sıçan Testisinde 3-Metil-4-Nitrofenol İle Oluşturulan Hasara Karşı *Morinda citrifolia* (Noni)'nın Koruyucu Etkisi

Özet

Bu çalışmada beş günlük deneysel bir periyotla *Morinda citrifolia* (Noni)'nın sıçan testislerinde 3-metil 4-nitrofenol (PNMC) toksisitesine karşı koruyucu etkisi araştırıldı. Ellialtı adet yetişkin erkek Sprague-Dawley sıçanı eşit sayıda yedi deneysel, bir kontrol grubuna ayrıldı (n:7). Bir gruba sadece Noni uygulandı. Altı deneysel grupta testiste doku hasarı farklı dozlarda (1, 10 and 100 mg/kg) subkutan PNMC enjeksiyonu ile indüklendi ve üç gruba Noni tedavisi uygulandı (her sıçan için 2ml gavaj ile). Altıncı günde tüm sıçanlar sakrifiye edilerek kan ve testis doku örnekleri toplandı. Serum testosteron, FSH ve LH seviyeleri değerlendirildi. Testis dokuları, tubuler çap, seminifer epitel yoğunluğu, lumen aralığı ve interstisyel doku açısından histomorfometrik olarak incelendi ve oksitatif hasarı değerlendirmek üzere indüklenebilir nitrik oksit sentaz (iNOS) ve endotelyal nitrik oksit sentaz (eNOS) belirteçleri ile immunohistokimyasal olarak işaretlendi. Histomorfometrik olarak en şiddetli doku hasarı 10 mg/kg'lık PNMC grubunda gözlendi. Doku hasarı bu gruba karşılık gelen tedavi (Noni ile) grubunda anlamlı ölçüde iyileşti. iNOS ve eNOS düzeyleri tüm PNMC gruplarında yükseldi ve Noni tedavisi ile aynı değerler düşüş gösterdi. Noni'nin oksidatif hasar bakımından en çok 100mg/kg'lık PNMC grubunda etkin olduğu izlendi. Serum hormon değerleri anlamlı sonuçlar vermedi. Sonuç olarak, Noni, sıçan testislerinde PNMC ile indüklenen doku hasarını azalttı.

Anahtar sözcükler: *3-metil 4-nitrofenol, Sıçan, Noni, Testis, iNOS, eNOS*

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INTRODUCTION

Nowadays, accelerating air pollution due to industrialization has become a serious threat for environmental health. With the increasing use of motor vehicles, diesel exhaust particles (DEP) found in motor vehicle emissions have posed a worldwide health-threatening problem [1].

Miscellaneous compounds found in DEP are among endocrine impairing chemicals, which exhibit estrogenic and anti-androgenic activities [2-5]. DEP compounds were reported to have adverse effects on reproductive system and impair both reproductive and endocrine functions in male mice and rats [6,7]. Watanabe and Kurita [8] indicated a decrease in the Sertoli cell count of rats, which were exposed to diesel exhaust in the fetal period while testosterone levels of the animals were found to have increased.

3-methyl-4-nitrophenol (PNMC) is a nitrophenol derivative of DEP and an important metabolite of fenitrotion, which is a widely used organophosphate insecticide in agriculture. This pesticide, however, was shown to have accumulated in water, air and soil [9,10], which inevitably has become a growing health threatening problem for all living things [11,12].

Nitric oxide (NO) is a free radical which is synthesized from L-arginin by nitric oxide synthase (NOS). There are 3 isoforms of NOS: inducible (i) NOS, endothelial (e) NOS and neuronal (n) NOS. They are found at high levels in tissues during inflammation [13]. NO is known to exhibit cytotoxic effects since it interacts with superoxide which results in tissue damage [14]. In previous experimental studies NO was shown to induce testicular tissue damage, as well [15]. In a study it was reported that NO exerted an inhibitory effect on testicular steroidogenesis. Although NO was reported to have shown preventive effect on testicular steroidogenesis, the specific site of action or its mechanism of action was not elucidated. Nonetheless, NO expression at high levels reduced testosterone production in testis [16].

Morinda citrifolia L. (Rubiaceae), which is an endemic and widespread plant species in the Pacific and tropical regions of Asia, is commonly named as Noni [17]. The fruit itself and its juice have been used in conventional medicine for prophylactic purposes and for the cure of miscellaneous diseases for many years [18]. Noni contains a number of compounds and enzymes which show antioxidant activity by supporting cellular functions [19,20].

Since there are limited numbers of studies with respect to utilizing antioxidants against PNMC-associated tissue injury, we designed this experimental model, in which tissue damage was induced in rat testes with PNMC, to investigate the putative protective effect of Noni owing to its antioxidant properties, by means of histomorphometric and immunohistochemical methods.

MATERIAL and METHODS

Animals

Fifty-six sexually-mature Spraque-Dawley male rats were purchased from the Institute of Experimental Medical Research, Istanbul University. These animals were housed in polypropylene cages with a 12-h light/dark cycle at 22- 24°C with 50% humidity. They were provided with standard laboratory chow and tap water ad libitum for at least 7 days of acclimation. All experiments were carried out according to the protocols approved by the Animal Care and Use (2013/53).

Administration of PNMC and Noni

3-methyl-4-nitrohenol (4-nitro-m-cresol, PNMC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA.) and was dissolved in phosphate buffered saline (PBS) containing 0.05% Tween 80 (Merck) before injection. Noni juice (99.5%) was provided from Alnoni Ltd. (Antalya, Turkey).

The animals were randomly divided into eight groups (n: 7). Noni juice was administered at a dose of 2 ml per rat, regardless of body weight via gastric gavage. The group designs were as follow:

Group 1: (G1; Control) received PBS containing 0.05% Tween 80 subcutaneously (s.c.)

Group 2: (G2) received Noni alone via gastric gavage

Group 3: (G3) received PNMC (1 mg/kg, s.c.)

Group 4: (G4) received PNMC (10 mg/kg, s.c.)

Group 5: (G5) received PNMC (100 mg/kg, s.c.)

Group 6: (G6) received Noni + PNMC (1 mg/kg, s.c.)

Group 7: (G7) received Noni + PNMC (10 mg/kg, s.c)

Group 8: (G8) received Noni + PNMC (100 mg/kg, s.c.)

Sample Collection

Animals in all groups were weighed and then sacrificed with an overdose of diethyl ether anesthesia 24 h after the last treatment. Blood samples were collected from each rat via cardiac puncture just prior to sacrifice and centrifuged at 2.500 x g for 15 min. Each serum sample was allocated into 1.5 mL microcentrifuge tube and stored at -80°C until further analysis. All samples were measured together centrally to avoid inter-assay variation. The right and left testes were excised, weighed separately and then processed for histological examination.

Hormone Assays

Rat serum follicle stimulating hormone (FSH) concentrations were determined by a commercial ELISA kit using a double antibody sandwich enzyme immunoassay technique (Cat. No. YHB0436Ra; Shanghai Yehua Biological Technology Co. Ltd, China). Each ELISA analysis was carried out according to the manufacturer's instructions. All tests showed intra-

assay and inter-assay coefficients of variations (CVs) below 10% and 12%, respectively. The analytical sensitivity of the test was 0.12 mIU/mL.

Rat luteinizing hormone (LH) levels were measured by a commercial ELISA kit (Cat. No. YHB0686Ra; Shanghai Yehua Biological Technology Co. Ltd, China). The analytical sensitivity was 0.051 µIU/mL. The intra- and inter-assay CVs were below 10% and 12%, respectively.

A commercial ELISA kit was used for rat testosterone measurements (Cat. no. YHB1031Ra; Shanghai Yehua Biological Technology Co. Ltd, China). The lowest limit of the assay was 0.25 nmol/L. The intra- and inter-assay CVs were under 10% and 12%, respectively.

Histomorphometric Evaluations

The testes were fixed in neutral buffered formalin (10%) for 24 h, routinely processed and embedded in paraffin. Paraffin blocks were sectioned at 4-5 µm thickness and then placed onto poly-L-lysine-coated slides. Finally, all slides were stained with hematoxylin and eosin (H&E) and examined by light microscopy for morphometric analyses.

For morphometric analysis, each testis of each animal was divided into four equal pieces. Fifteen randomly selected microscopic fields were evaluated on the sections obtained from each piece. For this purpose, we chose an area fraction approach with an area of an unbiased counting frame of 1.000 µm x1.000 µm. Meander sampling of each section was done in a 2.000 µm x 2.000 µm step size in a systematic-random manner.

The density of testicular tissue components was determined measuring by the density occupied by seminiferous epithelium, tubular lumen and interstitial tissue. The counting was performed by the standard point counting method. For this purpose, a 100-point grid printed on a transparency was placed over each unbiased counting frame field and the number of grid points over the seminiferous epithelium, tubular lumen and interstitial tissue was counted by a software program (stereo investigator, MBF Bio-science, version 9) associated to an Leica, DM400B microscope at x40 magnification. The arithmetic means of the values obtained were expressed as percentage values in 1 mm^2 area [21].

The diameter of seminiferous tubules was measured with a x100 magnification, using the software program (stereo investigator, MBF Bio-science, version 9) associated to light microscope (Leica, DM400B). Thirty tubular profiles that were round or nearly round were chosen randomly and measured for each animal [21,22].

Immunohistochemical Analysis

Testicular tissue samples were immunohistochemically examined for endothelial Nitric Oxide Synthase (eNOS) and inducible Nitric Oxide Synthase (iNOS) using the Streptavidin-Biotin Complex (Strep-ABC) method. Briefly, tissue sections from paraffin blocks were mounted on positively charged slides, deparaffinized and then subjected to antigen retrieval using citrate buffer solution, and endogen peroxidase and protein blocking procedures. They were incubated with commercially available, ready to use rabbit polyclonal primary antibodies for iNOS (clone RB-9242-R7; Thermo Scientific) and eNOS (clone RB-9279; Thermo Scientific) overnight at 4°C. Then they were treated with a commercial kit for secondary antibody (Thermo Scientific) and the reaction was visualized via AEC chromogen (3-amino-9-ethyl carbazole, Cat. No. TA-060-HA, Thermo Scientific). Finally, the sections were counterstained with Mayer's hematoxylin. The negative control sections were incubated with PBS instead of the primary antibody.

Immunohistochemical iNOS and eNOS staining were quantified using a histological scoring system (HSCOREs), which is a semiquantitative measurement of staining intensity and distribution. For this purpose, tissue sections were stained with antibodies against eNOS and iNOS and then observed under an Olympus microscope equipped with a special ocular grid. Cells were counted in at least 8-10 different regions at x400 magnification by two blinded observers. Based on staining intensity, positively stained cells were scored as: 0, no staining; 1, weak staining; 2, distinct staining; 3, intense staining. For each tissue, an HSCOREs was calculated using the equation: HSCOREs = S Pi (i + 1) where " i " is the intensity score and " Pi " is the corresponding percentage of stained cells with that score [23,24].

Statistical Analysis

All of the variables were analyzed using Kruskal -Wallis test. All calculations were carried out using the SPSS statistical software (version 13.0 for Windows, Chicago, IL, USA).

RESULTS

Body Weights and Testes Weights

Body weights, absolute weights of left and right testes and relative weights of both testes (testes /body weights) were shown in *Table 1*. There were no significant differences among the groups in terms of body weights, absolute testes weights and relative weights of both testes (P>0.05).

Histology and Histomorphometric Findings

There were no histopathological finding in the H&E stained sections of control and other groups *(Fig. 1)*.

Histomorphometric measurements of testes (diameter

Table 1. *Body weights, absolute testes weights, relative testes weights, and testicular morphometric parameters in the control and experimental groups*
Tablo 1. *Kontrol ve deney gruplarında vücut ağırlığı, absolut ve rölatif testis ağırlığı ve testisin morfometrik parametreleri*

Parameters	G1 Mean±SE	G2 Mean±SE	G3 Mean±SE	G4 Mean±SE	G5 Mean±SE	G6 Mean±SE	G7 Mean±SE	G8 Mean±SE	P Values
Body weight(g)	248±17	259±22	264±10	260±10	253±10	224±28	236±23	259±14	N.S
Left testis weight (mg)	1367±48	1538±82	1448±45	1516±44	1455±65	1338±58	1451±77	1467±56	N.S
Right testis weight(mg)	1375±34	1483±60	1418±55	1541±32	1413±64	1303±66	1456±104	1465±71	N.S
Relative testis weight (left) (mg/g b.w.)	5.5±0.2	6±0.3	5.4±0.2	5.8±0.3	6±0.1	6.3±0.5	6.3±0.4	5.7±0.1	N.S
Relative testis weight (right) (mg/g b.w)	5.6±0.2	5.7±0.3	5.4±0.3	5.9±0.2	5.8±0.2	6.1±0.5	6.3±0.5	5.7±0.1	N.S
Tubular diameter (µm)	278±5.5	285±3.1	282±5.5	282±2.8	267±6.1	278±6.9	273±5.5	274±7.2	N.S
Seminiferous epithelium (%)	$65.84±1.4^{ab}$	$64.53±1.5^{abc}$	$61.04±3.2^{bc}$	$59.33±0.7^{c}$	$62.56±3.3^{bc}$	$66.31±0.9^{ab}$	$70.38±1.1^{a}$	$63.05±1.1^{bc}$	<0.01
Luminal space (%)	13.74±0.6	14.27±0.9	15.08±0.8	14.53±0.6	15.56±1.2	14.71±1	15.07±1	15.07±0.9	N.S
Interstitial tissue (%)	$20.42±1.7^{b}$	$20.20±1.8^{b}$	$23.88±1.8^{ab}$	$26.14±0.4^{a}$	$21.88±1.3^{ab}$	$18.98±0.9^{bc}$	$14.55±1.5^{c}$	$21.88±3^{ab}$	<0.01

$^{a-c}$ Different superscripts within the same line demonstrate significant differences (P<0.01), NS: Not significant (P>0.05)

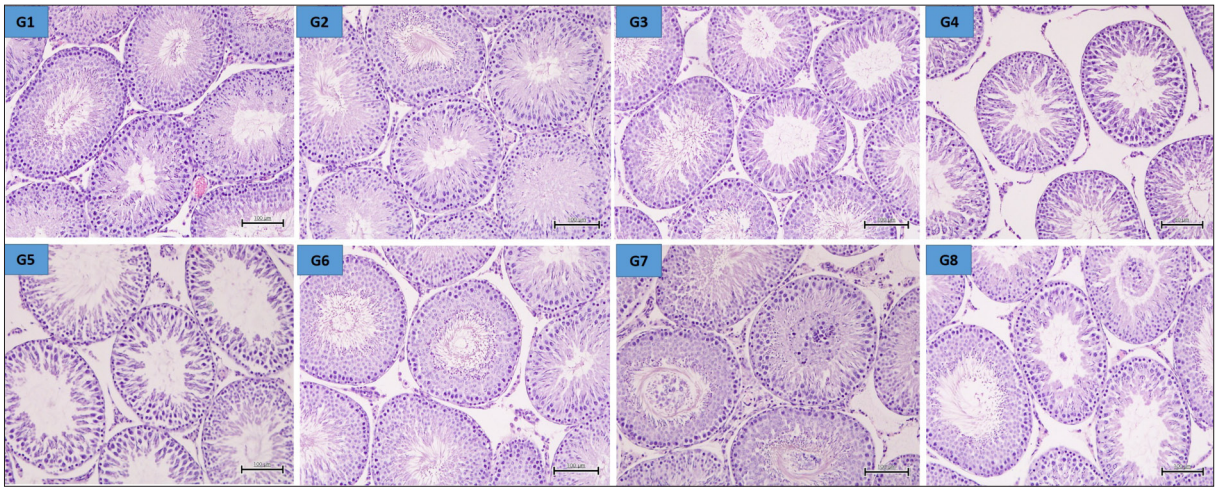

Fig 1. Histological architecture of the testes in all groups. **G1)** Control group, **G2)** Noni, **G3)** 1 mg/kg PNMC, **G4)** 10 mg/kg PNMC, **G5)** 100 mg/kg PNMC, **G6)** Noni + PNMC (1 mg/kg), **G7)** Noni + PNMC (10 mg/kg), **G8)** Noni + PNMC (100 mg/kg) H&E stain, Magnification x200.

Şekil 1. Tüm gruplarda testisin histolojik yapısı. **G1)** Kontrol grup, **G2)** Noni, **G3)** 1 mg/kg PNMC, **G4)** 10 mg/kg PNMC, **G5)** 100 mg/kg PNMC, **G6)** Noni + PNMC (1 mg/kg), **G7)** Noni + PNMC (10 mg/kg), **G8)** Noni + PNMC (100 mg/kg). H&E stain, x200 Büyütme

of seminiferous tubules, percentage values for density of seminiferous epithelium, luminal space and interstitial tissue) were given in *Table 1*.

On the basis of our findings no statistically significant difference was noted among groups in terms of tubular diameter and luminal space (P>0.05). Furthermore, the lowest value for tubular diameter was observed in G5. There was an increase in percentage value of luminal space in G3, G4 and G5 in comparison to those of G1 and G2 whereas there was a reduction in luminal density in G6, when compared with those of G3 and G5, which was not statistically significant (P>0.05).

In terms of seminiferous epithelium component, there was a statistically significant decrease (P<0.01) in G4 in comparison to G1 *(Table 1)*. Percentage value of density of seminiferous epithelium of G7 was found to have significantly increased (P<0.01) when compared with other groups *(Table 1)*.

When the groups were compared in terms of interstitial tissue component, the highest value was detected in G4, and this increase (P<0.01) was statistically significant compared to G1and G2. On the other hand, G7 exhibited the lowest value (P<0.01) and the difference was significant among groups except for G6 *(Table 1)*.

Biochemical Findings

Serum testosterone, LH and FSH levels were given in *Table 2*. Although serum testosterone levels increased in G3 when compared with other groups, this difference was far from being statistically significant (P>0.05).

FSH levels were observed to have increased in G4 in comparison to the control group while G5 revealed

Table 2. *Serum hormone levels for the control and experimental groups*
Tablo 2. *Kontrol ve deney gruplarında serum hormon düzeyleri*

Hormonal Parameters	G1 Mean±SE	G2 Mean±SE	G3 Mean±SE	G4 Mean±SE	G5 Mean±SE	G6 Mean±SE	G7 Mean±SE	G8 Mean±SE	P Values
Testosterone(nmol/L)	37.89±6.4	43.70±4.9	56.51±8.1	45.24±6.5	52.43±8.4	52.36±3.9	51.80±4.6	44.54±6.0	N.S
Follicle-stimulating hormone (mIU/mL.)	3.87±0.18	4.32±0.27	4.35±0.50	4.45±0.36	3.64±0.29	4.02±0.10	3.80±0.09	3.75±0.15	N.S
Luteinizing hormone (mIU/mL)	2.49±0.18	2.67±0.13	3.24±0.45	2.88±0.12	2.69±0.16	3.05±0.17	2.94±0.17	2.70±0.18	N.S

NS: *Not significant (P>0.05)*

Table 3. *iNOS and eNOS H-SCOREs values in the testicular tissue of the control and experimental groups*
Tablo 3. *Kontrol ve deney gruplarının testis dokusunda iNOS ve eNOS H-SKOR değerleri*

Parameters	G1 Mean±SE	G2 Mean±SE	G3 Mean±SE	G4 Mean±SE	G5 Mean±SE	G6 Mean±SE	G7 Mean±SE	G8 Mean±SE	P Values
iNOS	67.75±2.70^{a}	78.50±3.71^{a}	122.75±5.90^{c}	250.20±4.53^{e}	283.30±6.95^{f}	102.45±4.93^{b}	113.35±2.35^{bc}	190.75±3.48^{d}	<0.001
eNOS	45.50±2.90^{a}	57.40±2.74 ^{b}	70.70±1.58^{c}	145.40±1.67^{f}	177.95±2.73^{g}	63.05±2.81^{b}	87.30±2.27^{d}	132.90±2.02^{e}	<0.001

$^{a-g}$*Different superscripts in the same row indicate the significant difference (P<0.001)*

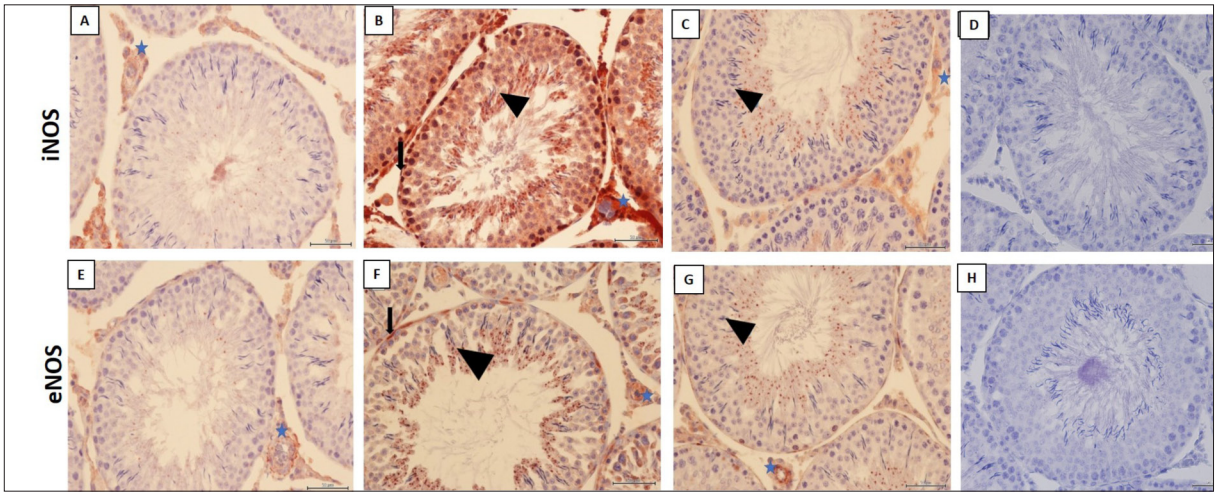

Fig 2. Immunohistochemical staining showing iNOS and eNOS expression in testes. **A,E)** Control grup, **B,F)** 100 mg/kg PNMC, **C,G)** Noni + PNMC (100 mg/kg), **D)** iNOS negative control, **H)** eNOS negative control. iNOS and eNOS immunostaining were seen within peritubular myoepithelial cells *(arrow)*, Leydig cells *(star)* and spermatids *(arrowheads)*, Magnification x400

Şekil 2. Testiste İNOS ve eNOS ekspresyonu gösteren immunohistokimyasal boyama **A,E)** Kontrol grubu; **B,F)** 100 mg/kg PNMC, **C,G)** Noni + PNMC (100 mg/kg), **D)** iNOS negatif kontrol, **H)** eNOS negatif kontrol. iNOS ve eNOS immun boyama peritubuler miyoepitelyal hücrelerde *(ok)*, Leydig hücrelerinde *(yıldız)* ve spermatidlerde *(okbaşı)* izlendi, x400 Büyütme

a reduction, which was not statistically significant (P>0.05).

Serum LH levels were higher in all experimental groups in comparison to the control. However, this change was statistically insignificant (P>0.05).

Immunohistochemical Findings

iNOS and eNOS H-SCOREs values were summarized in *Table 3*. iNOS and eNOS H-SCOREs values in G3, G4 and G5 were significantly higher (P<0.001) than those of G1 and G2). The relevant values showed a significant reduction (P<0.001) in G6, G7 and G8 when compared with those of G3, G4 and G5. Intensity of immunohistochemical staining for iNOS and eNOS was more prominent in the cytoplasm of peritubular myoepithelial cells, Leydig cells, and spermatids *(Fig. 2)*.

DISCUSSION

It is well known that DEP have serious adverse effects on male reproductive system. Numerous studies exhibited toxic effects of these particles on testicular tissue and spermatogenesis [6-8,25,26]. PNMC, one of the derivatives of DEP, is a degradation product of a widely used insecticide applied in agriculture and therefore it is quite likely to have been exposed to this chemical both in rural and residential environments [9,10]. Experimental animal models of PNMC-

induced tissue injury regarding male reproductive systems were carried out mostly with immature animals like rats, quails and mice and were mostly focused on serum hormone level changes of these animals [27-29].

Noni has been used in numerous animal models to demonstrate its protective and therapeutic effectiveness against cytotoxic agents including the chemicals which impair male reproductive system and its functions [30,31].

We designed this experimental model in mature rats since there is a limited number of studies with respect to PNMC toxicity on testicular tissue of adult animals. We evaluated its adverse effects with histomorphometric aspects which were also demonstrated by oxidative stress parameters with the anticipation that Noni might have been utilized in testicular injury, as well owing to its well-known therapeutic properties as an antioxidant.

On the basis of our findings PNMC administered at different doses did not have a significant impact on body weights. Likewise, no statistically significant difference was noted among groups in terms of testes weights. It was found that PNMC particularly at high doses decreased testes weights in immature rats [28,32]. Tsukue et al.[33] observed the adverse effects of DEP especially on accessory organs of male adult rats rather than on testes and they used fisher 344 rats in their research model unlike our design in which we used Sprague Dawley rats.

The difference in terms of tubular diameter and percentage value of luminal space was not statistically significant among our groups. Yue et al.[32] reported that tubular diameter increased significantly in immature rats. Bu et al.[34] showed that PNMC at low doses did not affect tubular diameter while high doses of the substance (100 mg/kg PNMC) increased tubular diameter and caused germ cell loss, which was compatible with our findings.

There is no data available with respect to the effects of PNMC on percentage values of seminiferous epithelium and interstitial tissue. In our study PNMC significantly reduced seminiferous epithelium component in the group which received PNMC at an intermediate dose (10 mg/kg) while yielding an increase in the density of interstitial tissue. This increase was considered to be associated with the thickening of interstitial region due to lymphatic dilatation and edema developed as a result of PNMC administration [35]. On the other hand, percentage value of seminiferous epithelium density increased while that of interstitial tissue decreased in G7 (Noni + 10 mg/kg PNMC). These findings revealed the effectiveness of Noni against the adverse effects of PNMC on seminipherous epithelium and interstitial tissue. It is clear that the inconsistency between the results of our histomorphometric measurements and those of the previous studies, in general, was associated with the age of our animals since most previous studies were performed on immature or growing animals of different breeds.

Testosterone and estradiol levels were shown to have significantly increased in the male offspring born from the females that were exposed to exhaust gas during pregnancy while FSH and LH levels were reduced [6]. Testosterone levels were increased in PNMC-treated castrated immature male rats while LH and FSH levels were decreased [27]. Li et al.[28] reported that PNMC decreased LH levels in immature male rats. Tsukue et al.[32] observed a marked and statistically significant increase in FSH, LH and testosterone levels in the male fisher 344 rats exposed to PNMC compared with the control group. On the basis of our findings these three hormone levels were observed to have increased though the difference was not statistically significant. The inconsistency in our findings when compared with those of the previous researches regarding the effects of PNMC on hormone levels might be associated with animal species, age, the duration of the experimental period and the individual susceptibility.

NOS, which is expressed also in male reproductive system under physiological conditions is essential for the maintenance of spermatogenesis and testicular androgen concentrations [36,37]. Pathogenic agents cause an enormous increase in both iNOS and eNOS activities, which lead to oxidative stress and thus increase apoptotic activity in germ cells [15,38]. PNMC was shown to induce oxidative stress by increasing free radical production [39]. No data is available with respect to elevation of iNOS and eNOS activities in testicular tissue due to tissue injury induced by PNMC. In our study, the expression of both iNOS and eNOS was increased in PNMC-treated groups. The immunoreactivity was manifested by intense staining with iNOS and eNOS antibodies. On the contrary, immunoreactivity for iNOS and eNOS was reduced in germ cells and Leydig cells of testes in Noni-treated animals, which was an evidence of beneficial effect of Noni against testicular tissue injury.

On the basis of histomorphometric and immunohisto-chemical findings, we may conclude that PNMC caused adverse effects on testicular tissue of adult male rats, which was markedly alleviated by the administration of Noni.

In conclusion, our findings suggest that Noni treatment would be able to alleviate the oxidative damage caused by the PNMC in the kidney of rats

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Research Article

The Effects of Cyclical Higher Incubation Temperatures on Body and Organs Weights, Thyroid Hormones and HSP70 Gene Expression of Newly Hatched Broiler Chicks

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Abstract

The objective of present study was to evaluate the effects of cyclical higher incubation temperatures in different embryonic ages on liver HSP70 gene expression, plasma T3, T4 and triglyceride (TG) levels, hatchability, body, heart and liver weights of newly hatched chicks. For this purpose, 2.700 fertile eggs (average weight, 58±1 g) were obtained from Arian broiler breeder at 34 weeks age. In a completely randomized design, eggs were assigned to three incubation temperature treatments at 60% relative humidity with 6 replicate per each. The treatment groups were: 1) control group that eggs were incubated at 37.6°C during incubation period, 2) incubation temperature was increased to 39°C for 3 h daily at embryonic ages from 12 to 14 in treatment 1 (TT1) and 3) incubation temperature was increased to 39°C for 3 h daily at embryonic ages from 15 to 17 in treatment 2 (TT2). All above-mentioned parameters were assessed at the day of hatch. The results showed that cyclical higher incubation temperatures did not affect body and liver weights, plasma T3, T4 and TG levels (P>0.05), while heart weights of chicks in TT1 and TT2 were lower (P<0.05) than control group. Furthermore, TT1 significantly increased liver HSP70 gene expression compared to the control group and TT2. It was concluded that cyclical higher incubation temperatures at embryonic ages from 12 to 14 could increase liver HSP70 gene expression with no effect on body and liver weights, plasma T3, T4 and TG levels at day-old chicks.

Keywords: *Incubation temperatures, Embryonic ages, Gene expression, Newly hatched chicks*

Yumurtadan Yeni Çıkmış Broiler Civcivlerde Döngüsel Yüksek İnkübasyon (Kuluçka) Sıcaklıklarının Vücut ve Organ Ağırlıkları, Tiroit Hormonları ve HSP70 Gen Ekspresyonu Üzerine Etkileri

Özet

Bu çalışmanın amacı; farklı embriyonik yaşlardaki döngüsel yüksek inkübasyon sıcaklıklarının, yumurtadan yeni çıkmış civcivlerde karaciğer HSP70 gen ekspresyonu, plazma T3, T4 ve trigliserid (TG) düzeyleri ile çıkış gücü, vücut, kalp ve karaciğer ağırlıkları üzerine etkisini değerlendirmekti. Bu amaçla, 34 haftalık etçi Arian damızlıklardan (ortalama ağırlık, 58±1 g) 2.700 adet döllenmiş yumurta elde edildi. Tamamen rasgele bir dizaynla seçilen yumurtalar, her biri 6 tekrarlı tarzda %60 nispi nemde üç farklı kuluçka sıcaklık uygulaması için ayrıldı. Tedavi grupları şunlardı: 1) kontrol grubu, yumurtalar inkübasyon süresi içinde 37.6°C'de inkübe edildi, 2) Tedavi 1 (TT1), inkübasyon sıcaklığı 12-14 arası embriyonik yaşta günde 3 saat süreyle 39°C'ye yükseltildi ve 3) Tedavi 2 (TT2), inkübasyon sıcaklığı 15-17 arası embriyonik yaşta günde 3 saat süreyle 39°C'ye yükseltildi. Yukarıda belirtilen tüm parametreler, kuluçka çıkış günü değerlendirildi. Sonuçlar, döngüsel yüksek inkübasyon sıcaklıklarının TT1 ve TT2'deki civcivlerin vücut ve karaciğer ağırlıkları ile plazma T3, T4 ve TG düzeylerini etkilemezken (P>0.05), kalp ağırlıklarının ise kontrol grubuna göre daha düşük (P<0.05) olduğunu gösterdi. Ayrıca, TT1 karaciğer HSP70 gen ekspresyonunu kontrol grubu ve TT2'ye göre anlamlı düzeyde artırdı. Sonuç olarak, 12-14 arası embriyonik yaşta döngüsel yüksek inkübasyon sıcaklıklarının, günlük civcivlerin karaciğer HSP70 gen ekspresyonunu artırabilmesine karşın, vücut ve karaciğer ağırlıkları ile plazma T3, T4 ve TG seviyeleri üzerine bir etkisi olmadı.

Anahtar sözcükler: *İnkübasyon sıcaklıkları, Embriyonik yaş, Gen Ekspresyonu, Yumurtadan yeni çıkmış civciv*

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INTRODUCTION

When living organisms are exposed to thermal and non-thermal stressors, the synthesis of most proteins is retarded; however, a group of highly conserved proteins known as heat shock proteins (HSPs) are rapidly synthesized [1]. These proteins are essential for organisms living at the edge of their thermal range. It is well documented that one of the most important functions of HSPs is to protect organisms from the toxic effects of heating [1,2]. HSPs may play important roles in protein assembly and disassembly, protein folding and unfolding, protein translocation and the refolding of damaged proteins [2]. Of the many expresses HSPs, those with a molecular weight of approximately 70 kDa appear to be most closely associated with heat tolerance. Heat shock 70 kDa proteins (HSP70s) are ubiquitous molecular chaperones that function in a myriad of biological processes [2].

Several investigations have demonstrated that heat shock response occurs in a variety of tissues [1,3]. Amongst all members of 70 kDa family, the one that has attracted most attention is HSP70. However, the mechanisms regulating HSP70 gene expression in broiler chicken have not been extensively studied. Being physiologically important tissue, the liver was selected in order to determine the changes in the gene expression levels of HSP70 after the process of cyclic thermal increase.

Embryonic temperature is considered to be the most important factor during incubation [3]. Since embryo is not capable of regulating its own temperature until hatching, the embryo temperature is regulated by the incubator air temperature [4]. Consequently, the temperature that the embryo encounters will depend on the incubator temperature, the ability of heat to transfer between the incubator and the embryo, and by its own metabolic heat production [5].

There are two parts involved in converting the yolk and albumen in a fertile egg into an embryo: 1. Differentiation and 2. Growth [6]. During the first half of incubation (embryonic ages from 1 to 10.5) chicken embryos go through a differentiation phase involving organ formation. They are capable of absorbing heat from the surrounding air, since they have a lower temperature than the incubator. The second half of incubation (embryonic ages from 10.5 to 21) is characterized by growth, and embryos must lose heat and metabolic rate and heat production increases, thereby increasing oxygen consumption and CO_2 levels [7].

The thermal conditions during embryonic period may have a great impact on development of physiological systems and maybe inducing epigenetic adaptations by having an effect on the chicks' development [8]. Thus, improving incubation conditions may have positive long-term effects on chicks. During the differentiation phase, the tissues are formed, and by embryonic ages 12 of incubation 90% of the organs are already present. Hyperthermia in developing tissues or cells has detrimental consequences for embryonic organogenesis. At a cellular level, hyperthermia may cause loss of protein function, which can be attributed to the normal misfolding of proteins with elevated temperature. A decrease in protein function, together with abnormal modifications that include changes in the phosphorylation state of eukaryote initiation factors and ribosomal proteins, will contribute to disruption of protein synthesis [9]. Thus, hyperthermia is a stress factor that, if present during embryonic development, will disrupt the plasma membrane and membrane protein function, altering cell structure and negatively impacting DNA, RNA, and protein synthesis [9]. Elevated temperatures during the avian embryo's early stage of development may have serious detrimental effects on its development and viability. For this reason, we used cyclical higher incubation temperatures in second half of incubation.

Some previous studies suggested that cyclical higher incubation temperatures can result in long-lasting modification to cellular and molecular neuronal mechanisms of temperature regulation [1,10,11]. Daily cyclical higher incubation temperatures, depending on the length of exposure and days of temperature modification, appear to improve tolerance of chicks to higher ambient temperatures [12]. Plasma T_3, which has an important role in the hatching process and thermoregulatory mechanisms [13], and plasma triglyceride (TG), which are the most abundant lipid present in birds, may be changed by thermal conditioning [10]; so, we evaluated these two factors.

The objective of this study was to evaluate the effect of daily higher incubation temperatures between embryonic ages from 12 to 17 on body, heart and liver weights and HSP70 relative gene expression in liver. Plasma T_3, T_4 and TG levels of chicks were also evaluated.

MATERIAL and METHODS

The study was approved by the Ethics Committee of Islamic Azad University, Science and Research Branch (approval date: 25.05.2013; no: 11096).

Eggs and Incubator Condition

A total of 2.700 fertile eggs (with average weight of 58±1 g) were obtained from a 34-wk old Arian broiler breeder flock. Eggs were incubated in single-stage incubators. From embryonic ages from days zero to 11, eggs were incubated under the same condition of 37.6°C and 60% relative humidity, while being turned once per h. At day 11 of incubation, dead embryos were identified by candling and removed from the experiment.

In a completely randomized design, eggs with live embryo (no: 2628) were assigned to three incubation temperature treatments at 60% relative humidity with 6

replicate and 146 eggs per each. The treatment groups were: 1) control group that eggs were incubated at 37.6°C during incubation period, 2) incubation temperature was increased to 39°C for 3 h per day at embryonic ages from 12 to 14 in treatment 1 (TT1) and 3) incubation temperature was increased to 39°C for 3 h per day at embryonic ages from 15 to 17 in treatment 2 (TT2). On the 18th day of incubation, the eggs were transferred to a hatcher. The hatchability was recorded for each treatment. Immediately after hatching completed (day 21 of incubation, with 8 h interval between the first and the latest hatched chick) male chicks were selected by vent sexing method in each treatment. All newly hatched male chicks were weighed and then three male chicks from each replicate (18 birds per treatment) were randomly selected. Blood and tissue sampling was started about two h and finished four h after hatching completed. Selection of chicks for sampling and sampling method was the same for all groups.

Sampling and Measurements

The blood sample of selected chicks was collected in EDTA-gel containing vacuum tubes directly from heart. Plasma was separated after centrifugation at 2.000 × *g* for 10 min and stored at -20°C for subsequent measurements of T_3, T_4 and TG.

The concentration of plasma T_3 and T_4 were determined using commercially available ELISA kits according to manufacturer recommendations (Biocheck Inc., Foster City, CA, USA). Plasma total TG level was measured enzymatically using photometric method by autoanalyser (BS-120 model, Minbray Co., USA) and commercial kits (Pars Azmon Co., Tehran, Iran).

Immediately after blood sampling, three chicks from each replicate were randomly chosen and sacrificed by cervical dislocation, then liver and heart were removed and weighed. Liver sample was taken, then frozen quickly in liquid nitrogen and stored at -70°C until further analysis for gene expression of HSP70.

Liver HSP70 Relative Gene Expression

Total RNA was extracted using Accuzol reagent (10 ml/g of tissue) from the ground liver segments according to the manufacturer instructions (Bioneer, Cat. No. K-2102). Total RNA purity was determined by calculating the ratio of the absorbance readings at 260 and 280 nm. Additionally, the quality of RNA was assessed by visualization of distinct 28S and 18S rRNA bands after gel electrophoresis with ethidium bromide staining. A quantity of 1 ug of each RNA sample was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcriptase kit (Bioneer Co., Seoul, South Korea). The 20 µL cDNA synthesis reaction contained in addition to the RNA template, 2 µL of 10x RT buffer, 0.8 µL of 25X dNTP mix, 2 µL 10x RT Random Primers and 1 µL MulitScribe Reverse Transcriptase. A volume of 10 µL of nuclease-free water was added bring the reaction up to final volume. The resulting cDNA was stored at -20°C prior to use.

Quantitative PCR was performed with a specific primer pairs (*Gallus gallus*, AY; 372 bp-763790; forward: 5′-AGC GTAACACCACCATTCC-3′; reverse: 5′-ACGCTCCTGCAAGAT AGTGAT-3′) using Quanti Fast SYBER Green PCR kit (QIAGEN, Cat. No. 204052). GAPDH (M-32599; 230 bp; forward: 5′-TGAAAGTCGGAGTCAACGGAT-3′, reverse: 5′-ACGCTCCTG GAAGATAGTGAT-3′) was chosen as a reference gene. Amplification of the day-old chicks liver HSP70 gene was performed for 45 cycles, which consisted of an initial activations step (95°C, 5 min), denaturation cycle (95°C, 10s) and combined annealing and extension (60°C, 30s). The GAPDH reference gene was amplified at 45 cycles under the same conditions in a different tube. After each run, preparation of standard curve was performed by serial dilution of pooled cDNA from samples. The relative expression ratio of HSP70 as a target gene was normalized to GAPDH gene using $2^{-\Delta\Delta ct}$ method as previously described by Livak and Schmittgen [14]. Quantification for each treatment group was performed in triplicates.

Statistical Analysis

Statistical analysis was performed using SAS software (version 9.1; SAS Institute, Cary, NC, USA [15]). Statistical analysis were performed using the one-way ANOVA to determine the effects of treatments on HSP70 gene expression, body and organs weights, hatchability, plasma T_3, T_4 and TG levels on newly hatched male broiler chicks. Mean comparison was done using the Duncan's multiple range test. Probability values of less than 0.05 ($P<0.05$) were considered significant.

RESULTS

The effects of cyclical higher incubation temperatures on body, liver and heart weights and hatchability are presented in *Table 1*. The results showed that cyclical higher incubation temperatures did not significantly affect body and liver weights ($P>0.05$), while heart weight of chicks in TT1 and TT2 were significantly lower than control group ($P<0.05$). Also, there was no significant difference in hatchability among treatments ($P>0.05$).

The effects of thermal manipulation during incubation on plasma TG, T_3, T_4 levels are presented in *Table 2*. TG, T_3 and T_4 levels at the day of hatch were not influence by thermal treatments ($P>0.05$).

The effects of different cyclical thermal manipulation during incubation period on HSP70 gene expression are shown in *Fig. 1*. The expression of liver HSP70 gene were quantified by qPCR assay and expressed relative to expression of the GAPDH gene. TT1 significantly increased gene expression compared to TT2 and control group

Table 1. *Effect of cyclical higher incubation temperatures on body, liver and heart weights and hatchability on newly hatched male broiler chicks*

Tablo 1. *Yumurtadan yeni çıkmış erkek etlik civcivlerde döngüsel yüksek inkübasyon sıcaklıklarının vücut, karaciğer ve kalp ağırlıkları ve kuluçka randımanı üzerine etkisi*

Parameter	Experimental Treatments			SEM	P-Value
	C	TT1	TT2		
Body weight (g)	40.33	40.20	40.48	0.24	0.8309
Liver weight (g)	1.255	1.194	1.178	0.01	0.2653
Heart weight (g)	0.332[a]	0.293[b]	0.295[b]	0.01	0.0474
Hatchability (%)	98.67	98	98	1.58	0.7247

[a,b] Means in row that possess different superscripts differ significantly (P<0.05); ***C:*** *Incubated at 37.6°C throughout;* ***TT1:*** *exposed to heat at 39°C 3 h/d from EA 12 to 14 of incubation;* ***TT2:*** *exposed to heat at 39°C 3 h/d from EA 15 to 17 of incubation*

Table 2*. Effect of cyclical higher incubation temperatures on some blood parameters levels on newly hatched male broiler chicks*

Tablo 2. *Yumurtadan yeni çıkmış erkek etlik civcivlerde döngüsel yüksek inkübasyon sıcaklıklarının bazı kan parametreleri üzerine etkisi*

Parameter	Experimental Treatments			SEM	P-Value
	C	TT1	TT2		
T_3 (ng/ml)	1.65	1.15	1.78	0.09	0.31
T_4 (ng/ml)	1.88	1.55	2.51	0.19	0.37
TG (mg/dl)	42.67	47.83	45.17	13.51	0.28

Means with a row superscript letter did not differ (P>0.05); ***C:*** *incubated at 37.6°C throughout;* ***TT1:*** *exposed to heat at 39°C 3 h/d from EA 12 to 14 of incubation;* ***TT2:*** *exposed to heat at 39°C, 3 h/d from EA 15 to 17 of incubation;* ***TG:*** *Triglycerides,* ***T_3:*** *3,5,3′-triiodothyronine,* ***T_4:*** *L-thyroxine*

(P<0.05). The expression of HSP70 mRNA was numerically lower in TT1 than control group.

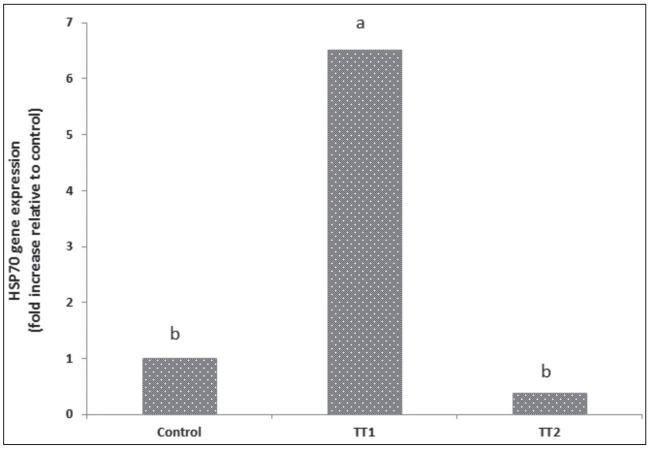

Fig 1. Result of the HSP70 gene expression data (mean ± SD) using real time qPCR according to $2^{-\Delta\Delta ct}$ method. Means with different superscripts differ significantly (P<0.05). Control group (incubated at 37.6°C throughout); TT1: exposed to heat at 39°C, 3 h/d from EA (embryonic age) 12 to 14 of incubation; TT2: exposed to heat at 39°C, 3 h/d from EA 15 to 17 of incubation. [a,b] Means that possess different superscripts differ significantly (P<0.05)

Şekil 1. HSP70 gen ekspresyon verilerinin (ortalama ± SD) 2-ΔΔct yöntemine göre gerçek zamanlı qPCR kullanım sonucu. Farklı üst harf taşıyan ortalamalar arasındaki fark önemlidir (P<0.05). Kontrol grubu (37.6°C'de daimi inkübasyon); TT1: 39°C sıcaklığa maruziyet, EY (embriyonik yaşı) 12-14 arası 3 saat/gün inkübasyon; TT2: 39°C sıcaklığa maruziyet, EY 15-17 arası 3 saat/gün inkübasyon. [a,b] Farklı harf taşıyan ortalamalar arasındaki fark önemlidir (P<0.05)

DISCUSSION

The objective of this study was to evaluate the effect of cyclical higher incubation temperature on hatchability, body and organs weights. A broiler chick spends up 30-40% of its life inside the egg. Thus, each factor or condition that enhance the growth and development during embryonic period can positively affect the post hatch performance of the broiler chicks [16,17]. There are some evidences that showed the embryonic temperature during the last 5 days of incubation could significantly affect post hatch growth and performance [16,18-20]; however, previous studies reported that thermal manipulation had no effect on body weight of both sex [21,22].

In the present study, cyclical higher temperature had no effect on hatchability, body and liver weights of chicks. These observations are in agreement with the findings of Lournes et al.[4] and Leksrisompong et al.[23], who showed that slight changes in incubation temperature cannot affect body weight of chicks and hatchability. The application of higher temperature in this study had no effect on liver weight, whereas application of higher temperature in the study of Leksrisompong et al.[23] resulted in change of liver weight. In our study, cyclical higher temperature was applied, whereas in previous studies [4,18,23] constant higher temperature was applied throughout incubation. This difference may be the reason of conflict between our study and other studies [4,18,23] concerning liver weight. It was reported [20] that deviation from optimum incubation temperature suppresses the development of organs and growth, but in our study, deviation from optimum incubation temperature was cyclical and low, which could not affect body and liver weights.

In this study, heart weight of chicks in control group was significantly higher than heart weight of chicks in TT1 and TT2. Inconsistent with our finding, Yalcin and Siegel [24] reported that heart weight of chicks exposed to higher temperature during embryonic ages from days 10 to 18 of incubation was higher than control numerically. The findings of Leksrisompong et al.[23] and Yalcin et al.[25] concerning heart weight of chicks exposed to higher temperature are in agreement to our result. The lower heart weight in chicks exposed to higher incubation temperatures compared to control group may be explained by low rate of cell division in heart of embryos. These data suggested that certain organs in a critical period during second half of incubation was more sensitive to high temperatures, whereas heart exhibited sensitivity from embryonic ages from 12 to 17 of incubation.

Thyroid hormones are essential for the normal development and differentiation of the embryo. These hormones also have an important role in temperature regulation in chicks by regulating basal metabolic rate. Thyroid hormones are important in several organismal level processes such as hatching [26]. The high perihatch concentrations of thyroid hormones appear to be stimulating a variety of developmental and metabolic processes necessary for successful hatching [13,26]. Plasma thyroid hormone concentrations increase dramatically during late stages of incubation [13,27]. After perihatch peak, plasma thyroid hormones decrease markedly, and then gradually increase during post-hatch life to reach adult concentrations [27]. Thermoregulation is under the control of thyroid hormones. One the mechanisms that induce thermotolerance involves the modulation of heat production through changes in circulating T_3. The ability to reduce plasma T_3 concentration, especially during a thermal challenge, has been suggested to be an improvement in thermotolerance [11,12].

In our study, cyclical higher incubation temperatures had no significant effect on plasma T_3 and T_4 concentrations. These findings are in agreement with the study of Tona et al.[28]. Also, an interesting study [10] showed that plasma T_3 and T_4 concentrations were not affected by high incubation temperatures from embryonic ages from 16 to 18.5 of incubation. Of course, they reported that higher thermal condition resulted in decrease of plasma T_3 concentration at embryonic ages 18 of incubation. Our results were in conflict with previous reports of Wineland et al.[29], who observed a reduction in plasma T_3 at day of hatch in chicks exposed to higher constant incubation temperatures from embryonic age from 17 to hatch. It was demonstrated [22] that continuously or 12 h/day higher temperature during embryonic age from 7 to 16 could significantly reduce thyroid hormones concentrations.

The difference in length of exposure and level of heat leaded to different responses. It can be speculated that thermal challenge caused a decrease in plasma thyroid hormones concentrations but in our study heat challenges were slight and cyclical. In other hand, time interval in the end of heat challenge and day of hatch, in both treatments, was sufficient to compensate this decline. Also the half-lives of T_3 and T_4 are essentially identical and are short (3-9 h) in birds [30]. Response of thyroid hormones to heat takes much time and in this study the length of exposure was not enough for stimulating thyroid hormones. It suggests that slight and cyclical thermal changes during second half of incubation may have no significant effect on plasma T_3 and T_4 concentrations in newly hatched chicks.

Plasma TG levels were slightly higher at the day of hatch in heat treated groups, which were in agreement with Yalcin et al.[31]. Cyclical higher incubation temperature in our study did not affect plasma TG levels of day-old chicks significantly. Our results were in conflict with the findings of Yalcin et al.[32] and Willemsen et al.[10]. Sensitivity of plasma TG levels to changes in thyroid hormones is high [32], hence decrease in plasma T_3 levels leaded to increase of plasma TG levels. As plasma thyroid hormone concentrations had no differences among treatments, hence there were no changes in plasma TG levels.

Of the many HSPs, HSP70 appear to be most closely associated with heat tolerance [2]. Under normal growth conditions, HSP70 is synthesized constitutively; however, its expression increases following thermal challenge. Heart, liver and kidney of broilers are more sensitive to heat stress and HSP70 gene expression increases after 2 h exposure to elevated temperatures [1]. It was showed that the stress-induced responses vary among different tissues [1]. Also, It is clear that after heat preconditioning HSPs are induced during the recovery from the heat stressed period and can work as a repair system in the recovery phase [1]. It was indicated that enhancement in HSP70 expression is evident for periods up to four weeks after termination of the daily heat conditioning episodes [33].

Cyclical higher temperature at embryonic age of 12 to 14 significantly increased liver HSP70gene expression. This indicates embryos in earlier ages have higher capability for synthesis of HSP70 than embryos near hatching. HSP70 gene expression varies in different tissues [1,34], therefore HSP70 gene expression in broiler chicken embryos is tissue and age dependent. In contrast to our result, Givisiez et al.[3] reported that heat-stress had no effect on liver HSP70 gene expression; however, significant effect on HSP70 gene expression of brain and kidney was observed.

The results of this study suggest that cyclical higher incubation temperatures (39°C) for 3 days (embryonic age from 12 to 14) could induce the HSP70 gene expression without negative effects on day-old chicks. It is advisable to industry; however, from the practical viewpoint, the application of cyclical higher temperature need to fully automated, programmable incubators. More detailed studies taking account of different incubation conditions are needed to define the effects of these conditions on thermotolerance and other important health parameters, especially immunity and its related gene expression.

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Short Communication

Comparative Analysis of Aflatoxin M1 in Marketed Butter by ELISA and HPLC

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Abstract

The aim of this study was to examine the presence of aflatoxin M1 (AFM1) in butter samples marketed in the Black Sea Region of Turkey with two complementary analytical methods to improve accuracy. A total of 40 samples were analysed by enzyme-linked immuno absorbent assay (ELISA) as the screening test and high performance liquid chromatography (HPLC) with fluorescence detection (FLD) after immunoaffinity column (IAC) clean-up as the confirmatory method. Results indicated that butter samples in the Black Sea region were of good quality with respect to AFM1, with no contamination detected. Furthermore, the accurate and sensitive IAC/HPLC-FLD method was confirmed as being appropriate for the detection of AFM1 in butter.

Keywords: *Butter, Aflatoxin M1, Black Sea, Immunoaffinity column, HPLC, ELISA*

Tereyağlarında Aflatoksin M1 Düzeylerinin ELISA ve HPLC ile Karşılaştırmalı Analizi

Özet

Bu çalışmada Karadeniz bölgesinde tüketime sunulan tereyağlarında iki farklı analitik yöntem karşılaştırarak aflatoksin M1 varlığının araştırılması amaçlandı. Bu amaçla, toplam 40 tereyağı numunesi ELISA testi ile tarandı ve ardından doğrulayıcı bir yöntem olannumunelerin temizleme aşamasında immunoafinite kolonların kullanıldığı floresan detektörlü yüksek performanslı sıvı kromatografi (IAC/HPLC-FLD) ile analizleri yapıldı. Yapılan çalışma ile herhangi bir tereyağı numunesinde AFM1 tespit edilmemiş olup, Karadeniz bölgesinde üretilen tereyağlarının AFM1 açısından iyi kalitede olduğu sonucuna varıldı. Ayrıca, elde edilen sonuçlara göre tereyağında AFM1 tespitinde kullanılan IAC/HPLC-FLD metodunun doğrulayıcı ve hassas bir yöntem olduğu ortaya konulmuştur.

Anahtar sözcükler: *Tereyağı, Aflatoksin M1, Karadeniz, İmmunoafinite kolon, HPLC, ELISA*

INTRODUCTION

Aflatoxin M1 (AFM1) is a hydroxylated metabolite of aflatoxin B1 (AFB1) that can be found in the milk of dairy cattle fed with AFB1-contaminated feeds [1].

The stability of AFM1 determines its persistence in foodstuffs such as butter, yogurt, cheese, cream and ice cream [2]. However, this toxin is not inactivated by the thermal processing (pasteurization and ultra-high-temperature (UHT) treatment) used in the dairy industry [3]. As milk and milk products are an important sources of nutrients, the contamination of these products with AFM1 is a potential risk for human health worldwide [4]. However, due to their low concentration in foods and feedstuff, analytical methods for detection and quantification of aflatoxins have to be specific, sensitive, and simple to carry out [5]. For the qualitative, quantitative and accurate determination of mycotoxin levels in food and feed products, the methods include thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) with flourescence (FLD) or diode array detector (DAD), gas chromatography coupled with mass spectrometry (GC-MS) or electron capture detection (GC-ECD), liquid

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chromatography-tandem mass spectrometry (LC-MS/MS) enzyme-lynked immunosorbent assays (ELISAs) and a combination of immunoaffinity column (IAC) techniques [6]. In general, fast and easy-to-use ELISA based aflatoxin screening kits are commercially available for all major types of aflatoxins. However, cross reactivity with related mycotoxins is a drawback of this method [7]. Therefore, quantification is predominantly done with HPLC-FLD due to the excellent native fluorescence activity of aflatoxins. In addition, IAC clean-up has shown great potential to increase method specificity and sensitivity [8]. The objective of this study was to investigate the occurrence of AFM1 in butter in the Black Sea Region of Turkey by screening with ELISA, followed by the comfirmatory method, HPLC-FLD.

MATERIAL and METHODS

Reagents and Materials

AFM1 analytical standard solution, phosphate-buffered saline (PBS) and immunoaffinity columns (IAC) were sourced from R-Biopharm Rhône Ltd. (Glasgow, Scotland). Acetonitrile and methanol were of HPLC grade (Sigma-Aldrich Co., USA). Whatman filter paper (No. 4) and Whatman 934-AH glass microfiber filter were from GE Healtcare (Buckinghamshire, UK).

Sampling

A total of 40 butter samples were purchased from supermarkets in the East, Middle and West Black Sea Regions of Turkey. They were produced in October-November, 2009 (first period) or May-June, 2010 (second period).

ELISA Procedures

The samples were prepared according to the application note, Ridascreen® Aflatoxin M1 (Art. No. R1121, R-Biopharm AG, Darmstadt, Germany) and analysed as described in the instructions of the AgraQuant® Aflatoxin M1 Sensitive kit (COKAQ7100, Romer Labs® Inc., U.S.A).

IAC/HPLC procedures

Aflatoksin M1 levels were determined in the butter samples by the method described in Sakuma et al.[9].

RESULTS

AFM1 was not detected in any of butter samples collected from the Black Sea region of Turkey. Mean recovery of AFM1 spiked into butter at 0.5 µg/kg was 84%. The limit of detection (LOD) was 0.023 ng/g and the limit of quantification (LOQ) was 0.077 ng/g. The HPLC standard calibration curve was linear over the range of concentrations of AFM1 injected and the R-squared (R^2) value was 0.99. The method exhibited great accuracy and reproducibility for detecting AFM1 in butter.

The HPLC chromatogram of the AFM1 spiked sample of butter is shown in *Fig. 1*. There are no interferences in the section where AFM1 was eluted.

DISCUSSION

In Turkey, many surveys have examined AFM1 content in milk products but only a few have been done with butter, especially with HPLC-FLD. Aydemir Atasever et al.[10] investigated AFM1 levels with ELISA in 80 butter samples obtained from supermarkets between September 2007 and September 2009 in Erzurum, Turkey, and reported that 66 samples were contaminated with AFM1 ranging from 10 to 121 ng/kg and 13 samples exceeded the maximum legal limit. Tekinşen and Uçar [11] were scanned AFM1 by ELISA method using with IAC in 92 butter and 100 cream cheese samples obtained from retail outlets in five major cities of Turkey. They found 100% of the butter samples and 99% of the cream cheese samples were contaminated with AFM1, ranging from 10 to 7.000 ng/kg and from 0 to 4.100 ng/kg, respectively. In another study, 223 samples of dairy products (27 samples of butter) marketed in Ankara, Turkey during September 2002-September 2003, were analysed for AFM1, total aflatoxin and AFB1 with ELISA. The AFM1 contamination rate was 90.58% in dairy products, including 92.6% in butter [12]. Var and Kabak [4] investigated AFM1 by ELISA in 70 dairy products (10 butter samples) purchased in different supermarkets in Adana, Turkey. They detected AFM1 in three of 10 butter samples. In addition, Tosun and Ayyildiz [13] studied AFM1 levels with ELISA in organic milk and dairy products marketed in Turkey between February 2010 and February

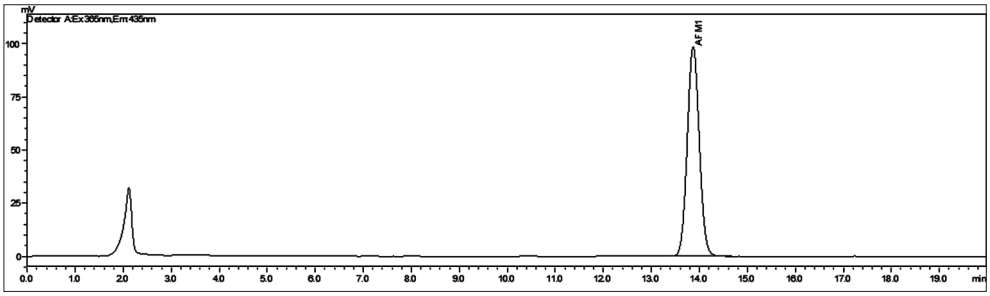

Fig1. HPLC-FLD chromatogram of spiked butter sample
Şekil 1. Tereyağı geri kazanım çalışmasına ait HPLC-FLD kromatogramı

2011. No AFM1 contamination was detected in organic butter samples.

During butter processing, the protein membrane around fat globules is broken down and the serum phase is separated. Due to the chemical structure of AFM1 and its affinity for casein, it adsorbs on this fraction of protein. Therefore, cream contains less AFM1 than milk and butter contains less AFM1 than cream [14]. As a result of the associated effects of these factors, less AFM1 occurs in the lipid phase (butter and cream) because it is concentrated in the serum phase and protein fraction. This data may be promote to our results. However, it is probable that good manufacturing practices and good storage prevented butter samples from getting mouldy.

Most of the current analytical techniques used for detection and quantification of aflatoxins involve sampling, extraction and clean-up, followed by an appropriate detection method, depending on the level of precision required of the result [7].

ELISA (test kits) are often used to screen and quantify mycotoxins as a rapid and practical method [15] but the drawbacks of the method are cross reactivity and matrix dependence, which often result in extreme over-estimation [16]. Therefore, comfirmatory methods such as LC, GC, LC/MS, GC/MS are needed for a sensitivity and accuracy. In the present study, the HPLC-FLD method used with an immunoaffinity column was highly advantageous, including rapidity, when used specifically for determination of the level of AFM1 in butter. Moreover, we achieved lower LODs and LOQs than several studies [17,18] by using the method described by Sakuma et al.[9].

In conclusion, the results of our survey showed that butter collected in a specific period from Black Sea Region was of excellent quality with respect to AFM1 contamination because none was detected. However AFM1 levels in dairy products such as butter can vary from season to season and year to year because they are related to levels of aflatoxins in feed. Therefore, regular surveillance studies are necessary to compile datum for a more comprehensive evaluation of AFM1 contamination in dairy products. The IAC/HPLC-FLD method that we applied to butter could be used more broadly as a sensitive and accurate detection method for AFM1 detection. Moreover, the systematic use of new analytical techniques to measure AFM1 levels with great precision can help to improve public health standards.

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Case Report

Marbofloxacin Overdose: The Culprit for Acute Blindness in a Dog

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Abstract

In this case report, a 4 year old female terrier crossbred dog was presented with acute blindness following accidental high dose marbofloxacin administration was discussed. On initial referral to the clinic, a respiratory disease was evident, requiring antibiotic therapy and marbofloxacin (2.5 mg/kg) was prescribed. Meanwhile, patient was transferred to another veterinary practitioner specialized in large animal diseases, marbofloxacin had administered at an over dose (1050 mg/total dose through 3 days). The dog was then introduced back to our clinic with acute bilateral blindness. A diagnosis of marbofloxacin toxicity related to an overdose was made based on clinical examination and laboratory findings, excluding other relevant etiology. Dexamethasone was applied as initial treatment. In the following weeks, vision loss was eminent even without responding to bright light and not able to negotiate the obstacle course. According to the current literature, this is the first report on marbofloxacin overdose related acute blindness in dogs.

Keywords: *Blindness, Marbofloxacin overdose, Dog*

Aşırı Doz Marbofloksasin: Bir Köpekte Akut Körlük İçin Olağan Şüpheli

Özet

Bu olgu sunumunda yanlışlıkla yüksek doz marbofloksasin uygulaması sonucunda 4 yaşlı, dişi, terrier melezi bir köpekte gelişen akut körlük vakası irdelenmiştir. Olgu kliniğe ilk başvurduğunda antibiyotik sağaltımı gerektiren bir solunum yolu enfeksiyonu olduğu belirlendi ve marbofloksasin (2.5 mg/kg) reçete edildi. Bu süre içerisinde, büyük hayvan hastalıkları konusunda uzmanlaşmış bir veteriner hekime götürülmüş ve marbofloksasin 3 gün boyunca aşırı dozda (1050 mg/3 gün boyunca toplam doz) uygulanmıştır. Köpek, uygulama sonrasında tekrar kliniğimize getirildiğinde akut körlük mevcuttu. Marbofloksasin toksisite tanısı, diğer ilişkili etiyoloji haricinde klinik, labaratuvar bulgular ve ilacın aşırı doz alımı temel alınarak konuldu. İlk sağaltım uygulaması olarak deksametazon uygulandı. Takip eden haftalarda görüş bozukluğunun olduğu, parlak ışığa tepki vermediği ve engellere takılmadan yürüyemediği tespit edildi. Son literatürlere göre köpeklerde marbofloksasinin aşırı doz uygulanması sonucu gelişen akut körlük ilk kez bildirilmiştir.

Anahtar sözcükler: *Körlük, Marbofloksasin yüksek doz, Köpek*

INTRODUCTION

Adverse drug reactions are not commonly observed in pet ophthalmology, more commonly detected in the dog in contrast to the cat. The latter side effects vary from mere annoyance to life-threatening conditions.

As the dog is often interpreted to analyze the toxicity of several drugs, many agents were tested and reported to cause retinal and chorioretinal changes [1,2] in cats. In the present case report a Terrier dog with acute blindness occurring following high dose marbofloxacin was described, to those of which, in the present authors' practice have never been reported.

CASE HISTORY

A 4 years old, unneutered crossbred Terrier breed dog, weighing 10 kg, was presented to the clinic with upper respiratory clinical signs. On initial referral to our clinic

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marbofloxacin with a dose of 2.5 mg/kg for at least 1 week and an upper airway anti-inflammatory drug, oxolamine were prescribed. The owner declined further therapy at the University Clinic, and indicated that the recommended drugs would be used at private veterinary practice in Nazilli, Aydin in Turkey. Afterwards the private veterinary surgeon who was specialized in large animal diseases, administered marbofloxacin at an over dose of 35 mg/kg (a total dose of 1050 mg) for 3 days. The dog was then referred back to our clinic with acute blindness on day 4. Hematological and serum biochemical analysis were within reference ranges (data not shown). At physical examination the dog was reluctant to walk, and eyelids were quite open. When the dog reaches the color differentiation area on the flat ground, the dog was lifting its limbs more than normal *(Fig. 1)*. This was evaluated as the dog could recognize the color differentiation on the ground.

During ophthalmic examination in both eyes, while cornea and iris were normal, vascularization in conjunctiva and pupillary dilatation were identified. Edema and papillomatosis on optical nerve, and dilatation in the optical nerve vessels in both eyes were determined *(Fig. 2)*. A preliminary diagnosis of marbofloxacin toxicity was made, excluding any other apparent reasons, as the owner reported no other drug usage. A blood sample was with-drawn and forwarded to Pharmacology and Toxicology Department for analyzing plasma marbofloxacin level.

Plasma marbofloxacin level was determined by high performance liquid chromatography (HPLC) with a fluorescence detector following the extraction procedure. A marbofloxacin standard was used from Fluca (Batch No. 34039, 98% putiry; Fluka, China). Plasma extraction of marbofloxacin and the analysis by HPLC was according to the method of Karademir et al.[3] which was validated in our laboratory conditions.

Dexamethasone (Maxidex ophthalmic pomad® Alcon) was suggested 4 times a day (4x1). A telephone call following 2 weeks later indicated that the dog was unable to orient, without responding to bright light. Three weeks later a physical examination along with ocular investigation revealed that the dog was still not negotiate an obstacle course.

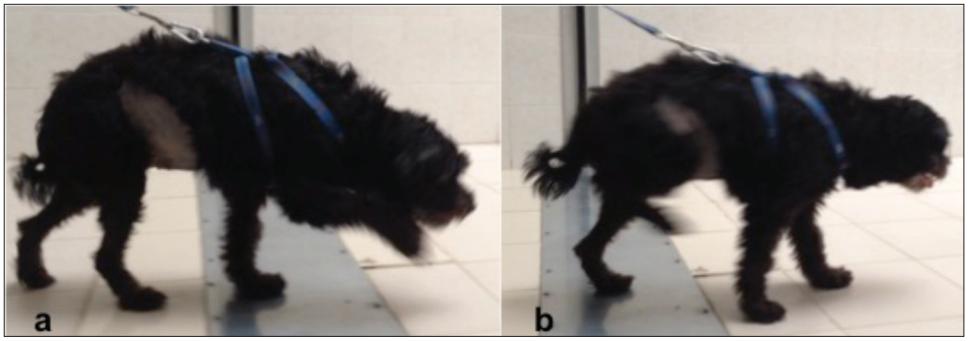

Fig 1. Realized of color differentiations on the ground, during the walking and lifting limbs (a. forelimb, b. hindlimb) more than normal

Şekil 1. Zemindeki renk değişiklikleri sebebiyle yürüme ve adım atışı sırasında ekstremitelerin (a. ön ekstremite, b. arka ekstremite) normalden daha fazla kaldırılması

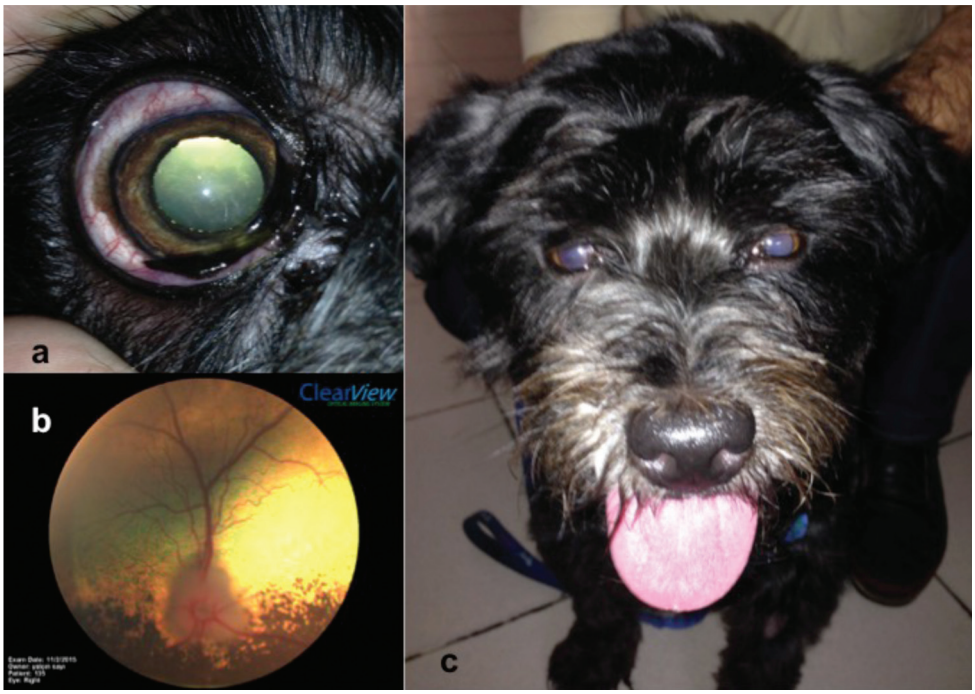

Fig 2. Pupillary dilatation (a, c), vascularization in conjunctiva (a), edema and papillomatosis on optical nerve, and dilatation in the optical nerve vessels (b)

Şekil 2. Pupillar dilatasyon (a, c) konjuktivada vaskülarizasyon (a), optik sinirde ödem, papillamatozis ve optik sinir damarlarında dilatasyon (b)

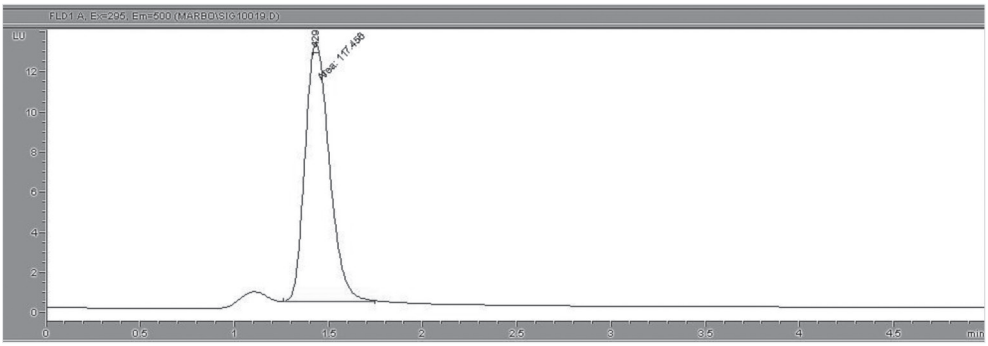

Fig 3. HPLC chromatogram of analyzed plasma sample

Şekil 3. Analiz edilen plazma örneğinin HPLC kromatogramı

The level of marbofloxacin in the plasma sample was found as 0.5 µg/ml *(Fig. 3)*.

DISCUSSION

Dogs are target species for testing toxicity of several drugs; where several compounds have been found to cause retinal or chorioretinal alterations [4]. To this content it was previously described that quinine produced rapid vasoconstriction of the retinal arterioles, optic disc atrophy and retinal ganglion loss [5]. Taking into account quinolones were derived from quinine [6], and there exists structural relationship between fluoroquinolones and quinine differing via an oxygen molecule [7-9], it may be speculated that similar ocular reactions may occur for both drugs. Excluding other probable reasons, besides marbofloxacin was the solely used injectable drug at the onset of clinical signs, it safely appeared that the usage of marbofloxacin was responsible for the vascularization in conjunctiva, dilation on pupilla and optical nerve vessels, edema and papillomatosis on optical nerve contributing to the blindness that developed in the present case.

In the current report, we hypothesized that it may be a canine case of sudden acquired retinal degeneration [10], although, to the present authors knowledge, at the time of writing and since then a canine case of retinal degeneration has not been documented elsewhere. A toxic insult to the retina was proposed similar to what have been reported previously [10], the only other drug, oxolamine phosphate, had not been associated with retinopathy. However the association of fluoroquinolones and visual disturbances [11] in cats [2,10,12] has now been well established. The use of enrofloxacin has been attributed to visual impairment with growing numbers of anecdotal reports [10]. Fluoroquinolones have long been recognized to cause acute retinal degeneration in cats [13]. Although unclearly explained, it has been suspected that the mechanism is dose dependent. Higher doses cause more severe side effects [1,14,15].

Given the molecular findings for fluoroquinolone-induced retinal degeneration and consecutive blindness in cats, ABCG2 transport protein deficiency was suggested as the responsible reason in feline retina [16]. Since previously, no case reports were presented for dogs, the present authors may briefly suggest that species difference might be a confounding factor involving the expression of ABCG2 transporters in dogs [16,17].

In the present report, a high dose of the drug was evident, 4 days prior to the retinal degeneration being detected. Marginal renal disorders may be related to further increase in the plasma level of the drug [10]. However in the present study, the case presented no renal dysfunction based on normal levels of urea, creatinine and cystatin-C levels. Moreover, elevated plasma level for marbofloxacin was not present; since the dog was referred to the clinic 4 days after exposure to high dose exposure, indicating low plasma level as 0.5 mg/ml.

In summary, it may be suggested that marbofloxacin overdose may result in acute blindness in dogs, similar to what have been previously described in cats.

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Case Report

Amorphus globosus in a White Galloway Cattle

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Abstract

In this article, a case of an amorphus globosus is presented. A three years old White Galloway dam gave birth in her second pregnancy a normal calf and a malformed amorphic co-twin. The malformed co-twin was slightly flattened with an ovoid covered by white hairy skin form of 21.0x10.0x6.0 cm in size and a weight of 1600 g. The present case had an umbilical cord like structure of 20 cm in length and 2-3 cm in diameter. A small opening, resembling am oral structure, contain a tongue-like structure with lips and three milk teeth. Histological examination showed a solid mass of connective tissue, skeletal muscles, lymph, arterial vessel and glandular units and focal by cartilage tissue. The radiography clearly showed lower-upper jaw, small bone similar to a tail-like-structure and a cartilage tissue. The inbreeding coefficient of the case was 6.25% due to a common ancestor on the maternal and paternal side. This is the first report on amorphus globosus in White Galloway cattle including pathological, histopathological, radiological findings and pedigree analysis.

Keywords: *Amorphus globosus, Galloway, Twin, Cattle*

Beyaz Galloway Sığırda *Amorphus globosus* Olgusu

Özet

Bu makale de bir Amorphus Globosus olgusu sunulmuştur. Üç yaşlı beyaz Galloway ineğin ikiz olarak şekillenen ikinci gebeliğinde bir normal buzağı ve bir de onun amorfik kusurlu ikiz partnerini doğurmuştur. Kusurlu ikiz partner, şekli hafif basık ve oval ,derisi beyaz tüylerle kaplı, 21.0x10.0x6.0 cm boyutlarında ve 1600 gr agırlığına sahibdi. Sunulan olgu, 20 cm uzunluğunda ve 2-3 cm genişliğinde göbek bağına benzer bir oluşuma sahipti. Ağız şeklinde küçük bir açıklık ve bu açıklıkta 3 adet diş ve dudaklarla birlikte küçük dil benzeri yapı görüldü. Histolojik incelemede ise olgunun sağlam bir bağ dokusu, iskelet kasları, lenf ve arteriyel damarlar, salgı bezleri ve kıkırdak dokuya benzeyen odaklardan meydana geldiği gözlemlendi. Radyolojik incelememizde net olarak alt ve üst çenenin, kuyruğa ait olduğu düşünülen küçük kemiksi yapıların ve kıkırdak dokunun varlığı tespit edildi. İnek ve boğanın ebeveynlerinin ortak olması nedeniyle olgunun akrabalı yetiştirme katsayısı %6.25 idi. Bu olgu galloway ırkı sığırda görülen ilk amorphus globosus vakası olup patolojik, histopatololojik, radyolojik bulgular ve pedigri analizini içerir.

Anahtar sözcükler: *Amorphus Globosus, Galloway, ikizlik, Sığır*

INTRODUCTION

Amorphus globosus (AG) is an imperfectly formed free twin fetus lacking a heart and invariably lacking other body parts as well. The incidences of twin births are 3.9% [1] and 4.2% [2] in dairy herds. Hossein-Zadeh et al.[1] reported calf stillbirths with 18.8% of twin calving events resulting in one or both calves as stillborn, compared with 4.0% for singleton births.

Several cases on this AG have been reported a rate of 0.4% in cattle, 0.003% humans and very rarely other domestic animals [3-13]. We present the first case of AG in Galloway cattle.

CASE HISTORY

Case Description

A case of amorphus was notified in a farm located in Bavaria, Germany, on April 16, 2009. A normal female calf and its malformed co-twin were delivered after full term gestation from a White Galloway cattle dam. The female normal calf had normal body size and weight. The amorphous co-twin was completely covered with white hair and white skin. The present case was sired by a natural service bull kept in this herd. The dam of the case was a 3-years-old White Galloway cow with a previous normal parturition.

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Necropsy Findings

The malformed co-twin was slightly flattened with an ovoid form, a dimension of 21.0x10.0x6.0 cm and a weight of 1600 g. The present case had an umbilical cord like structure of 20 cm in length and 2-3 cm in diameter *(Fig. 1A)*. The body of the present case had a small opening, resembling an oral structure, in which a tongue-like structure with deformed lips and three milk teeth were present *(Fig. 1B)*. In addition, the case had a tail formation on the opposite side to oral-like-structure. We observed ventral and dorsal median notches in the upper and lower jaw. The cross section of the body showed abundant connective tissue, glandular units, blood vessels and cartilage tissue *(Fig. 1C)*. The oral cavity ended blindly.

Histopathological Findings

Tissue sample from the oral-like-structure showed cutaneous mucosal cells indicative for tongue tissue. Body tissue samples contained connective tissue, skeletal muscles, lymph tissue, arterial vessels, cartilage tissue as well as tubular and glandular structures.

Radiological Findings

The radiography of the present case showed a lower and upper jaw *(Fig. 2)*, a small bone similar to a tail-like-structure and cartilage.

Pedigree Analysis

The autosite twin of the dam showed no congenital abnormalities. The inbreeding coefficient for the case was 6.25% due to a common ancestor on the maternal and paternal side *(Fig. 3)*.

DISCUSSION

The present case of an amorphus globosus (AG) co-twin is the first reported in White Galloway cattle. The main criteria for an AG including no specific shape of a body but covered by skin and hairs, were confirmed for this case. In addition, a small tongue with an oral cavity and a rudimentary bone formation were observed. Other forms of acardiac twins could be clearly excluded. The acardius anceps shows a partly developed head and a deformed

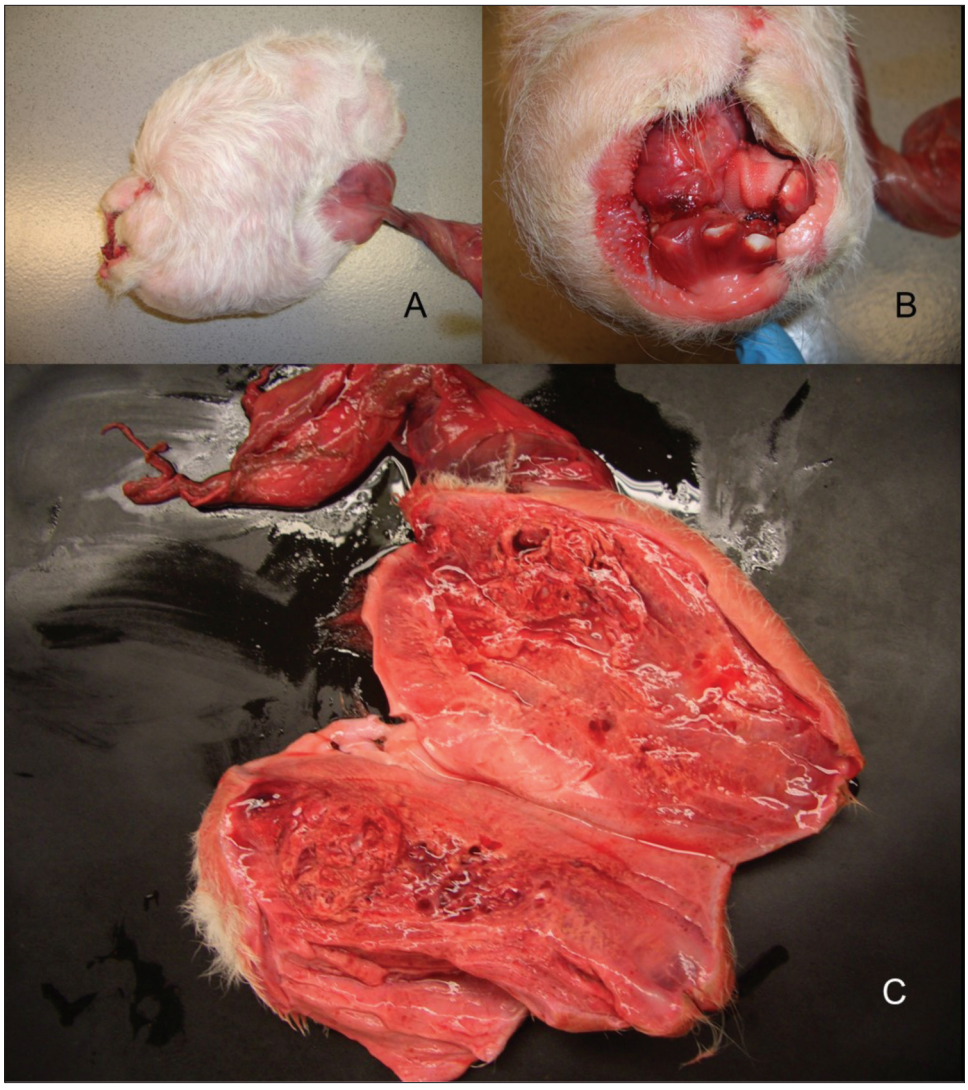

Fig 1. Present case of amorphous globosus (AG). A: Appearance of AG, B: Mouth of present case with teeth, lip and tongue, C: The cross section of the case showed abundant connective tissue, glandular units, blood vessels and the focal detection of cartilage tissue

Şekil 1. Sunulan olgu. A: Amorphus globosus dış görünüm, B: Diş, dudak ve dil ile birlikte sunulan olgunun ağızı, C: Olgunun enine kesitinde görülen bol miktarda bağ dokusu, lenf bezi üniteleri, kan damarları ve kıkırdak dokusundan oluşmuş odak tespiti

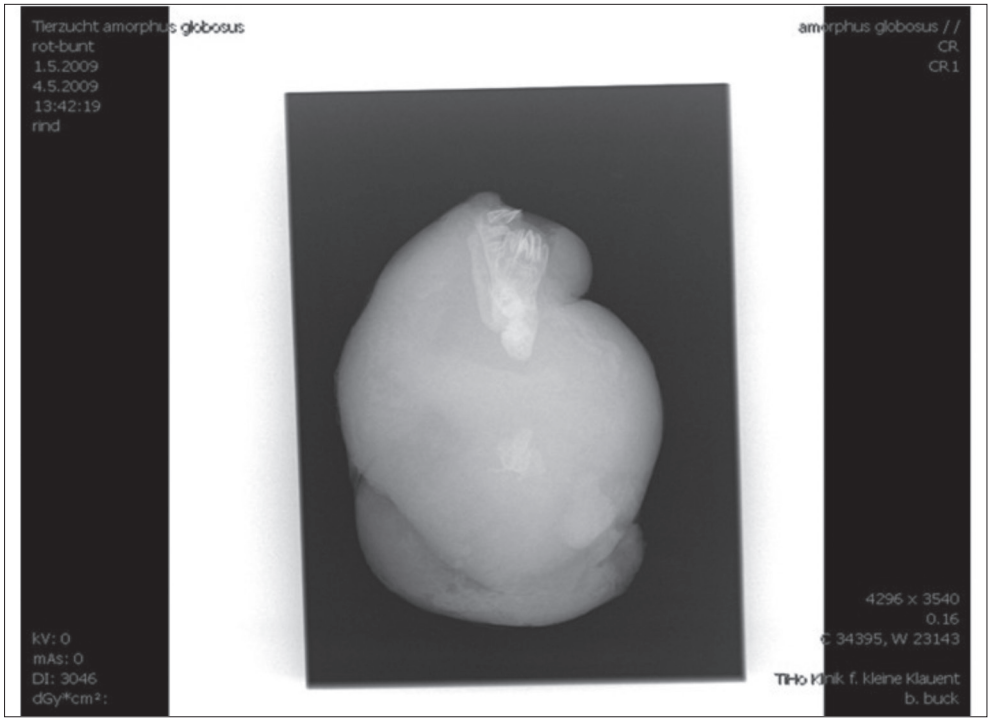

Fig 2. The radiography shows lower and upper jaws as well as several small bones in the body of the amorphous globosus

Şekil 2. Radyolojik incelemede Amorphous globosus'un vücudunda görülen, alt ve üst çene oluşumu ve bunun yanı sıra küçük kemik parçaları

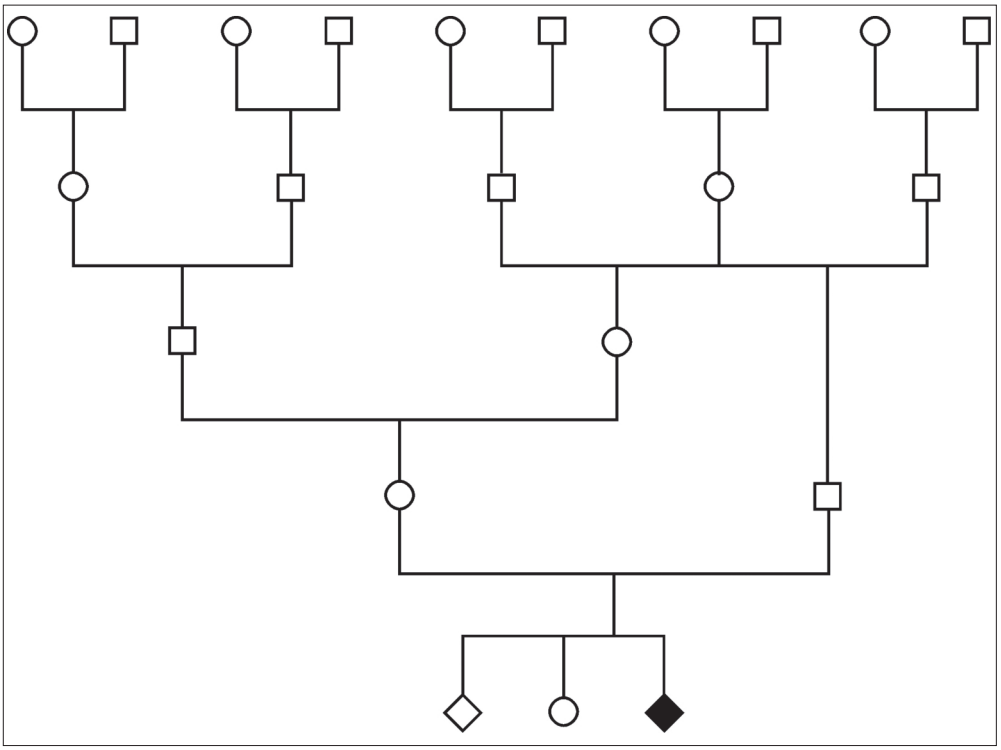

Fig 3. The pedigree of the amorphous globosus

Şekil 3. Amorphus globosus'un soyağacı

body with extremities. The acardius acormus consists of a partially developed head and brain. The acardius acephalus is lacking the head and thoracic organs [4,14].

Macroscopical findings in AG are usually a spherical or ovoid form of the body, covered with skin and hairs, and the umbilical cord [8,11,15]. Moreover, an imperfect tongue, oral or/and anal cavity, digestive organs or their vestiges were reported [11,13]. The present case had a small oral-like-structure, in which a tongue-like structure with lips and three milk teeth were present.

Histopathological findings in AG showed different tissues with changeable degrees of development [7,8,11,13]. In agreement with previous reports we found connective tissue, skeletal muscles, lymph tissue, arterial vessels, cartilage tissue as well as tubular and glandular structures. Further, the present case had no adipose and necrotic tissues.

In agreement with previous reports showing various bone formations in the spherical body forms [10,12,16], we found lower and upper jaws with teeth and remnants of a bony tail with cartilage in the present case.

There are three different hypotheses on the etiopathogenesis of amorphous fetus. First, failure of heart formation probably due to insufficient splanchnic mesodermal elements, second anastomosis of the umbilical vessels causing a backward circulation in one of the twins and an obliteration of the heart anlage [7] and third interference with the return flow of blood from the placenta to the twin. The second hypothesis is generally believed as the cause of most amorphous anomalies. The inbreeding coefficient for the present case was 6.25% due to a common ancestor on the maternal and paternal side.

In conclusion, an anastomosis of artery to artery or vein to vein could not be found in the present amorphous case and thus, this hypothesis could be ruled out. Two large blood vessels and many small vessels were observed in the umbilical cord-like structure in the present case. However, only very few blood cells were noted in the lumen suggesting that the present case may have been caused by insufficient blood supply and in consequence to an obliteration of the heart anlage. Support was obtained for this assumption from previous reports with similar findings [8,10].

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Review

How Can Better Anaesthetic Combinations Be Performed? A Review of Current Knowledge

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Abstract

In the ever-developing world, the recent years have brought about a wide range of novelties and significant developments in the fields of veterinary anaesthesia and analgesia. In the not-too-distant past, it was believed that the pain threshold of animals was high and that pain was not felt by animals during surgery, when they were anaesthetised. It was considered that the only analgesic drugs that could be used during operation were non-steroidal anti-inflammatory drugs. Today, these suppositions are all accepted as fallacies across the world, and it is well-known that the administration of analgesic drugs should be started in the preoperative period. This article describes pain in animals, how pain perceived by the operated animal can be recognised, and if not treated, to which complications pain may lead. Furthermore, in the last part of the manuscript, complications associated with the use of atropine and α2-agonist combinations, and the adverse effects of anaesthesia-induced hypotension are also explained. This review is intended to provide an insight into recently developed novel practices and to elucidate some main issues, which may be confusing for the veterinary practitioner.

Keywords: *Anaesthesia, Analgesia, Pain, Cat, Dog*

Nasıl Daha İyi Anestezi Kombinasyonları Yapılabilir? Güncel Bilgilerin İncelenmesi

Özet

Gelişen dünya ile beraber son zamanlarda veteriner anestezi ve analjezi alanında da pek çok değişiklikler, yenilikler ve ilerlemeler kaydedilmiştir. Eskiden hayvanların ağrı eşiklerinin yüksek olduğuna ve operasyon sırasında anestezi altında iken ağrının hissedilmediğine inanılırdı. Operasyon sırasında tek kullanılabilecek analjezik ilacın nonsteroidal antiinflamatuvar olabileceği düşünülüyordu. Bu inanışların yanlış olduğu artık tüm dünyada kabul edilmektedir, analjezik ilaçların uygulanmasına preoperatif dönemde başlanması gerekliliği bilinmektedir. Bu makalede, hayvanlardaki ağrı, operasyon sırasında gelişen ağrının nasıl anlaşılabileceği ve tedavi edilmediği takdirde gelişebilecek komplikasyonlar anlatılmıştır. Ayrıca, makalenin sonunda atropin ile α2-agonist kombinasyonlarının komplikasyonları ve anestezi sırasında oluşan hipotansiyonun yan etkileri üzerinde de durulmaya çalışılmıştır. Bu derleme ile veteriner hekimlerin kafalarında karışıklık yaratan bazı konulara ışık tutulması, son yıllarda gelişen yeni uygulamalara ve ilaç kombinasyonlarına yer verilmesi amaçlanmıştır.

Anahtar sözcükler: *Anestezi, Analjezi, Ağrı, Kedi, Köpek*

INTRODUCTION

Anaesthesia means "loss of sensation in the entire body or any part of the body". Most people think that humans or animals do not feel pain during anaesthesia. However, today it is known that without the use of analgesics both humans and animals feel pain during anaesthesia. Most popular general anaesthetics do not have analgesic properties. They provide unconsciousness, but cannot inhibit the nociceptive pathways. No matter how high a dose they are administered at, these agents do not provide analgesia. The animal can feel pain during surgery, however, cannot react because of a surgical plane of anaesthesia. The aim of this review is to present the recognition of pain in animals, basis for analgesic use during anaesthesia, the appropriate time and analgesic combinations for administration, adverse effects of pain, some analgesic medications, methods to overcome possible complications during anaesthesia and the contraindication of using α2-agonists with anticholinergics.

PAIN IN ANIMALS

The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or

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described in terms of such damage [1]. Although great importance is attached to pain management and analgesic therapy today [2], unfortunately, research on analgesic therapy in veterinary medicine does not go back too far in history. The main reason for veterinary research having started late is fallacies. In the not-too-distant past, it was believed that animals did not suffer from pain as much as humans and it was considered that the pain threshold of animals was higher. Today, it is well known that these arguments are false, and it is well established that all procedures that cause pain in humans also cause pain in animals and require analgesic therapy. In the past, the majority of veterinary practitioners believed that pain was protective in that it limited the movement of the animal, and thereby, reduced the risk of tissue lesions. Furthermore, it was thought that, as analgesia masks disease symptoms, analgesic therapy would misdirect treatment. However, today, it is accepted that pain provides very little benefit, and in fact, causes great damage to the body [3-5].

Why Should Analgesics Be Used?

Apart from the ethical perspective and moral compass, treatment of pain has major physiological and biological implications. Pain increases the secretion of catabolic hormones (cortisol, glucagon, and catecholamines) and decreases the secretion of anabolic hormones (insulin, testosterone). Furthermore, pain increases gluconeogenesis, glycogenolysis, proteolysis, and lipolysis, and causes protein loss and the weakening of muscles. Due to reduced collagen synthesis, pain is also associated with delayed wound healing. Stress induces the secretion of the adrenocorticotropic hormone (ACTH), which in return, leads to the secretion of cortisol. As a result of immunosuppression, both immunoglobulin synthesis and the resistance of the individual to diseases, decrease [6,7]. Catecholamines make the heart sensitive to arrhythmia and cause hypertension. The cardiac output and load, heart rate, and the oxygen consumption of the myocardium, all increase. This increase in cardiac parameters is dangerous if the cardiac reserves are low. Increased cardiac load requires a higher level of oxygen, and if the coronary arteries do not pump the required amount of blood to the heart, myocardial infarction occurs [4,8-10].

Renal failure may develop as a result of the vasoconstriction of the renal arteries. Hyperglycaemia occurs. While the respiratory rate increases, the tidal volume and cough reflex decrease, which eventually lead to pulmonary atelectasis. Small airways closed, intrapulmonary shunts and hypoxia are observed. These complications are particularly important in operations performed in the thoracic cavity and in the proximity of the diaphragm. Elevated carbon dioxide levels lead to respiratory acidosis. Acidosis causes arrhythmia and may even result in cardiac arrest [7,11,12].

When in pain, animals avoid movement, and long-term immobility increases the risk of embolism [5]. Pain often results in a prolonged hospital stay, immune suppression and secondary illness, inappetence, and cachexia. This is especially important in cats, in which hepatic lipidosis may occur as a result of inadequate caloric intake [5]. In particular, neuroendocrine responses to posttraumatic and postoperative pain may cause shock and death [4,10].

As there is a link between acute pain and chronic pain, it can be suggested to control acute pain in order to prevent the development of chronic pain [5,9].

Physiological pain is protective. It plays an adaptive role in the body's normal defence mechanism like touching a potentially damaging fire and initiating reflex avoidance strategies. Physiological pain alone is not important in clinical settings. On the other hand, if pain becomes permanent and is associated with severe tissue inflammation and nerve injury, then this type of pain is referred to as "pathological or clinical" pain. The clinical objective should be to minimize pathological pain while maintaining physiological pain [3,4]. It is useful to characterize clinical pain according to its duration, such as acute (recently occurring) and chronic (long-lasting) pain. Acute clinical pain arises from soft tissue trauma or inflammation, with the most common example being postoperative surgical pain. Actually, acute pain plays an adaptive role by facilitating tissue repair. If not promptly treated, acute pain turns into chronic pain. Chronic pain is maladaptive and offers no useful biological function or survival advantage. Cases of very severe acute pain or the development of chronic pain result in the manifestation of a hypersensitivity phenomenon referred to as "hyperalgesia". Hyperalgesia refers to an overly increased sensitivity to painful stimuli, and can be defined as the stimulation of both inactive nociceptive fibers, and high threshold nociceptive fibers, in healthy individuals [10,13]. The most advanced forms of hyperalgesia are allodynia, which refers to pain produced by a stimulus that is not normally noxious, and phantom pain, which is described as perceptions experienced by an individual in relation to a limb or an organ that is not physically part of the body [4]. The pain experienced by a soldier, who receives long-term treatment before an amputation, and even after an amputation he feels as if his arm was not amputated is a good example of phantom pain. Furthermore, the crying of a dog with an osteosarcoma in one of its limbs, when patted on its head, is due to the perception of being touched as pain,as a result of hyperalgesia.

ADDITION OF ANALGESIA IN ANAESTHESIA PROTOCOLS: IS IT NECESSARY OR NOT?

Loss of consciousness does not mean that the pain pathways are inactivated. Most general anaesthetics, including isoflurane, sevoflurane, propofol, and thiopental,

do not have analgesic properties. They provide unconsciousness, but cannot inhibit the nociceptive pathways. No matter how high a dose they are administered at, these agents do not provide analgesia. The patient can feel pain during surgery, however, cannot react because of a surgical plane of anaesthesia. It is still beneficial to inhibit pain pathways by using some analgesic drugs. Nowadays, preemptive and multimodal analgesia have become popular techniques used for this purpose. When general anaesthesia is used for surgery, the administration of drugs with analgesic properties should be included in the anaesthesia protocol [14-17]. Intraoperative use of multimodal analgesic therapy also reduces total anaesthetic requirements and autonomic responses to painful surgical stimulation [10,18-20].

The perception of intraoperative pain by an anaesthetized animal can be understood from an increase in the heart rate and blood pressure. While the anaesthesia continues and the monitored parameters remain stable, a sudden increase observed in the cardiac rhythm and blood pressure (approximately 30%) with the onset of the painful phase of the surgical operation (movement of the fracture ends during osteosynthesis, the distention of the peritoneum during laparotomy or surgical intervention performed near nerves etc.) shows the inadequacy of the analgesia procedure that has been followed as part of the anaesthesia protocol [11,15,21]. A medical malpractice performed in such cases is to administer a higher anaesthetic dose to the operated animal during increased heart rate caused by the perception of pain. In fact, the veterinary surgeon should only reduce the sympathetic stimulus, by deepening the anaesthesia, and should also decrease tachycardia. It should be noted that, if not administered with any analgesic, an anaesthetized animal shall perceive pain, even if unconscious. The adverse effects of pain on the body start during the surgical operation. An animal with postoperative pain is most likely to recover from anaesthesia poorly. In such cases, the animal may even damage the operation site while awakening. While the anaesthetic agents used before the 1980s provided a slow and slumbery recovery from anaesthesia, today, preparations (i.e. propofol, thiopental, isoflurane, sevoflurane), which enable the animal to recover from anaesthesia within only a few minutes after the end of the surgical operation, are available. If adequate analgesia is not achieved, then recovery from the anaesthesia established with these novel agents occurs fast, but also painfully.

In order to minimize intraoperative and postoperative pain, analgesics need to be administered at the appropriate time intervals and at the appropriate doses. In this context, recently, the clinically evolving concept "preemptive analgesia" has gained importance. Preemptive analgesia involves the administration of analgesics before the exposure of the animal to the painful stimulus, and in a sense, implies the elimination of pain even before it occurs. Thereby, pain management can be achieved without the development of hyperalgesia. The most appropriate stage to administer analgesics to an animal scheduled for surgical operation is the preoperative period [8,15,22,23].

Both the mechanism of action and the time course of the effects of all analgesics, which are routinely used in clinical practice today, are well known. The prior administration of the analgesic preferred for the surgical operation to be performed, and the repetition of the dose at the half-life of the analgesic, followed by an appropriate use of the agent in the postoperative period, will ensure that the patient recovers with little or no pain. It should be borne in mind that no analgesic can set pain to zero. Therefore, the goal of the administration of analgesics should be to eliminate pain or, at the very least, make the animal comfortable [14,15].

ANALGESICS THAT CAN BE USED DURING OPERATION

As each animal presents with varying levels of injury or illness, and experiences different levels of pain, individual drug selection and dosage are essential rather than the application of a standard protocol for all patients. Several groups of analgesic drugs are available for use in cats and dogs, which include opioids, α2-agonists, ketamine, NSAIDs (nonsteroidal antiinflamatory drugs) and local anaesthetics [9,10,24,25] that have been explained in below.

Opioids

Opioids are the most commonly preferred drugs for intraoperative and early postoperative analgesia. Their analgesic effect is strong as they block several pain pathways [10,26]. The most commonly used opioid drugs in Turkey are morphine, butorphanol and fentanyl. Some of the adverse effects associated with the use of these drugs are bradycardia, respiratory depression, hypotension, agitation and emesis. Although opioids are generally feared to have serious side effects, in contrast to human beings, cats and dogs seem to be remarkably resistant to these side effects, and serious side effects are surprisingly rare when these agents are used with care [8,11,27]. For example, opioid induced-bradycardia is generally not life-threatening and usually does not require treatment. Often it can be ameliorated with fluid therapy. If the patient is hypovolemic, hypovolemia should be treated before opioid-induced bradycardia, because no benefit has been shown to be achieved with anticholinergics in the hypovolemic patient [9]. As opioid induced-bradycardia is vagally mediated, when necessary, treatment can be accomplished with anticholinergic drugs (atropine or glycopyrrolate) [28]. Although clinically significant respiratory depression from opioids is uncommon, it can occur when opioids are combined with other drugs that have respiratory depressant effects. Bradycardia and hypotension are mostly observed when

opioids are administered intravenously [11,13,19]. The greatest advantage of opioids is the ability to titrate their dose to effect; and if an overdose or side effects occur, naloxone, which is an opioid antagonist, can also be titrated to remove these adverse effects as the analgesic effect continues [5,28].

The cheapest opioid in our country, and in the world, is morphine. It is a very effective analgesic in dogs and cats, and its dose should be calculated precisely when used in cats. If the clinician is not familiar with the use of opioids, and fears about side effects, a safeway of use is to administer effective low doses of opioids to healthy and young animals for the desired effect (e.g. sedation, relaxation, termination of pain, and maintenance of tranquilization during manipulation) [11]. If the desired effects do not occur within 3 minutes after intravenous administration then the dose is repeated until the desired effect is achieved. Once the analgesic and relaxing effects of the opioid is observed, the veterinary clinician may easily prefer to widen the scope of use of opioids. Occasionally, agitation and anxiety can be recorded and many of these patients vocalize continuously while treated with morphine. If a dog develops this type of response, the clinician has 5 options: (1) administering higher doses of morphine as higher doses cause sedation, (2) administering α2-adrenergic agonists both for sedation and additive analgesic effect, (3) combining the first and second options, (4) reversing the effect with the intravenous administration of butorphanol (as butorphanol is a mixed agonist-antagonist opioid, its analgesic effect continues while anxiety disappears as a side effect) and (5) administering naloxone for the reversal of the effect of morphine. As naloxone is an opioid antagonist, dose calculation (0.001-0.02 mg/kg, IM, IV) should be made carefully, because high doses of naloxone antagonize both the analgesic and its side effects [11,29]. Matthews [30] recommended using the naloxone dilution technique to reverse the side effects of opioids while their analgesic effects continue. When this technique is used for larger animals, 0.25 ml of naloxone (0.4 mg/ml) diluted in 10 ml of saline is used for titration. Once the side effects are reversed and a state of rest is achieved, the administration is terminated.

The first choice for animals, which are referred to the clinics for trauma or acute pain (e.g. peritonitis, pancreatitis) and cannot be treated due to this pain, should be opioids. These patients may also present with premature ventricular contractions (PVCs) or tachyarrhythmia as a result of trauma and/or pain. Attempts to control pain with the administration of sedatives or anaesthetics may cause further cardiovascular deterioration. The administration of opioids to these patients for analgesia can also be advantageous by enhancing the vagal tone [9,11].

Cats have a low tolerance to high doses of morphine and they more often show disorientation and agitation, and overdose often produces delirium. Hansen [11] recorded that marked mydriasis occurs after the administration of an adequate analgesic dose, and suggested that further administration of opioids after the onset of mydriasis is much more likely to produce agitation. Because of the common side effects of morphine in cats, butorphanol might be the first choice for cats with mild to moderate pain.

Adjuvant Analgesics

Adjuvant analgesic drugs are not first-choice analgesics and are generally used in combination with other known traditional analgesics as a part of the multimodal analgesic approach. Multimodal analgesia refers to the blockade of different pain pathways by the combined use of various analgesics and techniques. Thereby, an effective analgesia is achieved at low doses and with little side effects through the synergistic effects of different pharmaceuticals [25]. The pharmaceuticals most commonly used for multimodal analgesia are ketamine, α2-agonists, lidocaine, and tricyclic antidepressants like gabapentin.

Ketamine has been used as an anaesthetic agent for more than 30 years in veterinary medicine. Ketamine belongs to the group of dissociative anaesthetics and its high doses are combined with other agents for general anaesthesia. In the 1980s, it was discovered that ketamine was an antagonist of the N-methyl D-aspartate (NMDA) receptors in the spinal cord. Since this discovery, ketamine has been used as an adjunctive analgesic in humans. In clinical practice, it is suggested that low doses of ketamine be used for analgesia. When administered at low doses, ketamine can enhance analgesia and eliminate the hypersensitivity phenomenon [31]. Thereby, it also aids in the management of acute postoperative pain, acute posttraumatic pain, neuropathic pain, preoperative analgesia, and chronic pain [13,14,30,32,33]. Slingsby and Waterman-Pearson [34] reported that the preoperative and postoperative administration of 2.5 mg/kg of ketamine by intramuscular route to animals anaesthetized for ovariohysterectomy reduced the total pain score, and decreased both the need for rescue analgesia and the risk of postoperative wound hyperalgesia, in comparison to the control animals. In another study [35], it was shown that, when administered to cats as an analgesic agent at different doses, ketamine reduced the minimum alveolar concentration (MAC) of isoflurane by 45-75%. Muir et al.[36] reported that the administration of a ketamine infusion of 0.6 mg/kg/h to dogs reduced the MAC of isoflurane by 25%. Furthermore, Wagner et al.[37] indicated that, the preoperative and postoperative administration of ketamine to dogs, which underwent forelimb amputation, reduced the pain score. These researchers also observed that the operated animals, which were administered with ketamine, were more active during the first 3 days after operation, in comparison to the animals, which were not administered with ketamine. In many other similar studies, ketamine has been suggested to be administered as a constant rate infusion (CRI) and to be included in balanced anaesthesia protocols. Like α2-adrenergic agonists, ketamine

can also be combined with opioids to reduce the total opioid dose for the management of pain [21,29,32].

α2-*adrenergic agonists* (xylazine, medetomidine) are preferred for preemptive analgesia, yet their analgesic effects are not as strong as those of opioids. Therefore, they are considered to be inadequate for producing the intraoperative analgesia required for very painful surgical operations (osteosynthesis, cervical disk operations, spinal cord operations, amputations, neurosurgery, total ear canal operations, etc.). The combined administration of α2-adrenergic agonists with opioids may result in additive or synergistic drug interactions, and thereby, reduce the dose of the analgesic drug used [10,14,25,32,38,39]. The combination of medetomidine and ketamine is mostly preferred for castration and ovariohysterectomy in cats and dogs in many countries. However, it is important to recognize that although adequate analgesia is achieved with the use of α2-adrenergic agonists and ketamine, the duration of this analgesia is limited to, and often less than that of the sedative effect [24,32,40].

Lidocaine, in addition to its local anaesthetic effects, has been shown to alleviate hyperalgesia and to reduce opioid requirements during and after surgery when administered as a constant rate infusion [9,32]. While the mechanism underlying the nervous transmission block caused by the peripheral administration of lidocaine has been completely resolved, the analgesic mechanism of the intravenous administration of lidocaine is yet not solved. Several researches conducted in humans have shown that, following major abdominal operations, lidocaine increases gastrointestinal functions, reduces postoperative pain and the need for the use of opioids, and also shortens the hospitalization and rehabilitation periods [32,41,42]. A single dose of 2 mg/kg lidocaine should result in a short period of analgesia and MAC reduction. For this reason, it may be added to anaesthesia induction protocols [9]. While it has been shown that the administration of lidocaine, as a CRI of 0.05 mg/kg/min, reduces the MAC of isoflurane by 19-29% in dogs anaesthetized with isoflurane [36,43], it has also been reported that the use of lidocaine infusions up to 0.12 mg/kg/min are safe in dogs anaesthetized with isoflurane [44]. Pypendrop and Ikliw [45] have reported that cats are sensitive to lidocaine toxicity, and have suggested this agent not to be used in cats as it causes cardiovascular depression during anaesthesia.

Constant rate infusions (CRI) have recently gained popularity in perioperative (pre-, intra-, and post) analgesia [17,46,47]. There are several advantages to this technique, as a steady level of analgesia is more likely to be achieved and the mountains and peaks associated with intermittent analgesic use are avoided. Morphine, lidocaine and ketamine can be added to the intravenous solution. 12 mg of morphine, 150 mg of lidocaine and 30 mg of ketamine in 500 ml of surgical fluid, dripped at the standard surgical fluid rate (10 ml/kg/h), provide analgesia and MAC reduction during surgery. Multimodal analgesia can be achieved with this cocktail solution. If prepared under sterile conditions, it can be stored for use for a long time. In addition, this cocktail can be administered for postoperative analgesia at a dose of 2 ml/kg/h [24]. The author frequently uses triple combinations in dogs and dual combinations (morphine and ketamine) in cats due to their sensitivity to lidocaine.

Nonsteroidal Anti-Inflammatory Drugs (NSAIDS)

Systemic inflammatory response sensitizes the peripheral nervous system and can turn a normal somatic stimulus into a painful stimulus (remember how you felt last time when you had a fever!). This is because of the systemic release of inflammatory mediators [11]. As well as having central analgesic effects, NSAIDs may also reduce the peripheral inflammatory response associated with surgery, and thus, may provide sufficient analgesia for mild to moderate pain [25,29].

Once acute severe pain is controlled with an opioid, NSAIDs may be administered. The NSAIDs most commonly used in cats and dogs are ketoprofen, meloxicam, tolfenamic acid, flunixin meglumine, and carprofen (carprofen is registered only for dogs and can be used only once in cats). NSAIDs should be avoided until the renal, hepatic, circulatory, and coagulation status of the patient is known [5,14,28,29].

COMBINATION OF ALPHA2-AGONISTS WITH ANTICHOLINERGICS FOR ANAESTHESIA: IS IT RIGHT OR NOT?

α2-agonists, including xylazine, medetomidine and dexmedetomidine, are the most widely used sedatives in veterinary medicine [3,21,48-51]. These drugs are also administered during general anaesthesia to improve analgesia and to decrease the dose of the anaesthetics used. The most commonly observed clinical side effects after the administration of α2-agonists are peripheral vasoconstriction and reflex bradycardia [32,51,52]. There is no doubt that if bradycardia becomes profound, it should be treated. The question is: "Should we use anticholinergics for the treatment of bradycardia to save life or not?" Formerly, atropine had been frequently used to prevent bradycardia induced by α2-agonists [53], and the data sheet (package insert) for Rompun (commercial xylazine preparation) states that premedication with atropine may be advantageous in dogs. However, data reported in previous studies is based on the assessment of only the heart rate. For the past 20 years, it is known that such a correction is not always advantageous [53,54]. The administration of anticholinergics before α2-agonists effectively prevents bradycardia. However, the administration of anticholinergics either prior to, at the same time with, or after high doses

of medetomidine has been shown to result in severe hypertension, tachycardia, tachyarrhythmia, atrioventricular blockade, and pulsus internans [1,53,55-59]. There are contradictory opinions with respect to the prevention and treatment of α2 agonist-induced bradycardia. Some authors have recommended reversal with α2-agonists as the safest remedy for α2 agonist-induced bradycardia [1,53,58].

The underlying mechanism of α2 agonist-induced bradycardia can be explained as follows: α2-agonists show biphasic effects on blood pressure. Their first effects are vasoconstriction, caused by the activation of the peripheral α2-receptors of blood vessels, and resulting hypertension. Systolic blood pressures of approximately 200-250 mmHg have been recorded in dogs [32,52,53,57,58,60]. The vasoconstriction observed in the periphery also occurs in the coronary arteries, which decreases the blood flow, and thus, the amount of oxygen transported, to the heart. Due to the subsequent deepening of anaesthesia and the decrease of the sympathetic tone, the hypotensive phase occurs, during which bradycardia develops [1]. The occurrence of bradycardia when the blood supply of the heart decreases, is in fact a compensatory response, as it reduces the activity of the heart muscle, and thereby, decreases the oxygen and energy requirements of the myocardium.

Although anticholinergics alleviate the decrease in heart rate following the administration of α2-agonists, these drugs also increase the magnitude and duration of the hypertensive phase caused by α2-agonists. It has also been reported that, in dogs, hypertension is even greater when higher doses of α2-agonists are used [54]. The administration of anticholinergics, including atropine and glycopyrrolate, disrupts this compensation mechanism and causes an increase in heart rate and myocardial oxygen consumption. The administration of anticholinergics when the coronary arteries are constricted, in other words, when the myocardial blood supply is decreased, is a fatal mistake, as the oxygen and energy requirements of the myocardium increase if cardiac perfusion is not improved [1,59].

In clinical practice, α2-agonists are mostly used in combination with ketamine to establish anaesthesia, as ketamine has sympathomimetic effects. In other words, ketamine not only increases heart rate and cardiac output, but also arterial pressure and myocardial oxygen consumption. Therefore, hypotension and bradycardia induced by α2-agonists can be ameliorated, to some degree, by subsequent ketamine administration. The concurrent administration of anticholinergics with α2 agonist-ketamine combinations should also be avoided, because a prolonged high heart rate may occur. Xylazine-ketamine combinations are usually restricted to healthy animals and should not be used in patients with myocardial disease or reduced cardiopulmonary reserve [1].

HYPOTENSION DURING ANAESTHESIA

Hypotension is the most common perianaesthetic complication in veterinary patients. Normal systolic, diastolic, and mean arterial blood pressures in nonanaesthetized small animals are 100-160, 60-100, and 80-120 mm Hg, respectively. If systolic and mean blood pressures are measured below 80 and 60 mm Hg, respectively, this condition is referred to as hypotension. Hypotension, if not treated, can result in decreased perfusion to vital organs and death. Various anaesthetic agents such as α2-agonists, propofol, thiopental, isoflurane, sevoflurane, and opioids have hypotensive effects. Intravenous crystalloid fluid solutions at a dose of 10 ml/kg/h should be given to alleviate anaesthesia-induced hypotension. Treatment of acute blood loss due to haemorrhage includes crystalloid fluid replacement at a dose of three times the blood volume lost [61-63]. If intraoperative anaesthesia is deeper than required, side effects resulting from hypotension and hypertension increase.

When the animal is hypotensive, nonsteroidal anti-inflammatory drugs should not be used because they prevent the production of prostaglandins. Prostaglandins (PGs) have an important role in the auto-regulation of renal blood supply. As NSAIDs inhibit the production of PGs, their use in hypotensive, bleeding and dehydrated animals, may cause nephron damage and lead to complications as severe as acute renal failure. For this reason, if there is a risk of intraoperative hypotension (in view of the most anaesthetic drugs is hypotensive and some clinicians performing intraoperative fluid administration at an almost negligible level, which both imply that the majority of anaesthesia procedures are associated with the occurrence of hypotension), it is suggested that NSAIDs should not be used [5,14,28,29].

CONCLUSION and RECOMMENDATION

None of the drugs used to establish anaesthesia and analgesia are perfect, and further research is conducted with an aim to achieve the best intraoperative anaesthesia and analgesia with the least complications. Combination of drugs is essential for multimodal analgesia and balanced anesthesia. Without multimodal treatment, we cannot achieve a perfect anaesthesia. It should also be noted that, the addition of analgesic drugs to anaesthetic combinations not only lowers the amount of anaesthetics required, but also reduces the risk of complications that may result from the use of high doses of anaesthetics. Clinicians must be prepared to determine the best choice.

In conclusion, if we do not try novel drugs and persist with using conventional drugs, we can never do “better”.

Various analgesic and anaesthetic protocols are available for cats and dogs, and it is recommended that "tried and true" guidelines be used rather than "sticking to traditional or outdated dogma".

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YAZAR İNDEKSİ için tıklayınız

YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup ve derleme türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.

2- Dergide yayımlanması istenen yazılar Times New Roman yazı tipi ve 12 punto ile A4 formatında, 1.5 satır aralıklı ve sayfa kenar boşlukları 2.5 cm olacak şekilde hazırlanmalı ve şekil ve tablo gibi görsel öğelerin metin içindeki yerlerine Türkçe ve yabancı dilde adları ve gerekli açıklamaları mutlaka yazılmalıdır.

Dergiye gönderilecek makale ve ekleri (şekil vs) *http://vetdergi.kafkas.edu.tr* adresindeki online makale gönderme sistemi kullanılarak yapılmalıdır.

Başvuru sırasında yazarlar yazıda yer alacak şekilleri online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış *Telif Hakkı Devir Sözleşmesi* editörlüğe gönderilmelidir.

3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.

4- ***Makale Türleri***

Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır.

Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 12 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.

Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.

Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.

Gözlem (Olgu Sunumu), uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri Giriş, Olgunun Tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.

Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 2 sayfayı geçmemelidir.

Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri Giriş, Metin, Sonuç ve Kaynaklar bölümlerinden oluşmalı ve 12 sayfayı geçmemelidir.

5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.

6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır.

Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.

Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.

Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.

Örnek: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI numarası bulunan kaynaklarda bu bilgi ilgili kaynak künyesinin sonuna eklenmelidir.

Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi, kaynak bilgilerinin sonuna eklenmelidir.

Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.

Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.

7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.

8- Editörlük, dergiye gönderilen yazılar üzerinde gerekli görülen kısaltma ve düzeltmeleri yapabileceği gibi önerilerini yazarlara iletebilir. Yazarlar, düzeltilmek üzere yollanan yazıları online sistemde belirtilen sürede gerekli düzeltmeleri yaparak editörlüğe iade etmelidirler. Editörlükçe ön incelemesi yapılan ve değerlendirmeye alınması uygun görülen makaleler ilgili bilim dalından bir alan editörü ve iki raportörün olumlu görüşü alındığı takdirde yayımlanır.

9- Yayınlanan yazılardan dolayı doğabilecek her türlü sorumluluk yazarlara aittir.

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1- The Journal of the Faculty of Veterinary Medicine, University of Kafkas (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly, covers original papers, short communication and preliminary scientific reports, case reports, observation, letter to the editor, and review and on all aspects of veterinary medicine and animal science (clinical and paraclinical sciences, biological and basic sciences, zoonoses and public health, animal feeding and nutritional diseases, animal breeding and genetics, hygiene and technology of food from animal sources, exotic animal science). Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table must clearly be written in both Turkish and foreign language and their appropriate position should be indicated in the text.

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Manuscripts consist of the title, abstract and keywords, Introduction, Material and Methods, Results, Discussion, and References and it should not exceed 12 pages including text, tables and illustrations. Abstract should contain 200±20 words.

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Preliminary Scientific Reports are short description (maximum 4 pages) of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles.

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Letters to the Editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases without exceeding 2 pages.

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Example: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4^{t}h ed. ,339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must be complied with the other references.

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