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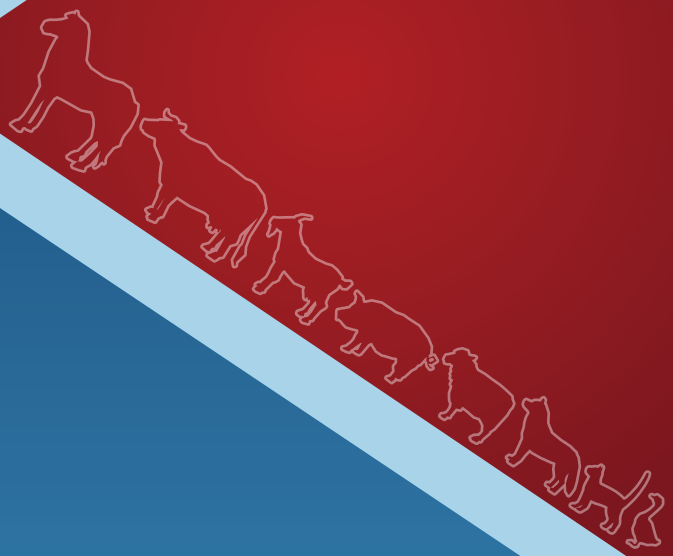

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RESEARCH ARTICLE

Role and Importance of Cardiac Biomarkers in Diagnosis and Prognosis of Feline Arterial Thromboembolism

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Abstract

Feline arterial thromboembolism (FATE) is a common complication of myocardial disease, often having poor prognosis. The purpose of this study is to evaluate the diagnostic and prognostic value of serum levels of cardiac biomarkers (N-terminal prohormone of brain natriuretic peptide [NT- proBNP], creatine kinase isoenzyme-MB [CK-MB], and cardiac troponins [cTnI and cTnT] in cats with hypertrophic cardiomyopathy (HCM) that was complicated by FATE. Two groups were constituted in the study. Cats with a diagnosis of HCM were included in group I (n=10) and cats with HCM having acute episodes of FATE were included in group II (n=10). Results of cardiac biomarkers and echocardiographic measurements of left ventricle related parameters were compared between groups. The ratio of left atrium to aorta ($P<0.05$), fractional shortening ($P<0.05$), left ventricular dimensions ($P<0.05$), and stroke volume ($P<0.01$) were found statistically significant between groups. Serum CK-MB and cTnI levels in group II were higher ($P<0.001$ and $P<0.05$ respectively)) than those in group I. Serum levels of cTnI and cTnT were found respectively under 3.0 ng/mL and 0.1 ng/mL in the cats (n=3) which have survived. Our data demonstrate that remarkably elevated serum levels of cardiac biomarkers could be associated with the diagnosis of HCM with acute onset of FATE in cats, however cTnI and CK_MB might have a role in the risk assessment.

Keywords: *Cardiac troponins, CK-MB, Feline arterial thromboembolism, Hypertrophic cardiomyopathy, NT-proBNP*

Feline Arteriyel Tromboembolizm Tanısı ve Prognozunda Kardiyak Biyobelirteçlerin Rolü ve Önemi

Öz

Feline arteriyel tromboembolizm (FATE), miyokardiyal hastalıkların en sık görülen ve genellikle kötü bir prognoza sahip olan bir komplikasyonudur. Bu çalışmanın amacı, FATE gelişmiş kedilerde, N-terminal prohormon beyin natriüretik peptid (NT-proBNP), kreatinin kinaz (CK_MB) ve cTnI, cTnT serum seviyelerinin diyagnostik ve prognostik önemini belirlemektir. Çalışma için iki grup (I ve II) oluşturuldu. HCM teşhisi almış kediler grup I'e (n=10) dahil edildi. Akut FATE gelişimi olan HCM'li kediler ise grup II'e (n=10) dahil edildi. cTnI, cTnT, NT-proBNP, CK-MB seviyeleri tüm grupta analiz edildi ve kardiyak belirteçlerin sonuçları ile sol ventrikül ölçümleri gruplar arasında Mann-Whitney U non-parametrik test ile karşılaştırıldı. Kardiyak belirteçlerin seviyesi, ekokardiyografik ölçümler ve yaşam süreleri Pearson korrelasyon testi ile karşılştırıldı. Gruplar arasında sol atriyum çapı ($P<0,05$), sol atriyum aorta oranında ($P<0.05$), fraksiyonel kısalma ($P<0.001$), sol ventrikül çapı ($P<0.01$) ve atım hacmi ($P<0.01$) ölçümlerinde istatistiksel farklılık saptandı. Grup II'e ait CK-MB ($P<0.001$), ve cTnI ($P<0.05$) ölçümlerinde anlamlı artış bulundu. Yaşamakta olan kedilerde (n=3), cTnI ve cTnT seviyeleri sırasıyla 3.0 ng/mL ve 0.1 ng/mL'nin altında bulundu. Çalışma verileri, belirgin şekilde artış gösteren kardiyak belirteçlerin akut olarak FATE gelişen HCM'li kedilerin tanısında önemli olduğunu göstermektedir. FATE için kardiyak biyobelirteçlerin diagnostik faydası bulunmaktadır, bununla birlikte cTnI ve CK_MB FATE'ye bağlı risk değerlendirilmesinde önemli bir role sahip olabilir.

Anahtar sözcükler: *CK-MB, Feline arteriyel tromboembolizm, Hipertrofik kardiyomiyopati, Kardiyak troponinler, NT-proBNP*

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is the most commonly diagnosed disease of the cardiac sarcomere and is diagnostically and therapeutically challenging for veterinarians [1,2]. It is reported that feline arterial thromboembolism (FATE) is a common complication of myocardial disease, often having poor prognosis [2]. Besides there is more interest in

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feline HCM, as it constitutes a model for study of human HCM [1,3].

For detection of myocardial damage, the cardiac biomarkers are useful parameters. Cardiac troponin I and T (cTnI and cTnT, respectively) are also highly sensitive and specific for myocardial damage [2,4-8]. It was shown that especially an increase in cTnI was also a reliable method for differentiating cats with moderate to severe HCM from normal cats with a sensitivity of 85% and specificity of 97% [2]. In another study it was reported that cTnI has a sensitivity of 62.0% and specificity of 100% when used to distinguish healthy cats from asymptomatic cats with HCM but without left atrial dilatation and high sensitivity and specifity (95% and 77.8%, respectively) for assessing heart failure [9]. It was determined that NT-proBNP is reliable on differentiating cardiac and respiratory causes of respiratory distress but it appears to be inadequate when it comes to prognostic information [6]. Also, it is known that creatine kinase isoenzyme-MB (CK-MB) is a useful cardiac biomarker to detect myocardial injury in cats although it is less sensitive than cTnI [2]. However, diagnostic and prognostic utility of troponin, NT-proBNP and CK-MB measurements in FATE remains unknown. Therefore, the purpose of this study was to evaluate the diagnostic and prognostic values of serum levels of cardiac biomarkers; N-terminal prohormone of brain natriuretic peptide (NT-proBNP), CK-MB, cTnI and cTnT in cats with HCM that was complicated by FATE. Although there are other cardiac biomarkers suitable for assessing myocardial status, this study focused on these biomarkers which are the most common and accessible cardiac biomarkers to evaluate myocardial stress and injury.

Material and Methods

Ethical Statement

Ethical approval of this study was obtained from Istanbul University-Cerrahpasa Faculty of Veterinary Ethical Committee (2021/17). The animals were treated in compliance with ethical standards.

Animal Selection and Groups

Two groups (I and II) were constituted in the study; cats with HCM (n=9) in group I, and that was complicated by acute episodes of FATE (n=24) in group II. Average age of group I was 6.9 and group II was 5.5. Approximately 70% of both groups were male cats. A diagnosis of HCM was established based on echocardiographic examination. Cats have an end-diastolic left ventricular wall (LVWd) thickness ≥6 mm, considered as HCM. Cats in group I were subclinical and classified as cardiomyopathy stage B1 and group II were classified as cardiomyopathy stage C according to ACVIM classification criteria [10]. The cats in group I were classified as stage B1 according to their LA size. All the cats that included in group I had normal or mild LA dilatation with ≥6 mm LVWd [10] *(Table 1)*.

All the cats have been evaluated for some other diseases or conditions that can cause a HCM phenotype such as anemia, systemic hypertension, hyperthyroidism, viral diseases, hydration status, kidney and liver function, blood glucose level, and recently used medications. Cats that have abnormal or inadmissible data in terms of these diseases and conditions have been excluded from this study.

Diagnosis of FATE was based on the presence of dysfunction in one or two limbs and clinical evidence of decreased perfusion (paresis, coldness, lack of palpable arterial pulses, pallor of the nail beds) in the affected limb(s). Cats with FATE were accepted in case they were brought to clinic within 12 h after the start of the episode. Presence of dilated, restrictive or unclassified cardiomyopathy and FATE diagnosis more than 12 h later constituted the exclusion criteria of the study. Survival time and affected limbs were recorded.

Radiography and Echocardiography

Radiographic evaluations were performed in all animals. The heart size was measured by vertebral heart score system in radiographs [11].

Echocardiography was performed in all groups using a micro convex probe running at 7-9.3 mHz. The cats were restrained in right lateral recumbency for echo-cardiographic examination which was performed from right chest wall using the parasternal long and short axis view. Interventricular septal end diastolic dimension (IVSd), interventricular septal end systolic dimension (IVSs), left ventricular diastolic diameter (LVd), left ventricular systolic diameter (LVs), left ventricular posterior wall end-diastolic diameter (LVWd), left ventricular posterior wall end-systolic diameter (LVWs), left atrium (LA), fractional shortening (FS), left ventricular outflow tract (LVOT), the ratio of left atrium to aorta (LA/AO) were measured [12]. HCM was defined in case of left ventricular wall and septal wall thickness with normal or elevated left ventricular fractional shortening. Symmetrical (concentric) or asymmetrical (eccentric) enlargements were noted.

Cardiac Biomarker Analysis

Cardiac biomarkers (cTnI, cTnT, NT-proBNP, and CK-MB) were analyzed in the serum samples of animals of two groups. CK-MB was analyzed by kits from Spinreact (Spain). For determination of cTnI and cTnT, Immulite 2000 Systems (TPI, TPT, Siemens, UK) were used following manufacturer's instructions. NT-proBNP was also measured in plasma lithium heparin using Immulite 2000 (Siemens, UK). Human reagents were used in the assays. Several human immunoassays cross-react to canine and feline samples and have appropriate sensitivity for the diagnosis of heart

diseases common to veterinary medicine. There is a great deal of homology between human and nonhuman cardiac troponin isoforms [13]. It was also reported that the interspecies homology of proANP is much greater than that of proBNP [14,15]. However, while evaluating, the differences among the groups were considered.

Treatment

Atenolol (6.25 mg/cat) was started to two groups. Treatment with enoxaparin sodium (1 mg/kg subcutaneously q6h) was performed in cats of group II. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured weekly. After 10 days of treatment with enoxaparin sodium, low dose aspirin (5 mg/cat q72h) was administered for prophylaxis. For supportive therapy, heat sources, physical therapy, oxygen supplement and analgesics (chlorpromazine hydrochloride with a dose of 0.4 mg/kg intravenously q6h) were also used. Amputation was performed when necrosis and erosions were occurred in those cases.

Statistical Analysis

The results of serum cardiac biomarkers and echocardiographic measurements of left ventricle were compared between the groups by Student's T test. Echocardiographic measurements and serum levels of cardiac biomarkers were compared within a group by Pearson correlation test.

Results

The vertebral heart score was more than 8 vertebral bodies in all animals. Eccentric hypertrophy was found in 2 cats of group I and 7 cats of group II. Concentric hypertrophy was detected in 7 cats of group I and 15 cats of group II and echocardiographic examination couldn't be performed on one of the cats from group II *(Fig. 1)*. The results of echocardiographic measurements and cardiac biomarkers were given in *Table 1*. The ratio of left atrium to aorta ($P<0.05$), fractional shortening ($P<0.001$), left ventricular dimension ($P<0.01$) and left atrium diameter ($P<0.05$) between the groups were found statistically significant. Significant increases were determined in CK-MB ($P<0.001$) and cTnI ($P<0.01$) in group II, compared with those in group I. Difference in cTnT and NT-proBNP levels between two groups was not statistically significant.

The cats included in the study were predominantly male (24/33). Only nine cats of group II survived of which two of them were known to be alive for more than one year. The other cats were followed for more than 3 months. Survival time and cardiac biomarker results of group II were given in *Table 2*. Serum levels of cTnI were found under 3.0 ng/mL in the cats (n=9) which have survived and serum levels of cTnT were found under 0.1 ng/mL in 5 survival cats. However, NT-proBNP couldn't be analyzed in two cats of group II and also cTnT couldn't be measured in one cat of

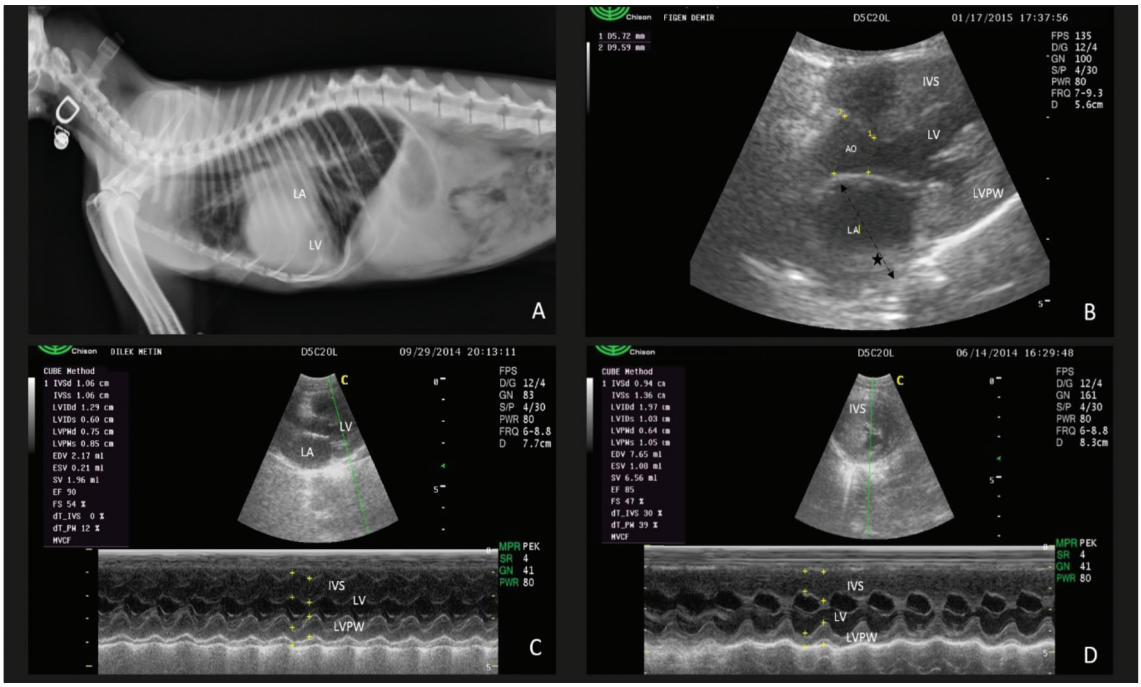

Fig 1. X-ray and echocardiographic images. **A-** Right lateral thoracic radiography of 4 years old short haired cat (case 5) of group II. Generalized cardiomegaly (vertebral heart score >11), peribronchial pattern and pulmonary congestion are present, **B-** Right parasternal long axis view of 3 years old long haired cat (case 7) of group II. Left atrial dilation (18.1 mm) and smoky view of the thrombus (marked by star), left ventricular outflow tract obstruction, hypertrophy in septum and left ventricular posterior wall are visualized, **C-** Right parasternal long axis view of 5 years old cat of group I. Left atrial dilation in 2-D image and concentric hypertrophy in septum and left ventricular posterior wall are seen in M-Mode image, **D-** Right parasternal short axis view of 7 years old short haired cat (case 1) of group II showing eccentric septal hypertrophy in 2-D and M-Mode echocardiogram

Table 1. *Comparison of echocardiographic measurements, cardiac biomarkers and age*

Group I (ACVIM Stage B1) (n=9)		Group II (ACVIM Stage C) (n=20)	
Parameters	**Mean±Std. Error Mean**	**Mean±Std. Error Mean**	**Significance**
Age (years)	6.9±1.206	5.50±0.778	NS
IVSd (cm)	0.775±0.0529	0.904±0.0730	NS
IVSs (cm)	1.218±0.0409	1.321±0.1109	NS
LVd (cm)	0.796±0.0379	1.160±0.1626	P<0.01
LVs (cm)	0.441±0.0308	0.669±0.1043	P<0,05
LVWd (cm)	0.877±0.0582	0.957±0.0786	NS
LVWs (cm)	1.225±0.1041	1.444±0.1149	NS
FS (%)	76.20±3.463	63.10±4.691	P<0.001
LVOT (m/sn)	0.672±0.0434	0.598±0.0459	NS
LA (cm)	1.538±0.0844	1.826±0.1301	P<0,05
LA/AO	1.649±0.12525	2.1278±0.1282	P<0.05
cTnI (ng/mL)	0.559±0.3229	13.860±64.203	P<0.05
cTnT(ng/mL)	0.1505±0.1188	1.695±10.657	NS
CK-MB (U/L)	45.67±7.269	2490.20±1204.601	P<0.001
NT-proBNP (pg/mL)	33.33±10.953	99.13±15.099	NS

NS: *not significant*

Table 2. *Levels of carciac biomarkers, survival time and number of affected limbs in cats of group II*

Cats of Group II	Age (years)	cTnI (ng/mL)	cTnT (ng/mL)	CK-MB (U/L)	NT-proBNP (pg/mL)	Survival Time	Number of Affected Limbs
1	10	9	NA	12310	NA	5 days	2
2	8	8.8	1.3	1202	NA	4 days	2
3	7	1.7	0.092	1020	130	Alive	2
4	3	32.6	0.28	1050	40	3 days	2
5	4	11.8	0.14	1050	58	10 days	2
6	3	0.95	0.057	1050	71	Alive	1
7	4	10	0.25	380	80	7 days	2
8	6	5.9	2.1	990	20	5 days	1
9	3	65	1.2	1510	62	2 days	2
10	7	2.6	0.094	810	32	Alive	1
11	4	1.1	0.86	120	20	Alive	1
12	5	1.3	0.092	277	25	Alive	1
13	13	0.57	0.41	53	10	Alive	1
14	4	0.43	0.075	380	20	6 months	1
15	2	0.87	0.41	159	19	Alive	1
16	3	0.87	0.13	149	29	Alive	1
17	10	0.27	0.044	215	10	Alive	1
18	6	4.3	1.14	470	35	5 days	2
19	4	14.7	0.15	498	34	1.5 days	2
20	7	3.7	0.071	175	18	18 days	2
21	11	2.4	0.12	1245	24	11 months	2
22	5	18	0.17	155	31	1 day	2
23	6	10.3	0.14	1729	28	1 day	2
24	5	175	2	418	37	8 days	2

NA: *not available*

group II. As an adverse effect of enoxaparin sodium, gastric bleeding was occurred in one cat. It was managed by classic treatment and prolonged intervals of enoxaparin sodium treatment. Amputation was performed to 4 cats at 4th, 5th, 11th and 14th days of treatment. Three of them survived. The one amputated at 4th day died at the day after.

Left atrial thrombus was visualized in echocardiographic examinations of 3 cats *(Fig. 1-B)*. According to statistical analysis of all data with Pearson correlation test, positive correlation was found between IVSs and LVWs (P<0.05), FS and IVSs (P<0.01), LVs and LVd (P<0.01), LA and LVd (P<0.05), LVWd and LVWs (P<0.01), LVWs and FS (P<0.01) and negative correlation was found between FS and LVd (P<0.01), LVd and LVWs (P<0.05), LVs and LVWd (P<0.05), LVs and LVWs (P<0.01) FS and LVs (P<0.01), LVWd and LVd (P<0.05), FS and LA (P<0.05). Also, positive correlation was found between CK-MB and LVd (P<0.01), cTnI and LA/AO (P<0.05), LA and CK_MB (P<0.01), LA and NT-proBNP (P<0.05), cTnI and CK-MB (P<0.05) and a negative correlation was found between LVOT and NT-proBNP (P<0.05) in all animals. Depending on the statistical analysis of group II by Pearson correlation test, positive correlation was detected between IVSs and IVSd (P<0.05), LVs and LVd (P<0.01), LVWd (P<0.01), FS and LVWs (P<0.05), LVOT and FS (P<0.05, CK_MB and IVSd (P<0.05), CK-MB and FS (P<0.05), NT-proBNP and IVSs (P<0.05), and negative correlation was found between LVWs and LVd (P<0.01), LVWs and LVs (P<0.05), LA and LVOT (P<0.01).

Discussion

Feline arterial thromboembolism is a considerable complication of feline cardiomyopathy which is having a poor prognosis [2,16-18]. Thromboembolism was seen in 20-50% of cats with cardiomyopathy [17,19]. It is known that the blood serum levels of cardiac biomarkers such as CK-MB, TnI, TnT and NT-proBNP are being elevated in cats with cardiomyopathies [2,4-6,8]. One of the cats (no: 4 in group II) involved in the current study was brought 6 h before the acute onset of FATE, and the obviously elevated laboratory results in this study depended on the prior examination. This case also inspired the creation of the current study to investigate the role of cardiac biomarkers in FATE by comparing the data of cats with HCM that didn't develop FATE.

The gender of the animals was predominantly male (24/33). Compatible with our results, most of the cats with FATE and HCM were found to be male in many studies and it indicates that males are at increased risk for FATE [8,16,18,20-22].

The echocardiographic examinations are important in feline medicine. As no auscultable abnormalities suggesting of an underlying cardiac disease could be detected in more than 40% of the cats diagnosed with FATE [18]. Severe left atrial enlargement and identification of smoke contrast or thrombus inside the left atrium are known to be considerable risk factors of FATE [18]. In one study conducted on cats with FATE, LA/AO was measured as 2.06±0.52 in 30 cats. However, no difference was found in survival based on LA/AO (P=0.780) [17]. It was reported that most of the cats with FATE were having LA enlargement while 45% had a LADs greater than 2.0 [23]. In our study, the mean result of LA/AO of group II was measured as 2.158 (1.47-2.50) cm and significance was found between the groups (P<0.05). It was considered that a correlation between left atrial dilation and thrombus formation was existent. Compatibly, Fuentes [16] reported that the blood stasis in left atrium results in local platelet activation and thrombus formation.

The survival rate was known to be between 20% and 50% independently from the type of the treatment used. The treatment protocol depends on supportive care and antithrombotic, surgical thrombolectomy or administration of thrombolytic agents [19]. Streptokinase, which is an expensive prescription and not easily available in our country, is one of the preferred choices in the treatment [17]. However, 100% mortality rate was reported in 8 streptokinase treated cats [24]. Short term survival rate of 33% was found in the cats treated with streptokinase in another study [17]. In recent years, low molecular weight heparin is suggested for treatment. However; in brief, survival rates differ through 35%-39% in conservative therapy or 33% in thrombolytic therapy; whereas natural death rates show similarity to euthanasia with the rates of 28-40%, 25-35%, respectively [25]. Also, tissue plasminogen activator is a new drug used in human medicine, whereas high rate of side effects occurred in administration to cats [19]. It is observed that complications related to TPA are fever (33%), minor hemorrhage (50%) and reperfusion injury (33%) [26]. The survival rate is not much different from these studies; however only antithrombotic agents were used. Also, it is reported that there is no statistically different results in terms of treatment outcomes and complications between the cases that treated with and without TPA [27]. Recently, some researchers recommended the antithrombotic drugs as a first-treatment choice, as the thrombolytic therapy could lead to adverse effects and high mortality rates [16]. Additionally, in one study, cats with FATE and HCM had a mean survival time of 61 days [28]. Cats with single limb episodes had a better prognosis than bilateral involvement [18]. In our study; bilateral involvement was observed in 16 of 24 cats. Also in one study, cats that died between 24 h and 7 days had a median age of 11 years (2-19) and 73.6% of them had two or more limbs affected whereas 33.3% of them had one limb effected. In our study; the median age of cats that died between 24 h and 7 days was 5.66 years (3-10) and it was similar to median age of cats that survived for 7 days and more (5.93 years, 2-13). However, there were fewer cats that had one limb affected in the group of animals that died between

24 h and 7 days (11% versus 40%) [29]. According to those information, it is deliberated that the high mortality rate in our study also could be depended on the number of the affected limbs.

Hertzsch et al.[30] have reported that cTnI is a sensitive and specific indicator for asymptomatic cats and a cutoff of >0,06 ng/ml for cTnI can be used as a screening test to detect the cats that have asymptomatic HCM. Langhorn et al.[5] have found that cTnI and especially cTnT are remarkably sensitive and specific indicators for detecting myocardial damage in cats with HCM, however their sensitivity and specificity can be changed between individual cases. However, non-survival cats (17 of 36) that suffering from HCM has shown remarkably higher concentrations of cTnI and cTnT, the cTnI and cTnT concentrations have not been elevated in survival cats as much as non-survivals but difference between healthy cats and cats with HCM was greater for cTnT. Two of the non-survival cats were euthanized from development of FATE. Similarly, in our study, serum levels of cTnI and cTnT were remarkably high in non-survival cats. Also, in our study; although cTnI levels were under 3.0 ng/mL in all survival cats (n=9), that kind of identity couldn't be observed on serum cTnT levels. Serum cTnT levels were higher than 0.1ng/mL in some of the survival cats (4 of 9) which was below in the rest of the cats. This information may indicate that serum cTnI and cTnT levels can be minimally variable on cats with FATE that has better prognosis and serum levels of cTnI and cTnT may help to determine the severity of the disease beside complete cardiac examination. Since the mentioned study was performed on cats with HCM (only 2 of them was developed ATE during the study) more studies with more cats with FATE must be performed to support this study.

In one study conducted on the cats with HCM, no correlation was found between cTnI and echocardiographic parameters. But only weak correlation was detected between cTnI and LVWd [2]. Ironside et al.[31] have reported that cats having a LA:Ao ≥1.5 are approximately four times more at risk of a cardiac-related event such as congestive heart failure and FATE. Also, the risk level is the same for the cats with ≥700 pmol/l NT-proBNP concentrations. A week correlation was found on cTnI with LA/AO ($P<0.05$) in the current study. However, positive correlation was found between CK-MB and LVD and LA ($P<0.01$). Similar but weaker correlation was also valid between NT-proBNP and LA. These correlations of CK-MB and NT-proBNP with LA might indicate the possible damage that occurred by dilation in the left atrial wall due to HCM. Also, negative correlation was found between NT-proBNP and LVOT and positive correlation between NT-proBNP and LA ($P<0.05$). As NT-proBNP is substantially known to be produced in atrial myocardiocytes [32], our findings are consistent and significant.

Herndon et al.[2] found an extreme increase in cTnI in two cats with HCM and FATE (10.93 ng/ml and 2.98 ng/mL). No macroscopic evidence of myocardial infarction was detected in postmortem examination of one of these cats. The researchers offered two explanations for these apparent increases. One was the possible cross-reaction. Although cTnI was a highly specific indicator for myocardial damage, it was not clear in animals. The second opinion depended on the formation of an additional small thrombi that embolized to the coronary vessels [2]. The other hypothesis might be depended on left ventricular damage due to increased pressure in left ventricle occurred by the thrombus in distal aorta. In the present study, however, lowest value of cTnI was detected in three cats which had survived. Also, an extreme result of cTnI was detected in the cat no: 4 of group II, 6 h before the acute onset in this study. These findings may lead to possible diagnostic and prognostic value of cTnI. In our study, positive correlation was also found between CK-MB and cTnI ($P<0.05$). Significant increases were detected in NT-proBNP and cTnI in human patients with pulmonary thromboembolism [33-35]. As a prognostic value, it was found that the patients with elevated cTnI levels had more serious vital parameters [34].

In conclusion; our data demonstrate that remarkably elevated serum levels of cardiac biomarkers (CK-MB, cTnI, cTnT and NT-proBNP) are associated with the cats diagnosed as having acute onset of FATE with HCM. Especially, detection of extremely high results of cTnI and cTnT in one case 6 hours before acute onset of FATE indicates the diagnostic utility of cardiac biomarkers for FATE. cTnI and cTnT may also play a role for the risk assessment of cats with FATE. Although serum cTnI and cTnT levels can be variable on cats with FATE that has better prognosis, remarkably high levels of both cTnI and cTnT can indicate poor diagnosis. It is considered that the cats with remarkably high levels of cTnI (>3.0 ng/mL) and cTnT (>0.1 ng/mL) may have poor prognosis with survival days shorter than 10 days despite antithrombotic treatment.

Limitations

Since it is known that interspecies homology is much greater for proANP than pro BNP [14,15], assessing the samples with human reagents for NT-proBNP must be considered as a limitation for this study. Although only the difference between the groups have been evaluated for NT-proBNP, it must be compared with the data that acquired by assessing the same samples on animal and/or cat reagents. In future studies, comparison of NT-proBNP levels that assessed with human and animal reagents between similar groups may also be considered.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Design of the study: BAKIREL U. and ULGEN SAKA S. Preparation of the study, management of the patients and data collection: BAKIREL U., ULGEN SAKA S. and YILDIZ K. Article writing, data analysis and editing: BAKIREL U., ULGEN SAKA S. and YILDIZ K.

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RESEARCH ARTICLE

Protective Effects of Chrysin in Rats with Ovarian Torsion

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Abstract

The objective of this study is to assess the protective effect of chrysin (CH) against ovarian torsion-detorsion injury. Thirty-two female albino rats were divided into 4 groups as Control, Torsion/Detorsion (T/D), TD-CH, and CH. Ovarian torsion was done for 3 hours on TD and TD-CH groups and then detorsion was performed. 50 mg/kg of CH was administered to the TD-CH group via oral gavage half an hour before the detorsion. Subsequently, 50 mg/kg of CH was administered via oral gavage to TD-CH and CH groups for 14 days. At the end of the experiment, blood samples were collected and ovarian tissue were taken. SOD and GPx activities and GSH and 8-OHdG levels were studied in serum and ovarian tissue. Also, IL-18, KIM-1, NGAL, Cys-C levels were studied in serum samples. GSH levels and GPx, SOD activities in both serum and ovarian tissue were significantly lower in TD group compared to the control and TD-CH groups ($P<0.05$), whereas the 8-OHdG level was significantly higher ($P<0.05$). Treatment with CH resulted in a decrease in 8-OHdG level and an increase in GSH level, GPx and SOD activities in both serum and ovarian tissue in the TD-CH group compared to TD group ($P<0.05$). Chrysin could ameliorate ovarian injury. Essentially, this outcome is thanks to the antioxidant, anti-inflammatory, and antiapoptotic effects of chrysin. Furthermore, it also has significant effects on DNA oxidative damage.

Keywords: *Ovary torsion, Chrysin, 8-OHdG, Antioxidant*

Ovaryum Torsiyonu Uygulanmış Ratlarda Chrisinin Koruyucu Etkisi

Öz

Bu çalışmadaki amacımız ovaryum torsiyon detorsiyon hasarına karşı chrysinin (CH) koruyucu etkisini değerlendirmektir. Otuz iki adet dişi wistar albino rat 4 gruba ayrıldı. Grup 1. Kontrol grubu, grup 2: TD grubu; grup 3: TD-CH grubu; grup 4: CH grubu. TD ve TD- CH gruplarına 3 saat torsiyon uygulandı ve 3 saatin sonunda detorsiyon yapıldı. TD-CH grubuna detorsiyondan yarım saat önce 50 mg/kg CH oral gavaj ile verildi. Daha sonra TD-CH ve CH gruplarına 14 gün boyunca 50 mg/kg CH oral gavaj ile verildi. Deneme sonunda ratlar sakrifiye edilerek kan örnekleri ve ovaryum dokusu alındı. Serum ve ovaryum dokusunda SOD ve GPx aktiviteleri, GSH ve 8-OHdG seviyeleri, serum örneklerinde ise 8-OHdG, IL-18, KIM-1, NGAL, Cys-C seviyeleri çalışıldı. TD grubunda hem serum hem de ovaryum dokusunda, GSH seviyeleri, GPx ve SOD aktiviteleri kontrol ve TD-CH gruplarından anlamlı olarak düşükken ($P<0.05$), 8-OHdG seviyesi anlamlı olarak yüksekti ($P<0.05$). TD-CH grubunda, CH uygulaması TD grubu ile karşılaştırıldığında hem serum hem de ovaryum dokusunda 8-OHdG seviyesini anlamlı olarak düşürürken, GSH seviyesi, GPx ve SOD aktivitelerini anlamlı olarak arttırmıştır ($P<0.05$). Chrysin ovaryum hasarını düzeltebilir. Bu sonuç esas olarak chrysinin antioksidant, antiinflammatuvar ve antiapoptotik etkilerinden kaynaklanmaktadır. Dahası DNA hasarı üzerinde de önemli etkileri vardır.

Anahtar sözcükler: *Ovaryum torsiyon, Chrysin, 8-OHdG, Antioksidant*

INTRODUCTION

Ovarian torsion, which is defined as the twisting of the ovary and vascular stem around the axis of the suspensory ligament, accounts for 3% of all gynecological emergencies [1]. Albeit ovarian torsion occurs in all women and primarily in women of reproductive age [2]. Depending on the degree of torsion, venous return to the ovarian tissue decreases, and

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subsequently, stromal edema and internal bleeding occur. If arterial blood flow stops, ischemic and necrotic processes begin in the tissue. Early diagnosis and management is indispensable to preserve ovarian function [3]. Due to non-specific symptoms such as vomiting, nausea, and pelvic pain, a delay is experienced in diagnosis almost always. Diagnostic difficulty in ovarian torsion leads to loss of ovarian tissue and function [4]. Ovarian torsion/detorsion (T/D) or ischemia/reperfusion (I/R) is a pathophysiological incident in which histological damage, which is associated with decreased perfusion following the lack of oxygen in the ovarian, occurs [5].

It releases reperfusion in the tissue following ischemia, and subsequent reactive oxygen species [4]. As a result of the reperfusion process, an excessive amount of molecular oxygen supplementation occurs in the ovary tissue. These reactive oxygen species (ROS) attack the cell membrane through the peroxidation of polyunsaturated fatty acids and lead to cellular damage [6].

Thus, oxidation has devastating effects on the ovarian tissue [5]. In healthy state, the ROS level is kept under control by antioxidants such as glutathione (GSH), glutathione peroxidase (GPx), and superoxide dismutase (SOD). However, in the event of oxidative stress, uncontrolled production of ROS damages biomolecules such as proteins, DNA, RNA and lipids, and cellular structure [7]. It has been revealed that I/R causes DNA strand breaks, oxidative DNA damage, and mutations [8]. 8-hydroxy-2' deoxyguanosine (8-OHdG) is a marker used to assess oxidative DNA damage and is a risk factor for various diseases [9].

Several antioxidants have been found to be effective in preventing oxidative damage and inflammation in ovarian tissue, which is exposed to I/R injury [10]. Chrysin is a natural flavonoid, found in honey, propolis, and various plant extracts. Antioxidant, anti-inflammatory, and anti-diabetogenic effects of chrysin have been reported in numerous studies [11,12]. It has been also revealed that it has protective effects in testicular/IR injury [13].

Kidney injury molecule-1 (KIM-1), cystatin C (Cys-C), and Neutrophil gelatinase-associated lipocalin (NGAL) have emerged as a sensitive marker in the early diagnosis of glomerular damage [14]. KIM-1 is a type 1 transmembrane glycoprotein and is significantly upregulated from proximal tubular cells following renal stress such as ischemia or nephrotoxicity [15]. Neutrophil gelatinase-associated lipocalin (NGAL) is a stress protein released from damaged tubular cells following various damaging stimuli, and it is known as one of the promising biomarkers of acute kidney injury [16]. Cys-C is eliminated only by the kidneys, and early-stage renal lesions might lead to a change in serum Cyc-C level [14].

Interleukin-18 (IL-18) is the proinflammatory cytokine of the IL-1 superfamily and mediates the proinflammatory response and ischemic proximal tubular damage [17]. Chrysin alleviates renal impairment and morphological anomalies caused by ischemic reperfusion injury. Chrysin suppresses tubular apoptosis and inflammation in renal I/R injury [18].

Thus, to reveal these effects, we aimed in this study to investigate the effects of CH on oxidant and oxidant enzymes, in the event of ovarian T/D.

Material and Methods

Ethical Statement

This study was approved by the Van Yuzuncu Yil University Animal Experiments Local Ethics Committee (YUHAD-YEK, Date: 17/06.2020; Decision number: 2020/06-15).

Animal and Experimental Design

Thirty-two non-pregnant female Wistar albino rats, aged between 8 to 10 weeks, weighing between 150 and 200 g were used in the study. Animals were kept in polypropylene cages at 21°C with a 12-h light and 12-h dark cycle. Food and water were provided to the animals via *ad libitum* intake.

Rats were divided into 4 groups, with 8 rats in each group.

Control group (C group): No treatment was performed on the rats in this group.

Torsion/Detorsion (TD) group: Torsion was performed on ovaries of the rats in this group for 3 h, and subsequently, detorsion was performed.

Torsion/Detorsion-Chrysin (TD-CH) group: Torsion was performed on ovaries of the rats in this group for 3 h, and then 50 mg/kg of Chrysin was administered via oral gavage for 14 days [2].

Chrysin (CH) group: 50 mg/kg of Chrysin was administered to this group via oral gavage for 14 days [2].

The rats were sacrificed following the 14-day study.

Surgical Procedure

All surgical interventions were performed in a sterile setting and under appropriate laboratory conditions. Ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg) were administered intraperitoneal for anesthesia. The abdomen was entered through a 2 cm longitudinal incision in the lower abdominal region of the rats. The vascular clip was applied approximately 1 cm above and below the left ovary, and reperfusion was achieved via relaparotomy 3 h later [19]. Half an hour before reperfusion, 50 mg/kg of chrysin was administered via oral gavage, and they were kept at 4°C for 15 min to coagulate. Subsequently, they were centrifuged at 3000 RPM for 15 min. Serums were stored at -20°C until the study day. The ovary was removed meticulously and stored at -20°C for biochemical assessment.

Collection of Samples

At the end of the study, in order to assess biochemical and pathological parameters, blood and ovary samples were taken 24 h after the administration of the last dose. Blood samples were taken into tubes without anticoagulant by cardiac route.

Biochemical Analysis

Following the surgical procedure, ovary tissue was removed and used for biochemical analysis. For biochemical analysis, ovary tissue was homogenized. The obtained supernatant and serum were stored at -20°C until they were studied. Ovary tissue SOD (Catalog No: SG-20188; Sinogeneclon Co., Hangzhou, China), GPx (Catalog No: SG-20976; Sinogeneclon Co., Hangzhou, China) , 8-OHdG (Catalog No: YLA0061RA, YL Biotech Co. Ltd. Shamghai, China) and GSH (Catalog No: SG-20391; Sinogeneclon Co., Hangzhou, China), serum IL-18 (Catalog No: SG-20281, Sinogeneclon Co., Hangzhou, China), 8-OhdG (Catalog No: YLA0061RA, YL Biotech Co. Ltd. Shamghai, China), GSH (Catalog No: SG-20391; Sinogeneclon Co., Hangzhou, China), GPx (Catalog No: SG-20976; Sinogeneclon Co., Hangzhou, China), SOD (Catalog No: SG 20188; Sinogeneclon Co., Hangzhou, China), KIM-1 (Catalog No: SG 20751; Sinogeneclon Co., Hangzhou, China), NGAL (Catalog No: SG-20801; Sinogeneclon Co., Hangzhou, China), Cys-c (Catalog No: SG-20197; Sinogeneclon Co., Hangzhou, China) levels were measured using ELISA kits via following the instructions of the manufacturer.

Histopathological Examination

Necropsy of rats was performed at the end of the trial. Ovarian tissue samples were taken and fixed in 10% buffered formaldehyde solution. The routine follow-ups of the tissues were performed. They were embedded in paraffin blocks and 4 µm sections were taken with a microtome. The sections were stained with hematoxylin-eosin (H&E), examined under a light microscope (Nikon 80i-DS-RI2), and photographed.

Statistical Analysis

The software of SPSS 20.0 (SPSS for Windows Chicago, IL, USA) was used for statistical analysis. All data was presented as mean and standard deviation. The Kolmogorov-Smirnov test was used to determine whether the data were normally distributed or not. One-way ANOVA was used for comparison of biochemical parameters between groups, and different groups were compared with post hoc Tukey's test. The results were considered statistically significant at $P<0.05$.

Results

Ovarian tissue SOD and GPX activities and GSH and 8-OHdG levels are presented in *Table 1*. It was determined that in TD Group, whereas ovarian tissue GPx and SOD activity and GSH levels were significantly lower compared to TD-CH and the control group, the 8-OHdG level was significantly higher ($P<0.05$). Whereas the TD-CH group ovarian tissue GSH and 8-OHdG levels were significantly higher than the control group, SOD activity was found to be lower ($P<0.05$). The ovarian tissue GPx activity was similar in the control group and TD-CH groups ($P>0.05$).

It was found out that serum 8-OHdG and IL-18 levels of TD group were significantly higher compared to the control, TD-CH, and CH groups ($P<0.05$) *(Table 2)*. Serum GSH level, GPx, and SOD activities were significantly lower in TD group compared to TD-CH, control, and CH groups ($P<0.05$). Serum KIM-1 level was determined to be significantly lower in CH group compared to the other groups ($P<0.05$). It was found out that serum NGAL level was significantly higher in TD group compared to C, TD-CH, and CH groups ($P<0.05$). It was determined that serum CYC-C level was significantly higher in TD and TD-CH groups compared to C and CH groups ($P<0.05$).

As a result of the examinations, a normal histological appearance was observed in the ovary tissues of the control group *(Fig. 1-A,B)*. However, venous congestion and interstitial edema in the cortex of the ovary, capillary hyperemia in the secondary follicle, degenerative-necrotic changes in the luteal cells in the corpus luteum, and accumulation of hemosiderin pigment were detected in TD group *(Fig. 1-C,D,E)*. Besides, a lesser venous congestion, degenerative and necrotic cells in the corpus luteum, and accumulation of hemosiderin pigment in macrophages were detected in ovarian tissues in D-CH group compared

Table 1. *Ovary tissue SOD, GPx, activities and GSH and 8-OhdG levels*

Parameters	Grops			
	Control	TD	TD-CH	CH
8-OHdG (ng/mL)	2.01±0.02^{c}	2.5±0.07^{a}	2.31±0.05^{b}	1.73±0.9^{d}
GPx (IU/mL)	27.28±1.4^{a}	24.83±1.1^{b}	28.39±0.8^{a}	27.17±0.1^{a}
SOD (ng/mL)	204.54±12.3^{a}	150.54±6.5^{c}	179.11±10.7^{b}	168.8±7.2^{b}
GSH (ng/mL)	123.03±4.9^{b}	100.11±3.2^{c}	148.83±4.8^{a}	128.04±8.5^{b}

*^{a,b,c,d} Values within a row with different superscripts differ significantly at $P<0.05$; **TD:** Torsion/Detorsion; **TD-CH:** Torsion/Detorsion-Chrysin; **CH: Chrysin; GPx:** Glutathione peroxidase; **SOD:** superoxide dismutase:. **GSH:** Glutathione*

Table 2. *Serum biochemical parameters of the groups*

Parameters	Grops			
	Control	TD	TD-CH	CH
8-OHdG (ng/mL)	1.63±0.1[d]	2.09±0.07[a]	1.87±0.06[b]	1.77±0.06[c]
GPx (IU/mL)	29.25±1.4[a]	18.93±1.2[d]	21.52±0.8[c]	22.92±0.1[b]
SOD (ng/mL)	252.43±9.0[a]	226.31±5.9[b]	251.74±5.7[a]	242.33±6.4[b]
GSH (ng/mL)	125.49±5.0[b]	95.16±4.7[c]	135.87±8.3[a]	119.10±4.1[b]
IL-18 (pg/mL)	121.31±7.1[b]	141.35±7.2[a]	108.35±3.8[b,c]	99.26±29.0[c]
KIM-1 (pg/mL)	87.36±3.0[a]	87.65±2.5[a]	88.53±3.6[a]	86.20±5.22[b]
NGAL (ng/mL)	1.43±0.07[b]	1.32±0.05[c]	1.52±0.05[a]	1.51±0.07[a]
CYS-C (ng/mL)	16.15±1.1[b]	18.87±0.4[a]	18.85±0.4[a]	13.95±0.6[c]

[a,b,c,d] Values within a row with different superscripts differ significantly at P<0.05; ***TD:*** *Torsion/Detorsion;* ***DT-CH:*** *Torsion/Detorsion-Chrysin;* ***CH:*** *Chrysin;* ***GPx:*** *Glutathione peroxidase;* ***GSH:*** *Glutathione;* ***SOD:*** *superoxide dismutase;* ***IL-18:*** *interleukin-18;* ***KIM-1:*** *Kidney İnjure molecule;* ***NGAL:*** *Neutrophile gelatinase-associated lipocalin;* ***CYC-C:*** *Cystatin-C.*

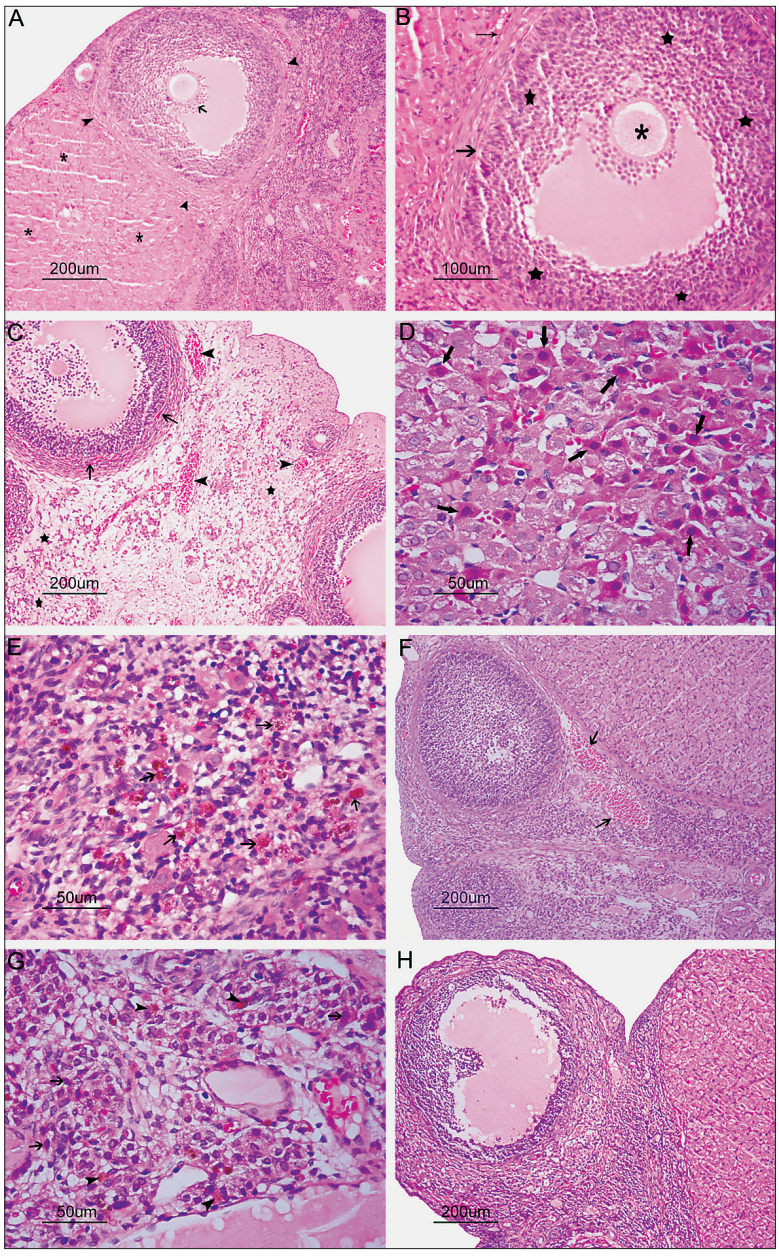

Fig 1. Histopathological assessment of the rat ovaries among the groups. **Control group (Group I): (A)** Secondary follicle (†), corpus luteum (*) and cumulus oophorus cells (→); **(B)** Primary oocyte (*), granulated cells (★), theca interna (➤), and theca externa (') in the ovary with histopathologically normal appearance. TD group (Group II): **(C)** Venous congestion (†) and interstitial edema (★) in the cortex of the ovary. Capillary hyperemia in the theca interna and externa in the secondary follicle (→), **(D)** Degenerative and necrotic changes in the luteal cells in the corpus luteum (→), **(E)** Accumulation of hemosiderin pigment in macrophages in the corpus luteum (→). **TD-CH group (Group III): (F)** Less venous congestion (→) is seen compared to Group II, **(G)** Lesser degenerative and necrotic cells (→) and hemosiderin pigment accumulation in macrophages (†) are seen in the corpus luteum compared to Group II. **CH group (Group IV): (H)** Normal histological appearance of the follicle and corpus luteum is seen in the ovary; H&E

to TD group *(Fig. 1-F,G)*. The normal histological appearance of the ovaries was observed in CH group similar to the control group *(Fig. 1-H)*. As a result of all findings, it was concluded that the pathological findings significantly decreased in the ovaries of the rats which were administered with Chrysin by oral route following the torsion-detorsion procedure.

Discussion

Ischemia-reperfusion injury is explained by the hypothesis that the accumulation of neutrophils and platelets occurs around the inflammation site due to activated complement and other inflammatory components. This accumulation of inflammatory cells increases the production of reactive oxygen species. Moreover, glycolysis, increased lactic acid concentration, and accumulation of intracellular Ca lower the intracellular pH and ends up with acidosis. This results in an increase in the intracytoplasmic lysosomal enzymes, which leads to injury in protein and cell membranes [4]. Antioxidant and oxidant balance is disturbed in the event of ischemia-reperfusion injury. Hence, it has been suggested that it might be beneficial to use antioxidant pharmacological agents during or before reperfusion to prevent I/R injury [20].

Ovarian detorsion without ovariectomy may preserve ovarian function, yet prophylactic measures against subsequent I/R injury are required. Thus, animal models have focused on antioxidant and anti-inflammatory pharmacological agents for protecting the ovary in the event of I/R injury [2,21]. One of these agents is Chrysin (CH). The antioxidative property of Chrysin has been attributed to the inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression, and the inhibition of nuclear factor kappa B activity has been suggested to be CH antioxidative and anti-inflammatory [22]. It has been demonstrated that CH protects rat ovaries from I/R injury through improving histopathological scores, increasing antioxidative activity, and decreasing lipid peroxidation [2].

Glutathione is an abundant non-thiol protein that plays a key role in the coordination of antioxidant defense mechanisms. GSH acts as a substrate for several enzymes containing glutathione peroxidase and can capture reactive oxygen species directly. Decreased GSH level in tissue not only deteriorates cellular defense but also ends up with increased oxidative damage [23]. It has been revealed that Chrysin could increase GSH concentration by upregulating the gene transcription of glutamate-cysteine ligase (GCL), which catalyzes the rate-limiting step in glutathione synthesis, via ERK2/Nrf2 signaling [24]. In line with our study, Oral et al.[25] demonstrated in their study that the GSH level of the ovary tissue decreased significantly in torsion group compared with the control groups. It has been revealed that the decrease in GSH level following detorsion in rats with ovarian torsion might be stemming from the consumption during oxidative stress [26]. In the current study, while GSH level of ovarian tissue in TD group was determined to be significantly lower compared to the control group, CH administration caused an increase in GSH level.

The increase in GSH level and increase in SOD, CAT, and GPx activities indicate tissue healing after oxidative damage [27]. It has been revealed that 75 mg/kg/day CH administration protects against focal cerebral I/R injury effectively; also, SOD, GPx activities, and GSH levels significantly increased in the treatment group upon the administration of CH following the I/R injury [28]. It has been revealed that compared to I/R group a significant increase was detected in SOD activity in I/R-CH group, which were administered with a single dose of 50 mg/kg CH [2].

The antioxidant property of GSH has been attributed to its ability to increase antioxidant enzyme activities as well as its powerful feature of free radical capture [23,29]. In the current study, while GSH level of ovarian tissue in TD group was found to be significantly lower compared to the control group, CH administration caused an increase in GSH level.

Enzymatic antioxidants play a crucial role in protecting cells from oxidative damage [30]. SOD is the most significant enzyme that catalyzes superoxide radicals ($O_2^{\cdot-}$) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). GPX is an enzyme that catalyzes H_2O_2 and lipid peroxides into water [30]. It has been shown in the ovarian torsion studies that SOD and GPX activities decreased in serum [5,31] and ovarian tissue [31]. In the current study, GPx and SOD activities in ovarian tissue and serum were determined to be significantly lower in TD group compared to C, CH, and TD-CH groups. CH administration caused decreasing SOD and GPx activities to increase. This mechanism of action is considered to stem from the hydroxyl groups in the 5th and 7th positions of the CH molecule, which directly eliminates free radicals [32]. It has also been determined that CH inhibits oxidative stress indirectly through regulating antioxidant enzyme activities [33]. It has been revealed in various studies that CH medication protects tissues against oxidative stress and induces an increase in antioxidant enzyme activities [13,34].

Reactive oxygen species cause deformation of DNA-protein bonds and changes in bases [35]. Biologically, I/R has been found to induce DNA strand breaks, oxidative DNA damage, and mutations [36,37]. 8-OHdG is one of the most stable DNA bases and is a well-established biomarker of oxidative damage in DNA [35]. 8-OHdG has been shown to be immunoreactive in ovarian I/R injury [8]. It has been revealed that ovarian tissue concentration of 8-OHdG significantly reduced in rats treated with 3-h torsion and 24-h detorsion compared to the control group [35]. Eken et al.[38] revealed in their study that ovary 8-OHdG concentration increased significantly in T/D group compared

to the control group. In the current study, serum and ovarian tissue 8-OHdG levels were determined to be significantly higher in TD group compared to TD-CH and CH groups. CH treatment significantly decreased both serum and ovarian 8-OHdG levels. CH antioxidant activity might reduce tissue damage, leading to a decrease in 8-OHdG level.

IL-18 is a pro-inflammatory cytokine and is also known as the interferon-gamma inducing factor. IL-18 is crucial in supporting host defense [39]. IL-18 is involved in the pathophysiology of various inflammatory diseases including I/R injury, transplant rejection, and autoimmune diseases [40]. It has been revealed that IL-18 whose expression is stimulated in cardiomyocytes by ROS could play a crucial role in myocardial I/R injury [41]. Administration of IL-18 binding proteins to human myocardiocytes improves cardiac function [42]. and has been shown to play a role in the cardiac inflammatory response to I/R injury in mice [40]. It was found out that IL-18 knockout mice with simulated kidney I/R injury were highly protected against I/R injury, and tubular damage was reduced [43]. CH displays various biological effects on the immune system. CH suppresses the inflammatory response and displays an anti-inflammatory response [44]. Pro-inflammatory cytokines are modulators of host responses in trauma and immune response; hence, the anti-inflammatory response serves to reduce inflammation and promote healing while acting to exacerbate the disease [44]. CH with a double bond at C2-3 and a hydrogen group at R3 could inhibit pro-inflammatory cytokines [45]. In the current study, serum IL-18 level was found to be significantly higher in TD group compared to TD-CH, CH, and control groups. CH administration caused serum IL-18 level to decrease. The decrease in serum IL-18 level upon the CH administration might be due to the inhibition of CH proinflammatory cytokines.

Neutrophil Gelatinase-Associated Lipocalin (NGAL) induces epithelial protection and epithelial development following ischemia [46]. It has been demonstrated that NGAL expression increases following renal ischemia [47]. NGAL is commonly found in numerous biological fluids and various cell types in humans. It serves to protect against bacterial infections in normal tissue and to modulate the oxidative system [48]. Kidney Injury Molecule-1 (KIM-1) has been suggested to play a significant role in removing damaged epithelial cells and dead cells via phagocytosis [46]. It has been revealed that ovarian tissue and serum NGAL levels in ovarian torsion are higher in T/D group compared to the control group, but this elevation is not significant [49]. It has been reported that the concentrations of KIM-1 and NGAL in urine are increased compared to the control group in the event of kidney I/R injury [50]. In the current study, serum KIM-1 level in TD group was akin to the control and TD-CH groups. Serum NGAL level was determined to be significantly higher in TD group compared to C, TD-CH, and CH groups. Serum Cyc-C level was found to be significantly higher in TD and TD-CH groups compared to C and CH groups. It has been revealed that NGAL uses the BCL2/BAX signaling pathway in renal tubular epithelial cell apoptosis [51]. It has been demonstrated that NGAL is reduced in apoptotic tubular cells, and this renoprotective effect is thanks to the inhibition effect of caspase-3 activation [52]. Thus, the fact that the serum NGAL level in the presented study was significantly lower in TD group compared to the other groups may be due to ovarian torsion increasing apoptosis in the kidney, and it can be stated that CH in TD-CH group has a protective effect on the kidney, since the serum NGAL level is significantly higher than TD group.

In TD group, venous congestion and interstitial edema in the ovarium cortex, capillary hyperemia in the secondary follicle, and degenerative necrotic cells in luteal cells in the corpus luteum were detected when we evaluated the histopathologic findings and compared them with the control group. In the TD-CH group, lesser venous congestion, lesser degenerative and necrotic cells in the corpus luteum were detected in ovarium tissues compared to TD group. Turkoz et al.[26] found that vascular congestion, hemorrhage, and edema in the ovarium tissue increased in the torsion detorsion group compared to the control group. When the I/R process occurs, inflammatory cells cause an increase in free oxygen radicals that lead to tissue damage by the mechanism of inflammation [53]. Compression of ovarian vessels due to stromal edema and ovarian enlargement prevents lymphatic and venous outflow first and subsequently arterial inflow. Ovarian arterial pressure is then blocked due to stromal pressure, resulting in infarction and necrosis in addition to microscopic bleeding in the ovarian tissue [21]. Consistent with our study, Hortu et al.[19] revealed a higher incidence of histopathological scars such as vascular congestion and hemorrhage and increased cellular damage in the torsion group compared to the control group. It has been demonstrated that Chrysin improves histological changes such as vascular congestion, hemorrhage, edema, and inflammatory cell infiltration in ovarian tissue, which underwent ovarian torsion [2]. In the current study, chrysin treatment significantly reduced the pathological findings in the ovaries. Anti-inflammatory and antioxidant properties of Chrysin could prevent the effects of I/R injury

In conclusion, it is considered in this study that CH administration could be effective in reducing ovarian injury during the TD procedure. The antioxidant and anti-inflammatory properties of CH could protect the ovary and lower injury during torsion-detorsion. Furthermore, CH has considerable effects on oxidative DNA damage.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

DD, AUK, UO, VK, YB, PK and OFK experimental Study, AUK and UO laboratory study, OFK pathological examination, AUK statistic, DD, AUK, UO, VK, YB, PK and OFK literature search and article writing.

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RESEARCH ARTICLE

The Effects of Different Mydriatics on Intraocular Pressure and Central Corneal Thickness in New Zealand White Rabbits [1]

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Abstract

This study was aimed to compare the effect of 1% atropine, 1% cyclopentolate, 0.5% tropicamide, and 10% phenylephrine eye drops on intraocular pressure (IOP) and central corneal thickness (CCT) in New Zealand White rabbits. Adult male, eight, New Zealand White rabbits were randomly received each of four mydriatic eye drops separately on left eye at a one-week washout period. Each rabbit received all of five different treatments (sterile saline solution, four mydriatic drugs) on the left eye, whereas no measurements were performed on the right eyes during the experiment. The IOP and CCT recordings of rabbits were performed until the pupil returned to normal diameter. The mean CCT values of sterile saline, 1% atropine, 0.5% tropicamide, 1% cyclopentolate, and 10% phenylephrine were 370±15, 368±17, 372±15, 364±18, and 360±17 µm, respectively, and no statistically significant differences (P>0.05) were observed among groups. The mean IOP values of control (sterile saline), 1% atropine, 0.5% tropicamide, 1% cyclopentolate, and 10% phenylephrine as, 9.7±2.1, 10.4±1.8, 10.3±2.1, 11.0±2.1, and 10±1.8 mmHg, respectively, and these were not statistically significant among groups (P>0.05). In conclusion, topical 1% atropine, 0.5% tropicamide 1% cyclopentolate, and 10% phenylephrine do not have significantly effect on IOP and CCT in New Zealand white rabbits.

Keywords: *Central corneal thickness, Intraocular pressure, Mydriatic, New Zealand white rabbit, Tropicamide*

Beyaz Yeni Zelanda Tavşanlarında Farklı Midriyatiklerin Göz İçi Basıncı ve Merkezi Kornea Kalınlığı Üzerine Etkileri

Öz

Bu çalışma, Beyaz Yeni Zelanda tavşanlarında %1 atropin, %1 siklopentolat, %0.5 tropikamid ve %10 fenilefrin göz damlasının göz içi basıncı (GİB) ve merkezi kornea kalınlığı (MKK) üzerindeki etkilerini karşılaştırmayı amaçladı. Sekiz, yetişkin erkek Beyaz Yeni Zelanda tavşanının sol gözüne dört ayrı midriyatik göz damlası bir haftalık arınma periyoduyla rastgele uygulandı. Her tavşanın sol gözüne beş farklı tedavinin (steril serum fizyolojik, dört midriyatik ilaç) tamamı uygulanırken, sağ gözlerde ise deney boyunca ölçüm yapılmadı. Tavşanların GİB ve MKK verileri pupil normal çapa dönene kadar alındı. Steril salin, %1 atropin, %0.5 tropikamid, %1 siklopentolat ve %10 fenilefrinin ortalama MKK değerleri sırasıyla 370±15, 368±17, 372±15, 364±18 ve 360±17 µm idi ve gruplar arasında istatistiksel olarak anlamlı farklılık (P>0.05) gözlenmedi. Kontrol (steril salin), %1 atropin, %0.5 tropikamid, %1 siklopentolat ve %10 fenilefrinin ortalama GİB değerleri sırasıyla 9.7±2.1, 10.4±1.8, 10.3±2.1, 11.0±2.1 ve 10±1.8 mmHg'idi ve gruplar arasında istatistiksel olarak önem yoktu (P>0.05). Sonuç olarak, Beyaz Yeni Zelanda tavşanlarında topikal %1 atropin, %0.5 tropikamid, %1 siklopentolat ve %10 fenilefrinin GİB ve MKK üzerinde anlamlı bir etkisi yoktur.

Anahtar sözcükler: *Beyaz Yeni Zelanda tavşanı, Göz içi basıncı, Merkezi kornea kalınlığı, Midriatik, Tropikamid*

INTRODUCTION

Mydriatics are commonly used in human and veterinary ophthalmology to examine the posterior segment and treat uveitis and corneal ulceration by providing pupil dilation [1,2].

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Mydriatics may alter the intraocular pressure (IOP) by obstructing the iridocorneal angle [3] or decreasing traction on trabecular meshwork that effects aqueous outflow drainage or widening on anterior chamber angle [4]. So many researches have been conducted to find the relationship between mydriatics and IOP in different species such as sheep [3], monkeys [5], horses [6], goats [7], dogs [1,8], cats [1,9] and rabbits [10].

Measuring the central corneal thickness (CCT) is important for laser corneal refractive surgery for the maximum safety of performance [11]. Mydriatic drugs are commonly used for ocular surgery and they may elevate the CCT because of the impairment of corneal physiological metabolism or function of epithelial barrier [12] Several studies have investigated the relationship between IOP and CCT in humans [13,14], cats [15] and dogs [16]. It has been reported that low CCT may lead to underestimation of IOP [17].

This study was aimed to compare the effects of 1% atropine, 1% cyclopentolate, 0.5% tropicamide, and 10% phenylephrine on CCT and IOP values of New Zealand White rabbits.

Material and Methods

Ethical Statement

Atatürk University Local Board of Ethics Committee for Animal Experiments has approved the study protocol of this research (HADYEK decision no: 2021/22).

Animals

Eight, adult New Zealand white rabbits with average weights of 2.9 kg were used. The animals were housed in individual cages (60x50x60 cm height) without bedding material and received water and a standard pellet diet *ad libitum*. The humidity ranged between 40 and 60%. A uniform temperature of 22±2°C was maintained throughout with a 12:12 h light: dark cycle.

Study Design

The rabbits were checked for pre-existing ocular disorders by measuring the IOP (Tonovet®, Icare, Finland), Schirmer tear test - I (STT-1; Eye Care Product Manufacturing LLC, Tucson, USA), fluorescein staining (Flu-Glo® ophthalmic strips USP 1.0 mg, Akorn, USA), and indirect ophthalmoscopy (Aesculap AC-635 C, Braun, Germany).

The treatment procedure was randomized, and each animal received all of five treatments on the left eye (sterile saline solution, four mydriatic drugs) with a minimum one-week washout period. No measurements were performed on the right eyes during the experiment.

The treatment protocols were one drop of sterile saline solution, 1% atropine ophthalmic solution (Atrosol® %1, Sanovel, Turkey), 0.5% tropicamide ophthalmic solution (Mydriaticum Stulln® 0.5%, Pharma Stulln GmbH, Germany), 1% cyclopentolate ophthalmic solution (Sikloplejin® %1, Abdi Ibrahim, Turkey), and 10% phenylephrine ophthalmic solution (Fenilefrin® 10%, Sanovel, Turkey). All mydriatics were administered by the same person who was unaware of the experimental design.

During the each IOP measurement, the rabbits were gently handled to prevent any pressure on the animal's neck which might effect IOP [18]. All measurements were recorded at predefined time points [9]. No anesthetic eye drops were used throughout the experiment. Before the experiment, the rabbits were adapted to the study for two weeks period to prevent false recordings.

Measurement

Intraocular pressure and CCT measurements were discontinued until the two independent researchers were not able to examine posterior segment of the eye by direct ophthalmoscopy. The IOP and CCT measurements were recorded in an examination room with the same light circumstances. The IOP and CCT recordings were collected by rebound tonometer (Tonovet, Icare, Vantaa, Finland) and ultrasound pachymetry (Ipac, Reichert, NY, USA), respectively.

All IOP and CCT measurements were performed by the same person at the predefined time points (32 h for 1% atropine, 14 h for 0.5% tropicamide, 40 h for 1% cyclopentolate, 6 h for 10% phenylephrine, 40 h for saline group. IOP and CCT measurements of sterile saline, 1% atropine, 0.5% tropicamide, 1% cyclopentolate and 10% phenylephrine were recorded 19, 17, 12, 19 and 8 times, respectively. On the first day, IOP and CCT were measured at 8 a.m., 8.30 a.m., 9 a.m., 9. 30 a.m., 10 a.m., 11 a.m., 12 p.m., 2 p.m., 4 p.m., 6 p.m., 8p.m., 10 p.m., 12 a.m. On the second day, IOP and CCT were measured at 4 a.m., 8 a.m., 12 p.m., 4 p.m., 8 p.m., 12 a.m. The time point of 30 min after mydriatic administration was chosen based on the previous study [9].

Statistical Analysis

All data were analyzed using the SPSS 19.0 (IBM, SPSS Inc, USA, 2010) statistical package. Data are reported as mean±standard deviation. To evaluate the differences in IOP levels among groups, a Repeated Measures-ANOVA followed by Bonferroni multiple comparisons Post-Hoc test was performed. A P-value of <0.05 was considered statistically significant.

Results

No signs of ocular irritation or pain were encountered during the experiment. The mean CCT values of sterile saline, 1% atropine, 0.5% tropicamide, 1% cyclopentolate, and 10% phenylephrine were 370±15, 368±17, 372±15,

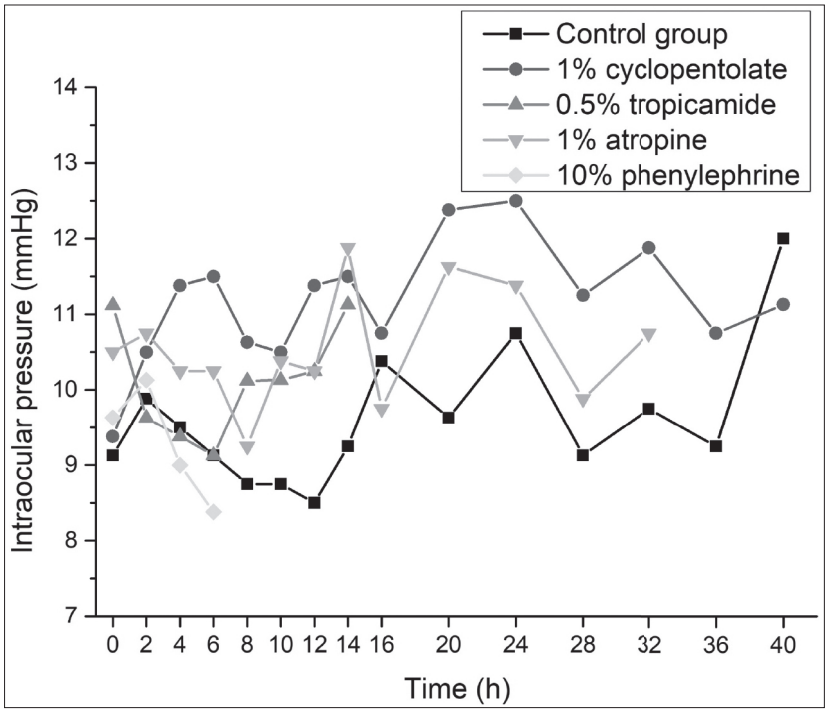
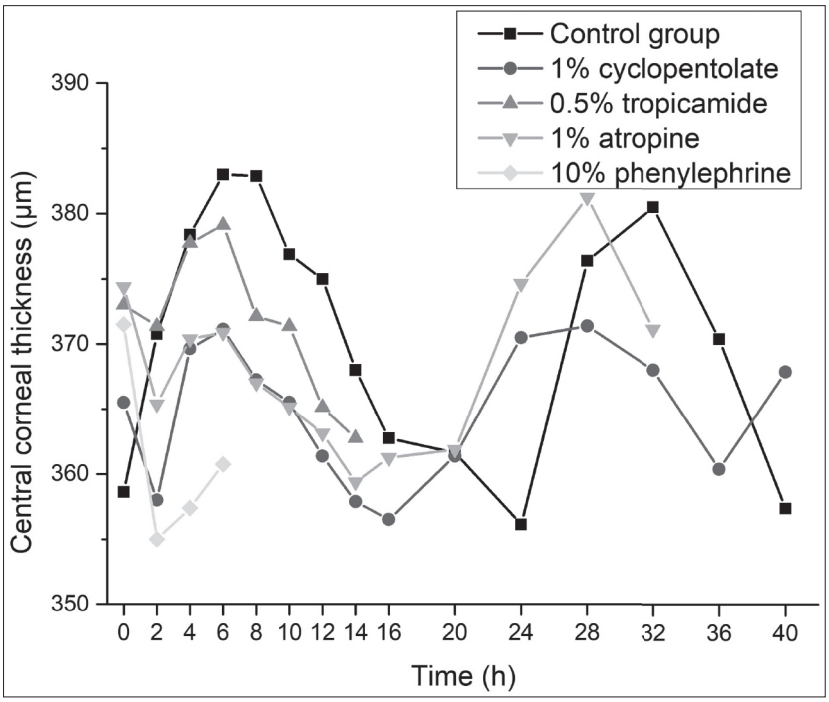


Fig 1. The effect of mean central corneal thickness values during the predefined time points after the topical sterile saline, 1% atropine, 0.5% tropicamide 1%, cyclopentolate, and 10% phenylephrine administrations

Fig 2. The effect of mean intraocular pressure values during the predefined time points after the topical sterile saline, 1% atropine, 0.5% tropicamide 1%, cyclopentolate, and 10% phenylephrine administrations



364±18, and 360±17 µm, respectively, and no statistically significant differences (P>0.05) were observed among groups *(Fig. 1)*.

The mean values of IOP in sterile saline, 1% atropine, 0.5% tropicamide, 1% cyclopentolate, and 10% phenylephrine as, 9.7±2.1, 10.4±1.8, 10.3±2.1, 11.0 2.1, and 10±1.8 mmHg, respectively and no significant differences (P>0.05) were observed among groups. No significant differences were observed on IOP levels in all groups at all-time intervals except for at the 4th h and 20th h *(Fig. 2)*. The IOP level was significantly increased (P<0.05) in 1% cyclopentolate group (11.4±1.8 mmHg) at 4th h compared to the 10% phenylephrine (9±1.2 mmHg). And also, 1% cyclopentolate (12.4±2.0 mmHg) resulted with a significant increase in IOP at 20th h compared to the control group (9.6±1.7 mmHg, P<0.05).

Discussion

Mydriatics are regularly administered to the eye to assist the clinician in the routine evaluation of ocular structures

located in the posterior segment of the eye [19,20]. In our study, 1% atropine, 0.5% tropicamide, 1% cyclopentolate and 10% phenylephrine were used for mydriatic effect. Atropine, tropicamide, and cyclopentolate are a parasympatolitic agents that have anti-muscarinic activity, which causes pupillary dilatation followed by ciliary paralysis [21]. However, phenylephrine shows its mydriatic effect by activating sympathetic receptors on the iris dilator muscle by inhibiting iris sphincter muscle action [22]. A previous study has reported that 1% atropine ointment may reduce the IOP in horses, possibly due to the large capacity of aqueous humor outflow [6]. However, in this study, 1% atropine did not cause any significant effect on IOP and CCT values of New Zealand white rabbits. Similar results were previously reported in sheep [3] and horses [23]. In the current study, no significant differences were observed in IOP and CCT levels between cyclopentolate and atropine groups. This may occur due to similar pharmacological effect of both drugs [1,8]. We observed an increase in IOP levels at some time points in the 1% cyclopentolate group compared to the saline and phenylephrine group. This finding was consistent with a previous report [10]. Therefore, 1% cyclopentolate must be used carefully in rabbits especially when the higher IOP levels are suspected.

Tropicamide is a commonly used ophthalmic solution to inhibit the action of acetylcholine on the iris sphincter [24]. It has been stated that tropicamide-induced mydriasis may cause an increase in IOP recordings of dogs [1] cats [9] and humans [4]. We observed an insignificant increase in IOP level after the 0.5% tropicamide application compared to the saline group, however, this was clinically acceptable. Because phenylephrine is sympathomimetic drug that causes less humor aqueous production and thereby lower IOP levels. However, in this study phenylephrine did not cause a significant effect on IOP. Similar results were also reported in cats [9], monkeys [25], and humans [26].

In this experiment, no topical anesthetics were used, which may affect the IOP [27] and CCT [28,29]. The IOP is dynamic so it changes during the day [30,31]. Environmental changes and stress-related factors may increase IOP [32]. Based on these premises, IOP and CCT measurements of the current study were recorded at the same time of the day with two weeks acclimation period. In our study, we did not serve the right eyes as a control because previous studies have shown that unilateral application of mydriatic drugs may affect both eyes [9].

Intraocular pressure values of healthy rabbit with Tonovet is ranged between 9.51±2.62 mmHg [32]. In the current study, the mean IOP recordings of the saline group were within the reference values. CCT measurements were easily obtained from rabbits' eyes' using an ultrasound pachymeter; however, it involves contact with the surface of the cornea epithelium [33]. Chan et al.[34] reported that CCT values of New Zealand white rabbits with ultrasound pachymeters were 407±20 µm. In this study, the mean CCT value was 370±15 µm, and the results were consistent with Wang et al.[35]'s report, which used optical coherence tomography for measuring the CCT.

In conclusion, topical application of 1% atropine, 0.5% tropicamide, 1% cyclopentolate, and 10% phenylephrine do not have a significant effect on IOP and CCT values of New Zealand white rabbits. 10% phenylephrine is less likely to affect IOP in rabbits among the mydriatics used in this study. Moreover, future studies that focus on the relationship between CCT and IOP levels of glaucomatous eyes may reveal the possible interactions of both parameters.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

LEY, SO, UE, MGS, ED, ZO: Study design. LEY, SO, UE, MGS, ED, ZO: Data collections. MGS: Data analysis. LEY, SO: Writing the manuscript.

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RESEARCH ARTICLE

Predicting The Growth Curve of Body Weight in Madura Cattle

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Abstract

The growth curve of livestock animals is important to evaluate the biological development managed with a farming management system. This study aimed to estimate the growth curve of body weight (BW) in Madura cattle *(Bos indicus)* kept at the breeding management. Three non-linear models of Logistic (L), Gompertz (G) and Von Bertalanffy (B) were performed in this study using 186 records data and computed with SPSS 16.0 package. Research showed that the asymptotic weight (male/female) was reached of 220.80/218.02 kg (L), 277.72/274.13 kg (G) and 333.92/329.83 kg (B). The weight of inflection (male/female) was reached of 110.40/109.01 kg (L), 102.10/100.78 kg (G) and 98.94/97.73 kg (B). The time of inflection (male/female) was reached of 10.89/10.47 months (L), 10.09/10.23 months (G) and 9.80 months (B). Moreover, the coefficient of determination (R2) in all models included of high category i.e. 0.68 (male) and 0.70 (female). However, three goodness-of-fit parameters of root mean squared error (RMSE), Akaike's (AIC) and Beyesian (BIC) values revealed that G and B models were more accurate than the other models for male and female, respectively. It can be concluded that about 68-70% of body weight of animals in a study can be explained by non-linear models of L, G and B.

Keywords: *Body weight, Growth curve, Inflection, Madura cattle, Non-linear models*

Madura Sığırlarında Vücut Ağırlığı Büyüme Eğrisinin Tahmini

Öz

Çiftlik hayvanlarının büyüme eğrisi, bir çiftlik yönetim sisteminde biyolojik gelişimin değerlendirilmesi için önemlidir. Bu çalışmada, damızlık amaçlı yetiştirilen Madura sığırlarında *(Bos indicus)* vücut ağırlığının (BW) büyüme eğrisinin tahmin edilmesi amaçlanmıştır. Bu çalışmada, 186 veri kaydı ve SPSS 16.0 paket programı eşliğinde doğrusal olmayan üç model, Lojistik (L), Gompertz (G) ve Von Bertalanffy (B), kullanılmıştır. Çalışma, asimptotik ağırlığın (erkek/dişi), 220.80/218.02 kg (L), 277.72/274.13 kg (G) ve 333.92/329.83 kg (B)'a ulaştığını göstermiştir. Büküm ağırlığı (erkek/dişi), 110.40/109.01 kg (L), 102.10/100.78 kg (G) ve 98.94/97.73 kg (B)'a ulaşmıştır. Büküm zamanı (erkek/dişi), 10.89/10.47 ay (L), 10.09/10.23 ay (G) ve 9.80 ay (B) olarak belirlenmiştir. Ayrıca, tüm modellerde determinasyon katsayısı (R2), yüksek, 0.68 (erkeklerde) ve 0.70 (dişilerde) saptanmıştır. Bununla birlikte, Hata Kareler Ortalamasının Karekökü (RMSE), Akaike Bilgi Kriteri (AIC) ve Bayesci Bilgi Kriteri (BIC) üçlü uyum indeks analiz değerleri, G ve B modellerinin sırasıyla erkek ve dişiler için diğer modellere göre daha doğru olduğunu ortaya koymuştur. Hayvanların vücut ağırlığının yaklaşık %68-70'inin doğrusal olmayan L, G ve B modelleri ile tahmin edilebileceği sonucuna varılabilir.

Anahtar sözcükler: *Vücut ağırlığı, Büyüme eğrisi, Büküm, Madura sığırı, Doğrusal olmayan modeller*

INTRODUCTION

Madura cattle is one of Indonesian native cattle that originated from Madura Island and is a composite breed that has undergone hundreds of years of selection and domestication. Based on its history, Madura cattle were formed from product cross mating between *Bos javanicus* (wild banteng) and *Bos indicus* that occurred thousands of years ago. Until now, Madura cattle had diverged into 3 functions: sonok, karapan and commercial Madura cattle. Sonok cattle are female Madura cows that have beautiful body shape, coat color, conformation and skills which are the result of selection and specific maintenance from generation to generation. Karapan cattle is a bull that has the ability to run fast and has a lighter and smaller body. Karapan cattle are widely used for the traditional arts of the Madura community [1].

The Madura cattle was kept for meat production and

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drought by smallholders. The weaning weight (205 days of age) and yearling weight (365 days of age) of Madura cattle were 97.00±13.77 kg and 120.00±10.86 kg, respectively [2]. Despite the slaughter weight, carcass weight and carcass percentage of Madura bull were 248.00±71.00 kg; 128.90±45.16 kg and 51.08±4.50%, respectively [3]. In addition, the reproductive traits of Madura cow such as service per conception, conception rate and gestation length were 1.46 time, 58.80%, and 283.35±17.15 days, respectively [4]. According to Cytochrome Oxidase I (COI) gene (mtDNA), the Madura cattle had similar genetic characterization with Zebu *(Bos indicus)* cattle [5].

Madura cattle had good of genetic potency as the beef cattle in Indonesia. The genetic improvement in these cattle is being carried out through a selection program. The early selection in Madura cattle can be performed with growth curve of body weight. The growth curve of body weight was used to evaluate the growth performance in several Indonesian cattle breeds i.e. Friesian Holstein [6-12], Bali [13], Brahman cross [14], Madura [15,16], Aceh [17] and Ongole grade [18]. In addition, the growth curve was also used in many cattle breeds such as Belgian Blue [19], N'Dama [20], Nellore [21,22], Dhofari [23], Parda de Montana [24], Aberdeen Angus [25], Podolica [26] and Friesian Holstein [27,28] to evaluate the growth performance in the world.

The growth in livestock can be defined as an increase of body size per time unit [29]. Growth is a continuous function during the animal's life from embryonic stages up to adult age and it is mathematically explained by growth curve models. Growth curve for poultry generally have the characteristics: an accelerating phase of growth from hatching, a point of inflection in the growth curve at which the growth rate is maximum, a phase where growth rate is decelerating and a limiting value (asymptote) mature weight [30]. In addition, the growth curve is to describe the regular change generated by the live weight or some part of the animal with the age increasing, which commonly is a S-shaped (sigmoid) curve [31].

The application of mathematical model on growth curve will provide a set of parameters that could be used to describe growth pattern overtime. Furthermore, it will enable the breeders to expect the weight of animals at a specific age and to detect the stage that associated with the reduction in growth rate [32]. Moreover, growth curves of livestock have been used to dynamically observe the growth course, to forecast the poultry growth law and to instruct the feeding and management programs to improve the selection and breeding effects [33].

The growth curve of livestock can be estimated with several nonlinear regression model of Brody, Von Bertalanffly, Gompertz, Logistic, Richards and Weibull. However, the Logistic, Gompertz and Von Bertalanffy models have fixed growth forms with point of inflection at about 50%, 30% and 30% of the asymptote in animals, respectively [34]. Moreover, many models were used to obtain the growth curve model in cattle [10,12]. Unfortunately, the study of growth curve estimation in Madura cattle using non-linear models so far is not reported. This study was aimed to estimate the growth curve of body weight with non-linear model of Logistic, Gompertz and Von Bertalanffy. The result of this study can be used as the basic information to keep Madura cattle for breeding or feedlot purposes.

Material and Methods

Ethical Approval

The following experiment was conducted under the guidelines of the Indonesian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the Indonesian Ministry of Agriculture Animal Ethics Committee (Balitbangtan/Lolitsapi/Rm/14/2019).

Animals Data and Research Site

The records of body weight (BW) in Madura cattle *(Bos indicus)* were collected from 186 animals (95 males and 91 females) between the year 2014 to 2019. The weighing time of each animal was performed every month from birth to 20 month of age using electronic digital weight scale. The animals were kept in the colony stall at Indonesian Beef Cattle Research Station *(Loka Penelitian Sapi Potong Grati)*. This station located at longitude112°33'55" to 113°30'37" E and latitude 70°32'34" to 80°30'20" S. This area located at 2 to 2770 m above the sea level with air temperature of 24-32°C and rainfall of 0.2-30.9 mm/year.

Animals Management

The animals were kept in the colony stall with natural mating system. Each stall consisted of 1 bull and 15 to 20 cows. The forages feed consisted of 97.98% of Elephant grass *(Pennisetum purpureum)* and 2.02% of rice straw. Thus, the concentrate feed consisted of chalk (1.89%), salt (1.89%), rice bran (24.75%), slamper corn (20.51%), coffee peel (4.98%), palm kernel cake (9.70%), copra cake (10.16%), cassava flour (10.16%), destillers dried grains with soluble (7.98%) and corn gluten feed (7.98%). Thus, the standard nutritional content of feed for Madura cattle was presented in *Table 1*. Moreover, the fresh water was given *ad libitum* with regular medical examination and vaccination.

Data Analysis

The individual BW data in male (1.876 datasets) and female

Table 1. *The standard nutritional content (DM) of feed for Madura cattle*

Physiological Status	CP (%)	TDN (%)	CF (%)
Birth to weaning	9-10	58-60	19-22
Weaning to adult	10-11	58-60	17-19

DM: *dry matter;* ***CP:*** *crute protein;* ***TDN:*** *total digestible nutrient;* ***CF:*** *crude fiber*

(1.737 datasets) cattle were used to estimate the growth curve with Logistic (L), Gompertz (G) and Von Bertalanffy (B) models using SPSS 16.0 software. The non-linear regression equations that used in the present study were presented in *Table 2*. The goodness-of-fit model in this study was selected based on coefficient of determination *(R^2)* and mean root squared error (*RMSE*), Akaike's information criterion *(AIC)* and Beyesian information criterion *(BIC)* using the mathematical formula as follow [35]:

$$R^2 = 1 - \left(\frac{SSE}{SST}\right)$$

$$RMSE = \sqrt{\frac{SSE}{n-p-1}}$$

$$AIC = NLn\left(\frac{SSE}{N}\right) + 2p$$

$$BIC = NLn\left(\frac{SSE}{n}\right) + pLn(n)$$

where, R2 is the coefficient of determination; *SSE* is the sum of square error; *SST* is the total sum of square; *AIC* = Akaike's information criterion; *BIC* = Beyesian information criterion; N is number of observations (data points); p is the number of parameters.

Results

The average of body weight in Madura cattle from birth to adult ages were presented in *Table 3* (male) and *Table 4* (female). Mostly, the CV value in each age group were included of high category (>20%). Therefore, the growth parameters in Madura cattle were presented in *Table 5*. According to *Table 5*, the A value in B model was higher than the other models in both sexes. Thus, the highest of W_i and t_i values were reached by L model. Meanwhile, the lowest of W_i and t_i values were reached by B model. In addition, A, W_i and t_i values in male were higher than female animals. In general, the A value in animals in the study was 218.02-333.92 kg. The W_i value in animals in the study was 97.73-110.40 kg. While, the t_i value in animals

Table 2. *The growth curve function of Logistic, Gompertz and Von Bertalanffy models (24)*

Model	Y_t	W_i	t_i
Logistic	$A(1+Be^{-kt})^{-1}$	A/2	(Ln.B)/k
Gompertz	$A\exp(-Be^{-kt})$	A/e	(Ln.B)/k
Von Bertalanffy	$A(1-Be^{-kt})^3$	A(8/27)	(Ln.3B)/k

Y_t: *body weight (kg) of cattle at t week of age;* ***A:*** *the asymptotic weight (kg) when times goes to infinity;* ***B:*** *scaling parameters (constant of integration);* ***k:*** *maturing rate (kg/month);* ***e:*** *constanta (2.72);* ***t:*** *time (month);* ***W_i:*** *weight of inflection (kg);* ***t_i:*** *time of inflection (month)*

Table 3. *The descriptive statistics of body weight in male Madura cattle at different ages*

Age (month)	N	Mean (kg)	SD	CV (%)	Min.	Max.
0 (birth)	95	16.81	3.51	20.88	11.00	25.00
1	95	25.86	4.52	17.48	16.80	33.62
2	95	34.93	7.08	20.27	20.72	50.85
3	94	43.99	10.07	22.89	24.58	68.27
4	91	53.00	13.26	25.02	28.44	85.69
5	91	61.08	15.82	25.90	32.30	103.12
6	89	69.94	18.92	27.06	36.00	120.54
7	87	78.36	22.05	28.14	39.10	137.96
8	87	86.90	25.41	29.25	41.25	155.38
9	87	95.67	28.59	29.88	43.41	172.81
10	87	104.43	31.77	30.42	45.57	190.23
11	87	113.20	34.96	30.88	47.73	207.65
12	87	121.97	38.15	31.27	49.88	225.08
13	87	130.74	41.34	31.62	52.04	242.50
14	87	139.51	44.53	31.92	54.20	259.92
15	87	148.27	47.72	32.19	56.35	277.35
16	87	157.04	50.92	32.42	58.51	294.77
17	87	165.81	54.12	32.64	60.67	312.19
18	87	174.58	57.32	32.83	62.82	329.62
19	87	183.35	60.51	33.01	64.98	347.04
20	87	192.11	63.71	33.16	67.14	364.46

N: *number of animals;* ***SD:*** *standard deviation;* ***CV:*** *coefficient of variation;* ***Min.:*** *minimum;* ***Max.:*** *maximum*

Table 4. *The descriptive statistics of body weight in female Madura cattle at different ages*

Age (month)	N	Mean (kg)	SD	CV (%)	Min.	Max.
0 (birth)	91	15.85	2.92	18.43	11.00	25.00
1	91	24.74	4.41	17.83	15.97	37.53
2	91	33.74	7.04	20.87	20.91	51.25
3	91	42.74	9.95	23.29	23.87	66.87
4	91	51.74	12.96	25.04	26.82	82.49
5	86	59.82	15.75	26.33	29.78	98.12
6	85	68.34	18.67	27.32	32.73	113.74
7	84	76.89	21.72	28.25	35.69	129.36
8	79	85.53	24.76	28.95	38.64	144.99
9	79	94.28	27.79	29.47	41.60	160.61
10	79	103.04	30.82	29.92	44.55	176.23
11	79	111.79	33.86	30.29	47.51	191.86
12	79	120.54	36.90	30.61	50.46	207.62
13	79	129.30	39.94	30.89	53.42	223.92
14	79	138.05	42.98	31.13	56.37	240.22
15	79	146.80	46.02	31.35	59.33	256.52
16	79	155.56	49.07	31.54	62.28	272.82
17	79	164.31	52.11	31.71	65.24	289.12
18	79	173.06	55.15	31.87	68.19	305.42
19	79	181.81	58.20	32.01	71.15	321.73
20	79	190.57	61.24	32.14	74.10	338.03

N: *number of animals;* ***SD:*** *standard deviation;* ***CV:*** *coefficient of variation;* ***Min.:*** *minimum;* ***Max.:*** *maximum*

Table 5. *The growth curve parameters for body weight of Madura cattle*

Model	Sex	A	B	k	W_i	t_i	Iteration
Logistic	Male	220.80±8.12	7.10±0.33	0.18±0.01	110.40	10.89	6
	Female	218.02±7.92	7.31±0.34	0.19±0.01	109.01	10.47	6
Gompertz	Male	277.72±18.85	2.48±0.05	0.09±0.01	102.10	10.09	5
	Female	274.13±18.40	2.51±0.05	0.09±0.01	100.78	10.23	5
Bertalanffy	Male	333.92±33.08	0.60±0.01	0.06±0.01	98.94	9.80	5
	Female	329.83±32.35	0.60±0.01	0.06±0.01	97.73	9.80	5

A: *the asymptotic weight (kg) when times goes to infinity;* ***B:*** *scaling parameters (constant of integration);* ***k:*** *maturing rate (kg/month);* ***t:*** *time (month);* ***W_i:*** *weight of inflection (kg);* ***t_i:*** *time of inflection (month)*

Table 6. *The goodness-of-fit criteria for the studied growth models*

Sex	Model	R^2	RMSE	AIC	BIC
Male	Logistic	0.68	36.80	13.531.09	13.547.70
	Gompertz	0.70	35.23	13.518.02	13.534.63
	Bertalanffy	0.68	36.67	13.514.44	13.531.05
Female	Logistic	0.70	35.09	12.376.97	13.393.35
	Gompertz	0.68	36.64	12.363.37	12.379.75
	Bertalanffy	0.70	35.05	12.359.64	12.376.02

R^2: *coefficient of determination;* ***RMSE:*** *root mean squared error;* ***AIC:*** *Akaike's information criterion;* ***BIC:*** *Beyesian information criterion*

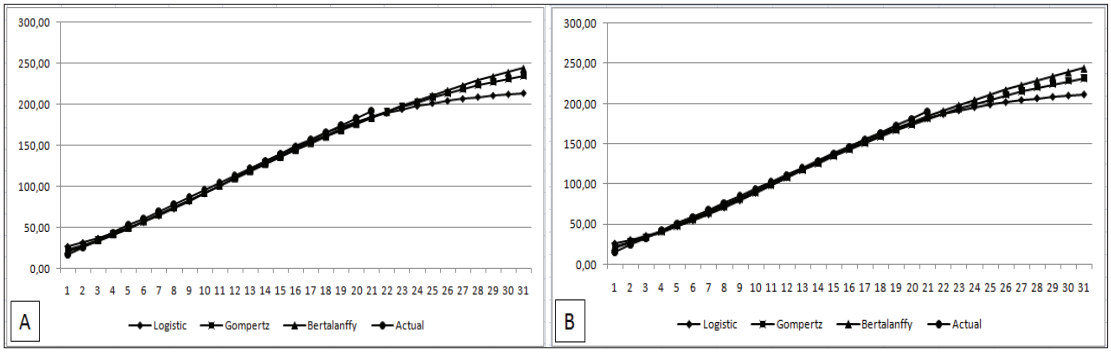

Fig 1. The growth curve of body weight in Madura cattle. A-male, B- female

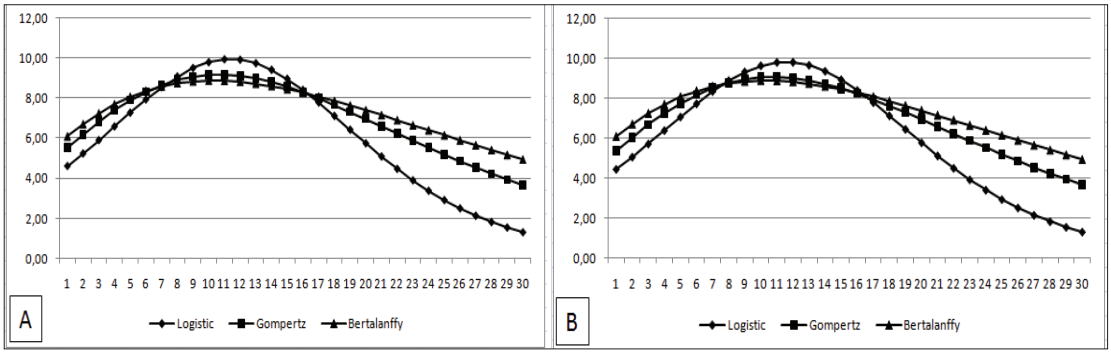

Fig 2. The growth rate of body weight in Madura cattle. A- male, B- female

in the study was 9.80-10.89 months. The R^2 value in each model included of high category ($0.60<R^2<0.80$). However, the goodness-of-fit criteria showed that the G model ($R^2=0.70$) can be selected as the best function to describe growth of male Madura cattle with the lowest of RMSE, AIC and BIC values *(Table 6)*. Meanwhile, the B model as the best function to describe growth of female Madura cattle with the lowest of RMSE, AIC and BIC values. The growth curve and the growth rate of BW in Madura cattle were presented in *Fig. 1* and *Fig. 2*, respectively. According to the growth curve and the growth rate illustration, both sexes have similar growth characteristics. In addition, the number of iterations in the estimated non-linear model were reached of 5 (L) to 6 (G and B),

Discussion

The A values (L/G/B) in dairy cows *(Bos taurus)* were 343.60/354.50/369.90 kg [8], 213.00/543.40/1084.00 kg [27] and 672.94/986.44/1565.60 kg [28]. In addition, the A values (L/G/B) in several *Bos taurus* cattle breeds were 431.00/481.00/517.00 kg in Belgian Blue [19]; 437.10/444.70/448.90 kg in Angus [25] and 778.50/936.90/1098.00 kg in Podolica [26]. Meanwhile, the A values (L/G/B) in some *Bos indicus* cattle breeds were 306.60/311.52/314.04 kg in Brahman cross [14] and 317.00/319.00/322.00 kg in Dhofari [23]. In general, the A value of L model in Madura and Turkish Holstein (TH) cows [27] were under similar range (218.02 vs 213.00). Therefore, the A value of B model in Madura and Dhofari cattle [23] were under similar range (329.83 vs 322.00 kg). The mature weight of cattle can be influenced by farming system and genetics factor.

The W_i value (L/G/B) in Indonesian Holstein (IH) and TH cows were 145.45/130.41/109.60 kg [8] and 336.47/362.89/463.88 kg [28], respectively. Hence, the W_i value in observed Madura cattle was lower than IH and TH cows. Moreover, the W_i values (L/G/B) in several *Bos taurus* cattle breeds were 215.50/176.84/153.19 kg in Belgian Blue [19]; 218.55/163.49/133.01 kg in Angus [25] and 389.30/344.70/325.30 kg in Podolica [26]. Therefore, the W_i values (L/G/B) in some *Bos indicus* cattle breeds were 153.30/143.03/93.05 kg in Brahman cross [14]; 174.00/132.00/108 kg in Ongole grade [18] and 158.00/117.00/95.40 kg in Dhofari [23]. The W_i value of B model in Madura and Dhofari cattle [23] were under similar range (98.84/97.73 vs 85.40). The W_i value in cattle can be influenced by farming system and genetics factor.

The t_i values (L/G/B) in IH and TH cows were 7.55/6.45/4.99 months [8] and 10.92/10.23/13.22 months [28], respectively. Therefore, the t_i value (L/G/B) in some *Bos taurus* cattle breeds were 8.25/6.61/5.56 months in Belgian Blue [19]; 13.24/7.36/19.57 months in Angus and 13.87/12.39/11.70 in Podolica [26]. Therefore, the t_i values (L/G/B) in some *Bos indicus* cattle breeds were 7.81/6.57/2.33 months in Brahman cross [14]; 10.90/6.32/7.26 months in Ongole grade and 7.00/5.00/9.00 months in Dhofari [23]. The t_i value of L modelin Madura, TH [28] and Ongole grade [18] were under

similar range (10.89/10.47vs 10.90 vs10.92). The inflection point indicates several things of the presence of maximum growth of livestock, age at puberty and the lowest point in mortality.

The t_i value in observed Madura cattle reveals that the puberty age of Madura cattle was reached at about 11 months (L) or about 10 months (G). A previous study reported that the puberty age in cattle was reached at 8-12 months [36]. Puberty age in cattle indicates that the sexual organs of cattle developed and signed by sexual behaviors of estrous (female) and flehmen libido (male). In addition, cattle at 11 months of age were mentioned as yearling age and used as the selection criteria for breeding cow and bull. Hence, the heritability (h^2) value of yearling weight (YW) in Madura cattle was 0.54±0.18 (high category) and suggested that the YW trait can be increased with selection program [37]. In cattle, the body weight at puberty age was about 40% of adult weight [38]. Commonly, the puberty age in *Bos indicus* cattle was higher than *Bos taurus* cattle [39]. Puberty age can be affected by genetic (breed) and environmental (climate, nutrition, season) factors [40]. However, the puberty age in cattle can be increased by selection, crossbreeding, and feed (nutrition) improvement [41].

The G and B models are able to describe the growth of observed male and female Madura cattle accurately with high R^2 value (0.70) and lowest of RMSE, AIC and BIC. A similar finding has been reported in Brahman cross cows with B model as the best function rather than L and G models [14]. In conclusion, the non-linear G and B models can be used to predict the body weight of male and female Madura cattle.

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Conflict of Interest

Authors declares that there is no conflict of interests.

Author Contributions

HH and WPBP planned and designed the study, methods, data analysis and manuscript preparation. All authors participated in the study and concurred with the submission and subsequent revisions submitted by the corresponding author.

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RESEARCH ARTICLE

Comparison of the Focused Assessment with Sonography for Trauma Protocol and Animal Trauma Triage Scoring System in Traumatized Dogs

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Abstract

FAST (focused assessment with sonography for trauma) is an ultrasound protocol developed to assess conditions such as pneumothorax, hemothorax, hemoabdomen, bladder rupture, and organ damage due to trauma. It is performed at the time of presentation of a trauma patient for rapid diagnosis. The aim of this study is to compare the results of FAST and ATT (Animal Trauma Triage) scoring system, which are among the first triage examinations, and to determine whether they support each other. Material of the study consisted of 50 dogs (different breed, sex and age) presented in years between 2019-2020 to Selcuk University, Faculty of Veterinary Medicine, Department of Surgery Clinics with trauma history within 24 h. FAST and ATT scoring system were performed for each dog. ATT score of 50 traumatized dogs was evaluated by dividing into 3 categories; ATT scores of 21 dogs were good (0-5), 19 dogs were moderate (6-11), and 10 dogs were poor (12-18). Thoracic and abdominal abnormal findings were detected by T-FAST in 12 dogs (24%) and by A-FAST in 10 dogs (20%). Spearman correlation analysis showed there was no significant association between ATT score and A-FAST (r=0.04, P=0.75), and ATT score and T-FAST (r=0.140, P=0.33) in traumatized dogs. In conclusion, FAST should be performed for urgent diagnosis of possible thoracic or abdominal pathological findings in traumatized dogs, regardless of ATT score.

Keywords: *Dog, FAST, Focussed sonography, Trauma, ATT*

Travmalı Köpeklerde Travma İçin Sonografi İle Odaklı Değerlendirme Protokolü ve Hayvan Travma Triyajı Skorlama Sisteminin Karşılaştırılması

Öz

FAST (travma için odaklanılmış sonografi) travmaya bağlı pnömotoraks, hemotoraks, hemoabdomen, idrar kesesi rupturu, organ hasarı gibi durumların değerlendirilmesi için geliştirilmiş bir ultrasonografi protokolüdür. Hızlı teşhis için travma hastasının sunumu sırasında uygulanır. Çalışmada travmatize olmuş köpeklerde ilk triyaj muayenelerinden FAST ve ATT (Animal Trauma Triage) skorlama sistemi sonuçları karşılaştırılarak, bu önemli triyaj muayene yöntemlerinin birbirlerini destekleyip desteklemediklerinin belirlenmesi amaçlanmaktadır. Çalışma materyalini; 2019-2020 yılları arasında Selçuk Üniversitesi Veteriner Fakültesi Cerrahi Kliniği'ne travma şikayeti ile getirilmiş farklı ırk, cinsiyet ve yaştaki 50 köpek oluşturdu. Travma sonrası üzerinden 24 saat geçmemiş köpekler çalışmaya dahil edildi. FAST ve ATT skorlama sistemi her bir hayvan için uygulandı. Travma geçiren 50 köpeğin ATT skorlaması 3 kategoriye ayrılarak değerlendirildi; 21 köpeğin ATT skoru iyi (0-5), 19 köpeğin ATT skoru orta (6-11) ve 10 köpeğin ATT skoru kötü (12-18) bulundu. 12 köpekte (%24) T-FAST muayenesinde toraks bölgesinde, 10 köpekte (%20) A-FAST muayenesinde abdominal bölgede anormal bulgular belirlendi. Spearman korelasyon analizi, travmalı köpeklerde ATT skoru ve A-FAST (r=0.04, P=0.75) ile ATT skoru ve T-FAST (r=0.140, P=0.33) arasında önemli bir ilişki olmadığını gösterdi. Sonuç olarak, travma geçiren köpeklerde ATT skoru ne olursa olsun, olası torasik veya abdominal patolojik durumların acil olarak saptanması için FAST uygulanması gerektiği sonucuna varıldı.

Anahtar sözcükler: *FAST, Köpek, Odaklı sonografi, Travma, ATT*

INTRODUCTION

Since the 1990s, focused assessment with sonography for trauma (FAST) has been the primary care, standard care and screening technique in many algorithms for both blunt and penetrating trauma in humans [1-4]. FAST aims to diagnose the lesions that require urgent intervention such as pneumothorax, hemothorax, hemoabdomen, bladder

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rupture and organ damage due to trauma [2,5-9]. The advantage of FAST is a rapid diagnosis with minimal manipulation. Previous studies have shown clinical utility in diagnosing and managing intraabdominal and intrapleural injuries due to trauma in dogs and cats [2,5,6,10-14]. The increasing range of critical care qualifications available and expectations of patient owners have led to the use of FAST in animals as well as in humans [15]. In veterinary medicine, the term "T-FAST" is used for the thoracic region, and "A-FAST" for the abdominal region [16].

Animal trauma triage (ATT) score is used to measure illness severity that numerically classifies the degree of trauma in an attempt to quantify mortality risk probability in veterinary medicine [17]. ATT has been widely utilized in veterinary medicine, both clinically and in clinical research settings [18]. ATT score is based on a 0-3 scale (0 being slight or no injury, 3 indicating severe injury) with assessment of 6 independent components (perfusion, cardiac, respiratory, eye/muscle/skin, skeletal, and neurologic) that contribute equally to the overall predictive score [17].

In veterinary medicine, FAST and ATT scoring system are performed during the first triage at the time of presentation of a trauma patient and provide information about the prognosis [19,20]. The aim of this study is to compare the results of FAST and ATT scoring system, which are among the first triage examinations, and to determine whether they support each other.

Material and Methods

Ethical Statement

This study was approved by Selcuk University, Faculty of Veterinary Medicine, Laboratory Animal Production and Research Center Ethics Committee (SUVDAMEK) (2019/43). Patient owners of all cases signed an informed consent form.

Animals and Examinations

Material of the study consisted of 50 dogs (different breed, sex and age) presented in years between 2019-2020 to Selcuk University, Faculty of Veterinary Medicine, Department of Surgery Clinics with a trauma history within 24 h.

Initially, traumatized dogs were scored with ATT [21]. Appropriate stabilization was provided in order to ensure the care of the patient. Six independent components (perfusion, cardiac, respiratory, eye/muscle/skin, skeletal, and neurologic) were evaluated in ATT for each patient [17,21]. Scores between 0 and 3 are given for each category. According to the urgency of their clinical condition, following intravenous catheter insertion, one-fourth of the shock dose (20-30 mL/kg/h) intravenous (IV) fluid lactated Ringer's solution (over 15 min), methylprednisolone (4 mg/kg, IV) (Prednol-L®, Mustafa Nevzat, Turkey) administered for the initial medical management and patient reassessed. O_2 therapy (5 L/min flow rate) was performed to patients via facemask and cefazolin (25 mg/kg, IV) (Iespor®, Ibrahim Etem, Turkey) was administered as antibiotic therapy. Also, tightly fitting mask was switched out periodically to prevent rebreathing of carbon dioxide. Normalized patients were moved for radiographic examination. According to performed examinations; thoracocentesis, thoracostomy tube insertion or surgery was performed [22,23].

FAST (A-FAST, T-FAST) Procedure

During these therapies, sonographic examination (EDAN DUS 60/USA) was performed for A-FAST and T-FAST of the traumatized dog in lateral recumbency. Left or right lateral recumbency was determined depending on rib or vertebral fractures [5,24]. For A-FAST, the ultrasound probe was set to 5-7.5 megahertz (MHz) and the transducer depth to 5-10 cm depending on the size of the patient. For T-FAST, the probe was set to 7.5 MHz and the transducer depth to 4-6 cm [24]. In the sonographic examination, the imaging areas were opened by separating the hair only and using alcohol. However, the area was clipped in cases where the animal's coat was impairing the imaging.

A-FAST examination started from the diaphragmatico-hepatic (DH) site by placing the ultrasound probe on the subxiphoid area and possible evaluations were made. Following DH site, the splenorenal (SR) view was examined by placing the probe on the left side of the dog under the last rib, cysto-colic (CC) view was examined by placing the probe on the bladder and hepato-renal (HR) view was examined by placing the probe close to the umbilical region on the right side. In all these views, the probe was oriented in several directions at different angles until the target organs were identified. Right and left kidneys, spleen, intestine, bladder rupture and peritoneal free fluid (intra-abdominal hemorrhage) were evaluated and noted in the sonographic examination of these views.

In T-FAST examination, bilateral chest tube site (CTS) (both sides of the chest between 7-9th intercostal spaces), bilateral pericardial chest site (PCS) (bilaterally on the heart between 5-6th intercostal space) and DH site (from subxiphoid area) were examined. The probe was moved between every few centimeters on the intercostal spaces to increase the chance of detection of pericardial and pleural fluid. The short and long axes of the heart were visualized by directing the probe "towards the elbow" and "towards the spine". Pneumothorax, hemothorax, pulmonary contusion and hemopericardium were evaluated in T-FAST.

Radiographic Examination

Normalized dogs were moved to radiographic examination after the IV fluid and O_2 therapies. Examinations (Konica Minolta, Regius 110, JAPAN) were performed for abdomen and thorax in the latero-lateral, dorso-ventral and ventro-

dorsal positions according to the clinical conditions of the dog.

Statistical Analysis

Statistical analysis was performed using the statistical package (SPSS 25.0, IBM Corp. 2017). ATT scoring was divided into 3 categories (good 0-5, moderate 6-11, poor 12-18) in terms of prognosis. The Spearman correlation test was used for detection of correlation between A-FAST, T-FAST and ATT. Statistical significance was considered as $P<0.05$.

Results

In the study, 50 traumatized dogs of different breeds, ages (mean age 12±1 months) and gender (29 males-21 females) were evaluated. All dogs had trauma due to a motor vehicle accident. A-FAST and T-FAST procedures were generally performed in 10-25 min. The fastest and easiest examination areas were the diaphragmatico-hepatic, cysto-colic and chest tube sites in the FAST procedures.

The sign of abdominal effusion was observed in 10 dogs by A-FAST of 50 traumatized dogs. In the remaining 40 dogs, the sign of effusion was not established. However, because of reduced abdominal serous detail in radiographic examination, development of effusion was suspected in 15 dogs *(Fig. 1, Fig. 2)*. Also, no rupture was detected, and normal anatomical structure of the bladder was seen in the A-FAST of 50 dogs.

According to the T-FAST examinations of 50 traumatized dogs; B-Line (interstitial-alveolar pulmonary pathology) was detected in 9 dogs. There was no glide sign (pneumothorax positive) in 2 dogs. The step sign (partial PTX, hemothorax, rib fractures, intercostal muscle tear, pulmonary contusions) was detected in 1 dog *(Fig. 3)*. In the radiographic examination of the thorax; pneumothorax in 10 dogs, pleural effusion in 1 dog and pulmonary contusion *(Fig. 4)* in 7 dogs were diagnosed. In a patient with multiple pathological findings, the step sign was also detected by FAST examination *(Table 1)*. The step sign, which is observed in pulmonary contusion, was confirmed by the observation of pathology in the lungs during necropsy *(Fig. 5)*.

The ATT scores of the 50 traumatized dogs were divided into 3 categories and evaluated. Results of ATT scoring system for 21 dogs were good (0-5), 19 dogs were moderate (6-11) and 10 dogs were poor (12-18). A Spearman correlation analysis demonstrated that there was no significant

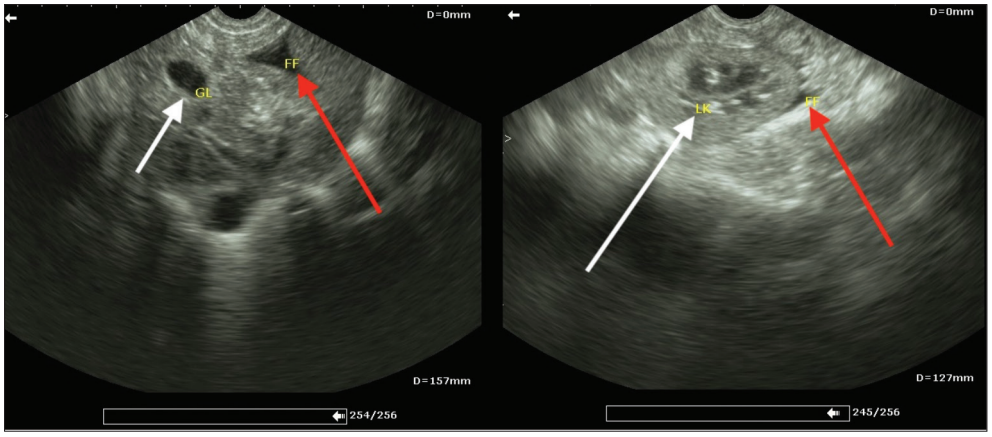
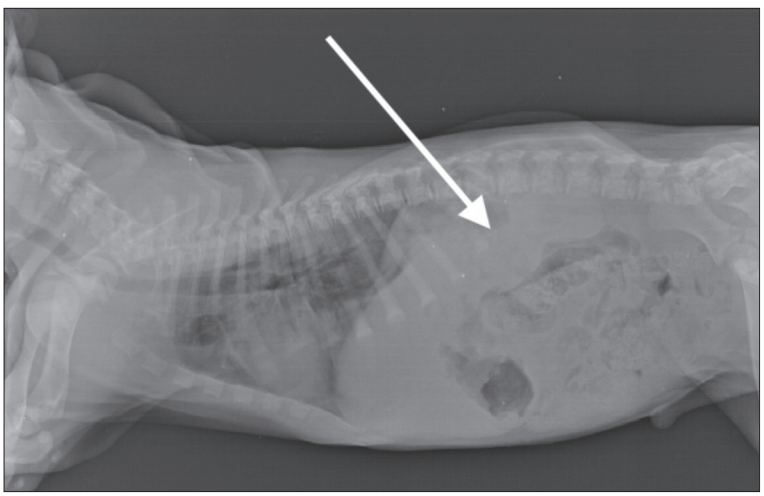


Fig 1. Abdominal effusion in A-FAST. Free fluid *(red arrows)* image above the gallbladder *(white short arrow)* and left kidney *(white long arrow)*

Fig 2. Loss of serous detail *(white arrow)* in abdominal organs in radiographic image



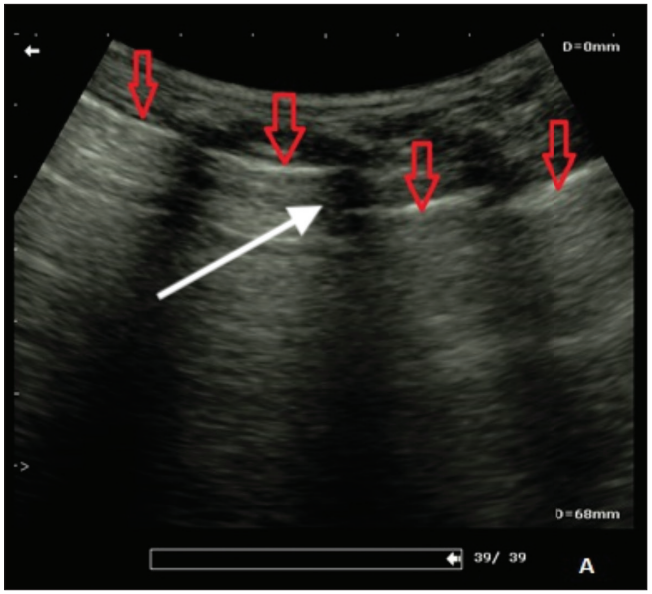


Fig 3. Step Sign *(white arrow)* in T-FAST *(red arrows: pleural lines)*

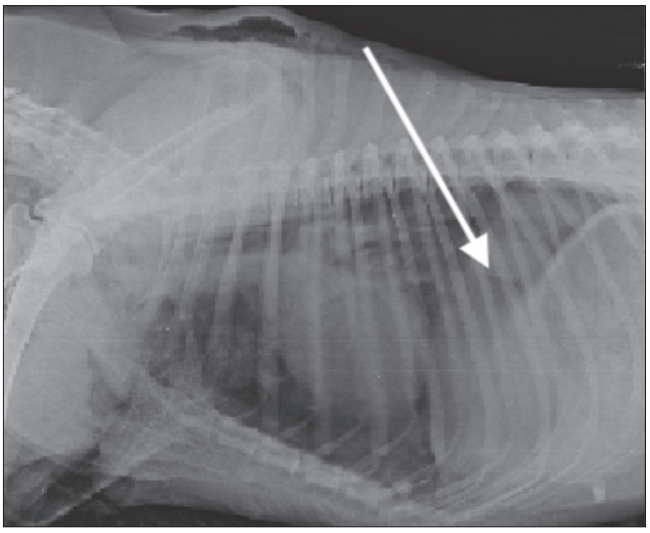


Fig 4. Radiographic view in case of pulmonary contusion *(white arrow)*

association between ATT score and A-FAST (r=0.04, P=0.75), and ATT score and T-FAST (r=0.140, P=0.33) in traumatized dogs.

Discussion

In recent years, motor vehicle accidents cause high mortality rates for both people and animals due to excessive urbanization and advancing technology. Particularly dogs, that live on the street and can establish close relationships with people, are highly affected [18,25,26]. For this reason, traumatized animals with motor vehicle accident history in the emergency clinics are increasing day by day. Therefore, it is not surprising that all traumatized dogs, which were evaluated in our study, had a "motor vehicle accident" history. The fact that post-traumatic A-FAST and T-FAST procedures can be performed in 10-25 min indicates that the diagnosis of pathologies can be made rapidly in order to begin the correct treatment as soon as

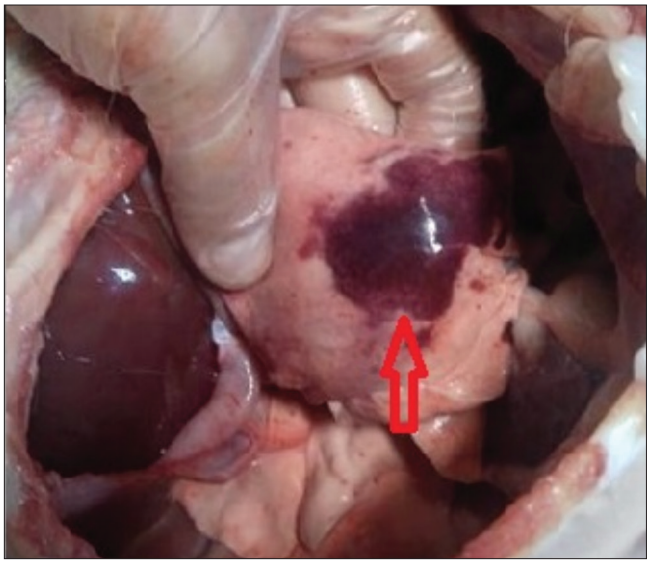


Fig 5. Pulmonary contusion *(red arrow)* during the necropsy

Table 1. *Examination results by FAST, Radiography and ATT scores in 50 dogs following motor vehicle accident trauma*

Procedures (n=50)	**Results**
A-FAST	Abdominal effusion (n=10, 20%)
Abdominal Radiography	Loss of serous detail (n=15, 30%)
T-FAST	Interstitial-alveolar pulmonary pathology (B-Line) (n=9, 18%) Pneumothorax (No glide sign) (n=2, 4%) Pulmonary contusion (step sign) (n=1, 2%)
Thorax Radiography	Pneumothorax (n=10, 20%) Pleural effusion (n=1, 2%) Pulmonary contusion (n=7, 14%)
ATT Score	Good (0-5) (n=21, 42%) Moderate (6-11) (n=19, 38%) Poor (12-18) (n=10, 20%)

A-FAST: *Abdominal focused assessment with sonography for trauma;* ***T-FAST:*** *Thoracic focused assessment with sonography for trauma;* ***ATT:*** *Animal trauma triage*

possible [5,6,16,27]. In addition, unlike humans, dogs have a dense hair coat, which requires clipping for the FAST procedures. In the study, although the examination time of the FAST procedures took a long time at the beginning, with increasing experience, the procedure shortened. However, independent from experience, it has been observed that dogs with complex, long and dirty hair absolutely need shaving for proper FAST procedure.

Veterinary studies have also confirmed the applicability of the FAST procedure in the diagnosis of abdominal and pleural free fluid due to blunt trauma in dogs [11]. In the evaluation of A-FAST of traumatized dogs, intra-abdominal damage, specifically hemoabdomen, was reported more frequently [5,6]. In addition, the high sensitivity rate of T-FAST in traumatized dogs has shown that T-FAST can be used as the first screening method in blunt and penetrating traumas. It has been shown in previous studies to have a sensitivity of more than 75% in detecting pneumothorax in dogs after blunt trauma [28,29]. However, it has been reported that this sensitivity rate may be higher when performed by a specialist physicians [5]. The A-FAST examination requires more attention and experience in terms of fluid presence, according to our study. It is thought that determination of free fluid is more possible to interpretation since the presence of free fluid in thoracic radiographs conceals the apex of the heart due to gravity. In addition, radiography is one of the frequently used imaging methods in the diagnosis of pathologies that are common in dogs with trauma such as pneumothorax and pulmonary contusion.

The use of A-FAST has also been helpful in determining the integrity of the bladder. In FAST studies, it was stated that the probability of rupture is quite low when the bladder is viewed with a normal contour [5,6]. In the subsequent radiographic examinations, the integrity of the bladder was confirmed, and this was supported by the literature data [5,6].

Observation of a step sign referred to pleuropulmonary line continuity pathologies, such as intercostal tears, rib fractures, and subcostal hematoma [16,28]. According to our study, allowed the confirmation of multiple pathologies, which were diagnosed with FAST procedures, by necropsy and showed that FAST procedures could be used significantly in complicated cases. In the FAST procedures, examination of the diaphragmatico-hepatic and chest tube sites was encountered as the most comfortable examination areas.

In the veterinary medicine, the "T" in "FAST" not only "Trauma", but also stands for "Triage" and "Tracking" [16]. For this reason, it was investigated the ATT scoring system and FAST protocols, which are triage methods in veterinary emergency, whether superior to each other or whether complement each other. Many retrospective studies have been conducted on the ATT scoring system, and positive prognostic data have been reported in the evaluation of dogs after trauma (survival rate, diagnosis and treatment protocols) [17,21,25,30]. Ash et al.[21] 599 dogs with complete data entries recruited into the Veterinary Committee on Trauma patient registry. Interventions: None. Measurements and Main Results: We compared the predictive power (area under receiver operating characteristic [AUROC] reported that each 1-point increase in ATT score was associated with 2.07 increase in mortality rates. The observation of life-threatening multiple pathology findings in animals with high ATT scores in our study is consistent with the literature data. Rapid diagnosis and treatment of these pathologies can be achieved with FAST, and positive responses can even be obtained in animals with low probability of survival. However, in the ATT scoring system, only perfusion, respiratory and neurologic categories can show an equivalent performance. The findings obtained in our study are also similar to the literature, and it is recommended examining only the perfusion, respiratory and cardiac categories. Because the basic pathological signs (abdominal effusion, pleural effusion, pulmonary contusion, pneumothorax) determined in the FAST protocol are directly related to the respiratory, perfusion and cardiac systems.

Fast is thought to be a procedure that should be performed before radiography in determining rapid diagnosis and intervention methods (such as thoracocentesis, abdominocentesis) in dogs with trauma. It is important to gain experience by performing multiple abdominal and thoracic sonographies for successful FAST results. In addition, FAST does not prevent emergency interventions such as fluid-electrolyte therapy, administration of respiratory and circulatory analeptics to the traumatized dog during examination. This is especially important in emergency medicine for teamwork and rapid response.

As a result, in order for the FAST examination to be evaluated with ATT, it is thought that only respiratory, cardiac and perfusion categories should be considered and examined in the ATT scoring system for the traumatized dogs. Although FAST and ATT are considered as first triage examinations in emergency, FAST should be performed to determine the possible thoracic or abdominal pathological conditions, regardless of the ATT score.

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Author Contributions

KP and NY, drafting and critically revising the study for important intellectual content. Final approval of the version to be published. NZ, EOU and ETA, make a significant contribution to the study concept or design; data collection, analysis or interpretation.

Declaration of Conflict of Interest

The authors declared that there is no conflict of interest.

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RESEARCH ARTICLE

Effects of Gossypin on Fracture Healing in Experimental Femur Fractured Mouse Mechano-Bioregulatory Model

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Abstract

This paper aimed to research the possible effects of gossypin in a mechano-bio-regulatory mouse model for bone fracture healing. A total of 28 male *Mus musculus* BALB/c mice were randomly selected. Four groups were created. Each group consisted of 7 mice. The Control group was called Group 1. Group 2 was a femur fractured group of mice without any medication. Group 3 was the dose group of 5 mg/kg gossypin to mouse with femur fractures. Group 4 was the dose group of 10 mg/kg gossypin to mouse with femur fractures. An open fracture model was created in the right femur of the animals in Groups 2, 3, and 4. The radiological views for all groups were taken on postoperative 1st day, 6th week, and 12th week. All samples were obtained, collected, and prepared for biomechanical features, histopathological examinations, biochemical tests, and PCR tests. In terms of radiological and histological results, gossypin showed a significant difference depending on the dose in Group 4 to Group 3. Gossypin had sufficient antioxidant and anti-inflammatory effects in mice with femur fracture in bone healing in Groups 3 and 4. Biomechanical tests showed enough hardness levels and high thresholds in braking forces.

Keywords: *Femur fracture, Mouse, Gossypin, Antioxidant, Anti-inflammatory*

Gossypin'in Fare Mekano-Biyoregülatör Modeli Deneysel Femur Kırıklarında Kırık İyileşmesi Üzerine Etkileri

Öz

Bu çalışma, gossypin'in kemik kırık iyileşmesi için fare mekano-bioregülatör modelinde olası iyileştirici etkilerini araştırmayı amaçladı. Toplam 28 erkek *Mus musculus* BALB/c faresi rastgele seçildi. Dört grup oluşturuldu. Her grup 7 fare içermekteydi. Kontrol grubu Grup 1 olarak adlandırıldı. Grup 2 herhangi bir ilaç uygulaması yapılmayan femur kırıklı fare grubuydu. Grup 3, 5 mg/kg gossypin uygulanan femur kırıklı fare doz grubuydu. Grup 4, 10 mg/kg gossypin uygulanan femur kırıklı fare doz grubuydu. Grup 2, 3 ve 4'teki hayvanların sağ femurlarında açık kırık modeli oluşturuldu. Postoperatif birinci gün, altıncı ve onikinci hafta sonunda tüm gruplar için radyolojik tetkikler yapıldı. Tüm örnekler alındı, toplandı ve biyomekanik testler, histopatolojik incelemeler, biyokimyasal testler, PCR testleri için hazırlandı. Radyolojik ve histolojik sonuçlar açısından gossypin, doza bağlı olarak önemli bir farklılık göstermiştir. Gossypin, kemik iyileşmesinde femur kırığı olan farelerde yeterli antioksidan ve antiinflamatuar etkilere sahipti. Biyomekanik testler, yeterli kemik sertlik dereceleri ve kırılma kuvvetlerinde yüksek eşik değerleri gösterdi.

Anahtar sözcükler: *Femur kırığı, Fare, Gossypin, Antioksidan, Anti-inflamatuar*

INTRODUCTION

Gossypin is a bioflavonoid that is found naturally in plants (Malvaceae, *Hibiscus vitifolius)*. It has some protective effects against various diseases that have been declared in studies. These effects can be listed as antioxidant, anti-inflammatory, and analgesic effects. In an earlier study, gossypin was evaluated to establish analgesic effects in mice [1]. In rats, the neuroprotective effects were shown *in-vivo* studies [2,3]. In an experimental sepsis model of

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rats, the effects of gossypin against acute lung injury were demonstrated [4]. Similarly, gossypin was found effective in the treatment of rats with gentamicin-induced nephrotoxicity [5]. Similarly in rats, Tanyeli et al.[6] defended that gossypin had protective effects in the renal ischemia. However, gossypin had ameliorative effects in rats with the formation of galactose-induced cataracts [7]. The antioxidant effects were shown in a study in beta-amyloid-induced toxicity [8].

As a flavonoid, gossypin had potent antioxidant and free radical scavenging effects on different in-vitro systems. Ganapaty et al.[9] demonstrated in their study as an in-vitro evaluation. Reactive oxygen species (ROS) are products of the normal oxidation process. ROS is counted as singlet oxygen, hydroxyl radical, superoxide anion, free oxygen radicals (FOR), hydroperoxyl radical, and hydrogen peroxide. FORs cause cell damage in the ischemic limb such as nitric oxide (NO) overproduction and their participation in the bloodstream causes. FORs accumulation during fracture healing reaches the maximum level on the 15th day. At the same time, the inflammatory process is on the summit point. The increase of FORs levels negatively affects fracture healing [10]. As known as literature knowledge, gossypin protects many tissues from the effects of FORs [9,11]. On the other hand, gossypin may be important because of the abrogation of osteoclastogenesis in the bone fracture healing process [12].

In rat and mouse fracture models, many studies were performed to study out the healing process in bone fracture healing [10]. Recently, mice and rats have become popular as transgenic models for bone fracture healing studies. These models have the available tools for molecular analysis [9]. In the literature, there are some studies about bone fracture healing in rats and rabbits related to biocompatibility and experimental bone defects [13,14]. But, there is no study about the effects of gossypin in mice with femur fracture as a mechano-bioregulatory model.

In our study, mice were the correct choice as a mechano-bioregulatory model to investigate the effects of gossypin on the bone fracture healing process. This paper aims to search the possible effects of gossypin as a new alternative agent for bone fracture healing because of its antioxidant, anti-inflammatory, analgesic effects. We used radiological, biomechanical, histopathological, and biochemical examination methods to detect the healing effects of gossypin in the experimental femur fracture model of mice.

Material and Methods

Ethical Approval

The study with Kafkas University Animal Experiments Local Ethics Committee approval (dated 25.06.2019, with the decision no: 2019/96) was conducted at the Kafkas University Experimental Animal Production and Research Center. All steps of experimental procedures in this study were performed in line with the ethics committee protocols.

Experimental Animals and Creating Groups

A total of 28 male *Mus musculus* BALB/c mice (10-12 weeks, 31.7±3.4 g) were used in the study. During the experiment, mice were conserved in light and dark cycle (12 h/12 h; at 20-22°C), and the *ad libitum* feeding standard chow and normal tap water was performed in mice.

Four groups with 7 mice in each were randomly formed. Group 1 was the control group without any surgical or medical treatment. Group 2 was the femur fractured group of mice without any medical administration. Group 3 was the dose group of 5 mg/kg gossypin to mouse with femur fractures. Group 4 was the dose group of 10 mg/kg gossypin to mouse with femur fractures.

Chemicals

Gossypin (Biovision, USA) was dissolved in dimethyl-sulfoxide (DMSO, Amresco, Canada) and administered intraperitoneally. A 4.5 mg gossypin was dissolved with 16 mL DMSO (Dimethyl sulfoxide, Reagent Plus®, ≥99.5%, Sigma Aldrich).

Operative Technique

Anesthesia protocol was performed by intraperitoneal injection of 2% Xylazine (Rompun®, Bayer AG, Leverkusen, Germany) and 10% Ketamine (Ketalar®, Pfizer Inc., NY, USA) which provided approximately 20 min of deep anesthesia in mice. The surgical anesthesia of mice with 3-10 mg/kg Xylazine and 80-100 mg/kg Ketamine by using the intraperitoneal route of administration was performed [15]. The right femurs were scrubbed with a 10% povidone-iodine solution to prepare them for open femur fracture surgery. The dose adjustment for anesthesia and treatment was performed to pharmacological guidelines and studies about gossypin [15,16]. This group was opened with the help of a bistoury, and the all-right femur fracture was created with a bone cutter. A modification of the method by Manigrasso et al.[17] with a 3-point bending, the device was used for the experimental model. Antibiotic prophylaxis from surgery 30 min ago intramuscular 5 mg/kg cefazolin sodium and an additional dose at the 8th h postoperatively antibiotic prophylaxis was terminated. An open fracture model was created in the right femur of the animals with a 3 cm incision in Groups 2, 3, and 4. The medullary femoral channel was carved and reamerized with a 21 gauge needle. 0.8 mm stainless steel wire (TST, Turkey) was inserted into the canal to obtain a reduction of the fracture. All fractured femurs were reducted by intramedullary methods. By bending the wire proximal part was cut and the patella was reducted and the incisions were sutured. The mice were kept in the supine position at 24°C room temperature in the heating blanket. After the operation, 1-2 mL 0.9% NaCl isotonic solution was

given to animals intraperitoneally to prevent dehydration. Intramuscular narcotics (Buprenorphine 1 mg/kg) were given for controlling the postoperative pain. Desai et al.[18] showed that buprenorphine did not affect bone fracture healing.

Postoperative Period

For mice in all groups, X-rays were taken on the 1st day and the end of 6th and 12th weeks after osteotomy and bone formation in the osteotomy area was evaluated by Lane and Sandhu [19] radiological evaluation system. It is shown in *Table 1*.

Gossypin Application

The basic solution was given by gavage with 18-22 gauge tubes generally in mice from Group 3 and Group 4, with smaller tubes (higher gauge) used for smaller mice with doses. After the operation, gossypin was given to each group for 4 weeks by the doses in the literature. At the end of the study, femur bone tissues in all groups were taken for necessary analyses.

Sample Collection

The mice sacrification was a continued method of euthanasia, such as cervical dislocation. After the scarification blood samples were obtained from all mice for biochemical and PCR tests. Biomechanical tests were performed as soon as the animals were sacrificed. All operated right femurs were cleared from soft tissues and they were stored at -20°C. Tissue sampling was then performed for histo-pathological examinations. The femur bone tissues were placed in a 10% formaldehyde solution for histo-pathological examinations and the other was preserved at -80°C. The samples of right femurs were collected with fixation in 10% formaldehyde in neutral buffered formalin for 48 h, 10% formic in 0.1 M citrate for decalcification kept in acid for 12 h. After the decalcification process longitudinal sections were taken from the femur. Paraffin blocks of samples were created. 5 µm sections were taken. The deparaffinization and then rehydration procedures were performed. Hematoxylin-Eosin (H&E) staining protocol was used for all sections. And these sections were examined by blinded two histologists by the light microscope (Olympus Bx43). The histo-pathological images were taken using a digital camera (Olympus DP21). Grading of Femur Bone Fracture Healing [size of chondrocytes (mm^2) values, trabecular area (percent) values, and trabecular thickness (µm) values] were made.

Table 1. *Radiological Evaluation System for mechano-bioregulatory femur fracture healing model of mouse*

Evaluation	Score
Bone Formation	
Bone formation loss	0
Bone formation (filling of 25% in defect)	1
Bone formation (filling of 50% in defect)	2
Bone formation (filling of 75% in defect)	3
Bone formation (filling of 100% in defect)	4
Bone Union	
Bone non-union	0
Bone union (initiation)	1
Complete radiological bone union	2
Remodeling	
Lack of remodeling	0
Intramedullary canal formation	1
Cortical formation	2

Determination of Biomechanical Features

Biomechanical evaluations were made with three points bending machine (Hounsfield H50KM Surrey, UK). Femur samples, after the removal of intramedullary wires, were placed on the tip of the applicator of the machine. Two mm/min constant speed fracture healing site, to form a fracture again force applied. The broken force as Newton/m^2 was recorded as a unit. After intramedullary wires were removed, femur specimens were placed on the machine towards the end of the force applicator. 2 mm/min constant speed was applied to the fracture healing area to re-form a new fracture. The level for re-fracture force was recorded as a Newton unit. Hardness grade scores were created based on breaking strength (N) and hardness (N/mm).

Histo-pathological Examination

Histo-pathological examination obtained from the fracture line callus area 5 µm thick paraffin sections were prepared. It was stained using the H&E staining protocol for histological examinations. Light microscopy was used for evaluation. Histological evaluation was made according to the histologic evaluation system of Huddlestone et al.'s method [20]. The H&E staining method was used to determine the damage levels. According to this method, some criteria were used: Size of Chondrocytes (SOC) (mm^2) is a definition based on measuring the area occupied by fibrocyte cells at the fracture site. The definition of Trabecular Area (TA) (percent) values described the trabecular microstructure formed during callus tissue formation in the union region. The definition of Trabecular Thickness (TT) (µm) values define the measurable thickness of the trabecular microstructure formed during callus tissue formation at the union site. The number of chondrocytes (NOC) definition signed the measurable number of the chondrocytes formed during callus tissue formation at the union site.

Biochemical Tests

Superoxide dismutase (SOD), Glutathione (GSH), and Malondialdehyde (MDA) levels were measured with blood serum samples of mice. The linear GSH, MDA, and SOD concentrations were determined according to the standard

equation as expressed in nmol/mg, nmol/mg, and U/mg, respectively. The values of mean±standard deviation (SD) were obtained. The analytical grade from Sigma-Aldrich (Germany) was used for all measurements.

PCR Tests for TNF-α and IL-1β

TNF-α and IL-1β analyses were performed (Step One Plus Real-Time PCR System, Applied Biosystems; Primer Design Ltd., Southampton, UK). The relative expression analyses of TNF-α and IL-1β were performed using cDNA synthesized from the blood serum of mice. The plates were heated in suitable conditions.

Statistical Analysis

Statistical studies were performed by SPSS 22.0 program (Windows). Using the One-Way ANOVA method for statistical analysis, p values under 0.05 were considered statistically significant. Databases were expressed as values in mean ± standard deviation. Continuous variables used in the study Kolmogorov Since it shows normal distribution according to the Smirnov test one-way comparisons between 4 groups variance analysis (ANOVA) was used. Between groups in binary comparisons, variance homogeneity Sheffe and Tamhane tests were used. Continuous variables with arithmetic mean and the standard deviation was expressed. Less than 0.05 in statistical evaluation p values were considered significant. SPSS (Windows, IL, USA) software package was used. The statistical analyzes for the histological examination, Descriptive statistics for numeric variables median, minimum-maximum values were measured. Due to the sample number being less than 30, normality analysis was not performed and non-parametric test procedures were conducted directly. In this context, Kruskal-Wallis Variance Analysis, which is a nonparametric alternative to One-Way Variance Analysis was used to determine relationships between parameters. Dunn's test was used in post hoc analysis. The statistical results were interpreted in a 95% confidence interval. P values were considered as statistically significant under 0.05.

Results

This experimental study was completed with a total of 28 mice. Radiographic, biomechanical, histopathological, biochemical, molecular results were collected.

Radiographic Results

In the radiological evaluation on the postoperative 1st day, 6th week, and 12th week, all bones in four groups were evaluated according to the radiologic evaluation system of Lane and Sandhu. In the 6th week, average radiologic evaluation scores performed between groups showed significant differences as statistically (P<0.05). Scheffe test was used to determine significance between Groups 3 and 4. It had a higher statistical difference between these dose groups (P<0.05). Between the other two groups (Group 2 and 3; Group 2 and 4), there was a difference (P<0.05 and P<0.05). They were shown in *Table 2* and *Fig. 1, Fig. 2, Fig. 3*.

Biomechanical Tests and Results

When the maximum braking forces were comparing, the peak values were detected in Group 1. The deep values were detected in Group 2. The braking force levels from the lowest to the highest were put in order. The order was as follows: Group 2 < Group 3 < Group 4 < Group 1. The differences in groups were significant (P<0.05). Hardness grade scores were higher in Group 3 and 4 than Group 2 (P<0.001). The lowest level of Hardness grade scores was detected in Group 2, and the highest level in Group 1. It was shown in *Table 3*.

Histo-pathological Results

In the histopathological examination of Groups 2, 3, 4;

Table 2. *Radiological Evaluation scores of mechano-bioregulatory femur fracture healing model of mouse*

Groups	Radiology Score (mean±SD)
Group 2 (*ff+*abs. of med.)	6.6±0.43
Group 3 (5 mg/kg gossypin)	8.9±0.69
Group 4 (10 mg/kg gossypin)	9.1±0.48
p	0.036
ANOVA test	p<0.05

***ff:** femur fracture, **abs. of med.:** absence of medication*

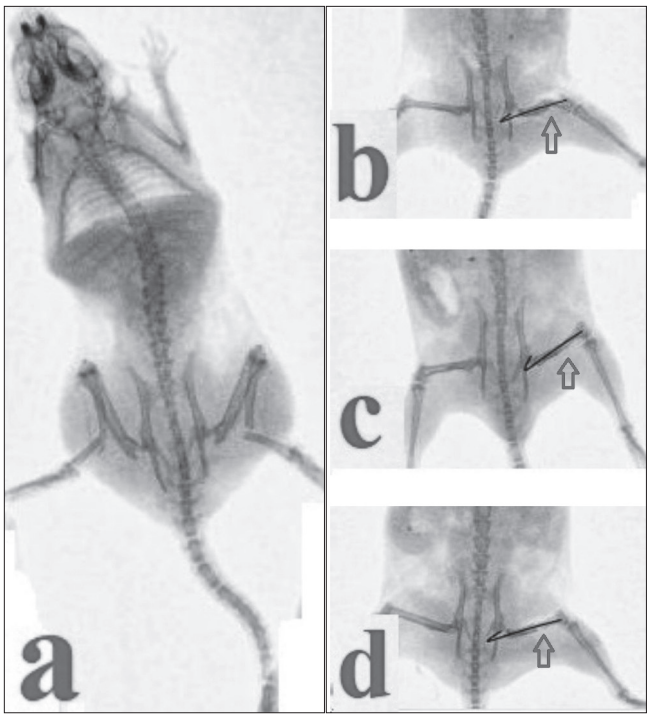


Fig 1. Postoperative 1st day X-rays of right femurs of mice in study groups

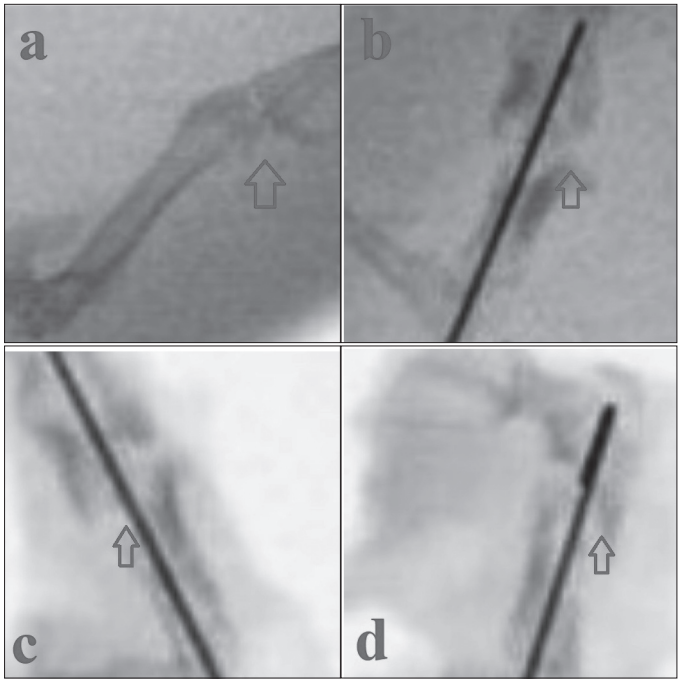


Fig 2. Postoperative 6th week X-rays of right femurs of mice in study groups

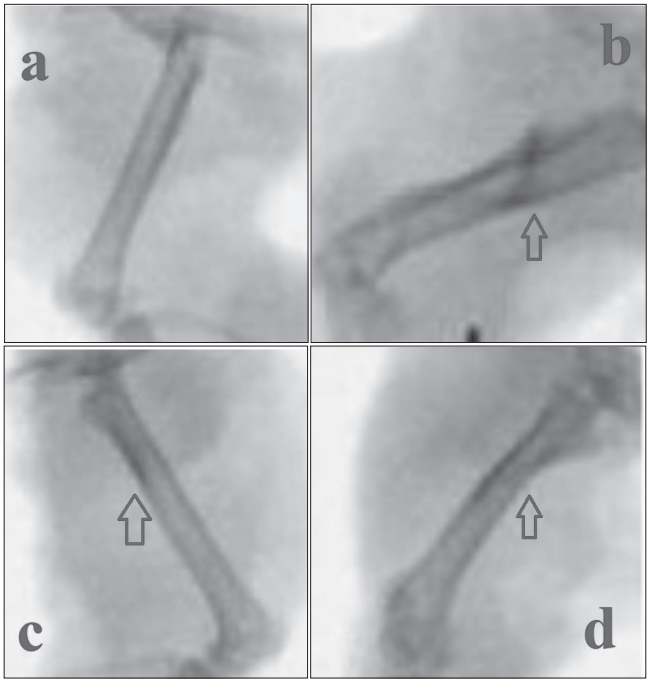


Fig 3. Postoperative 12th week X-rays of right femurs of mice in study groups

Table 3. *Biomechanical tests results of mechano-bioregulatory femur fracture healing model of mouse*

Group	Count	Braking Force (N)	Hardness (N/mm)
Group 1 (control group)	7	22.1±12.3	51.8±74.8
Group 2 (*ff+*abs. of med.)	7	11.2±8.7	15.7±11.7
Group 3 (5 mg/kg gossypin)	7	18.3±9.6	47.2±19.7
Group 4 (10 mg/kg gossypin)	7	19.2±12.3	49.3±20.1
Significance		P<0.05	P<0.001

***ff:** femur fracture, **abs. of med.:** absence of medication*

the fracture lines were rich with osteoblasts, common neovascularization images, and mature compact bone islets. In Groups 3 and 4, the predominance of the fracture site hyaline cartilage tissue rich healing tissue, mature compact in areas adjacent to hyaline cartilage bone tissue sites were located. The osteoblastic activity was observed to be more intense in Groups 3, 4 comparing to Group 2. In Group 2, compact bone healing was watched in which osteo-blastic activity is weaker. They were shown in *Table 4*. In *Fig. 4*, it is shown that the staining of the groups by the H&E method. Differences and similarities between the groups have been expressed in various symbols.

In the histological examination, groups were found significant differences statistically in the sixth week in terms of scores, in favor of Group 3 and 4 (P<0.05). From which group of differences Scheffe test was used to determine if it was caused. The mean scores of Group 3 and Group 4 were higher than Group 1 and Group 2 statistically (P=0.001, P=0.002, P=0.21, P=0.34).

Since the sample size was below 28, nonparametric Kruskal-Wallis Variance analysis was used. The relationship between the SOC value and the groups and whether this relationship was significant or not was examined. According to the test results, there is a significant relationship between the SOC value and the Groups (0.022<0.05). "Dunn's Test" was used to examine the parameters of this significant relationship. According to this; there is a significant difference in terms of SOC values between Group 2 and Group 3 in favor of Group 3 (0.017<0.05). There is a significant difference in terms of SOC values between Group 2 and Group 4 in favor of Group 4 (0.012<0.05). A significant difference was not found in terms of SOC values between Group 3 and Group 4 (0.608≥0.05).

The relationship between the groups with TA per value and whether this relationship was significant or not was examined. According to the test results, there is a significant relationship between the TA per value and the Groups (0.003<0.05). "Dunn's Test" was used to examine the parameters of this significant relationship. According to this; there is a significant difference in terms of TA per values between Group 2 and Group 3 in favor of Group 3

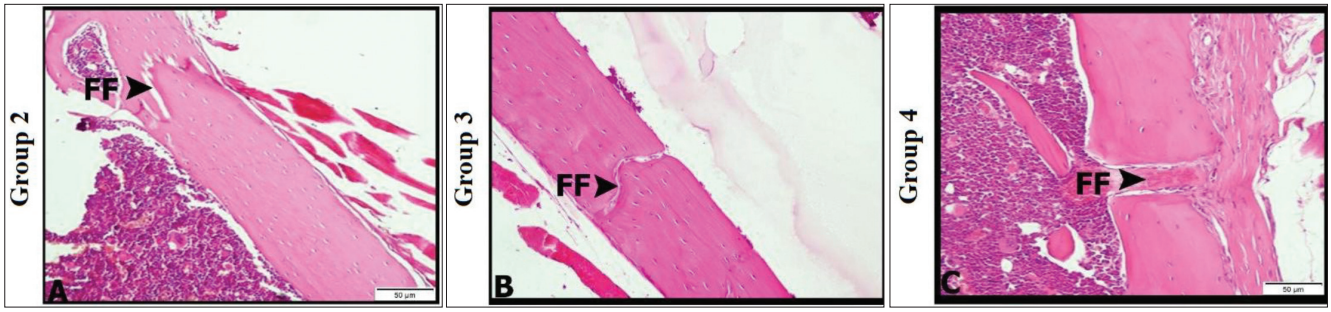


Fig 4. Histological samples from groups on femur fracture healing of mice

Table 4. *Histological evaluation score results of mechano-bioregulatory femur fracture healing model of mouse*

Groups	**6th-week Histological Score**
Group 2 (*ff+*abs. of med.)	6.3±0.47
Group 3 (5 mg/kg gossypin)	7.6±0.53
Group 4 (10 mg/kg gossypin)	8.1±0.23
P	0.001
ANOVA test	P<0.001

***ff:** femur fracture, **abs. of med.:** absence of medication*

(0.003<0.05). There is a significant difference in terms of TT values between Group 2 and Group 4 in favor of Group 4 (0.03<0.05). A significant difference was not found in terms of TT values between Group 3 and Group 4 (0.537≥0.05).

Biochemical Test Results

In Group 3 and 4, at both dose groups, GSH had higher levels (P<0.05). GSH levels were lesser in Group 2 than in Group 1 (P<0.05). MDA levels were higher in three groups with femur fractures than in the control group. MDA levels were detected low in Groups 3 and 4 (P<0.05). SOD levels

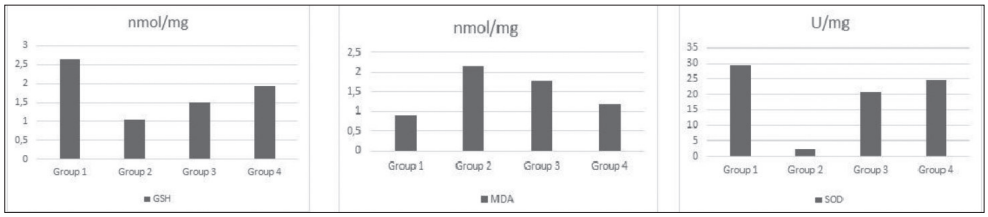
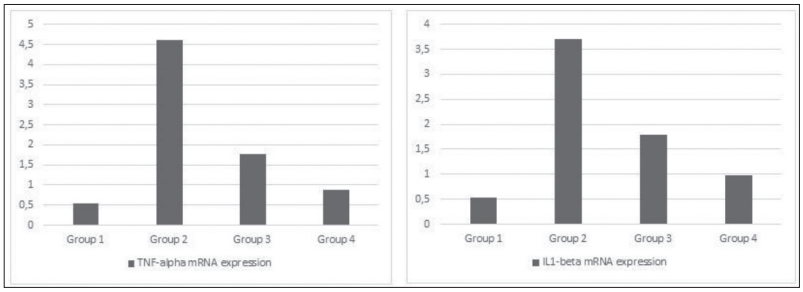


Fig 5. GSH, MDA, SOD levels of mice in all groups

Fig 6. IL-1β and TNF-α levels of mice in all groups



(0.013<0.05). There is a significant difference in terms of TA per values between Group 2 and Group 4 in favor of Group 4 (0.002<0.05). A significant difference was not found in terms of TA per values between Group 3 and Group 4 (0.169≥0.05).

The relationship between the values and the groups with TT per value and whether this relationship was significant or not was examined. According to the test results, there is a significant relationship between the values and the groups (0.004<0.05). "Dunn's Test" was used to examine the parameters of this significant relationship. According to this; there is a significant difference in terms of TT values between Group 2 and Group 3 in favor of Group 3

were in a dose-dependent manner. SOD activity was lower in Group 1 comparing to the control group (P<0.05). It was shown in *Fig. 5*.

Results of PCR Tests

TNF-α and IL-1β mRNA expressions were found to increase in Group 2 comparing to Group 1 (P<0.05). In Group 3 and 4, gossypin reduced TNF-α and IL-1β mRNA expressions in comparison to Group 2 (P<0.05). It was shown in *Fig. 6*.

Discussion

The orthopedic researches to find the optimal model for human bone diseases determined that the reproducible

animal models were an effective choice [21]. Lu et al.[22] researched the tibia fractures in rats. The study was aimed to detect the fracture healing process in non-stabilized and stabilized conditions. 10-14 weeks-old, male rats were used in the study. The ischemia was induced by femoral artery resection in mice. They found three important results: i) ischemic conditions in fractures led to a delayed union or a nonunion, ii) formation of cartilage over bone is not related to this condition, iii) this model was suitable to test new therapeutic regimens in fracture healing [22]. Terjesen [23] studied a rabbit tibia fracture model to evaluate bone healing after external fixation of rabbit tibial osteotomies. The various groups were created according to different periods for removal of external fixation and sacrifice. The optimum time for removal of external fixation was found as 6 weeks [23]. Recently, the small animal models are becoming favorable to make a study in some clinical conditions like vascularization and ischemia on fracture healing. Mouse models, one of the small animals, are special for studying metaphyseal bone fracture healing in osteoporotic conditions [21]. As the number of animal species studied in bone fracture models increases, the chance of testing environmental and internal factors affecting the fracture increases accordingly [24]. The mice have some advantages for the animal bone fracture modeling as listed: 1) easy handling, 2) low husbandry costs, 3) short reproductive cycle, 4) transgenic features, 5) specific analytic tools; monoclonal antibodies, antigens [25]. Also, human and mouse genomes have similar orthologs/homologs genes. This feature makes this animal model is appropriate for the process of human bone fracture healing. However, the bone structure and remodeling process are different from humans [26]. To obtain the most appropriate approach to the physio-pathological process of bone fracture healing in orthopedic practice, we used mice as the most appropriate model.

The mechano-regulatory models, bioregulatory models, and coupled mechano-bioregulatory models were studied in some papers to search the simulation of fracture healing outcomes [27]. Exploring the new models of the bone fracture healing process may help us to find new therapeutic drugs/agents that effective on this complex process. The mesenchymal progenitors and their accumulation in the fracture gap is the first step of the bone fracture healing. And then, the proliferation and differentiation into the osteoblastic cell lineage are the basic processes [28]. In the general view, ischemia, the impaired vascularization, osteoporosis, and complications with soft tissue injuries are real factors for the bone fracture healing process [21]. In the fracturing method, the classifications of the fracture line (metaphyseal or diaphyseal) affect the results of the union in some health problems (ovariectomized/estrogen depleted animal models) [24]. Our mechano-bioregulatory model was in full compliance to research the possible effects of gossypin in the bone fracture healing process of mice as a mechano-bioregulatory model.

In 2014, Erdem et al.[29] performed an experimental study on a rat bone fracture model to investigate the effects of melatonin and caffeic acid phenethyl ester (CAPE) antioxidant molecules against ischemic factors. At the end of the 6th week, two blinded observers evaluated the radiological parameters of rats and the mean in radiological scores. All radiological images were evaluated according to the rating system Lane and Sandhu [19] Radiological findings of bone fracture union were detected in all experimental groups. The mean radiological scores had significant differences at the end of 6 weeks between groups statistically ($P<0.05$). To determine the difference the score of the CAPE-ischemia group comparing to the ischemia-fracture group Scheffe test was statistically different significantly higher ($P<0.05$). Other groups had no statistically significant differences between each other ($P>0.05$) [29]. In our radiologic evaluation, on the postoperative 1st day, 6th week, and 12th week, all bones in four groups were evaluated according to the radiological evaluation system of Lane and Sandhu [19]. At the end of the 6th week, average radiological evaluation scores performed between groups showed significant differences statistically ($P<0.05$). Scheffe test was used to determine significance between Groups 3 and 4. It had a higher statistical difference between these dose groups ($P<0.05$). Between the other two groups (Group 2 and 3; Group 2 and 4), there was a difference ($P<0.05$ and $P<0.05$). In terms of radiological and histological results, gossypin showed a significant difference depending on the dose.

To gain an orthopedic approach, the biomechanical evaluation is made by some measurement components as stress, strain, loading, forcing, displacement, ultimate strength, fracture stiffness, and healing time. The three-point bending test (TTPT) is applied to measure biomechanical features of fracture healing [30]. Wang et al. made an overview of current computational healing models in a study. They discussed the limitations, solutions, and potentials of animal bone models. They presented three kinds of animal bone fracture models (mechano-regulatory models, bioregulatory models, and coupled mechano-bioregulatory models). They emphasized the multiscale models and the coupled mechano-bioregulatory models as new investigations on bone fracture healing. All these experimental studies will conduct optimum treatment strategies [27,29]. In our study, all biomechanical tests were started with TTPT. All biomechanical components as stress, strain, loading, forcing, displacement, ultimate strength, fracture stiffness, and healing time were evaluated for mice of all groups. Hardness grade scale was the highest level in groups with gossypin administration. The lowest level was in Group 2 without any gossypin medication. According to biomechanical tests, gossypin yielded significant results depending on the dose. Biomechanical tests in this mechano-bioregulatory model showed that gossypin provided enough hardness levels, and high thresholds in braking forces.

Erdem et al.[29] used the method of Huddlestone et al.[20] as a histological evaluation system in their rat bone fracture model. At the end of 6 weeks, in terms of scores, a statistically significant difference was found between groups (P<0.05). They performed Scheffe test to determine which group differs from others. The mean in scores of fracture group (p=0.002), melatonin-ischemia group (P=0.021), CAPE-ischemia group (P=0.001) was found statistically higher than the mean score of the ischemia-fracture group. At the fracture line of these groups, there are full images of the osteoblasts and neo-vascularity with the mature bone islets. In the fracture-ischemia group, the fracture site is predominantly full of healing tissues rich in hyaline cartilage tissue and mature compact in areas adjacent to the hyaline cartilage bone tissue areas. Especially, the fracture group and the CAPE-ischemia group had more intense osteoblastic activity. In the fracture-ischemia group, compact bone with weaker osteoblastic activity recovery was observed [29]. As the result of our histo-pathological studies, there is a significant relationship between the SOC values and groups. There is a significant relationship between the groups with TA per value. There is a significant relationship between the values of the TT and groups. A significant difference was not found between groups in terms of NOC values. There is a significant relationship between the SOC value and groups. There is a significant relationship between the groups with TA per value. There is a significant relationship between the values of the TT and groups. A significant difference was not found between groups in terms of NOC values. At the evaluation of SOC, TA, and TT; gossypin showed a healing effect in a dose-dependent manner. There were no statistical differences between groups in terms of the NOC.

The antioxidant effects of gossypin have been demonstrated in publications published in 1998 [31], 2004 [8], 2007 [9], 2017 [32], and 2020 [11]. In 1998 [31], Jornot et al.[31] showed potent antioxidant and free radical scavenging effects of gossypin in different *in-vitro* studies. According to Yoon et al.[8], gossypin inhibited oxidative stress- and Aβ (25-35)-induced toxicity. Also, it inhibited lipid peroxidation and scavenge DPPH radicals. Gossypin inhibited the toxicity induced by X/XO, in this study [8]. Ganapaty et al.[8] demonstrated gossypin and BHT inhibited free radical-mediated deoxyribose damage. Antioxidant activity of gossypin was investigated as good antioxidant activity levels at the tested concentrations. Also, gossypin had enough *in-vitro* lipid peroxidation inhibitory potential [32]. The oxidative stress-induced hydrogen peroxide (H_2O_2) is used commonly to show the antioxidant activity in cells. In the L929 cells, the oxidative stress levels induced by H_2O_2 were evaluated for gossypin. The antioxidative capacity was decreased, increased levels of MDA, and the lower levels of SOD were determined. The doses groups of 25 ve 50 µg/mL gossypin increased the antioxidative activity but decreased the levels of MDA. Especially, the levels of SOD in the 50 µg/mL dose group were higher than in the H_2O_2 group. These effects may be related to the alterations of ROS (reactive oxygen species) by gossypin in L929 cells. Gossypin's protective effects include two factors: free radical scavenging activities and some signals by the genes related to antioxidant and anti-inflammatory bio-molecules [11]. In our study, Groups 3 and 4, at both dose groups, GSH had higher levels (P<0.05). GSH levels were lesser in Group 2 than in Group 1 (P<0.05). MDA levels were higher in three groups with femur fractures than in the control group. MDA levels were detected low in Groups 3 and 4 (P<0.05). SOD levels were in a dose-dependent manner. SOD activity was lower in Group 1 comparing to the control group (P<0.05). Considering the SOD, MDA, and GSH values, we found that gossypin showed an antioxidant effect depending on the dose. Gossypin had sufficient antioxidant and anti-inflammatory effects in mice with femur fracture in bone healing.

As an injury, bone fracture creates an inflammatory response. It reaches to peak on the first day [33,34]. At this time, neutrophils reached the inflammatory area. Acute inflammation and hematoma have critical roles in fracture healing [35,36]. The hematoma followed acute inflammation [33,37,38]. Suppressed or dysregulated response elicits chronic inflammation that may be detrimental to fracture healing. TNF-α, IL-1β, IL-6, and CCL2 are pro-inflammatory cytokines and they were secreted by macrophages. The alterations in the levels of pro-inflammatory molecules have major effects. After the bone fracture, TNF-α and IL-1β reach two times to peaks (at 24 h and 3rd week) following the injury [39]. They promote bone resorption by some activities of osteoclasts and/or osteoblasts. Chronic inflammation has an imbalance related to the formation and resorption of bone [40]. TNF1 acts a pro-inflammatory and apoptotic role [41,42]. Generally, TNF-α suppresses osteoblasts and stimulates osteoclast proliferation and differentiation [43-45]. On the regulation of bone metabolism, TNF-α has some roles. TNF-α follows a biphasic pattern with its receptors, TNFR1 and TNFR2. By the functions of TNFR1 and TNFR2, TNF-α regulates osteogenic cells. TNFR1 is seen in the bone. The expression of TNFR2 followed the bone injury. TNF-α has some paradoxical effects on osteogenesis and bone formation-related MSCs. By the way, as an acute-phase protein after trauma, TNF-α can initiate blood clotting [46]. As a pro-inflammatory cytokine, IL1b takes charge in cell proliferation, differentiation, and apoptosis. IL-1 has similar effects with TNF-α. IL-1 gets a role in the callogenesis and angiogenesis with roles of IL-6 in osteoblasts. Even if IL-1 exists, fracture healing is not affected adversely. IL-1 has a biphasic pattern in the expression phase and its source is macrophages, like TNF-α. In a study, TNF-α and IL-1β inhibit NaBu-induced IAP (intestinal alkaline phosphatase) gene expression. Pro-inflammatory molecules have infamous features with high circulating levels of TNF-α and IL-1 is linked to joint and bone destruction [47,48]. In our experimental study, the results had compliance with the

literature. When looking at IL-1β and TNF-α levels, gossypin affected the recovery with its anti-inflammatory effects by inhibiting the chronic inflammation procedure.

The new pharmaceutical agents can develop the regimen for the healing process of human bone. Klontzas et al.[24] searched some keywords as fracture, drugs, bone, and healing in Medline and Scopus databases between 2010 and 2016. 5310 results were found. Investigational drugs for fracture healing can be listed as anti-osteoporotic drugs (Bi-phosphonates), parathyroid hormone (PTH), strontium ranelate, estrogens and selective estrogen receptor modulators (SERMS), sclerostin Ab & DKK-1 Ab, bone morphogenetic proteins (BMPs), statins, antihypertensive drugs, lithium, proteasome inhibitors, melatonin, botulinum toxin, erythropoietin (EPO), sildenafil, sphingosine 1-phosphate receptor-targeted drugs, G-CSF (Granulocyte Colony Stimulating Factor), FGF (Fibroblast Growth Factor), VEGF (Vascular Endothelial Growth Factor), local vanadium. The TGF-b superfamily members as G-CSF, VEGF, FGF, BMPs, and other substances such as EPO were used to evaluate bone regeneration widely in the preclinical and clinical tests [49-51].

The drugs used in the treatment of bone fracture healing aim to provide this in three ways as i) osteoblastic activity increasing, (indirectly progenitor cells that will mature to osteoblasts), ii) inhibiting of osteoclastic activity (favoring osteoblastic aspect of the process), or iii) by stimulating the vascularization of tissue. According to the results of our study, besides its antioxidant and anti-inflammatory effects, we can state that gossypin had therapeutic effects on bone fracture healing in the latter two ways, although the exact level of its effect on osteoblastic activity is not known, yet.

The mice have some advantages for animal bone fracture modeling as a mechano-bioregulatory model in full compliance with the human bone fracture healing model. The studies in mechano-bioregulatory models may be performed with mice. We recommended the use of gossypin in the treatment of bone fracture healing with its antioxidant, analgesic, and anti-inflammatory effects with the trinity effects of osteoblastic activity, inhibiting of osteoclastic activity, stimulating the vascularization as bone fracture healer agents.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Author Contributions

KY: conceived and designed the analysis, collected the databases, contributed databases or analysis tools, performed analysis, wrote the paper, other contribution; MFT: contributed databases or analysis tools, performed the analysis; VT: collected the databases, contributed databases or analysis tools (veterinary, biomechanical examinations), performed analysis, wrote the paper, other contribution; FB: collected the databases, contributed databases or analysis tools (biochemistry), performed analysis, wrote the paper, other contribution; SY: collected the databases, contributed databases or analysis tools (histological examination), performed analysis, wrote the paper, other contribution.

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RESEARCH ARTICLE

Comparative Analysis of the Heart Tissue Transcriptomes Between Low-altitude Reared and High-altitude Reared Bar-headed Geese *(Anser indicus)*

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Abstract

The bar-headed geese *(Anser indicus)* are renowned for high-altitude migratory flights and they must fly over the Qinghai-Tibetan Plateau for their annual migration. Through comparing the high-altitude bar-headed geese with the other closely related low-altitude species, many efforts have been made to reveal the unique adaptations at physiological, biochemical, and behavioral levels that help bar-headed geese living and flying in high-altitude conditions. Nonetheless, little is known about the transcriptome level changes of the bar-headed geese adaptation to low-altitude environment. To explore the variations of gene expression that were induced by low-altitude environment in the bar-headed geese, we conducted the first comparative transcriptomic analysis of heart tissues between bar-headed geese reared in high-altitude regions (~3000 m), and the bar-headed geese reared at the low-altitude regions (~30 m) for nearly three years. A total of 76 differentially expressed genes (DEGs) were detected in the low-altitude bar-headed geese compared with the high-altitude bar-headed geese. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that these DEGs were mainly involved in the focal adhesion, extracellular matrix (ECM) - receptor interaction, the mammalian target of rapamycin (mTOR) signaling pathway, wingless-type (Wnt) signaling pathway, and glycosaminoglycan degradation etc. The results will be useful for understanding the divergent adaptation of the bar-headed geese to different altitude environment, and the transcriptome data provides a valuable resource for future functional studies.

Keywords: *Anser indicus, Transcriptome, Adaptation, Altitude, Heart*

Düşük ve Yüksek İrtifalı Alanlarda Yetiştirilen Çubuk Başlı Kazlarda *(Anser indicus)* Kalp Dokusu Transkriptomlarının Karşılaştırmalı Analizi

Öz

Çubuk başlı kazlar *(Anser indicus)*, yüksek irtifada göçmen uçuşlarıyla ünlüdür ve yıllık göçleri için Tibet Platosu üzerinden uçmaları gerekir. Yüksek irtifaya adapte çubuk başlı kazları, diğer yakından ilişkili alçak irtifalı türlerle karşılaştırarak, bu kazların yüksek irtifa koşullarında yaşamasına ve uçmasına yardımcı olan fizyolojik, biyokimyasal ve davranışsal seviyelerde benzersiz adaptasyonlarını ortaya çıkaracak birçok çalışma yapılmıştır. Bununla birlikte, çubuk başlı kazların alçak irtifalı ortamlara adaptasyonlarının transkriptom seviyesi değişiklikleri ile ilgili çok az şey bilinmektedir. Çubuk başlı kazlarda düşük irtifa ortamı tarafından indüklenen gen ekspresyon varyasyonlarını araştırmak için, yüksek irtifa bölgelerinde (~3000 m) yetiştirilen çubuk başlı kazlar arasındaki kalp dokularının ilk karşılaştırmalı transkriptomik analizini gerçekleştirdik ve bu kazlar yaklaşık üç yıl boyunca alçak irtifalı bölgelerde (~30 m) yetiştirildi. Yüksek irtifalı çubuk başlı kazlarla karşılaştırıldığında, alçak irtifalı çubuk başlı kazlarda farklı eksprese edilmiş (DEG) toplam 76 gen tespit edildi. Gen ontolojisi (GO) ve Kyoto Genler ve Genom Ansiklopedisi (KEGG) analizi, bu DEG'lerin temel olarak fokal adezyon, ekstrasellüler matris (ECM) - reseptör etkileşimi, rapamisin protein kompleksinin memeli hedefi (mTOR) sinyal yolu, wingless-tip (Wnt) sinyal yolu ve glikozaminoglikan degredasyonu vb. ile ilişkili oduğunu göstermiştir. Çalışma sonuçları, çubuk başlı kazların farklı irtifa ortamlarına farklı adaptasyonlarını anlamak için faydalı olacaktır ve elde edilen transkriptom verileri gelecekteki fonksiyonel çalışmalar için değerli bir kaynak imkanı sunacaktır.

Anahtar sözcükler: *Anser indicus, Transkriptom, Adaptasyon, İrtifa, Kalp*

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Introduction

The bar-headed goose *(Anser indicus)* is endemic to Asia, breeding in the high-altitude plateau wetlands of central Asia [1-4], wintering mainly in the south-central Tibet (approximately 56.88% of the world populations) and India (around 27.84% of the world populations) [1,4]. This species is famous for extremely high-altitude flying over the Himalayan Mountains twice a year on the Central Asian flyway between the wintering areas in India subcontinent and the summering grounds on the Qinghai-Tibetan Plateau [5]. The bar-headed geese were tracked flying as high as 7.290 meters up [6], and mountaineers even reported seeing them fly over the summit of Mount Everest (8.850 meters above sea level) [7]. At these heights, the partial pressure of oxygen is one-third that of sea level. Incredibly, bar-headed geese are able to maintain the high metabolic and oxygen consumption rates necessary for flapping flight under severe hypoxia conditions [8]. Not surprisingly, there has been increasing interest among researchers in understanding the physiological, molecular, and behavioral adaptations of bar-headed geese that allow for the exceptional high-altitude flight performances [9,10].

Through comparing the high-altitude bar-headed geese with the other closely related low-altitude species, a growing body of literature has identified several unique adaptations that help bar-headed geese flying in low oxygen conditions. For example, physiological adaptations have evolved at every step in the oxygen transport cascade of bar-headed geese to help them accomplish the high-altitude success. Such adaptations include an effective breathing pattern [11], larger lungs [12], an enhanced hypoxic ventilatory response [13], hemoglobin (Hb) with a higher oxygen affinity [14], an increase in flight and cardiac muscle capillarity [15], and subcellular redistribution of the mitochondrial reducing oxygen diffusion distances [15]. The increased affinity between Hb and O_2 plays a critical role in adaptation of bar-headed geese to hypoxia through enhancing pulmonary O_2 loading. A few studies have found that in the bar-headed goose the major isoform of Hb has significantly higher O_2-affinity than that of the closely related low-altitude species due to the single and large-effect substitutions in the hemoglobin gene [16,17]. In addition, a roller coaster strategy was reported to take by bar-headed geese, rising and falling with the relief of the terrain, to minimize energy expenditure during their trans-Himalayan flights [18]. Overall, these studies have greatly improved our understanding of the adaptive mechanism of bar-headed goose for high-altitude conditions.

Recent advances in genomics have opened a huge opportunity to study the genetic basis of high-altitude adaptation characteristics [19,20]. In our previous study, we reported the first *de novo* whole genome sequencing, assembly, gene prediction and annotation of the bar-headed goose [21]. Transcriptome is a useful tool to analyze changes in gene expression, and has been widely used to explore high-altitude adaptations in a group of birds [22]. Therefore, in this study, we aimed to compare the transcriptome profiles of heart tissues between bar-headed geese reared in low- and high-altitude environment. Unlike previous studies that compared high-altitude bar-headed geese with the other lower-altitude birds, our current study focused on comparison between the low-altitude reared bar-headed geese, where these geese were transported to and there is no distribution of wild bar-headed geese, with the high-altitude reared bar-headed geese. The major advantage of this experimental setup is that the genetic backgrounds of the subjects being compared are the same. The results will enrich the transcriptomic resources of bar-headed geese and laid a foundation for the functional study of genes related to low altitude adaptation in this species.

Material and Methods

Ethics Statement

This study conformed to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398). The research protocol was reviewed and approved by the Ethical Committee of Qinghai University.

Animal Sample Collection

Eggs of wild bar-headed geese were collected and hatched at the Fei Yan specialized breeding and rearing farming cooperative (elevation 3.000 meter) in Huangzhong District, Xi'ning City, Qinghai Province, China. After hatch, three individuals were reared at this cooperative for three years until the experiment began and labeled as high-altitude group (HA group, n=3) *(Fig. 1)*. Another three individuals were transported to and reared at the He Ming breeding Co., LTD. (elevation 30 meter) in Hengshui City, Hebei Province, China. The latter three individuals were also reared for three years until the experiment began and labeled as low-altitude group (LA group, n=3) *(Fig. 1)*.

These bar-headed geese lived freely in both wild and artificially reared environments. These birds were healthy during experimental period. The heart tissues were sampled rapidly from each carcass, and immediately frozen in the liquid nitrogen. All heart samples were stored at -80°C until used.

RNA Extraction and Transcriptome Sequencing

For each sample, total RNA was isolated using Trizol reagent (Invitrogen, California, USA) following the manufacturer's instructions. Residual genomic DNA was digested by RNase-free DNase (Qiagen, Germany). The RNA concentration and overall quality were assessed using a Qubit 2.0 Fluorometer (Invitrogen, California, USA), and an Agilent 2100

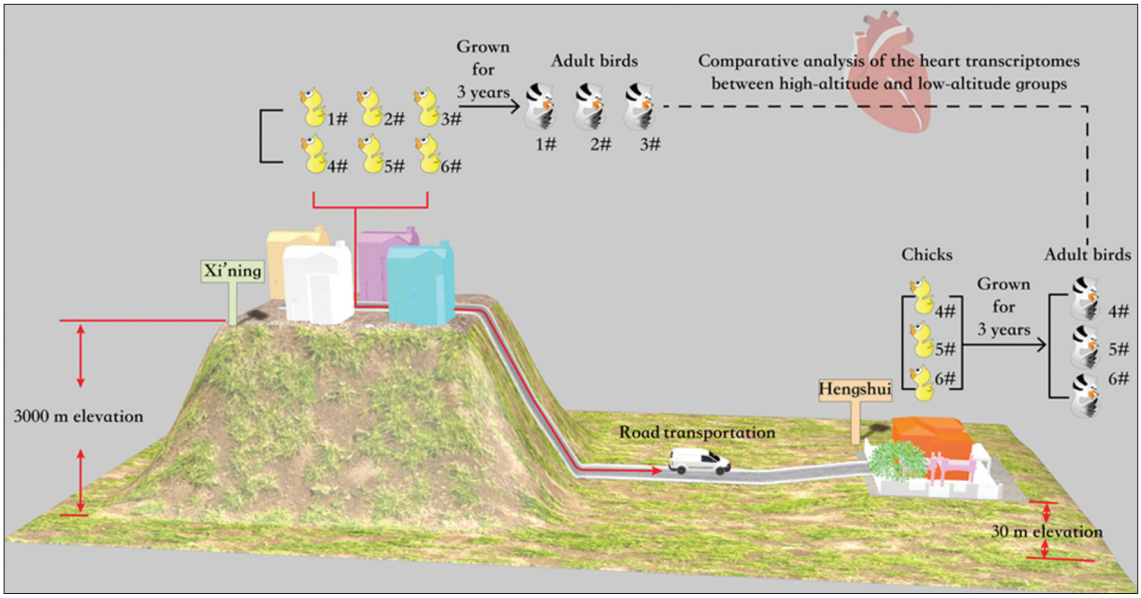

Fig 1. The schematic diagram of our experiment

bioanalyser (Agilent Technologies, USA), respectively. The RNA integrity number threshold was set at 7.0 for the construction of library. mRNA was further purified using poly-T oligo-attached magnetic beads. Finally, six libraries with 350 bp insert sizes were constructed using the NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA). The constructed libraries were then sequenced by the Illumina NovaSeq 6000 platform at Novogene Bioinformatics Technology Co. Ltd (Beijing, China). The whole process followed a standardized procedure and was monitored by Novogene's Quality Control System.

Data Accessibility

The raw sequencing data has been deposited in the National Center for Biotechnology Information (NCBI) sequence read archive (SRA) with the bioproject number PRJNA612653.

Bioinformatics Analyses

- Quality Control

To produce the high-quality clean reads, raw reads were assessed for quality using FastQC v.0.11.9 *(https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)* and filtered for low-quality reads according to the following rules: 1) removing the reads with adapter contamination; 2) removing the reads with unknown nucleobases; 3) removing the low-quality reads (reads with a Q_{PHRED} <= 20 base number accounting for more than 50% of the total read length). At the same time, Q20, Q30 and GC content of the clean reads were counted. The clean reads with high quality were used for further analysis.

- Genome Mapping

The bar-headed goose reference genome (DDBJ/ENA/GenBank under the accession VDDG01000000) [21] was used as the reference genome for our transcriptome sequencing data. Genome mapping was conducted using HISAT2 v2.0.5 software [23].

- Quantification of Gene Expression Level

FeatureCounts v1.5.0-p3 [24] was used to calculate the number of mapped reads to each gene, which were then normalized for the gene length and library size. FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) of each gene was counted based on the length of the gene and reads count mapped to this gene.

- Differential Expression Analysis

Genes with very low expression levels were filtered out of the dataset. Then, differentially expressed genes were analyzed using DESeq2 R package (1.16.1) [25] between HA and LA groups. The DEGs were identified using the thresholds of |log2 fold change| ≥1.0 and Padj <0.05. Clustering analysis of the identified DEGs was implemented by the heatmaps in R software.

- GO Function and KEGG Pathway Enrichment Analysis

GO functional enrichment and KEGG pathway analysis of DEGs was conducted by the clusterProfiler R package [26], in which gene length bias was corrected. GO terms or KEGG pathways with P<0.05 were significantly enriched.

- Real-time Quantitative PCR (RT-qPCR) Verification

To verify the repeatability of DEGs derived from transcriptome data, 16 DEGs were selected randomly for RT-qPCR validation. The primer sequences were shown in *Table 1*. RNAiso Pure RNA Isolation Kit (TaKaRa, Dalian, China) was used to extract RNA. A PrimeScript RT reagent

Table 1. *Primers used in this study for real-time quantitative PCR (RT-qPCR)*

Gene	Regulation	Primer Sequences (5′-3′)	Amplicon Size (bp)
DUSP1	Up	Forward: CGATGGAGGAAGGGTGTTTG	152
		Reverse: TGAAGTTTGGGGAGATGATGC	
NR4A1	Up	Forward: CCCTCCAAGCCCAAGCA	238
		Reverse: CGGCGAAGCCCTGAATC	
FOS	Up	Forward: TTCTATGCGTCGGACTGGG	113
		Reverse: AAGGTGGAGGTGTAGGTGCTG	
JUN	Up	Forward: GGAAAAGGAAGTTGGAAAGGAT	155
		Reverse: CTGGCACCCGCTGTTGA	
HSP70	Up	Forward: AGCGTAACACCACCATTCCC	94
		Reverse: TCACCCTCATACACCTGGACC	
FABP	Up	Forward: CCAAGCCCAATGTAACTATCAGC	237
		Reverse: CGACCAGGTTCCCATCCAC	
APOA1	Up	Forward: GACCCTCGCCCTGCTCTT	209
		Reverse: GCGTGTCCAGGTTGTCGG	
SCD	Up	Forward: TCAACCCACGGGAGAACC	213
		Reverse: TCCGCATTTTCCGAGCC	
ACACB	Down	Forward: TGAGGTGGGGATGGTTGC	96
		Reverse: TTGTGCGTGATGTCGTTGC	
TNC	Down	Forward: CCAAGGGGCACCAAACAA	103
		Reverse: CGGAAGCCGTCTGGAGTAGC	
COL4A1	Down	Forward: ACAAGGCAATGAAAGAGCACA	150
		Reverse: GGCGTTGACAGCCAGTAGG	
MYLK	Down	Forward: CGCCTACCAGCCCGATAA	177
		Reverse: CAGGGTCAGGATAGCCTTCAA	
FLNC	Down	Forward: CATCAAGAACGACAACGACACC	108
		Reverse: AACGGGCTGCTGGGGAT	
GK	Down	Forward: ATGTAACCAATGCCAGTAGAACG	185
		Reverse: GTCACCCAAGCACCCAGAA	
GPCPD1	Down	Forward: TGGATGCTGGGTGGCTGAC	193
		Reverse: TTCTGAGGAACTGTAGGGGATGT	
SLC9A1	Down	Forward: GCAGTCGCCAGAGTCCGT	110
		Reverse: GCTCCTTCACCCGCATCA	
TUBULIN	-	Forward: ATCAGGTGGTCCCCAAGAGC	143
		Reverse: GGTGATGAGATGGCGGAGG	

DUSP1: *Dual Specificity Phosphatase 1;* ***NR4A1:*** *Nuclear Receptor Subfamily 4 Group A Member 1;* ***FOS:*** *Fos Proto-Oncogene, AP-1 Transcription Factor Subunit;* ***JUN:*** *Jun Proto-Oncogene, AP-1 Transcription Factor Subunit;* ***HSP70:*** *Heat Shock Protein 70;* ***FABP:*** *Fatty Acid Binding Protein;* ***APOA1:*** *Apolipoprotein A1;* ***SCD:*** *Stearoyl-CoA Desaturase;* ***ACACB:*** *Acetyl-CoA Carboxylase Beta;* ***TNC:*** *Tenascin C;* ***COL4A1:*** *Collagen Type IV Alpha 1 Chain;* ***MYLK:*** *Myosin Light Chain Kinase;* ***FLNC:*** *Filamin C;* ***GK:*** *Glycerol Kinase;* ***GPCPD1:*** *Glycerophosphocholine Phosphodiesterase 1;* ***SLC9A1:*** *Solute Carrier Family 9 Member A1*

kit (TaKaRa, Dalian, China) was used to synthesize the single stranded cDNA. qPCR was conducted on Gene9600 RT-PCR detection system (Hangzhou Bioer Technology Co. Ltd., Hangzhou, China) and TB Green Fast qPCR Mix (TaKaRa, Dalian, China). The 6 samples were run in triplicate for the 16 genes. *TUBULIN* was used as an internal control in this study. The method of $2^{-\Delta\Delta CT}$ [27] was used to count the relative expression of the 16 genes.

RESULTS

Overview of Transcriptome Sequencing Data

Six sequencing libraries from the two distinct groups (high- and low-altitude) were constructed and sequenced using the Illumina HiSeq platform. The overall sequencing results were shown in *Table 2*. A total of 391,970,972 raw

Table 2. *Summary of read statistics from the transcriptome sequencing*

Samples	Raw Reads	Clean Reads	Clean Bases (Gb)	Error Rate (%)	Q20 (%)	Q30 (%)	GC (%)
HA_h1	59,914,010	58,248,934	8.74	0.03	96.34	90.93	50.84
HA_h2	64,648,766	62,867,252	9.43	0.03	96.62	91.39	50.56
HA_h3	67,105,524	65,327,764	9.80	0.03	96.05	90.13	50.42
LA_h1	68,834,484	67,275,492	10.09	0.03	96.76	91.65	50.20
LA_h2	66,169,684	64,443,694	9.67	0.03	96.29	90.65	50.56
LA_h3	65,298,504	63,595,928	9.54	0.03	96.49	91.15	50.46

Table 3. *Reads coverage mapping to the reference genome of bar-headed goose*

Samples	Total Reads	Total Map	Unique Map	Multiple Map	Exon	Intron	Intergenic
HA_h1	58,248,934	36,857,118(63.28%)	36,275,462(62.28%)	581,656(1.0%)	3,699,777,153(67.2575%)	571,953,754(10.3974%)	1,229,188,619(22.3451%)
HA_h2	62,867,252	40,369,734(64.21%)	39,737,652(63.21%)	632,082(1.01%)	4,014,112,346(66.5661%)	614,605,303(10.192%)	1,401,548,436(23.2419%)
HA_h3	65,327,764	40,977,873(62.73%)	40,401,956(61.85%)	575,917(0.88%)	4,066,706,910(66.433%)	654,328,150(10.689%)	1,400,487,751(22.8781%)
LA_h1	67,275,492	41,840,766(62.19%)	41,197,140(61.24%)	643,626(0.96%)	4,245,606,572(67.9315%)	648,989,314(10.3841%)	1,355,233,098(21.6843%)
LA_h2	64,443,694	40,178,319(62.35%)	39,525,864(61.33%)	652,455(1.01%)	4,097,059,115(68.2738%)	607,198,221(10.1184%)	1,296,665,508(21.6078%)
LA_h3	63,595,928	39,515,638(62.14%)	38,940,133(61.23%)	575,505(0.9%)	3,805,155,967(64.4771%)	696,974,108(11.81%)	1,399,431,085(23.7129%)

reads were generated and were deposited in the NCBI SRA database (accession number SRP252835). All error rates were less than 0.04%, and the quality of the base values were Q20 ≥ 96% and Q30 ≥ 90% in the six samples, suggesting that the transcriptome data were technically qualified. After filtering the adapter sequences, the ambiguous N nucleotides, and the low-quality reads, we got a total of 381,759,064 clean reads. These clean reads were then used for subsequent analysis. The percentage of clean reads among raw reads ranged from 97.22% to 97.74% in each sample. The guanine + cytosine (GC) values ranged from 50.20% to 50.84%.

The mapping results showed that more than 62% of the clean reads (total mapped reads) matched to the bar-headed goose reference genome *(Table 3)*. Among the mapped reads, more than 61% of the clean reads were matched to a unique genomic location, and less than 1% of the clean reads were matched to multiple genomic locations *(Table 3)*. For the high-altitude group and low-altitude group, 66.75% and 66.89% were mapping to the exon region, 10.43% and 10.77% belonged to the intron region, and 22.82% and 22.34% were in the intergenic sequence respectively *(Table 3)*. These results suggested that the transcriptome data were good enough for further analyses.

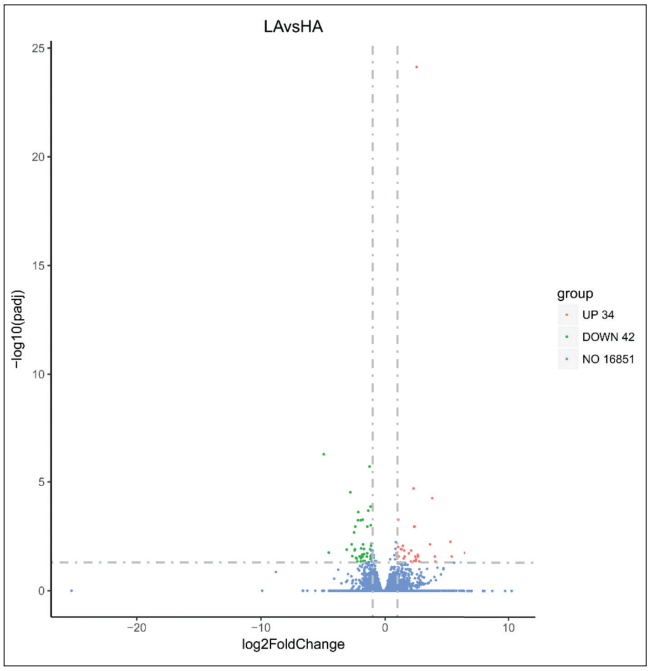

Fig 2. Differentially expressed genes (DEGs) in low-altitude hearts (LA_h) versus high-altitude hearts (HA_h). Red indicates up regulated genes, green indicates down regulated genes, and blue indicates genes with unchanged expression

DEGs Analysis and RT-qPCR Validation

DEGs between the low- and high-altitude bar-headed geese hearts were analyzed by the DESeq2 package, with the criteria of |log2 fold change| ≥ 1.0 and Padj < 0.05. A total of 76 DEGs were identified, of which 34 showed up-regulation, and 42 showed down-regulation in the low-altitude group compared with the high-altitude group *(Fig. 2)*.

A heat map of the DEGs between the low- and high-altitude groups was shown with hierarchical clustering of the samples and genes in *Fig. 3*. Hierarchical clustering result clearly separated the high- and low-altitude groups, while samples within each group showed strong correlations *(Fig. 4)*.

In order to verify the reliability of the candidate DEGs, we selected and quantified the expression levels for 16 DEGs (8 up-regulated DEGs and 8 down-regulated DEGs)

by using the RT-qPCR technique *(Fig. 5)*. We successfully confirmed the differential expression for all the selected DEGs *(Fig. 5)*. The results revealed the tendency of gene expression was in line with the transcriptome results, suggesting the transcriptome sequencing results were reliable for following functional analysis.

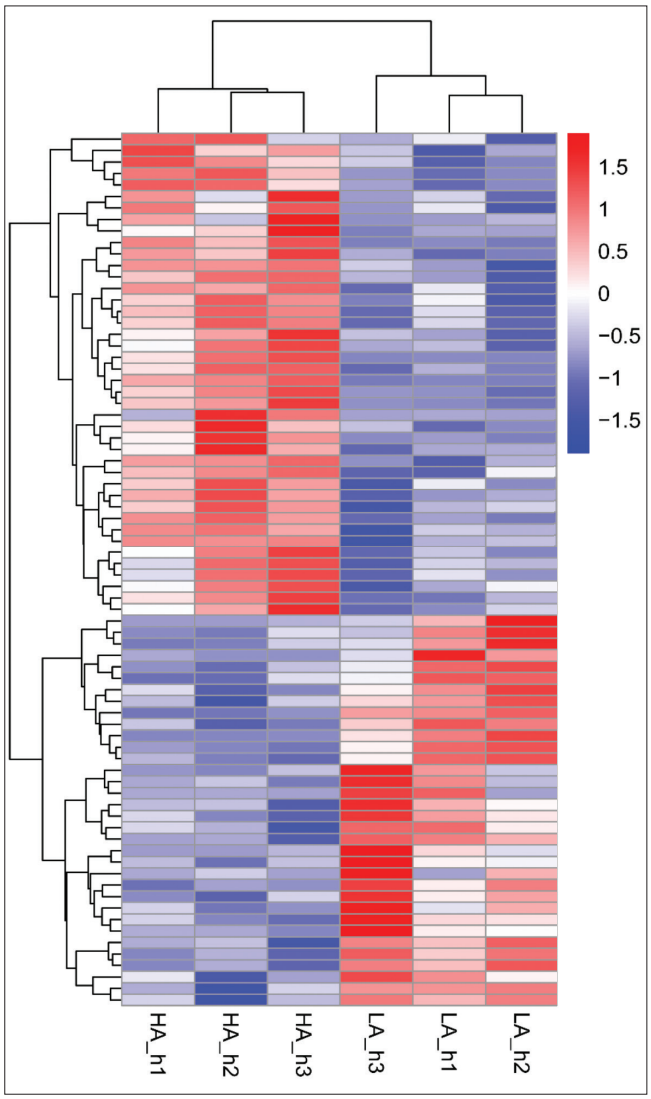

Fig 3. Heat map of the 76 differentially expressed genes (DEGs) in the two groups. Colors represent the normalized gene expression values of DEGs

Functional Analysis for DEGs

To further explore the possible biological functions and metabolic pathways of the identified DEGs, GO and KEGG enrichment analyses were performed.

The DEGs in the comparison group (LA_h vs. HA_h) were first annotated by the GO function database. The DEGs were significantly enriched in 1 terms of BP (biological process), 1 terms of CC (cellular component), and 11 terms of MF (molecular function) *(Fig. 6)*. Under the molecular function category, the genes mainly enriched in the functions involved in "extracellular matrix structural constituent", "GTPase activity", "hydrolase activity", and "nucleotide/ nucleoside binding" *(Fig. 6)*.

We then performed a KEGG analysis of the DEGs. In our study, 76 DEGs were mapped to 32 KEGG pathways. A total

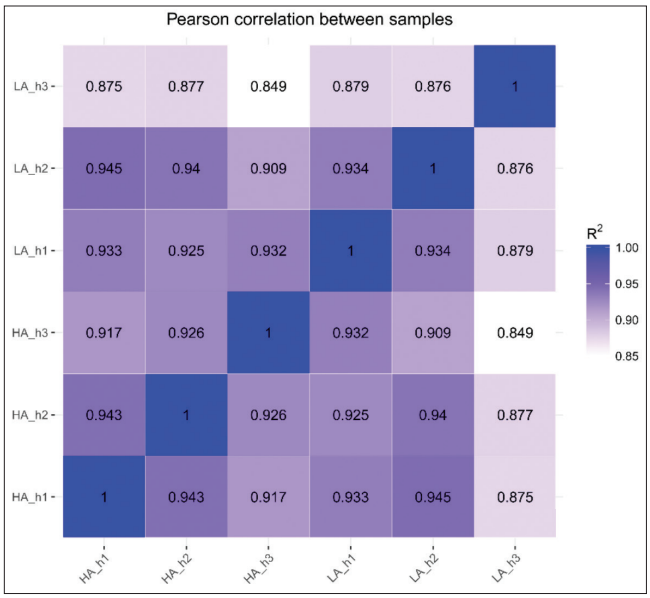

Fig 4. Hierarchical clustering (Pearson correlation) of transcriptome sequencing results of low-altitude hearts (LA_h, n=3) and high-altitude hearts (HA_h, n=3)

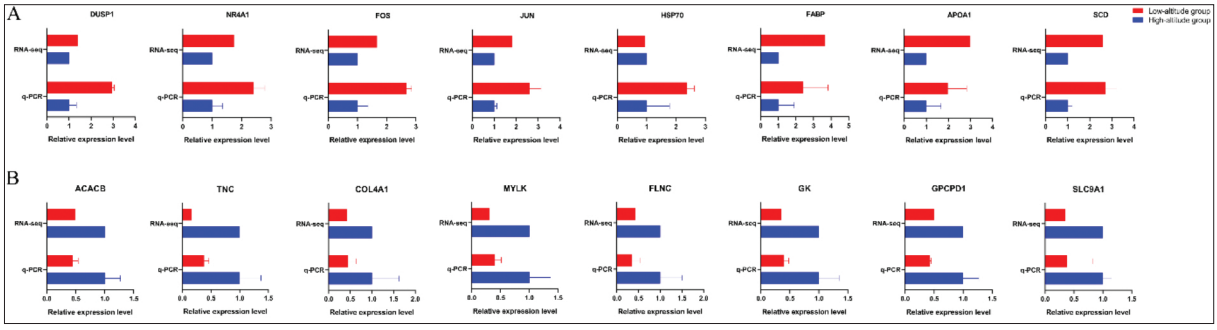

Fig 5. Validation of RNA-seq results by Real-time quantitative PCR (RT-qPCR). **(A)** validation of up-regulated differentially expressed genes (DEGs) by RT-qPCR, **(B)** validation of down-regulated differentially expressed genes (DEGs) by RT-qPCR. The RT-qPCR results are the means ± standard deviations (± SDs) of three replicates

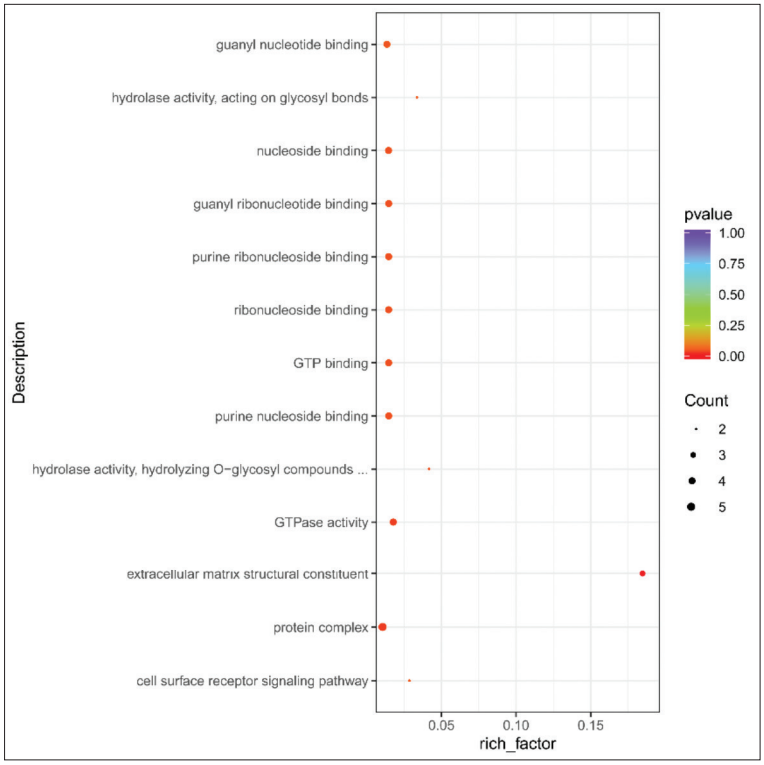

Fig 6. Gene Ontology enrichment scatter plot for differentially expressed genes between low-altitude hearts (LA_h) and high-altitude hearts (HA_h)

Table 4. *Differentially expressed genes for the six significant KEGG signal pathways*

KEGG Signal Pathway	Up_regulated Genes	Down_regulated Genes
ECM-receptor interaction	-	TNC, COL4A1, COL4A2, THBS1, COL4A5
Focal adhesion	-	TNC, COL4A1, MYLK, COL4A2, THBS1, COL4A5
AGE-RAGE signaling pathway in diabetic complications	EGR1	COL4A1, COL4A2, COL4A5
Glycosaminoglycan degradation	-	HYAL2
mTOR signaling pathway	FZD8, FZD2	ULK2, ATP6V1C2
Wnt signaling pathway	FZD8, FZD2, AXIN2	-

of 6 statistically significant pathways were identified when comparing LA_h group with HA_h group, including three in environmental information processing, one in cellular processes, one in human diseases, and one in metabolism categories, respectively *(Table 4)*. Within the upregulated DEGs, the most enriched pathway term was "Wnt signaling pathway" *(Table 4)*. For the downregulated DEGs, the dominant pathways were as follows: "focal adhesion", "ECM - receptor interaction","Advanced glycation end-product (AGE) - role of receptor for advanced glycation end-product (RAGE) signaling pathway in diabetic complications", and "glycosaminoglycan degradation" *(Table 4)*.

Discussion

The bar-headed goose provides an extraordinary opportunity to investigate the mechanism of high-altitude adaptation for their migratory flight at extreme altitude, and attracted many experts from various fields [5,9,10,12]. However, the adaptive mechanism of bar-headed geese migrating to low altitude environment has not been reported. We newly sequenced a bar-headed goose genome to provide a useful resource for detection of genomic adaptive changes at the DNA sequence level associated with this species [21]. Transcriptome intermediate between DNA sequences and physiological traits can extend the genomic information by concentrate on gene expression and molecular pathways involved in different altitude adaptation. Here, for the first time, we performed transcriptome analysis in bar-headed geese reared in both high-altitude and low-altitude environment for one hypoxia-sensitive tissue, heart. In this study, bar-headed geese were transported to low-altitude areas for artificial rearing, and then comparative transcriptome analysis was conducted with high-altitude bar-headed geese. This kind of comparison, rather than comparing high-altitude bar-headed geese with the relative geese species living at low-altitude, will better explore the adaptability of bar-headed geese to low-altitude environment.

In this study, each sample obtained more than 8.74 GB of high-quality clean sequencing data, and the base quality

value (error rate, Q20, and Q30) indicating good quality and high reliability. Eight up-regulated DEGs and 8 down-regulated DEGs were chosen for RT-qPCR test to confirm the expression levels calculated by FPKM values. The similar changing trends were found between RT-qPCR validation and transcriptome sequencing. Overall, these results indicate that the results of our transcriptome data are reliable.

In this work, GO annotation results showed that most DEGs were found to be related to cell surface receptor signaling pathway, extracellular matrix structural constituent, GTPase and hydrolase activity, and nucleotide / nucleoside binding. This suggests that when bar-headed geese migrate from high altitude to low altitude, the interactions between membrane receptors and extracellular ligands of the heart were changed. The results were similar to those of Qi et al. in the study of yak heart transcriptome [28]. The effects of these changes on the health status of low-altitude bar-headed geese need to be studied and determined with the help of more morphological, physiological, and pathological data in the future. The annotation analysis of metabolic pathways of DEGs is helpful for further interpretation of gene function. KEGG database is the main public database on metabolic pathways, and six KEGG pathways were significantly enriched in this study. "Wnt signaling pathway" *(FZD8, FZD2, AXIN2)* was the only upregulated KEGG pathways in the hearts of low-altitude bar-headed geese. A large body of evidence shows that the Wnt signaling pathway plays an important role in various stages of heart development [29]. While this pathway is quiescent in adult hearts, and activation occurs upon pathological stress [30]. In the future, it is necessary to further study the correlation between the activation of this signaling pathway and heart disease of bar-headed geese reared at low altitude. "focal adhesion" and "ECM-receptor interaction" were found to be downregulated in the hearts of low-altitude bar-headed geese. Collagens that encode cell-ECM (including downregulated *COL4A1*, *COL4A2*, and *COL4A5)* are the major structural components of the basement membrane and are important during cardiac morphogenesis by promoting cardiomyocyte proliferation [31]. We also identified three DEGs in focal adhesion pathways (including *TNC*, *MYLK*, and *THBS1)* with significantly downregulated in the hearts of low-altitude bar-headed geese. Those genes usually provide a force-transmitting physical link between the EMC and cytoskeleton, and are helpful for the species to adapt to high altitude [28]. But we hypothesized that the expression of these genes was suppressed in low-altitude environment where oxygen was abundant.

Glycosaminoglycans are a class of anionic linear periodic polysaccharides, which play a key role in tissue homeostasis not only by providing mechanical load resistance but also signaling mediators that play an important physiological function in cell proliferation, differentiation, metastasis, and recognition [32]. The glycosaminoglycan degradation pathway in the hearts of low-altitude bar-headed geese is involved in the regulation of the structure and function of glycosaminoglycans in the hearts. The down-regulated expression of *HYAL2* in this pathway was detected by transcriptome data. Hyaluronidase is an endogenous glycosidase that can degrade glycosidic bonds in hyaluronic acid polymers (one form of glycosaminoglycans). We hypothesized that high-altitude bar-headed geese need hyaluronidase and other hyaluronidase to maintain the permeability of extracellular matrix of heart, so as to facilitate oxygen exchange and transportation in anoxic environment. However, when high-altitude bar-head geese migrate to low-altitude environment for growth, oxygen supply is sufficient, and hyaluronic acid in the extracellular matrix of heart does not need to be greatly degraded, so the glycosaminoglycan degradation pathway is inhibited. All these differentially expressed signal pathways and metabolic pathways in the hearts of bar-head geese intercrossed with each other, and thus forming a huge and complex network. The specific regulatory mechanism and biological effects of these differentially expressed signaling pathways remain to be further studied. Our study has a limitation that also suggests direction for further research. We did not do biochemical, physiological, and histological tests, and how these observed differently expressed genes and pathways may translate into functional and phenotypic changes remains unclear. However, the transcriptome data obtained in this study provides us with a useful candidate pathway and gene resource for future functional studies of altitude adaptation.

In conclusion, this study lays the first foundations for comparative analysis of transcriptional and signaling pathway changes in bar-headed geese heart tissue under low-altitude environment. A total of 76 DEGs were identified, of which 34 showed up-regulation and 42 showed down-regulation in the low-altitude bar-headed geese compared with the high-altitude bar-headed geese. Nine KEGG pathways and 13 GO terms were significantly enriched in low-altitude bar-headed geese. Further studies are required to confirm the functions of these different pathways and genes reported here between low-altitude bar-headed geese and high-altitude bar-headed geese, and the extent to which these differences may contribute to the low-altitude adaptation.

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Declaration of Conflicting Interests

The authors declare that they have no conflicts of interest.

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Author Contributions

WW conceived and designed the study. WW, and KS critically revised the manuscript. YL, FW, XLG, and LLZ performed the experiments, analyzed the data. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Cytotoxic and Apoptotic Effects of Curcumin on D-17 Canine Osteosarcoma Cell Line [1]

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Abstract

Cancer is a major health problem in dogs. Types of cancer seen in dogs include melanoma, Non-Hodgkin lymphoma, osteosarcoma, soft tissue sarcomas and prostate, breast, lung and colorectal carcinomas. Osteosarcoma (OSA) is the most common malignant primary bone tumor in domestic dogs. It constitutes 85% of skeletal tumors. It is derived from primitive bone cells that occur in both the appendicular (~75%) and axial (~25%) skeleton. The present study was intended to determine the cytotoxic and apoptotic effects of curcumin administration at certain doses and in certain periods on D-17 canine osteosarcoma cells. Canine osteosarcoma cells were treated with curcumin and the effects of it on proliferation were determined by WST-1, apoptosis by caspase 3/7 activity (MuseCaspase 3/7) and the ratio of proapoptotic Bax gene to antiapoptotic Bcl-2 gene expression level by qRT-PCR. Our data demonstrated that curcumin decreased cell proliferation and viability, ultimately inducing caspase 3/7 mediated apoptosis in treated D-17 canine osteosarcoma cells. Furthermore, the application of curcumin on canine osteosarcoma cells downregulated the expression of Bcl-2 and upregulated the expression of proapoptotic gene Bax. Thus, these results may provide a basis for further study of curcumin in the treatment of canine osteosarcoma.

Keywords: *Apoptozis, bax/bcl-2, Canine osteosarcoma, Caspase 3/7, Curcumin*

Kurkuminin D-17 Köpek Osteosarkom Hücre Hattı Üzerindeki Sitotoksik ve Apoptotik Etkileri

Öz

Kanser, köpeklerde büyük bir sağlık sorunudur. Köpeklerde görülen kanser türleri arasında melanom, Non-Hodgkin lenfoma, osteosarkom, yumuşak doku sarkomları ve prostat, meme, akciğer ve kolorektal karsinomlar bulunur. Osteosarkom (OSA) evcil köpeklerde en sık görülen kötü huylu birincil kemik tümörüdür. İskelet tümörlerinin %85'ini oluşturur. Hem apendiküler (~%75) hem de aksiyal (~%25) iskelette meydana gelen ilkel kemik hücrelerinden türemektedir. Bu çalışmanın amacı, belirli dozlarda ve belirli periyotlarda kurkumin uygulamasının ardından D-17 köpek osteosarkom hücreleri üzerindeki sitotoksik ve apoptotik etkilerini saptamaktır. Kurkumin uygulanan köpek osteosarkom hücrelerinde proliferasyon üzerindeki etkileri WST-1 analiziyle, apoptotik etkileri ise kaspaz 3/7 aktivitesi (MuseCaspase 3/7) ve proapoptotik Bax geninin antiapoptotik Bcl-2 gen ekspresyon düzeyine oranı ile belirlendi. Verilerimiz kurkuminin D-17 köpek osteosarkom hücrelerinde hücre proliferasyonunu ve canlılığını azalttığını, kazpaz 3/7 aracılığıyla apoptozu indüklediğini göstermektedir. Bunun yanında kurkuminin köpek osteosarkom hücrelerine uygulanması Bcl-2'nin ekspresyonunu azalttı ve proapoptotik gen Bax ekspresyonunu arttırdı. Bu nedenle, bu sonuçlar, köpek osteosarkoma tedavisinde kurkumin ile ilgili daha fazla çalışma için bir temel sağlayabilir.

Anahtar sözcükler: *Apoptoz, bax/bcl-2, Köpek osteosarkom, Kaspaz 3/7, Kurkumin*

INTRODUCTION

Osteosarcoma (OSA) is the most common type of malignant bone cancer found in dogs, and is about 10-50 times more common in dogs than in humans [1]. OSA, which constitutes approximately 85% of primary canine bone tumors, arises from mesenchymal cells and is mostly seen in long bones such as knee, hip and shoulder [2,3]. The tumor grows rapidly inside the bone and becomes more painful as it grows outward [4]. In humans, it is predominantly seen

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in adolescents and young adults and the most common incidence is between the ages of 10-15. Unlike human OSA, canine OSA is more common in older dogs [5] and it accounts for 80-85% of bone tumors in dogs 2 to 15 years old [6]. It is locally invasive and highly metastatic and this case makes it difficult to treat [4]. While it usually metastasizes to the lungs, it also spreads to other bones. In dogs, tumors spread hematogenously into the lungs earlier than in humans and they begin to develop micro-metastases [7]. While lower than 45% of the affected dogs can survive only in the first year despite appropriate surgical and chemotherapeutic protocols [8], less than 20% of them can survive more than 2 years after diagnosis [9].

Treatment of OSA is very difficult both in humans and in dogs. For this reason, despite the advanced cancer treatment methods, the annual survival rate in dogs is around 45%, while this rate in people with OSA has not changed lately [10]. The low survival rate in dogs with OSA highlights the need for new therapeutic approaches [11,12]. Today, osteosarcoma is usually treated with the preoperative chemotherapy, with the amputation or tumor resection for limb salvage and with the postoperative chemotherapy [3]. However, the use of chemotherapeutic agents is limited due to severe toxicity. Therefore, the ultimate goal of this study is to demonstrate the need for new studies to discover effective chemotherapeutics with appropriate and minimal toxicity.

Apoptosis is a physiological process responsible for the elimination of cells that have completed certain functions or that damage the growth and development of the organism. Cell apoptosis plays a central role in the control of cell proliferation and therefore has a significant role in preventing tumor growth [13]. Two characteristic apoptotic pathways, called as extrinsic and intrinsic pathways, have been identified [14]. The intrinsic pathway is activated as a result of death signals that may occur due to DNA damage, growth factor deficiency and oxidative stress, and these signals are carried to the mitochondria by two proapoptotic members of the Bcl-2 family (Bax, Bad), creating a large pore formation on the surface of the mitochondria [15,16]. Thus, there is a large increase in mitochondrial outer membrane permeability and cytochrome-c release is performed by breaking the mitochondrial outer membrane. The Bcl-2 family contains some pro-apoptotic (eg: BAK and BAX) and some anti-apoptotic proteins (eg: BCL-2, Bcl-xl) [17,18]. Among these, Bcl-2 and Bax are key proteins for apoptosis or survival [19].

Curcumin, [(1E, 6E) -1, 7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione or diferuloylmethane] is a yellow -orange phenolic compound obtained from the ribosomes of the turmeric plant *(Curcuma longa* L.) [20]. This compound has anti-inflammatory, antioxidant, and chemopreventive properties, and chemotherapeutic potential with no obvious side effects [21,22]. It exhibits beneficial effects on numerous diseases such as diabetes, allergies, asthma, hepatic diseases, arthritis, Alzheimer's disease, cardio-vascular diseases and cancer [23-25]. Curcumin has been shown to modulate multiple cell signaling pathways such as apoptosis, proliferation, angiogenesis, and inflammation [19].

Preclinical studies have proven that curcumin inhibits cell proliferation in various cancer cell lines, including breast, cervical and pancreatic cancers [19]. In addition, recent studies have shown that curcumin, alone or in combination with other anticancer agents, can effectively induce apoptosis [26]. Curcumin has been proven to display antitumoral effects on a wide variety of human cancer cell lines and to induce apoptotic cell death [27-29].

Chemotherapeutic agents used in veterinary oncology are generally applied by making use of the information obtained in human medicine. However, this practice is controversial due to the interspecies differences in pharmacokinetic parameters and the sensitivity of tumor cells to cancer therapeutic compounds [30]. Consequently, for the treatment of canine osteosarcoma, it is of great importance to develop and/or discover low cost, less toxic and highly efficient therapeutic agents that increase survival rates. Although the antiproliferative effect of curcumin has been shown in many cell lines, our study will contribute to the literature since it is a specific dose study that can be used in veterinary oncology, since it was performed with a dog cell line.

In our study, we were going to explore that curcumin exhibits antineoplastic potency in a metastatic canine osteosarcoma cell line (D-17) *in vitro*. To assess this hypothesis we exposed canine OSA cell lines to curcumin and determined *in vitro* measures of proliferation. Furthermore Bax/Bcl-2 expression ratio was determined by qRT-PCR as an indicator of apoptosis, and apoptosis was confirmed by determination of caspase 3/7 activity.

Material and Methods

Cell Culture and Treatment

The canine osteosarcoma cell line D-17 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Osteosarcoma cells were cultured in Minimum Essential Medium Eagle medium (MEM; Sigma, M4655) supplemented with 10% heat in-activated fetal bovine serum (FBS, Sigma, F0804), 0.1% 10.000 U/mL penicillin/ streptomycin (Gibco), 1% nonessential amino acids, 0.11 g/L pyruvic acid (sodium salt) and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air. The medium was refreshed every 2-3 days. After about 90% of confluence, the cultured cells were detached with 0.25% trypsin-EDTA (Sigma T4049) and sub-cultured.

Curcumin (Sigma, C1386) was dissolved in culture medium with 1% DMSO, the solution was added to the culture medium to reach final concentrations of 2.5, 5, 10, 25,

40, 50, 75, 100 µM. Control group (concentration 0) were cultured without curcumin and medium containing 1% DMSO.

Cell Proliferation Assay

The effect of curcumin on D-17 canine OSA cell proliferation was initially determined by cell proliferation analysis using commercial cell proliferation kit 2-(4-iodofenil)-3-(4-nitrofenil)-5-(2,4-disülfofenil)-2H-tetrazolyum or WST-1 which is one of the proliferation tests based on metabolic activity. Cells ($1x10^4$ cells per 200 µL medium per well) were seeded in 96-well plates and allowed to attach for 24 h. There after, medium was changed and cells of the experimental groups were treatment with 2.5-5-10-25-40-50-75 or 100 µM curcumin whereas the control group was treated with DMSO (1%). After 24, 48, and 72 h incubation, 100 µL WST-1 solution was added to each well and the cell was incubated for 3 h at 37ºC in a humidified atmosphere of 5% CO_2 in air. After incubation and mixing gently for one minute on an orbital shaker, the absorbance of each well was measured using a microplate-reader (Multiskan™ FC Microplate Photometer, Thermo Fisher, Finland) in absorbance mode at a wavelength of 450 nm. The mean absorbance of the four-time repeated measurements per curcumin concentration was related to the mean absorbance of the control and expressed as the percentage of control. Dose–response curves and IC_{50} (The half maximal inhibitory concentration) were established with Graphpad Prism.

RNA Isolation and cDNA Synthesis

Total RNA isolation was performed according to the manufacturer's instructions with a Hybrid-RTM RNA Isolation kit (GeneAll R Biotechnology, South Korea) after 24 and 48 h incubation times from canine OSA D-17 cells treated with curcumin at specified concentrations (0, 10, 25, 40 and 50 µM). cDNA synthesis was achieved using 2 µg total RNA using the HyperScript First Strand Syntesis Kit (GeneAll Biotechnology, South Korea). Then, 1/20 of the resultant cDNA was used for each PCR reaction in a total volume of 20 µL.

Real-Time Quantitative Polymerase Chain Reaction (RT-PCR)

Gene expression was evaluated by Quantitative real-time PCR (qPCR). qRT-PCR was carried out using SYBR® Green PCR Master Mix (Applied Biosystems kit) and LigthCycler®480 (Roche Diagnostics GmbH, Mannheim, Germany). Specific primer sets designed and utilized for canine bax, bcl-2 and house keeping gene gapdh were listed in *Table 1*. The annealing temperature for this primer was 60°C. Fluorescence was determined by Step One Plus (Applied Biosystems) at each amplification cycle and analyzed by Step One Software 2.1 (AppliedBiosystems). C_t values representing the number of amplification cycles were obtained for all samples. The expression levels for all the genes analyzed were normalized to GAPDH. The protocol was performed in triplicate.

Table 1. *Primers for RT-PCR*

Gene	Primer Base Sequences (5´—>3´)	Amplicon Length (bp)
Bax	F: TTCCGAGTGGCAGCTGAGATGTTT R: GCTGGCAAAGTAGAAGAGGGCAA	79
Bcl-2	F:CATGCCAAGAGGGAAACACCAGAA R: GTGCTTTGCATTCTTGGATGAGGG	76
Gapdh	F: AGTCAAGGCTGAGAACGGGAAA R: TCCACAACATACTCAGCACCAGC	114

Caspase 3/7 Activity Assay

Canine OSA D-17 cells were seeded in 12-well plates at a density of $2.5x10^5$ cells/well and incubated overnight. Cells were treated with various curcumin concentrations (5, 10, 25, 40, 50 µM) for 24 and 48 h. Wells with media only were included as controls. All experiment were done in triplicates for each dose treatment. Apoptotic and necrotic cell ratios in the cell suspension obtained after incubation were determined by Muse Cell Analyzer (Merck Millipore) using the MuseCaspase 3/7 (MCH100108) kit according to the manufacturer's protocol.

Statistical Analysis

All experiments were done in triplicates for each dose treatment. All data were presented as mean values ± standard error (SE). A P value of <0.05 was considered as significant. A Shapiro-Wilk test was used for evaluation of the normality of distributions. The one-way analysis of variance (ANOVA) test (for Windows Release 22.0 Standart Version Copyright© SPSS Inc. Chicago, IL, USA) was used for evaluating the differences in the data. Statistical significance ($P<0.05$) was established by the post hoc Tukey's pairwise comparison.

The IC_{50} values were calculated by four-parameter non-linear regression using GraphPad Prism v.5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The data were performed replication in four-time for each dose in cell proliferation assay, and data are presented as mean ± standard error (SE).

Results

Curcumin Inhibits Population Growth in Canine OSA Cells

Effect of curcumin on canine OSA cell viability was evaluated by WST-1 method. Canine OSA D-17 cells exposed to curcumin resulted in a significant decrease in viable cells in a time and dose-dependent manner *(Fig. 1-A,B,C)*. A dose-effect curve was created using the GraphPad Prism 5 program with the dose-dependent viability results obtained by WST-1 viability analysis *(Fig. 2-A,B,C)*. IC_{50} values of canine OSA D-17 cells were 61.51, 47.80 and 39.41 µM

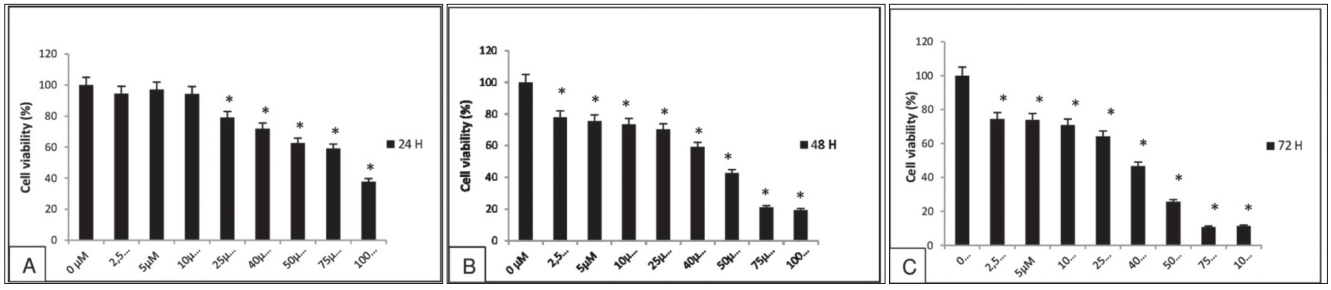


Fig 1. Effect of curcumin on cell viability of canine OSA D-17. Cells were treated with different concentrations of curcumin (0, 2.5, 5, 10, 25, 40, 50, 75, 100 µM) A- 24 h, B- 48 h, C- 72 h. The results represent the mean±standard deviation, * P<0.001 relative to control

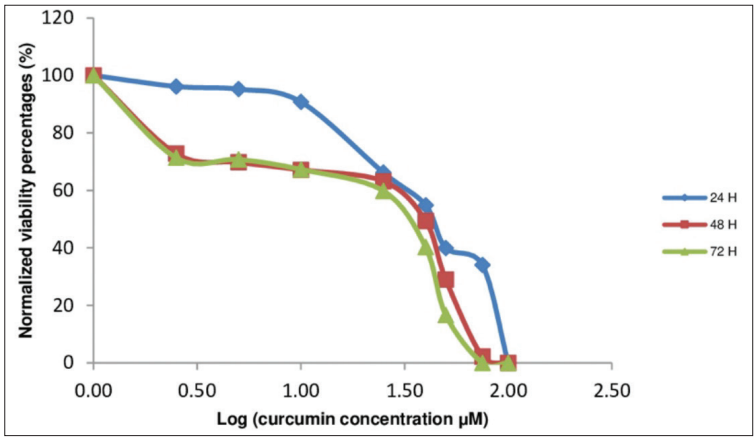
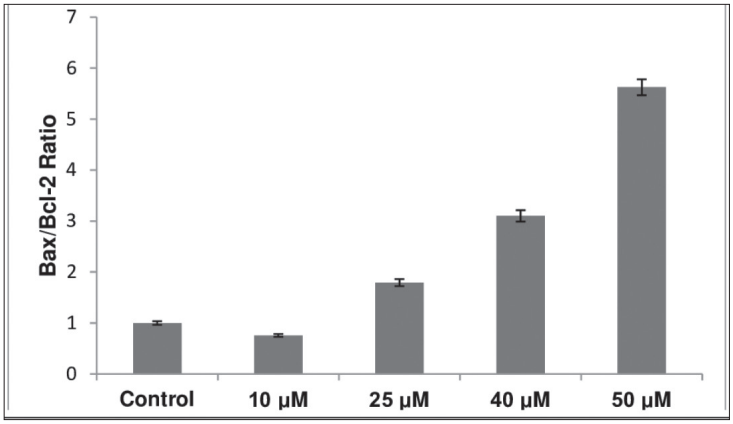


Fig 2. A dose-effect curve was created using the GraphPad Prism 5 program with the dose-dependent viability results obtained by WST-1 viability analysis of curcumin-administered D-17 canine osteosarcoma cell

Fig 3. Effect of curcumin on the Bax/Bcl 2 ratio in canine OSA D-17 cells. Cells were treated with different concentrations of curcumin (10, 25, 40 and 50 µM) for 48 h



at 24, 48 and 72 h, respectively. There was observed no significant effect on cell proliferation at 0-10 µM curcumin concentration during the 24 h incubation period (P>0.05). After 48 and 72 h incubation times, there was a decrease in cell viability in low doses (2.5, 5 and 10 µM) compared to the control group, but no statistically significant difference was found between the doses. Curcumin caused significant decreases in cell viability due to dose and time in increasing doses, and this decrease was found to be statistically significant (P<0.001).

Curcumin Increases the bax/Bcl2 Ratio in Canine OSA D-17 Cells

Expression of Bax (proapoptotic) and Bcl-2 (antiapoptotic) proteins were determined by RT-PCR method to investigate the apoptotic effect of curcumin on canine OSA D-17 cells and thus bax/bcl-2 ratios were evaluated. Canine OSA D-17 cells were treated with different concentrations of curcumin (0, 10, 25, 40, 50 µM) for 24 and 48 h. There was no significant change in Bax/Bcl-2 ratios compared to the control (0 µM) in 24-h incubation period (P>0.05), (Data not shown). End of 48 h incubation with curcumin, bax/bcl-2 ratio was found to be 1.79, 3.10 and 5.6 fold increase compared with the control cells for 25, 40 and 50 µM curcumin doses, respectively (P<0.05), *(Fig. 3)*.

Curcumin Toxicity is Associated with Caspase Activation in Canine OSA D-17 Cells

Cell viability in canine OSA D-17 cells treated with 5, 10 and 25 µM curcumin during 24 h was not significantly

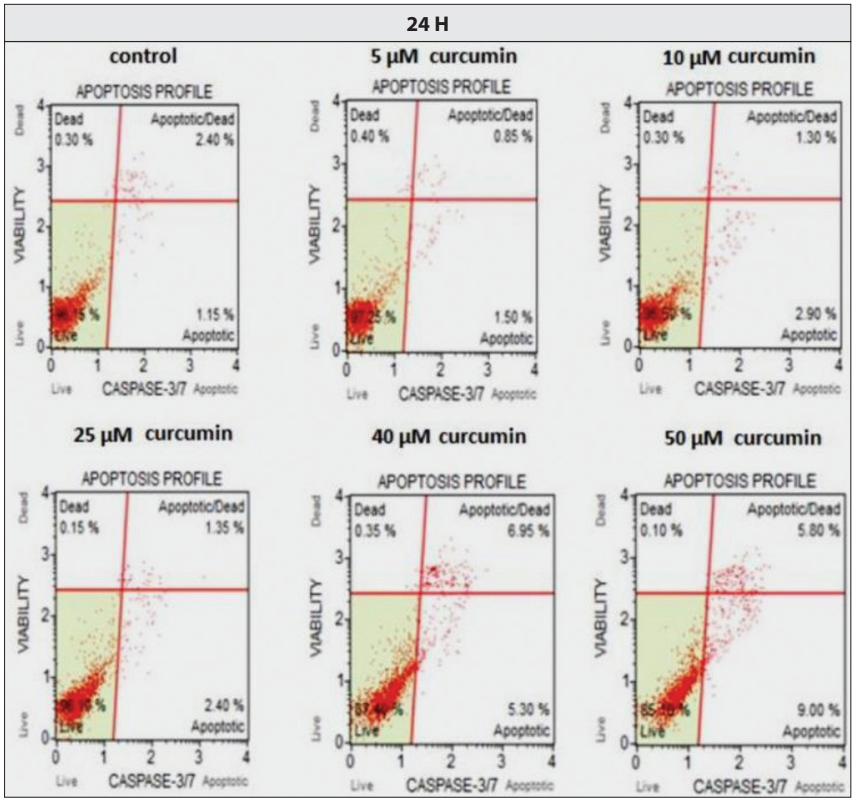


Fig 4. Caspase 3/7 activity after treatment with different concentrations of curcumin (0, 5, 10, 25, 40 and 50 µM) for 24 h in canine OSA D-17 cells-

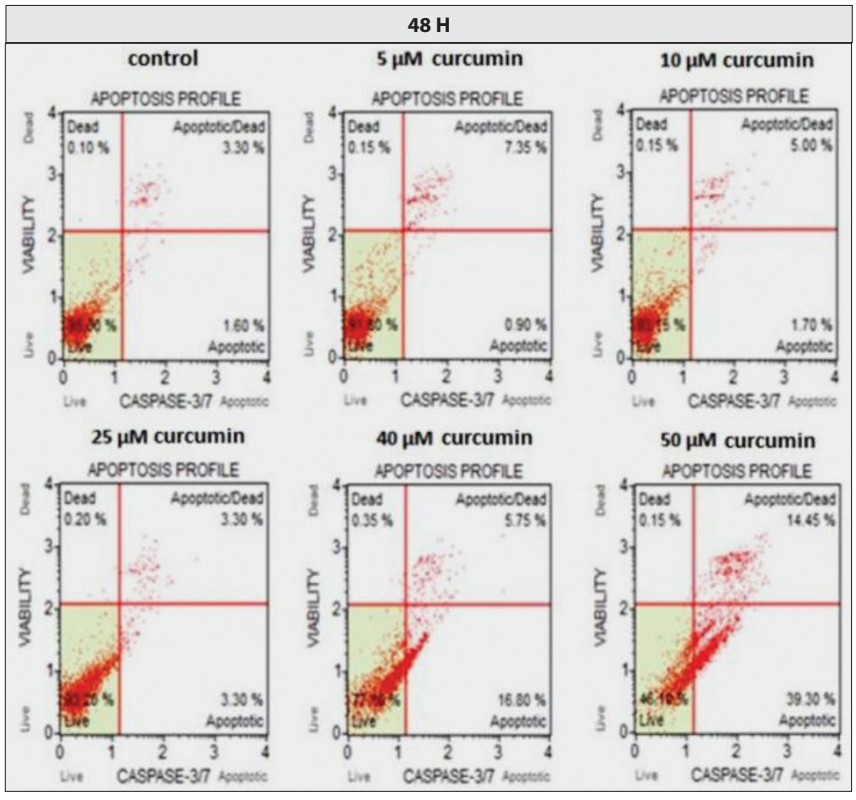


Fig 5. Caspase 3/7 activity after treatment with different concentrations of curcumin (0, 5, 10, 25, 40 and 50 µM) for 48 h in canine OSA D-17 cells

changed that due to caspase 3/7 activity compared to the control group (P>0.05). The percentage of early apoptotic cells induced by 40 µM and 50 µM curcumin increased to 5.73% and 9.51%, and the percentage of late apoptotic cells increased to 6.75% and 6.25%, respectively (P<0.05). The application of 5, 10 and 25 µM curcumin for 48 h did not affect viability significantly (P>0.05) however the percentage of early apoptotic cells 15.27% and 39.85% and late apoptotic cells 5.25% and 13.95% at 40 µM and 50 µM curcumin doses, respectively during 48 h (P<0.05). As a

Table 2. *Live/apoptotic/necrotic cell ratios in canine D-17 OSA cells treated for 24 h with 5, 10, 25, 40 and 50 µM of curcumin and in control group cells*

Group	% Live	% Apoptotic	% Dead
Control	95.45±1.26[a]	3.25±0.74[a]	0.68±0.19
5 µM	97.25±1.61[a]	2.58±0.87[a]	0.43±0.13
10 µM	96.27±1.43[a]	3.60±0.52[a]	0.25±0.048
25 µM	96.65±0.41[a]	2.77±0.81[a]	0.32±0.062
40 µM	87.25±0.89[b]	12.48±1.15[b]	0.36±0.14
50 µM	83.56±2.56[c]	15.76±2.03 [c]	0.10±0.011

[abc] *Statistical difference between groups with different letters in the same column is significant*

Table 3. *Live/apoptotic/necrotic cell ratios in canine D-17 OSA cells treated for 48 h with 5, 10, 25, 40 and 50 µM of curcumin and in control group cells*

Group	% Live	% Apoptotic	% Dead
Control	95.00±1.16[a]	4.95±1.24[a]	0.10±0.007
5 µM	94.65±1.23[a]	5.28±1.27[a]	0.13±0.013
10 µM	94.27±1.78[a]	5.60±1.50[a]	0.15±0.008
25 µM	93.65±0.91[a]	6.17±0.21[a]	0.12±0.043
40 µM	79.15±1.19[b]	20.62±1.15[b]	0.33±0.13
50 µM	47.16±1.56[c]	53.8±1.12 [c]	0.15±0.03

[abc] *Statistical difference between groups with different letters in the same column is significant*

result of the analysis based on the measurement principle of caspase 3/7 activity, it was observed that 40 and 50 µM curcumin application decreased viability and caused apoptosis in proportion according to total apoptotic ranges as 12.48% and 15.76% for 24 h and 20.62% and 53.8% for 48 h ($P<0.05$) *(Fig. 4, Fig. 5; Table 2, Table 3)*.

Discussion

Today, veterinarians' current options in the treatment of osteosarcoma are palliative therapy, limb-sparing anti-cancer therapy, preparation for surgery by reducing the size of the tumor to remove it, or prevent metastasis to other tissues. In addition, post-operative treatments prevent recurrence of the tumor [9,11,12,31]. The purpose of chemo-therapy and radiation treatments among these applied cancer therapies is to kill tumor cells because these cells are more sensitive to the effects of these drugs and methods, at least in adults, since they grow at a much faster rate than healthy cells [32]. However, losses occurring in healthy cells as well as tumor cells are a disadvantage of the treatments applied. For this reason, new treatment searches are required for healthy cells in the treatment of cancer that provide the induction of apoptosis by targeting molecules that have less toxicity but are located on apoptotic pathways in cancer cells [33]. It has been proven in many studies that phytochemicals affect gene expression of many different target molecules that modulate the signal transduction pathway, cell cycle, cell metabolism and apoptosis, and have antitumor effects. Therefore, more emphasis has been placed on phytochemicals and their antitumor effects in recent years [33,34].

Turmeric *(Curcuma longa)* is a plant that has medicinal value in both human and animal health [35]. Curcumin is a phytochemical compound in the class of flavonoids with various pharmacological properties and is obtained from the roots of the Curcuma longa plant that is used as a herbal diet and medicine [33]. Curcumin has a wide pharmacological action and low toxicity and is well tolerated by humans [3,36]. Curcumin is a highly pleiotropic molecule that modulates the activation of transcription factors, kinases, cytokines, various enzymes (eg, MMP, iNOS, GST, and ATPase), cell cycle (Cyclin D1 and cyclin E), growth factors (eg, EGF, NGF, HGF, and PDGF) and numerous targets involved in invasion, migration, angiogenesis, and apoptosis [37-39]. Curcumin can affect the skeletal system as many of the mentioned curcumin targets participate in the regulation of bone remodeling [20].

The effects of curcumin on osteoarcoma have been investigated in human osteosarcoma cell lines such as MG63, U2OS, MNNG/HOS, SAOS-2 and KHOS, and it has been determined that it has antiproliferative and apoptotic effects [3,20,40-43]. However, studies on canine osteosarcoma and specifically the D-17 cell line are limited. When our data is evaluated, it will be seen that curcumin inhibits the proliferation of canine OSA D-17 cells in a dose and time dependent manner.

It was found that D-17 canine OSA cells treated with curcumin had an IC_{50} value of 61.51 µM in the first 24 h and in short-term exposure to curcumin, cytotoxic activity increased at high doses. At 48 and 72 h, IC_{50} values were determined as 47.80 and 39.41 µM, respectively, and it was

concluded that the cells were more sensitive to long-term curcumin exposure. Our results are in line with the results of some studies based on the antiproliferative efficacy of curcumin with human and canine OSA cells. Lee et al.[41] reported that when they treated human osteosarcoma (HOS) cells with 0-20 µg/mL curcumin for 48 h, a dose-dependent reduction in cell proliferation occurred. Fossey et al.[44] investigated the anti-proliferative and apoptotic effects of FLLL32 compound obtained by modification of curcumin in order to improve the potential and biochemical properties of curcumin and after administration of FLLL32 particularly at concentrations above 0.75 µm (2.5 and 7.5 µm), there were significant reductions in the proliferation of both canine (OSA-8, 16 and D-17) and human (SJSA and U2OS) OSA cell lines. Canine cell lines have been found to be somewhat resistant, while human cell lines are more sensitive to curcumin treatment. Yu et al.[45] found that the proliferation of MG-63 cells, which were incubated with curcumin for 24 h, 36 h and 48 h in the range of 0-40 µM and at different concentrations, were inhibited significantly in a dose and time dependent manner. In another study comparing the effects of curcumin on the viability of MG-63 osteosarcoma cells and healthy human osteoblasts on different concentrations, when exposed to 5 and 10 µM curcumin, no statistical difference was observed in the density of healthy osteoblast cells, while the density of osteosarcoma cells decreased 0.6 and 5.3 times, respectively, compared to the control group [42]. The effect of Lipocurc™ formulation, designed to enhance the bioavailability of curcumin, on cell viability in canine OSA, melanoma and mammary carcinoma cell lines was investigated. A concentration-dependent decrease in cell viability was determined in all cell lines at concentrations above 2000 ng/mL, and the D-17 cell line was found to be significantly less sensitive to Lipocurc™ than both MG-63 and U2OS cell lines [46].

Induction of apoptosis, an actively regulated cellular process that causes cell death after specific stimuli are received in tumor cells, without cytotoxic effect in healthy cells in cancer therapy [47], is an approach on which many studies have been conducted in recent years [15,26]. Regulation of the level of some pro-apoptotic and anti-apoptotic proteins involved in the apoptotic process or activation of apoptotic enzymes causes apoptosis to occur in cells. This is achieved by the Bcl-2/Bax gene family and the activation of caspases [48,49]. While proapoptotic Bax protein interacts with membrane pore proteins to increase cytochrome c release, antiapoptotic Bcl-2 inhibits apoptotic signals by restricting proapoptotic Bax and thus the cytochrome c release [15]. Cells having a high Bax/Bcl-2 ratio are more sensitive to certain apoptotic stimuli [50]. Therefore, changes in Bax and Bcl-2 levels are important in determining whether cells will undergo apoptosis. The effects of apoptosis induction are more dependent on the ratio between these two proteins (Bax/Bcl-2) rather than the amount of Bcl-2 or Bax [51]. In our study, a dose-dependent increase in the Bax/Bcl-2 ratio was observed 24 h after treatment with curcumin in canine OSA D-17 cells. However, after 48 h of incubation, a significant increase in Bax/Bcl-2 ratio was detected, especially at 40 and 50 µM concentrations. Our results show that the strong anticancer activity of curcumin is associated with the concordant modulation of the expression rate (Bax/Bcl-2) of two major proteins that play a critical role for apoptosis induction by the intrinsic pathway. Curcumin has been reported to cause a decrease in cellular levels of Bcl-2 and an increase in cellular levels of Bax in various cancer cells [52,53]. Shankar et al.[52] and Anto et al.[54] determined that the regulation of Bcl-2 and Bax proteins by curcumin administration is a major factor for apoptosis caused by curcumin. Jin et al.[43] showed that curcumin can induce apoptosis in U2OS cells by increasing mitochondrial membrane permeability by downregulation of Bcl-2 and upregulation of Bax, Bak and p-Bad, and the treatment of U2OS cells with curcumin resulted in a significant inhibition of cell growth. Curcumin has been proven to induce apoptosis associated with the regulation of Bax/Bcl-2 protein expression in human hepatoma cells (SMMC7721) [33]. In addition, Jun et al.[3] found that there was a significant increase in Bax/Bcl-2 ratios depending on the dose after the human osteosarcoma cell line U2OS and MG-63 had been exposed to curcumin for 24 h.

Effector caspases (caspase 3, 7) also act on a range of different target proteins that play important roles in mediating the apoptotic response [55]. The results we obtained show that high doses of curcumin (40-50 µM) can induce apoptosis based on Caspase 3/7 activation. The highest apoptotic rates were determined for a dose of 50 µM curcumin in 48 h, indicating the long incubation time and high dose caspase-induced apoptotic pathway. Subramaniam et al.[56] found that the activation of effector caspase 3 and caspase 7 increased within 24 h in esophageal adenocarcinoma TE-7 cell line treated with curcumin. Another study showed that curcumin could activate caspase 3/7 in a dose-dependent manner (0, 10, 20, 30 µmol/L) within 24 h, thereby inducing apoptosis in human colon cancer HCT116 and SW480 cell lines [57]. Levine et al.[58] determined that caspase 3/7 activation was also induced in 3 different canine cell lines (C2 mastocytoma, CMT-12 mammary carcinoma and D-17 OSA) to which they applied turmeric root, which is the source of curcuminoids. They determined that there was a significant increase in 2 and 2.5 times in apoptotic cells, especially in C2 and CMT-12 cell lines. McNally et al.[59] determined that when doses of curcumin above 25 µm were administered to human liver cancer HUH7 cells, it reduced vitality depending on the increased concentration within 24 h. Furthermore, it was detected that caspase 3/7 activity increased at doses of 50 µM and above, and the decrease in survival was associated with caspase activation. In a study, it was observed that caspase-3, JNK and AMPK molecules were activated as a result of the application of curcumin at various

concentrations (20, 50 and 100 µM) to D-17 OSA cells, and the extrinsic apoptosis pathway mediated by caspase, JNK and cAMP/AMPK was induced [60]. The conducted studies reveal that curcumin exhibits an effective antitumoral potential by inducing the apoptotic pathways.

As a result, we demonstrated that canine OSA D-17 cells treated with curcumin decreased cell proliferation in a dose and time dependent manner, inducing apoptosis by increasing Bax/Bcl-2 protein expression and caspase 3/7 activation. Although various treatments are available, curcumin is predicted to be a candidate with a promising chemorherapeutic effect by inducing apoptosis for canine osteosarcoma with poor clinical results.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Author Contributions

FK funded acquisition and conceptualizationed the study. GSEA and İB performed laboratory analysis and drafted manuscript. GSEA also analyzed data with specific software, wrote the manuscript. AB and PAU investigationed that literature and methodology, revised the manuscript.

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RESEARCH ARTICLE

Preparation and Evaluation of Alum Precipitate and Oil Adjuvant Multivalent Vaccines Against *Clostridium perfringens* [1]

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Abstract

Enterotoxaemia is one of the hazardous diseases of the livestock. In Pakistan prophylaxis failure is due to the vaccination with type D monovalent vaccine. There is a need to develop a cost effective multivalent vaccine against enterotoxaemia using characterized toxinotypes isolated from field. Indigenously (Punjab, Pakistan) characterized *Clostridium perfringens* toxinotypes A (MW551947.1), B (MW332247.1) and D (MW332258.1) (n=1 each) were used. These toxinotypes were used to produce higher amount of alpha, beta and epsilon toxin units under culture conditions. Colony forming units (CFU) of each bacterium was determined through the standard plate count method and 10^6CFU/mL bacteria were used for vaccine dose. Monovalent, bivalent and multivalent oil adjuvant and alum precipitate vaccines were prepared. Formulated vaccines were passed the stability, sterility and safety test. Bacterin plus toxoid oil adjuvant vaccine produced higher (868.25±3.54 IU/mL) antibody titer at 28th day post vaccination in rabbits and 100% protection was observed after challenge. Multivalent bacterin plus toxoid oil adjuvant vaccine was used in field trials. Increased antibody response was detected after 4 months in sheep (1294.81±1.90 IU/mL) and goats (1091.85±2.51 IU/mL). During the experimental and field trials commercial vaccine did not produced higher antibody titer. Multivalent bacterin plus toxoid oil adjuvant vaccine proved as an excellent candidate for vaccination of animals against *C. perfringens* diseases, and it produced specific and efficient immune response to be used in field.

Keywords: *Alpha, Bacterin, Beta, Epsilon, Toxoid, Vaccine*

Clostridium perfringens'e Karşı Alum Presipite ve Yağ Adjuvanlı Multivalan Aşıların Hazırlanması ve Değerlendirilmesi

Öz

Enterotoksemi, çiftlik hayvanları için tehlikeli hastalıklardan birisidir. Pakistan'daki profilaksinin başarısızlığı tip D monovalan aşıdan kaynaklanmaktadır. Sahadan izole edilen ve karakterize edilmiş toksinotipler kullanılarak enterotoksemiye karşı uygun maliyetli bir multivalan aşı geliştirmeye ihtiyaç vardır. Yöreye özgü (Punjab, Pakistan) karakterize edilmiş *Clostridium perfringens* toksinotipleri, A (MW551947.1), B (MW332247.1) ve D (MW332258.1) (n=1 her biri için) kullanıldı. Bu toksinotipler, kültür ortamında yüksek miktarda alfa, beta ve epsilon toksinlerinin üretiminde kullanıldı. Her bakterinin koloni oluşturan birimleri (KOB) standart plak sayım yöntemiyle belirlendi ve aşı dozu olarak 10^6 CFU/mL kullanıldı. Yağ adjuvanlı ve alum presipite monovalan, bivalan ve multivalan aşılar hazırlandı. Formüle edilen aşılar stabilite, sterilite ve güvenlik testlerinden geçirildi. Bakterin + toksoid yağ adjuvanlı aşı, tavşanlarda aşılamadan sonraki 28. günde yüksek (868.25±3.54 IU/mL) antikor titresine yol açtı ve eprüvasyon sonrası %100 koruma gözlendi. Saha çalışmalarında multivalan bakterin + toksoid yağ adjuvanlı aşı kullanıldı. Aşılamadan 4 ay sonra koyun (1294.81±1.90 IU/mL) ve keçilerde (1091.85±2.51 IU/mL) antikor yanıtında artış saptandı. Deneysel ve saha çalışmaları sırasında ticari aşının daha yüksek antikor titresi üretmediği gözlendi. Hayvanların *C. perfringens* enfeksiyonlarına karşı aşılanmasında multivalan bakterin + toksoid yağ adjuvanlı aşının mükemmel bir aday olduğu kanıtlandı ve bu aşı sahada kullanılmak üzere spesifik ve etkili bir bağışıklık yanıtı üretti.

Anahtar sözcükler: *Alfa, Bakterin, Beta, Epsilon, Toksoid, Aşı*

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Introduction

Clostridium perfringens is a Gram positive, rod shaped, nonmotile, spore forming pathogenic anaerobic bacteria of human and domestic animals [1]. *C. perfringens* is divided into 5 types as A, B, C, D and E on the basis of major toxins (a, b, e, i) and minor toxins [1,2]. Gas gangrene, necrotic enteritis and enterotoxaemia are the most common symptoms of alpha toxin, which causes hemolysis, platelet aggregation, blood vessels contractions, superoxide generation, cytokine storm and ultimately cause death [3]. Lethal dose 50 (LD_{50}) of the alpha toxin per kilogram of mouse after intravenous injection was 3 mg [4]. Epsilon toxin of *C. perfringens* is the third most dangerous toxin among the clostridial toxins. Its 50% lethal dose (LD_{50}) was 50-110 ng per kg in mice [5]. The epsilon toxin was classified as the potential bio war and bioterrorism agent by the Center of Disease Control and Prevention (CDC) of theUnited States [6]. In sheep and goats, the epsilon toxin is a causative agent of enterotoxaemia [7]. Beta toxin is an etiological agent in necrotizing enterocolitis and also involves in enterotoxaemia [8]. It was reported that this toxin had a LD_{50} as 310 ng/kg [9].

Enteric infections caused by *C. perfringens* in sheep, goats and other ruminants are called enterotoxaemia because of the absorption of the toxins via intestine into the circulation. It is a frequent disease of sheep and goats and its prevalence rates are between 24.13 and 100% [10]. Enterotoxaemia is distributed worldwide and endemic in Pakistan as well [11]. Bacteria are normally present in intestinesin low concentration, however dietary changes (diet rich in carbohydrate) may lead to proliferation of the microorganism and ultimately toxin production [12].

Enterotoxaemia is one of the dangerous infectious diseases of the livestock. This disease has 2-8% incidence rates and 100% case fatality rate [13]. Most of the factors are responsible for the disease outbreak e.g. improper vaccination, change in diet and poor feed management [14]. Vaccination with accurate antigens and improvements on feed management are the only ways to struggle with the disease [15]. At present the disease is being handled by treatment of animals using antibacterial drugs but prognosis is poor. A monovalent clostridial vaccine is being practiced in the country for prophylaxis. This vaccine is prepared using type D which is not providing complete cover. There are genetic differences between field types and vaccine type of Clostridia, the major reason for prophylaxis failure in Pakistan. Dealing with the high economic impact of clostridiosis, prevention of it, is a big challenge for farmers [13].

The development and production of conventional clostridial vaccines involves expensive, time consuming and dangerous processes of detoxification, purification and antigen concentration steps [16]. Furthermore, the continued selection of the toxigenic strains that produce high titers of toxin is necessary [17]. There is a need to develop a cost effective vaccine using indigenously characterized toxinotypes isolates from field. The production of vaccine will help to combat enterotoxaemia in Pakistan. For vaccination of huge number of animals in Pakistan there is need for the development of new production units. Currently existing units are insufficient to fulfill the requirements [18]. An effort was done for preparation of a cost effective multivalent vaccine using prevailing toxinotypes of *C. perfringens* in sheep and goats in Pakistan. An effective vaccine from indigenous *C. perfringens* isolates will be available for sheep and goats to protect them against *C. perfringens* diseases.

Material and Methods

Indigenously characterized *C. perfringens* (field isolates from Punjab, Pakistan) toxinotypes, A (MW551947.1), B (MW332247.1) and D (MW332258.1) (n=1 each) were procured under the project TDF 02-028, from the Institute of Microbiology University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. These toxinotypes were used for preparation of multivalent toxoid and bacterin + toxoid clostridia vaccine(s). Type A, B and D isolates were previously confirmed on the basis of the 16SrRNA gene polymerase chain reaction (PCR) after sequence analysis and sequence submission to GenBank NCBI, these (MW551947.1, MW332247.1 and MW332258.1) accession numbers were received.

Vaccine Preparation

Vaccine was prepared by following the method described by Saadh et al.[18] with minor modifications. One Liter reinforced clostridial medium (RCM) broth (6.5 pH) was inoculated separately with bacterial inoculum (10% v/v) (concentration was adjusted as McFarland No. 1) of *C. perfringens* type A, B and D. The RCM broth was supplemented with glucose (0.2%), vitamin mixtures, mineral mixture (0.2%), tween 80 (0.3%) along with sodium chloride (0.75%) and sodium acetate (0.3%). Inoculated culture flasks were incubated at 37°C for 24 h in an CO_2 incubator with ~80-90% CO_2. Following the incubation, representative (100 mL) volumes were taken from broth culture. It was centrifuged at 8000 rpm for 10 min. Supernatant was separated and purified trypsin (10 µg per milliliter) was added for epsilon toxin activation and incubated for 60 min at 37°C. After the incubation, it was stored at 4°C. Alpha and epsilon toxins hemolytic units per milliliter (HU/mL) were quantified through a hemolytic system by using washed 1% sheep RBCs [19] and beta toxin cytotoxic units per milliliter (CU/mL) were estimated on BHK 21 cell line following the method of Nagahama et al.[20]. Bacteria colony forming units (CFU) were counted by the standard total plate count method [21]. Bacteria and toxins were inactivated using formaldehyde (0.4% v/v). After 2 weeks of incubation, inactivation was checked by inoculating the bacteria on blood agar and injecting 0.5 mL of each toxin into a healthy rabbit. Oil (Montanide ISA

70) and alum adjuvant (20% stock solution) was added into the inactivated culture and toxins up to 50% and 3% concentration, respectively. After proper mixing, vaccine mixtures were filled in sterile labelled bottles and stored at 4°C. Each vaccine was prepared with standard CFU per mL of bacterin and toxins units quantified through assays. Toxin units and bacterial CFU per vaccine dose is represented in *Table 1-A* and *B*.

Evaluation of Vaccine

Vaccine(s) were evaluated for sterility, safety and stability according to the OIE manual of diagnostic tests and vaccines for terrestrial animals (chapter 1.01.08, 2018) [22]. For sterility testing fluid thioglycolate medium (FTM) with 0.5% beef extract and soybean casein digest medium (SCDM) were used for bacterial (37°C) and fungal (25°C) growth, respectively and the media were incubated for 14 days. At intervals during the incubation and after 14 days of incubation, inoculated media tubes were examined for the evidence of microbial growth. For safety testing of vaccine, recommended dose of vaccines was administered intramuscularly (IM) and subcutaneously (SC) to rabbits. For adverse localized reaction, the rabbits were observed for 30 days. For stability testing, vaccine vials were stored at 4°C. Emulsions were stored for 6 months without any significant loss of potency. Efficacy testing was conducted in rabbits following the method of Saadh et al.[18]. Oil and alum adjuvant vaccines were injected to rabbits (n=5 for each vaccine) intramuscularly (IM) and subcutaneously (SC), respectively.

Experimental Trials in Rabbits

All animals experiments were approved by the institutional Ethical Review Committee for the Animals, University of Veterinary and Animal Sciences, Lahore, Pakistan and carried out according to the International Ethics Law and Regulations. All efforts were made to minimize the animal sufferance.

Experimental trials were conducted in rabbits (n=70, 1 kg) according to the report of Saadh et al.[18] with some modifications. A total of 14 groups of rabbits were constituted according to the type of vaccine and control *(Table 2)*. Each group contained 5 rabbits. One mL of each vaccine was injected to rabbits and blood was collected at day 0 before the vaccination, 14th, 21st and 28th day after the vaccination. Serum was separated and anti-toxin (antibody) titer (IU/mL) was determined by an indirect enzyme linked immune sorbent assay (ELISA). Commercially available imported vaccine and sterile saline solution was injected to positive control and negative control groups, respectively. Grouping of rabbits for vaccine experimental trials was represented in *Table 2*.

Challenge Test

The immunized rabbits were challenged with a particular toxinotype of *C. perfringens* broth culture containing toxin (having double amount of toxin without formalin inactivation) post vaccination at 28th day and they were kept under observation for three days for mortality. Multivalent bacterin and toxoid oil adjuvant vaccine found to be the most effective in this experiment and selected for field trials in sheep and goat.

Field Trials in Sheep and Goats

Field trials were conducted in sheep and goats (n=80, 40/each). These sheep and goats were screened for the presence of any parasitic infestation and each of them was divided into two groups (Goats A, Goats B, Sheep A and Sheep B). One mL of vaccine was injected subcutaneously to Goat A and Sheep A group (n=20 each). Similarly a commercially available imported vaccine was injected to Goat B and Sheep B group (n=20 each) by the same route. Blood of the animals was collected before and after the vaccination. Anti-toxin (antibody) titer was measured by ELISA and continuously monitored till the 6-9 months with an interval of one month [18]. Indirect ELISA for anti-bodies detection was performed following the method of Bentancor et al.[23]. Optical density (OD) was measured immediately after the addition of the stop solution at 450 nm.

Statistical Analysis

Data obtained from antibody titer was analyzed through one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMR) using statistical package for the social sciences (SPSS) version 20.0.

Results

Titers of toxins (in culture supernatant) produced under culture conditions (as mentioned above) were 2048.22±0.11 HU/mL (alpha toxin) (MW551947.1), 1052.46±0.18 CU/mL (beta toxin) (MW332247.1) and 512.25±0.06 HU/mL (epsilon) (MW332258.1) observed. These toxins titers were used in vaccine formulation. Toxins units per vaccine dose were presented in *Table 1-A* and *B*. Alpha toxin inactivation test result represented that toxin titer was 64.89 HU/mL after one week. Epsilon toxin inactivation result represented that toxin titer decreased to 35.54 HU/mL. After 14 days, titers of the alpha and epsilon toxins were observed zero. For the beta toxin inactivation, cytotoxicity assay represented that the beta toxin titer after 1 week was 45.67 CU/mL and zero at 14 days post inactivation. Colony forming units of *C. perfringens* types A, B and D were calculated by anaerobic plate count. Bacterial CFU was adjusted to 10^6CFU/mL. Bacteria were inactivated by 0.4% formalin (v/v) and after 36 h of the incubation the inactivated bacterial culture was inoculated on Perfringens agar base. No growth was observed indicating complete inactivation of the bacteria by formalin. *C. perfringens* toxoid and bacterin plus toxoid vaccines were prepared using formulations as already

Table 1 A. *Formulation of toxoid vaccines (oil adjuvant and alum precipitate)*

Sr. No	Vaccines	Antigen (toxins)	Toxins Units/ Vaccine Dose	Volume of Antigen Containing CFS(µL)/ Vaccine Dose	Volume of Oil Adjuvant (µL)/ Vaccine Dose	Volume of Alum Adjuvant (µL)/ Vaccine Dose	Volume of PBS (µL) in Alum Vaccine/ Vaccine Dose	Volume of Single Dose (µL)
1	Monovalent Toxoid (oil adjuvant vaccine)	Alpha	1024.11±0.05	500	500	-	-	1000
2	Monovalent Toxoid (alum precipitate vaccine)				-	150	350	
3	Bivalent Toxoid (oil adjuvant vaccine)	Alpha + Epsilon	1024.11±0.05 + 256.12±0.03	500	500	-	-	
4	Bivalent Toxoid (alum precipitate vaccine)				-	150	350	
5	Multivalent Toxoid (oil adjuvant vaccine)	Alpha + Beta + Epsilon	1024.11±0.05 + 256.12±0.03 + 526.23±0.09	500	500	-	-	
6	Multivalent Toxoid (alum precipitate vaccine)				-	150	350	

CFS: *cell free supernatant;* ***PBS:*** *phosphate buffer saline*

Table 1 B. *Formulation of bacterin plus toxoid vaccines (oil adjuvant and alum precipitate)*

Sr. No	Vaccines	Antigen				Volume of Antigen/ Vaccine Dose	Volume of Oil Adjuvant (µL)/ Vaccine Dose	Volume of Alum Adjuvant (µL)/ Vaccine Dose	Volume of PBS (µL) in Alum Vaccine/ Vaccine Dose	Volume of Single Dose (µL)
		Toxin Units/Vaccine Dose		Bacteria CFU/ Vaccine Dose						
1	Monovalent Bacterin and Toxoid (oil adjuvant vaccine)	Alpha	1024.11±0.05	Type A	10^6	500	500	-	-	1000
2	Monovalent Bacterin and Toxoid (alum precipitate vaccine)						-	150	350	
3	Bivalent Bacterin and Toxoid (oil adjuvant vaccine)	Alpha + Epsilon	1024.11±0.05 + 256.12±0.03	Type A + Type D	10^6 + 10^6	500	500	-	-	
4	Bivalent Bacterin and Toxoid (alum precipitate vaccine)						-	150	350	
5	Multivalent Bacterin and Toxoid (oil adjuvant vaccine)	Alpha + Beta + Epsilon	1024.11±0.05 + 256.12±0.03 + 526.23±0.09	Type A + Type B + Type D	10^6 + 10^6 + 10^6	500	500	-	-	
6	Multivalent Bacterin and Toxoid (alum precipitate vaccine)						-	150	350	

CFU: *colony forming units;* ***CFS:*** *cell free supernatant;* ***PBS:*** *phosphate buffer saline*

described *(Table 1B)*. Vaccines were proved to safe as there was no localized reaction observed at site of the injection in rabbits. Vaccines remained stable after storage at 4ºC. Vaccines were pure, white, and stick to glass like paint. Emulsions did not display the signs of cracking. All vaccines passed the sterility test. No growth of bacteria and fungi was observed in fluid thioglycolate broth and soya bean casein digest medium, respectively after 14 days of incubation at 37ºC and 25ºC.

Vaccine experimental trial was conducted in groups of rabbits and serum samples were collected as already described in materials and methods section. The cutoff OD value of antibody detected by ELISA was calculated 0.124±0.03. Optical density values greater and less than this cutoff value were considered positive and negative, respectively. The anti-toxin titer in rabbit sera at day 0 was lower and ranged from 2.67±1.08 to 5.89±1.02 IU/mL. Non-significant differences (P>0.05) were observed among the anti-toxin titer of all groups at day 0 of the experimental trial. At day 14th of the experimental trial, a higher anti-toxin titer (75.86±2.95 IU/mL) was observed in multivalent toxoid vaccine (alum based) followed by (71.86±2.29 IU/mL) of monovalent toxoid vaccine (alum based). There were significant differences (P<0.05) observed among the anti-toxin titers of different groups at day 14th of the experimental trial. At day 21st of the experimental trial, a higher anti-toxin titer (181.97±4.57 IU/mL) was observed in multivalent toxoid vaccine (oil based). Meanwhile, the positive control depicted (8.71±1.63 IU/mL) anti-toxin titer. There were significant differences (P<0.05) observed among the anti-toxin titers of different groups at day 21st of experimental trial. At day 28th of the experimental trial, a higher anti-toxin titer (868.25±3.54 IU/mL) was observed in multivalent bacterin and toxoid vaccine (oil based) followed by (851.14±3.72 IU/mL) of multivalent toxoid vaccine (oil based). There were nonsignificant differences (P>0.05) observed among the anti-toxin titers

Table 2. *Humoral immune response in different experimental groups of rabbits at different time intervals measured by ELISA*

Rabbits Groups (n=5)	Type of Vaccine	Adjuvant	Anti-Toxin (Antibody) Titer (IU/mL)			
			Day 0	Day 14th	Day 21st	Day 28th
A	Monovalent Toxoid	Alum	3.39±1.02[a]	71.86±2.29[d]	6.76±1.38[a]	7.41±1.62[a]
B		Oil	3.89±1.01[a]	28.18±2.09[b]	30.90±2.69[c]	457.09±6.16[b]
C	Bivalent Toxoid	Alum	5.89±1.02[a]	64.56±2.45[c]	17.37±3.16[b]	9.33±1.35[a]
D		Oil	4.68±1.01[a]	57.54±1.86[c]	109.65±4.17[d]	776.25±3.09[b]
E	Multivalent Toxoid	Alum	4.57±1.01[a]	75.86±2.95[d]	32.36±2.82[c]	17.78±3.02[a]
F		Oil	4.57±1.01[a]	37.15±2.75[b]	181.97±4.57[e]	851.14±3.72[c]
G	Monovalent Bacterin plus Toxoid	Alum	2.69±1.02[a]	60.26±2.04[c]	11.48±2.29[b]	5.89±1.54[a]
H		Oil	3.24±1.00[a]	20.42±1.99[b]	87.54±2.51[d]	363.08±5.62[b]
I	Bivalent Bacterin plus Toxoid	Alum	5.13±1.01[a]	51.28±2.29[c]	13.80±2.95[b]	7.41±1.31[a]
G		Oil	2.95±1.00[a]	29.51±2.63[b]	93.32±3.98[d]	616.59±2.81[b]
K	Multivalent Bacterin plus Toxoid	Alum	3.63±1.00[a]	58.88±2.81[c]	25.70±2.63[c]	14.12±2.81[a]
L		Oil	2.88±1.01[a]	45.71±1.77[b]	144.54±4.36[e]	868.25±3.54[c]
M	Positive Control	Alum	4.57±1.01[a]	17.15±1.61[b]	8.71±1.63[a]	5.48±2.95[a]
N	Negative Control	----	2.67±1.08[a]	2.37±1.09[a]	2.32±1.04[a]	2.29±1.01[a]

[a,b,c,d,e] *Values with these different superscripts in column differ significantly (P<0.05) and with same differ non-significantly (P>0.05); IU/mL: international units per milli liter*

of multivalent toxoid and monovalent bacterin plus toxoid oil adjuvant vaccines at day 28th of the experimental trial. Positive control revealed 5.48±2.95 IU/mL anti-toxin titer. There were significant differences (P<0.05) observed among the anti-toxin titers of different groups at day 28th of the experimental trial. Humoral immune responses in different experimental groups of rabbits were presented in *Table 2*.

There was a 100% protection in rabbits (multivalent toxoid and bacterin plus toxoid vaccines groups) after the challenge. In case of multivalent, bivalent and monovalent oil adjuvant vaccines 98%, 95% and 85% protection was observed, respectively.

Multivalent bacterin plus toxoid vaccine (oil based) produced a higher anti-toxin titer in experimental trial thus selected for the field trial in sheep and goats. The anti-toxin titer was determined by indirect ELISA *(Table 3)*. The anti-toxin titer in sheep and goats at day 0 was lower and ranged from 39.98±2.43 to 47.01±2.93 IU/mL. Non-significant differences (P>0.05) were observed among the anti-toxin titer of all groups at day 0 of the field trial. At one month, a higher anti-toxin titer (654.59±3.12 IU/mL) was demonstrated by the sheep A group. A slightly decreased anti-toxin titer (642.23±3.01 IU/mL) was observed in the goat A group. The goat B and sheep B groups produced relatively lower anti-toxin titer (474.76±1.23, 577.91±2.44 IU/mL). There were significant differences (P<0.05) observed among the anti-toxin titers of different groups at one month of the field trial. At two months, a higher anti-toxin titer (887.07±4.03 IU/mL) was demonstrated by the sheep A group. A slightly decreased anti-toxin titer (859.84±1.81 IU/mL) was observed in the goat A group. The goat B and sheep B groups produced relatively lower anti-toxin titer (589.76±1.30, 741.20±1.35 IU/mL). A higher anti-toxin titer was observed in the Sheep A (1294.81±1.90 IU/mL) at four) months of the field trial followed by the sheep A (1192.54±1.79 IU/mL) at five) months of the field trial. A lower anti-toxin titer was observed in the goat A (445.32±0.72 IU/mL) at nine months of the field trial. The anti-toxin titer of the commercial vaccine under study was higher (948.70±1.59 IU/mL) at three months of the field trial in the sheep B whereas, the lower anti-toxin titer (131.54±1.32 IU/mL) was observed in the goat A at nine months of the field trial. There were significant differences (P<0.05) among the anti-toxin titers of different groups throughout the course of the field trial *(Table 3)*.

Discussion

C. perfringens is an anaerobic bacterium that produces several toxins. Of these, alpha, beta, and epsilon toxins are responsible for causing the most severe *C. perfringens*-related diseases in farm animals. The best way to control these diseases is through the vaccination [24]. Entero-toxaemia is an important disease of sheep, and this disease causes severe economic losses to sheep farmers [25]. The development and production of conventional clostridial vaccines involves expensive, time consuming and dangerous processes of detoxification, purification and antigen concentration steps [16]. Alternatively, the use of recombinant vaccines against the clostridial infections has yielded promising results in other animal species [26]. Furthermore, the continued selection of the toxigenic strains that produce high titers of toxin is necessary [17].

Table 3. *Humoral immune response in different experimental groups of sheep and goat at different time intervals measured by ELISA*

Time Period	Anti-Toxin Antibody Titer (IU/mL)			
	Multivalent Bacterin and Toxoid Vaccine (Oil Based)		Commercial Vaccine	
	Goat A	Sheep A	Goat B	Sheep B
Day 0	45.49±2.64^{a}	47.01±2.93^{a}	42.99±1.58^{a}	39.98±2.43^{a}
Month1	642.23±3.01^{c}	654.59±3.12^{c}	474.76±1.23^{a}	577.91±2.44^{b}
Month2	859.84±1.81^{c}	887.07±4.03^{c}	589.76±1.30^{a}	741.20±1.35^{b}
Month3	943.83±3.17^{b}	987.20±1.25^{b}	886.87±1.01^{a}	948.70±1.59^{b}
Month4	1091.85±2.51^{b}	1294.81±1.90^{c}	601.91±1.79^{a}	633.51±1.16^{a}
Month5	1014.87±2.13^{b}	1192.54±1.79^{c}	435.03±1.71^{a}	474.92±1.36^{a}
Month6	826.83±2.55^{b}	928.11±1.19^{c}	324.55±2.44^{a}	386.28±1.23^{a}
Month7	674.56±1.85^{b}	776.67±2.87^{c}	201.42±2.10^{a}	212.66±1.83^{a}
Month8	587.33±1.27^{b}	623.74±3.05^{c}	165.34±1.10^{a}	178.45±1.34^{a}
Month9	445.32±0.72^{b}	457.50±1.77^{b}	131.54±1.32^{a}	142.65±1.24^{a}

^{a,b,c,d,e} *Values with these different superscripts in column differ significantly (P<0.05) and with same differ non-significantly (P>0.05); IU/mL: international units per milli liter*

In the present study, toxoid and bacterin + toxoid oil and alum adjuvanted vaccines were prepared. Experimental and field trials were performed on rabbits, sheep and goats, respectively. 0.2 mg of *C. perfringens* type D epsilon toxoid expressed by *Escherichia coli,* was administered to rabbits, goats, sheep, and cattle to evaluate the potency of the vaccine [27]. Higher antibody titers in rabbits were 776.25±3.09 and 616.59±2.81 IU/mL in the bivalent toxoid and bacterin plus toxoid oil adjuvant vaccines, containing epsilon toxin respectively. These antibody titers were higher than that of evaluated by Morcrette et al.[27] in the rabbit (40 IU/mL) serum pools. The Type B bacterin plus toxoid vaccine potency titer in sheep (1294.81±1.90 IU/mL) and goat (1091.85±2.51 IU/mL) was higher than as reported by Morcrette et al.[27] in goat, sheep, and cattle serum pools, 14.3, 26 IU/mL respectively. In vaccinated ewes, titer was peaked at 1st week with 15 IU/mL. The titer was dropped after lambing [28]. Commercial enterotoxaemia vaccine evaluation was done in goats and a great majority of the vaccinated animals had titers below the protective level, arbitrarily set at 0.25 IU/mL, by day 98 [29]. In the present study, antibody titers at 28th day post vaccination in rabbits and after 4 months post vaccination in sheep and goats were observed to be higher than as observed by de la Rosa et al.[28] and Uzal et al.[29] among rabbits, sheep and goats. The serological response to monovalent epsilon toxoid alum hydroxide adjuvant vaccine against *C. perfringens* type D enterotoxaemia was evaluated in goats. Mean antibody titers was 0.6 and 1.1 IU/ mL at 40th day after the first vaccination, 1.8 IU/mL at 40th day after the booster dose, respectively [30]. These titers were not in agreement with the titer observed in goats after 1st and 2nd month post vaccination with multivalent bacterin plus toxoid vaccine. The potency value of the recombinant epsilon toxoid with aluminum hydroxide as an adjuvant in sheep was determined >5 IU being protective. Further, the use of this construct in a combination vaccine against sheep pox resulted in the sheep being protected against enterotoxaemia [25]. Epsilon -beta fusion toxin was used as vaccine candidate to evaluate potency in rabbits. There was 6 and 10 IU/mL epsilon and beta antitoxin titer observed in rabbits [31]. ELISA test represented that antibody titer against multivalent toxoid vaccine was observed greater than titers observed by Langroudi et al.[31]. Recombinant trivalent vaccine (alpha, beta and epsilon) given to cattle, sheep, and goats and generated respectively, 5.19±0.48, 4.34±0.43, and 4.70±0.58 IU/mL against alpha toxin, 13.71±1.17 IU/mL (for all three species) against beta toxin, and 12.74±1.70, 7.66±1.69, and 8.91±2.14 IU/mL against epsilon toxin. These levels were above the minimum recommended by the international protocols. Vaccines represent an interesting alternative for the prevention of *C. perfringens* related intoxications in farm animals [24]. Moreira and colleagues study results were in contrast to the present study that there were increase in antibody titer in sheep and goats up to 4 months post vaccination. Recombinant monovalent alpha toxoid (200 mg/dose) vaccine potency evaluated against the yellow lamb disease in rabbit and sheep. This vaccine induced 13.82 IU/mL antitoxin in rabbits. In sheep, antibody titer was 4 IU/mL after 56 days of the vaccination [32]. In the present study, monovalent toxoid alum and oil adjuvant vaccine antibody titers in rabbits were observed higher at 14th and 28th day post vaccination, respectively, in contrast to titers observed by Ferreira et al.[32]. The rTA and rTB proteins produced and tested, induced an immune response (9.6 and 20.4 IU/mL, respectively) and can be regarded as candidates for the development of a commercial vaccine against *C. perfringens* type A and C induced diarrhea in pigs [19]. Present study observations were in agreement with that Montanide vaccinated groups exhibited a highest protection percentage (100%) post

challenge [18]. At 31st day post vaccination experimental units, rabbits were challenged with double dose of antigen and there was 100% protection against multivalent toxoid and bacterin plus toxoid oil adjuvant vaccines. Antibody titers of the bivalent and trivalent oil adjuvant vaccine (Montanide ISA 70 oil adjuvant) were in contrast to Saadh et al.[18] observations because at 28th day antibody titer was observed higher. In the present study, antibody titers results post vaccination at 28th days were also not in agreement with the results observed by by Hu et al.[19] and Moreira et al.[24].

For effective vaccine production against *C. perfringens* diseases, selection of toxigenic strains that produce high titers of toxin is necessary. *C. perfringens* toxinotype A, B and D produced higher amount of a, b and e toxins can be used at industrial scale for antigen production. Multivalent bacterin plus toxoid oil adjuvant vaccines produced a specific and efficient immune response during the experimental and field trials; proved an excellent candidate to use in the field to vaccinate animals to protect against *C. perfringens* diseases.

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Conflict of interest

There is no conflict of interest.

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Author's Contributions

MT, TA and AAS conducted the research on vaccines preparation. Vaccine evaluation and experimental trials were conducted by AAA, MAA and ARA. Field trial of vaccine on sheep and goat was performed by MMKS and SH.

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RESEARCH ARTICLE

Determinate of ECG, Oxidative Stress, and Angiogenesis in APAP-Induced Toxicity in Rats

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Abstract

This study was aimed to evaluate the effects of N-acetyl-p-aminophenol (APAP) toxicity on the cardiovascular system as there exist relatively a few studies on this matter. The study included 14 female Wistar rats divided into two groups having 7 rats in each (control-APAP). Control group received no medication and APAP group was given single oral dose of 1g/kg APAP. ECG measurements of each animal in either group were obtained before the administration of APAP (0 h) and at the 1st, 6th, 12th and 24th h after the APAP administration. All animals were sacrificed at the end of the study. Heart tissue samples were obtained for biochemical and histopathological analyses. The levels of MDA, GSH, Apelin, Elabela, Meteorin, Endoglin, Keap1, and Nrf2 were measured in the tissue samples. Results revealed a statistically significantly prolonged QTc and QRS intervals and increased heart in the APAP group. A notable increase in MDA and Endoglin, and a significant decrease in GSH, Elabela, and Nrf2 levels occurred in the APAP group. Histopathologically, necrotic lesions were found in the APAP group. The use of high doses of APAP as an analgesic may cause permanent damage in the cardiovascular system.

Keywords: *N-acetyl-p-aminophenol, ECG, Apelin, Elabela, Meteorin, Endoglin*

Sıçanlarda APAP Kaynaklı Toksisitede EKG, Oksidatif Stres ve Anjiyogenezin Belirlenmesi

Öz

Bu çalışma, N-acetyl-p-aminophenol (APAP) toksisitesinin kardiyovasküler sistem üzerindeki etkilerini değerlendirmeyi amaçlamıştır çünkü bu konuda nispeten az sayıda çalışma bulunmaktadır. Çalışma kapsamında her grupta 7 rat (kontrol ve APAP) olacak şekilde 14 adet dişi wistar rat kullanıldı. Kontrol grubuna herhangi bir uygulama yapılmazken APAP grubuna tek doz 1 g/kg N-acetyl-p-aminophenol oral olarak verilmiştir. Apap uygulaması öncesi 0. saat ve uygulama sonrası 1. saat 6. saat 12. saat ve 24. saatte iki gruptaki tüm hayvanlara EKG ölçümü yapıldı. Çalışma sonunda tüm hayvanlar sakrifiye edildi. Biyokimyasal ve histopatolojik analizler için kalp dokusu örnekleri alındı. Doku örneklerinden MDA, GSH, Apelin, Elabela, Meteorin, Endoglin, Keap1 ve Nrf2 ölçümleri yapıldı. Yapılan analizlere göre ekg verilerinde QTc, kalp atım sayısı ve QRS'de APAP grubunda istatistiksel anlamda artış belirlendi. Biyokimyasal verilerde ise APAP grubunda MDA ve Endoglinde anlamlı artış bulunurken GSH, Elabela ve Nrf2'de ise anlamlı bir azalma belirlenmiştir. Histopatolojik olarak APAP grubunda nekroze lezyonlara rastlanmıştır. Sonuç olarak analjezi olarak kullanılan APAP yüksek doz alımlarında kardiyovasküler sistemde kalıcı hasarlara yol açabilmektedir.

Anahtar sözcükler: *N-acetyl-p-aminophenol, EKG, Apelin, Elabela, Meteorin, Endoglin*

INTRODUCTION

Acetaminophen (paracetamol) is a commonly used analgesic-antipyretic drug in the US since 1955. Paracetamol overdose accounts for a significant number of emergency service admission [1,2]. When taken in therapeutic doses, the major quantity of paracetamol is conjugated with glucuronic acid and sulphate, and the remaining small quantity is converted to the N-acetyl-p-benzoquinoneimine (NAPQI) metabolite by hepatic cytochrome P-450 dependent mixed-function oxidases in the liver [3,4]. Under normal circumstances, NAPQI is rapidly converted to its non-toxic

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metabolites by glutathione (GSH). However, under GSH-deficient conditions NAPQI cannot be converted to non-toxic metabolites leading to liver damage. Paracetamol overdose can clinically manifest by metabolic acidosis, elevated lactate, hypoglycaemia, and acute nephrotoxicity and hepatotoxicity. There exist few studies on paracetamol cardiotoxicity. Several studies have focused on the direct toxic effects of acetaminophen on the heart [1,5].

Apelin, a cardiovascular system associated hormone, is particularly effective in regulating the blood flow and lowering the blood pressure. Elabela and Apelin antagonize the renin-angiotensin system; thus, playing a role in preventing the development and slowing the progression of cardiovascular diseases. Furthermore, apelin and related peptide receptors have also cardioprotective effects in atherosclerosis, myocardial infarction, heart failure, and pulmonary arterial hypertension [6].

Meteorin-like (Metrnl) is a recently discovered adipokine that acts on insulin sensitivity favourably. Adipokines actively take part in lipid metabolism and inflammation. Adipokines are involved in cardiometabolic diseases including coronary diseases. The most important cause of coronary diseases is atherosclerosis. In such cases, Metrnl causes increased levels of anti-inflammatory cytokines [7].

Endoglin is an essential co-receptor for transforming growth factor β (TGF-β) family, playing an important role in angiogenesis. Increased levels of circulating endoglin have been found in hypertensive or diabetic patients, in early stages of preeclampsia, and in some cancer patients. These suggest that endoglin can be a predictive biomarker in these pathological conditions [8].

The nuclear factor erythroid 2–related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) are proteins that play an active role in apoptosis. Simultaneous depletion of Nrf2 and Keap1 is known to prevent apoptosis. Nrf2 and Keap1 are also reported to have a protective effect against oxidative stress [9].

In this study, we aimed to find out whether cardiotoxic damage and cardiac rhythm disturbances develop in paracetamol toxicity, and to identify new biomarkers that could be used for early diagnosis.

Material and Methods

This study was approved by Kafkas University Animal Experiments Local Ethics Committee (Approval No: KAÜ-HADYEK 2019/122) Kars, Turkey. The study included 14 female, 4-6-month-old Wistar-Albino rats with an average weight of 190-250 g. All animals were fed ad-libitum, maintained at a room temperature of approximately 25°C, and kept under 12-h light-dark cycles. Animals were divided into two equal groups (7 rats in each). Paracetamol was obtained from Atabay Pharmaceuticals Inc.

The Experimental Groups

Group I (Control): Rats were administered only 0.9% NaCl orally.

Group II (Toxicity): Toxicity was induced in rats by administering a single oral dose of Paracetamol (1 g/kg) [10].

Electrocardiography (ECG) measurements were obtained from each animal in the experiment under anaesthesia [ketamine HCl (75 mg/kg) (Ketalar, Pfizer®), and xylazine HCl (10 mg/kg) (Rompun, 2%, Bayer®) intramuscular] [11] before the induction of toxicity (0 h) and at 1st, 6th, 12th, and 24th h after inducing toxicity using Nihon Kohden cardiofax S ECG-1250 device. Digital ECG records were obtained using the leads I, II, III, aVR, aVL, and aVF at a velocity of 50 mm/s, at a calibration of 1 mV=10 mm, and using a 50-Hz filter *(Fig. 1)*. The device calculates the QTc data automatically; therefore, no further calculations were performed [12].

At the end of the study, animals were not fed overnight and cardiac tissue samples were collected after sacrificing via cervical dislocation under anaesthesia [11] in compliance with ethical principles.

Biochemical Measurements

The tissue samples were homogenized in phosphate buffer (pH 7.4) and centrifuged at 3000 rpm for 5 min. The obtained homogenates were kept at -20°C until analysis. Apelin, Elabela, Meteorin, Endoglin, Keap1, and Nrf2 levels were determined in the cardiac tissue by using commercially available Enzyme-Linked Immunosorbent Assay (ELISA-YL Biotech Company, Shanghai) kits in compliance with the manufacturer's instructions. The GSH and MDA levels in the cardiac tissue were analysed applying the methods described by Beutler et al.[13] and Yoshioka et al.[14], respectively.

Histological Analysis

The cardiac tissues were fixed in 10% buffered formaldehyde solution. The tissues were subjected to tissue processing in an automated device (Leica TP 1020). Sections of 5-µm thickness were taken from the tissues that were manually embedded in paraffin and the sections were stained with Hematoxylin & Eosin. The sections were examined and photographed under a light microscope (Olympus BX46) [15].

Statistical Analyses

Before the study, power analysis was performed using G-Power 3.1.9.7. According to the analysis, the sample size was decided according to the test power of 0.95 and the significance level of 0.05. Analysis of independent samples t-test was conducted for all the biochemical and ECG parameters to test if there is a difference between the two groups. Analysis of repeated-measures ANOVA was conducted for ECG parameters obtained on different time.

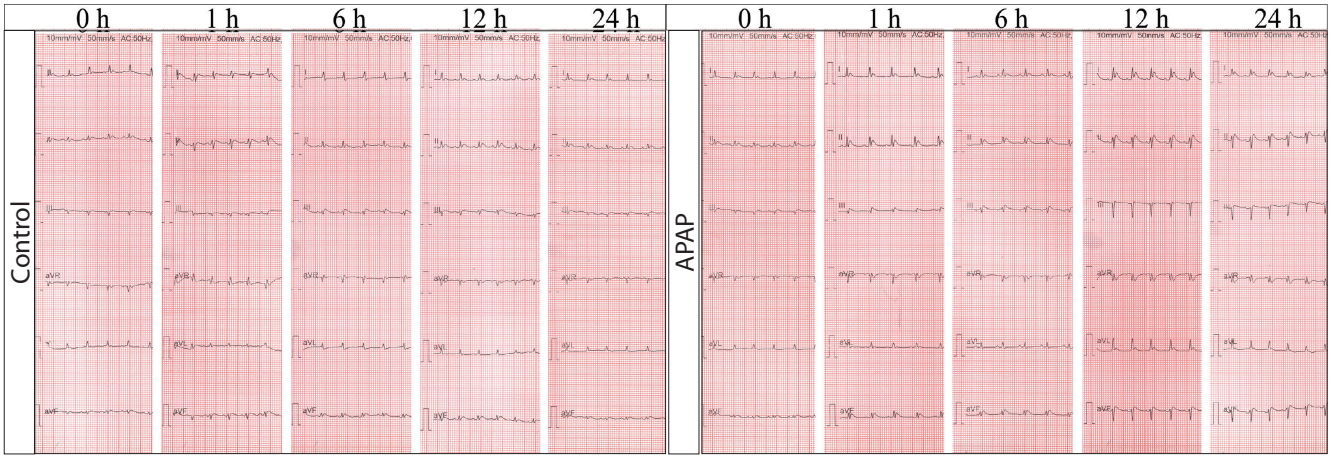

Fig 1. ECG changes according to time interval in control and APAP groups

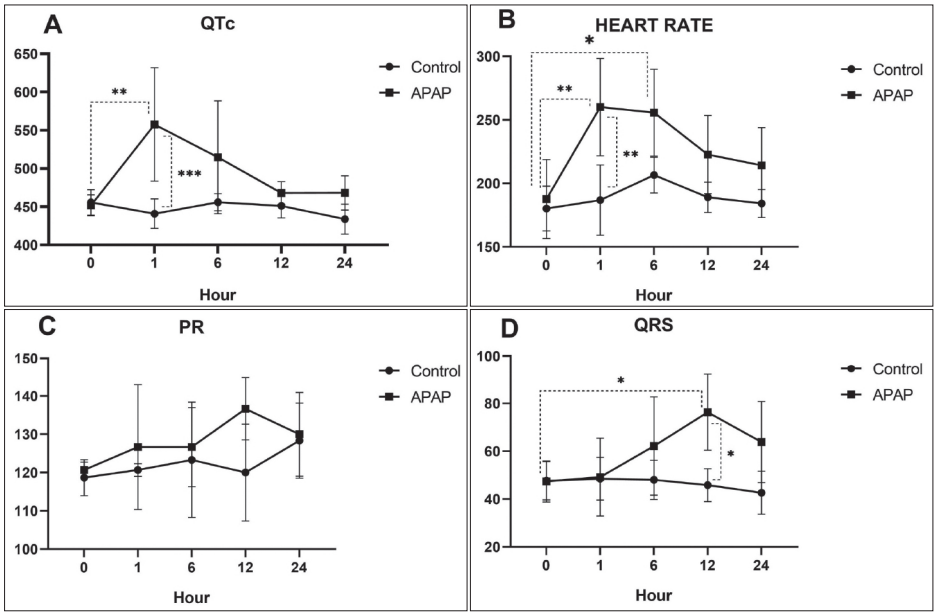

Fig 2. Means and Std. deviation of the two groups for ECG (A, B, C, D) parameters; * P<0.05, ** P<0.01, *** P<0.001

A P-value of <0.05 was accepted as significant. GraphPad 8.1 (San Diego, CA, USA) was used for statistical analyses.

Results

ECG Results

The QTc interval *(Fig. 2-A)* significantly increased at the 1st h in the APAP group compared to 0 hour (P<0.01) and to that of the control group at the 1st h (P<0.001).

The heart rate *(Fig. 2-B)* significantly increased both at the 1st (P<0.01) and the 6th h (P<0.05) in the APAP group compared to 0 h. When the APAP group was compared with the control group, a significant increase in the heart rate was observed in the APAP group at the 1st compared to the control group.

The PR interval *(Fig. 2-C)* did not change throughout the study between and within the groups.

A significant difference in the QRS complex *(Fig. 2-D)* was detected in the APAP group between the values at 0 h and the 12th h (P<0.05). Similarly, a significant difference was determined between the APAP group and the control group in terms of QRS complex at the 12th h (P<0.05).

Biochemical Results

Malondialdehyde levels significantly increased in the APAP group compared to the control group (P<0.05) while GSH levels markedly decreased between the groups (P<0.01, *Fig. 3-A,B)*.

The analysis of Apelin, Meteorin, and Keap1 levels revealed no differences between the APAP and the control groups *(Fig. 3-C,D,E,F,G,H)*. However, a statistically significant decrease in Elabela (P<0.001) and Nrf2 (P<0.05) levels, and a notable increase in Endoglin (P<0.05) levels were noted between APAP and control groups.

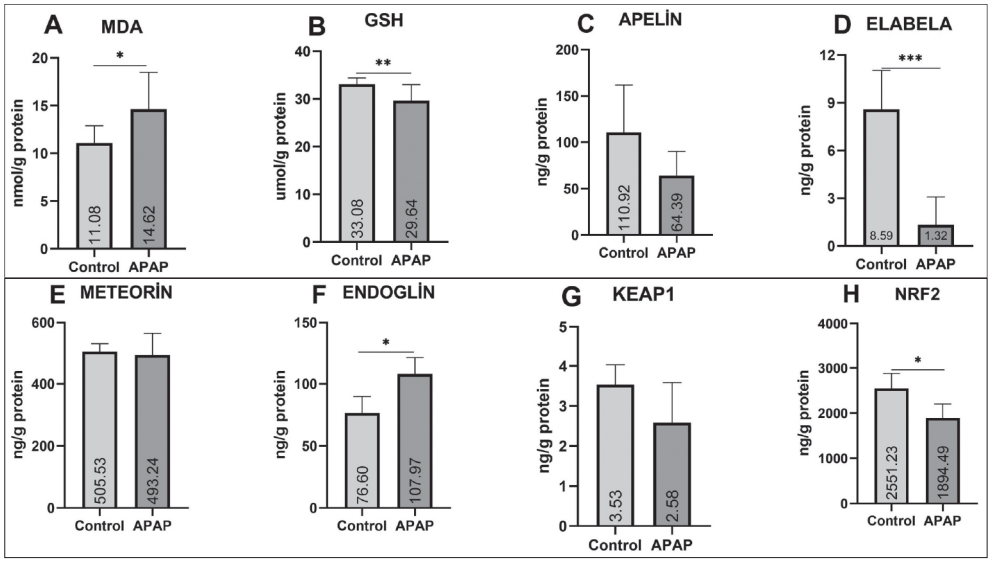

Fig 3. Means and Std. deviation of the two groups for biochemical (A, B, C, D, E, F, G, H) parameters. *P<0.05, **P<0.01, ***P<0.001

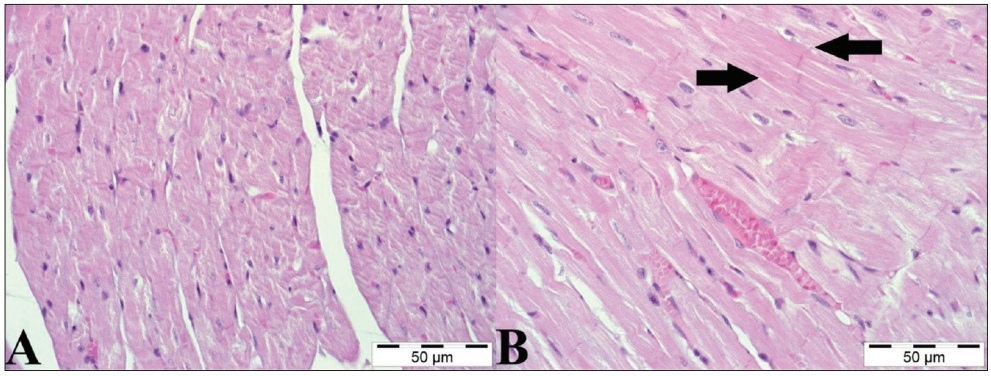

Fig 4. A: Control group, heart tissue, H&E, Bar = 50 µm **B:** Paracetamol group, heart tissue, degeneration in muscle fibers (arrows), H&E, Bar = 50 µm

Histological Results

On histopathological examinations, cardiac tissue was normal had no pathological changes in control group. In APAP group, muscle fibers were swollen and homogeneous in pink color, the transverse striation of the muscle fibers disappeared and in addition to degenerative changes, the presence of necrotic cells with pycnotic nuclei was remarkable *(Fig. 4)*.

Discussion

Raising awareness about recognizing new and early symptoms, signs, and biochemical markers in paracetamol overdose is important in the early diagnosis and treatment in order to reduce mortality and morbidity.

Cardiac autonomic dysfunction can be detected by various tests and some ECG findings (PR, QT interval, QTc, QRS complex). The relationship between a prolonged QT interval and sudden cardiac death in various diseases such as coronary artery disease and heart failure is known [16]. In our study, the increase in the length of the QTc interval and the heart rate has been demonstrated at the 1st h after paracetamol overdose compared to the control group. Furthermore, cardiac tissue damage in paracetamol overdose has been shown histopathologically. The development of these findings after paracetamol overdose can be explained by the degeneration of cardiac cells leading to delays in cardiac conduction and changes in cardiac conduction pathways resulting in delayed action potentials.

N-acetyl-p-benzoquinoneimine formed in paracetamol overdose cases causes cellular injury and cell death by binding to cellular proteins [1]. We expect that the underlying mechanism of cardiac injury in paracetamol overdose is the cardiac cell damage resulting from the transport of NAPQI to the cardiac blood supply by the venous system.

An intraventricular conduction delay or a bundle branch block is considered in depolarization disorders of the cardiovascular system [17]. Afshari et al.[18] conducted a study and found out that paracetamol overdose was associated with a prolonged QRS complex detected via ECG monitoring.

This study found significant prolonged QRS complex at the 12th h compared to the zero h in the APAP group, suggesting a conduction delay consistent with previous studies. We assumed that the underlying reason was the impairment in the signal transduction pathway; affected by secondary to cardiac tissue damage. This condition is

supported by the levels of the inflammatory, oxidant, and antioxidant parameters determined in our study.

N-acetyl-p-benzoquinoneimine, induced by high-dose paracetamol, causes the formation of reactive oxygen species in cells resulting in lipid peroxidation. Lipid peroxidation leads to GSH deficiency and protein synthesis in hepatocytes, finally resulting in intracellular calcium (Ca^{+2}) imbalance in liver cells [19]. The GSH levels in our study significantly reduced in the APAP group compared to the control group. This may be attributed to high NAPQI causing reduction in GSH levels in cardiac cell injury resulting from impaired protein synthesis and changes in intracellular Ca levels. Another indicator of tissue damage is the increased MDA levels caused by lipid peroxidation [20] as was the case in the cardiac tissue in our study consistent with earlier studies where paracetamol induced increased MDA levels and reduced GSH levels [4,21]. Zhao et al.[22] reported elevated MDA levels and reduced GSH levels in doxorubicin-induced cardiotoxicity similar to the results of our study.

Both Apelin and Elabela are suggested to have cardioprotective, vasodilator, hypotensive, and strong positive inotropic effects [23]. In our study, the changes in apelin levels were not statistically significant but a notable decrease in Elabela levels was found in the paracetamol group. Elabela is heavily expressed in the cardiovascular endothelium. Elabela is required for normal heart development and angiogenesis and is available in the heart tissue more abundantly compared to apelin [24]. The reduction in Elabela levels may suggest the occurrence of damaged cells. Furthermore, Elabela and Apelin are reported to play an active role in preventing cardiovascular diseases by antagonizing the renin-angiotensin system [6]. Considering this feature, the numerical reduction in Apelin levels and the statistically significant reduction in Elabela levels in our study may represent a challenge to eliminate the effects of paracetamol toxicity.

Dadmanesh et al.[7] reported that coronary artery patients had significantly reduced Meteorin levels compared to the control group in their study. However, a numerical decrease in Meteorin levels was found in the cardiac tissue of the rats in the toxicity group in our study but no statistically significant differences were observed.

Endoglin is a transmembrane co-receptor involved in the transformation of the growth factor-β (TGF-β) expressed predominantly on proliferating endothelial cells. Endoglin-knockout mice die of cardiovascular defects at mid-gestation. Endoglin is expressed at low levels in cells of normal endothelial tissue, but its over activity can be observed in infected tissue during embryogenesis [25]. In our study, we expect that the Endoglin levels were found to be significantly high due to histopathologically confirmed cardiac cell damage. Jacob et al.[26] reported focal necrosis in myocard due to APAP as was the case in our study. Kapur et al.[27] investigated endoglin levels in cardiac fibrosis and reported the isolation of very high levels of endoglin in the fibrosis group. We found statistically high Endoglin levels in the toxicity group of our study consistent with the findings of the study reported by Kapur et al.[27]. Considering the effective role of endoglin in angiogenesis, the high levels of endoglin in the toxicity group in our study support the toxic effect of paracetamol on the heart. This finding also suggests that endoglin levels can potentially be used as a marker of the effects of paracetamol intoxication on the heart tissue.

The radicals produced in a variety of physiological conditions are neutralized by antioxidative defence mechanisms [8,28]. Free oxygen radicals are also reported to play an active role in apoptosis. Nrf2 and Keap1 are involved in cell regenerating processes together. However, their levels are significantly reduced during these processes [9]. Under the light of this information, the respectively numerical and statistical reductions in the Nrf2 and Keap1 levels in the toxicity group indicate the activation of apoptotic mechanisms in response to cellular injury. Zhao et al.[22] found a significant reduction in Nrf2 levels in the toxicity group, similar to the results of our study.

It is very important to detect early tissue and organ damage in paracetamol overdose. Our study shows the importance of the early recognition of ECG changes including the lengths of QTc interval and QRS complex, and the heart rate, especially at the first hours of intoxication. Paracetamol overdose induced heart injury might be associated with some novel biomarkers as such studied here.

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Availability of Data and Materials

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

Consent for Publication

N/A

Consent to Participate

Not applicable.

Competing Interests

The authors declare that they have no competing interests

Authors' Contributions

MM and TD analyzed and interpreted the data and was a major contributor in writing the manuscript. EK and HFG analyzed and interpreted the data. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

L-Carnitine Supplemented Extenders Improve Post-Thawing Quality of Honey Bee Drone *(Apis mellifera)* Spermatozoa [1]

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Abstract

The study aimed to evaluate the effects of L-carnitine on honey bee *(Apis mellifera)* drone spermatozoon at post-thaw. Semen samples obtained from sexually mature drones were pooled. Then, pooled semen was diluted with different concentrations of L-carnitine (2.5 mM/ 5 mM/ 10 mM) and without L-carnitine (control) supplemented extenders. Motility, plasma membrane functional integrity, acrosomal integrity and mitochondrial function were negatively affected by the cryopreservation process but DNA integrity was not affected. Malondialdehyde (MDA) concentration was used for analyzing the membrane lipid peroxidation status and a better result was obtained in the LC5 group compared with the control group at post-thaw. L-carnitine groups had a positive effect on sperm motility, plasma membrane integrity, and mitochondrial function compared to the control group ($P<0.05$). Acrosomal integrity was better preserved in the LC5 group compared to the control group. The study shows that LC supplemented extenders have beneficial effects on honey bee drone sperm parameters. The results of the present study demonstrated the beneficial effect of using a 5 mMol LC supplemented extender.

Keywords: *Apis mellifera, Cryopreservation, Drone semen, Honey bee, L-carnitine*

L-Karnitin İlave Edilmiş Sulandırıcılar Bal Arısı *(Apis mellifera)* Spermatozoası'nın Çözdürme Sonrası Kalitesini Arttırır

Öz

Bu çalışmada, L-karnitinin eritme sonrası bal arısı *(Apis mellifera)* spermatozoonu üzerindeki etkilerini değerlendirme amaçlandı. Cinsel olarak olgun erkek arılardan elde edilen sperm örnekleri birleştirildi. Daha sonra birleştirilen semen, farklı konsantrasyonlarda L-karnitin içeren (2.5 mM/5 mM/10 mM) ve içermeyen (kontrol) sulandırıcılar ile seyreltildi. Motilite, plazma membranı fonksiyonel bütünlüğü, akrozomal bütünlük ve mitokondriyal fonksiyon kriyoprezervasyon sürecinden olumsuz etkilenmiş, ancak DNA bütünlüğü etkilenmemiştir. Malondialdehit (MDA) konsantrasyonu, membran lipid peroksidasyon durumunu analiz etmek için kullanıldı ve çözdürme sonrası kontrol grubuna kıyasla LC5 grubunda daha iyi bir sonuç elde edildi. L-karnitin grupları, kontrol grubuna kıyasla sperm motilitesi, plazma membran bütünlüğü ve mitokondriyal fonksiyon üzerinde olumlu bir etkiye sahipti ($P<0.05$). Akrozomal bütünlük, kontrol grubuna kıyasla LC5 grubunda daha iyi korunmuştur. Çalışma, LC takviyeli sulandırıcıların bal arısı sperm parametreleri üzerinde faydalı etkilere sahip olduğunu göstermektedir. Mevcut çalışmanın sonuçları, 5 mMol LC takviyeli sulandırıcı kullanmanın faydalı etkisini gösterdi.

Anahtar sözcükler: *Apis mellifera, Bal arısı, Erkek arı semen, Kriyoprezervasyon, L-karnitin*

INTRODUCTION

Beekeeping is indispensable in ensuring the integration of animal and plant production models. As a result of a better understanding of the contribution of honey and other bee products (royal jelly, propolis, pollen, beeswax, and bee venom) to human health in recent years, the areas of use for food, treatment, and cosmetic purposes have

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expanded [1]. Increasing honey production in the world can be achieved through productive and genetically improved colonies [2,3]. It is known that drones have an important effect on colony productivity in terms of genetic potential as well as that of the queen. Queen bees that cannot mate with a sufficient number of qualified drones will not be able to store enough sperm, so their economic use will be shorter [4]. Therefore, colony productivity can be increased by artificial insemination of queens with the genetically superior drone semen.

The genetic diversity within colony populations can be increased with cryopreservation of drone semen [5-7]. Although drone spermatozoon has been successfully cryopreserved in recent years, this process has a detrimental effect on spermatozoon because of temperature change and cold shock [2,8]. These undesirable effects cause a decrease in the fertilizing ability of spermatozoon [9-11]. Besides, during the freezing-thawing process, reactive oxygen species (ROS) emerge as a result of lipid peroxidation in the cytoplasm membrane. Excessive ROS formation can induce oxidative stress, leading to cell damage that can reduce motility and fertilizing ability. Therefore, antioxidants have been used to get rid of these undesirable effects of cryopreservation in recent years [9,12].

L-Carnitine (LC) is an endogenous compound maintaining cellular homeostasis, limiting the β-oxidation pathway, and acting in the transport of fatty acids to mitochondria [13,14]. Moreover, antioxidant characteristics and anti-apoptotic activities of LC may protect the mitochondrial membrane and DNA structure against ROS [14,15]. The use of L-carnitine in the extenders for buffalo, sheep, goat, rabbit, and rooster semen cryopreservation enhanced post-thawing sperm quality.

The cryopreservation of drone spermatozoon without losing its ability of fertilization contributes to the conservation of gene lines. Our hypothesis was that LC supplementation in the semen extender could improve the post-thawing drone sperm viability and its longevity. Hence, the present study was designed to compare different concentrations of LC supplemented extenders for the cryopreservation of drone sperm using quality tests.

Material and Methods

Chemicals

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

Experimental Design

In the design of this study, the efficiency of LC supplementation extenders for drone semen cryopreservation was evaluated. For this purpose, we used various concentrations of LC (0 mM, 2.5 mM, 5 mM, and 10 mM) supplemented extenders for post-thaw quality of drone spermatozoon.

Extender Preparation

According to the experimental design, various LC concentrations were added in certain proportions to each extender group. We prepared the experimental groups as LC 2.5 (with 2.5 mM LC), LC5 (with 5 mM LC), LC10 (with 10 mM LC), and control (non-LC). Extenders contained Na citrate (82.21 mMol), catalase (1.59 mMol), KCl (5.34 mMol), NaHCO3 (24.87 mMol), amoxicillin (0.82 mMol), and DMSO (10%). The pH value of the diluents prepared was scaled to be 8.1.

Production of Drones

Healthy and strong honey bee colonies reared in Bursa Uludag University, Beekeeping-Development-Application and Research Center (artificial insemination of queens have been performed over 10 years and drones are also reared as part of the breeding studies) were used for semen collection.

Semen Collection and Dilution

Sexually mature drones (16 days and older) were selected for semen collection and at least five colonies were used for this research. Drone semen was collected five times every other day and at least 250 bees were used in each application. During the sperm collection, pressure was applied to the thorax to induce ejaculation, and then the abdominal area was gently squeezed. Approximately 1 µL semen was collected from per drone using the Schley syringe under a stereo microscope. Besides, to eliminate individual differences all semen was pooled. The volume of each pooled semen was portioned into four equal volumes. Each group of the extender was individually diluted with control or LC supplemented extenders to a final concentration of about 150×10^6 (spermatozoa/mL).

Semen Freezing and Thawing

The method of cryopreservation and thawing was based on Alcay et al.[12]. According to this method, equilibrated drone sperm was filled into 0.25 mL straws. After the filling process, straws were frozen in a programmable freezing device (Air Liquide, Marne-la-Vallée Cedex 3, France). Then the sperm-filled straws were immersed in liquid nitrogen and then stored in a liquid nitrogen tank. In each group, three straws were used for post-thaw semen parameters.

Semen Evaluation

In the evaluation of post-thaw semen, plasma membrane integrity, acrosome integrity sperm motility, and DNA integrity parameters were examined. A hypoosmotic swelling test (HOST) was used for plasma membrane integrity. FITC-Pisum sativum agglutinin (PSA-FITC) was used for acrosome integrity. Terminal deoxynucleotidyl transferase-

mediated dUTP nick-end labeling (TUNEL) is used to assess DNA integrity. Evaluations were made by the same person during the study.

- Motility

Drone semen motility assessment was performed using a phase-contrast microscope (Olympus BX51-TF - Olympus Optical Co., Ltd., Japan) with the slide heated to 37°C.

- Membrane Functionality

For the assessment of the plasma membrane integrity, the hypo osmotic swelling test method is used, which was previously described by Alcay [12]. Following this method, the membrane integrity of the drone sperm was evaluated by observing the frizzled tails.

- Acrosomal Integrity

For this evaluation, a sample of 10 µL spermatozoa was added into 100 mL of PBS and centrifuged for 5 min. The sperm pellet obtained after centrifugation was suspended again in 100 mL PBS. The spermatozoa obtained after these procedures were smeared. Smeared-slides were allowed to dry after being smeared. After the drying process is over, the smears were left in acetone fixation at 4°C for 15 min in a glass chalet (vertical, Hellendahl type). After fixation, smears were stained with FITC PSA solution for 1 h at 37°C in a light-proof sample kit. After the staining process was completed, at least 200 drone spermatozoa emitting fluorescent light were evaluated under a fluorescent attachment microscope [16].

- Mitochondrial Activity

Fluorescent stains, PI, and Rhodamine (R123) combination were used to examine mitochondrial integrity. For this analysis, Fareser's method was used [17]. Results are expressed as a percentage.

- DNA Fragmentation

DNA fragmentation was evaluated by the TUNEL technique using In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with slight modifications [18].

- Malondialdehyde (MDA) concentrations

For the assessment of MDA concentrations, the method described by Sharafi is used [19]. As per the method, 0.25 mL of diluted semen sample was treated with 0.25 mL of cold 20% (w/v) trichloro aceticacid to precipitate the protein. During the centrifugation, the precipitated protein was pelleted and the supernatant was incubated with (w/v) thiobarbituric acid for 10 min in a 100°C boiling water bath. After the incubation in the hot water bath, the sample was allowed to cool. Absorbance was determined using a spectrophotometer (Mannheim Boehringer Photometer 4010). MDA concentrations were expressed as nmol/mL.

Statistical Analysis

All data were analyzed using IBM SPSS version 23 (Chicago, IL, USA). Shapiro Wilk test was used as normality test. Data were represented as mean ± standard deviation (SD). Statistical significance of differences between subdivided groups were analyzed with one-way ANOVA followed by Tukey.

Results

The percentages of motility, plasma membrane functional integrity, acrosomal integrity, mitochondrial function, and DNA fragmentation rates of pooled semen samples were 89.00±2.24, 93.80±1.30, 95.40±0.89, 93.00±2.00, and 0.40±0.89, respectively. In the study, sperm quality were negatively affected the cryopreservation process compared with the fresh pooled semen (P<0.05). The *Table 1* shows the effects of different concentrations of LC on drone sperm parameters at post-thawed.

The sperm motility and plasma membrane functional integrity was better-preserved in LC groups compared with the control group at post-thaw (P<0.05). Besides, the highest percentage of motility and membrane integrity rates were obtained from the LC5 group (P<0.05). The better acrosomal integrity was obtained in the LC5 group compared to the control group (P<0.05). The percentage of acrosomal integrity was not found significantly different among other groups (P>0.05). Mitochondrial function rate was higher in the LC5 group compared with the other

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

Variable	L-Carnitine Concentrations (mM)			
	0	2.5	5	10
Motility (%)	49.67±2.96^{a}	52.67±2.58^{b}	62.00±2.54^{c}	57.66±2.58^{d}
HOST (%)	59.33±3.62^{a}	63.40±2.53^{b}	70.13±2.07^{c}	66.27±1.62^{d}
Acrosomal Integrity (%)	80.67±3.67^{a}	81.27±2.96^{a}	84.27±2.60^{b}	82.47±2.83^{ab}
Mitochondrial function (%)	58.00±3.05^{a}	61.80±2.48^{b}	67.93±3.10^{c}	64.73±2.49^{d}
DNA fragmentation (%)	0.33±0.62^{a}	0.40±0.63^{a}	0.40±0.63^{a}	0.47±0.64^{a}

Data is presented in Mean± S.D.; Different letters within the same rows show significant differences among the groups (P<0.05)

Table 2. *Malondialdehiyde (MDA) levels in frozen-thawed drone sperm*

Parameter	Groups			
	Control	LC2.5	LC5	LC10
MDA (nmol/mL)	3.22±0.67[a]	3.00±0.71[ab]	2.33±0.50[b]	2.78±0.67[ab]

Data is presented in Mean± S.D.; Different superscripts (a and b) in the same line indicate significant differences (P<0.05)

LC and control groups (P>0.05). The percentage of DNA damaged spermatozoa were not significantly different in all groups at post-thaw (P>0.05).

As shown in *Table 2*, it was found that MDA levels in the LC5 groups were lower than the control group (P<0.05). Also, there were no significant differences among the LC groups.

Discussion

Cryopreservation of spermatozoa ensures that genetic material is preserved for a long time. However, it is known that the freezing-thawing process has negative effects on the fertilization ability of spermatozoa [9,16,20]. These undesired effects decrease viability, motility, plasma membrane, and acrosomal integrities of spermatozoa [12]. Besides, poor sperm quality generates poorer quality of queens which is one of the main causes of colony loss [21].

LC is a water-soluble amino acid that has a beneficial effect on spermatozoa during the freeze-thaw process because of antioxidant characteristics and anti-apoptotic activities. In the study, we evaluated the effect of exogenous addition of LC in extender on drone sperm quality at post-thaw [14].

Motility is essential for sperm migration to the queen's spermathecal and subsequent egg fertilization. Therefore, it is one of the most widely used spermatological parameters to evaluate sperm quality in drone sperm similar to mammals [22]. In the study, the presence of LC in the extenders increased drone sperm motility compared to the control group at post-thaw (P<0.05). LC facilitates the transport of fatty acids across the inner membrane of mitochondria for ATP production. Therefore, this characteristic of LC may be responsible for improving the motility of drone sperm. Also, the motility values of drone spermatozoa cryopreserved with various semen extenders ranged between 25% and 62% in the different studies [1,10,12,23]. Our study shows that post-thaw motilities in the LC groups are in good agreement with the findings of the previous studies.

Plasma membrane integrity is one of the most frequently evaluated sperm quality parameters for semen analysis in the honey bee because the loss of membrane integrity is considered incompatible with sperm viability [24]. However, cold shock, ice crystallization, osmotic stress, and lipid peroxidation have negative effect on membrane permeability and integrity during cryopreservation [7,25]. Therefore, it is crucial to keep the integrity during the cryopreservation process to avoid cellular damage. HOST is the optimized test for detecting the subtle changes of spermatozoon membrane functionality [26,27]. In the study, the plasma membrane functional integrity values in the LC5 group was higher than those of in the other groups at post-thaw (P<0.05). The HOST values are in agreement with the previous studies [10,12,16,28].

During oviposition, the queen releases few spermatozoa from the spermatheca, and then the acrosome reaction releases lytic enzymes that aid in the penetration of the vitelline membrane to fertilize the egg. Therefore, acrosomal integrity is crucial for the fertility of spermatozoon at post-thawed [9,11,28]. In the honey bee, the acrosomal integrity could be evaluated by Pisum sativum agglutinin (PSA) lectin staining method [12]. In the study, there was no statistical difference among LC2.5, LC10, and the control groups. LC5 group preserved acrosomal integrity better than LC2.5 and the control groups. These results are in agreement with the previous researches [12,16].

Spermatozoon needs energy to carry out its functions and it can mostly obtain ATP through the glycolytic and oxidative phosphorylation pathways [29,30]. Mitochondria play an essential role in regulating sperm function [22]. Therefore, it is important to investigate the mitochondrial function for spermatozoon quality. In the study, mitochondrial function was better preserved in LC groups compared to the control group (P<0.05). Only one study is present evaluating the mitochondrial function of honey bee drone spermatozoa [31]. Similar results were obtained in our study.

During cryopreservation, protecting the DNA integrity also has great importance not to disrupt the early development of the embryo [18]. In this study, it was observed that drone spermatozoa were resistant to the freeze-thaw process. Besides, our DNA integrity rates statistically the same at post-thaw for all groups (P>0.05).

Oxidative damage may be evaluated by MDA levels which is a key product of polyunsaturated fatty acid's peroxidation in the cells. In our study, MDA levels in the LC5 group were lower than that of the control group (P<0.05).

The results of this study indicated that the 5 mM LC supplemented extender was the optimal for the drone semen cryopreservation process. However, the fertilizing ability of spermatozoon is crucial, and further studies must

be focusing on the effect of LC on reproductive success (viable off spring) when used to fertilize the queens.

Conflict of Interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Author Contributions

SA, EA, MA, HS designed the experiment. SA, SC, IC, AA, MY, performed the experiment. BU, ST, ZN analyzed the data. HS, ZN made tables, and wrote the paper. AA, SA revised the manuscript. All authors reviewed and approved the final manuscript.

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RESEARCH ARTICLE

Activity of Disinfecting Biocides and Enzymes of Proteases and Amylases on Bacteria in Biofilms

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Abstract

The presence of microbial biofilms on the surfaces of medical instruments, operating equipment, prostheses, catheters, technological lines in the food industry is a fact that contributes to the infection of the macroorganism and contamination of raw materials and products. The aim of the work was to investigate the effect of disinfecting substances Vantocilu TG and Catamine AB and their combination with enzymes on bacteria in biofilms. In the experiments, we used disinfecting substances Vantocil TG (Arch Biocides LTD, Great Britain) and Catamine AB (Intersintez, Ukraine). Enzymes: Everlase 16 L and Termamyl 300 L (Novozymes, Denmark). It was found that bacteria in biofilms withstood the minimum bactericidal concentration of Vantocil and Catamine, which was set on their planktonic forms. From one mL of wash from the biofilm after exposure to Vantocil were isolated from 1.9×10^3 to 4.3×10^3 microbial cells, and after treatment with Catamine from 5.6×10^3 to 1.7×10^4. At the same time, after treatment of biofilms with Vantocil and Catamine together with enzymes, a decrease in the number of *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* cells was observed, on average by two orders up to 10^1 CFU/mL, compared with treatment with biocides only. That is, there is a clear synergy of enzymes and biocides, which ultimately has a more detrimental effect on bacteria in biofilms.

Keywords: *Vantocil TG, Catamine AB, Enzymes, Biofilm degradation*

Dezenfektan Biyositler ve Proteaz ve Amilaz Enzimlerinin Biyofilmlerdeki Bakteriler Üzerine Aktivitesi

Öz

Gıda endüstrisinde tıbbi cihazların, ameliyat ekipmanlarının, protezlerin, kateterlerin, teknolojik alanların yüzeylerinde mikrobiyal biyofilmlerin varlığı, makroorganizma enfeksiyonlarına ve hammadde ve ürünlerin kontaminasyonuna katkıda bulunan bir gerçektir. Bu çalışmanın amacı, dezenfektan maddeler Vantocilu TG ve Catamine AB'nin ve bunların enzimlerle kombinasyonlarının biyofilmlerdeki bakteriler üzerine etkisini araştırmaktır. Deneylerde, Vantocil TG (Arch Biocides LTD, İngiltere) ve Catamine AB (Intersintez, Ukrayna) dezenfektan maddeleri ile Everlase 16 L ve Termamyl 300 L (Novozymes, Danimarka) enzimlerini kullandık. Biyofilmlerdeki bakterilerin, planktonik formlarına karşı uygulanan Vantocil ve Catamine'nin minimum bakterisidal konsantrasyonlarına dayanıklılık gösterdiği saptandı. Vantocil ile sağaltımdan sonra bir mL biyofilm yıkantısından $1.9\text{x}10^3$ ile $4.3\text{x}10^3$ arası mikroorganizma ve Catamin ile sağaltımdan sonra $5.6\text{x}10^3$ ile $1.7\text{x}10^4$ arası mikroorganizma izole edildi. Aynı zamanda, biyofilmlerin enzimlerle birlikte Vantocil ve Catamine ile sağaltımından sonra, *Staphylococcus aureus, Escherichia coli* ve *Pseudomonas aeruginosa* bakteri sayılarında sadece biyositlerin kullanıldığı sağaltım ile kıyaslandığında ortalama olarak 10^1 CFU/mL'ye kadar iki kat bir azalma gözlendi. Sonuçta, biyofilmlerdeki bakteriler üzerine daha hasar verici bir etkiye sahip açık bir enzim ve biyosit sinerjisi mevcuttu.

Anahtar sözcükler: *Vantocil TG, Catamine AB, Enzimler, Biyofilm degradasyonu*

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Introduction

Disinfection, as a component of all hygienic measures, in medical, veterinary and food industries is aimed at the destruction of opportunistic and infectious pathogen microorganisms to prevent infection of humans, animals and to produce safe food. Therefore, the pharmaceutical industry is constantly working to create ideal disinfectants that have a wide range of antimicrobial action in minimal concentrations, and not cause resistance in bacteria, are non-toxic, non-corrosive, non-allergenic, cheap, etc. [1-4]. However, despite the large number of disinfectants on the market, an ideal drug does not exist, as microorganisms adapt quite quickly to new antibacterial substances [5-7].

Bacterial resistance to biocides may be associated with their presence in the biofilm [8-16]. The modern generalized term "biofilm" is used to define the set of bacteria and products of their metabolism at the interface between solid and liquid phases attached to the surface in an aqueous or water-saturated medium [14,17]. Today, most scientists recognize that a significant number of microorganisms in natural and artificial environments exist in the form of structured, attached to the surface formations-biofilms [18-20]. Bacteria in the biofilm are surrounded by their own producing matrix (EPS), which consists of polysaccharides, proteins, uranium acid and humic substances [21-23]. It is due to the matrix, which acts as a barrier that protects bacterial cells inside, many antimicrobial agents cannot penetrate the biofilm [18,24,25].

The presence of bacteria in the biofilm creates serious problems with infection of various surfaces in human and veterinary medicine and the food industry [26,27]. Bacteria in biofilms are much more difficult to destroy with antimicrobial drugs, which can potentially lead to the accumulation and spread of dangerous pathogens. It is reported that the concentration of biocide, which is necessary to kill microbial cells in the biofilm, should be several times higher than the working for this agent [5,28,29]. Therefore, efforts are constantly being made to improve the performance of existing disinfectants or to develop new ones to affect microorganisms in the biofilm state.

Studies found that disinfection with chlorine dioxide and chlorine-containing agents reduced the number of planktonic bacteria in a good way, but had little effect on the content of bacteria in biofilms [18,30]. Perumal et al.[31] found that disinfectants based on hydrogen peroxide in working concentrations did not affect clinical isolates of *Acinetobacter spp., Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, which were in biofilms and were isolated in medical institutions. However, planktonic forms of these bacteria were sensitive to these biocides. The authors argue the need to test the effectiveness of disinfectants on biofilm bacteria, rather than planktonic, as this poses a threat for the use of such agents to control the spread of these pathogens.

Therefore, given the role of the matrix in protecting microbial cells from the action of biocides, researchers are looking for different methods for its destruction [32,33]. One such method is the use of enzymes to destroy the extracellular matrix of the biofilm. Studies have shown that enzymes have been significantly effective in reducing the density of *P. aeruginosa* biofilm and its degradation from various surfaces [8,34]. In particular, there were used synthetic polysaccharides to destroy the matrix of biofilms formed by pseudomonads [34-37], used microbial amylase and proteases for destruction of biofilms of gram-positive and gram-negative bacteria. However, researchers are inclined to the opinion that due to the heterogenicity of the composition of the biofilm matrix, the use of mono-enzymes has a limiting potential.

Therefore, for the effective use of enzyme agents in practice, it is necessary to comprehensively study the process of growth and development of biofilm in a particular object with knowledge of the approximate composition of possible microflora. In addition, it is advisable to combine different classes of enzymes with biocidal substances for better contact of the latter with bacterial cells. Therefore, the use of enzymes in combination with antibacterial substances to degrade the biofilm and reduce the content of microorganisms is promising and important in many sectors of the economy. The purpose of the study was to investigate the effect of disinfectants Vantocil TG and Catamine AB and their combination with enzymes on bacteria in biofilms.

Material and Methods

The study contained disinfectants Vantocil TG-20%-an aqueous solution of polyhexamethylenebiguanidine hydrochloride (Arch Biocides LTD, UK) and Catamine AB - a solution containing 49-51% of alkyldimethylbenzylammonium chloride (Intersynthesis, Ukraine), proteolytic enzyme-Everlase 16 L and amylolytic enzyme-Termamyl 300 L (Novozymes, Denmark), strains of test cultures of *Escherichia coli* (055K59 No.3912/41), *Staphylococcus aureus* (ATCC 25923) and *P. aeruginosa* (27/99). Stainless steel plates of the AISI 321 brand in the size of 30×30 mm for cultivation of biofilms.

The minimum bactericidal concentration of disinfectants was determined by the standard suspension method [3].

The density of microbial biofilms and the effect of disinfectants and enzymes on them were determined according to the guidelines [16]. Briefly: Biofilms of bacterial test cultures were grown on sterile stainless-steel plates in petri dishes for 24 h in plain broth with 1% glucose concentration. The plates with biofilms were then washed three times with sterile phosphate buffer to remove planktonic cells and the plates were dried. Disinfectants or enzymes were added to petri dishes with plates and kept for 15 min. The plates were removed, washed with

phosphate buffer and the biofilms were fixed with 96° ethyl alcohol for 10 min. Then the biofilms were stained with a solution of crystalline violet for 10 min. After that, the plates with biofilms were washed three times with phosphate buffer to remove paint residues. Then 5.0 cm^3 of 96° ethyl alcohol was added to a petri dish with a plate and left for 20-30 min, shaking periodically. The optical density of the alcohol solution was measured spectrophotometrically at a wavelength of 570 nm. At the optical density of the washing solution up to 0.5 units, the density of the formed biofilm was considered low, from 0.5 to 1.0 units -average and at a density of solution more than 1.0 units the density of the formed biofilm was considered high [19].

To determine the number of bacteria in the biofilm after exposure to biocides and enzymes, washes were removed from the plates using a sterile swab. Ten-fold dilutions of the wash were then prepared and 1.0 cm^3 of each dilution was sown in petri dishes, plated with plain broth and incubated at 37°C for 24-48 h. Before use, the enzymes were dissolved in 0.1 M phosphate buffer, pH 8.3.

Statistical Analysis

Statistical processing of the results was carried out using methods of variation statistics using the program Statistica 9.0 (StatSoft Inc., USA). Non-parametric methods of research were used (Wilcoxon-Mann-Whitney test). The arithmetic mean (x) and the standard error of the mean (SE) were determined. The difference between the comparable values was considered to be significant for $P<0.05$.

Results

At the first stage of the study, we determined the minimum bactericidal concentration of Vantocil TG and Catamine AB in the suspension method on planktonic forms of bacteria during 15 min of action at a solution temperature of 20±1°C. It was found *(Table 1)* that Vantocil TG showed a better antimicrobial effect on gram-negative bacteria *(E. coli* and *P. aeruginosa),* compared with gram-positive bacteria *(S. aureus)*. In particular, the minimum bactericidal concentration of Vantocil against *S. aureus* was 4.5 times higher, compared with test cultures of *E. coli* and *P. aeruginosa*.

At the same time, Catamine AB had a better effect on gram-positive microflora than on gram-negative. The minimum bactericidal concentration of Catamine relative to test cultures of *S. aureus* was 2.0 times lower compared to the cultures of *E. coli* and 4.0 times compared to *P. aeruginosa*.

It was also found that Vantocil TG acts bactericidal in much lower concentrations compared to Catamine. In particular, the minimum bactericidal concentration of Vantocil relative to test cultures of *S. aureus* was 6.9 times lower than that of Catamine. To inhibit *E. coli* and *P. aeruginosa* cells, the minimum bactericidal concentration of Vantocil was 62 and 125 times lower, respectively, than the concentration of Catamine.

It is believed that the planktonic state of bacteria is intended for the colonization of other surfaces or substrates, and microorganisms are mainly in the biofilm state in the synthesized matrix, which performs a protective function. In fact, the presence of bacteria in the peptide glycolytic matrix of the biofilm and in the depressions of the surface roughness prevents the penetration of disinfectants into the cells [16,18]. Therefore, for effective antimicrobial action of biocides, it is necessary to destroy the bacterial biofilm and ensure maximum contact of the microbial cell with the disinfectant [25]. Given this phenomenon, the next step in our work was to investigate the effect of disinfectants Vantocil TG and Catamine AB in combination with enzymes on bacteria in biofilms. Vantocil TG and Catamine AB were used in concentrations that provided a bactericidal effect on planktonic bacteria *(Table 1)*. Enzymes were used at a concentration that provided maximum proteolytic and amylolytic activity at a temperature of +20±1°C for 15 min of exposure.

The results of studies of the effect of Vantocil TG and enzymes on biofilms formed by *S. aureus* are shown in *Fig. 1*.

It was found that under the action of Vantocil the matrix of the *S. aureus* biofilm was destroyed, which is indicated by a 1.5-fold decrease ($P<0.05$) in the optical density of the biofilm washing solutions. However, the biofilm was still of high density - more than 1.0 unit. Treatment of the biofilm with the proteolytic enzyme Everlase 16 L more intensively destroyed the matrix compared to Vantocil, as the density decreased by 2.4 times ($P<0.05$), i.e. to medium density. This indicates the presence in the matrix of the biofilm of a significant number of peptide components. The effect on biofilms with amylase Termamyl 300 L also significantly destroyed the matrix, its density decreased by 2.1 times ($P<0.05$) relative to the average density. However, the degradation of the biofilm under the influence of Vantocil in combination with the enzymes Everlase 16 L and Termamyl 300 L was the most intensive - the optical density of the washing solutions decreased 4.1 times ($P<0.05$) and the biofilm was considered of low density (less than 0.5 units).

Table 1. *Minimum bactericidal concentration of Vantocil TG and Catamine AB on test cultures of S. aureus, E. coli, P. aeruginosa at an exposure of 15 min and a solution temperature of 20±1°C*

Test Cultures	Concentration of Solutions, %	
	Vantocil TG	Catamine AB
S. aureus	0.009	0.062
E. coli	0.002	0.125
P. aeruginosa	0.002	0.250
n =15		

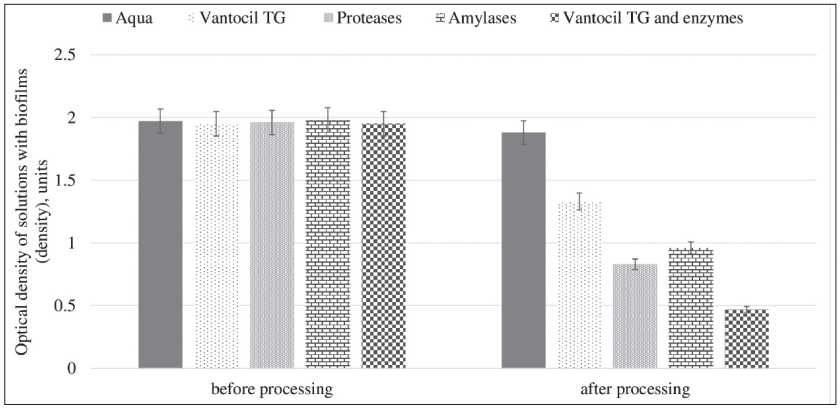
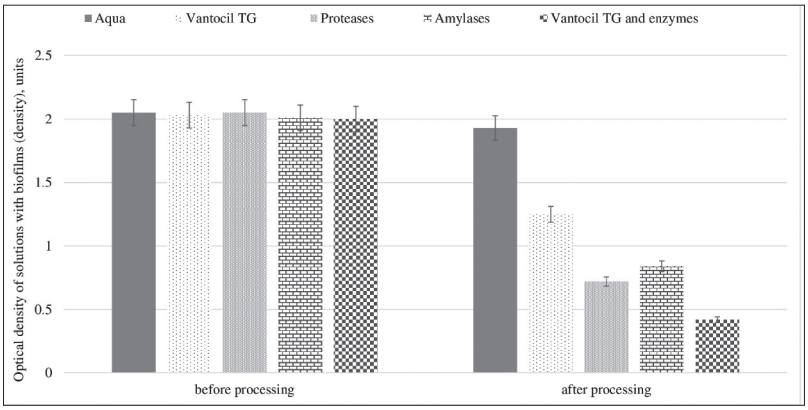


Fig 1. The effect of Vantocil TG and enzymes on biofilms formed by *S. aureus* (action for 15 min at a solution temperature of 20±1°C)

Fig 2. The effect of Vantocil TG and enzymes on biofilms formed by *E. coli* (action for 15 min at a solution temperature of 20±1°C)



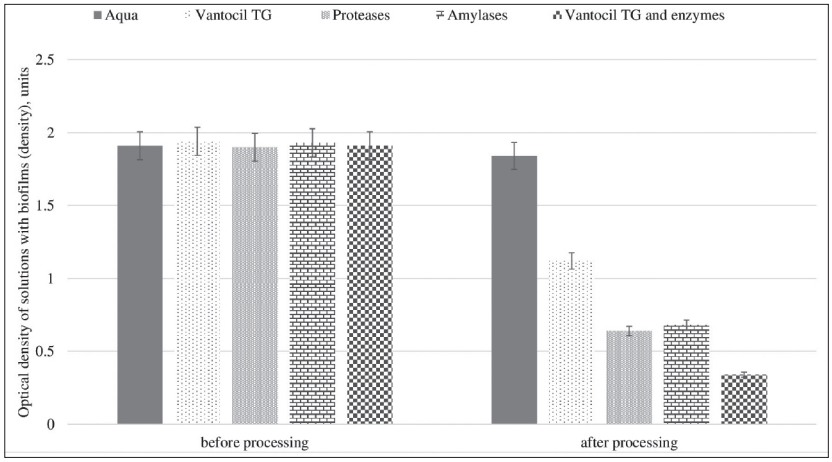


Fig 3. The effect of Vantocil TG and enzymes on biofilms formed by *P. aeruginosa* (action for 15 min at a solution temperature of 20±1°C)

The study of the effect of Vantocil TG and enzymes on biofilms formed by *E. coli* is shown in *Fig. 2*.

A more intensive degradation process of *E. coli* biofilm under the influence of Vantocil and enzymes than *S. aureus* biofilm was revealed. In particular, under the action of Vantocil, the optical density of the biofilm decreased 1.6 times (P<0.05), and under the influence of enzymes Everlase 16 L and Termamyl 300 L 2.8 and 2.4 times (P<0.05), respectively. In this case, after the action of enzymes, the biofilms became of medium density. However, the greatest degradation of the matrix of the biofilm of *E. coli* was observed under the simultaneous influence of Vantocil and enzymes - the optical density of solutions from the biofilm decreased by 4.8 times (P<0.05) and the biofilms became of low density.

The effect of Vantocil TG and enzymes on biofilms formed by *P. aeruginosa (Fig. 3)* showed a similar pattern as the effect on biofilms of *S. aureus* and *E. coli*. However, the matrix of the biofilm of *P. aeruginosa* was more susceptible to destruction than *S. aureus* and *E. coli*. In particular, under the influence of Vantocil, the optical density of biofilm solutions decreased 1.7 times (P<0.05), and under the action of proteolytic and amylolytic enzymes 3.0 and 2.8 times (P<0.05), respectively. However, biofilms of *P. aeruginosa* became of low density only when simultaneously treated with Vantocil and enzymes - 0.34±0.2 units.

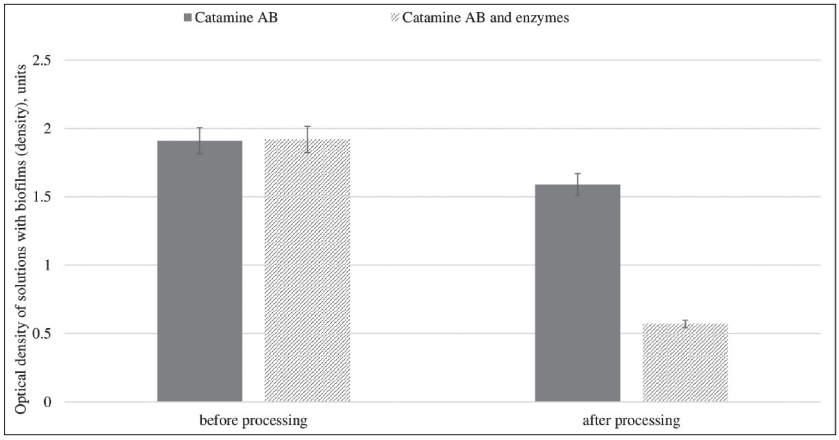
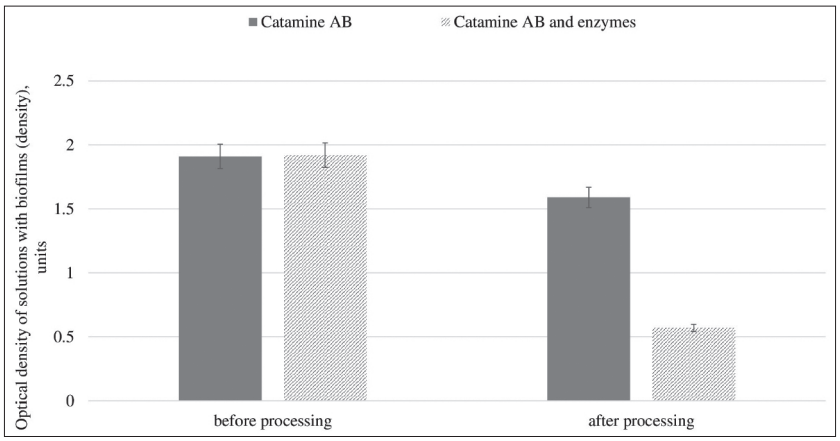


Fig 4. The effect of Catamine AB and its combination with enzymes on biofilms formed by *S. aureus* (action for 15 min at a solution temperature of 20±1°C)

Fig 5. The effect of Catamine AB and its combination with enzymes on biofilms formed by *E. coli* (action for 15 min at a solution temperature of 20±1°C)



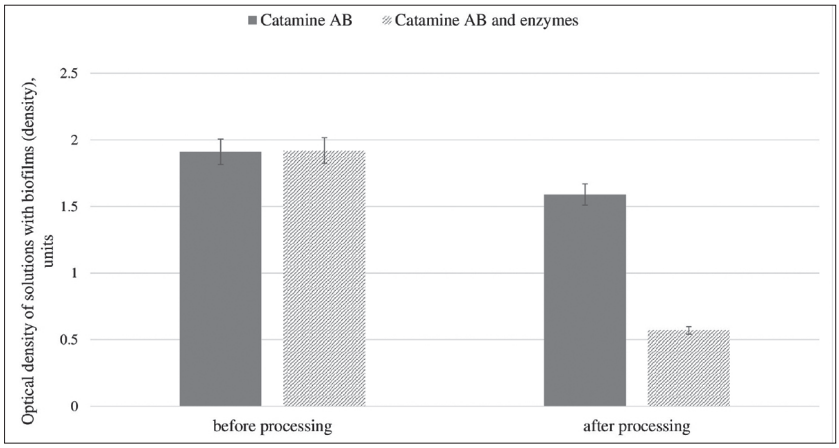


Fig 6. The effect of Catamine AB and its combination with enzymes on biofilms formed by *P. aeruginosa* (action for 15 min at a solution temperature of 20±1°C)

Therefore, the obtained experimental data indicate that the disinfectant Vantocil TG weakly destroys the matrix of biofilms formed by bacteria *S. aureus, E. coli* and *P. aeruginosa*. At the same time, the simultaneous use of Vantocil with proteolytic and glycolytic enzymes leads to significant degradation of the biofilm in the studied bacteria.

In addition to disinfectants based on polyhexamethylen-ebiguanide hydrochloride, drugs, containing quaternary ammonium compounds, in particular Catamine AB, are widely used in Ukraine and abroad. Therefore, the next part of the work was to determine the effect of Catamine and its action with enzymes on microbial biofilms. The results of the study are shown in *Fig. 4, 5, 6*.

It was found that Catamine in the minimum bactericidal concentration for planktonic cultures to a lesser extent destroyed the biofilms of *S. aureus, E. coli* and *P. aeruginosa*, compared with Vantocil. It was found that biofilms of *S. aureus* were more intensively degraded by Catamine than biofilms of *E. coli* and *P. aeruginosa*. In particular, the optical density of solutions from *S. aureus* biofilms after Catamine treatment decreased 1.4 times ($P<0.05$), and in *E. coli*

Table 2. *Influence of disinfectants and enzymes on the quantitative content of microbial cells in biofilm (action for 15 min at a solution temperature of 20±1°C)*

Studied Bacteria	Bacterial Status	Bacterial Count in 1 cm³ Suspension from Biofilm, CFU				
		Control	Vantocil	Vantocil with Enzymes	Catamine AB	Catamine AB with Enzymes
S. aureus	plankton	$1.1\pm0.1\times10^{7}$	0	0	0	0
	biofilm	$5.2\pm0.2\times10^{8}$	$4.3\times10^{3*}$	$5.1\times10^{1*}$	$5.6\times10^{3*}$	$4.4\times10^{1*}$
E. coli	plankton	$3.4\pm0.2\times10^{7}$	0	0	0	0
	biofilm	$4.9\pm0.1\times10^{8}$	$2.5\times10^{3*}$	$1.7\times10^{1*}$	8.2×10^{3}	$7.8\times10^{1*}$
P. aeruginosa	plankton	$2.8\pm0.1\times10^{7}$	0	0	0	0

** – P< 0.05 – concerning control*

and *P. aeruginosa* biofilms 1.3 and 1.2 times, respectively. In addition, the biofilms of all bacteria sampled after Catamine treatment remained of high density.

The combination of the action of Catamine with enzymes Everlase 16 L and Termamyl 300 L significantly increased the degradation of the biofilm in both gram-positive and gram-negative bacteria. In particular, under this effect on the biofilms of *S. aureus*, the optical density of the washing solutions decreased 4.1 times (P<0.05) and the biofilms became of low density (0.47±0.2 units). Biofilms of gram-negative bacteria *E. coli* and *P. aeruginosa* degraded less even under the influence of Catamine with enzymes than biofilms of *S. aureus*. The decrease in the optical density of solutions from biofilms in these bacteria was 3.7 and 3.4 times, respectively (P<0.05). The density of biofilms was on the border between low and medium - 0.54-0.57 units, respectively.

In general, the obtained data show that Catamine has a weaker effect on the matrix of the biofilm of *S. aureus, E. coli* and *P. aeruginosa*, compared with Vantocil. However, when combining disinfectants Vantocil TG, Catamine AB with proteolytic and glycolytic enzymes, synergism is manifested in more intensive degradation of biofilms of gram-positive and gram-negative bacteria, their density decreases from high to low.

It is believed that the concentration of antibacterial substance, required for the destruction of bacteria in the biofilm, should be several times higher than the minimum bactericidal value determined on planktonic bacteria. It was important to investigate the effect of disinfectants at the minimum bactericidal concentration found on planktonic bacteria and in combination with enzymes on the quantitative content of microorganisms in the biofilm. The research results are given in *Table 2*.

It was found that bacteria in biofilms withstood the minimum bactericidal concentration of Vantocil and Catamine, which was established on their planktonic forms. From 1.9×10^{3} to 4.3×10^{3} microbial cells were isolated from one ml of biofilm wash after exposure to Vantocil, which is almost five orders less than in the control. At the same time, after the action of Vantocil with enzymes, a decrease in the number of *S. aureus, E. coli* and *P. aeruginosa* cells was observed, on average by two orders up to 5.1×10^{1} CFU/mL, compared with treatment with Vantocil alone.

After treatment of biofilms with Catamine, slightly more bacteria were isolated than after treatment with Vantocil, in particular, the content of *S. aureus* cells was 1.3 times higher (P<0.05), *E. coli* 3.3 times (P<0.05), and *P. aeruginosa* by almost one order (1.7×10^{4} CFU/mL of wash). The simultaneous action of Catamine with enzymes caused a decrease in the number of bacteria in the biofilm by two orders, compared with the action of Catamine alone. However, 10^{1} microbial cells were isolated from *S. aureus* and *E. coli* biofilms and 10^{2} from *P. aeruginosa* biofilms, indicating less destruction of the biofilm matrix by disinfectant and enzymes and protection of cells from contact with the biocide.

Discussion

The presence of microbial biofilms on the surfaces of medical instruments, operating equipment, prostheses, catheters, production lines in the food industry is an obvious fact that contributes to microorganism infection and contamination of raw materials and products [19,26,27]. Therefore, the use of biocides is aimed at the destruction of planktonic and biofilm forms of microorganisms on various surfaces [1,3,4,8]. However, successful control of microorganisms, present in biofilms, is possible with the use of disinfectants that destroy the exopolysaccharide matrix and promote closer contact of bacteria with the biocide [34]. Among the significant range of disinfectants, a significant part of them contains as active substances - biguanides and quaternary ammonium compounds. In this study, we determined the effect of disinfectants Vantocil TG and Catamine AB and enzymes Everlase 16 L and Termamyl 300 L on the degradation of biofilm matrix. It was found that Vantocil TG in the minimum bactericidal concentration, which was determined on planktonic bacteria, reduced the density of the biofilm of *S. aureus* by 1.5 times, *E. coli*-1.6 times and *P. aeruginosa*-1.7 times, comparing with the control before processing. This indicates that the

exopolysaccharide matrix of biofilms contains components that are poorly degraded by this biocide. At the same time, treatment of biofilms with proteolytic and amylolytic enzymes significantly reduced their density. In particular, after treatment with enzyme Everlase 16 L, the density of the biofilm of *S. aureus* decreased 2.4 times, *E. coli* - 2.8 times and *P. aeruginosa* - 3.0 times. Matrix degradation was less effective with Termamyl 300 L biofilms than with Everlase 16 L. In particular, the density of *S. aureus, E. coli,* and *P. aeruginosa* biofilms decreased 2.1, 2.4, and 2.8 times, respectively. This indicates the heterogeneous chemical composition of the biofilm in different bacteria and for their destruction it is necessary to use enzymes of different classes [34,35]. According to [14,17,21-23] the composition of the biofilm matrix depends on many factors, the availability of nutrients, species composition of microflora, pH of the medium, type of surface, etc. Due to this, the protective function of even one species of bacteria in the biofilm will be different. In addition, a study [36] reported that the degradation of the biofilm of *P. aeruginosa* by the Savinase enzyme was stronger than with Alphamylase treatment, with better proteolytic enzyme matrix destruction. When treating biofilms with Vantocil with enzymes revealed a synergism of action, in particular, the optical density of solutions from biofilms of *S. aureus, E. coli* and *P. aeruginosa* decreased by 4.1, 4.8, 5.6 times compared with the control, and the biofilms became of low density. Synergism of different enzymes in the fight against heterogeneous biofilms was reported [21-23,34]. Despite the fact that Catamine in the minimum bactericidal concentration for planktonic cultures destroyed the biofilms of *S. aureus, E. coli* and *P. aeruginosa* to a lesser extent, compared with Vantocil, the general patterns of exposure to biofilms of Catamine with enzymes were the same as for treatment with Vantocil.

During the study of the effect of disinfectants on the quantitative content of microorganisms in the biofilm, it was found that from one mL of wash from the biofilm after exposure to Vantocil were isolated from 1.9×10^3 to 4.3×10^3 microbial cells, and after treatment with Catamine- from 5.6×10^3 to 1.7×10^4. The results confirm the data of many researchers [18,28-31] that the determined minimum bactericidal concentration on planktonic bacteria does not have a bactericidal effect on biofilm forms. At the same time, after treatment of biofilms with Vantocil and Catamine together with enzymes, a decrease in the number *of S. aureus, E. coli* and *P. aeruginosa* cells was observed, on average by two orders to 10^1 CFU/mL, compared with treatment with biocides only.

There is a clear synergy of enzymes and biocides, which ultimately has a more detrimental effect on bacteria in biofilms. In this case, it can be argued that enzymes destroy the matrix of the biofilm, which promotes better contact of antibacterial substances with target cells. Therefore, we believe that the combination of antibacterial substances with enzymes is a good prospect in the fight against bacteria in biofilms on the surfaces of various materials. When choosing a disinfectant, it is necessary to evaluate its effectiveness against bacteria in biofilms under conditions close to production.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

MK, VK and VH conceived and executed the idea, designed experiments, analyzed results and a deep revision of the manuscript. ZM, YH, TY and SK collected samples, performed experiments, contributed to tand implementation of the research. All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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RESEARCH ARTICLE

Evaluation of the Analytical Efficiency of Real-Time PCR in the Diagnosis of Brucellosis in Cattle and Sheep

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Abstract

Brucellosis is an important infectious disease that affects animal and public health in many developing countries, including Turkey. The control and eradication of brucellosis are contingent upon methods that provide a fast and reliable diagnosis. In this context, molecular methods which enable the enzymatic amplification of bacterial conserved gene regions are advantageous. In this study, samples of cattle and sheep blood serum sent to our laboratory from enterprises at risk of brucellosis in different settlements of the Central Anatolia and the Black Sea Regions of Turkey were analyzed. In our study, we aimed to investigate brucellosis using serological methods and Real-Time PCR (RT-PCR), and to analyze these methods comparatively. To this end, RBPT, SAT and CFT tests, as well as the Brucella RT-PCR, which enables the amplification of the BCSP31 gene found in all Brucella species, were used. In the 368 serum samples analyzed, Brucella positivity was determined as 11.41%, 10.05% and 9.8% by RBPT, SAT and CFT, respectively, while the RT-PCR gave the same rate of positivity (9.8%) as CFT. When CFT is taken as a reference test, the sensitivity, specificity, positive and negative predictive value and diagnostic accuracy of the RT-PCR were determined as 100%; and it was found to be perfectly compatible with CFT, with a kappa value of 1.000. The number of bacterial genomes that could be detected by the RT-PCR in the presence of DNA of the Brucella melitensis biotype 3 (Ether) reference strain was determined as 3.94x103 copies. Linear regression analysis revealed that the amplification efficiency (92.71%) of the RT-PCR was within the desired limits (90-110%) and that the RT-PCR was repeatable (CV 2.9%) and reproducible (CV 1.8%). This study indicates that the RT-PCR is a useful method for application in the diagnosis of bovine and sheep brucellosis thanks to its high analytical efficiency; it also emphasizes the importance of blood serum samples as preferable clinical materials in this context.

Keywords: *Brucella spp., Brucellosis, Cattle, Sheep, Real-Time PCR, Analytical competence*

Sığır ve Koyunlarda Brusellozisin Tanısında Real-Time PCR'nin Analitik Yeterliliğinin Değerlendirilmesi

Öz

Brusellozis, ülkemizle birlikte gelişmekte olan birçok ülkede hayvanlarda ve halk sağlığı açısından önem arzeden infeksiyöz bir hastalıktır. Brusellozisin, kontrol ve eradikasyonunda hızlı ve güvenilir teşhis imkânı sunan yöntemlere her zaman ihtiyaç duyulmaktadır. Bu kapsamda, bakterilerin korunaklı gen bölgelerinin enzimatik amplifikasyonunu sağlayan moleküler yöntemler avantaj sağlamaktadır. Bu çalışmada, İç Anadolu ve Karadeniz Bölgelerine ait farklı yerleşim birimlerindeki brusellozis riski taşıyan işletmelerden laboratuvarımıza gönderilen sığır ve koyunlara ait kan serum örnekleri incelenmiştir. Çalışmamızda, brusellozisin serolojik yöntemler ve Real-Time PCR (RT-PCR) ile araştırılması ve bu yöntemlerin karşılaştırmalı analizi amaçlanmıştır. Bu amaçla, RBPT, SAT ve KFT testleri ile birlikte tüm Brucella türlerinde bulunan BCSP31 geninin amplifikasyonunu sağlayan Brusella RT-PCR metodu kullanılmıştır. İncelenen 368 serum örneğinde RBPT, SAT ve KFT ile Brucella pozitifliği sırayla %11.41, %10.05 ve %9.8 belirlenirken, RT-PCR analizi sonucu, KFT ile aynı oranda (%9.8) pozitiflik elde edilmiştir. KFT referans test olarak dikkate alındığında, RT-PCR'nin sensitivite, spesifite, pozitif ve negatif prediktif değerleri ve tanı doğruluğu %100 ve 1.000'lık kappa değeri ile KFT ile mükemmel derecede uyumlu olduğu saptanmıştır. Brucella melitensis biyotip 3 (Ether) referans suşuna ait DNA eşliğinde RT-PCR ile saptanabilen bakteriyel genom sayısı 3.94x103 kopya olarak belirlenmiştir. Gerçekleştirilen linear regresyon analizi ile RT-PCR'nin amplifikasyon verimliliği, istenen sınırlar (%90-110) arasında (%92.71) yer almış ve tekrarlanabilir (CV %2.9) ve üretilebilir (CV %1.8) olduğu belirlenmiştir. Bu çalışma ile yüksek analitik verimliliği sayesinde RT-PCR'nin sığır ve koyun brusellozisin teşhisinde başvurulabilir bir yöntem olduğu öngörülmüş ve bu kapsamda kan serum örneklerinin tercih edilebilir klinik materyaller olarak önemi vurgulanmıştır.

Anahtar sözcükler: *Brucella spp., Brusellozis, Sığır, Koyun, Real-Time PCR, Analitik yeterlilik*

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Introduction

Brucellosis, caused by bacteria of the *Brucella* genus, is a disease that affects many animal species such as ruminants, pigs, dogs, foxes, and some marine mammals. Capable of being transmitted directly or indirectly to humans, it poses a risk to public health and is known as the most common zoonotic disease in the world. The predominant *Brucella* species in cattle is *Brucella abortus*, and in sheep *Brucella melitensis* [1]. These agents mostly settle in the uterus, placenta, fetus, and breast tissue in animals, and cause necrotic and inflammatory infections which lead to abortion, infertility and mastitis in affected animals. In addition to causing significant economic losses in the livestock industry through reduced fertility, decreased animal product and quality, and expensive protection and control practices [2].

Definitive diagnosis is important for the control of *Brucella* infection in animals and humans. Clinical diagnosis is generally based on the presence of reproductive system infections in livestock. However, this hypothetical diagnosis must be confirmed by laboratory methods. In this context, culture methods have been reported as the "gold standard" in the diagnosis of brucellosis. However, since the culture method is time-consuming and the *Brucella* agents have to be studied in biosafety level 3 laboratories, culture procedures are not always available. Serological tests are frequently used in the implementation of control and eradication programs of brucellosis. Some of them (Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT), etc.) are used as screening tests, while others (2-Mercaptoethanol, Complement Fixation Test (CFT) and ELISA) are used as confirmation tests. With its high diagnostic accuracy, CFT is used to confirm the diagnosis of *B. abortus* and *B. melitensis* infections and is recommended by the World Animal Health Organization (OIE) as a reference test for international animal mobility [3]. Although they have been continuously improved in terms of sensitivity and specificity, these tests have some disadvantages, such as their high cost, the need for special equipment and expert personnel, and the inability to distinguish between vaccinated and naturally infected animals. Molecular techniques are advantageous tools for the direct diagnosis of brucellosis and for the verification of agents identified by conventional methods, and can also be used in the investigation of bacterial variants and vaccine candidates, and to perform virulence assessment and epidemiological analysis [4]. Recently, several PCR methods have been reported that enable the amplification of conserved gene regions of *Brucella* agents, such as the 31-kDa surface protein *(BCSP31), 16S rRNA*, and the insertion sequence *IS711* [4-6]. Among these, Real-Time PCR (RT-PCR) method, which provides results simultaneous with the enzymatic amplification cycle of the target gene region, offers a more advantageous diagnosis with features such as high sensitivity and amplification efficiency, a short turnaround time, and no need for electrophoresis imaging [6,7].

This study aimed to evaluate the analytical efficiency of an *in-house* RT-PCR for the rapid and sensitive diagnosis of the *Brucella* genome in bovine and sheep blood sera. In this context, a comparative analysis was carried out between the test and the RBPT, SAT and CFT methods.

Material and Methods

Ethical Permission

The ethical permission of the study was ensured by the decision of The Republic of Turkey Ministry of Agriculture and Forestry, Veterinary Control Central Research Institute, Animal Experiments Local Ethics Committee with the code of "2021-01".

Study Material and Sampling

This study was carried out on blood serum samples from 368 animals, 311 cattle and 57 sheep, sent to the laboratories of the Veterinary Control Central Research Institute from enterprises with a recent history of abortion in the Central Anatolia and Black Sea Regions of Turkey *(Table 1)*. There was no history of vaccination against *Brucella* agents in the cattle and sheep whose blood samples were taken in the study. Abortion cases usually correspond to the last few

Table 1. *DNA concentrations and predicted genome numbers of positive control subdilutions*

Dilution	dsDNA Concentration (ng/µL)	The Number of Copies in 1 µL	The Number of Copies in 2 µL	Average Ct (±SD)	Coefficient of Variation (CV, %)	
					Intra-assay	Inter-assay
Stock	19.2	$5.4x10^6$	$1.08x10^7$	20.79±0.14	0.4	0.3
10^{-1}	1.3	$3.66x10^5$	$7.32x10^5$	21.88±1.21	6.4	6.4
10^{-2}	0.9	$2.53x10^5$	$5.06x10^5$	27.44±0.56	2.7	0.7
10^{-3}	0.1	$2.81x10^4$	$5.62x10^4$	31.26±0.26	0.8	1.2
10^{-4}	0.01	$2.81x10^03$	$5.62x10^3$	33.82±0.35	1.2	1.1
10^{-5}	0.007	$1.97x10^3$	$3.94x10^3$	35.83±0.93	3.6	1.1
10^{-6}	0.0004	$1.12x10^2$	$2.24x10^2$	ND*	-	-

** **Not determined:** Ct value was taken as ≥36 in regression analysis for samples that do not form an amplification curve and are considered negative*

months of pregnancy in animals, and blood samples were taken at 2 to 4 weeks following the abortion. The blood samples were centrifuged at 3.000 rpm for 10 min; one aliquot of the obtained serum samples was stored at -80°C for serological analysis and another for the RT-PCR.

RT-PCR Analysis

DNA extraction from samples: DNA extraction from serum samples was carried out using a commercially available nucleic acid purification kit (QIAampDNA Mini Kit, Qiagen, Germany) in accordance with the manufacturer's instructions. DNA concentrations were determined by measuring at 260 and 280 nm wavelengths with a NanoDrop (ND-1000 spectrophotometer, Marshall Scientific, USA).

Analysis of samples: The RT-PCR analysis of the serum samples was performed on a RT-PCR Detection System (CFX96, BioRad, USA). A RT-PCR test (Bioeksen Ar-Ge Teknolojileri Ltd.® Istanbul) that enables the amplification of the *BCSP31* gene region of *Brucella* species was used. The reaction mixture prepared in a volume of 11 µL for each sample was composed of 5 µL RT-PCR master mix, 3 µL *Brucella* spp. oligo mix, 1 µL internal control DNA and 2 µL template DNA components. The thermal cycle was set as 5 min pre-denaturation at 95°C, 45 cycles consisting of denaturation at 95°C for 15 sec, and binding and elongation at 60°C for 40 sec. In the RT-PCR, FAM-labeled *Brucella* spp. targeted oligonucleotides and HEX-labeled internal control (IC), which provides sample-based inhibition control and kit reagent control, were used. Thus, two amplification curves were obtained for each positive sample and only IC results for negative samples. All positive and negative serum samples were studied in duplicate. The RT-PCR was performed in the presence of negative control (RNAse/DNAse free water) and positive control (in the kit). Samples with no amplification or a cycling threshold (Ct) of 36 or greater were considered negative.

Analytical capability of the RT-PCR: The *Brucella melitensis* biotype 3 (Ether) reference strain (NCTC 10505) was used to test the analytical ability of the RT-PCR. Bacterial DNA extraction was performed with a commercially available nucleic acid purification kit (QIAampDNA Mini Kit, Qiagen, Germany). The total amount of double-stranded DNA (dsDNA) was determined using a NanoDrop ND-1000 spectrophotometer and the total number of DNA copies was then calculated using an interactive program [8] with the formula: DNA copy number=(DNA amount (ng) x $6.022x10^{23}$)/(DNA length (bp) x $1x10^{9}$ x 650 Dalton). In this calculation, the DNA length of *B. melitensis* was taken as 3.294.931 bp [9]. Intra-assay repeatability was tested by calculating Ct values following three amplifications of stock and 10^{-5} DNA copies of the *B. melitensis* biotype 3 (Ether) by the RT-PCR. Inter-assay reproducibility was determined by the Ct values obtained as a result of the amplification of positive control DNA and its sub-dilutions on two different days by the RT-PCR.

Serological Analysis

Rose Bengal Plate Test (RBPT): The RBPT was conducted according to the method reported by Alton et al.[10]. The test was carried out using the Brucella RBPT antigen produced at the İstanbul Pendik Veterinary Control Institute of the Ministry of Agriculture and Forestry of Turkey. The antigen was used to detect IgG specific for Smooth *Brucella* species *(B. abortus, B. melitensis* and *B. suis)*. 15 µL of the RBPT test antigen was dropped on a clean slide. 15 µL of the serum sample to be tested was taken and dropped next to the antigen. After mixing the antigen and serum samples, the reaction that occurred within 4-5 min on the slide was evaluated with the naked eye. Large/small precipitate (agglutination) like sand grains formed on the slide was evaluated as positive. The absence of any agglutination on the slide with the serum-antigen mixture remaining as a homogeneous suspension was evaluated as negative.

Serum Agglutination Test (SAT): The SAT was performed according to the method reported by Alton et al.[10]. In the test, Brucella Tube Agglutination Test Antigen, produced at the İstanbul Pendik Veterinary Control Institute of the Ministry of Agriculture and Forestry of Turkey, was used. The antigen was used to detect IgM and IgG in blood serum for smooth *Brucella* species *(B. abortus, B. melitensis* and *B. suis)*. The SAT was performed in the presence of positive and negative control sera. The lace-like precipitate (agglutination) formed at the bottom of the glass tubes was evaluated as positive. Dilution in the last tube in which the precipitate was seen was considered the serum antibody titer. During the evaluation, the degree of agglutination was expressed in IU and a serum sample containing 30 or more IU was considered positive.

Complement Fixation Test (CFT): The CFT was conducted according to the method reported by Alton et al.[10]. After preparing a 5-fold sub-dilution of the serum samples to be tested in strip tubes with Veronal buffer (VB), the samples were inactivated for 50 min in a 58°C water bath. 25 µL of VB was added to the wells on the microplate and 25 µL of serum samples were added to the wells. 25 µL of test antigen (CFT antigen, Istanbul Pendik Veterinary Control Institute of the Ministry of Agriculture and Forestry of Turkey) was added to wells from A to G. 50 µL of complement (Freeze-dried Guinea Pig Complement ID. Vet, France) was added to the wells from A to H. After incubation, 50 µL hemolytic system consisting of sheep erythrocytes (2%) and amboceptor (Virion/Serion 1: 1500 AMB) was added to the wells. The test limit titer was determined by observing the lysis occurring in the microplate wells. Serum dilutions containing 20 or more International Complement Fixation Units (ICFTU) per milliliter were accepted as positive [11].

Statistical Analysis

Programs enabling interactive calculation were used in the statistical analysis of the data. The sensitivity, specificity and

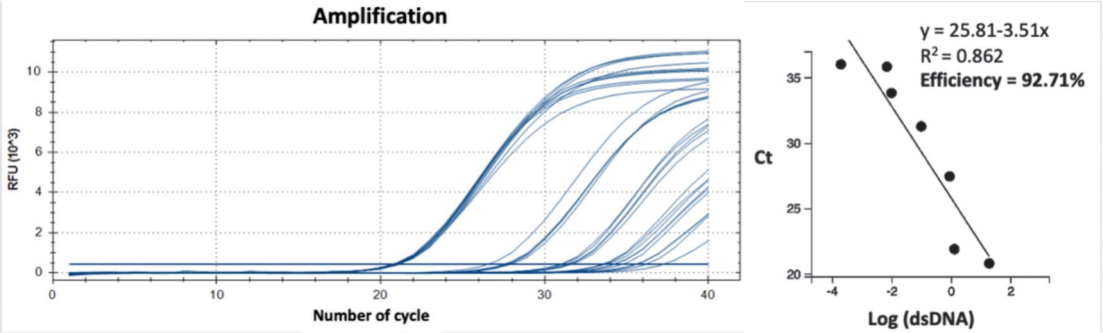

Fig 1. Amplification curves and linear regression analysis of the DNA dilutions of *B. melitensis* biotype 3 (Ether) strain

Table 2. *Distribution of samples according to settlements*

Province	Number of Samples	RT-PCR Positivity		P- Value
		n	%	
Çankırı	48	6	12.5%	>0.05
Ankara	189	19	10.05%	
Çorum	31	3	9.68%	
Kırşehir	12	0	0%	
Yozgat	37	3	8.11%	
Kastamonu	21	3	14.29%	
Karabük	5	1	20%	
Bartın	9	0	0%	
Nevşehir	7	0	0%	
Kırıkkale	9	1	11.11%	
Total	368	36	9.78%	

diagnostic accuracy analyzes of the tests were performed with the reported program [12]. Linear regression analysis was carried out with the qPCR Library Quantification program [13]. The coefficients of variation for the test repetitions (CV) were calculated with the reported program [14]. The Chi-square test was carried out with the reported program [15].

Results

RT-PCR Findings and Analytical Capability

The results of the RT-PCR revealed that 36 (9.78%) of 368 serum samples contained *Brucella* spp. DNA *(Fig. 1)*. The amount of template DNA (2 µL) used in the RT-PCR, the gene copy number of the *B. melitensis* biotype 3 (Ether) DNA dilutions between the stock and 10^{-6} varied $1.08x10^7$ to $2.24x10^2$. Thus, the number of bacterial genomes detectable (in other words limit of detection (LOD)) by the RT-PCR was calculated as $3.94x10^3$ copies, with a standard deviation of 0.93 and a variation coefficient of 0.026. In the linear regression analysis performed, the amplification efficiency (92.71%) of the RT-PCR was determined to be within the desired amplification efficiency limits (90-110%) with a y-cutpoint of 25.81 and a determination coefficient (R^2) of 0.862 *(Fig. 1)*. The intra-assay repeatability of the RT-PCR was determined by calculating the average coefficient of variation (CV), obtained by amplifying the *B. melitensis* biotype 3 (Ether) strain three times using different DNA dilutions between the stock and 10^{-5}; the average CV was determined as 2.9%. The inter-assay reproducibility of the RT-PCR was determined by calculating the average coefficient of variation (CV), obtained by amplifying the *B. melitensis* biotype 3 (Ether) strain two times on two different days with the stock to 10^{-5} sub-dilutions of control DNA; the average CV was determined as 1.8% *(Table 1)*.

Serological Analysis Findings

In this study, 42 (11.41%) of 368 blood serum samples were found to be positive in terms of *Brucella* spp. by RBPT, 37 (10.05%) by SAT and 36 (9.78%) by CFT *(Table 2)*. It was found that there was no significant difference between the serological tests in determining *Brucella* antibodies in cattle and sheep blood serum samples (Chi-square=0.602 and P=0.740). All samples that were found to be positive with CFT were also found positive by the RT-PCR. When CFT was taken as the reference test, the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of the RT-PCR were determined as

Table 3. *Diagnostic values of tests for cattle and sheep sera*

Test		Serological Tests		Analytic Diagnostic Values												
		Positive	Negative	Sensitivity		Specifity		PPD		NPD		Diagnostic Accuracy		Kappa		
				%	%95 CI	%	%95 CI	%	%95 CI	%	%95 CI	%	%95 CI	%	%95 CI	SE
RT-PCR	**RBPT**															
	Positive	36	0	85.71	71.46-94.57	100	98.87-100	100		98.19	96.28-99.13	98.37	96.49-99.40	0.914	0.846-0.982	0.035
	Negative	6	326													
	SAT															
	Positive	36	0	97.30	85.84-99.93	100	98.89-100	100		99.70	97.95-99.96	99.73	98.50-99.99	0.985	0.955-1	0.015
	Negative	1	331													
	CFT															
	Positive	36	0	100	90.26-100	100	98.90-100	100		100		100	99-100	1.000		
	Negative	0	332													

PPD: *positive predictive value,* ***NPD:*** *negative predictive value,* ***CI:*** *confidence interval,* ***SE:*** *standard error*

100%. It was observed that a perfect match was achieved, with a kappa value of 1.000, between the RT-PCR and CFT, which is used as a reference test in the detection of *Brucella* infections *(Table 3)*.

Discussion

Brucellosis is a zoonotic disease that is endemic in many parts of the world, including Turkey, and adversely affects animal production and public health. Brucellosis, which is rarely fatal, is highly contagious. In this respect, there is always a need for fast and reliable diagnostic methods to be used in the follow-up and eradication of the disease. Although the culture method that allows isolation of the agent is still known as the "gold standard" in the diagnosis of brucellosis, it is not widely used due to the time-consuming and biohazardous characteristics. The practical diagnosis of brucellosis in animals is mainly based on antibody-based serological methods. However, these methods have many negative features such as cross-reactions, which caused by vaccinated animals or close bacteria harbouring the same antigenic structures, restrict their use in routine diagnosis as mentioned [1,7]. The 31-kDa surface protein *(BCSP31)* gene is the most protected gene region among the *Brucella* members and has found widespread use in molecular diagnosis of infection in humans and animals [6,16]. In this study, an *in-house* RT-PCR kit was found to be lower ($3.94x10^3$ copies) than in the other studies [7,16,17]. This can be interpreted with reference to the use of low cut-off values of the RT-PCR that allows the detection of DNA with a lower copy number as well as the kit differences. The average coefficients of variation (CV) of the intra-assay repeatability and the inter-assay reproducibility of the RT-PCR were determined as 2.9% and 1.8%, respectively, and these values are below the acceptable level (<10%) for the RT-PCR [18]. This indicates that the test repetitions are consistent, hence the method is reproducible. In an optimized RT-PCR, it is desirable that the coefficient of detection is greater than 0.980, the amplification efficiency ranges between 90% and 110%, and the reaction repeats are consistent [19]. In this study, a value (0.862) close to the ideal determination coefficient (R^2) was observed and the amplification efficiency (92.71%) of the RT-PCR was found to lie within the acceptable limits [19]. Moreover, the amplification efficiency of the test is very similar to those high efficacy was reported for the BCSP31 gene region of *B. melitensis* bacteria [20-22].

Many studies have been reported on the diagnostic capability of the RT-PCR, which enables amplification of the *BCSP31* gene. In these studies, performed in humans and animals, analytical calculations such as diagnostic sensitivity and specificity were performed and compared with cultural and/or serological methods [21,23]. In this study, the diagnostic efficiency, sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of the RT-PCR were determined as 100%, when compared with the sample-focused approach and CFT. A perfect agreement (kappa was 1.000) was found between CFT and RT-PCR. Although there are some small proportional differences in diagnostic competence, it is generally similar to the other studies [20,21]. When compared to the RBPT and SAT, the diagnostic competence values of the RT-PCR PCR are slightly lower, except for the specificity and positive predictive value. However, the agreement between the RT-PCR and these tests is still close to the perfect agreement (kappa was 0.914 and 0.985, respectively).

Brucella spp. are facultative intracellular microorganisms and they settle in macrophages. After completing replications in these cells, they migrate to the lymphoid tissues of the reproductive system as a result of a primary bacteremia. The agent causes a secondary bacteremia from these tissues, which subsequently leads to a generalized infection and then abortion. Therefore, these persistent bacteremia phases, which almost always contain the microorganisms in the bloodstream, are repeated in the next gestational period [24]. Modern PCR methods are

capable of detecting both living bacteria and bacteria that have been phagocyted or killed by macrophages in different compartments of the blood during the periods of bacteremia [25,26]. Also, PCR techniques have the ability to determine the course of the infection. In this context, blood in which bacteria-laden macrophages are constantly circulating is a useful clinical material that can be used for diagnosis as a source of DNA belonging to the infectious agent [25]. Different diagnostic advantages of different blood compartments such as whole blood or serum have been reported. Although the use of a whole blood compartment containing leukocytes is essential for the obligate intracellular agents, there is no such requirement for the facultative intracellular bacteria such as *Brucella* species. In addition, the analytical sensitivity of the serum in the diagnosis of brucellosis in humans and animals is comparable with the whole blood phase. Blood serum is the preferred DNA source in PCR because it provides quick test results and simplifies the method, and does not contain inhibitory structures such as anticoagulants and hemoglobin [25,26]. In this respect, the use of samples of blood serum from animals at risk of brucellosis in this study has once again confirmed it as an evaluable clinical material in the diagnosis of brucellosis. Based on the above-mentioned high diagnostic characteristics of the RT-PCR and the usability of the blood serum samples as a diagnostic clinical material, *Brucella* positivity in cattle and sheep was found to be 9.78% for the region studied. Due to the heterogeneous sample distribution, there was no statistical relationship between the disease prevalence and the provinces sampled (P>0.05). Nevertheless, the positivity for *Brucella* obtained on the provincial basis is similar to that [27] reported by the Veterinary Control Central Research Institute between 2007 and 2011, and values close to 2011 seropositivity were obtained in particular.

The control and eradication of brucellosis in animals in Turkey is based on the vaccination of susceptible animals and the slaughtering of infected animals [11]. In this respect, it is important to use reliable and validated test methods that provide rapid laboratory diagnosis. In this study, the analytical competence of an *in-house* RT-PCR was tested and it was determined that this method with its high diagnostic efficiency can be of benefit in the diagnosis of brucellosis in cattle and sheep populations. In addition, the importance of blood serum as a preferable clinical material in the molecular diagnosis of brucellosis in animals was reinforced.

Conflict of Interest

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

Author Contributions

DKY and DA planned, designed, and supervised the research procedure, carried out the experiments and the analytic tests and wrote the article.

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RESEARCH ARTICLE

Effect of Exercise on Electrocardiography and Stress Behavior of Kangal Shepherd Dogs with Ankyloglossia

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Abstract

Effect of exercise has been studied on electrocardiography and stress behavioral parameters of 12 male Kangal shepherd dogs with ankyloglossia and 15 male Kangal shepherd dogs without ankyloglossia were compared. In this study, electrocardiography recordings were obtained from Kangal shepherd dogs with the holter device at resting state and during 15-min exercise. All data collection were conducted in the same environmental conditation. In addition, behavioral parameters captured with a video camera during resting state and the end of 15-min exercise period. While no statistical difference was detected in two groups in terms of all electrocardiographic parameters at resting state, statistical differences were detected in both groups in the P wave amplitude, T wave amplitude, duration of PR interval, duration of QT interval and heart rate parameters during exercise. Following 15-min of exercise, all stress parameters were observed in dogs with ankyloglossia, while only two parameters were observed at resting state. Following a 15-min exercise, without ankyloglossia dogs showed only two stress parameters, while no parameters were observed at resting state. This study will have an important area in the literature in terms of examining the electrocardiographic data and stress behavioral parameters of Kangal shepherd dogs with ankyloglossia.

Keywords: *Ankyloglossia, Behavior, Exercise, Holter, Kangal shepherd dog*

Ankloglossia Olan Kangal Çoban Köpeklerinde Egzersizin Elektrokardiyografi ve Stres Davranışı Üzerine Etkisi

Öz

Ankyloglossia olan 12 adet erkek Kangal köpeği ve ankyloglossia olmayan 15 adet erkek Kangal köpeğinde elektorkardiyografi ve stress davranış parametreleri üzerine egzersizin etkisi karşılaştırılmıştır. Bu çalışmada elektrokardiyografi kayıtları Kangal çoban köpeklerinden aynı çevre şartlarında dinlenim ve 15 dakikalık egzersiz sırasında holter cihazı ile alınmıştır. Ayrıca dinlenim ve 15 dakikalık egzersiz sonundaki davranış parametreleri video kamera ile kayıt edilmiştir. İki grupta dinlenme durumunda elektrokardiyografik parametrelerin tamanında istatistiksel olarak fark bulunmazken, egzersiz sırasında P dalgası amplitüdü, T dalgası amplitüdü, PR aralığı süresi, QT aralığı süresi ve dakika kalp atımı sayısı parametrelerinde istatistiksel olarak farklılık bulundu. Ankyloglossialı köpeklerde dinlenme durumunda sadece iki stres parametresi gözlenirken 15 dakikalık egzersizi takiben tüm stres parametreleri gözlendi. Ankyloglossia olmayan köpeklerde dinlenme durumunda stres parametreleri gözlemlenmezken 15 dakikalık egzersizi takiben sadece iki stres parametresi gözlendi. Bu çalışma ankyloglossialı Kangal çoban köpeklerinin elektrokardiyografik verilerini ve stres davranış parametrelerini incelemesi yönüyle literatürde önemli bir yere sahip olacaktır.

Anahtar sözcükler: *Ankyloglossia, Davranış, Egzersiz, Holter, Kangal çoban köpeği*

INTRODUCTION

Kangal shepherd dogs are highly valued as livestock guard dogs because of their large size, agility, imposing appearance and the fact that they are brave enough to confront greater savage animals than themselves. Moreover, they are highly valued as guard dogs because they are loyal to their owners and harmless and well-disposed toward children and weaker animals [1-3]. As guard and shepherd dogs, Kangal Shepherd dog breed is present in variety of different countries in the world and, in some countries like in the US, Belgium, and France, breeding is also present [2]. Due to their

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loyalty and excellent awareness the Kangal Shepherd Dog breed is thoroughly preferred as guard dogs. This breed is known to have a profound adaptability to various climatic changes by their excellent thermoregulatory mechanisms. The tongue is of particular importance as a sensory organ for this breed, which primarily takes key roles in regulation of the body temperature. Also the tongue plays a major role in eating, sucking, making sounds and adjusting the body temperature in dogs [4]. Congenital and acquired diseases of the tongue include aglossia, microglossia, ankyloglossia, and lingua bifida. Of these, the most common congenital disease is ankyloglossia [5]. Ankyloglossia is a rare condition observed in humans and Kangal dogs [6]. The frenulum linguae, a small fold of mucous membrane that connects the tongue to the base of the mouth, cannot be completely separated from the base of the mouth or it cannot be thickened by cell proliferation, and this, prevents the separation of the tongue [7]. Electrocardiography (ECG) is a method that includes the recording and interpretation of action potentials during cardiac activity and provides information on the current electrical activity of the heart, and to some extent, its functional status [8]. The ECG method can be used in dogs to obtain information about many physiological conditions related to the heart [9,10].

Therefore, the aim of this study was to compare ECG data and stress behavioral parameters during resting state and exercise periods in Kangal shepherd dogs with and without ankyloglossia.

Material and Methods

Ethical Statement

This study was approved by the Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (Approval no: 65202830-050.04.04-226 and date 06.12.2018).

Animal Materials

The animal material of the study was composed of 27 healthy male Kangal shepherd dogs; 12 of which had ankyloglossia *(Fig. 1)* and 15 did not have ankyloglossia. The dogs used in the study, total to 27, of which 24 were in the Kangal Shepherd Dog Breeding Center and 3 were in the hands of independent breeders. Among the 24 dogs in the center, 15 were without ankyloglossia and 9 with it, whereas the 3 dogs from breeders were all with ankyloglossia. All dogs were older than 18 months of age.

Exercise Program

Dogs were run with their owners at a pace of 3.2 km/h in average for 15 min during the air temperature was 24°C [11].

Electrocardiographical Parameters

In this study, ECG recordings were obtained with a Holter device (Televet II) from the Kangal shepherd dogs during resting state and during a 15-min exercise at the same environmental condition (temperature (24°C), altitude (5050 ft=1540 m), standard dog diet and *ad libitum* watering). In ECG recordings of all dogs, the last 1 min of the 15-min exercise was considered to determine the effect of the exercise. The ECG data were recorded in resting state dogs for 5 min and the last 1 min was taken into evaluation. The Holter device and electrodes were fixed with net bandages, and a test was conducted to prevent stress in the dogs.

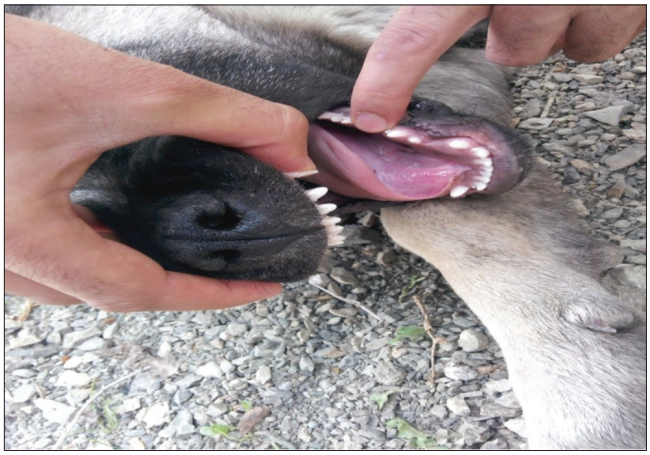

Fig 1. A Kangal shepherd dog with ankyloglossia

Aqua-wet gel electrodes and a recording device (KRUUSE ECG-HOLTER) were placed in the thorax of the animals by the procedure proposed by the manufacturer and fixed with net bandages, and standard derivations I, II, III were printed. Second derivation of the obtained electrocardiograms, P wave duration and amplitude, QRS complex duration, Q and R wave amplitudes, T wave time and amplitude, the duration of PR, QT and ST intervals and heart rate were determined.

Stress Behavioral Parameters

Both the resting state and at the end of the 15-min exercise periods, stress behavioral parameters of all dogs were captured with a video camera. Subsequently, these recordings were analyzed and evaluated using an ethogram that described stress behavioral parameters in dogs [2]. In the study, the "focal animal sampling" method was used [12]. The method was based on following a particular dog for a certain period of time and counting the behavior shown. Both the ECG and video recordings of the dogs were taken at the same environmental condition while the Kangal shepherd dogs followed their daily routines.

Statistical Analysis

For the statistical analyses of study parameters the Mann-Whitney U test was used. Also the summary statistics was calculated. All tests were calculated by SPSS v.15 package

program [13]. Observed stress behavioral parameters in each group were expressed as a percentage of the number of animals.

Results

The difference between the resting state ECG data of Kangal shepherd dogs with ankyloglossia and without ankyloglossia was shown in *Table 1*. No statistically significant difference was detected in the resting state ECG data of Kangal shepherd dogs with ankyloglossia or without ankyloglossia. The difference between the ECG data during exercise status of Kangal shepherd dogs with and without ankyloglossia was demonstrated in *Table 1*. When the ECG data during exercise of Kangal shepherd dogs with and without ankyloglossia were compared; statistically significant differences were found in the P wave amplitude, T wave amplitude, duration of PR interval, duration of QT interval and min heart rate. It was determined that P wave amplitude was significantly increased in Kangal shepherd dogs with ankyloglossia ($P<0.001$). It was detected the T wave amplitude was significantly increased more in Kangal shepherd dogs without ankyloglossia ($P<0.05$). It was verified the duration of PR interval was significantly shorter in Kangal shepherd dogs with ankyloglossia ($P<0.01$). It was also determined the duration of QT interval was significantly shorter in dogs with ankyloglossia ($P<0.01$). It was determined that in dogs with ankyloglossia the increase in heartbeats per minute was statistically significant compared to without ankyloglossia ($P<0.001$).

Stress behavioral parameters at resting state of Kangal shepherd dogs in both groups at resting state were shown in *Table 2*. Some of the stress behavioral parameters that included hind between legs, lowering the body position were observed in Kangal shepherd dogs with ankyloglossia. However, no stress behavioral parameters were observed in Kangal shepherd dogs without ankyloglossia. Stress behavioral parameters exhibited by Kangal shepherd dogs in both groups at the end of 15-min exercise duration were

Table 1. *Effect of resting state and exercise on ECG findings of Kangal shepherd dogs with and without ankyloglossia*

Resting ECG With Ankyloglossia (n=12)	Resting ECG		Exercise ECG	
	Without Ankyloglossia (n=15)	With Ankyloglossia (n=12)	Without Ankyloglossia (n=15)	With Ankyloglossia (n=12)
P wave duration (ms)	0.03807±0.00043	0.03767±0.00035	0.03800±0.00042	0.03758±0.00036
P wave amplitude (mV)	0.1760±0.00321	0.1767±0.00355	0.2013±0.00350	0.2383±0.00386***
QRS complex duration (ms)	0.0500±0.00324	0.0492±0.00229	0.0507±0.00330	0.0508±0.00313
R wave amplitude (mV)	1.6000±0.05024	1.6292±0.04240	1.6033±0.04987	1.6625±0.03899
Q wave amplitude (mV)	0.2680±0.01079	0.2692±0.00484	0.2713±0.01041	0.2692±0.00484
T wave duration (ms)	0.0500±0.00324	0.0500±0.00302	0.0500±0.00324	0.0508±0.00313
T wave amplitude (mV)	0.3487±0.02070	0.3375±0.02863	0.3560±0.01997	0.3083±0.00944*
PR intervals duration (ms)	0.1207±0.00316	0.1200±0.00302	0.1000±0.00378	0.0792±0.00288**
QT intervals duration (ms)	0.2067±0.01436	0.2058±0.00783	0.1840±0.01013	0.1425±0.00566**
ST intervals duration (ms)	0.1400±0.00239	0.1392±0.00336	0.1407±0.00248	0.1383±0.00271
Heart rate (BPM)	114.80±0.863	114.92±0.543	120.67±0.826	125.58±0.763***

*Mean±SEM; Mann-Whitney U test * P<0.05, ** P<0.01, *** P<0.001; mV: millivolt; ms: milliseconds*

Table 2. *Stress behavioral parameters during resting state and exercise (at the end of 15 min exercise) on Kangal shepherd dogs with and without ankyloglossia*

Stres Behavior	Resting Phase		Exercise for 15 min	
	Without Ankyloglossia (n, %)	With Ankyloglossia (n, %)	Without Ankyloglossia (n, %)	With Ankyloglossia (n, %)
Tail between hind legs	0	1 (8.33)	2 (13.33)	5 (41.66)
Lowering the body position	0	1 (8.33)	3 (20)	6 (50)
Yawning	0	0	0	3 (25)
Groovy appearance below the eyes	0	0	0	2 (16.66)
Strained lips	0	0	0	3 (25)
Avoidance	0	0	0	2 (16.66)

Fig 2. Some stress behaviour parameters in Kangal shepherd dogs with ankyloglossia after exercise

shown in *Table 2*. Stress related behavior was observed as increased in both groups at the end of 15-min exercise period. Notably, stress behavioral parameters (strained lips, avoidance, yawning, lowering the body position, tail between hind legs and groovy appearance below the eyes) increased in dogs with ankyloglossia at the end of 15-min exercise *(Fig. 2)*. In addition, it was determined that the number of Kangal shepherd dogs with ankyloglossia exhibiting the behavior in the same stress behavioral parameter is higher than the number of Kangal shepherd dogs without ankyloglossia.

Discussion

This work will have an important area in the literature in terms of examining the electrocardiographic data and stress behavioral parameters of Kangal shepherd dogs with ankyloglossia.

The data obtained from the resting and exercise periods in our study were found to be inside the normal range, which is similar to the data from other researches [10,14-17]. It can be stated that having ankyloglossia did not have a pronounced effect on heart parameters during resting state as both groups did not provide significant changes in parameters. In this study, P wave amplitude and the count of heart beats per minute were significantly increased in dogs with ankyloglossia compared to the dogs without ankyloglossia during the exercise period. The duration of the PR interval, the duration of the QT interval, and T wave amplitude were significantly lower and this indicated that the workload in the heart was higher in dogs with ankyloglossia during exercise. In this case, dogs with ankyloglossia were insufficient to set the thermoregulation, thus suggesting that the workload on the heart increased. As in other studies, the increase in the number of heartbeat per minute caused the amplitude of the P wave to increase, and the duration of the PR interval and the duration of the QT interval were both shortened [16,18,19].

The type and intensity of exercise can cause stress in dogs [20-22]. While conducting research for the investigation of stress behavioral parameters, there are debates on whether behavior data should be coupled with the physiological parameters. Certain researchers support that the data should be evaluated altogether [23]; however some researchers insist that these two data would not comply with each other in case of acute stress conditions, and therefore the behavior should get the priority [24,25]. In this study, ECG parameters and stress behavioral parameters are evaluated together. At the end of a 15-min exercise, strained lips, avoidance, yawning, lowering the body position, tail between hind legs and groovy appearance below the eyes behaviors were observed in dogs with ankyloglossia. At the end of a 15-min exercise, lowering the body position and tail between hind legs behaviors were observed in dogs without ankyloglossia.

Body position lowering and keeping tail between hind legs were reported to be associated with stress in behavioral changes for dogs as stated in recent studies [26,27]. In a study, it was stated that dogs would have changed behaviors under stress conditions [28]. By the notion of changed behaviors, independent activities exhibited during the behavioral incident and by the motivation is being referred [29]. Yawning behavior was detected in dogs included to our study thus can be considered as one of the stress associated changed behavior in dogs with ankyloglossia.

In a study, it was stated that avoidance, groovy appearance below the eyes were behaviors that emerged when the animal was under stress [26]. Similarly, in the present study, avoidance and groovy appearance below the eyes were observed in dogs with ankyloglossia. In a study, it was stated that strained lips could be observed in dogs when they were under stress [30]. Similarly, in the current study, strained lips behavior were observed in dogs with ankyloglossia.

Ankyloglossia is a cause of increased workload on the heart during exercise in Kangal shepherd dogs and detection of all stress behavioral parameters during exercise of Kangal shepherd dogs with ankyloglossia suggest that these dogs are under stress. It is recommended that these data should be evaluated in clinical, surgical and exercise studies on dogs with ankyloglossia.

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RESEARCH ARTICLE

Presence and Importance of Oxidative Stress Parameters in Malignant Mammary Gland Tumors in Dogs

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Abstract

This study aimed to evaluate the presence of oxidative stress based on lipid peroxidation and the DNA damage markers malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in canine mammary gland carcinomas using immunohistochemistry techniques. A total of ten malignant and six normal canine mammary tissue samples were evaluated. The specimens were fixed in 10% buffered formaldehyde solution, processed routinely, embedded in paraffin wax, sectioned at 5 µm, stained with hematoxylin and eosin, examined under a light microscope, and photographed to detect histopathological changes. For immunohistochemistry, the avidin-biotin-peroxidase method was performed. All canine mammary gland tumors were immunopositive for MDA and 8-OHdG expression. There was a statistically significant increase in MDA and 8-OHdG expressions in the tumor group compared to the control group. Based on this study data, in the context of oxidative stress, it is proposed that lipid peroxidation and reactive oxygen species (ROS)-induced DNA damage are significantly associated with canine mammary gland tumor development. In addition, antioxidants may be useful in the treatment of canine mammary gland tumors.

Keywords: *Canine, Carcinoma, Mammary gland, Oxidative stress*

Köpeklerde Malign Meme Bezi Tümörlerinde Oksidatif Stres Parametrelerinin Varlığı ve Önemi

Öz

Bu çalışmada, kanin meme bezi karsinomlarında lipid peroksidasyonuna dayalı oksidatif stres varlığının ve DNA hasar belirteçleri malondialdehit (MDA) ve 8-hidroksi-2'-deoksiguanozin (8-OHdG)'nin immünohistokimya teknikleri kullanılarak değerlendirilmesi amaçlanmıştır. Toplam on malign ve altı normal köpek meme dokusu örneği değerlendirildi. Örnekler %10'luk tamponlu formaldehit solüsyonunda tespit edildi, rutin olarak işlendi, parafin blok içine gömüldü, 5 µm kalınlığında kesitler alındı, Hematoksilen ve Eozin ile boyandı, ışık mikroskobu altında incelendi ve histopatolojik değişiklikleri saptamak için fotoğraflandı. İmmünhistokimya için Avidin-Biotin-Peroksidaz yöntemi uygulandı. Tüm köpek meme bezi tümörleri, MDA ve 8-OHdG ekspresyonu yönünden immünopozitifti. Kontrol grubuna göre tümör grubunda MDA ve 8-OHdG ekspresyonlarında istatistiksel olarak anlamlı bir artış vardı. Bu çalışma verilerine dayanarak, oksidatif stres bağlamında, lipid peroksidasyonu ve reaktif oksijen türleri (ROT) kaynaklı DNA hasarının köpek meme bezi tümörü gelişimi ile önemli ölçüde ilişkili olduğu önerilmektedir. Ek olarak, köpek meme bezi tümörlerinin tedavisinde antioksidanlar faydalı olabilir.

Anahtar sözcükler: *Köpek, Karsinom, Meme bezi, Oksidatif stres*

INTRODUCTION

Mammary gland tumors are very common in female dogs as well as women; however, the prevalence rate in dogs is three times higher. The majority of these canine mammary tumors are malignant and cause significant clinical problems [1-3]. The incidence of tumors, found primarily in adult female dogs, increases with age (average 8-11 years) [4,5]. Malignant mammary gland tumors have been reported more often in Poodles, English Springer Spaniels, Brittany Spaniels, Cocker Spaniels, English Setters, Pointers, Maltese, Yorkshire Terriers, and Dachshunds

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compared to other breeds [6]. The most common histopathological tumor types found in dogs are tubular, papillary, solid, and complex carcinomas and carcinosarcomas [7,8]; whereas, the most common type of breast tumors seen in humans are invasive ductal carcinomas [9]. Prominent mammary gland regions such as the fourth (caudal abdominal) and fifth (inguinal) glands are more predisposed to tumor formation, with the incidence of tumors in these areas ranging between 65% and 70% [4,6]. Generally, pet owners become aware of tumors when macroscopic changes in the mammary glands become apparent [7]. Canine mammary tumors tend to metastasize to nearby lymph nodes and lungs, while metastases to bones, adrenal glands, kidneys, heart, liver, brain, and skin are extremely rare [4]. Death is primarily attributable to lung metastasis [5]. These tumors have a wide range of clinical behaviors. A definitive diagnosis based on a tumor classification and grade is essential for developing optimal individualized treatment plans [10,11]. In controversial cases, immunohistochemical markers can also be evaluated for a more accurate diagnosis [4]. Risk factors such as ovariohysterectomy performed after 2.5 years of age, an intact reproductive status, treatment with progesterone and estrogen, and early obesity are known to play important roles in tumor formation [12]. The primary treatment for canine mammary tumors is mastectomy, but chemotherapy is also a complementary method for more aggressive or recurrent and metastasizing tumors [1,3].

The etiology of canine mammary tumors is multifactorial. Xeno-estrogens present in water, food, and air are known to accumulate in mammary tissue due to prolonged and continuous exposure [13,14]. Mammary epithelial cells convert xeno-estrogens to highly toxic reactive oxygen species (ROS) [15] known to cause serious structural changes in proteins, lipids, and DNA. These changes can result in cell degeneration and aging [16,17]. In addition, these changes may lead to suppression or activation of some signaling pathways and gene expression, thus leading to cell death (apoptosis) or activation of protooncogenes and/or activation/inactivation of tumor suppressor genes. These events can be important in the initiation and promotion of carcinogenesis [18,19]. An imbalance between oxidative and antioxidative reactions causes excessive ROS production, also called oxidative stress, known to play a significant role in the pathogenesis of many illnesses such as cardiovascular diseases, neuropathies, inflammatory diseases, AIDS, diabetes mellitus, renal disorders, and various cancer types, including breast cancer [17,19-21]. Oxidative stress is also associated with carcinogenesis in dogs [16,22]. The primary target of ROS is polyunsaturated fatty acids in cell membranes, causing lipid peroxidation [23], which in turn cause nuclear damage and consequently mutagenesis and carcinogenesis [24]. Malondialdehyde (MDA) is an end product of lipid peroxidation and an important marker for determining oxidative stress [16,25,26]. Increased lipid peroxidation and MDA-DNA adducts have been found in canine mammary tumors and human breast cancers [21]. An increase in the production rate of ROS leads to various modifications in the nucleotide base of DNA. As a crucial risk factor for many pathological conditions, including breast cancer, the ROS product 8-hydroxy-2′-deoxyguanosine (8-OHdG) is a biomarker widely used to detect DNA damage due to oxidative stress [27-29].

This study aimed to evaluate the presence of oxidative stress parameteres with lipid peroxidation and the DNA damage markers MDA and 8-OHdG in canine mammary gland carcinomas.

Material and Methods

Ethical Approval

The ethics committee approval for this study was obtained from Kafkas University Animal Experimentals Local Ethics Committee (No: KAU-HADYEK-2020/076).

Animals

Malignant mammary gland carcinoma samples taken from ten female dogs (Kangal, n=6; Setter, n=4; average age: 8.3 years) brought to Pathology Department for routine diagnosis, and six normal canine mammary tissues (Crossbreed, n=6, average age: 5.5 years) were evaluated.

Histopathology

Mammary tissue samples were fixed in 10% buffered formaldehyde solution, processed routinely, embedded in paraffin wax, sectioned at 5 µm, stained with hematoxylin & eosin (H&E), examined under a light microscope (Olympus Bx53), and photographed via the Cell^P program (Olympus Soft Imaging Solutions GmbH, 3,4) to detect histopathological changes. Tumor sections were classified according to the modified World Health Organization classification of canine mammary tumors [30]. The malignancy grade of the tumors was determined according to the Nottingham method [31]. Accordingly, tubule formation, nuclear polymorphism, and mitotic cell counts were evaluated and scored from 1-3. Tumor grades were defined as follows: 3-5 points = well-differentiated (Stage 1), 6-7 points = moderately differentiated (Stage 2), 8-9 points = poorly differentiated (Stage 3).

Immunohistochemistry

The Avidin-Biotin-Peroxidase method was used for immunohistochemistry. Slides were deparaffinized and rehydrated in graded alcohols. The sections were treated with 3% hydrogen peroxide solution in phosphate-buffered saline (PBS) for 15 min to prevent endogenous peroxidase activity, then boiled in citrate buffer solution (pH 6) for 25 min in an 800-watt microwave oven for antigen retrieval. The sections were incubated for ten min with non-immune

serum (Thermo Scientific Histostain-Plus IHC Kit, HRP, broad-spectrum, REF: TP-125-HL) at room temperature to prevent nonspecific staining. Diluted antibodies (8-OHdG: Bioss Antibodies, bs-1278R, dilution:1/800; MDA: Abcam, ab6463, dilution:1/250) were incubated overnight in a refrigerator at 4°C after which the sections were washed three times in PBS for three min. The biotinylated secondary antibody (Thermo Scientific, Histostain-Plus IHC Kit, HRP, broad-spectrum, REF: TP-125-HL) was applied at room temperature for ten min. After washing in PBS for three min, all sections were incubated with peroxidase-bound streptavidin (Thermo Scientific, Histostain-Plus IHC Kit, HRP, broad-spectrum, REF: TP-125-HL) for ten min at room temperature. A solution of 3,3'-diaminobenzidine (DAB) tetrahydrochloride (Thermo Scientific, REF: TA-125-HD) was used as a chromogen for 15 min. The sections were treated with Mayer's Hematoxylin for 30 second and washed in running water for 5 min, dehydrated in graded alcohols, cleared in xylene and coated with entellan. Primary antibodies were omitted from the negative control sections and were treated with diluted normal serum. The prepared slides were examined under a light microscope (Olympus Bx53) and photographed via the Cell^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Analyses of the images were accomplished with the Image J Program (1.51j8).

Immunopositivity was evaluated with a 20× objective using a semiquantitative grading scheme based on the determination of 8-OHdG and MDA markers in five representative fields as (+) mild labeling of 1%-10% of cells, (++) moderate labeling of 11%-59% of cells, or (+++) severe labeling of >60% of cells [32].

Statistical Analysis

Before the study, a power analysis was performed using G-Power 3.1.9.7. As a result, the sample size was based on a test power of 0.8 and a significance level of 0.05. A Mann-Whitney U test was used to compare mammary tumor and control groups according to immune-positive cell scoring. The obtained results were given as mean ± standard error (SE) and median. Statistical analyses were performed using the SPSS® program (Version 26.0, Chicago, IL, USA). Differences between groups were considered significant at the P<0.05 level.

Results

Macroscopic Results

Lobular single or multiple tumor masses with hemorrhagic and ulcerative surfaces were observed. The generally round and oval-shaped masses were grayish-white in color. While the incision faces of some masses had soft and spongy areas, others were quite hard and difficult to cut due to the formation of bone and cartilage tissue *(Fig. 1-a,b)*. Metastases to regional lymph nodes and lungs were observed in only two cases.

Microscopic Results

Histological classification and grade information of all cases are provided in *Table 1*. In the histopathological examination of 10 mammary gland tumor samples, 1 tubular carcinoma (10%), 1 solid carcinoma (10%), 4 intraductal papillary carcinomas (25%), and 4 mix carcinoma variants (25%) were identified. Of the 10 cases, 5 were Grade 1 (50%), 3 were Grade 2 (30%), and 2 were Grade 3 (20%). In the tubular carcinoma variant, one or two cell thick tubular formations, pleomorphism, pronounced hyperchromasia, and an increase in mitotic figures were remarkable. In the solid carcinoma variant, neoplastic cells in the form of solid layers/clumps separated by thin fibrous capsules were detected. The increase in the ratio of nuclei to cytoplasm in these neoplastic cells in favor of the nucleus was remarkable. Mitotic figures were rare in some areas and quite numerous in others. In the intraductal papillary carcinoma variant, finger-like projections, formed by neoplastic cells extending towards the lumen and supported by a fibrovascular layer, were prominent. In addition to these findings, nuclear pleomorphism and increased mitotic activity were detected similar to the other variants. In the mixed carcinoma variant, neoplastic epithelial cells, spindle-shaped myoepithelial cells, as well as cartilage and bone tissue formations in the tumor area were observed. Other significant histopathological findings included disorganized glandular structures formed by neoplastic epithelial cells, mitotic figures, and pleomorphism *(Fig. 2-ab,c,d,e,f,g,h)*.

Immunohistochemical Results

All canine mammary gland tumors (CMGT) were immunopositive for MDA and 8-OHdG expression. Mean ± SE values

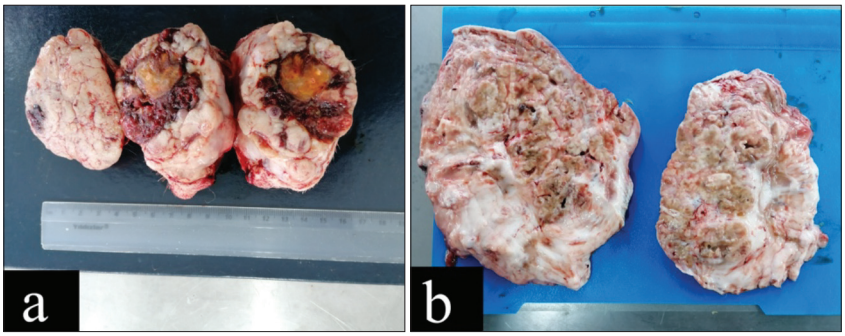

Fig 1. a-b: Macroscopic view of tumor masses

Table 1. *Breed, age information and tumor characteristics of dogs in CMGT and control groups*

Groups	Case No	Breed	Age	Histological Classification	Grade
CMGT	1	Kangal	4 years	Mix carcinoma	1
CMGT	2	Kangal	15 years	Intraductal papillary carcinoma	3
CMGT	3	Setter	4 years	Intraductal papillary carcinoma	2
CMGT	4	Setter	17 years	Solid carcinoma	3
CMGT	5	Kangal	6 years	Tubular carcinoma	2
CMGT	6	Kangal	8 years	Mix carcinoma	3
CMGT	7	Kangal	7 years	Mix carcinoma	2
CMGT	8	Setter	5 years	Intraductal papillary carcinoma	1
CMGT	9	Setter	7 years	Intraductal papillary carcinoma	1
CMGT	10	Kangal	10 years	Mix carcinoma	1
Control	11	Cross breed	4 years	-	-
Control	12	Cross breed	6 years	-	-
Control	13	Cross breed	5 years	-	-
Control	14	Cross breed	6 years	-	-
Control	15	Cross breed	4 years	-	-
Control	16	Cross breed	8 years	-	-

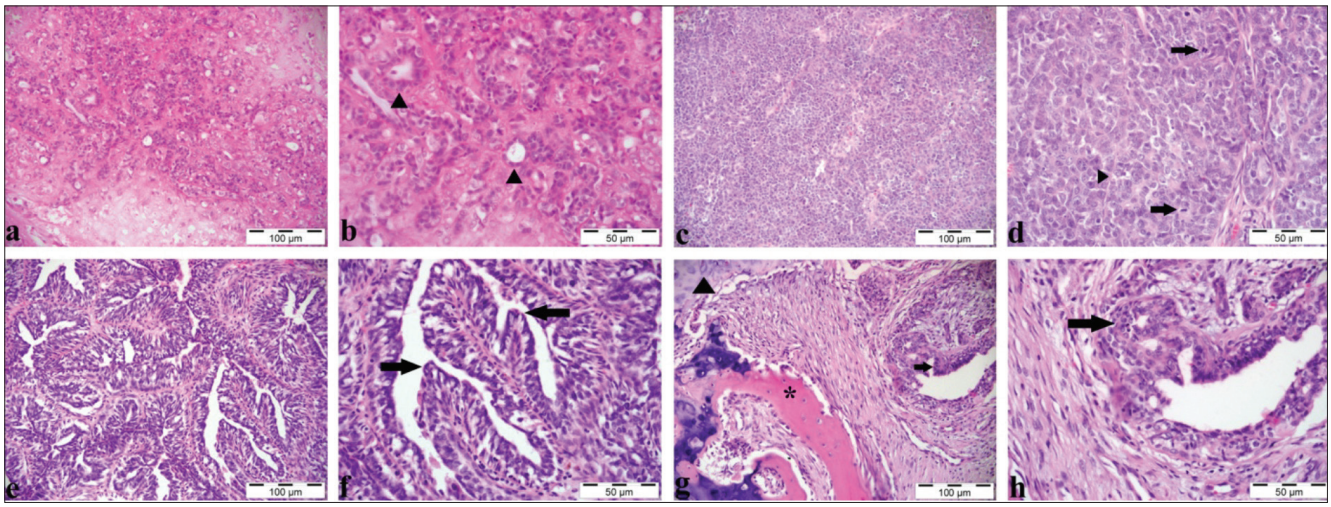

Fig 2. Histological classifications, H&E staining, **a-b:** Tubular carcinoma, tubular formations *(arrowheads);* **c-d:** Solid carcinoma, clusters of solid cells separated by a thin fibrous capsule *(arrowhead),* mitotic figures *(arrows);* **e-f:** Intraductal papillary carcinoma, finger-like extensions towards the lumen *(arrows);* **g-h:** Mixed carcinoma, epithelial tumor cells *(arrows),* bone *(star),* and cartilage formations *(arrowhead)*

Table 2. *Mean ± SE values of all groups*

Groups	MDA Mean±SE (Median)	8-OHDG Mean±SE (Median)
CMGT	2.00±0.30[a] (2)	2.10±0.23[a] (2)
Control	0±0[b] (0)	0±0[b] (0)

[a,b] Different letters in each column show the statistical differences of the groups, P<0.001; Mean ± standard error and median values of cell scoring are given; ***MDA:*** *Malondialdehyde,* ***8-OHDG:*** *8-Hydroxy-2′-deoxyguanosine*

of all groups are provided in *Table 2*. No MDA or 8-OHdG immunoreactivity was found in the healthy mammary gland tissues of control animals. It was found that a statistically significant increase in MDA and 8-OHdG expressions in the tumor group compared to the control group. MDA positive reactions were particularly strong in intraductal papillary carcinomas and tubular carcinomas. The MDA expression intensity increased in Grades 2 and 3 compared to Grade 1. Intracytoplasmic yellow-brownish MDA immune reactivity was apparent in tubular carcinomas, especially in tubular structures formed by tumor cells and in areas where pleomorphism was evident. Intracytoplasmic MDA expression

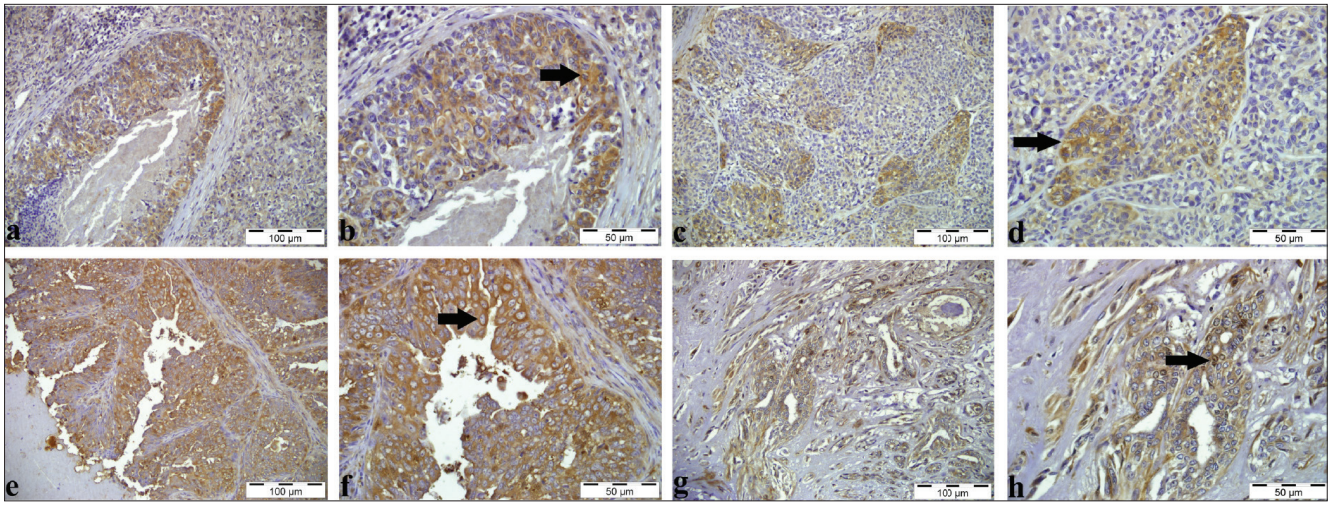

Fig 3. MDA, IHC, **a-b:** Tubular carcinoma, intracytoplasmic MDA immunopositive expressions *(arrow);* **c-d:** Solid carcinoma, MDA expressions in the cytoplasm of tumor cells *(arrow);* **e-f:** Intraductal papillary carcinoma, severe MDA reactions in the cytoplasm of neoplastic cells forming finger-like extensions *(arrow);* **g-h:** Mixed carcinoma, intracytoplasmic MDA immunoreactivity in tumor cells forming glandular structures *(arrow)*

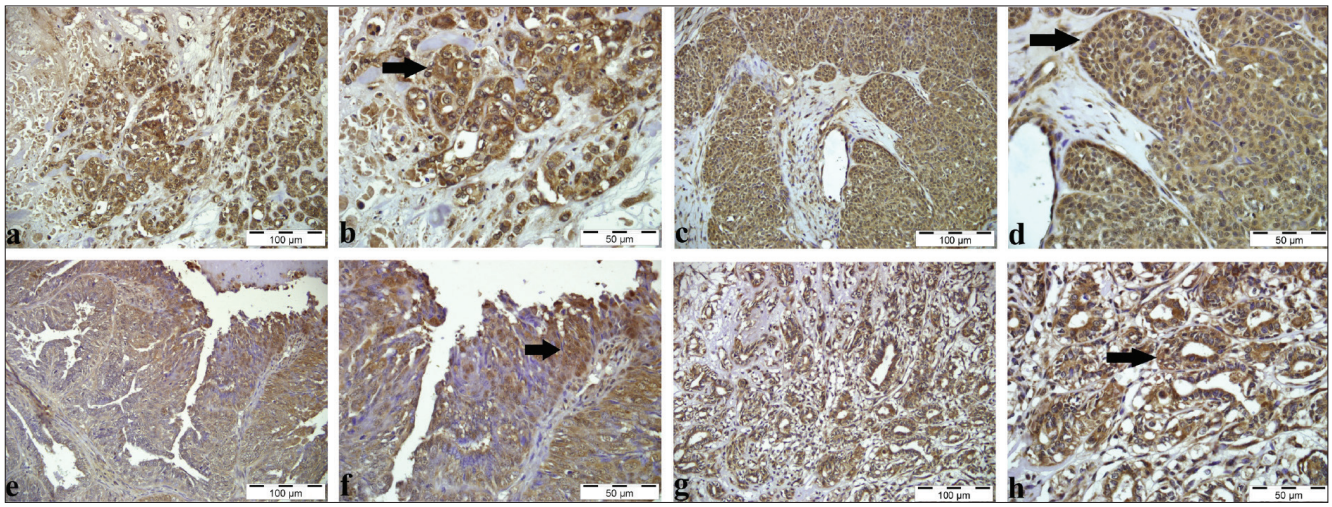

Fig 4. 8-OHdG, IHC, **a-b:** Tubular carcinoma, intranuclear and intracytoplasmic 8-OHdG immunopositive reactions *(arrow);* **c-d:** Solid carcinoma, 8-OHdG expression in both the cytoplasm and nucleus of tumor cells *(arrow);* **e-f:** Intraductal papillary carcinoma, intracytoplasmic and intranuclear dark-brown 8-OHdG positive reactions in cells located at the periphery of the finger-like projections extending towards the lumen *(arrow);* **g-h:** Mixed carcinoma, 8-OHdG immunopositive expression in the cytoplasm of neoplastic epithelial cells forming glandular structures *(arrow)*

occurred especially in tumor cells localized as clusters in solid carcinomas. The reaction was much more severe in the cells located in the periphery of these clusters. Intracytoplasmic dark-brown MDA positive reactions were apparent in papillary structures extending towards the lumen in intraductal papillary carcinomas. MDA immunoreactivity was determined in the cytoplasm of tumor cells forming glandular structures in mixed carcinomas. There was no positive reaction in bone or cartilage tissue formations *(Fig. 3-a,b,c,d,e,f,g,h)*. Solid carcinomas and tubular carcinomas had particularly strong 8-OHdG immune positive reactions. The intensity of 8-OHdG expressions was increased in Grades 2 and 3 compared to Grade 1. Expression of 8-OHdG was also localized similarly to foci where MDA expression was observed in the different tumor variants. In addition to dark-brown intracytoplasmic reactions, positive immune reactions in the nucleus were also observed *(Fig. 4-a,b,c,d,e,h,g,h)*.

Discussion

High ROS levels or failure to remove ROS results in oxidative stress, which causes severe metabolic disturbances and damage to biological macromolecules such as lipids, proteins, and DNA [18,33]. Excessive ROS production causes cytotoxicity, membrane damage, lipid peroxidation, and mutagenesis, as well as initiation and promotion of multistage carcinogenesis [13,14]. Lipids are macromolecules most susceptible to the toxic effects of ROS. Products such as MDA are formed as a result of ROS-induced lipid peroxidation [21]. The determination of the amount of MDA in biological systems is an important parameter used to

evaluate cellular oxidative stress [16,25]. Various researchers have concluded that MDA is highly cytotoxic and genotoxic and that oxidative damage should be seen as more than a biomarker due to its interaction with DNA and other proteins [26]. Controversial results exist as to whether there is any significant difference in lipid peroxidation values between clinically healthy dogs and dogs with malignant tumors. Some researchers noted that there was no significant difference when comparing lipid peroxidation levels (MDA, thiobarbituric acid reactive substances [TBARS]) between healthy animals and those with tumors [15,20]. Contrary to these reports, there are also those that indicate significant differences in lipid peroxidation values between normal tissues and canine mammary tumors [13,14,16,17,21,24]. Present study revealed a significant increase in lipid peroxidation values between dogs with malignant tumors and dogs in the control group. It is attributed that the increase in MDA expression in the tumor group to the overproduction of ROS.

Reactive oxygen species production can lead to DNA damage, double-strand breaks, rearrangements resulting in point mutations and deletions, and gene amplification in the early stage of carcinogenesis [17]. Oxidative damage of DNA induced by ROS causes the production of 8-OHdG, an oxidized form of deoxyguanosine nucleoside [34,35]. Although there are more than 20 oxidative DNA damage products, 8-OHdG has been concentrated on due to its sensitivity and mutagenicity potential. A serious association between 8-OHdG and carcinogenesis has been reported [20,29], and 8-OHdG is known to cause GC to TA transversions. Measurement of 8-OHdG levels is used to detect oxidative stress-mediated DNA damage [28,36]. Various researchers have noted that 8-OHdG levels are significantly increased in various types of cancer, such as gastric cancer, epithelial ovarian carcinoma, colorectal carcinoma, and esophageal cancer, and may be associated with a poor prognosis [37]. A literature search failed to find any studies in which 8-OHdG levels were used to evaluate canine mammary tumors or different types of cancer. However, similar to human cancers, it was found that 8-OHdG expression was more severe in tubular and solid carcinomas, which have a worse prognosis among canine mammary tumors and advanced Grades 2 and 3 [11,37].

In conclusion, based on these results, in the context of oxidative stress, lipid peroxidation and ROS-induced DNA damage are significantly associated with tumor development. The use of antioxidants in the treatment of these tumors may be beneficial. Since there are no reports in the literature detailing the evaluation of oxidative stress markers MDA and 8-OHdG in canine mammary tumors by immunohistochemical methods, this study represents novel data. Additional studies are needed to determine the value of incorporating oxidative markers in the grading and prognosis of CGMTS.

Author Contributions

Surgical operation and sample collection: MK, HO, Histopathological and immunohistochemical stainings: HN, AY, Tumor classification and staging: SD, EB, EK, Statistical analyses: MK, EK, Idea, concept and writing the article: EK.

Conflicts of Interest

The authors declared that there is no conflict of interest.

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RESEARCH ARTICLE

Determination of Hemato-Biochemical Biomarkers, Associated Risk Factors and Therapeutic Protocols for Pregnancy Toxemia in Beetal Goats

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Abstract

This study was aimed at evaluating the risk factors, alterations in blood β-hydroxybutyrate (BHB), hemato-biochemical biomarkers for earlier detection of pregnancy toxemia (PT) and comparative efficacy of therapeutic protocols in Beetal goats. A total of (N=100) goats between 120-150 days of gestation were examined. Goats having BHB >3 mmol/L were considered positive for PT by employing Freestyle™ Optium Kit. Risk factors parity, age, body weight, number of fetuses, grazing and housing, were significantly (P<0.05) associated with incidence of PT. Packed cell volume (PCV), WBCs, neutrophils, monocytes and lymphocytes were significantly higher in affected animals. Total protein and albumin were low while ALT, AST, ALP, GGT creatinine and BUN were significantly elevated. Twenty-four diseased goats were divided into two groups. Animals in group A were administered with 10% dextrose and propylene glycol orally, twice a day (BID) for three days. Whereas, in group B aforementioned treatment was supplemented with 0.15 mg/kg/SC recombinant bovine somatotropin (rbST), once a day (OID). Treatment efficacy was 75% and 83.3% in group A and B, respectively. Significant improvement in BHB, hemato-biochemical parameters were observed in goats receiving rbST. This study highlighted the significance of risk factors and hemato-biochemical biomarkers for earlier diagnosis of PT. Treatment with rbST, 10% dextrose and propylene glycol had significant effect on improvement of hemato-biochemical parameters in PT in Beetal goats.

Keywords: *Beetal goats, Hemato-biochemical biomarkers, Pregnancy toxemia, Recombinant bovine somatotropin, rbST, β-hydroxybutyrate*

Beetal Keçilerinde Gebelik Toksemisi İçin Hemato-Biyokimyasal Biyobelirteçlerin, İlgili Risk Faktörlerinin ve Terapötik Protokollerin Belirlenmesi

Öz

Bu çalışmada, Beetal keçilerinde gebelik toksemisinin (PT) erken teşhisi için risk faktörlerinin, kan β-hidroksibütirattaki (BHB) değişikliklerin ve hemato-biyokimyasal biyobelirteçlerin değerlendirilmesi ve terapötik protokollerin karşılaştırmalı etkinliğinin analizi amaçlandı. Çalışmada, 120-150 günlük gebe toplam (N=100) keçi incelendi. Freestyle™ Optium Kit kullanılarak gerçekleştirilen analizde β-hidroksibutirat (BHB) seviyesi >3 mmol/L olan keçiler, PT için pozitif kabul edildi. Risk faktörleri paritesi, yaş, vücut ağırlığı, fetüs sayısı, otlatma ve barındırma, PT insidansı ile anlamlı (P<0.05) ilişkili saptandı. Hematokrit değer (PCV), beyaz kan hücreleri (WBCs), nötrofiller, monositler ve lenfositler, hasta hayvanlarda önemli ölçüde daha yüksekti. ALT, AST, ALP, GGT kreatinin ve BUN önemli ölçüde yüksek iken, toplam protein ve albümin düşüktü. Yirmi dört hasta keçi iki gruba ayrıldı. Grup A'daki hayvanlara, üç gün boyunca günde iki kez (BID) %10 dekstroz ve propilen glikol oral yoldan uygulandı. Grup B'de ise yukarıda bahsedilen tedaviye, günde bir kez (OID) 0.15 mg/kg/SC rekombinant sığır somatotropini (rbST) ilave edildi. Tedavi etkinliği grup A ve grup B için sırasıyla %75 ve %83.3 olarak saptandı. rbST uygulanan keçilerde BHB ve hemato-biyokimyasal parametrelerde önemli iyileşme gözlendi. Bu çalışma, PT'nin erken teşhisi için risk faktörlerinin ve hemato-biyokimyasal biyobelirteçlerin önemini vurguladı. Beetal keçilerinde rbST, %10 dekstroz ve propilen glikol tedavisi, PT'de hemato-biyokimyasal parametrelerin iyileştirilmesinde önemli etkiye sahipti.

Anahtar sözcükler: *Beetal keçisi, Hemato-biyokimyasal biyobelirteçler, Gebelik toksemisi, Rekombinant sığır somatotropini, rbST, β-hidroksibütirat*

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Introduction

Pregnancy toxemia known as gestational ketosis is quite common in ewes and goats during last trimester, due to ever-growing negative energy balance [1]. Small ruminants become extremely susceptible to various metabolic diseases as they come closer to parturition [2]. Pregnancy toxemia often develops during last 4 to 6 weeks of gestation, mainly in pregnancies with more than one fetus [3]. About 60% of fetal growth takes place in last days of gestation period [4]. During this time approximately 33 to 36% of the circulating glucose is directed towards feto-placental unit to satisfy its energy demands [5]. Enormous glucose demand of growing fetuses during pregnancy is fulfilled by the dam. Disparity in fetal glucose demands and supply by dams occurs either due to reduced dietary intake of mother or exponential increase in glucose demands by developing fetuses usually in last trimester, which create negative energy balance and ultimately severe hypoglycemia [6]. Older animals carrying multiple fetuses and having high production under severe stress are most frequently prone to develop pregnancy toxemia [7]. Hyperketonemia and hypoglycemia are the most common biochemical manifestations of this disease. However, biochemical investigations have revealed a marked impact on the functionality of liver and kidney as well [8]. Pregnancy toxemic goats have β-hydroxybutyrate persistently high in the blood, as most of acetoacetate produced in liver cells is consistently reduced to β-hydroxybutyrate by the enzyme hydroxybutyrate dehydrogenase [9]. Unfortunately, PT is endemic amongst all species of goats across the world, causing high morbidities and high mortalities [10]. Absence of the early detection of the disease due to the insufficient information about its pathogenesis and the lack of efficient diagnostic tools are the foremost obstacles in improving our prophylactic as well as therapeutic policies against this disease [11]. Biochemical parameters are integral to early diagnosis of pregnancy toxemia in goats [12]. Affected animals are generally recumbent in 3-4 days, and frequently manifest clinical complications [13]. The glucose, propylene glycol and rbST are being commonly used in treatment therapies of the pregnancy toxemia [14-16]. Whereas, in advance cases the augmentation of treatment therapy with some other compound such as insulin may also be required [16]. Despite vigorous treatment prognosis is generally poor and mortality rates are high in affected animals. Approximately, up to 20% decline in health has been observed in individuals birthed by affected mothers [11]. There is always undue delay in diagnosis of pregnancy toxemia in early stages due to an absence of reliable detection biomarkers of pregnancy toxemia in goats [17]. So, this study was designed, aiming to evaluate the risk factors associated with PT and identify hemato-biochemical biomarkers for early disease detection, alteration in β-hydroxybutyrate (BHB) levels in the blood. A comparative efficacy of rbST supplementation to pregnancy toxemia treatment involving administration of 10% dextrose and propylene glycol was evaluated as well.

Material and Methods

Ethical Considerations

The designed study was submitted to and approved by animal ethics committee and departmental board of studies (BOS) of Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore.

Experimental Animals

A total of 100 pregnant (N=100) Beetal goats aged between 1-6 years, weighing around 35-55 kg body weight and gestational duration ranging from 120 to 150 days were inducted into this study. Animals showing signs of illness were examined and sampled for pregnancy toxemia from different villages.

Ultrasonography

The pregnant does were subjected to ultrasonography to confirm the stage of gestation and assess the viability of the fetuses. The estimated gestational age of the fetus in weeks was calculated using the formula:

$$Y=4.712+0.445\ X$$

where Y=Gestational age (wks) and X=Fetal parameter (cm) in case of crown rump length and Y=2.675+3.229 X where Y=Gestational age (wks) and X=Fetal parameter (cm) in case of bi-parietal diameter [18].

Blood Sampling and Parameters Measured

Blood samples (3 mL) were aseptically collected in EDTA and non- EDTA coated vacutainers by jugular vein puncture for screening of PT. The blood samples were subjected to analysis of BHB (β-hydroxybutyrate) level in mmol/L via automated Freestyle™ Optium kit for BHBA (Abbot Pharma, Neo-H) [19]. Goats with BHB>3.0 mmol/L were considered as positive for pregnancy toxemia [20]. Hematological parameters were determined from whole blood by using auto hematology analyzer (Rayto, RT-7600™). Whereas, serum samples were obtained from blood collected in non EDTA coated vacutainers and analyzed for serum biochemical parameters by clinical chemistry analyzer (Seamaty, SD1 Sichuan™). Pregnancy toxemic group consisted (N=24) and hemato-biochemical parameters were measured in these animals' pre-treatment. These pregnancy toxemic animals were then subjected to treatment trials for the improvement of clinical signs, increase in fetus's livability and life of dam before and after parturition.

Treatment Trials

Total twenty-four (N=24) pregnancy toxemic goats were equally divided into two groups A and B. Goats in group A were injected with 10% dextrose 500 mL IV, 60 mL propylene glycol orally twice a day, for three consecutive days and while in group B recombinant bovine somatotropin

(rbST) was given additionally at the dose rate of 0.15 mg/kg sc, once daily for three consecutive days [14-16]. Blood samples were taken again from both groups after three consecutive days of treatment and efficacy of treatment was determined on the basis of reduction in BHB in blood and improvement of hematologic and biochemical parameters.

Statistical Analysis

Data regarding prevalence of pregnancy toxemia (PT) was subjected to chi-square analysis with significance level ($P<0.05$). Data regarding hemato-biochemical changes was analyzed through One-Way ANOVA whereas, data regarding comparative therapeutics efficacies was analyzed using paired t-Test, keeping level of significance ($P<0.05$). All the statistical analyses were carried at SPSS version 26.0 (version 26, IBM, Chicago, IL).

Results

Correlation of Risk Factors Associated with Pregnancy Toxemia

Goats with higher parity and multiple fetuses had more tendency to develop pregnancy toxemia. Goats with 3rd and 4th parity while carrying 3 fetuses experienced highest incidence of pregnancy toxemia at 60.71% and 68.29%, respectively. Similarly, 'age' was also significantly associated ($P<0.05$) with pregnancy toxemia. Therefore, highest percentage 80.0% was seen in goats of age ≤5 years. Higher body weight was positively associated with the development of pregnancy toxemia whereby 41.17% of affected dams weighed between 46-55 kg. In present study, 63.6% goats with pregnancy toxemia were mal-nourished. Whereas, incidence of PT in goats with other concurrent infections was 61.20%. Similarly, 57.80% PT affected animals were self- medicated by the farmers. The 46.20% pregnancy toxemic animals were reared in tethered systems and not allowed to graze. Improper housing played a pivotal role in development of this ailment hence, 57.1% animals positive for pregnancy toxemia were with improper housing. Importance of good sanitary measures was reinforced when 68.7% animals reared in abominable conditions were affected with pregnancy toxemia *(Table 1)*.

Correlation of Hemato-biochemical Changes Pre- and Post-treatment

- Hematological parameters

Values of total WBCs, packed cell volume, neutrophils, lymphocytes, and monocytes, were significantly ($P<0.05$) higher in pregnancy toxemic goats whereas, red blood cells count (RBCs), hemoglobin (Hb) were significantly ($P<0.05$) lower in pregnancy toxemic animals depicting the hematological disturbances. However, treatment had a positive effect on these parameters leading to normalization of hematological. Whereas, no significant discrepancies were found in other measured hematological parameters as shown *(Table 2, Fig. 1)*.

- Biochemical parameters

In regard to the biochemical analysis, values of AST, ALT, ALP, and GGT were significantly ($P<0.05$) higher in pregnancy toxemia reflecting the hepatic damages. However, there was considerable improvement in these parameters and were in normal ranges after implication of treatment *(Table 3)*. On the other hand, total protein (TP) and albumin, were also significantly ($P<0.05$) lower in pregnancy toxemic goats. Similarly, BUN and creatinine was considerably higher in the animals suffering from PT which was considerably ($P<0.05$) reduced after treatment *(Table 3)*.

- BHBA (β-hydroxybutyrate)

In both group A and B, mean value of BHB was significantly declined after treatment. The findings declared, additional use of rbST with 10% Dextrose 500 mL IV and 60 mL propylene glycol is more effective than only dextrose and propylene glycol. However, comparison of treatment groups using t-independent test revealed insignificant difference ($P>0.05$) between both groups. Chi-square analysis of survival rates of dam in both treatment groups revealed insignificant difference ($P>0.05$), which indicated almost equal efficacy of both treatments approaches against pregnancy toxemia in Beetal goats *(Table 4)*.

Discussion

Pregnancy toxemia is inability of dams to fulfill glucose requirements of developing fetuses [21] Caprine pregnancy toxemia is diagnosed based upon the stage of gestation, number of fetus dam carried, physical signs and hemato-biochemical indices. Current study revealed considerable 35% prevalence of pregnancy toxemia in Beetal goats which was close to 40% [3] whereas, contrary to 88.9% [22]. Association of number of fetuses with pregnancy toxemia was in agreement with previous findings [3]. Failure to cope energy drain in higher number of fetuses stimulated fats metabolism leading to hyperketonemia and pregnancy toxemia [23]. Age of animal corroborates earlier findings [24] that reported older animals to be more susceptible to PT than the young dams. The older animals have comparatively less active basal metabolic rate (BMR) to provide the sufficient energy to developing fetuses [25]. Similarly, as in current study it has been previously established that animals with higher body weight are more prone to PT [26]. Poor feeding and lack of sustained grazing have proven to be significant risk factors for pregnancy toxemia [27]. Malnourishement in pregnant dams produces ketone bodies. These ketone bodies reduce the ruminal motility thereby causing reduced intake and ruminal contractions which further deteriorate body condition [11]. Stress factors

Table 1. *Chi-square analysis of associated risk factors of pregnancy toxemia*

Variables	Variables Level	Positive/Total	Prevalence%	P-Value
Parity number	1-2	08/52	15.38	0.001
	3-4	17/28	60.71	
	5-6	10/20	50.00	
	Total	35/100	35.00	
No. of fetuses carrying	2	04/51	07.84	0.001
	3	28/ 41	68.29	
	4	03/8	37.5	
	Total	35/100	35.00	
Age(years)	≤2	01/08	12.50	0.001
	≤3	09/55	16.36	
	≤4	10/17	58.82	
	≤5	12/15	80.00	
	≤6	03/05	60.00	
	Total	35/100	35.00	
Body weight	35-45 kg	28/83	33.73	0.001
	46-55 kg	07/17	41.17	
	Total	35/100	35.00	
Onset of clinical signs/days	1-2	15/56	26.79	0.052
	3-4	20/44	45.45	
	Total	35/100	35.00	
Concurrent infection	Infection	19/31	61.29	0.001
	No infection	16/69	23.19	
	Total	35/100	35.00	
Self-medication by farmers	Medicated	24/81	29.63	0.020
	Not medicated	11/19	57.89	
	Total	35/100	35.00	
Abortion history	Abortion	03/04	75.00	0.087
	No abortion	32/96	33.33	
	Total	35/100	35.00	
Season	Winter	31/82	37.80	0.209
	Summer	04/16	25.00	
	Total	35/100	35.00	
Stall feeding/restricted feeding	Stall feeding	29/73	39.73	0.103
	No stall feeding	06/27	22.22	
	Total	35/100	35.00	
Grazing	Grazing	04/33	12.12	0.001
	Non- grazing	31/67	46.27	
	Total	35/100	35.00	
Housing	Confined	23/79	29.11	0.017
	Open	12/21	57.14	
	Total	35/100	35.00	
Feeding quality	Good	07/56	12.50	0.001
	Poor	28/44	63.64	
	Total	35/100	35.00	
Space availability	Enough	09/18	50.00	0.141
	Overcrowded	26/82	31.71	
	Total	35/100	35.00	
Sanitation	Good	13/68	19.12	0.001
	Poor	22/32	68.75	
	Total	35/100	35.00	
Water quality	Fresh/tap water	26/84	30.95	0.052
	Canal/pond	09/16	56.25	
	Total	35/100	35.00	

P<0.05 indicates significant difference

Table 2. *Hematological analysis of different parameters*

Parameters	Before Treatment	After Treatment	
	Pregnancy Toxemia (Mean±SD) (n=24)	Group A (Mean±SD) (n=12)	Group B (Mean±SD) (n=12)
RBCs (x10^6/µL)	13.90±0.12^a	14.49±0.13^b	14.40±0.25^b
Hb (g/dL)	9.83±0.29^a	9.83±0.31^a	10.25±0.62^b
PCV (%)	44.41±0.11^a	38.58±0.19^b	28.16±0.62^c
MCV (fL)	25.00±0.16^a	24.83±0.17^a	24.83±0.29^a
MCH (pg)	7.50±0.07^a	7.56±0.09^a	7.48±0.11^a
MCHC (g/dL)	34.50±0.63^a	34.64±0.56^a	34.79±0.54^a
WBCs (x10^3/µL)	16.90±0.24^a	15.15±0.23^b	7.31±0.13^c
Neutrophils (x10^3/µL)	8.96±0.20^a	8.21±0.07^b	7.46±0.10^c
Monocytes (x10^3/µL)	0.28±0.02^a	0.28±0.01^b	0.15±0.02^c
Lymphocytes (x10^3/µL)	8.30±0.17^a	8.12±0.22^b	7.21±0.15^c
Eosinophils (x10^3/µL)	0.23±0.01^a	0.20±0.01^a	0.18±0.008^a
Thrombocytes (×10^9/µL)	433±11.54^a	436±13.30^a	423±16.06^a
Fibrinogen (g/L)	5.25±0.18^a	4.87±0.14^a	5.34±0.11^a

^{abc} *Different superscripts shows significant variation in the same row*

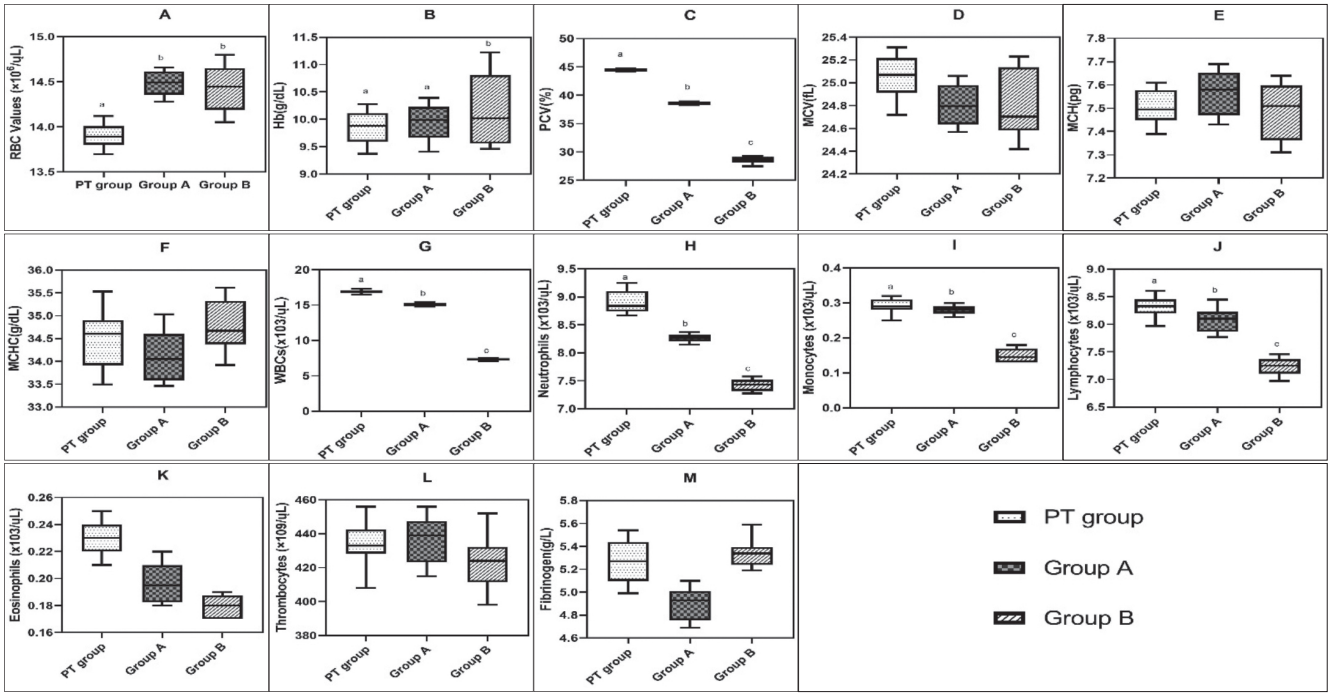

Fig 1. Interleaved Box and Whisker plot (Graph Pad Prism Ver.8.4.3) depicting comparative mean±SD values for hematological parameters, whereby significance between pre-treatment (Pregnancy toxemia, PT group) and (post-treatment) group A (10% dextrose 500 mL IV+ 60 mL propylene glycol) and group B (10% dextrose 500 mL IV+ 60 mL propylene glycol + recombinant bovine somatotropin (rbST) at 0.15 mg/kg s/c) are indicated by different superscripts

including concurrent infections, open housing and poor sanitary conditions have also cause decline in feed intake amongst goats leading to a failure in coping with energy requirements of fetuses [28].

Decrease in RBCs and Hb whereas, elevation in PCV in pregnancy toxemic goats indicated electrolyte imbalance which was attributed to stress of starvation, dehydration and kidney failure. These symptoms have been concurrently associated with pathogenesis of caprine pregnancy toxemia [29,30]. In present study decrease in number of RBCs and Hb concentration corroborated previous reports [29,31] while a sharp decline in hemoglobin (Hb) concentration refuted previously published findings in goats. Elevation

Table 3. *Biochemical analysis of different parameters*

Parameters	Before Treatment	After Treatment	
	Pregnancy Toxemia (Mean±SD) (n=24)	Group A (Mean±SD) (n=12)	Group B (Mean±SD) (n=12)
Total Proteins (g/dL)	4.83±0.25[a]	6.04±0.23[b]	7.20±0.22[c]
Albumin (g/dL)	1.98±0.06[a]	2.43±0.18[b]	2.85±0.10[c]
ALT (IU/L)	55.75±1.20[a]	45.91±0.80[b]	30.08±0.85[c]
AST (IU/L)	306.33±1.35[a]	246.16±2.13[b]	153.00±1.71[c]
ALP (IU/L)	414.9±3.46[a]	349.00±3.23[b]	173.08±2.99[c]
GGT (IU/L)	65.08±0.93[a]	39.58±1.01[b]	29.33±0.54[c]
BUN (mg/dL)	24.58±1.01[a]	23.16±0.73[a]	13.54±0.61[b]
Creatinine (mg/dL)	3.93±0.41[a]	2.92±0.34[b]	1.69±0.33[c]

[abc] *The different superscripts show significant variation in the same row*

Table 4. *Efficacy of treatment in group A and B*

Groups	Before Treatment BHBA (mmol/L) (n=12)	After Treatment BHBA (mmol/L) (n=12)	P-Value	Survival (%)
A	5.008±1.41	2.875±1.62	0.00019	75
B	5.1±1.33	2.08±1.62	0.0000008	83.33
p-value (between treated groups)		0.185	-	0.615

P<0.05 indicates significant difference

in PCV (hematocrit) observed by authors was similar to the descriptions of previous studies [29,30,32] but contrary to the findings of Tharwat and Al-Sobayil [31]. MCH, MCV and MCHC varied insignificantly in this study which agreed with the findings of previous study [31].

Leukocytosis and lymphocytosis in pregnancy toxemia could be attributed to the presence of acute and chronic inflammations [33]. Increases in WBCs, neutrophils, monocytes, eosinophils and lymphocytes are in agreement with results as described previously by Abba et al.[29] and Tharwat and Al-Sobayil [31] who postulated that this increase was due to metabolic acidosis (ketoacidosis), infection, localized inflammatory process and tissue necrosis of liver. Neutrophilia could be due to hepatic lipidosis in which exposure of hepatocytes to fatty acids elicits inflammation, increase in oxidative stress, and production of fibrogenic cytokines [32]. Neutrophilia in present findings was in agreement with the description of Smith and Sherman [32] but was contrary to the Tharwat and Al-Sobayil [31]. Lymphocytosis in the present study was similar as described by the previous study [31]. Whereas, lymphopenia in pregnancy toxemic goats was also determined by Abba et al.[29] and Smith and Sherman [32]. Thrombocytes did not show any significant variation in current findings which is similar to the previous study [33]. Similarly, fibrinogen was not affected by pregnancy toxemia and corroborated by the previous findings [34].

Decrease in total protein and albumin recorded was similar as described by the previous study [35,36]. It clearly indicates that adequate quantity of proteins is not being produced by the hepatic system or being lost from the body of diseased animals. This is might be due to increased protein catabolism, decomposing fetuses or terminal kidney failure which causes the decrease in the total protein and albumin [35,36]. The higher levels of AST, ALT, and GGT activities in the pregnancy toxemic, may be attributed to hepatic damage or hepatic lipidosis due to fat mobilization [30,35,37]. These elevated levels of AST, ALT, and GGT are similar to the descriptions of previous studies [30,36,38,39] who found a significantly higher and positive correlation with the rise of ketonemia. In current study the higher GGT level in blood was same as reported by [36] which is an indicator of liver damage in PT in goats [40]. An elevation in the release of alkaline phosphate (ALP) in the circulation from the epithelium of the bile ducts is associated with severe liver damage [41]. In this study elevated level of ALP was similar to the descriptions of previous studies [30,36]. However, current results were contrary to the descriptions of previous studies [35,42] who did not find any changes in blood ALP activity. Higher concentrations of BUN and creatinine may be considered as indicator of involvement of the kidney in caprine pregnancy toxemia due to increased catabolism and severe kidney dysfunction [43]. The increase in BUN and creatinine was similar as reported previously [35,36,44] whereas these findings were not in line with Tharwat and Al-Sobayil [31]. After treatment results show improvement in liver and kidney functioning to prevent the organs from further damages.

Treatment protocols for PT consisted using glucose and other products that trigger glucose utilization [15]. The administration of the i.v. glucose infusion, propylene glycol causes the glycaemia in the blood [15] whereas, rbST triggers glucose utilization via the gluconeogenesis. In current study the therapeutic efficacy of 75%, and 83.3% in both groups was similar to survival rate of 73%, 75%, and 86.7% as described by the previous studies [42,45,46] where i.v. glucose infusion, propylene glycol and rbST was given as treatment protocol [47]. Pregnancy toxemia sometimes might not respond well to a glucose challenge in the advance cases probably due to glucose intolerance caused by decreased insulin levels [48]. However, in animals at the early stages of pregnancy toxemia, treatment with i.v. glucose is useful to reverse the process [14,16,45,46]. Treatment protocols showed better response in the current study probably because of diagnosis of the disease at the early stages and initiation of treatment with dextrose along with propylene glycol and rbST in both groups, which is similar to the descriptions of previous studies [14,16,42,45,46]. Furthermore, propylene glycol treatment was repeated twice daily in this study which subsequently improved the treatment response [26]. Whereas, in the advance cases the supplementation with insulin would be required to enhance the glucose utilization [14,16].

In present study the comparative efficacy of treatment was higher in animals receiving rbST, which is similar to the findings of Anoushepour et al.[44]. Difference in results in both groups indicated that rbST has beneficial effects in the treatment of pregnancy toxemia in goats. So the findings of this study declared that, rbST should preferably be used with dextrose and propylene glycol while treating pregnancy toxemia in goats [45]. However, the treatments regimens may be studied more intensely with other protocols in the Beetal goats to get an ultimate conclusion.

Animals suffering from pregnancy toxemia showed various hemato-biochemical changes which can be used as biomarkers in early detection. Risk factors parity, age, fetuses carried, grazing, housing, were significantly ($P<0.05$) associated. Animals suffering from pregnancy toxemia treated with dextrose, propylene glycol and recombinant bovine somatotropin presented significant decrease in beta hydroxybutyric acid and improvement in other hemato-biochemical parameters which is an indication of successful therapy. Treatment of group A with 10% dextrose 500 mL and 60 mL propylene glycol bid orally for three days expressed survival rate of 75% while group B treated additionally with rbST (0.15 mg/kg) S/C expressed survival rate of 83.3%. Comparison of treatment A and B was non-significant but with higher survival rate of both dams and fetuses in group B. Assumed risk factors have positive association with pregnancy toxemia.

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Conflict of Interest

There are no conflicts of interest in our present study.

Author Contributions

YRK, AZD and MI planned, designed, and supervised the research procedure. Data was collected by YRK and AA. Statistical analysis was conducted by AA, KH and AHR. Original draft was written by YRK, RLK. All authors have contributed to the revision and final proof-reading of the manuscript.

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SHORT COMMUNICATION

Diagnostic Efficacy of Copro-ELISA for Detection of Fasciolosis in Cattle and Buffaloes in Punjab Province, Pakistan

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Abstract

Fasciolosis is a food borne zoonotic trematode disease that causes liver damage in ruminants and humans. The information recorded on the prepatent diagnostic methods is limited with low sensitivity. The study aimed to investigate the diagnostic efficacy of coproantigens by using in house developed indirect enzyme linked immunosorbent assay (ELISA). Adult helminths were collected from the bile duct of buffaloes for the extraction of excretory secretory (ES) and somatic (SA) antigens. The polyclonal antibodies were produced by immunization of rabbits with SA and ES antigens of Fasciola. Kappa value of developed ELISA was calculated to check the diagnostic performance of the test. The mean absorbance values at different concentrations of coproantigens were significantly different (P≤0.001) from controls. However, the difference was not significant among the concentrations of coproantigen. A positive linear relationship was observed to the concentration of antigens used in fecal supernatant and the absorbance values. The sensitivity and specificity of diagnostic test with ES-polyclonal antibodies were 100% (95% CI: 89.42%-100.00%) and 76.19% (95% CI: 52.83.30%-91.78%), respectively. Kappa value revealed that the strength of agreement is almost substantial. The SA-polyclonal antibodies showed the specificity and sensitivity of diagnostic test were 100% (95% CI: 89.42%-100.00%) and 90.00% (95% CI: 68.30%-98.77%), respectively. However, Kappa value of the test revealed that the strength of agreement is perfect. The result provides information that will add sensitive diagnostic methods for the detection of fasciolosis.

Keywords: *Copro-ELISA, Fascioliasis, Ruminants, SA antigens, ES antigens*

Pakistan'ın Pencap Eyaletinde Sığır ve Mandalarda Fasciolozis'in Tespitinde Copro-ELISA'nın Tanısal Etkinliği

Öz

Fasciolozis, geviş getiren hayvanlarda ve insanlarda karaciğer hasarına neden olan gıda kaynaklı zoonotik bir trematod hastalığıdır. Prepatent tanı yöntemleri ile elde edilen diagnostik bilgiler düşük hassasiyetli ve sınırlıdır. Bu çalışma, in house geliştirilen indirekt Enzyme Linked Immunosorbent Assay (ELISA) kullanılarak koproantijenlerin tanısal etkinliğinin araştırılmasını amaçlamıştır. Ekskretuar sekretuar (ES) ve somatik (SA) antijenlerin ekstraksiyonu için kullanılacak erişkin helmintler bufaloların safra kanallarından toplandı. Fasciola'nın SA ve ES antijenleri ile tavşanların immunizasyonunu takiben poliklonal antikorlar üretildi. Geliştirilen ELISA testinin tanısal performansını kontrol etmek için Kappa değeri hesaplandı. Koproantijenlerin farklı konsantrasyonlardaki ortalama absorbans değerleri, kontrollerden önemli ölçüde farklı saptandı (P≤0.001). Fakat, koproantijen konsantrasyonları arasındaki fark önemli değildi. Fekal süpernatanda kullanılan antijenlerin konsantrasyonu ile absorbans değerleri arasında pozitif linear bir ilişki gözlemlendi. ES-poliklonal antikorlarla yapılan diagnostik testin duyarlılığı %100 (%95 CI: %89.42-100.00) ve özgüllüğü %76.19 (%95 CI: %52.83-91.78) saptandı. Kappa değeri, uyum gücünün neredeyse önemli derecede olduğunu ortaya koydu. SA-poliklonal antikorlarlarla yapılan diagnostik testin özgüllüğü %100 (%95 CI: %89.42-100.00) ve duyarlılığı %90.00 (%95 CU: %68.30-98.77) saptandı. Ancak, testin Kappa değeri, uyum gücünün mükemmel olduğunu ortaya koydu. Bulgular, fasciolozisin tespiti için hassas tanı yöntemlerine ek bilgi sağlayacak niteliktedir.

Anahtar sözcükler: *Copro-ELISA, Fasioliazis, Ruminant, SA antijenleri, ES antijenleri*

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Introduction

Fascioliasis is a food-born zoonotic infection having etiological agent *Fasciola* species, commonly acquired by eating metacercaria encysted on vegetation [1]. Fasciolosis is a critical problem to the livestock population, having high morbidity, considerable mortality, and tremendous economic losses to the livestock sector [2,3].

The fecal egg counting (FEC) techniques with zinc sulfate floatation and sedimentation methods are most frequently used for the diagnosis of chronic fasciolosis [4]. The limitations of FEC reduction tests (FECRT) includes the long pre-patent period, low worm burden, and irregular egg shedding of adult *Fasciola spp.* [5]. In live animals, for the detection of early-stage infection and precautionary control, preventive measures appropriate diagnostic methods are very pivotal. Various molecular, biochemical, and metabolic processes are necessary for these diagnostic tools [6]. The diagnostic tools like immunological and coprological methods not only detect the circulating antibodies and antigens but also help in quantifying the coproantigens. A highly sensitive antibody specific test is critical to help reduce the losses caused by fasciolosis in the livestock sector in different regions of the globe [7]. ELISA and immunoblots based on antibody detection are the preferred techniques for the diagnosis of fasciolosis on account of their relative simplicity and detection of seroconversion in primary infection. Serological and coprological detection of fasciolosis in live animals has an advantage over other techniques due to its low cost and high specificity [8]. Nevertheless, collecting sera is a tough routine with a large population of animals, and methods like these are of less diagnostic value in areas where the disease is highly prevalent because antibody concentration remained at high even after animals have been treated [9]. On the other hand, active fasciolosis can be diagnosed by the detection of eggs shed into feces. It is conclusive that the collection of feces at the herd level is a comparatively easier process than the collection of blood samples. Studies had centralized the idea of the establishment of ELISA tests for the diagnosis of trematode antigens in feces, i.e., coproantigens [10]. The present study aimed to evaluate the suitability of coproantigen ELISA established for the detection of fasciolosis.

Material and Methods

Adult Helminths and Feces Collection

Adult helminths were collected from the bile duct of cattle and buffaloes which were brought to the local abattoirs to slaughter. The study was conducted by following the ethical guideline approved by the Ethical Committee of Quaid-i-Azam University Islamabad, Pakistan. Slaughtered animals (n=252) were examined for confirmation of the *Fasciola* helminths among which thirty-three were found positive. Collected helminths were subjected to 0.01M Phosphate buffer saline (pH-7.2) washed several times to clear any contaminated material. Fecal samples from animals positive (n=33) and negative i.e. no history of prior infection or infected with other helminths (n=21) were collected.

Extraction of Excretory Secretory and Somatic Antigens

Fasciola excretory secretory (ES) antigen was prepared according to the method described by Mezo et al.[11]. Briefly, mature live helminths were kept in 0.01M PBS (1 helminth/5 mL) for 24 h. The medium containing the flukes was removed and centrifuged for 20 min at 10000 g and 4°C by adding protease inhibitors. To obtain somatic (SA) extract, the fluke was homogenized in chilled tissue lysis buffer, added according to the weight of tissue in a ratio of (1000 µL buffer/100 mg of tissue). The homogenate was centrifuged at 10.000g and the resultant pellet was removed, and the supernatant refrigerated at -70°C. The protein concentrations of the ES and SA products were calculated by the method described by Bradford [12].

Generation of Polyclonal Antibodies in Rabbits

Four New Zealand white rabbits were injected by *Fasciola* SA and ES extracts to produce polyclonal IgG antibodies. The rabbits were given four doses of 200 mg of *Fasciola* ES and SA antigens at a 3-week interval by subcutaneous, parenteral route. Freund's complete adjuvant was used as an immune stimulator and sera were collected from all four rabbits a week after the last booster dose and stored at -20°C for further analysis.

Extracting Antigens from Fecal Samples

To detect the *Fasciola* coproantigens, the collected fecal samples were mixed individually with distilled water at 1:1 (3 mL water containing 3 g of faces) and subjected to centrifugation for 15 min at 1000 g. Then supernatants were collected from each sample and maintained at -20°C until further investigation by ELISA.

Coproantigen ELISA

The assay was performed according to the method described by Ahmad and Nizami [13]. Briefly, 50 µL/well of fecal supernatant in coating buffer were added in microtiter plates and left overnight at 4°C. The plates were washed with PBS 0.1% Tween and blocked with bovine serum albumin (100 µL/well) for 2 h at room temperature. In the next step, plates were washed again and serially diluted 100 µL/well rabbit anti-*Fasciola* sera generated against ES and SA antigens (1: 2000) were incubated for 1 h at room temperature. The plates were washed three times and 100 µL/well goat anti-bovine IgG secondary antibodies (1:10.000), conjugated with alkaline phosphatase (Invitrogen™ Cat. nos. WP20006, WP20007) was incubated for 1 h at room temperature. After washing the plates 100 µL of the substrate

para-Nitrophenyle Phosphate (PNPP) (Thermo Scientific™ Cat. No. 37621) was incubated at room temperature for 20 min. Finally, the reaction was stopped by the addition of 50 µL of 3N NaOH solution, and OD was recorded at 405 nm on an ELISA reader.

Statistical Analysis

The online software 'QuickCalcs (https://www.graphpad.com/quickcalcs/) was used to calculate the Kappa value of developed ELISA. The sensitivity and specificity of indirect ELISA was computed by using Online Statistical Software MedCalc *(https://www.medcalc.org/calc/diagnostic_test.php)*. Univariate analysis was performed between concentration and absorbance values and the level of significance was set at P=0.001. The relationship between coproantigen concentration and absorbance values was calculated with a regression equation.

Results

The detectable level of coproantigens (OD value 0.2) by using indirect ELISA was observed in a total of 33 samples out of 252. The cut-off point ≥0.22 was considered positive for *Fasciola* coproantigens. The ELISA O.D. value for the buffalo population tested for the coproantigens of *Fasciola* was high with increasing the antigen concentrations, indicating a high level of coproantigens. From the 50 µL of fecal supernatant, the mean absorbance values were significantly different (P<0.001) from controls *(Table 1)*. However, the difference was not significant among the animals tested positive along with different concentrations of coproantigen *(Fig. 1-a,b)*. A positive linear relationship was observed to the concentration of antigens used in fecal supernatant and the absorbance values *(Fig. 2-a,b)*.

Coproantigen ELISA with ES Polyclonal Antibodies

The cut-off point was calculated by the average optical density (OD) of the negative reference feces, plus three times standard deviations (0.045+3*0.0168=0.095). The specificity of the assay was determined by using *Fasciola* negative feces from 21 buffaloes, although some of the buffaloes (7/20) harbored other gastrointestinal parasites. The cross-reactivity was observed with helminths parasitizing these domesticated animals.

Table 1. *OD values obtained with the Copro- ELISA testing fecal samples from animals infected with Fasciola species and controls. The absorbance values are presented a Mean± SD of different concentrations of coproantigens*

Concentration of Coproantigens	ES Polyclonal Antibodies		SA Polyclonal Antibodies	
	Mean± SD	95% CL	Mean±SD	95% CL
50 µL	0.42±0.08[a]	0.40±0.44	0.46±0.07[a]	0.44±0.48
80 µL	0.45±0.07[ab]	0.43±0.48	0.46±0.06[a]	0.44±0.48
100 µL	0.44±0.08[ab]	0.42±0.47	nc*	
120 µL	0.46±0.06[ab]	0.43±0.48	nc	
140 µL	0.48±0.05[b]	0.46±0.51	nc	
Cross reactive	0.11±0.04[c]	0.05±0.18	0.11±0.04[b]	0.05±0.17
Negative Controls	0.6±0.04[c]	0.03±0.09	0.08±0.04[b]	0.06±0.11
P-value	>0.001		>0.001	
F calculated	107.500		217.800	

**nc:* *not calculated*

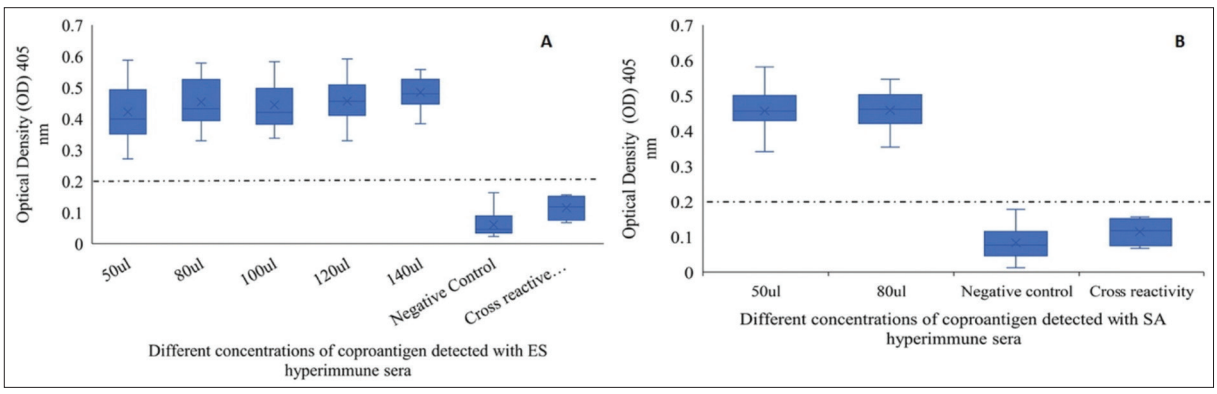

Fig 1. Detectability of Copro ELISA performed with **A)** ES polyclonal antibodies **B)** and SA polyclonal antibodies raised in rabbits from animals infected with *Fasciola* species. Cut-off point was set at 0.2

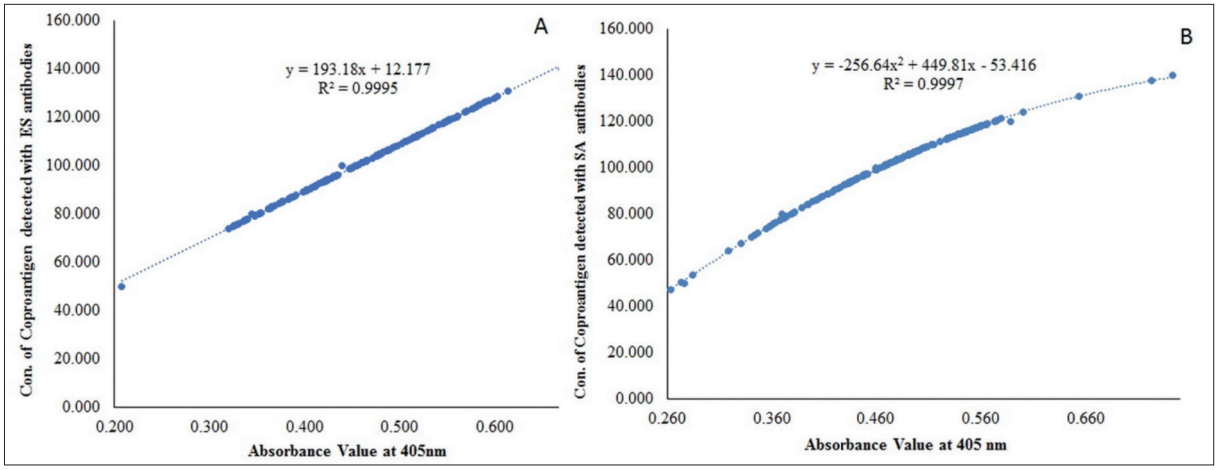

Fig 2. Correlation between absorbance values and different concentrations of coproantigens with Copro ELISA testing fecal samples **A)** with ES **B)** and SA polyclonal antibodies raised in rabbits from animals infected with *Fasciola* species

Table 2. *Diagnostic efficacy of copro-ELISA using hyperimmune sera of Fasciola ES antigens*

Diagnostic Methods	**ELISA Test**			**Sensitivity**	**Specificity**	**KAPPA Value**
Fecal/Postmortem Examination	**Positive**	**Negative**	**Total**	**95% CI**	**95% CI**	
Positive	33	0	33	100.00 (89.42-100.00)	76.19 (52.83-91.78)	Kappa = 0.796 SE of Kappa = 0.085 95% Confidence interval: 0630 to 0.963
Negative	5	16	21			
Total	38	16	54			

Table 3. *Diagnostic efficacy of copro-ELISA using hyperimmune sera of Fasciola somatic antigens*

Diagnostic Methods	**ELISA Test**			**Sensitivity**	**Specificity**	**KAPPA Value**
Fecal/Postmortem Examination	**Positive**	**Negative**	**Total**	**95% CI**	**95% CI**	
Positive	33	0	33	100 (89.42-100.00)	90 (68.30-98.77)	Kappa = 0.918 SE of kappa = 0.057 95% confidence interval: 0.807 to 1.00.
Negative	2	18	20			
Total	35	18	53			

The sensitivity and specificity of diagnostic test were 100% (95% CI: 89.42%-100.00%) and 76.19% (95% CI: 52.83.30%-91.78%), respectively *(Table 2)*. Kappa value of the test calculated that the strength of agreement is almost substantial.

Coproantigen ELISA with SA Polyclonal Antibodies

The cut-off point was calculated by the mean optical density (OD) of the negative reference feces, plus three times standard deviations (0.083+3*0.044=0.22).

The specificity and sensitivity of diagnostic test were 100% (95% CI: 89.42%-100.00%) and 90.00% (95% CI: 68.30%-98.77%), respectively *(Table 3)*. The results of Kappa value showed that the strength of agreement is approaching to perfect. A total of 20 negative control sera were used and two of the fecal samples showed cross-reactivity with *Fasciola* SA antigen polyclonal antibodies.

Discussion

In the present study, the efficacy of coproantigens ELISA based on polyclonal antibodies against SA and ES antigens was tested for the diagnosis of fasciolosis. The *Fasciola* coproantigens ELISA was found highly sensitive, which may reflect the stability of the antigens being excreted out along the feces. Several studies have been conducted on the detection of coproantigen in feces for a

number of helminths [14-17]. Monoclonal antibodies based coproantigen ELISA method was reported several times for detection of fasciolosis, which may compromise sensitivity due to high variability in the concentration of cathepsins in feces of infected animals [18].

In the present study 50 µL of fecal supernatant was used for detection of fasciolosis, the values containing 10 ng/mL of coproantigen in feces. The detection values in feces were found lower than reported previously [19,20]. These differences may be due to the capture antibody used since in our work a polyclonal serum was used whereas in the two other studies two different monoclonal antibodies were used.

The detection limits were different in the current study for the somatic and ES polyclonal antibodies based coproantigen ELISA, which may attribute to components present in the feces which resulted in an elevation of the non-specific limit of the assay [21]. The difference in detection limit as compared to previous studies may be because of diluting fecal samples in PBS containing BSA [17].

The cross-reaction occurred with other helminths when detected in the assay. However, the possibility of false positives would be very low, thus making the test highly specific. Similarly, 100% specificity was recorded by Espino et al.[22], however, Deplazes et al.[23] observed that a polyclonal antibody against ES antigen of tapeworms can detect different parasites of the same genus. The limitation of the current assay was, did not use helminth species i.e. *Schistosoma* and *Dicrocoelium* to check the cross-reactivity of the assay. Previous work had reported that *Fasciola* spp. shares antigens with these trematode parasites i.e. *Schistosoma* and *Dicrocoelium* [11,13,24]. Therefore, possible cross-reactions with antigens of these trematodes probably are not a factor that would affect the use of this test to diagnose fasciolosis in Pakistan.

In conclusion, the developed coproantigen detection methods are a good alternative to the conventional fecal examination microscopic techniques for fasciolosis detection in ruminants. However, further studies are required for field implementation of this indigenous coproantigen ELISA to detect fasciolosis and the cross-reactivity with other helminths species which may shares antigens with *Fasciola*.

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Conflict of Interest

Authors state no conflict of interest.

Author's Contribution

KA designed the study. KA, IA, MK performed the experiment. SF, IAK and MQ advised on methods, experimentation, and interpretation of findings. KA and MQ conducted literature search, data analysis and manuscript preparation. KA and MQ reviewed the manuscript. All authors participated in the study and concurred with the submission and subsequent revisions submitted by the corresponding author.

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CASE REPORT

Treatment of a Post-Operative Infected Wound of a Cat with Maggot Debridement Therapy

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Abstract

Maggot debridement therapy (MDT), which is an ancient remedy, has been reintroduced and commonly used to promote wound healing in humans. However, its use in the veterinary field has still remained limited. The aim of this case study was to evaluate the effectiveness of MDT on a non-healing post-operative infected wound covering the abdominal and inguinal regions of a 3-year-old male cat. For MDT, sterile first and second stage larvae of *Lucilia sericata* were applied to the infected wound for the first time on the 10th day after laparotomy. A total of five larva applications were performed during the MDT process. From the first to the last MDT application, the large and infected wound gradually shrank and healed. Sterile *L. sericata* larvae were successfully used in the treatment of a chronic and infected wound that did not respond to antibiotics in a cat in this case study. It is considered that the use of MDT for promoting healing in chronic necrotic and infected wounds will increase because of supplying effective, cost-efficient and simple wound care in future.

Keywords: *Biotherapy, Cat, Lucilia sericata, Maggot, Wound*

Maggot Debridman Tedavi İle Bir Kedinin Post-operatif Enfekte Yarasının Sağaltımı

Öz

Eskiden tedavi amacıyla kullanılan maggot debridman tedavi (MDT) yeniden gündeme gelmiş ve insanlarda yara iyileşmesini teşvik etmek amacıyla kullanılmaya başlanmıştır. Ancak, bu yöntemin veteriner hekimlik alanında kullanımı sınırlıdır. Bu vaka sunumu, 3 yaşındaki erkek bir kedinin abdominal ve inguinal bölgelerini kapsayan, iyileşmeyen postoperatif enfekte bir yarada MDT'nin etkinliğini değerlendirmek amacıyla hazırlandı. MDT için ilk uygulama laparotomiden sonraki 10. günde yapıldı. Bu amaçla *Lucilia sericata* türü sineklerin steril birinci ve ikinci dönem larvaları kullanıldı. MDT sürecinde toplam beş adet larva uygulaması yapıldı. İlk MDT uygulamasından son MDT uygulamasına kadar, büyük ve enfekte olmuş yara yavaş yavaş küçülerek iyileşti. Steril *L. sericata* larvaları ile bir kedinin antibiyotiklere yanıt vermeyen kronik ve enfekte yarası başarılı bir şekilde sağaltıldı. Gelecekte etkili, uygun maliyetli ve basit yara bakımı sağlaması nedeniyle kronik, nekrotik ve enfekte yaralarda iyileşmeyi teşvik etmek amacıyla MDT kullanımının artacağı düşünülmektedir.

Anahtar sözcükler: *Biyoterapi, Kedi, Lucilia sericata, Larva, Yara*

Introduction

Lucilia sericata (Phaenicia sericata) (Diptera: Calliphoridae) fly larvae have been known to remove necrotic tissues from wounds and accelerate wound healing for many years. The use of calliphorid fly larvae for the treatment of chronic wounds is known as larval treatment, maggot debridement therapy (MDT), biosurgery or biotherapy [1,2]. *Lucilia sericata* flies can be grown in special laboratories where appropriate space, temperature and humidity are provided, and sterile maggots can be obtained when necessary [3].

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Medicinal maggots have various beneficial effects on wounds. These can be listed as debridement, disinfection of the wound through microbial killing and promotion of wound healing [2,4].

Maggot debridement therapy was firstly used successfully in a patient with chronic osteomyelitis in 1931 [5]. Although MDT was used frequently in those times, the number of MDT applications decreased due to the widespread use of antibiotics and intensive surgical treatments. However, MDT has been started to reapply in countries such as the USA, Germany, Austria, England, Israel and Sweden to treat non-healing chronic wounds and has become popular again from the late 20th century [1,6]. Although MDT has been used to treat chronic wounds in humans for many years [7-9], its use in veterinary medicine is limited. However, it has also been widely applied in the field of veterinary medicine in recent years, and different types of wounds can be treated by MDT in animals [10-15].

This case study was conducted to evaluate the effectiveness of MDT in the treatment of a non-healing post-operative infected wound in a cat, despite an antibiotic treatment for a long time. Maggot debridement therapy has been used in the wound of humans for many years, but there is no study on the application of MDT in animals in Turkey.

Case History

The material of this case study consisted of a 3-year-old male cat with a post-operative infected wound covering the abdominal and inguinal region as a result of laparotomy performed for cryptorchitis. Systemic antibiotics such as ceftriaxone (Unacefin® 1 g IM Flakon, Yavuz İlaç San. Tic. A.Ş, İstanbul, Turkey) and enrofloxacin (Baytril-K® 5% 50 mL, Injectable solution Bayer) were used for wound care, but no improvement was observed. Thereupon, it was decided to benefit from MDT, which has commonly been used in human medicine in recent years. For this purpose, the approval of the owner of the cat was also obtained. Sterile I. and II. instars of *L. sericata* used in the case study *(Fig. 1)* were supplied from Selçuk University, Faculty of Veterinary Medicine, Maggot Production Unit.

On the 10th day after laparotomy, the first application of sterile I. and II. instars of *L. sericata* was applied to the infected wound. A light surgical debridement to prepare the wound was used before application. The size of the wound was determined (7-8 x 10-12 cm), and the wound edges were demarcated after shaving and disinfecting. The wound was washed with physiological saline solution and a total of almost 1000 sterile I. and II. instars of *L. sericata* were left on the wound. Approximately 8 to 10 maggots per square centimeter were used. Then, the wound was covered with a gauze patch, and the edges of the wound were pasted to prevent the escape of maggots from the wound. The second and third maggot applications were made by repeating the same procedures at the 24th and 48th h after the first maggot application. Later, the MDT application was paused for a week, and the fourth and fifth maggot applications were made at one-day intervals. Systemic antibiotic (ceftriaxone and enrofloxacin) administrations continued during the MDT procedures. The large and infective wound of the cat gradually shrank and healed during the process of MDT applications. After the last MDT application, there was a tiny opening in the wound, and this opening completely closed after one and a half months. During the treatment, the cat was supported every day with liquid electrolytes and vitamin, mineral and amino acid complexes. Moreover, some sprays such as Dr. Animal (Farmaso İlaç San. Tic. A.Ş., İstanbul/Turkey) and Acto® Vet Solution (Acto Pharma Hijyen San. Tic. A.Ş., İstanbul/Turkey) were daily used for wound care. The status of the wound and the maggot applications are indicated in *Fig. 2*.

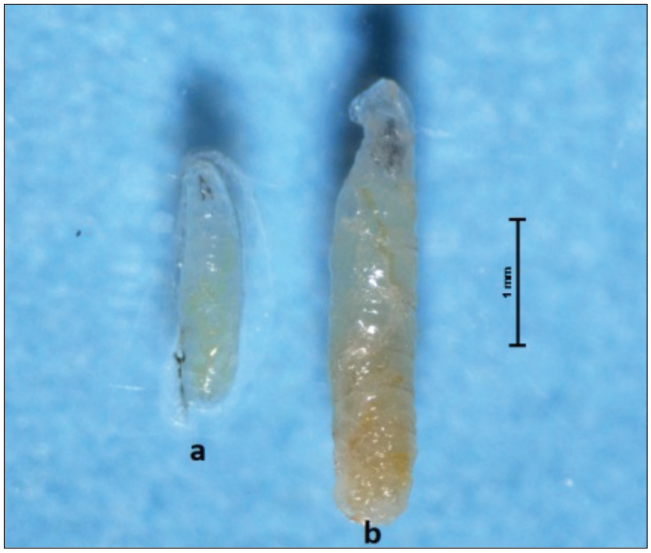

Fig 1. First and second stage larvae of *L. sericata*

Discussion

The fly larvae have been used to debride necrotic tissues since the beginning of civilisation. For this purpose, facultative calliphorids' larvae are the most commonly used fly larvae, with the sterile larvae of *L. sericata*, colloquially known as green bottle blowfly, being the most widely used fly species [7].

Freshly emerged germ-free maggots of *L. sericata* can be applied to a necrotic wound in two different ways: direct and indirect contact techniques. Free-range maggots (I. or II. instars of *L. sericata)* are deposited directly onto the wound's surface in the direct contact technique. In this technique, after the larvae are placed onto the surface of the wound, the wound is restricted with a nylon retention net or gauze bandage to prevent the escape of larvae from the wound. On the other hand, maggots are applied

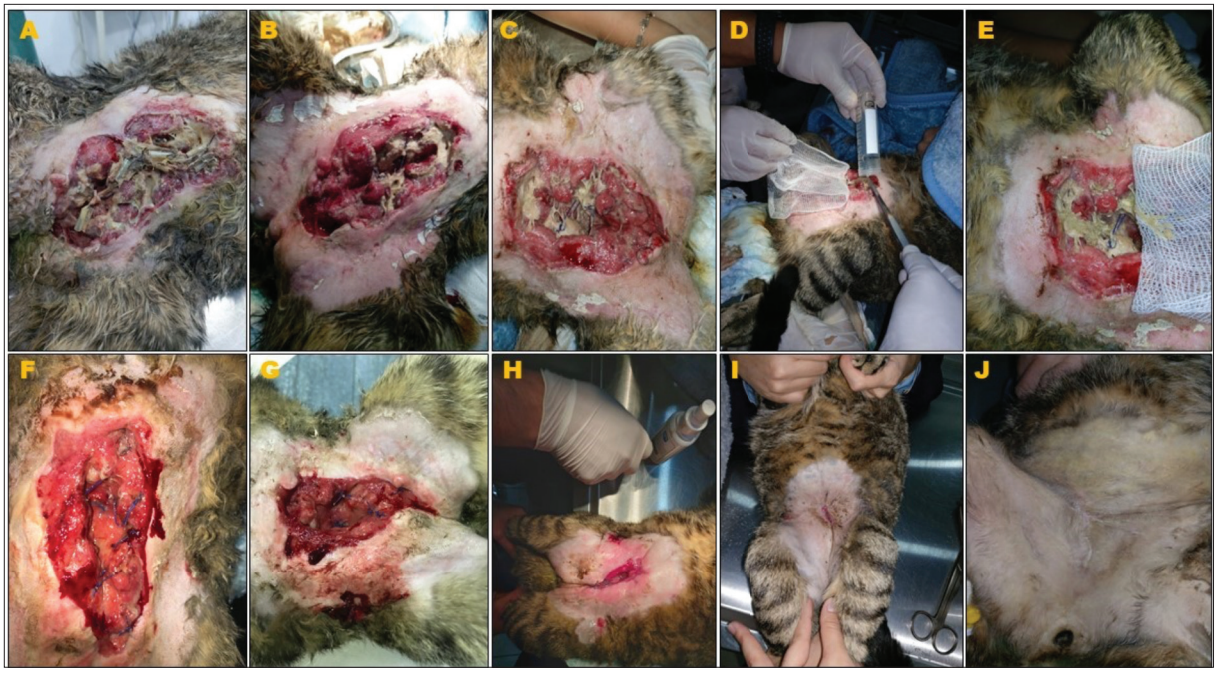

Fig 2. A: The status of the wound before MDT, **B and C:** The appearance of the wound after the First and second maggot application, **D and E**: Steps of third maggot application, **F:** The appearance of the wound after the third maggot application, **G:** The appearance of the wound after the fourth maggot application, **H:** The appearance of the wound after the fifth maggot application, **I and J:** The appearance of the wound area after MDT

on the wound's surface within a bio bag in the indirect contact technique [16]. Mumucuoğlu and Taylan Özkan [1] noted that the maggots within the bio bags could not thoroughly debride the necrotic tissues. Therefore, the direct contact technique was preferred considering the size of the wound and the condition of the necrotic tissues in the current case study.

Maggot debridement therapy is widely used in human medicine. Chan et al.[17] listed the types of wounds or lesions for which maggot therapy may be used in humans as follows; arterial/ischaemic ulcers, diabetic ulcers, neuropathic ulcers, venous ulcers, mixed arterial-venous ulcers, burns, excised abscess on malleolus, grossly infected toe, infected wound after forearm replantation, infected gunshot wound, infected wounds after breast surgery, osteomyelitis, malignant wounds, non-healing surgical wounds, subacute mastoiditis, Methicillin resistant *Staphylococcus aureus*-infected wounds, necrotising fasciitis, pilonidal sinus, pyoderma gangrenosum, post traumatic wounds/ulcers, pressure sores, thromboangiitis obliterans, wound of exposed knee prostheses. MDT has been used in the treatment of various types of wounds, especially chronic wounds caused by diabetes in humans in Turkey [7-9,18]. MDT has also successfully been applied in veterinary medicine, but the number of MDTs applied in veterinary medicine is lower than that of human medicine [19]. In the veterinary field, studies have reported that MDT has been used for the treatment of panniculitis in donkeys [10], laceration of the limbs, soft tissue abscess and wounds, fistulous withers, dehiscence of *linea alba*, and other musculoskeletal infections, laminitis and other foot pathology in horses [12,14,15], foot root and foot scald in sheep [11], pressure ulcer and gunshot wound in dogs [13], necrotic tumour and multiple bite wound in cats [13]. As a result of the literature search, MDT application in animals has not been encountered in Turkey. In the present study, MDT was used in a cat with a post-operative infected wound covering the abdominal and inguinal region, and the wound almost completely healed after five maggot applications. Sherman et al.[13] stated that MDT provides debridement of wounds of cats and dogs, which are expected to be amputated or euthanized, and these animals regained their health. This case study demonstrates once again that the cat with a chronic infected wound can be successfully treated with MDT. It should not be forgotten that MDT is an important alternative treatment option to be used to treat such wounds in the veterinary field.

Maggot debridement therapy can be supported with different additional applications by different researchers. Arshadniya et al.[20] stated that the combination of MDT with topical or oral antibiotics effectively provided the elimination of infected tissues and the regeneration of new tissues. Therefore, antibiotics and various supportive treatments were used to contribute to the healing process of the infected wound in the present study.

In conclusion, maggot debridement therapy is an effective, fast, and safe method for the debridement of wounds. Considering the increase in antibiotic resistance to infections, MDT is one of the most important and cost-effective

treatment options. It is thought that MDT will be used more for the treatment of non-healing chronic wounds of animals in Turkey in the future.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgments

This case report has not previously been presented at any scientific meeting, either in whole or in part, and no financial support has been received. All applications in the study were carried out with the approval of the Experimental Animals Production and Research Center Ethics Committee of Selçuk University, Faculty of Veterinary Medicine (SÜVDAMEK-2021/31).

Statement of Author Contributions

UU designed to apply maggot debridement therapy. OC and AK produced the sterile maggots for application. OC and HKA applied maggots to the wound. UU and OC wrote the manuscript. All authors read and approved the final form of the manuscript.

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